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Identification du mécanisme d'entrée du *Canid herpesvirus 1* dans les cellules épithéliales Madin-Darby Canine Kidney

Par

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عن أبي الدرداء رضي الله عنه قال سَمِعْتُ رَسُولَ اللَّهِ ـ صلى الله عليه وسلم ـ يَقُولُ **" مَنْ سَلَكَ طَرِيقًا يَلْتَمِسُ فِيهِ عِلْمًا سَهَّلَ اللَّهُ لَهُ طَرِيقًا إِلَى الْجَنَّةِ وَإِنَّ الْمَلاَئِكَةَ لَتَضَعُ أَجْنِحَتَهَا رِضًا لِطَالِبِ الْعِلْمِ وَإِنَّ طَالِبَ الْعِلْمِ يَسْتَغْفِرُ لَهُ مَنْ فِي السَّمَاءِ وَالأَرْضِ حَتَّى الْحِيتَانِ فِي الْمَاءِ وَإِنَّ فَضْلَ الْعَالِمِ عَلَى الْعَابِدِ كَفَضْلِ الْقَمَرِ عَلَى سَائِرِ الْكَوَاكِبِ إِنَّ الْعُلَمَاءَ هُمْ وَرَثَةُ الأَنْبِيَاءِ إِنَّ الأَنْبِيَاءَ لَمْ يُوَرِّثُوا دِينَارًا وَلاَ دِرْهَمًا إِنَّمَا وَرَّثُوا الْعِلْمَ فَمَنْ أَخَذَهُ أَخَذَ بِحَظٍّ وَافِرٍ** ».

Abu Darda said: 'I heard the Messenger of Allah say: "Whoever follows a path in the pursuit of knowledge, Allah will make easy for him a path to Paradise. The angels lower their wings in approval of the seeker of knowledge, and everyone in the heavens and on earth prays for forgiveness for the seeker of knowledge, even the fish in the sea. The superiority of the scholar over the worshipper is like the superiority of the moon above all other heavenly bodies. The scholars are the heirs of the Prophets, for the Prophets they did not leave behind money, rather they left behind knowledge, so whoever takes it has taken a great share.'"

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RÉSUMÉ

IDENTIFICATION DU MECANISME D'ENTREE DU CANID HERPESVIRUS 1 DANS LES CELLULES EPITHELIALES MADIN-DARBY CANINE KIDNEY

L'herpesvirus canin (CHV-1 pour *Canid herpesvirus 1*) est un varicellovirus qui est répandu chez les membres de la famille des canidés, infectant principalement les chiens. CHV-1 cause chez les chiots nouveau-nés des morbidités et de la mortalité, ainsi que chez les chiens adultes des maladies respiratoires, génitales et oculaires. En tant que parasite intracellulaire obligatoire, les virus doivent pénétrer dans les cellules hôtes pour établir une infection efficace et assurer leur propagation à un nouvel hôte. L'entrée virale représente une première étape essentielle dans le cycle de réplication virale. La voie d'entrée du CHV-1 dans les cellules épithéliales de l'hôte - le site primaire pour le réplication virale – est inconnue.

Nous avons étudié la voie d'entrée du CHV-1 dans les cellules épithéliales canines du rein Madin-Darby canine kidney (MDCK). En utilisant la microscopie électronique à transmission, nous avons détecté l'entrée du CHV-1 dans des grandes vacuoles non revêtues ainsi que l'association du virus avec des protrusions membranaires. Sur la base de ces observations, nous avons formulé l'hypothèse que l'entrée du CHV-1 peut être médiée par une voie endocytaire appelée macropinocytose, une voie utilisée par les cellules pour l'absorption de relativement grands volumes de fluides extracellulaires riches en nutriments.

Pour tester le rôle possible de la macropinocytose dans l'entrée du CHV-1, nous avons étudié l'impact du blocage de certains facteurs cellulaires importants pour la macropinocytose, sur la formation de plages de lyse virales. Nos résultats ont montré que l'entrée du CHV-1 était bloquée par les inhibiteurs ciblant les échangeurs Na^+/ H^+, la F-actine, la myosine, p21-activated kinase, la phosphatidylinositol-3-kinase, la dynamine et la focale adhésion kinase. Ces résultats indiquent que CHV-1 utilise la machinerie de la macropinocytose durant l'entrée. De plus, les résultats avec les inhibiteurs montrent que la signalisation via les intégrines et les récepteurs de type tyrosine kinase EGFR et PDGFR sont nécessaires pour le processus d'entrée. En plus, nos résultats ont montré que l'entrée du CHV-1 dans les cellules MDCK est indépendante de l'endocytose médiée par la clathrine et par la cavéoline, des microtubules et de l'acidification endosomale.

L'une des principales caractéristiques de l'entrée virale par macropinocytose est l'induction du protrusions membranaires et l'augmentation de l'absorption des fluides. En utilisant

un microscope électronique à balayage, nous avons constaté que CHV-1 induisent des protrusions de type lamellipodes. De plus, par microscopie confocale nous avons montré que le CHV-1 marqué à la fluorescence se colocalise avec un marqueur d'absorption des fluides (le dextran fluorescent). Cependant, nos résultats d'analyse par cytométrix en flux n'ont pas révélé une augmentation de l'absorption de liquide durant la première heure de l'infection par le virus. Ces résultats suggèrent que l'entrée du CHV-1 dépend d'un mécanisme lié à la macropinocytose mais non de la macropinocytose classique.

En conclusion, ces résultats décrivent pour la première fois le mécanisme d'entrée du CHV-1 dans les cellules épithéliales canines. Nos résultats ont identifié plusieurs facteurs cellulaires importants pour l'entrée du CHV-1, qui pourraient être des cibles potentielles pour le développement de futurs antiviraux contre cet important agent pathogène vétérinaire.

ABSTRACT

Canid herpesvirus (CHV-1) is a varicellovirus that is widespread in members of the *Canidae* family, infecting mainly dogs and causing morbidities and mortality in newborn pups as well as respiratory, genital, and ocular diseases in adult dogs. As obligate intracellular parasites, viruses have to enter host cells to establish a successful infection and ensure their replication in a new host. Viral entry represents an essential first step in the viral replication cycle. The pathway by which CHV-1 enters host epithelial cells – the initial site of viral replication – has not been identified.

We studied the entry pathway of CHV-1 into Madin-Darby canine kidney (MDCK) epithelial cells. Using transmission electron microscopy, we detected uptake of CHV-1 in large uncoated vacuoles, as well as the association of the virus with membrane protrusions. Based on these observations, we hypothesized that CHV-1 entry may be mediated by an endocytic pathway called macropinocytosis, a pathway that is used by the cells for the uptake of a relatively large volume of nutrient rich extracellular fluids.

To investigate the possible role of macropinocytosis in CHV-1 entry, we studied the impact of blocking key cellular factors for macropinocytosis on CHV-1 infection (plaque formation). Our results revealed that the entry of CHV-1 was blocked by inhibitors targeting Na^+/H^+ exchangers, F-actin, myosin light-chain kinase, protein kinase C, p21-activated kinase, phosphatidylinositol-3-kinase, dynamin, and focal adhesion kinase. These findings support the notion that CHV-1 hijacks macropinocytosis for its entry. Moreover, our inhibitor study also suggested that signaling via integrins and the receptor tyrosin kinases EGFR and PDGFR is required for the entry process. We found that the entry of CHV-1 in MDCK cells was independent of clathrin- and caveolin-mediated endocytosis, microtubules, and endosomal acidification.

One of the main characteristics of viral entry via macropinocytosis is the induction of cell-wide membrane ruffling and an increase in the uptake of fluids. Using scanning electron microscopy, we found that CHV-1 triggered extensive lamellipodial ruffles. Furthermore, the results from confocal microscopy showed that fluorescently-labeled CHV-1 colocalized with a marker of fluid uptake, fluorescent dextran. Nevertheless, using multiparametric FACS® analysis, we did not detect an increase in fluid uptake during the first hour of CHV-1 infection. These results suggest that CHV-1 entry depends on macropinocytosis-related mechanism, that deviates from classic macropinocytosis.

Taken together, these results describe for the first time the entry mechanism of CHV-1 into canine epithelial cells. Our results identified multiple cellular factors that are critical for CHV-1 cell entry, and thus could be a target for the design of future antivirals against this important veterinary pathogen.

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LISTE DES ABRÉVIATIONS

Ad35	**Adenovirus 35**
AAV2	**Adeno-associated virus 2**
AlHV-1	**Alcelaphine herpesvirus 1**
AJ	**Adherent junctions**
ASVF	**African swine fever virus**
BHK-21	**Baby Hamster Kidney fibroblasts**
BHV-1	**Bovine herpesvirus 1**
CAR	**Coxsackievirus and adenovirus receptor**
CAV-2	**Canine adenovirus type 2**
CHV-1	**Canid Herpesvirus 1**
CCP	**Clathrin-coated pit**
CCV	**Clathrin-coated vesicle**
CIRD	**Canine infectious respiratory disease complex**
CLIP-170	**Cytoplasmic linker protein 170**
CME	**Clathrin-mediated endocytosis**
CPIV	**Canine parainfluenza virus**
CPZ	**Chlorpromazine**
Cyt-D	**Cytochalasin D**
DC-SIGN	**Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin**
DCTN1	**Dynactin-1**
Dyn	**Dynasore**

EB1	**End-binding protein1**
EBV	**Epstein–Barr virus**
EHV-1	**Equine herpesvirus 1**
EIPA	**5-(*N*-ethyl-*N*-isopropyl) amiloride**
FAK	**Focal adhesion kinase**
FHV-1	**Feline herpesvirus 1**
GAGs	**Glycosaminoglycans**
gB	**Glycoprotein B**
gC	**Glycoprotein C**
gD	**Glycoprotein D**
gE	**Glycoprotein E**
gH/gL	**Glycoprotein H and L heterodimer**
HCMV	**Human cytomegalovirus**
HCV	**Hepatitis C virus**
HIV	**Human Immunodeficiency virus**
HPV16	**Human Papillomavirus 16**
HS	**Heparan sulfate**
HSPG	**Heparan sulfate proteoglycans**
HSV-1	**Herpes simplex virus 1**
HSV-2	**Herpes simplex virus 2**
HVEM	**Herpesvirus entry mediator**
IAV	**Influenza A virus**
ICP0	**Infected Cell Protein 0**
ICTV	**International Committee on Taxonomy of Viruses**

IDE	**Insulin-degrading enzyme**
KSHV	**Kaposi's sarcoma-associated herpesvirus**
LDL	**Low-density lipoprotein**
Man 6-P	**Mannose 6-phosphate**
MAG	**Myelin-associated glycoprotein**
MDCK	**Madin-Darby canine kidney**
MHC-1	**Major histocompatibility class I**
MPR^{ci}	**Cation-independent Mannose 6-phosphate receptor**
NHE	**Na^+/H^+ exchanger**
NMMHC-IIA	**Non-muscle myosin heavy chain IIA**
NMMHC-IIB	**Non-muscle myosin heavy chain IIB**
Noc	**Nocodazole**
Nyst	**Nystatin**
Pak1	**p21-activated kinase 1**
PI(3)K	**Phosphatidylinositol-3-kinase**
PILRα	**Paired immunoglobin like type 2 receptor alpha**
PKC	**Protein kinase C**
PPV	**Porcine Parvovirus**
PRV	**Pseudorabies virus**
RSV	**Respiratory syncytial virus**
RTKs	**Receptor tyrosine kinases**
SEM	**Scanning electron microscope**
SARS-CoV-2	**Severe acute respiratory syndrome coronavirus 2**
SFV	**Semliki Forest virus**

SV40	**Simian virus 40**
Tax	**Paclitaxel**
TEM	**Transmission electron microscope**
VSV	**Vesicular stomatitis virus**
VZV	**Varicella-zoster virus**
Wort	**Wortmannin**
ZF-3-OS HS	**Zebrafish encoded 3-O-sulfotransferase-2**

CHAPITRE UN: INTRODUCTION

1 CANID HERPESVIRUS 1 (CHV-1)

1.1 Taxonomy of herpesviruses

Canid Herpesvirus 1 (CHV-1) is an important veterinary virus that is widespread in dogs and other members of the *Canidae* family. According to the 2019 classification of virus taxonomy released by the «International Committee on Taxonomy of Viruses (ICTV)», CHV-1 is classified as a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae,* and the genus *Varicellovirus*.

The order *Herpesvirales* is a large group of double-stranded DNA viruses that infect a wide range of animal hosts (Andrade-Martínez *et al.*, 2019; Roizman, 2007). It contains three families; *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae. Herpesviridae* is divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. *Herpesviridae* includes 107 species most of which evolved over millions of years with their respective host species. Few herpesviruses are capable of infecting more than one host species (Azab & Osterrieder, 2017).

The subfamily *Alphaherpesvirinae* encompasses five genera including 37 different species, three of which infect humans: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) in the genus *Simplexvirus*, and varicella-zoster virus (VZV) in the genus *Varicellovirus*. The remaining 34 viruses including CHV-1, infect different animal hosts and some of which cause economical losses. (Azab & Osterrieder, 2017). The genus *Varicellovirus* contains 18 species, among which, only VZV infects human, while the remaining viruses infect different animals' hosts such as cattle (*Bovine alphaherpesvirus 1* (BHV-1)), Bubalus (*bubaline alphaherpesvirus 1*), goats (*Caprine alphaherpesvirus 1*), monkeys (*Cercopithecine alphaherpesvirus 9*), deer (*Cervid alphaherpesvirus 1*), whales (*monodontid alphaherpesvirus 1*), dogs (CHV-1), phocids (*phocid alphaherpesvirus 1*), cats (*feline herpesvirus 1* (FHV-1)), horses (*equine herpesvirus 1* (EHV-1)), and pigs (pseudorabies virus (PRV)). CHV-1 has phylogenetic similarity to other varicelloviruses including *phocid herpesvirus 1*, FHV-1, EHV-1, PRV, BHV-1, and VZV (Davison & Scott, 1986; Martina *et al.*, 2003; Papageorgiou *et al.*, 2016; Rémond *et al.*, 1996; Rota & Maes, 1990).

The subfamily *Betaherpesvirinae* comprises the genus *Cytomegalovirus* which is represented by human cytomegalovirus (HCMV). Infection by HCMV can lead to severe complications including congenital abnormalities and organ transplant rejection in immunocompromised individuals. Moreover, HCMV infection could also increase the risk of atherosclerosis (Söderberg-Nauclér, 2006; Streblow *et al.*, 2001). Members of the genus

roseoloviruses including Human herpesvirus 6A (HHV-6A), human herpesvirus 6B (HHV-6B), and human herpesvirus 7 (HHV-7) are genetically related to cytomegalovirus and also belong to *Betaherpesvirinae* subfamily. HHV-6A and HHV-6B infect over 90% of the world population during early childhood and can integrate their genomes into the host chromosomes as a way to establish latency (Flamand, 2018). Infection by HHV-6A has been associated with multiple sclerosis. While HHV-6B and HH7-7 cause exanthem subitem (roseola infantum) a common childhood disease that is characterized by fever, skin rash, and respiratory distress (Suga *et al.*, 1997; Tanaka *et al.*, 1994; Yamanishi *et al.*, 1988). Moreover, reactivation of HHV-6B can cause encephalitis, pneumonitis, and bone marrow suppression in hematopoietic stem cell transplant recipients (Miura *et al.*, 2018; Miura *et al.*, 2015; Quintela *et al.*, 2016).

β-herpesviruses are characterized by restricted host tropism and a relatively long replication cycle. Among members of the family *betaherpesvirinae* are *murine cytomegalovirus* (MCMV) which is a widely used model to study the immunology and pathogenesis of HCMV (Brune, 2013; Puhach *et al.*, 2020), and elephantid betaherpesvirus that causes lethal hemorrhagic disease in young elephants (Hoornweg *et al.*, 2021; Long *et al.*, 2016).

The third subfamily *Gammaherpesvirinae* includes Epstein–Barr virus (EBV) of the genus *Lymphocryptovirus*, and Kaposi's sarcoma-associated herpesvirus (KSHV) of the genus *Rhadinovirus*. EBV can infect B cells and epithelial cells (Chen & Longnecker, 2019; Tosato & Cohen, 2007), causes infectious mononucleosis (Dunmire *et al.*, 2015; Houen & Trier, 2020) and is linked to malignancies like Hodgkin’s lymphoma, diffuse large B-cell lymphoma, Burkitt lymphoma, and nasopharyngeal carcinoma (Ko, 2015; Neparidze & Lacy, 2014; Tsao *et al.*, 2015). KSHV infects B cells, fibroblasts, epithelial, and endothelial cells (Dollery, 2019) and can cause two major diseases: endothelial cell neoplasms (Kaposi’s sarcoma) and primary effusion lymphoma (Dupin, 2020). Examples of animal viruses in the *Gammaherpesvirinae* include *Alcelaphine herpesvirus 1* (AlHV-1), which causes malignant catarrhal fever, a fatal lymphoproliferative disease in wildebeest and cattle (Myster *et al.*, 2020).

1.2 CHV-1 structure and genomic organization

Like other members of the *Herpesviridae* family, CHV-1 is an enveloped virus of a relatively large size with a diameter that can reach 280 nm (Carmichael *et al.*, 1965; Lara *et al.*, 2016). The viral particle **(Figure 1.1)** consists of a linear double-stranded DNA genome surrounded by an icosahedral capsid. The capsid itself is surrounded by a relatively amorphous protein layer known as the tegument. The tegument is enveloped within a lipid bilayer viral envelope in which several

viral envelope glycoproteins are anchored. Many of these glycoproteins are proposed to be important for viral adsorption to the cell surface, receptor recognition, and membrane fusion leading to viral entry into the host cell.

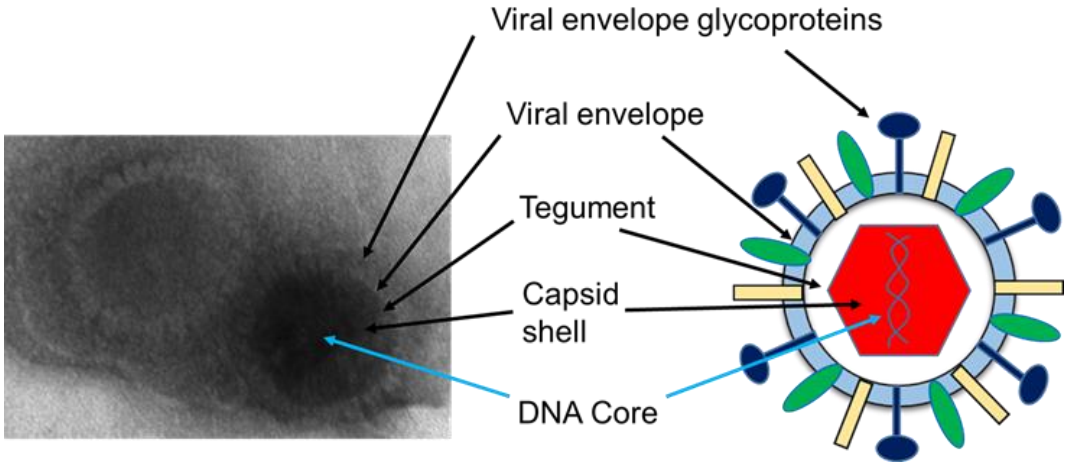

Figure 1.1 CHV-1 viral particle

Different components of CHV-1 particles, to the left, is an electron micrograph of CHV-1 strain V777 imaged by transmission electron microscope (TEM) (copyright© Mohamed Eisa 2021), to the right is a schematic representation of the CHV-1 viral particle (modified from (Davidson, 2014) (not to scale).

Several CHV-1 genomes have been recently sequenced (Kolb *et al.*, 2017; Lewin *et al.*, 2020; Papageorgiou *et al.*, 2016). The genome size is 125 kbp, a size that is at the lower limit for varicelloviruses and is similar to the size of VZV. The CHV-1 genome organization **(Figure 1.2)** includes a unique long (UL) segment (97.5 kbp) and a unique short (US) segment (7.7 kbp) each of which is flanked by a terminal and internal (38 bp and 9.9 kbp, respectively) inverted repeat sequence (Papageorgiou *et al.*, 2016). The genome contains 76 open reading frames that are predicted to code for functional proteins, all of which have counterparts in other alphaherpesviruses. Interestingly, the CHV-1 genome sequence lacks the herpesvirus orthologs for 39 UL and 40 UL which encode for the large and small subunits respectively of the ribonucleotide reductase. These two genes are conserved in other alphaherpesviruses that infect mammals and birds (Davison, 2010; Gandar *et al.*, 2015; Papageorgiou *et al.*, 2016). The CHV-1 genome has the lowest GC content (31.6%) among all of the completely sequenced alphaherpesviruses genomes. Although the two CHV-1 strains, V777 and 0194 were isolated over 15 years apart, they show very high sequence similarity (Papageorgiou *et al.*, 2016). Similarly, data from sequencing the genomes of fifteen CHV-1 isolates from USA have been shown a high degree of similarity to isolates from hosts in the UK, Australia, and Brazil, suggesting a transboundary transmission of CHV-1 in dog populations. However, two strains; BTU-1 and ELAL-

1 were different from the remaining strains and formed a separate clade (Lewin *et al.*, 2020). Moreover, the UL50 gene of the Brazilian CHV-1 strain BTU-1 is 12.2% distant from other CHV-1 isolates, that are hypothesized to have resulted from a recombination event between CHV-1 and an unknown varicellovirus (Kolb *et al.*, 2017).

Figure 1.2 CHV-1 genomic organization

Schematic representation of CHV-1 genome consisting of two unique sequences; a unique long UL (97.5 kbp) and a unique short US (7.7 kbp) segments, respectively flanked by the terminal inverted repeats TRL (38 bp), TRS (9.9 kbp), and internal inverted repeats IRL (38 bp) and IRS (9.9 kbp).

1.3 Replication cycle and infection

Compared to other alphaherpesviruses like HSV-1 and PRV, which have a wide tropism and can replicate in many cell lines derived from different vertebrate species, CHV-1 has a restricted host cell range in *vitro* and in *vivo*, as the virus replicates only in canine derived cells such as the canine epithelial cells Madin-Darby canine kidney (MDCK) (Nakamichi *et al.*, 2000).

Infection by CHV-1 starts by entry of the virus into epithelial cells of the upper respiratory tract mucosa where it replicates (Li et al., 2016), then—using varicella-zoster virus (VZV) as a model—the virions spread to the local lymphoid tissues to infect hematopoietic cells that disseminate the virus via the blood stream to the skin. During this acute phase of infection, the virus spreads by retrograde axonal transport to reach the sensory nerves of the trigeminal or dorsal root ganglia to establish latency, a phase in which the virus persists without replication (Gilden et al., 1983; Miyoshi et al., 1999). Reactivation of the virus from latency is usually triggered by stress or immunosuppression, then, newly formed virions travel via anterograde axonal transport to the epithelia where the virus replicates and causes new lesions (Zerboni et al., 2014).

The detailed molecular biology of CHV-1 replication is not known. Based on the information accumulated from the studies of the replication cycles of other alphaherpesviruses like HSV-1, VZV, and PRV, the hypothesized replication cycle **(Figure 1.3)** of CHV-1 starts by virion attachment to the cell surface which is followed by entry of the virus into the host cell possibly via direct fusion of the viral envelope with the host cell plasma membrane or via endocytosis (in cell type dependent manner). The mechanism of CHV-1 entry was unknown when

this thesis project was undertaken. Then, following the release of the viral capsid into the cytoplasm, it is transported on microtubules to the nuclear pore complex. The free capsid docks at the nuclear pores and injects the linear double-stranded viral DNA into the nucleus where it circularizes. Then, different stages of viral gene expression take place starting with immediate early genes, followed by early, then late genes. The viral genome is replicated and newly formed capsids package the newly synthesized DNA. Then viral capsids acquire a primary envelope by budding through the inner nuclear membrane. This membrane is lost by fusion with the outer nuclear membrane, which releases the capsids into the cytoplasm. The viral glycoproteins mature in the *trans*-Golgi network, and the tegument proteins assemble in vesicles that form the secondary envelope of the virus. Finally, the newly assembled virions move towards the cell membrane to be released by exocytosis.

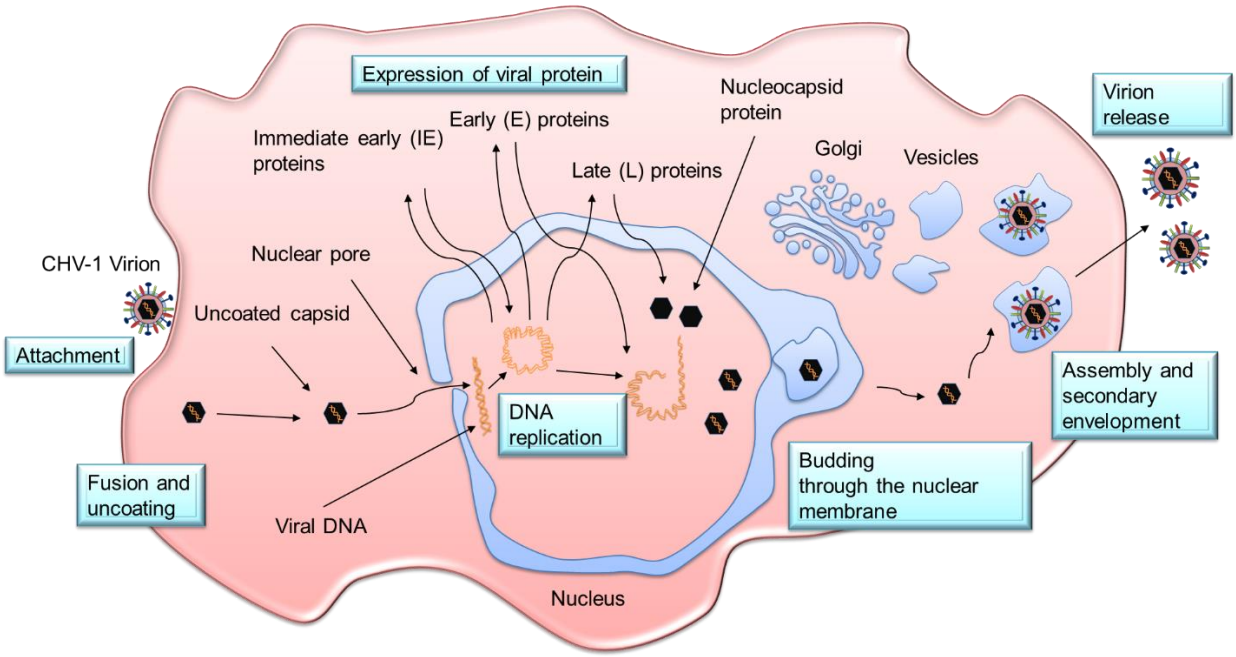

Figure 1.3 Model of CHV-1 replication cycle

Schematic representation of the proposed different stages of the CHV-1 replication cycle. The cycle starts with viral attachment to the cell surface, then entry and capsid penetration into the cytosol, viral DNA delivery into the nucleus, expression of immediate early and early genes, viral DNA replication, assembly of new capsids, budding of new capsid into the inner nuclear membrane, loss of the primary envelope during fusion with the outer nuclear membrane, secondary envelopment and tegumentation in the cytoplasm, and finally, the egress of newly formed viral particles by exocytosis. The figure is modified from (Zerboni *et al.*, 2014).

Viral attachment and entry

CHV-1 attachment to permissive MDCK cells involves two mechanisms. First, reversible binding of CHV-1 glycoproteins to cell surface heparan sulfate (HS), is likely mediated by gC, and second, irreversible binding to unidentified cellular receptors via viral glycoprotein(s) other than gC (possibly gB and gD) (Nakamichi *et al.*, 2000). The attachment of CHV-1 to HS is partially reduced by heparin treatment, whereas the virus retains the ability to attach to heparin-treated MDCK cells via the second mechanism. Interestingly, the attachment of heparin-treated MDCK is reduced by treatment with anti-CHV-1 gB and gD antibodies, suggesting that gB and gD may be involved in the HS-independent attachment via the second mechanism (Nakamichi *et al.*, 2000). Moreover, CHV-1 successfully attaches to non-permissive MDBK, RK13, CPK, and HmLu-1 cells, and this attachment is completely inhibited by treatment with heparin, suggesting that CHV-1 attaches to non-permissive cells only through HS, and those cells likely lack the receptors for gB and/or gD that are required for viral penetration (Nakamichi *et al.*, 2000).

The mechanism by which CHV-1 enters its host cells was not known at the beginning of this thesis. For the prototypic alphaherpesvirus, HSV-1, the entry process is cell-type dependent and occurs either through a direct fusion of the viral envelope with the plasma membrane or by endocytosis followed by fusion with the endosomal membrane in pH-dependent or independent way (reviewed in (Campadelli-Fiume *et al.*, 2012; Connolly *et al.*, 2011; Krummenacher *et al.*, 2013; Nicola, 2016)). The entry of HSV-1 in Vero cells and human neurons involves direct fusion with the plasma membrane (Lycke *et al.*, 1988; Wittels & Spear, 1991) whereas HSV-1 enters epithelial cells, HeLa, CHO cells expressing gD receptors by endocytosis (Devadas *et al.*, 2014; Nicola *et al.*, 2005; Nicola *et al.*, 2003). Moreover, the entry of HSV-1 in human keratinocytes involves both direct fusion and endocytosis (Rahn *et al.*, 2011). Viral entry by direct fusion with the plasma membrane or endocytosis is discussed in detail in section 2 of this introduction.

Capsid transport towards the nucleus

Following fusion of the viral envelope with cellular membranes (either the endosomal or the plasma membrane), the viral capsid is released into the cytoplasm. Herpesviruses replicate in the nucleus, this means that the viral capsid has to travel through the crowded environment of the cytoplasm to reach its final destination at the nuclear pores to release its DNA content into the nucleus. The molecular crowding in the cytoplasm due to the presence of cellular organelles hinders the passive diffusion of viral capsids. It is estimated that the HSV-1 capsid would travel by diffusion through the cytoplasm at a rate of 5 µm / hour, which means it would take about 231 years to move 1 cm, moreover, the retrograde transport of HSV-1 capsids over the several centimeters long neurons proceeds at a speed of 3–5 mm/hour, which is inconsistent with the fact

that HSV-1 viral gene expression is detected within 2 hours of viral entry (Lycke *et al.*, 1984; Sodeik, 2000). Thus, the transport of viral capsid within the cytoplasm is an active process rather than by passive diffusion.

The host cell cytoskeleton, including actin microfilaments and microtubules, plays an important role in the transport of HSV-1 capsid towards the nucleus, reviewed by (Wu *et al.*, 2019). The microtubule transport of HSV-1 is mediated by the motor proteins kinesin, and dynein (Döhner *et al.*, 2005; Lyman & Enquist, 2009). Moreover, HSV-1 exploits a plus end-tracking protein (+TIP) complex to initiate a retrograde transport towards the nucleus. This complex consists of end-binding protein1 (EB1), the cytoplasmic linker protein 170 (CLIP-170), and the dynactin-1 (DCTN1), and the depletion of these proteins completely inhibits postentry trafficking of HSV-1 (Jovasevic *et al.*, 2015). Several HSV-1 viral proteins including pUS3, pUL36, pUL37, ICP0, pUL14, pUL16, and pUL21 have been shown to recruit the motor proteins dynein, dynactin, kinesin-1, and kinesin-2 (Banerjee *et al.*, 2020). In addition, HSV-1 outer capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 to mediate minus-end-directed retrograde transport of viral capsids along the microtubules (Douglas *et al.*, 2004). Also, the HSV nuclear egress complex (NEC) protein UL34 interacts with the dynein intermediate chain (Reynolds *et al.*, 2002).

The alphaherpesviruses' tegument proteins have also been shown to play an important role in capsid transport towards the nuclear pores. For instance, HSV-1 UL14 tegument protein is required for nuclear targeting of viral capsids, and UL14-deficient HSV-1 mutants show a significant reduction in capsid transport to the nucleus (Yamauchi *et al.*, 2008). Also, the tegument protein UL36p (VP1/2) of the varicellovirus PRV, interacts through its N-terminal proline-rich sequence with the dynein/dynactin microtubule motor complex to promote microtubule-directed retrograde transport of the viral capsid in neuronal cells (Zaichick *et al.*, 2013). The binding of VP1/2 to the capsid protein pUL25 is required for the activation of the VP1/2 and capsid transport (Zaichick *et al.*, 2013). Moreover, the interaction of the PRV inner tegument protein UL36p with the C-terminal region of the inner tegument protein UL37p is required for retrograde trafficking of PRV capsids to the nucleus. pUL37-deleted PRV mutants show a delay in capsid translocation to the nucleus (Koenigsberg & Heldwein, 2018; Krautwald *et al.*, 2009). The HSV-1 Infected Cell Protein 0 (ICP0), known for playing a role in subverting anti-viral type I interferon (IFN) mediated immune responses (Shahnazaryan *et al.*, 2020), is another tegument protein implicated in viral capsid transport. Mutations in the N-terminal RING finger domain of ICP0 or deletion of ICP0 result in a reduction in nuclear targeting of HSV-1 capsids (Delboy & Nicola, 2011). Moreover, the

PRV tegument protein early protein EP0, which is a homolog of HSV-1 ICP0, has been demonstrated to be implicated in nuclear transport of PRV in a manner dependant on the cellular proteins Ran, importin α1, α3, α7, β1, and transportin-1 (Cai *et al.*, 2019).

Disruption of microtubule polymerization by nocodazole treatment has been shown to affect dynamic microtubules, but have little impact on the stable microtubules (Pietrantoni *et al.*, 2020). HIV has been found to induce the formation of acetylated and detyrosinated stable microtubules through the interaction of its matrix protein with end-binding protein, EB1 and Kif4, which recruits proteins that stabilize microtubules (Sabo *et al.*, 2013). The infection by HIV of nocodazole-treated cells was not completely abolished, suggesting a deferential role for the dynamic and stable microtubules in viral infection (Sabo *et al.*, 2013; Walsh & Naghavi, 2019).

To the best of our knowledge, neither the specific viral protein nor the cellular cytoskeleton components implicated in CHV-1 transport towards the nucleus have been determined.

1.4 Latency and reactivation

Like all alphaherpesviruses, CHV-1 is characterized by a biphasic life cycle, which includes a primary infection at the mucosal sites of the upper respiratory or genital tracts (Evermann *et al.*, 2011; Li *et al.*, 2016), that is likely followed by infection of hematopoietic cells that cause viremia and spread of the virus to the neurons of the trigeminal or lumbosacral ganglia where it establishes a latency (Evermann *et al.*, 2011). As for all herpesviruses, the latency phase is characterized by maintenance of the viral genome as an episome in the nucleus of the latently infected neurons, and silencing of lytic viral gene expression (Bloom, 2016; Kosz-Vnenchak *et al.*, 1990; Mellerick & Fraser, 1987). Periodically, as a result of stress or immune suppression, the virus reactivates from latency and travels down the axons to reach the epithelia where the virus may continue to replicate.

There have been few studies on the sites of latency for CHV-1 (Miyoshi *et al.*, 1999). To determine the sites and cell types for the latency of CHV-1, Miyoshi *et al* experimentally inoculated adult dogs with the virus by intranasal, intravaginal, or intravenous routes. Regardless of the inoculation site, the trigeminal ganglion was identified as the main latency site where the viral DNA was detected at 2-4 months post-inoculation. Latency in trigeminal ganglia of animals has also identified as the primary site for the latency of several other veterinary varicelloviruses including FHV-1 (Ohmura *et al.*, 1993), EHV-1 (Baxi *et al.*, 1995), BHV-1 (Rock *et al.*, 1986), and PRV (Gutekunst, 1979). In addition, the retropharyngeal lymph nodes were also identified as a primary latency site where CHV-1 was detected in 7 of 8 tested dogs. Moreover, CHV-1 latency

was also detected in the lumbosacral ganglia of the dogs inoculated with the virus intranasally or both intranasally and intravenously (Miyoshi *et al.*, 1999). In a study screening for the presence of CHV-1 DNA in twelve different sites that were known to be associated with latency of other herpesviruses, the viral DNA was detected mostly in the lumbosacral ganglia, tonsil, parotid salivary gland, and the liver of adult dogs with no recorded history of CHV-1 infection (Burr *et al.*, 1996).

Periodically, during times of stress, pregnancy, or administration of immunosuppressant drugs, the virus reactivates from the latency and sheds to the primary sites of infection (Greene, 2012). High prednisolone doses (600mg), have been shown to trigger the reactivation of CHV-1 in latently infected dams with a history of reproductive disorders caused by CHV-1 (Okuda *et al.*, 1993). Although infectious CHV-1 has been recovered from the nasal, oral, ocular, and vaginal mucosa after one to three weeks of prednisolone administration, the dams did not show any clinical symptoms of infection (Okuda *et al.*, 1993). The shedding of the virus following the reactivation is assumed to last for only a few days compared to the primary infection which is characterized by longer duration and higher viral titers (Okuda *et al.*, 1993; Ronsse *et al.*, 2005). Systemic administration of prednisolone has been shown to trigger reactivation and ocular viral shedding in latently infected dogs with a primary ocular CHV-1 infection (Ledbetter *et al.*, 2012).

In a prospective study, Ronsse *et al.* monitored CHV-1 reactivation, antibody patterns, and detection of the virus in the nasal and vaginal secretions during one reproductive cycle in 27 naturally infected breeding dams. About 60% of the seropositive dogs remained seropositive, while 40% became seronegative on one or two occasions, and all initially seronegative dogs seroconverted at one stage of the investigated reproductive cycle, suggesting that the virus infect the whole dog population in most of the breeding colonies (Ronsse *et al.*, 2005). There was a non-significant fluctuation in the mean antibody titers between the reproductive cycle stages, with a general low titer in the di-oestrus period (Ronsse *et al.*, 2005). Similarly, a low level of antibody titer against HSV-2 was also observed in the late stage of women's pregnancy (Eskild *et al.*, 2000).

1.5 CHV-1 pathogenesis

CHV-1 infection severity and outcome depend on the affected dog's age and immune status **(Figure 1.4)**. Different aspects of CHV-1 pathogenesis in adult dogs and puppies are summarized in the following sections.

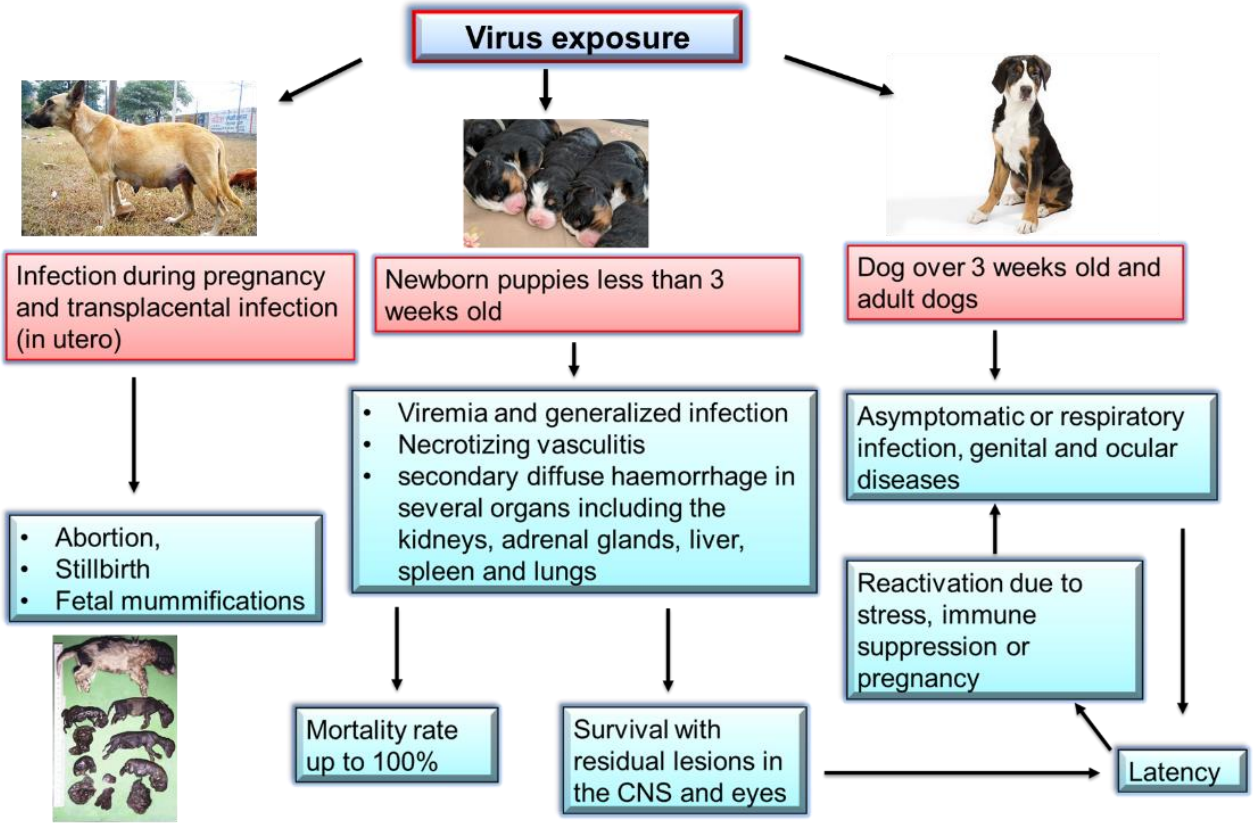

Figure 1.4 CHV-1 pathogenesis in pregnant dams, two-week-old puppies, and adult dogs

Pathogenesis due to CHV-1 infection. Dams' infection during pregnancy may result in abortion or stillbirth. Infection in puppies older than two weeks and adult dogs is usually asymptomatic or mild while the infection in puppies younger than two weeks may cause viremia and hemorrhage in many body organs in addition to mortalities. The figure is modified from (Greene, 2012; Hashimoto *et al.*, 1983; Ronsse *et al.*, 2003).

1.6 Infection during pregnancy

The transplacental transmission of CHV-1 to the fetus resulting in fetal mummification and death has been studied (Hashimoto *et al.*, 1983; Hashimoto *et al.*, 1982). Intravenous inoculation of CHV-1 in two pregnant dams at day 30 of gestation, followed by cesarean sections resulted in six mummified and two dead fetuses, and three unaffected live-born pups. Interestingly, the three living pups did not show clinical symptoms up to two weeks post-delivery, and no virus was isolated at postmortem. The inoculation of the virus in another dam on day 30 of gestation resulted in spontaneous abortion two weeks after the inoculation. Premature birth was reported for two dams inoculated with the virus at day 40 of inoculation, and the premature pups showed CHV-1 lesions in various organs including the liver, spleen, lung, and kidneys from which the virus was successfully reisolated (Hashimoto *et al.*, 1983).

In another study, seven pregnant dogs were experimentally inoculated intravenously with CHV-1 from 47th to 53rd day of gestation, which resulted in fetal infection for 28 of 33 pups. The spontaneous delivery at full term of four dams resulted in eleven pups that died post one week of delivery with CHV-1 lesions. While the delivery by cesarean section revealed two stillborn pups and thirteen live-born pups that died within nine days of birth (Matlin *et al.*, 1982). The virus was detected in the blood vessels of the placental tissues that showed a variable degree of necrotizing lesions. In both studies, the authors did not have an explanation for the birth of healthy uninfected pups (that did not show clinical symptoms of infection and from which no virus was isolated at postmortem) that were present in the same litter with severely infected littermates.

Preventive measures including vaccination of reproducing dams should be taken to protect it from CHV-1 infection that results in abortion and pups' death.

1.7 Infection in newborn puppies

Neonatal pups may become infected in utero via transplacental transmission, during delivery through exposure to the virus in the birth canal, or by direct contact with infected oronasal secretions from the dam or littermates (Carmichael *et al.*, 1965; Decaro *et al.*, 2008; Stewart *et al.*, 1965). The infection in pups older than two to three weeks at the time of infection is usually asymptomatic, although cases of CNS signs, blindness, and deafness have been reported (Appel *et al.*, 1969; Carmichael, 1970). The incubation period lasts between 6 to 10 days and the infection is generally fatal in puppies lacking maternally derived immunity. The mortality rate is high especially in pups younger than two to three weeks at the time of infection and mortality may reach up to 100% of the infected litters (Decaro *et al.*, 2008). The virus replicates in nasal epithelium and pharyngeal tonsils during the first 24 hours of infection, then spreads to the bloodstream through the infection of macrophages. The virus can replicate in the vascular endothelium of the small blood vessels causing necrotizing vasculitis with secondary diffuse hemorrhage in several organs including the kidneys, lung, liver, and CNS within 3 to 4 days post-inoculation (Decaro *et al.*, 2008; Greene, 2012; Poulet *et al.*, 2001; Wright & Cornwell, 1968).

Infected puppies appear pale and suffer from anorexia, loss of body weight, persistent crying, depression, vocalization, abdominal pain, loss of interest in nursing, dyspnea, serous or hemorrhagic nasal discharge, petechial hemorrhage on the mucous membranes, diarrhea, and passage of soft yellow-green feces (Carmichael *et al.*, 1965; Decaro *et al.*, 2008; Greene, 2012; Poulet *et al.*, 2001). CHV-1 viremia in naturally and experimentally infected puppies (via the nasal-oral route) resulted in CNS lesions including non-suppurative meningoencephalomyelitis

characterized by focal and segmental destruction of gray and white matter possibly through viral spread from the trigeminal ganglion, in addition to neuronal degeneration and neutrophil infiltration of the retina (Percy *et al.*, 1968). Pups that survive the generalized CHV-1 infection may suffer from persistent neurologic symptoms including ataxia, blindness, and cerebellar vestibular deficits in addition to eye, lung, and kidney lesions (Greene, 2012; Percy *et al.*, 1971; Percy *et al.*, 1968).

Among the factors that increase puppies' vulnerability to CHV-1 infection is poor development of the immune system and possibly a lack of core temperature control. Newborn pups do not develop temperature regulation until 2 to 3 weeks of age, and the rectal temperature of pups is normally 1^{o}C to 1.5 ^{o}C lower than that of adult dogs (38.4 ^{o}C to 39.5 ^{o}C), which has been reported to be an optimal temperature for the replication of CHV-1 (Carmichael & Barnes, 1969; Greene, 2012). In an experiment aiming to study the impact of puppies' body temperature on CHV-1 infection, four to eight-week-old pups with natural resistance to CHV-1 infection, were shown to develop systemic CHV-1 infection when their body temperature was artificially reduced after intraperitoneal inoculation, while their inoculated littermates that were kept at an elevated environmental temperature showed less severe infection (Carmichael & Barnes, 1969; Greene, 2012). Moreover, the fact that newborn pups are incapable of developing febrile and inflammatory responses before 2-3 weeks of age may also contribute to the increased susceptibility of newborn puppies to severe CHV-1 infection (Carmichael, 1970; Carmichael & Barnes, 1969; Day, 2007). Immunity acquired from seropositive mothers is an important factor in the survival of the infected pups. The puppies suckling seropositive mothers, showed asymptomatic infection and limited virus was recovered from the oropharyngeal region, while puppies nursed by seronegative mothers or from those with a low level of immunoglobulin developed fatal CHV-1 infection (Carmichael, 1970; Greene, 2012). The presence of protective antibodies in the serum of previously naturally infected mothers that gave birth to diseased puppies can protect future pregnancies, which usually results in healthy pups (Evermann *et al.*, 2011).

1.8 Infection in adult dogs

Respiratory tract infection

CHV-1 is one of multiple viruses thought to cause canine infectious respiratory disease complex (CIRD) also known as canine infectious tracheobronchitis (ITB) or “Kennel Cough”, although its role in CIRD still a subject of an ongoing debate (Binn *et al.*, 1979; Decaro *et al.*, 2008; Evermann *et al.*, 2011). CIRD is a contagious, acute-onset infection of the upper respiratory tract of dogs. The etiology of CIRD is complex and involves several viral and bacterial pathogens

acting alone or synergistically (Erles *et al.*, 2004). Most of the affected dogs suffer from a dry cough that lasts for a limited period but can develop into fatal bronchopneumonia in severe cases. In a two-year longitudinal study of viruses associated with CIRD in dogs from a rehoming center that received vaccination against distemper, canine adenovirus type 2 (CAV-2), and canine parainfluenza virus (CPIV), CHV-1 was detected in 12.8% of the tracheal samples and 9.6% of the lung samples (Erles *et al.*, 2004). Experimental inoculation of CHV-1 in puppies older than three weeks has been shown to cause mild clinical symptoms of rhinitis, pharyngitis, and tracheobronchitis (Appel *et al.*, 1969; Karpas *et al.*, 1968).

Ocular infection

CHV-1 has been detected in conjunctival and corneal samples collected from adult dogs (Ledbetter *et al.*, 2009a). When CHV-1 has been experimentally inoculated topically to the eyes of adult dogs, it induces bilateral conjunctivitis followed by latency eight months later. Following prednisolone-induced reactivation, dogs exhibited ocular viral shedding, conjunctival and corneal leukocyte infiltration, and bilateral ocular disease including conjunctivitis and keratitis (Ledbetter *et al.*, 2009b).

Genital infection

CHV-1 has been reported to replicate in the cooler regions of the dog's genital mucosa causing lymphofollicular lesions and vaginal hyperemia and similar lesions over the base of the male's penis and prepuce (Decaro *et al.*, 2008). Genital lesions at the time of birth represent a risk factor for the transmission of the virus to the puppies.

1.9 Treatment options

To date, there is no available treatment that can eradicate the virus from infected animals. Moreover, there is no available vaccine that can provide lifelong protection from CHV-1 infection.

As a preventative measure, animals should be raised in larger kennels with improved hygiene as CHV-1 antibody titers are correlated to kennels size and hygiene (Ronsse *et al.*, 2004). In addition, pregnant dams should be isolated in separate kennels to reduce the risk of viral transmission from other dogs. Newborn puppies should be kept at an elevated environmental temperature which is reported to reduce viral replication, morbidity, and mortality (Carmichael, 1970).

In adult dogs, treatment to alleviate symptoms may be required for respiratory, ocular, and genital infection otherwise, no treatment is necessary as the infection is usually self-limiting

(Evermann *et al.*, 2011). For puppies with symptoms of systemic CHV-1 infection, treatments that have been tested are rarely efficacious due to the rapid and fatal nature of the disease. The treatment of systemic infection with the antiviral 5-iodo-2-deoxyuridine has been unsuccessful. However, in a litter with a confirmed case of death of two 15-day-old pups due to CHV-1 infection, vidarabine was reported to protect 5 pups following identification of the causative agent of death. All survived pups had high titers of neutralizing antibodies two months later, suggesting that they had been infected (Greene, 2012).

Administration of 1-2 ml of intraperitoneal immune sera obtained from seropositive dogs has been demonstrated to reduce mortality in pups, although it is more effective if administered before infection. If the pups have already developed neurological symptoms, these symptoms will most likely persist (Greene, 2012). Lactoferrin, a multifunctional protein that can bind to viral particles and inhibits its entry into host cells (Kell *et al.*, 2020), has been shown to reduce CHV-1 infection in MDCK cells in vitro, but its effectiveness *in-vivo* has not been demonstrated (Tanaka *et al.*, 2003).

Nucleoside analogs including trifluridine (Spertus *et al.*, 2016), idoxuridine (Ledbetter *et al.*, 2006), and cidofovir (Gervais *et al.*, 2012) are effective in the treatment of ocular CHV-1 infection. However, resistance to idoxuridine has been reported in strains of CHV-1, that have mutations in the viral thymidine kinase gene (Yamada *et al.*, 2005). Systemic acyclovir administered orally as a 10-mg total dose every 6 hours can be used but residual CNS lesions may still occur (Greene, 2012). Limited success in treating ocular CHV-1 infection has been reported using topical administration of nucleoside analogs like idoxuridine and trifluridine (Ledbetter *et al.*, 2006). Both drugs are administered 6 to 8 times daily for the first two days of therapy followed by 4 times daily until the resolution of the symptoms and are well tolerated by dogs. Cidofovir ophthalmic solution twice-daily administration is an alternative effective therapy in CHV-1 ulcerative keratitis, however, it is reported to have a higher incidence of side effects (Gervais *et al.*, 2012). The administration of 0.15% ganciclovir ophthalmic gel to dogs with experimentally induced ocular CHV-1 infection has been shown to reduce both ocular tissue inflammation and duration of viral shedding (Ledbetter *et al.*, 2018). None of these treatments will prevent latent CHV-1 infection, which has no cure.

Eurican Herpes 205 (Merial, Lyon, France), is a CHV-gB subunit vaccine that is exclusively licensed in Europe. It is administrated subcutaneously to pregnant dogs in a two doses regimen, one dose 10 days after mating day followed by a second dose 6 weeks later. The vaccine has been reported to induce a strong humoral immune response in pregnant mothers that significantly

reduced the mortality in newborn pups (Poulet *et al.*, 2001). Pups from CHV-1 seronegative mothers that received the vaccine were protected from mortality and severe respiratory lesions upon oronasal experimental challenge with CHV-1 on the third day of birth (Poulet *et al.*, 2001). However, vaccination of latently infected dogs with Eurican Herpes 205 failed to prevent recurrent ocular CHV-1 infection and viral shedding induced by corticosteroid administration (Ledbetter *et al.*, 2016). To date, no approved vaccine provides lifelong protection from CHV-1 infection.

2 MECHANISMS OF VIRAL ENTRY

The main mission of a virus particle is to transport the viral genome in a replication-competent form from infected host cells to uninfected cells and to deliver it into the sites of replication, either in the nucleus (for most DNA viruses) or in the cytoplasm (for most RNA viruses) (Yamauchi & Helenius, 2013). Viral entry into host cells is an essential first step for the virus to establish a successful infection. For their entry, animal viruses exploit host cellular factors to transmit their genetic material to the sites of replication. The general program for the entry process of enveloped animal viruses involves multiple steps. It begins by viral attachment (adsorption) to the plasma membrane followed by binding to the entry receptors. Virus binding to its receptor(s) initiates a complex network of intracellular signaling that triggers virions' internalization either via fusion of viral envelope with the plasma membrane or -most often- by endocytosis. In this section, different steps and pathways involved in viral entry will be discussed.

2.1 Viral attachment to the cell surface

Viruses mainly infect the cells to which they can bind. However, in some instances, an infectious viral entry independent of direct binding of viral particles to the cell surface can be achieved. Examples include antibody-dependent enhancement (ADE) of virus infection, a phenomenon in which virus-specific antibodies promote viral entry into the target cells that express FcR on their surface such as monocytes, macrophages, dendritic cells, and certain granulocytes. ADE of viral infection has been reported for several viruses including Dengue virus (DENV), Respiratory syncytial virus (RSV), Human Immunodeficiency virus (HIV),and Coronaviruses (CoV) and represent a major challenge to vaccine development (Xu *et al.*, 2021). Another example is the transmission of viral proteins and nucleic acids via exosomes as has been reported for HCV (Bukong *et al.*, 2014) and HSV (Kalamvoki *et al.*, 2014). The classic receptor-mediated entry process starts by viral attachment to cell surface molecules that comprise a diverse spectrum of cell surface proteins, lipids and glycans. To a large extent, the identity and distribution of these cellular molecules determine which cell, tissue, and organisms that virus can infect (Yamauchi & Helenius, 2013).

Glycosaminoglycans (GAGs), mainly heparan sulfate proteoglycans (HSPG) act as binding receptors for many viruses including Adeno-associated virus 2 (AAV2) (Summerford & Samulski, 1998), human papillomavirus (Drobni *et al.*, 2003), dengue virus (Chen *et al.*, 1997), Sindbis virus (Byrnes & Griffin, 1998), tick-borne encephalitis virus (Kroschewski *et al.*, 2003), human

parainfluenza virus type 3 (Bose & Banerjee, 2002), HCV (Barth *et al.*, 2003), and HSV-1 (Spear *et al.*, 2000) . Viral attachment to HSPG is reversible and charge-based and involves an electrostatic attraction between the positively charged amino acid residues of viral envelope glycoproteins and the negatively charged heparan sulfate (HS) on the cell surface. HS is one of the most negatively charged biopolymers in nature and is highly expressed on the cellular surface of almost all cell types (Sarrazin *et al.*, 2011).

Cell surface lectins including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, also known as CD209) are other cellular molecules that act as receptors for viruses through binding to high-mannose N-linked glycans of viral glycoproteins (Yamauchi & Helenius, 2013). Viruses including SARS-CoV-2 (Amraie *et al.*, 2020), KSHV (van der Meulen *et al.*, 2021), HIV-1 (Geijtenbeek *et al.*, 2000; Pöhlmann *et al.*, 2001), Sindbis (Klimstra *et al.*, 2003), hepatitis C (Lozach *et al.*, 2003), phleboviruses (Lozach *et al.*, 2011), dengue (Tassaneetrithep *et al.*, 2003), Marburg, and Ebola (González-Hernández *et al.*, 2019; Simmons *et al.*, 2003) are reported to exploit DC-SIGN for their entry.

Viral binding to attachment factors functions to bind and concentrate the virions at the host cell surface but is not sufficient to mediate viral entry (Azab & Osterrieder, 2017). In contrast, attachment factors that act as true viral receptors can mediate viral binding, endocytosis, induction of conformational changes in the virus particle, and trigger signaling pathways that promote viral entry and infection (Yamauchi & Helenius, 2013). In some cases, a cellular factor may mediate both viral attachment and entry, for example, DC-SIGN which act as a receptor for KSHV in B cells, macrophages and dendritic cells (Hensler *et al.*, 2014; Rappocciolo *et al.*, 2008; Rappocciolo *et al.*, 2006; van der Meulen *et al.*, 2021).

For the prototypic herpesvirus HSV-1, initial attachment to the cell surface is mediated by binding of viral envelope glycoprotein(s) gB and/or gC with HSPG. Although gC has a prominent role in HSV-1 adsorption to the cell surface, gC-deficient HSV-1 virions are still infectious but show less efficient binding and infectivity compared to the wild type virus (Herold *et al.*, 1991; Spear, 2004). In the absence of gC, gB is suggested to be sufficient to mediate the binding of gC-negative virions to cells (Herold *et al.*, 1994).

2.2 Viral entry by direct fusion at the plasma membrane

While most enveloped animal viruses fuse with intracellular membranes after their entry via endocytosis (Barrow *et al.*, 2013), some fuse directly with the plasma membrane. Based on the host cell type involved, herpesviruses can trigger their entry via endocytosis or direct fusion

with the plasma membrane (Frampton *et al.*, 2007; Miller & Hutt-Fletcher, 1992; Nicola *et al.*, 2005; Nicola *et al.*, 2003; Raghu *et al.*, 2009; Van de Walle *et al.*, 2008). For instance, HSV-1 uses different entry pathways in a cell-dependent manner. The entry of HSV-1 into Vero cells and human neurons is mediated by fusion at the plasma membrane (Lycke *et al.*, 1988; Wittels & Spear, 1991), whereas entry by endocytosis is reported in epithelial cells, HeLa, and CHO cells expressing gD receptor (Nicola, 2016; Nicola *et al.*, 2005; Nicola *et al.*, 2003).

Four HSV-1 viral envelope glycoproteins, gB, gD, and the heterodimer gH/gL are required for HSV-1 fusion with the plasma membrane of Vero cells. The deletion or mutation of the genes encoding these glycoproteins block the entry of HSV-1 into Vero cells (Cai *et al.*, 1987; Forrester *et al.*, 1992; Ligas & Johnson, 1988; Roop *et al.*, 1993). Moreover, infectious entry of HSV-1 into Vero cells is blocked by neutralizing antibodies directed against gB, gD or gH (Cohen *et al.*, 1972; Fuller & Spear, 1987; Gompels & Minson, 1986; Highlander *et al.*, 1988; Highlander *et al.*, 1987; Navarro *et al.*, 1992; Nicola *et al.*, 1998; Peng *et al.*, 1998). Interestingly, deletion of HSV-1 gB, gD or gH/gL halts infectious entry by endocytosis of HSV-1 into CHO-nectin-1 cells, indicating that the four glycoproteins are also involved in the fusion of HSV-1 with endosomal membranes (Nicola & Straus, 2004). The HSV-1 viral envelope glycoproteins gC (Herold *et al.*, 1991; Spear, 2004), gE (Dingwell *et al.*, 1994; Komala Sari *et al.*, 2013), gI (Balan *et al.*, 1994; Komala Sari *et al.*, 2013), gM (Baines & Roizman, 1991; Komala Sari *et al.*, 2013; Striebinger *et al.*, 2016), gN (Striebinger *et al.*, 2016), UL45 (Dollery *et al.*, 2010; Komala Sari *et al.*, 2013), and Us9 (Komala Sari *et al.*, 2013) have been shown to be dispensable for HSV-1 fusion because null mutant viruses devoid of those proteins still enter cells via endocytosis or direct fusion.

Attachment of HSV-1 to HSPG on the cell surface initiates a cascade of interactions that trigger fusion and may be critical in vivo (Weed & Nicola, 2017), however, HSV-1 still infect cells lacking HSPG in cell culture, suggesting that HSPG binding is not an essential requirement for virus-cell fusion (Shukla & Spear, 2001). Following attachment, (i) gD binds to one of its receptors **(Table 1.1)**, which triggers conformational changes in gD (Krummenacher et al. 2005; Lazear et al. 2008), (ii) then activated gD complexes with and activates gH/gL to (iii) bind to and activate the fusogen gB **(Figure 1.5)** (Atanasiu *et al.*, 2010; Atanasiu *et al.*, 2016; Atanasiu *et al.*, 2007; Avitabile *et al.*, 2007; Chowdary *et al.*, 2010; Heldwein *et al.*, 2006; Zeev-Ben-Mordehai *et al.*, 2016). gB, is a class III fusion protein, and thus shares characteristics of both class I and II fusion proteins, moreover, it has high structural homology to the class III fusion protein, glycoprotein G of vesicular stomatitis virus (Maurer *et al.*, 2008).

Glycoprotein	Function	Cellular receptors
gC	Attachment	• Heparan sulfate • DC-SIGN
gD	Triggers fusion	• HVEM • Nectin-1 • Nectin-2 • 3-OS HS • ZF-3-OS HS
gH/gL	Regulates fusion Activators of gB	• αVβ3 integrin • αVβ6 integrin • αVβ8 integrin
gB	Attachment Fusogen	• Heparan sulfate • DC-SIGN • PILRα • MAG • NMMHC-IIA • NMMHC-IIB

Table 1.1 HSV-1 glycoproteins required for viral entry and their corresponding cellular receptors.

The table is modified from (Azab & Osterrieder, 2017).

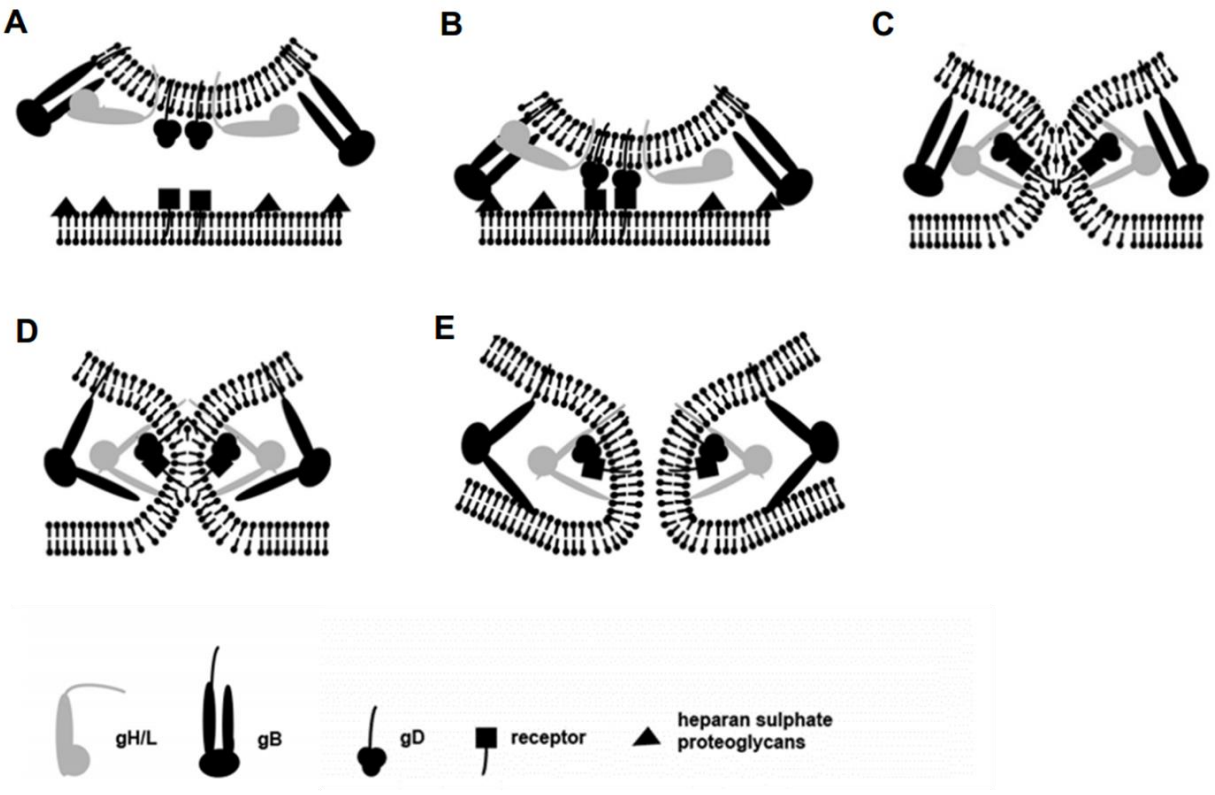

Figure 1.5 Model of fusion mechanism between the HSV-1 envelope and the plasma membrane

(A) HSV-1 gD must have an access to bind to its corresponding receptor on the cell surface without steric hindrance. **(B)** Non-essential binding of gB to HSPG can coincide or precede gD binding to its receptor. **(C)**The binding of gD to its receptors triggers conformational changes in gD to interacts with and activates gH/gL to also change conformation and binds to its receptor on the cell surface. This brings the two membranes close to each other and triggers lipid mixing **(D)** Conformational changes in gH/gL recruit gB to the gD/gH/gL complex and binds to its receptor. **(E)** The fusogenic gB stabilizes the fusion pore formation. The figure is modified from (Maurer *et al.*, 2008).

2.3 Viral entry by endocytosis

Endocytosis is a term that describes distinct cellular processes that mediate the uptake and internalization of cargos, fluids, nutrients, membranes, ligands, and particles (including pathogens like viruses and bacteria) into the cell within endocytic vesicles formed from the plasma membrane (Cossart & Helenius, 2014; Doherty & McMahon, 2009; Mercer *et al.*, 2010a). Endocytosis has an important role in controlling cell signaling through the regulation of the plasma membrane level of cellular receptors like growth factor receptors (Doherty & McMahon, 2009). The internalized receptors are either recycled back to the plasma membrane to maintain signaling or delivered to the lysosome for degradation to induce long-term desensitization of the cell

(Barbieri *et al.*, 2016; Grant & Donaldson, 2009; Watanabe & Boucrot, 2017). It also controls the recycling of synaptic vesicles to the plasma membrane following synaptic transmission to maintain the level of synaptic vesicles pool (Saheki & De Camilli, 2012; Soykan *et al.*, 2016; Watanabe & Boucrot, 2017). The endocytic machinery may be manipulated to mediate the delivery of therapeutics, proteins, and nanocarriers (Jones, 2007; Mercer & Helenius, 2012; Swanson, 2008). Many pathogens including viruses and intracellular bacteria hijack the endocytic machinery for penetration into the cytosol or other sites of replication.

Based on the cargos internalized and the cellular factors that mediate the endocytic process, distinct forms of endocytic pathways have been identified in mammalian cells. Viruses serve as valuable tools for the study and characterization of different endocytic pathways as the internalization of viruses can be traced and quantified. In this section, different common forms of viral entry by endocytosis will be discussed.

2.3.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is one of the best characterized and mechanistically understood endocytic pathways (Mettlen *et al.*, 2018). CME is a key pathway in all eukaryotic cells for the specific uptake of cell surface receptors and their ligands including transferrin, hormones, metabolites, proteins, and some viruses. It plays an important role in regulating the plasma membrane composition, cell signaling, morphology, adhesion, and migration (Haucke & Kozlov, 2018). In addition to the scaffolding protein clathrin, there are more than 50 different proteins known to be involved in CME (Kaksonen & Roux, 2018; Traub, 2011; Weinberg & Drubin, 2012), the main factors include the heterotetrameric adaptor protein AP2 complex, the scaffold epidermal growth factor receptor substrate 15 (EPS15), epsin, actin, myosin, the large GTPase dynamin, and phosphatidylinositol phosphates (PIPs) (Czech, 2000; Ford *et al.*, 2002; Kadlecova *et al.*, 2017; Kaksonen & Roux, 2018; Kelly *et al.*, 2014; Sun *et al.*, 2006; Taylor *et al.*, 2011a).

CME occurs through four highly organized main sequential steps: (A) initiation, which involves clathrin assembly to form clathrin-coated pit (CCP), (B) stabilization of nascent CCPs, (C) induction of membrane curvature and invagination, and (D) vesicle fission catalyzed by the GTPase dynamin II to form the clathrin-coated vesicles (CCV) (Haucke & Kozlov, 2018; Mettlen *et al.*, 2018; Ramanan *et al.*, 2011).

Studies using time-lapse movies have shown that following lateral movement along the membrane, viruses that exploit CME can be internalized within a pre-existing clathrin-coated area of the plasma membrane or induce assembly of clathrin at the site of their binding, in which case,

formation of CCP takes longer (1–6 min). Following formation of CCV, the clathrin coat dissociates within 5–20 sec (Cossart & Helenius, 2014; Ehrlich *et al.*, 2004; Johannsdottir *et al.*, 2009; Rust *et al.*, 2004; van der Schaar *et al.*, 2008). Similar to CME of physiological cargos, entry of viruses via CME requires clathrin and dynamin-2 and is usually blocked by inhibitors of clathrin (chlorpromazine) and dynamin-2 (dynasore and MiTMAB) (Cossart & Helenius, 2014; Sieczkarski & Whittaker, 2002; Wang *et al.*, 1993).

CME is widely known to mediate the entry of relatively small and medium-sized viruses like Semliki Forest virus (SFV) (Helenius *et al.*, 1980), hepatitis C virus (Helle & Dubuisson, 2008; Meertens *et al.*, 2006), dengue virus (Acosta *et al.*, 2009; van der Schaar *et al.*, 2008), and adenovirus 2 (Gastaldelli *et al.*, 2008). Although the clathrin-coated vesicles (CCV) typically have a diameter of 60-120nm (Kaksonen & Roux, 2018), some have been shown to accommodate large viruses such as African swine fever virus (ASVF) and vesicular stomatitis virus (VSV), both with average sizes up to 200 nm (Cureton *et al.*, 2010; Hernáez *et al.*, 2016). Interestingly, VZV enters into neonatal lung fibroblasts by CME (Hambleton *et al.*, 2007). A list of viruses that exploit CME is presented in **(Table 1.2)**. Different viral entry mechanisms are illustrated in **(Figure 1.6)**.

VSV has been reported to exploit CME for its productive infection (Matlin *et al.*, 1982). Studies using live-cell imaging and single-particle tracking have shown that VSV triggers the recruitment of clathrin, dynamin-2, and clathrin adaptor molecules such as AP2, Hsc70, and auxilin to its binding sites (Cureton *et al.*, 2009; Johannsdottir *et al.*, 2009). Due to the bullet shape and large size of VSV, the CCVs deviates from the classical round shape and are only partially coated by clathrin. Moreover, CME of VSV depends on plasma membrane PI(4,5)P2, AP-2, and components of the actin machinery (Cureton *et al.*, 2009).

Using lentivirus pseudotyped with SARS-CoV-2 spike glycoprotein, Bayati et al, concluded that entry of SARS-CoV-2 involves rapid CME that depends on an angiotensin-converting enzyme 2 (ACE2) receptor. Entry of the pseudotyped virus has been blocked by the knockdown of clathrin heavy chain and inhibitors of dynamin-2. Interestingly, CME of SARS-CoV-2 spike glycoprotein showed no colocalization with transferrin an established cargo for the entry by CME, suggesting that SARS-CoV-2 - spike glycoprotein-ACE2 complex follows a different trafficking itinerary from transferrin, which usually transports rapidly to early endosomes then recycles directly back to the plasma membrane or transported to Rab11-positive recycling endosomes before returning to the cell surface (Bayati *et al.*, 2021).

HSV-1 enters cells through different pathways based on the cell type involved. Recently, CME has been reported as an entry pathway for HSV-1 to enter the human oligodendroglial cell

line. Entry was found to require dynamin and to be independent of caveolin. The virus has been detected in clathrin-coated vesicles (using TEM) and the entry was blocked by chlorpromazine and dynasore but not nystatin (Praena *et al.*, 2020).

Table 1.2 Examples of the main endocytic entry pathways of viruses

Virus	Family	Receptor	Penetration	Cell type	References
Clathrin-mediated endocytosis					
Semliki Forest virus	*Togaviridae* Enveloped ss (+) RNA	Not known	pH < 6.2 Early endosome	BHK-21 (Baby Hamster Kidney fibroblasts)	(Doxsey *et al.*, 1987; Garoff *et al.*, 1994; Helenius *et al.*, 1980)
Vesicular stomatitis virus	*Rhabdoviridae* Enveloped ss (–) RNA	low-density lipoprotein (LDL) receptor	pH < 6.4 Early endosome	MDCK	(Cureton *et al.*, 2009; Johannsdottir *et al.*, 2009; Matlin *et al.*, 1982)
Influenza A virus	*Myxoviridae* Enveloped ss (–) RNA	Sialic acid containing glycoconjugates	pH < 5.4 Early endosome	MDCK, BS-C-1 (African green monkey kidney epithelial cells)	(Matlin *et al.*, 1981; Rust *et al.*, 2004)
Adenovirus 2	*Adenoviridae* Nonenveloped dsDNA	Coxsackievirus and adenovirus receptor (CAR) Integrins αvβ, αvβ5	Endosome	HeLa	(Meier *et al.*, 2002a; Wickham *et al.*, 1993)

Varicella-zoster virus	*Herpesviridae* Enveloped dsDNA	Mannose 6-Phosphate Insulin-degrading enzyme	Low pH Endosome	neonatal lung fibroblasts, MeWo U373MG	(Chen *et al.*, 2004; Hambleton *et al.*, 2007; Li *et al.*, 2006)
Macropinocytosis					
Vaccinia virus Mature particle (MV) Enveloped particle (EV)	*Poxviridae* Enveloped dsDNA	Heparan sulfate proteoglycan	Low pH for MV macropinosome	HeLa BS-C-1	(Mercer & Helenius, 2008; Schmidt *et al.*, 2011; Townsley *et al.*, 2006)
Ebolavirus	*Filoviridae* Enveloped ss (–) RNA	NPC-1, DC-SIGN and other host lectins	Cathepsins B and L. macropinosome	293T Vero	(Chandran *et al.*, 2005; Nanbo *et al.*, 2010b; Saeed *et al.*, 2010)
Kaposi sarcoma virus	*Herpesviridae* Enveloped dsDNA	Heparan sulfate proteoglycan Integrins α3(β1, αVβ3, and αVβ5)	Macropinosome	HMVEC-d	(Veetti *et al.*, 2010)
Human cytomegalovirus	*Herpesviridae* Enveloped dsDNA	α2β1, α6β1, and αVβ3 integrins	pH-independent early endosome	primary human fibroblasts	(Feire *et al.*, 2004; Hetzenecker *et al.*, 2016)
Caveolar/lipid-mediated endocytosis					

Simian virus 40	*Polyomaviridae* Nonenveloped dsDNA	GM1 ganglioside	Endoplasmic reticulum	CV-1 MEF Cav$^{-/-}$	(Anderson *et al.*, 1996; Damm *et al.*, 2005; Engel *et al.*, 2011; Stang *et al.*, 1997)
Polyomavirus (mouse)	*Polyomaviridae* Nonenveloped dsDNA	GD1A and GT1B gangliosides	Endoplasmic reticulum	NIH 3T6 NMuMG	(Richterová *et al.*, 2001; Tsai *et al.*, 2003)
Clathrin-mediated endocytosis and macropinocytosis					
Porcine Parvovirus (PPV)	*Parvoviridae* ss DNA	unknown	Low pH Late endosome	Porcine testis fibroblast	(Boisvert *et al.*, 2010)
African swine fever virus	*Asfarviridae* Enveloped dsDNA	Not known	pH, 5.0 late endosomes and lysosomes	Swine macrophage	(Hernáez *et al.*, 2016; Sánchez *et al.*, 2017)
Other mechanisms					
Human Papillomavirus 16 (HPV16)	*Papillomaviridae* Nonenveloped dsDNA	Heparan sulfate proteoglycans	Late endosome macropinosome	HeLa HaCaT	(Schelhaas *et al.*, 2012)
Polio virus	*Picornavirus* ss (–) RNA	Poliovirus receptor	pH-independent early endosome	HeLa S3	(Brandenburg *et al.*, 2007)

Table 1.2 is modified from (Cossart & Helenius, 2014).

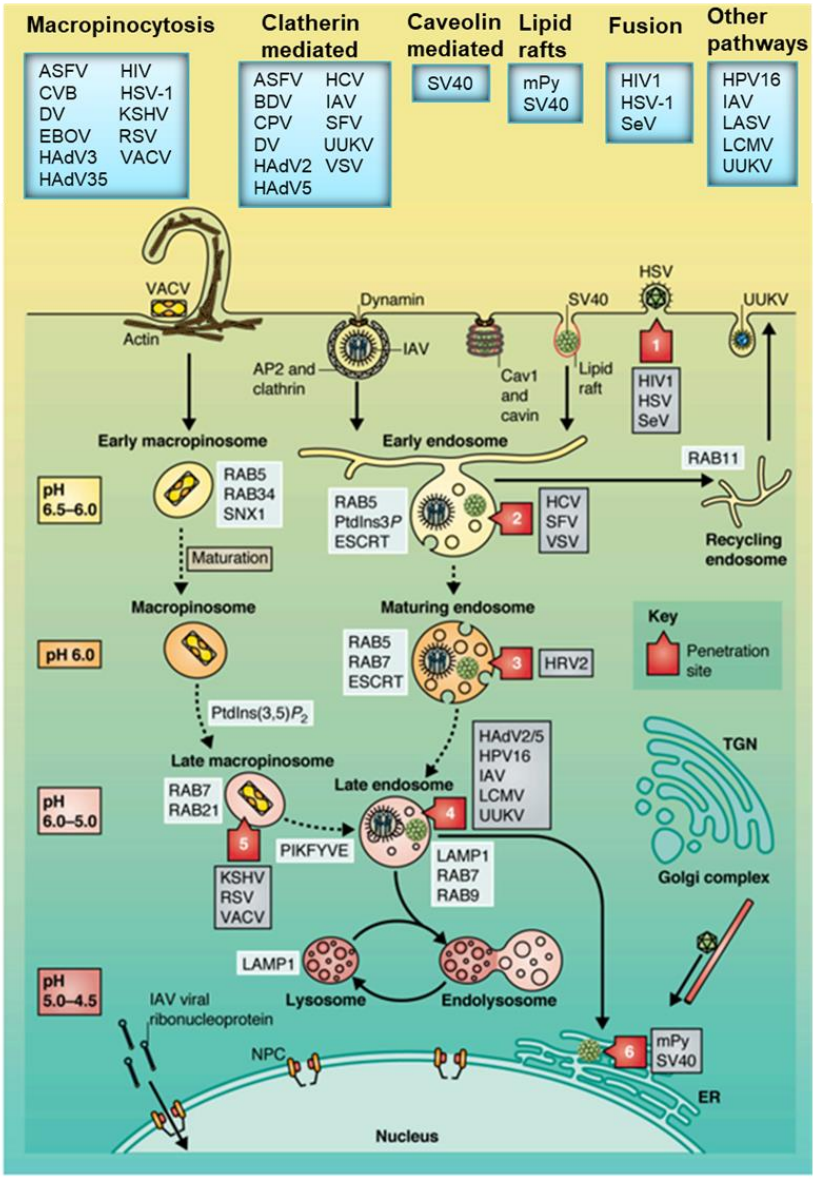

Figure 1.6 Snapshot of different endocytic entry pathways exploited by viruses

Summary of different endocytic mechanisms, cellular factors, and organelles that are required during viral entry.The penetration sites are presented within red arrows numbered 1 to 6. Abbreviations: ASFV = African Swine-fever virus, BDV= Borna Disease virus, CPV = Canine Parvovirus, CVB = Coxsackie virus Group B, DV = Dengue virus, EBOV = Ebola virus, ER = endoplasmic reticulum, ESCRT = endosomal sorting complex required for transport, FMDV = Foot-and-mouth Disease virus, HAdV = Human Adenovirus, HCMV = Human Cytomegalovirus, HCV = Hepatitis C virus, HIV = Human Immunodeficiency virus, HPV = Human Papillomavirus, HSV = Herpes Simplex virus, IAV = Influenza A virus, KSHV = Kaposi's sarcoma-associated herpesvirus, LASV = Lassa virus, LCMV = Lymphocytic Choriomeningitis virus, mPy = mouse Polyomavirus, NPC = nuclear pore complex, PtdIns(3,5)*P*2 = phosphatidylinositol 3,5- bisphosphate, PIKFYVE = phosphatidylinositol kinase FYVE, RSV = Respiratory Syncytial virus, SeV = Sendai virus, SFV = Semliki Forest virus, SNX = sorting nexin, SV40 = Simian virus 40, TGN = trans-Golgi network, UUKV = Uukuniemi virus, and VACV = Vaccinia virus, VSV = Vesicular Stomatitis virus. The figure is modified from (Yamauchi & Helenius, 2013).

2.3.2 Caveolar / lipid raft-mediated endocytosis

Caveolae are a specialized form of lipid rafts that generate flask-shaped membrane invaginations that dissociate to give small caveolin-coated vesicles with a diameter of 50-100 nm. Those vesicles are enriched with cholesterol, phospholipids, and sphingolipids (Parton, 2018; Stan, 2005; Xing *et al.*, 2020). Simian virus 40 (SV40), mouse polyomaviruses, and other members of the family *Polyomaviridae* were among the first viruses recognized to use caveolar and lipid raft-mediated endocytosis for their entry **(Table 1.2 and Figure 1.6)**, reviewed in (Mayberry & Maginnis, 2020).

SV40, a small (70 nm), nonenveloped DNA virus, is one of the best-studied viruses to be internalized via caveolar endocytosis (Cossart & Helenius, 2014). TEM and fluorescent microscopy studies have shown that SV40 is internalized within caveolae coated pits **(Figure 1.7)** (Kartenbeck *et al.*, 1989; Norkin *et al.*, 2002). The addition of SV40 to HeLa cells trigger an extensive tyrosine-phosphorylation-dependent formation of caveolar vesicles, and also increases microtubule-dependent caveolar vesicular transport (Tagawa *et al.*, 2005). siRNA knockdown of caveolin-1 decreases both SV40 endocytosis and infection (Pelkmans *et al.*, 2004; Stergiou *et al.*, 2013). The internalization of the virus in caveolae triggers the breakdown of actin stress fibers that are recruited to the virus-loaded caveolae as actin patches. In addition, dynamin II is recruited to mediate the scission of SV40-containing vesicles from the plasma membrane. These events were found to depend on cholesterol and activation of tyrosine kinases (Pelkmans *et al.*, 2002). Following its uptake, SV40 passes through a series of classical endocytic organelles including the early endosome, late endosome, and possibly endolysosomes to reach the endoplasmic reticulum where disassembly occurs (Engel *et al.*, 2011; Kartenbeck *et al.*, 1989; Norkin *et al.*, 2002). Surprisingly, SV40 can enter embryonic fibroblasts from a Cav-1 knockout mouse, and this uptake is cholesterol-and tyrosine kinase-dependent but clathrin, dynamin II independent (Damm *et al.*, 2005), suggesting redundant entry pathways.

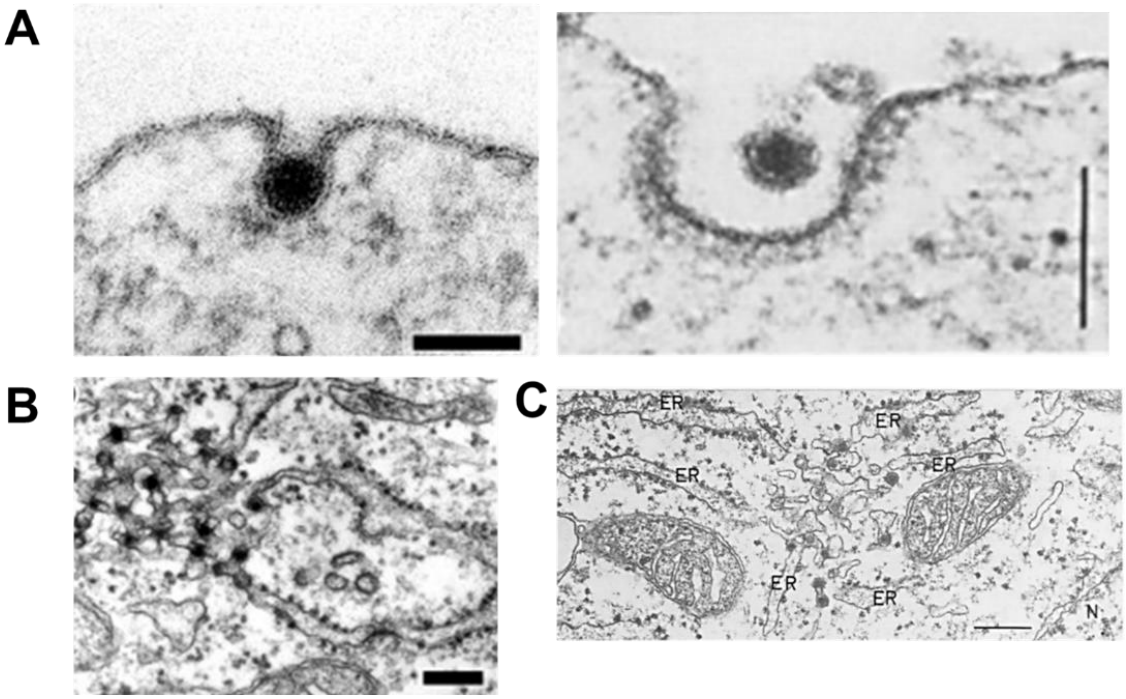

Figure 1.7 SV40 entry via caveolar / lipid raft-mediated endocytosis

Electron micrographs showing SV40 internalization within caveolin coated pits (A). The viruses are then transported via the endosomal system to the cisternae of the endoplasmic reticulum (ER), where virus disassembly takes place (B and C). N= nucleus, scale bar in (A) = 100 nm, (B) = 200 nm and (C) = 250 nm. The figure is adapted from (Kartenbeck *et al.*, 1989; Marsh & Helenius, 2006).

2.3.3 Phagocytosis

Phagocytosis is defined as particle-driven, receptor-mediated uptake of large particles (larger than 0.5 µm in diameter), including microorganisms and apoptotic cells, into tightly fitting vesicles (Uribe-Querol & Rosales, 2020). Although phagocytosis and macropinocytosis, which will be described further, share many characteristics including internalization of large particles into large vesicles, transient activation of actin rearrangement, and dependency on many similar cellular factors and regulators, phagocytosis is strictly particle-driven, and phagosomes are formed following the contour of the ingested particle (Mercer & Helenius, 2009). Moreover, macropinocytosis usually involves uptake of a large volume of extracellular fluids and solutes while phagocytosis does not, due to the tight association of phagosomal membrane with the ingested particles. Another difference is that macropinocytosis involves generalized actin rearrangement that results in a cell-wide plasma membrane ruffling in the form of lamellipodia, filopodia, circular ruffles, or or blebs, while phagocytosis includes localized actin modifications at

the phagocytic cup that forms around the ingested particle (Mercer & Helenius, 2009). Macropinocytosis can be triggered in cells that are not capable of phagocytosis, while phagocytosis is limited to specific cells like dendritic cells, neutrophils, macrophages, and amoeba, some cells, like dendritic cells, are capable of internalizing particles by both macropinocytosis and phagocytosis (Xiang *et al.*, 2006).

Mimivirus or *Acanthamoeba polyphaga mimivirus* (APMV), a giant double-stranded DNA virus with a diameter of 500 nm enters amoebae and macrophages via dynamin-, actin-, and phosphatidylinositol-3-kinase-(PI(3)K) dependent phagocytosis (Ghigo *et al.*, 2008; Suzan-Monti *et al.*, 2007). Marseillevirus is another large (250 nm) DNA virus of the family *Marseilleviridae,* enters Acanthamoeba cells via acidification-independent phagocytosis (Arantes *et al.*, 2016). In addition, HCV enters into macrophages by cholesterol-, actin-, and PI(3)K-dependent, and clathrin and dynamin independent phagocytosis (Liu *et al.*, 2019).

2.3.4 Macropinocytosis

Macropinocytosis is an actin-dependent process used by the cell for nonspecific uptake of extracellular nutrient-rich fluids (pinocytosis is a cell drinking), solutes, membranes, ligands, and membrane-associated particles including viruses and bacteria (Doherty & McMahon, 2009; Mercer & Helenius, 2009; Mercer & Helenius, 2012). In most cells, the process is transiently triggered by activation of the receptor tyrosine kinases, integrins, and phosphatidylserine receptors by ligands such as growth factors, integrin substrates, and phosphatidylserine (PS)-containing cell debris respectively (Haigler *et al.*, 1979; Hoffmann *et al.*, 2001; Meier *et al.*, 2002a; Mercer & Helenius, 2012). Although macropinocytosis is triggered - in most cells - by external stimuli for a short time, dendritic cells have been reported to constitutively macropinocytose a large volumes of external fluids as a part of their immune surveillance function (Norbury, 2006).

The activation of macropinocytosis induces a complex network of intracellular signaling that induces rearrangements of the actin cytoskeleton leading to global plasma membrane ruffling, which may take different forms such as lamellipodia, filopodia, circular ruffles, or blebs (Doherty & McMahon, 2009; Mercer & Helenius, 2009; Mercer & Helenius, 2012). These protrusions fold back to nonspecifically internalize extracellular fluids and membrane-associated particles within heterogeneous, large (0.5 to 10 µm diameter) uncoated, and irregularly shaped vacuoles called macropinosomes (reviewed in (Swanson, 2008)). The fission of macropinosomes from the cell surface is mediated by the action of CtBP1/BARS or dynamin II (Bonazzi *et al.*, 2005; Mercer & Helenius, 2012; Schlunck *et al.*, 2004). Macropinocytosis induction is usually associated

with a transient (30–60 min) increase in fluid phase uptake (5–10-fold over the basal rate) (Mercer & Helenius, 2009).

Recently, several intracellular pathogens including viruses have been shown to exploit macropinocytosis for cell entry. Examples include HSV-1, HCMV (Haspot *et al.*, 2012; Hetzenecker *et al.*, 2016), KSHV (Kumar & Chandran, 2016; Raghu *et al.*, 2009), HIV-1 (Liu *et al.*, 2002; Maréchal *et al.*, 2001; Yasen *et al.*, 2018), Echovirus 1 (Krieger *et al.*, 2013), Vaccinia virus (Mercer & Helenius, 2008; Schmidt *et al.*, 2011), Influenza A virus (de Vries *et al.*, 2011), Ebola virus (Nanbo *et al.*, 2010b; Saeed *et al.*, 2010), Adenovirus 2 (Meier *et al.*, 2002a), Adenovirus 35 (Kälin *et al.*, 2010b), and respiratory syncytial virus (Krzyzaniak *et al.*, 2013) (**Table 1.2 and Figure 1.6**) .

Viral entry by macropinocytosis involves five main steps that rely on different sets of cellular factors (reviewed in (Mercer & Helenius, 2012)) : (1) Binding of viral particles to cell surface non-specific attachment factors like glycosaminoglycans, this attachment concentrates the viral particles to the cell surface and sometimes brings the viral particles in contact with the specific entry receptors. Cell surface receptors that have been reported to activate the macropinocytosis machinery include integrins, phosphatidylserine receptors, and receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptors (PDGF-R), (2) Activation of complex intracellular signaling that involves cellular kinases, phospholipases, myosins, fission/fusion factors, Na^+/H^+ exchangers, and actin, (3) The signaling cascades trigger actin mediated cell-wide plasma membrane ruffling and protrusions, (4) Vacuole closure and formation - the closure of circular ruffles is mediated mainly by dynamin II, while lamellipodial ruffles closure requires CtBP1/Bars (5). The vesicle closure leads to the formation of macropinosomes followed by the trafficking of those vesicles deeper into the cytosol where they undergo acidification, maturation, and fusion with late endosomes or lysosomes (Mercer & Helenius, 2012; Yamauchi & Helenius, 2013).

There is no single specific test or cellular factor that by itself identifies macropinocytosis, however, the dependency of the viral entry process on actin dynamics and Na^+/H^+ exchangers can strongly suggest a role for macropinocytosis. Perturbing the activity of Na^+/H^+exchangers using 5-(N-ethyl-N-isopropyl) amiloride (EIPA), and of actin polymerization using latrunculin A or cytochalasin D, has been shown to inhibit the macropinocytotic uptake of KSH, HCMV, and HSV-1 (Devadas *et al.*, 2014; Hetzenecker *et al.*, 2016; Hilterbrand *et al.*, 2020; Raghu *et al.*, 2009). In addition, several other criteria should be tested to satisfy the definition of virus uptake by macropinocytosis (discussed in (Mercer *et al.*, 2010a)), these include (1) Induction of membrane

ruffling as a result of actin rearrangement. (2) Transient increase in the uptake of extracellular fluids, which could be evidenced by quantifying the uptake of the fluid phase marker fluorescent dextran. (3) The dependency of the infection on the Rho GTPases Rac1 or Cdc42. (4) The requirement of different kinases like p21-activated kinase 1 (Pak1), focal adhesion kinase (FAK), phosphatidylinositol-3-kinase (PI (3)K), and protein kinase C (PKC). (5) Other cellular factors like myosin II, microtubules, and dynamin II may also be required. The requirements of the different cellular factors for viral macropinocytosis vary for a specific cell type and/ or virus **(Table 1.3)**.

	Ebola	**Adeno 35**	**IAV**	**KSHV**	**HCMV**	**HSV-1**	**VV EV**	**VV MV IHD**
Genome	(-) ssRNA	dsDNA	(-) ssRNA	dsDNA	dsDNA	dsDNA	dsDNA	dsDNA
Cells	293T and Vero	HeLa/HK-2	Hela	HMVEC-d	fibroblasts	Hela, nectin-1-CHO, MDCK and Vero	Hela/DC	Hela
Ruffling	Lamellipodia	Lamellipodia	N.D.	Blebbing	CDRs	Filopodia	Blebbing	Filopodia
Fluid-uptake								
Fluid induction	Yes	Yes	Yes	Yes	N.D.	N.D.	Yes	Yes
Fluid colocalization	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cytoskeleton								
Actin	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Myosin II	N.D.	N.D.	Yes	Yes	Yes	N.D.	Yes	Yes
GTPases								
Rac1	Yes	Yes	NO	Yes/NO	N.D.	Yes	Yes	Yes/NO
Cdc42	Yes	N.D.	NO	Yes/NO	N.D.	Yes	NO	Yes
Kinases								
Ser/Thr	Yes	N.D.	Yes	N.D.	N.D.	N.D.	Yes	Yes
Tyr	N.D.	N.D.	Yes	Yes	Yes	NO	Yes/NO	Yes/NO
PKC	Yes	Yes	Yes	Yes	Yes	NO	Yes	Yes
PI3K	Yes	N.D.	Yes	Yes	N.D.	Yes/NO	NO	NO
PAK1	Yes	Yes	Yes	N.D.	Yes	Yes	Yes	Yes
Closure								
CtBP1	Yes	Yes	N.D.	N.D.	N.D.	NO	N.D.	N.D.
Dynamin-II	NO	NO	NO	NO	Yes	NO	NO	NO
Kinases								
Na^+/H^+ exchange	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cholesterol	Yes	N.D.	N.D.	Yes	N.D.	N.D.	Yes	Yes
Acidification	Yes	Yes	Yes	Yes	NO	Yes/NO	Yes	NO
Integrins	N.D.	Yes	N.D.	Yes	Yes	N.D.	N.D.	N.D.
PS dependence	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	NO	Yes

Table 1.3 Cellular factors involved in macropinocytic uptake of viruses

Different cellular factors and criteria used in defining macropinocytic entry of viruses. The cellular factors are listed based on their molecular functions, and their necessity is indicated as required (yes-green), not required (No-red), not determined (N.D.- grey), or required in a system-dependent fashion (yellow, Yes/No). The viruses presented include the Ebola virus (Brindley *et al.*, 2011; Hunt *et al.*, 2011; Nanbo *et al.*, 2010a; Saeed *et al.*, 2010; Shimojima *et al.*, 2006), adenovirus 35 (Ad35) (Kälin *et al.*, 2010a; Kälin *et al.*, 2010b), influenza A virus (IAV) (de Vries *et al.*, 2011; Eierhoff *et al.*, 2010), Kaposi's sarcoma-associated herpesvirus (KSHV) (Chakraborty *et al.*, 2011; Raghu *et al.*, 2009; Veetti *et al.*, 2010), Human cytomegalovirus (HCMV) (Hetzenecker *et al.*, 2016), Herpes simplex virus 1 (HSV-1) (Clement *et al.*, 2006; Devadas *et al.*, 2014; Hoppe *et al.*, 2006; Nicola *et al.*, 2005), Vaccinia virus extracellular virions (VV EVs), Vaccinia virus mature virions of the IHD-J strain (VV MV IHD-J) (Krijnse *et al.*, 2000; Mercer *et al.*, 2010a;

Sandgren *et al.*, 2010; Schmidt *et al.*, 2011). The table is modified from (Mercer & Helenius, 2009; Mercer & Helenius, 2012).

Vaccinia virus (VV) induces its macropinocytic uptake in HeLa cells by mimicking the uptake of apoptotic bodies through exposing phosphatidylserine in the viral envelope (Mercer & Helenius, 2008). Upon addition to the cells, VV moves along filopodial membrane extensions towards the cell body to induce extensive membrane blebbing. Macropinocytosis of VV has been found to depend on the activity of Na^+/H^+ exchangers, PAK-1, actin, myosin II, cholesterol, PKC, Rac1, Cdc42, PI(3)K, and endosomal acidification but is independent of dynamin, microtubules, and integrins. The expression of constitutive active Arf6 mutants blocks VV infection but at post-entry steps. Moreover, the addition of VV to the cells triggers a rapid increase in fluid uptake and the virus colocalizes with the fluid phase uptake marker, dextran (Mercer & Helenius, 2008). Interestingly, different strains of VV have been reported to induce different forms of macropinocytosis in HeLa cells (Mercer *et al.*, 2010a). While mature virions of the WR VV strain induce membrane blebs, IHD-J VV strain has been shown to trigger a rapid formation of filopodial ruffles. Moreover, the two strains have differences in the level of Rho GTPases activation. WR strain induction of membrane blebbing is mediated by activation of Rac1, while IHD-J strain triggers filopodial ruffles through activation of Cdc42. Unlike WR strain, the entry of IHD-J strain is independent of endosomal acidification, genistein-sensitive tyrosine kinase and PI(3)K activities. However, macropinocytosis entry of the two strains requires virion-exposed phosphatidylserine, cellular serine/threonine kinases, EGFR, PKC, and Pak1(Mercer *et al.*, 2010a). These findings indicate that different strains of the same virus may trigger different forms of macropinocytosis in the same cell.

HCMV induces macropinocytosis for its entry into primary fibroblasts. The virus rapidly internalizes within large uncoated vacuoles that containing early endocytic marker proteins, and the capsid penetrates into the cytosol as early as 15 minutes following endocytosis. The macropinocytosis entry pathway of HCMV is blocked by inhibitors of Na^+/H^+ exchangers, p21-activated kinase 1, PKC, myosin II, dynamin, tyrosine kinase, and FAK. Moreover, the entry depends on signaling via integrins and PDGFR but not EGFR. In addition, HCMV has been found to induce circular dorsal ruffles and colocalize with the fluid phase uptake marker, dextran (Hetzenecker *et al.*, 2016).

Ebolavirus (EBOV) entry into Vero cells involves viral glycoprotein-dependent macropinocytosis that requires NHE, actin polymerization, Rac1, PKC, Cdc42, Pak1, and PI(3)K. In addition, EBOV entry has been shown to trigger extensive lamellipodial membrane ruffling and

increased the uptake of the fluid phase marker, dextran with which the virus colocalizes. Following its uptake, the EBOV is transported to Rab7-positive late endosomes/lysosomes where low pH-dependent membrane fusion is triggered (Nanbo *et al.*, 2010b). Moreover, Inhibitors of tyrosine kinase have been reported to block the glycoprotein-dependent entry of both EBOV and Marburg virus in Vero cells. In addition, EBOV replication in Vero cells is blocked by the inhibitors of epidermal growth factor receptor (EGFR), tyrosine protein kinase Met (c-Met), and the insulin receptor (InsR)/insulin-like growth factor 1 receptor (IGF1R) (Stewart *et al.*, 2021).

HIV-1 has been reported to enter macrophages through a macropinocytosis-like mechanism that requires binding of viral gp120 to the CCR5 coreceptor. Following its macropinocytosis, HIV-1 virions are targeted via the endolysosomal pathway for degradation (Gobeil *et al.*, 2013). Moreover, HIV-1 interaction with the cellular surface proteins HSPG, GalCer, and TIM-1 is followed by viral entry via multiple endocytic pathways including clathrin- and caveolin/lipid raft-mediated endocytosis and macropinocytosis into polarized tonsil, cervical, and foreskin epithelial cells. HIV-1 internalization has been shown to result in the sequestration of virions into late endosomes of these epithelial cells. The spread of sequestered intraepithelial virions into lymphocytes is triggered by the interaction of activated CD4+ T lymphocytes with epithelial cells (Yasen *et al.*, 2017; Yasen *et al.*, 2018).

Recently, some viruses have been reported to hijack the host's apoptotic cell removal system to trigger their macropinocytosis uptake. In that apoptotic mimicry strategy, viruses expose the lipid phosphatidylserine (PS) on their surface to mimic apoptotic debris and activate the intracellular signaling used by the cells for the clearance of apoptotic bodies. Viruses reported using this strategy include hepatitis B virus (Vanlandschoot & Leroux-Roels, 2003), HIV (Callahan *et al.*, 2003), Ebola virus (Shimojima *et al.*, 2006), Marburg virus (Shimojima *et al.*, 2006), and vaccinia virus mature virions (VV MVs) (Laliberte & Moss, 2009; Mercer & Helenius, 2010; Morizono *et al.*, 2011).

2.3.5 Macropinocytosis-like viral entry mechanisms

There are some cases where the viral entry process deviates from the criteria identified for classical viral macropinocytosis, in this case, the mechanism is referred to ‘macropinocytosis-like’ entry (Mercer & Helenius, 2012). A clear example of macropinocytosis-like uptake of viruses is the entry of human papillomavirus type 16 (HPV-16) into HeLa and HaCaT cells. Here the entry process relies on many factors involved in classical macropinocytosis like tyrosine kinase signaling, actin dynamics, Na^+/H^+ exchangers, PAK-1, and PKC, however, the pathway also

differed in many aspects; the entry is inhibited by chlorpromazine, a known inhibitor of clathrin-mediated endocytosis that normally does not affect macropinocytosis. In addition, there was no evidence for the induction of any type of membrane ruffling or protrusions (Schelhaas *et al.*, 2012). Moreover, the virus is internalized in small, homogeneous uncoated endocytic vesicles, which differs from what is seen for classical macropinosomes, which are large and irregular in shape. In addition, the entry of HPV-16 did not depend on the Rho-like GTPases Cdc42 and Rac1, known regulators of actin dynamics during macropinocytosis (Schelhaas *et al.*, 2012).

Another example of macropinocytosis-like viral entry is the entry of Adeno-associated virus 2 (AAV2) into HeLa cells, which is inhibited by EIPA a known blocker of macropinocytosis, and the virus-induced extensive membrane blebbing and filopodial extensions and depended on Cdc42, Arf1, Graf1, and actin polymerization. However, the virus did not induce fluid-phase uptake (Nonnenmacher & Weber, 2011). The authors of this study concluded that the virus uses a pathway called clathrin-independent carriers / Glycosylphosphotidylinositol-anchored protein-enriched compartments (CLIC/GEEC), an endocytic pathway that had not been previously reported to internalize viruses (Nonnenmacher & Weber, 2011).

The phenomenon is also observed for the entry of influenza A virus (IAV) into A549 cells. Here too, the entry process met many of the conditions for macropinocytosis (Table 1.3), however, inhibitors of the Rho-like GTPases Cdc42 and Rac1 did not affect the entry, while Na^+/H^+ exchangers inhibitor EIPA, which inhibits activation of Rac1 and Cdc42 by reducing submembranous pH, effectively blocked IAV entry. In addition, the virus activated fluid uptake only in the presence of serum (de Vries *et al.*, 2011).

2.4 Small compound inhibitors targeting endocytosis

Endocytosis is a complex process regulated by a wide range of cellular proteins. Some of those proteins may be involved in more than one endocytic process while others are unique for a specific pathway. Chemical inhibitors targeting specific cellular factors are widely used to identify which endocytic pathway a virus might hijack to enter the target host cells. There are several advantages to using chemical inhibitors to study entry. They may be administered at specific time points of the replication cycle (i.e binding, entry, or post-entry) to dissect the role of their target at a specific stage of viral entry. Moreover, the reversibility of the block provides an extra advantage to be able to remove the inhibition pressure at a specific time point. This flexibility is in contrast to the permanent effect of a genetic approach such as expressing mutant forms of cellular factors or siRNA-mediated depletion of the target protein (Dutta & Donaldson, 2012; Ivanov, 2014).

However, the possibility of off-target effects that influence other factors involved in different endocytic pathways may sometimes complicate interpretations of the data. For example, inhibition of actin dynamics by cytochalasin D or latrunculin blocks both actin dependant phagocytosis and macropinocytosis (Dutta & Donaldson, 2012), which is not surprising since the two processes depend on actin dynamics. Also, inhibition of actin polymerization could have variable effects on the uptake of transferrin by clathrin mediate endocytosis based on the cell line studied (Fujimoto *et al.*, 2000). Another example of the problem of specificity when inhibiting endocytosis by pharmacological inhibitors is the inhibition of the small GTPase dynamin by dynasore which is reported to also block membrane ruffling and macropinocytosis in a dynamin-independent manner (Harper *et al.*, 2013; Park *et al.*, 2013; von Kleist & Haucke, 2012). The key regulators of endocytosis investigated in our study with their respective small compound inhibitors are described and summarized in Table 1.4

CME is the best-studied endocytic mechanism in part because the adaptor and coat proteins in addition to the scission factors are well characterized. Chlorpromazine (CPZ) inhibits clathrin pit formation by blocking the assembly of the clathrin adaptor protein AP2 (Wang *et al.*, 1993). The role of dynamin, which acts as a scission factor during vesicle fission from the plasma membrane in clathrin- and caveolin-mediated endocytosis, and in some forms of macropinocytosis, has been investigated using dynasore (Dyn), which inhibits all dynamin isoforms (Ferguson & De Camilli, 2012; Mercer & Helenius, 2012; Orth & McNiven, 2003; Schmid & Frolov, 2011). The entry via cholesterol/lipid-rafts and caveolin mediated endocytosis can be inhibited by nystatin (Nyst) which forms complex with cholesterol and prevents caveolin pit formation (Matveev *et al.*, 2001).

Many viruses that exploit endocytic pathways for their entry require endosomal acidification to trigger conformational changes in the viral fusion proteins to trigger fusion with the endosomal membrane which is followed by viral capsid penetration into the cytosol. Endosomal acidification can be blocked by monensin, a carboxylic ionophore, and the lysosomotropic weak base Ammonium chloride which raises endosomal pH (Nicola, 2016; White & Whittaker, 2016). The role of the transport of endocytic vesicle to late endosomes and trafficking from the Golgi to the ER can be studied using brefeldin A (BFA) (Klausner *et al.*, 1992). The role of microtubules in viral entry can be investigated using the inhibitors nocodazole (Noc) which inhibits the microtubule-dependent trafficking of endosomal vesicles to late endosomes (Bayer *et al.*, 1998), and paclitaxel (Tax) which inhibits microtubule dynamics by binding to and stabilizing microtubule polymers (Schiff *et al.*, 1979).

Macropinocytosis is triggered by the activation of the growth factor receptors by ligands as EGF or PDGF. The signaling via the receptors tyrosine kinases EGFR and PDGFR can be blocked by genistein (an inhibitor of tyrosine kinases), Iressa, and 324674 (inhibitors of EGFR), Gleevec (an inhibitor of PDGFR) (Buchdunger *et al.*, 2000; Woodburn, 1999). Alternatively, macropinocytosis could be triggered by the activation of integrins that involve signaling through the non-receptor tyrosine kinase, focal adhesion kinase (FAK). Signaling via FAK can be blocked via Y11 (Golubovskaya *et al.*, 2012). The role of other kinases in viral macropinocytosis may be explored using the following inhibitors, IPA-3 (an inhibitor of Pak1), and Calphostin C (an inhibitor of PKC), and wortmannin (Wort) a PI(3)K inhibitor. All forms of macropinocytosis involve dramatic actin rearrangement and membrane ruffling that require the activity of Na^+/H^+ exchanger (NHE) and actin polymerization (Mercer & Helenius, 2009; Mercer & Helenius, 2012). The amiloride analog 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) inhibits NHE activity through the reduction of submembranous pH (Koivusalo *et al.*, 2010) and cytochalasin D (Cyt-D) inhibits actin polymerization (Goddette & Frieden, 1986). The myosin light chain kinase (MLCK), which is required for myosin activity can be blocked by ML-7 (Makishima *et al.*, 1991).

Inhibitor	Cellular target	Concentrations used in this study
5-(N-ethyl-N-isopropyl) amiloride (EIPA)	Na^+/H^+ exchanger (NHE)	20-80 µM
Cytochalasin D (Cyt-D)	Actin polymerization	12.5-50 µM
ML7	myosin light chain kinase (MLCK)	5-25 µM
Wortmannin (Wort)	PI(3)K	5-20 µM
Genistein	Tyrosine kinases	50-200 µM
Iressa,	EGFR	12.5-50 µM
324674	EGFR	10-40 µM
Gleevec	PDGFR	12.5-50 µM
Y11	FAK	12.5-50 µM
IPA-3	Pak1	12.5-50 µM
Calphostin C	PKC	0.125-0.5 µM
Dynasore (Dyn)	Dynamin	40-160 µM
Chlorpromazine CPZ	CME	2-8 µg/ml
Nystatin (Nyst)	Caveolin/lipid rafts endocytosis	10-50 µM
Ammonium chloride	Endosomal acidification	10-30 mM
Brefeldin A (BFA)	translocation of endocytic vesicle to late endosomes and trafficking from the Golgi to the ER	10-40 µM
Nocodazole (Noc)	Microtubule polymerization	10-30 µM
Paclitaxel (Tax)	Microtubule depolymerization	10-50 µM

Table 1.4 Small compound inhibitors used in this thesis to study the entry of CHV-1

Different inhibitors with corresponding cellular targets and the concentrations used in this study.

2.5 Viral entry into polarized cells

2.5.1 Organization of polarized epithelial cells

Epithelia are made of a continuous sheet of tightly linked cells that form a barrier between the deep cell layers of organs and the external environment. The epithelial cells play an important role in many physiological processes including secretion, absorption, tissue homeostasis as well as regulation of the exchange of ions, nutrients, and molecules (Bryant & Mostov, 2008; Garcia *et al.*, 2018). The harsh environment at the interface where the epithelial cells are localized requires that epithelial structure be robust to maintain its function as a barrier to the external environment. This is mediated by different types of cell-to-cell junctions that firmly join the cells together and form cell polarity.

The main characteristics of epithelial polarization include the formation of intercellular junctions, asymmetric distribution of cytoskeleton, polarity complexes and membrane lipids, and the reposition of organelles (Garcia *et al.*, 2018). These components act as intermediate scaffolds to maintain an accurate cellular polarization and are precisely regulated by multiple signaling pathways including the Rho GTPases Rho, Rac 1, and Cdc42 (Garcia *et al.*, 2018; Mack & Georgiou, 2014). Polarized epithelial cells exhibit apicobasal polarity defined by three plasma membrane domains including apical, lateral, and basal domains (Rodriguez-Boulan & Macara, 2014). The apical domains face the lumen of biological tubes and mediate the exchange of materials into and out of tissues. The lateral domains connect adjacent cells via specialized intercellular junctional structures. The basal domains mediate the adherence to the extracellular matrix (ECM) and the underlying basement membranes. Both of the lateral and basal domains have similar components that are collectively named basolateral domains (Román-Fernández & Bryant, 2016).

The intercellular junctions that mediate epithelial cell polarization include tight junctions (TJ), adherent junctions (AJ), desmosomes, and gap junctions that are arranged sequentially from apical to basal regions at the intercellular space (**Figure 1.8**).

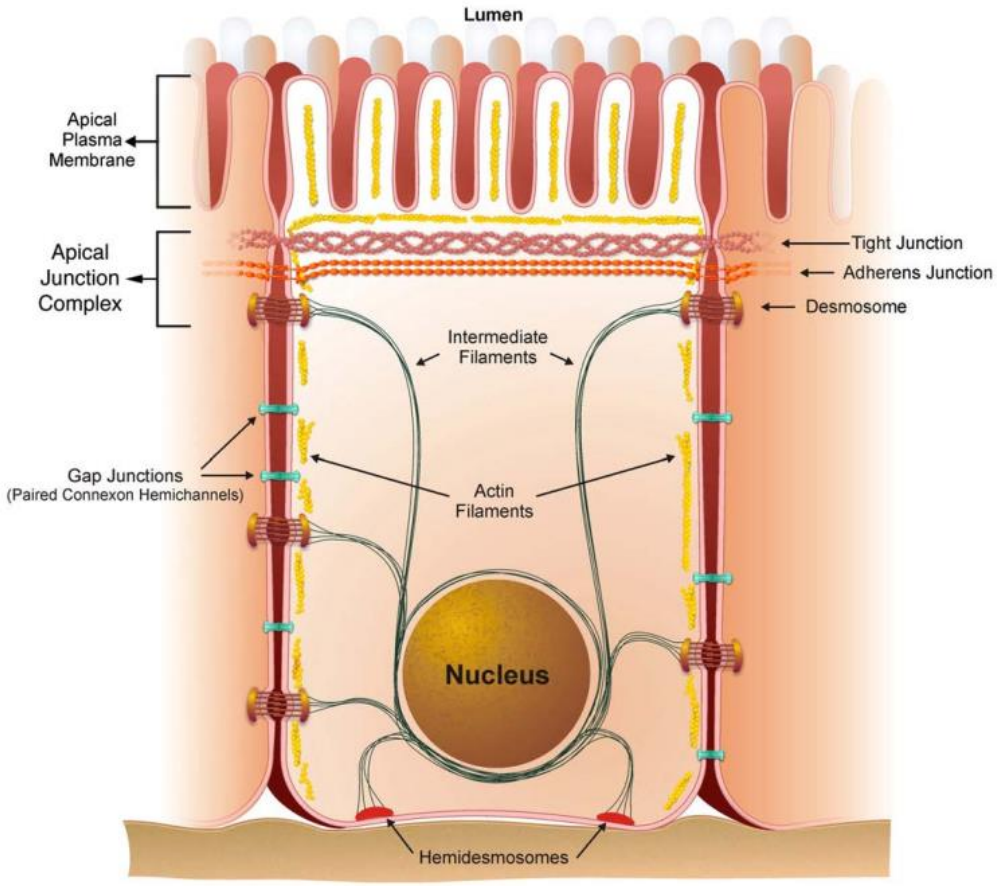

Figure 1.8 Schematic representation of cell-to-cell junctions in polarized epithelial cells

The apical junction complex (AJC) consists of tight junctions (TJ), adherens junctions (AJ), and apically localized desmosomes. Gap junctions and additional desmosomes are located beneath the apical junction complex and along the lateral cell membranes. Laterally, each AJC interacts with the AJC of the neighboring cells while hemidesmosomes interact with the basal lamina located at the base of cells. Moreover, AJCs will also interact with the cytoskeleton of the cell (actin filaments and intermediate filaments) to strengthen cell-to-cell interactions modified from (Guttman & Finlay, 2009).

Desmosomes are transmembrane, multiprotein complexes including cadherin proteins (Thomason *et al.*, 2010). They have a prominent distribution in tissues that are routinely subjected to physical forces, such as the heart and skin (Kowalczyk & Green, 2013). Desmosomes integrate cells within tissues and thereby function to resist mechanical stress and maintain intercellular junctions through the interaction of desmosomal cadherins with cytoskeleton intermediate filaments (Saito *et al.*, 2012).

Tight junctions (TJ) function as paracellular gates to occlude intercellular gaps and restrict diffusion based on size and charge (Zihni *et al.*, 2016). Disruption of the TJs will directly induce the loss of apical-basal polarity and have serious consequences on the impermeability of epithelium (Furuse, 2010). The TJs are located near the apical domain and separate it from the basolateral domain. The TJs are composed of multimeric transmembrane-protein complexes including zonula occludens (ZOs), tricellulin (also known as MARVEL domain-containing protein 2 (MARVELD2) and MARVELD3), occludins, and claudins (Chang *et al.*, 2019). The ZOs regulate TJ assembly, occludins maintain the stability and barrier function of a TJ, while claudins regulate the permeability properties of epithelial cells (Günzel & Yu, 2013). The ZO- proteins include ZO-11 (zonula occludens-1), the ZO-2 protein (zonula occludens-2), and the ZO-3 protein (zonula occludens-3). ZO-1 is the most important regulator of TJ formation through the assembling of occludins and claudins as scaffolding proteins, it also interacts with and regulates actin cortex components (Balda & Matter, 2016). The immunofluorescence detection of ZO proteins is often used to confirm the polarization of epithelial cells (Umeda *et al.*, 2006).

Adherent junctions (AJ) (or zonula adherens) are made of the Ca^{2+}-independent immunoglobulin-like intercellular adhesion molecule nectin, the actin filaments, and nectin binding protein afadin. By its interaction with the actin cytoskeleton, AJs play important roles in the organization of E-cadherin-mediated adherens junctions and claudin-based tight junctions in epithelial cells. In addition, AJs are involved in the formation of neuronal synapses, and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis (Nelson, 2008; Takai & Nakanishi, 2003). Cadherin proteins are essential components of AJs that promote their stability. The interactions between E-cadherin, β-catenin, and the filamentous (F)-actin-binding protein αE-catenin form a complex that binds directly to the actin cytoskeleton and supports the integrity of AJs (Buckley *et al.*, 2014).

2.5.2 Viral entry into polarized cells

Mutants of HSV-1 strain SC16 that lack glycoproteins C, G, E, I, or J have been shown to infect the apical and basolateral surfaces of the polarized Caco-2 epithelial cell line with comparable efficiency to the wild-type virus (Griffiths *et al.*, 1998). However, the infection of polarized MDCK cells by a gC-negative HSV-1(F) mutant was productive only when the virus was added to the basolateral surface, suggesting that the receptors for HSV-1 gC are asymmetrically distributed upon cell polarization and the role of gC in virion adsorption may be very strain-dependent (Sears *et al.*, 1991). Indeed, HSPG receptors of HSV-1 gC are mainly localized to the

basolateral cell surface (Caplan *et al.*, 1987), while chondroitin sulfate proteoglycans that are less efficiently utilized by HSV-1 are distributed mainly apically (Mårdberg *et al.*, 2002). Similarly, HSV-1 mutants that lacking gG showed defective entry through the apical surface of polarized MDCK cells, and the mutant virus only infects the cells from the basolateral surface (Tran *et al.*, 2000).

Productive infection by HSV-1 of polarized MDCK cells is lower than of nonpolarized cells suggesting that the tight junctions present in polarized cells might restrict viral entry. Indeed, the infection is enhanced when the integrity of the tight junctions of polarized cells is compromised by mechanical disruption of the junctions or by incubation in a low Ca^{2+}-containing medium. Moreover, under these conditions, the basolateral infection resulted in more virus spread compared to the apical infection where the release of the virus to the noninfected side was markedly reduced (Hayashi, 1995). Similar results for HSV-1 infection of polarized MDCK and Caco-2 cells have been reported, where partially polarized cells were more susceptible to infection from the apical chamber than fully polarized cells (Marozin *et al.*, 2004). The fully polarized cells become more susceptible to HSV-1 infection following the disruption of cell-cell contacts or using the Ca^{2+} chelator EGTA. The polarization of MDCK cells results in the sequestration of the HSV-1 gD receptor nectin-1 into the adherent junctions and the formation of tight junctions above the adherent junctions that ultimately restrict apical infection of HSV-1 (Marozin *et al.*, 2004).

The entry of HSV-1 into the human uterine (ECC-1), colonic (CaCo-2), and retinal pigment (ARPE-19) epithelial cells is preferentially from the apical surface, and the disruption of tight junctions using the Ca^{2+} chelator EGTA does not affect the level of apical infection. However, it does relieve the restriction of the basolateral infection (Galen *et al.*, 2006). The nectin-1 receptors of HSV-1 gD are preferentially distributed on the apical cell surface, and specific interfering RNAs directed against nectin-1 reduce the apical but not the basal infection suggesting that nectin-1 contributes to HSV-1 preferential apical infection. Moreover, the infection from the apical but not the basolateral surface triggers phosphorylation of focal adhesion kinase and the transport of viral capsid towards the nucleus (Galen *et al.*, 2006). A preferential apical infection has also been reported for HSV-2 entry into retinal pigment epithelial cells (Shukla *et al.*, 2009).

The entry of HSV-1 into polarized MDCKII cells transiently induces the phosphorylation of the Rho GTPases Rac1, Cdc42, which are key signaling proteins that regulate actin dynamics. The expression of a dominant-negative form of Cdc42 has been shown to reduce the infection by HSV-1, however dominant-negative Rac1 has no impact. Furthermore, constitutively active Cdc42 and Rac 1 inhibit HSV-1 infection. However, the expression of constitutively active or

dominant-negative Rac1/Cdc42 mutants did not affect the binding, internalization, and transport stages of HSV-1 infection. The expression of constitutively active or dominant-negative downstream effector protein p21-activated kinase (Pak1), also did not affect HSV-1 infection suggesting that the signaling via Rac1/Cdc42 during HSV-1 infection does not involve activation of Pak1(Hoppe *et al.*, 2006).

The cell adhesion molecule nectin-1 is the receptor for HSV-1 gD in epithelial cells and neurons, it is reported to have homophilic interactions at epithelial adherens junctions and heterophilic interaction with nectin-3 at neuronal synapses (Miyahara *et al.*, 2000; Mizoguchi *et al.*, 2002; Sakisaka *et al.*, 2007). HSV-1 gD is shown to disrupt the homophilic interactions of nectin-1 and redistribute it from the cell junctions (Bhargava *et al.*, 2016).

The asymmetric distribution of viral receptors in polarized cells that results in the preferential entry of the virus from the apical or basolateral surface has been described for several viruses. For Ebola virus, the viral receptors TIM-1 and Axl are distributed apically and the virus entry is more efficient from the apical surface (Hu *et al.*, 2019). For Zika virus, infection in polarized Caco-2 cells is preferentially from the apical side without affecting the paracellular permeability (Tamhankar & Patterson, 2019). In contrast, the expression of Nipah virus ephrin receptors and viral entry were bipolar in polarized MDCK, however, virus release was restricted to the apical side (Lamp *et al.*, 2013). The infection of bovine viral diarrhea virus was more efficient from the basolateral surface while the viral receptors bovine CD46 is distributed mainly apically and the use of an antibody directed against CD46 completely blocked the apical infection but had no effect on the basolateral infection suggesting that viral receptors are distinct on both cellular poles (Su *et al.*, 2021).

3 CELLULAR ENTRY MECHANISMS OF VARICELLOVIRUSES

The genus *Varicellovirus* of the subfamily *Alphaherpesvirinae* comprises a group of animal viruses that infect different animal hosts often causing economic losses. In addition, it includes VZV that causes chickenpox (varicella) and shingles in humans. Varicelloviruses are reported to exploit different entry pathways depending on the virus and host cell involved. In this section, the cell entry pathways of representative varicelloviruses will be discussed.

3.1 Varicella-zoster virus

VZV is a human-specific alphaherpesvirus of the genus *Varicellovirus* that infects about 90% of the human population worldwide (Laing *et al.*, 2018). Primary infection occurs during childhood causing chickenpox (varicella), while viral reactivation later in life from latently infected sensory ganglia in immune-compromised or elderly individuals causes zoster (shingles) (Arvin & Gilden, 2013; Cohen, 2008). The primary infection starts in the epithelial cells of the upper respiratory tract mucosa where the virus replicates and infects tonsils and other local lymphoid tissues. As the virus has a tropism for T cells, the infected T cells then disseminate the virus from the mucosal sites to the skin via the bloodstream (Ku *et al.*, 2004; Zerboni *et al.*, 2014). This acute phase of infection is known as chickenpox (varicella) and is characterized by fever and a generalized, pruritic vesicular rash. Then the virions migrate from the skin sites via retrograde axonal transport or T cell-mediated viremia to the sensory nerve cell bodies in ganglionic neurons where the virus establishes latency (Gilden *et al.*, 1983). Later in life, reactivation of latent virus replication due to stress or immune suppression leads to the migration of the virus from the affected neurons by anterograde axonal transport to the primary sites of infection in the skin innervated by those ganglia. This reactivation phase of infection is known as zoster (shingles).

The entry process of VZV is not completely understood (Zerboni *et al.*, 2014), this in part is related to the intensely cell-associated nature of VZV (Cook & Stevens, 1968; Weller *et al.*, 1958), which hinders the production of high titers of cell-free virus and hence the study of this important stage of the virus life cycle (Hambleton *et al.*, 2007).

Attachment of VZV to the cell surface heparan sulfate proteoglycans and Myelin-associated glycoprotein (MAG, Siglec-4) is mediated by gB (Jacquet *et al.*, 1998; Suenaga T, 2010). gB is the most conserved viral glycoprotein for cellular entry of herpesviruses (Connolly *et al.*, 2011). MAG is a type I transmembrane glycoprotein, a member of the sialic-acid binding Ig-like lectin (Siglec). MAG is expressed on the surface of myelin-forming cells like oligodendrocytes

of the central nervous system and Schwann cells of the peripheral nervous system. It functions in the differentiation, maintenance, and survival of oligodendrocytes, maintenance of myelinated axons, and regulation of axonal growth (Quarles, 2007). VZV gB binds directly to MAG expressed on the surface of oligodendroglia cells, and the co-expression of VZV gB and gH/gL was found to induce cell-cell fusion of MAG expressing 293T cells (Suenaga T, 2010). Sialic acid (a diverse group of monosaccharides found as components of oligosaccharide chains of glycoproteins and glycolipids) residues on VZV gB are essential for MAG binding as well as for membrane fusion during VZV infection (Suenaga *et al.*, 2015). It was shown that MAG that is mutated in the sialic acid-binding site does not bind to gB and does not support gB/gH-gL mediated cell-cell fusion or VZV entry. Also, mutations in the N-glycosylation sites of VZV gB hinder the binding to MAG and reduce membrane fusion efficiency (Suenaga *et al.*, 2015). However, the impact of those mutations on gB conformational changes, and consequently the fusion process is not excluded (Azab & Osterrieder, 2017). Interestingly, MAG has 12% amino acids sequence homology with the paired Ig-like type-2 receptor α (PILRα) protein that is a receptor for HSV-1 gB, suggesting that gB of different alphaherpesviruses might bind to similar classes of cell surface receptors (Oliver *et al.*, 2016; Suenaga T, 2010). Indeed, the transient expression of MAG in fibroblast was shown to enhance the infectivity of both HSV-1 and VZV, supporting the notion that cell surface homologs to MAG and PILRα are important for alphaherpesviruses attachment and entry (Oliver *et al.*, 2016; Suenaga T, 2010).

In addition to the role of MAG in VZV gB binding, HSPG was found to bind specifically to VZV gB but not gE, and this binding is inhibited by treatment with heparin (Jacquet *et al.*, 1998). Although gC has been reported to mediate HSV-1 and PRV viral attachment to the cell surface, VZV gC is not required for viral binding as mutant viruses lacking gC replicates at the same rate as the wild type virus (Cohen & Seidel, 1994; Zhang *et al.*, 2010).

VZV gE is reported to be essential for infection, viral entry, and cell to cell spread (Mo *et al.*, 2002). In addition to its role as a gB binding receptor, insulin-degrading enzyme (IDE) was found to interact with gE (Li *et al.*, 2006). Blocking or downregulation of IDE inhibited VZV infection and cell-to-cell spread, furthermore, overexpression of IDE in resistant cells increased viral entry and enhanced infection for both cell-free and cell-associated virus (Li *et al.*, 2006). However, it is reported that gE-IDE interaction is not required for viral entry in differentiated human T cells (Berarducci *et al.*, 2010). Cation-independent mannose 6-phosphate receptor (MPR^{ci}) proposed to act as an entry receptor for VZV, possibly via interaction with viral glycoprotein (gB, gH, gI, and gE) that contain phosphorylated N-linked complex oligosaccharides with mannose 6-phosphate

(Man 6-P) groups. Infection with cell-free VZV was blocked by Man 6-P, and cells deficient in MPR^{ci} were shown to be resistant to cell-free but not cell-associated VZV infection (Chen *et al.*, 2004).

In most cell types, both in vitro and in vivo, VZV spreads by cell-to-cell fusion. In contrast to most alphaherpesviruses which contain gD as the main receptor-binding glycoprotein that triggers viral entry and the cascade for membrane fusion, VZV does not express gD (Cole & Grose, 2003), instead, gB and gH-gL are necessary and sufficient for VZV membrane fusion (Oliver *et al.*, 2016; Suenaga T, 2010; Vleck *et al.*, 2011). Coexpression of VZV gH/gL alone using a recombinant vaccinia virus was sufficient to induce extensive cell-to-cell fusion with polykaryocytosis (Duus *et al.*, 1995; Maresova *et al.*, 2001), however, the expression of the cells in a vaccinia virus-free condition showed that the fusion required a combination of VZV gH/gL and gB (Suenaga T, 2010). Importantly, the crystal structure of the gH/gL ectodomains from other herpesviruses showed no homology to any of the known fusogenic proteins (Backovic *et al.*, 2010; Chowdary *et al.*, 2010; Matsuura *et al.*, 2010), and the potential fusion peptides from gH are buried (Chowdary *et al.*, 2010) suggesting a model in which gH/gL is not the fusogen but instead, it regulates membrane fusion via interaction with gB (Connolly *et al.*, 2011). Recently, X-ray crystallography of VZV gB suggested a functional role for the N-terminus domain in membrane fusion and viral propagation (Oliver *et al.*, 2021).

VZV enters into human neonatal lung fibroblasts, human embryonic lung fibroblasts, MeWo (melanoma-derived cells), and U373MG (astrocytoma-derived cells) by pH-dependent clathrin-mediated endocytosis that required HSPG and MPR^{ci} as attachment and entry receptors respectively (summarized in **Figure 1.9**). The virus is detected within coated pits and vesicles and the entry can be blocked by the clathrin assembly inhibitor, chlorpromazine. Moreover, the entry requires both viral and target cell membrane cholesterol. The depletion of cholesterol from target cells or virions using methyl-β-cyclodextrin (MβCD) was found to block VZV entry (Hambleton *et al.*, 2007). Also, the tyrosine kinases inhibitor genistein blocked the infection by both cell-free and cell-associated VZV but at post entry time points, suggesting a role for the tyrosine kinases activity in viral replication but not entry (Hambleton *et al.*, 2007). Furthermore, inhibition of endosomal acidification by ammonium chloride or bafilomycin blocked VZV entry, suggesting a role for the pH in viral membrane fusion within the endosomes (Finnen *et al.*, 2006; Hambleton *et al.*, 2007). Although phosphatidylinositol 3-kinase (PI3K) is involved in the endosomal sorting of MPR^{ci} (Gaffet *et al.*, 1997), it is not required for the endocytic entry of VZV (Hambleton *et al.*, 2007).

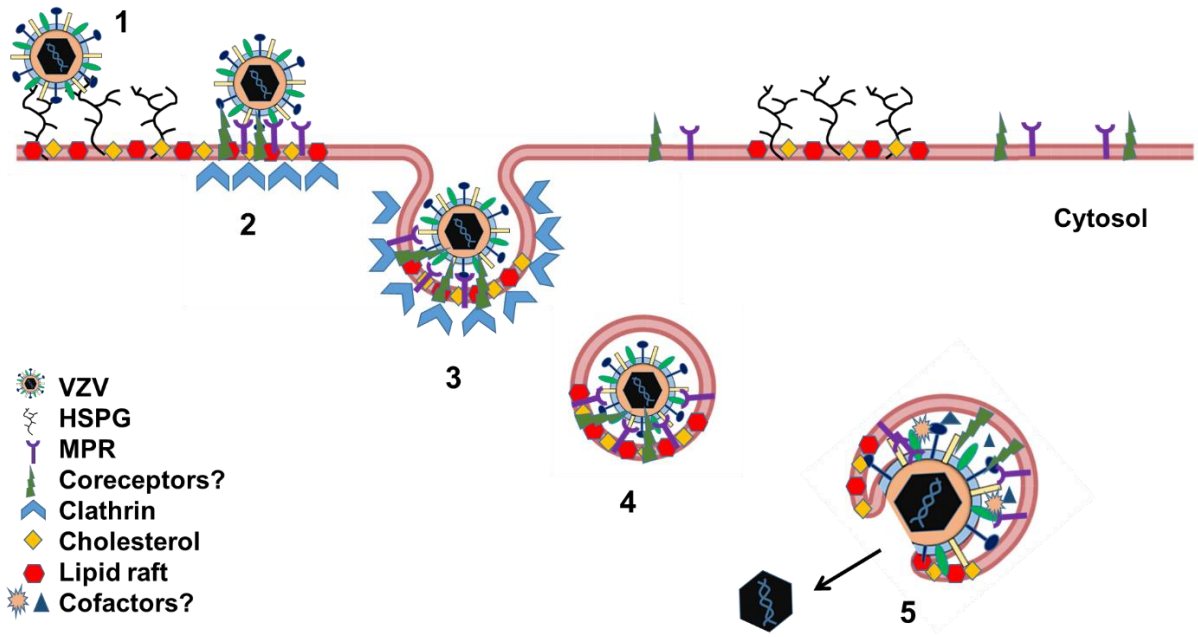

Figure 1.9 Model of VZV viral entry.

Proposed entry pathway of VZV. The virions attach to cell surface HSPG (step 1), which facilitates fixation of VZV to the plasma membrane and its interaction with the entry receptors MPR^{ci} (step 2). Then the virus enters the cell via clathrin-mediated endocytosis (step 3). The virion is then delivered to the compartments of the endosomal system, probably the early endosome (step 4). The exposure of virions to reduced pH in the endosome and possibly the binding to IDE receptors trigger the viral fusion glycoproteins to promote fusion of the viral envelope with the endosomal membrane (step 5). Following fusion, the viral capsid is released into the cytosol. Cholesterol may be involved in each step of the entry process. The figure is adapted from (Hambleton *et al.*, 2007).

3.2 Pseudorabies virus

PRV is an alphaherpesvirus that infects pigs, causing Aujeszky's disease (pseudorabies), which causes mortality of young and immunocompromised animals, and substantial economic losses to the swine industry (Müller *et al.*, 2011). The entry of PRV into HeLa cells has been shown to involve macropinocytosis that requires NHE, Rac1, Cdc42, PI3K, PaK1, and PKC (Lv *et al.*, 2018b). The virus is engulfed by cell membrane protrusions and detected within SNX5-positive macropinosomes together with the fluid phase marker, dextran. Moreover, the entry into HeLa cells is independent of dynamin, clathrin, and caveolin (Lv *et al.*, 2018b).

A hypertonic sucrose medium, which inhibits receptor-mediated endocytosis (Carpentier *et al.*, 1989; Daukas & Zigmond, 1985; Heuser & Anderson, 1989; Mellman, 1996), blocks the uptake of PRV. The entry is also blocked by the lysosomotropic agents, ammonium chloride and monensin, both are known to inhibit endosomal acidification (Miller *et al.*, 2019). When exposing

viruses that rely on a low-pH entry pathway, to a low pH buffer in the absence of cellular membranes, they prematurely undergo irreversible conformational changes in their fusion glycoproteins, rendering them unable to fuse with the host membranes and their infectivity will be blocked (Bron *et al.*, 1993; Dollery *et al.*, 2011; Edwards *et al.*, 1983; Rosenthal *et al.*, 1989; Weed *et al.*, 2017). Accordingly, the pretreatment of PRV with mildly acidic pH inactivated the infectivity of the virus. A role for the cellular proteasome in PRV entry has also been suggested, because MG132 (a competitive inhibitor of proteasome degradative activity) reduced the entry of PRV (Miller *et al.*, 2019). Although the precise mechanism is not fully understood, the proteasome-dependent entry mechanism has also been suggested for HSV-1 and BHV-1 (Delboy & Nicola, 2011; Delboy *et al.*, 2008; Pastenkos *et al.*, 2018; Schneider *et al.*, 2019).

In a screening study, the impact of a library of 44 FDA-approved drugs on *in vitro* and *in vivo* infection by PRV has been investigated (Fang *et al.*, 2020). The results showed that hydroquinone significantly reduced both PRV adsorption and internalization into mouse neuroblastoma N2a cells. The inhibitory effect of hydroquinone has been linked to activation of AKT phosphorylation. The activation of AKT phosphorylation stimulates the expression of genes associated with the PI3K-AKT signaling pathway including TNF-α (Sun *et al.*, 2019), which has been shown to inhibit viral replication (Schijns *et al.*, 1991; Seo & Webster, 2002). Importantly, pretreatment of N2a cells with LY294002 (inhibits AKT phosphorylation) has been shown to abolish the antiviral activity of hydroquinone on PRV (Fang *et al.*, 2020).

3.3 Bovine Herpesvirus 1

Bovine herpesvirus 1 (BHV-1) is a varicellovirus that infects cattle causing inflammatory disease in the upper respiratory tract, conjunctivitis, genital disorders, and suppression of the immune function that may predispose the animal to secondary infections by other pathogens (Fulton *et al.*, 2016; Hodgson *et al.*, 2005; Jones & Chowdhury, 2007). BHV-1 is one of the pathogens that cause bovine respiratory disease complex (BRDC) a poly-microbial disease that costs the U.S. cattle industry about 3 billion $ annually (Griffin, 1997). Furthermore, BHV-1 is the most frequently diagnosed pathogen associated with viral abortion in the North American cattle industry (Chase *et al.*, 2017; Jones, 2019).

BHV-1 entry into Madin Darby bovine kidney (MDBK) cells, bovine turbinate cells, and African green monkey kidney (Vero) cells is mediated by a low-pH endocytic pathway (Pastenkos *et al.*, 2018). The uptake of BHV-1 into MDBK cells is rapid, about 50% of the viral particles are endocytosed within 5 minutes, and 90% of virions are endocytosed within 30 minutes of infection.

The lysosomotropic agents ammonium chloride and monensin, which inhibit endosomal acidification, block BHV-1 infection suggesting that low endosomal pH is required for the penetration of viral capsid from the endosome. Similar to HSV-1 and PRV (Miller *et al.*, 2019; Schneider *et al.*, 2019), the proteasome inhibitor MG132 was found to block the entry of BHV-1 (Pastenkos *et al.*, 2018).

EGFR signaling is important in viral entry by macropinocytosis (Mercer & Helenius, 2009; Mercer & Helenius, 2012). Moreover, HSV-1 is known to hijack EGFR /PI3K / ERK/ ROCK/ cofilin signaling to initiate entry into neuronal cells (Zheng *et al.*, 2014b). Interestingly, BHV-1 infection of the human lung carcinoma cell line A549 and MDBK cells induced phosphorylation of EGFR at Tyr1068 (Qiu *et al.*, 2020). Importantly, the EGFR inhibitor Gefitinib (Iressa) reduced BHV-1 titers at both the entry and post-entry stages of infection. The activated EGFR has been reported to translocate to the nucleus where it acts as a transcription factor through binding to and activating AT-rich DNA sequences (Faria *et al.*, 2016; Wang *et al.*, 2010b). Of note, varicelloviruses including BHV-1 are known to have A–T rich sequences (Papageorgiou *et al.*, 2016; Qiu *et al.*, 2020). Activated EGFR may interact with the BHV-1 genome to regulate viral gene transcription, also late stages of BHV-1 replication cycle, such as viral particle assembly and virus egress, may be affected by EGFR inhibition, but further studies are needed to explore such hypotheses.

The binding of ligands like growth factors or viruses to EGFR induces downstream signaling cascades including the RhoGTPases (Rac1 or Cdc42), (PI3Ks)/Akt, and PLC (Mercer & Helenius, 2009; Mercer & Helenius, 2012; Zheng *et al.*, 2014a). BHV-1 Infection of A549 cells results in EGFR-dependent activation of phospholipase C-γ1 (PLC-γ1) and EGFR-independent activation of Akt. The chemical inhibition of PLC-γ1, but not of Akt reduced viral titers suggesting that BHV-1 infection stimulated in part PLC-γ1 via EGFR, and PLC- γ1 but not Akt is essential for the BHV-1 infection in A549 cells (Qiu *et al.*, 2020). Interestingly, BHV-1 titers in MDBK cells were significantly reduced by Akt inhibition, supporting the notion that viral entry pathways are cell type dependant.

A genome-wide CRISPR knockout screening identified about 41 pro-viral host genes critical to BHV-1 entry, the list included genes coding for cell surface receptors, enzymes involved in glycosaminoglycans (GAGs) biosynthesis, and proteins involved in trafficking within the Golgi apparatus (Tan *et al.*, 2020). Among the host factors identified are PVR (CD155) and PVRL2 (nectin-2) which proved to be receptors for BHV-1 entry in MDBK cells. The double knockout of both PVR and PVRL2 did not completely block BHV-1 infection in MDBK cells suggesting that the

virus uses additional host cell receptors as nectin-1 and/or the entry is mediated via receptor-independent mechanisms.

HSPG plays an important role in viral attachment to the host cell surface (Oh *et al.*, 2010). Both HS2ST1(2-O-sulfotransferase) and GLCE (an epimerase) enzymes are important for HS chain polymerization (Pinhal *et al.*, 2001). The knockout of HS2ST1 or GLCE from MDBK cells has been shown to reduce BHV-1 plaques formation, suggesting the significance of cell surface HS in mediating BHV-1 entry (Tan *et al.*, 2020).

3.4 Equine herpesvirus

Equine herpesvirus-1 (EHV-1) is a varicellovirus that infects equines, especially horses with a worldwide distribution (Khusro *et al.*, 2020). EHV-1 infection in horses causes respiratory and neurological diseases, as well as abortion in pregnant mares (Loh *et al.*, 2015; Oladunni *et al.*, 2019). The infection is highly contagious and represents major economic and welfare problems (Khusro *et al.*, 2020). Currently, nine different species of EHVs have been identified among them EHV-1, EHV-3, EHV-4, EHV-8, and EHV-9 are classified in the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*. While EHV-2 and EHV-5 are classified as *Percaviruses* in the subfamily *Gamaherpesvirinae*. Both EHV-6 and EHV-7 are temporarily placed in the subfamily *Alphaherpesvirinae* and *Gammaherpesvirinae*, respectively (Davison *et al.*, 2009; Khusro *et al.*, 2020).

The attachment of EHV-1 and EHV-4 to cell surface heparan sulfate is mediated via gC (Azab *et al.*, 2010; Osterrieder, 1999). The deletion of gC or HSPG reduces EHV-1 titers in rabbit kidney, equine dermal, and primary equine cells (Osterrieder, 1999). Furthermore, EHV-4 gC and gB were shown to bind heparin, and the infection of fetal horse kidney by EHV-4 was blocked by soluble heparin (Azab *et al.*, 2010).

The EHV-1 entry pathway was shown to be cell-type dependent in that the virus enters Chinese hamster ovary (CHO-K1) cells via endocytosis, while entry into equine dermal and rabbit kidney (RK13) cells involves direct fusion with the cell membrane (Frampton *et al.*, 2007). The endocytosis of EHV-1 in CHO-K1 cells requires endosomal acidification and activation of the serine/threonine Rho-kinase ROCK1 (upstream activator of FAK signaling), but is independent of clathrin and caveolin.

Although both EHV-1 and EHV-4 share a significant genetic and antigenic similarity (Davison *et al.*, 2009; Telford *et al.*, 1998), EHV-1 has been shown to use a unique gD receptor that allows it to have a wide tropism in several cell types, even for cells resistant to HSV-1 infection (CHO-K1 cells that devoid of gD entry receptors nectin 1 and nectin 2) (Frampton *et al.*, 2005), while EHV-4 infection is restricted mainly to primary equine cells (Azab & Osterrieder, 2012). The major histocompatibility (MHC) class I has been identified as a gD receptor for EHV-1 and EHV-4 in equine fibroblasts, epithelial cells, and endothelial cells (Azab *et al.*, 2014; Kurtz *et al.*, 2010; Sasaki *et al.*, 2011) and the expression of equine MHC-1 in mice increased its susceptibility to pulmonary infection by EHV-1 (Minato *et al.*, 2019). However, both viruses still infect non-equine cells independently of equine MHC-I (Azab *et al.*, 2014; Sasaki *et al.*, 2011) suggesting the entry is mediated via different receptors in those cells.

Although MHC-I molecules are expressed on the cell surface of all nucleated cells, EHV-1 has a restricted replication in equine CD172a$^+$ cells *in vitro* (Laval *et al.*, 2015), thus MHC-I is not responsible for the restricted tropism of EHV-1, indeed, antibodies directed against MHC-I did not efficiently block EHV-1 entry into equine PBMCs suggesting that entry may be mediated by different receptors (Azab & Osterrieder, 2012). This restriction is related in part to the low binding efficiency of the virus to the CD172a$^+$ cells (Laval *et al.*, 2016). The binding of the virus was found to be independent of heparan sulphate but required cell surface sialic acids. The entry of EHV-1 into CD172a$^+$ cells is mediated by an endocytic pathway that depends on cellular $\alpha_V\beta3$ integrins, cholesterol, tyrosine kinase, actin, dynamin, and endosomal acidification, but is independent of clathrin, caveolae, and macropinocytosis.

EHV-1 and EHV-4 use different entry pathways into equine epithelial cells which depend on viral glycoprotein H (gH) and the cellular α4β1 integrins (Azab *et al.*, 2013). The entry of EHV-1 into equine epithelial cells is mediated via a direct fusion at the plasma membrane that is insensitive to inhibitors of clathrin, cholesterol/lipid rafts, caveolin-mediated endocytosis, macropinocytosis, dynamin, tyrosine kinases, and endosomal acidification. On the other hand, the entry of EHV-4 into equine epithelial cells involves neutral pH endocytosis that is dependent on caveolin 1, cholesterol, dynamin II, and tyrosine kinase activity. Interestingly, replacing EHV-1 gH with that of EHV-4, or mutation of the SDI integrin-binding motif of EHV-1 gH to make it identical to that of EHV-4 gH or blocking of cellular α4β1 integrins receptors have been shown to change the route of EHV-1 entry from direct fusion to a caveolin/raft-mediated endocytosis (**Figure 1.10**). However, the exchange of EHV-4 gH with that of EHV-1 did not trigger viral fusion

with the plasma membrane suggesting that an additional viral factor is required to direct the fusion process (Azab *et al.*, 2013).

Signaling via the cellular receptors α4β1 integrins has been shown to activate phospholipase C (PLC) that triggers the release of Ca^{2+} stores of the endoplasmic reticulum (Kanner *et al.*, 1993; Van Seventer *et al.*, 1992). Activated PLC triggers the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binding to its receptors IP3-R on the cytoplasmic side of the ER induces the release of Ca^{2+} stores, while DAG triggers the activation of several signaling proteins including protein kinase C (PKC) (Rhee, 2001; Sekiya *et al.*, 2004). The binding of EHV-1 gH to its cell surface receptors α4β1 integrin has been shown to activate PLC and generation of IP3 that binds to IP3R and induce the release of ER Ca^{2+} stores (Azab *et al.*, 2015). The increase in the intracellular levels of Ca^{2+}results in the activation of phospholipid scramblase that triggers the exposure of phosphatidylserine (PS) (which are mainly located in the inner leaflet of the plasma membrane (Bretscher, 1972)) on the outer leaflet of the plasma membrane that may enhance the fusion of EHV-1 envelope with the plasma membrane (**Figure 1.10**). PS has been reported to enhance virus fusion with endosomal membranes, where the virus encounters anionic lipids for the first time during entry (Coil & Miller, 2005; Zaitseva *et al.*, 2010). Interestingly, blocking of increased cytosolic Ca^{2+} levels or PS exposure changed the entry pathway of EHV-1 in equine epidermal cells from the direct fusion at the plasma membrane to caveolin-mediated endocytic pathway (Azab *et al.*, 2015).

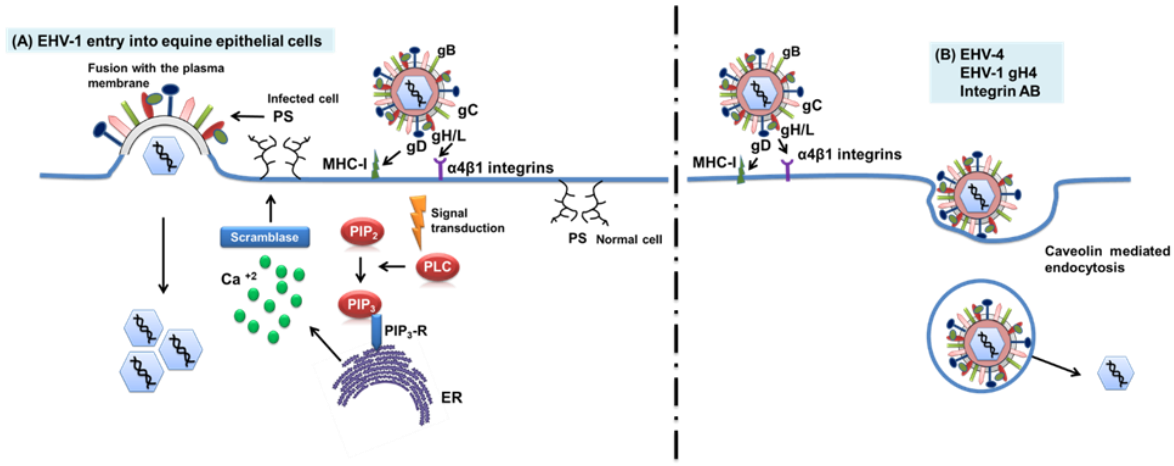

Figure 1.10 Entry model of EHV-1 and EHV-4

Proposed entry pathway of EHV-1 and EHV-4 into equine dermal cells. **(A)** EHV-1 attachment to heparan sulfate proteoglycans on the cell surface of target cells is mediated by gC and/or gB, which is followed by binding of gD to its receptor (MHC-I), triggering the activation of gH/gL to interact with α4β1 integrins receptors. Interaction of gH with α4β1 integrins induces the activation of PLC and generates PI3 that binds to PIP3-R on the cytosolic side of the ER to increase the release of Ca^{2+} from the ER stores. Increased cytoplasmic Ca^{2+} levels lead to activation of phospholipid scramblase that triggers the exposure of phosphatidylserine (PS) on the cell surface. Exposed PS may enhance the fusion of the viral envelope with the plasma membrane. **(B)** EHV-4 entry into equine dermal cells is mediated by caveolin-dependent endocytosis. The mutant EHV-1 gH4 in which the gH has been replaced with that of EHV-4, or blocking the receptors α4β1 integrins, have been shown to switch the entry pathway of the wild type EHV-1 from direct fusion to caveolin mediated endocytosis. The figure is adapted from (Azab *et al.*, 2015; Azab *et al.*, 2013).

Among the important signaling molecules that viruses hijack to promote their entry and infection is the signaling via the Rho family small GTPases, such as Rac1 and Cdc42 (Fujioka *et al.*, 2013; Hoppe *et al.*, 2006; Wang *et al.*, 2010a). EHV-1 infection in equine epidermal cells has been shown to activate both Rac1 and Cdc42, and the infection but not the entry of EHV-1, was blocked by the inhibition of Cdc42 (which delayed but did not inhibit the entry), while Rac inhibition had no impact on virus entry (Kolyvushko *et al.*, 2020). The disruption of Rac1 and Cdc42 signaling reduced EHV-1 induced acetylation of α-tubulin and viral transport towards the nucleus. Furthermore, activation of Rac1 and Cdc42 was found to be essential for EHV-1 to induce cell-to-cell fusion.

In summary, varicelloviruses exploit different entry pathways to infect host cells. The pathways that have been reported include direct fusion of the viral envelope with the host cell plasma membrane, CME, caveolin mediated endocytosis, and macropinocytosis. Viral envelope glycoproteins and cellular receptors dictate the entry mechanism that the virus uses, as has been

shown for the entry of EHV-1 and EHV-4. However, the pathway(s) and cellular factors that mediate CHV-1 entry into canine epithelial cells had not been identified prior to our study.

4 PROJECT OBJECTIVE

CHV-1 is a widespread pathogen that infects wild and domestic dogs causing fatal infection in newborn puppies. Currently, there is no effective treatment or vaccine that provides long-term protection from the virus. To date, the entry pathway of CHV-1 into the canine epithelial cells - which represent portal sites for the virus- is not known. Understanding of CHV-1-host cell interactions is critical for the development of novel therapeutics.

For other alphaherpesviruses like HSV-1, EHV-1and EHV-4 the entry pathway may involve a pH-independent direct fusion of the viral envelope with the plasma membrane, or pH-dependent or independent fusion with the endosomal membranes following the uptake of the viral particle by different endocytic mechanisms in cell type and viral strain-dependent manner. HSV-1 has been reported to enter the epithelial cells Vero, HeLa, HEp-2, and PtK_2 by a macropinocytosis-related mechanism (Devadas *et al.*, 2014), However, the entry of EHV-1 and EHV-4 into the equine epithelial dermal cells involves direct fusion at the plasma membrane and caveolin mediated endocytosis respectively (Azab *et al.*, 2013). The main goal of our study is to identify the entry pathway of CHV-1 into canine epithelial cells. Hence, this thesis aimed to investigate the entry pathway of CHV-1 and identify different cellular factors implicated in the entry process.

CHAPITRE DEUX: ENTRY OF THE VARICELLOVIRUS *CANID HERPESVIRUS 1* INTO MADIN-DARBY CANINE KIDNEY EPITHELIAL CELLS IS PH-INDEPENDENT AND OCCURS VIA A MACROPINOCYTOSIS-LIKE MECHANISM BUT WITHOUT INCREASE IN FLUID UPTAKE

Titre : L'entrée du varicellovirus Canid herpesvirus 1 dans les cellules épithéliales canines du rein Madin-Darby canine kidney (MDCK) est indépendante du pH et se produit via un mécanisme de type macropinocytose mais sans augmentation de l'absorption de liquide

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Résumé :

L'herpesvirus canin (CHV-1 pour *Canid herpesvirus 1*) est un varicellovirus qui cause des infections qui se résolvent spontanément chez les chiens adultes mais qui induisent de la morbidité et mortalité chez les chiots. En utilisant une approche à plusieurs volets, nous avons découvert la voie d'entrée du CHV-1 dans les cellules épithéliales du rein canines Madin-Darby (MDCK). Nous avons découvert que le CHV-1 induit des protrusions de type lamellipodes de la membrane plasmique de la cellule hôte, et est internalisé rapidement dans de grandes vacuoles non revêtues, suggérant la macropinocytose. Traitements avec des inhibiteurs ciblant les facteurs cellulaires importants pour la macropinocytose, comprenant les inhibiteurs des échangeurs Na^+/ H^+, la F-actine, la myosine, p21-activated kinase, la phosphatidylinositol-3-kinase, la dynamine et la focale adhésion kinase, ont réduit de manière significative la réplication virale. De plus, l'effet était limité à une exposition aux inhibiteurs au début de l'infection, confirmant le rôle de la machinerie de macropinocytose lors de l'entrée. Le profil des inhibiteurs a également suggéré un rôle de la signalisation via des intégrines et des récepteurs tyrosine kinases dans l'entrée virale. En revanche, les inhibiteurs de l'endocytose médiée par la clathrine ou la cavéoline, des microtubules et de l'acidification endosomale n'ont pas affecté l'entrée du CHV-1 dans les cellules MDCK. Nous avons montré que le virus se colocalise avec le dextran, un marqueur d'absorption des fluides; néanmoins, l'infection par le CHV-1 n'a pas mené à une augmentation de l'absorption du dextran. Ainsi, nos résultats indiquent que CHV-1 utilise une voie d'entrée de type macropinocytose dans les cellules MDCK qui est indépendante du pH, et qui n'est cependant pas basée sur la stimulation de l'absorption de liquide.

Contribution des auteurs :

Mohamed Eisa : Conception et réalisation de toutes les expériences. Analyse des résultats et création des figures. Rédaction du manuscrit.

Hamza Loucif : Analyse des résultats de cytométrix en flux.

Julien van Grevenynghe : Révision du manuscrit.

Angela Pearson : Acquisition de financement. Conception des expériences. Analyse des résultats. Révision et correction du manuscrit

Entry of the *varicellovirus Canid herpesvirus 1* into Madin-Darby canine kidney epithelial cells is pH-independent and occurs via a macropinocytosis-like mechanism but without increase in fluid uptake

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Running title

CHV-1 entry via a macropinocytosis-like process

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1 ABSTRACT

Canid herpesvirus 1 (CHV-1) is a *Varicellovirus* that causes self-limiting infections in adult dogs but morbidity and mortality in puppies. Using a multipronged approach, we discovered the CHV-1 entry pathway into Madin-Darby canine kidney (MDCK) epithelial cells. We found that CHV-1 triggered extensive host cell membrane lamellipodial ruffling and rapid internalization of virions in large, uncoated vacuoles, suggestive of macropinocytosis. Treatment with inhibitors targeting key macropinocytosis factors, including inhibitors of Na^+/H^+ exchangers, F-actin, myosin light-chain kinase, protein kinase C, p21-activated kinase, phosphatidylinositol-3-kinase, and focal adhesion kinase, significantly reduced viral replication. Moreover, the effect was restricted to exposure to the inhibitors early in infection, confirming a role for the macropinocytic machinery during entry. The profile of inhibitors also suggested a role for signaling via integrins and receptor tyrosine kinases in viral entry. In contrast, inhibitors of clathrin, caveolin, microtubules, and endosomal acidification did not affect CHV-1 entry into MDCK cells. We found that the virus colocalized with the fluid phase uptake marker dextran; however, surprisingly, CHV-1 infection did not enhance the uptake of dextran. Thus, our results indicate that CHV-1 uses a macropinocytosis-like, pH-independent entry pathway into MDCK cells, which nevertheless is not based on stimulation of fluid uptake.

Take a way:

CHV-1 enters epithelial cells via a macropinocytosis-like mechanism.

CHV-1 induces extensive lamellipodial ruffling.

CHV-1 entry into MDCK cells is pH-independent.

2 INTRODUCTION

Canine herpesvirus 1 (CHV-1) is a herpesvirus of the genus *Varicellovirus* (Pellett PE, 2012). CHV-1 is an important and widespread pathogen in wild and domestic canine populations. CHV-1 causes respiratory (kennel cough), ocular (conjunctivitis and keratitis), and genital infections in dogs as well as other canines such as foxes (Decaro *et al.*, 2008; Evermann *et al.*, 2011). It was first recognized in 1965 in the context of a fatal hemorrhagic septicemic disease in puppies (Carmichael *et al.*, 1965). The infection of adult dogs by CHV-1 is usually asymptomatic and self-limited; however, in neonatal puppies (1–2 weeks of age), the systemic infection is fatal and may affect the entire litter. The virus causes infertility and spontaneous abortions. It is transmitted mainly by direct contact with infected nasal, oral, and vaginal secretions. In addition, transplacental infection of puppies may occur during delivery (Evermann *et al.*, 2011). Like other α-herpesviruses, infection by varicelloviruses consists of an acute phase followed by a latent phase, where the virus persists in sensory ganglia (Miyoshi *et al.*, 1999; Okuda *et al.*, 1993). *In vivo,* CHV-1 infection begins with entry of the virus into epithelial cells of the upper respiratory mucosa where it replicates (Li *et al.*, 2016), then—using varicella-zoster virus (VZV) as a model—the virions likely infect hematopoietic cells such as T cells in the local lymphoid tissues leading to a cell-mediated viremia. During the acute phase of infection, there is spread of the virus to sensory nerves forming the trigeminal or dorsal root ganglia, wherein the virus establishes latency (Gilden *et al.*, 1983; Miyoshi *et al.*, 1999). Periodically, the virus reactivates from latency leading to the production of new infectious viral particles that travel via anterograde axonal transport and ultimately reach the epithelia, and cause recurrent lesions that promote the spread of the virus to new hosts (Zerboni *et al.*, 2014). A therapeutic vaccine that must be readministered with each pregnancy is available in Europe (Eurican™); however, there are no commercial vaccines currently available to provide life-long protection from CHV-1, and there is no treatment available to cure or block infection (Decaro *et al.*, 2008).

Because virus entry into host cells is a necessary first step for successful infection, identifying the underlying mechanism of how CHV-1 enters epithelial cells will provide important information for the basis of developing new therapeutic strategies. For the extensively studied herpes simplex virus 1 (HSV-1) (genus *Simplex*), the entry process varies depending on the cell type (reviewed in (Nicola, 2016)). Entry of HSV-1 into Vero cells and human neurons involves direct fusion of viral envelope with the plasma membrane (Lycke *et al.*, 1988; Wittels & Spear, 1991). In contrast, HSV-1 enters by endocytosis into epithelial cells, CHO cells expressing gD

receptors, and HeLa cells (Devadas *et al.*, 2014; Nicola *et al.*, 2005; Nicola *et al.*, 2003). Moreover, the entry of HSV-1 into human keratinocytes involves both direct fusion of the viral envelope with the cell membrane and endocytosis that is dynamin- and cholesterol-dependent (Rahn *et al.*, 2011). A cell-type dependent mode of entry has also been found for equine herpesvirus (EHV-1) (genus *Varicellovirus*) (Azab *et al.*, 2013). It is not known what pathways are used for CHV-1 entry into host cells.

Viruses have been found to exploit a variety of cellular entry pathways including the well-characterized uptake within clathrin- or caveolin-coated endocytic vesicles (Kaksonen & Roux, 2018; Mercer *et al.*, 2010b). Another endocytosis-mediated form of cellular entry is macropinocytosis, which is a transient, actin-dependent, nonspecific endocytic process used by cells for the non-selective uptake of large volumes of fluids, membranes, solutes, and membrane-bound particles. Viral entry via macropinocytosis starts with virus binding to cell surface receptors. This binding activates directly or indirectly intracellular signaling that leads to actin rearrangement and cell-wide plasma membrane ruffling. These ruffles, which may take different forms, fold back towards the cell body to form large, fluid-filled, irregularly shaped uncoated vacuoles that result in virus internalization (reviewed in (Mercer & Helenius, 2009; Mercer & Helenius, 2012)). Host factors that are common to all forms of macropinocytosis include actin and the Na^+/H^+ exchanger (NHE). In contrast, several other cellular factors are required only for certain forms of macropinocytosis, these include receptor tyrosine kinases (RTKs), integrins, focal adhesion kinase (FAK), phosphatidylinositol-3-kinase (PI(3)K), protein kinase C (PKC), the Rho GTPases Cdc42 and Rac1, myosin II, C-terminal binding protein (CtBP), dynamin and the endosomal vacuolar H^+-ATPase pump (King & Kay, 2019; Lim & Gleeson, 2011; Mercer & Helenius, 2009; Mercer & Helenius, 2012; Swanson, 2008).

In this study, we investigated the mechanism of entry of the varicellovirus CHV-1 into Madin-Darby Canine Kidney (MDCK) epithelial cells. Using a multipronged approach involving electron microscopy, a panel of chemical inhibitors, and confocal microscopy strategies with fluorescent-labeled virus, we identified multiple cellular factors critical for the entry of the virus into host cells. These results provide potential targets for the development of new antiviral strategies against this pathogen and will contribute to a better understanding of the entry processes of varicelloviruses in general.

3 RESULTS

3.1 CHV-1 is endocytosed into MDCK cells

To study the early events in CHV-1 infection of host cells, CHV-1 entry into an epithelial cell line was analyzed by transmission electron microscopy (TEM). The CHV-1 strain CHV-V777 (Papageorgiou *et al.*, 2016) was incubated with MDCK cells at a multiplicity of infection (MOI) of 2.5 at 4 ^{o}C for 2 h to allow binding to the cells. Next, cells were transferred to a 37 ^{o}C incubator for 30 sec or 2 min to trigger viral entry. The cells were then fixed and processed for TEM analysis to allow visualization of the subcellular structures with which the viral particles were associated immediately following entry. We found that at both 30 sec and 2 min post entry, enveloped viral particles were detected within large, irregularly shaped cytoplasmic vesicles (Fig 1A). These vesicles were devoid of cytoplasmic coat and ranged in diameter from 264 to 1943 nm (Fig. 1B). Throughout our experiments, our attempts to identify an event that could be construed to be a consequence fusion of the viral envelope with the plasma membrane, amounted to a single observation of what appeared to be a naked capsid in the cytoplasm early in infection. Thus, our results indicate an endocytic entry mechanism for CHV-1. Furthermore, we noted that external viral particles were often detected adjacent to plasma membrane protrusions (Fig. 1C). These observations led us to hypothesize that the virus enters MDCK cells via macropinocytosis.

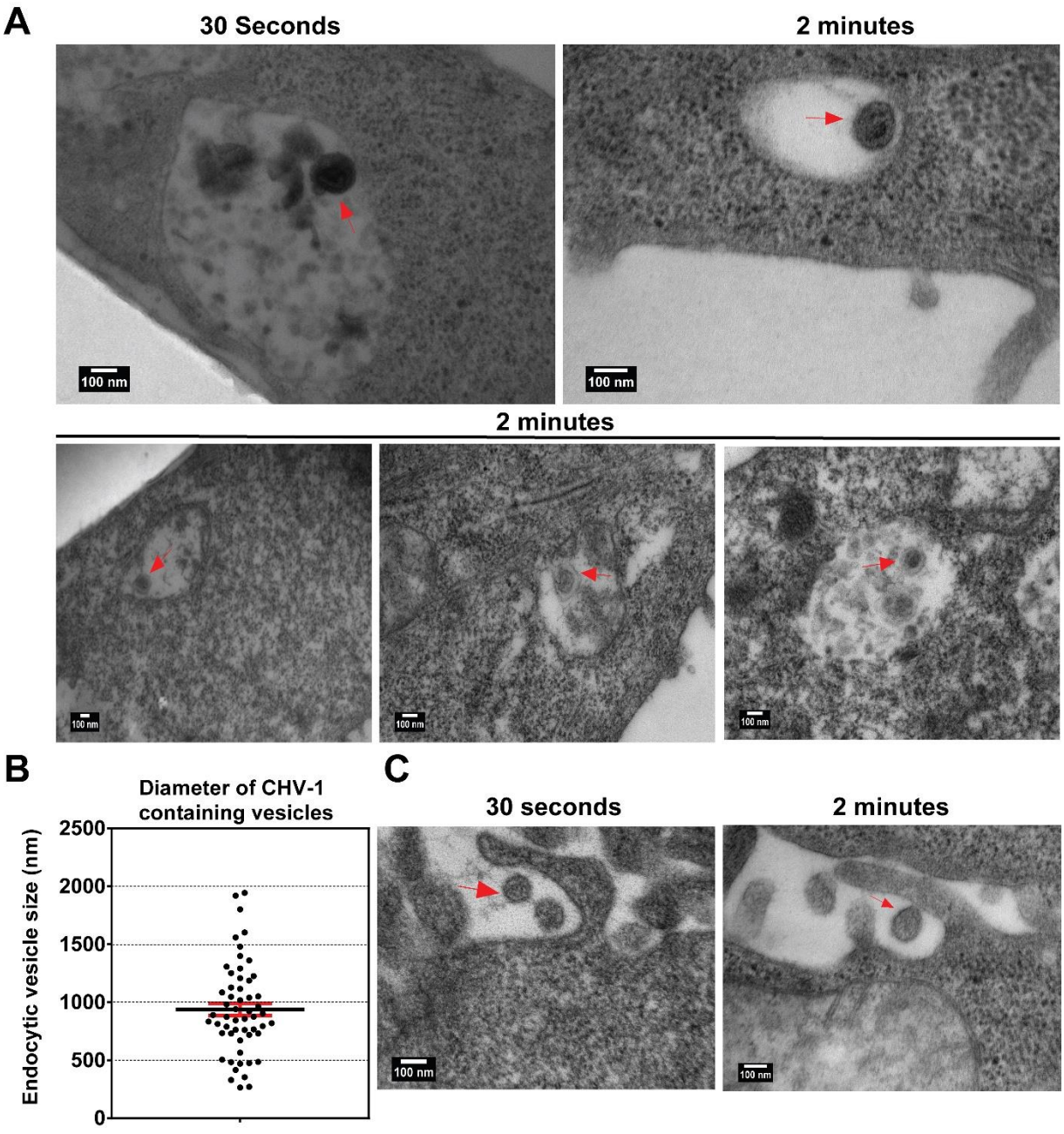


Fig 1. CHV-1 is endocytosed in MDCK cells

CHV-1 was pre-bound to MDCK cells at 4 ^{o}C for 2 h at a multiplicity of infection (MOI) of 2.5, followed by incubation at 37 ^{o}C for 30 sec or 2 min to trigger viral entry. The monolayer was then fixed with glutaraldehyde and processed for TEM. Shown in **(A)** is a representative electron micrograph of a virion within an endocytic vesicle. Red arrows highlight CHV-1 virions. **(B)** The graph shows the size of cytosolic vacuoles containing CHV-1 virions (n = 55). The black bar represents the mean vesicle diameter. **(C)** Representative TEM images showing virions adjacent to plasma membrane protrusions. Scale bars = 100 nm.

3.2 CHV-1 entry is blocked by inhibitors targeting macropinocytosis

To characterize the endocytic uptake of CHV-1 observed by TEM analysis, we used inhibitors targeting key players of different cellular endocytic pathways. The relatively large size of CHV-1 viral particles that must be endocytosed, and accordingly the relatively large size of virus-containing vacuoles, as well as the absence of cytosolic coat, are all consistent with CHV-1 exploiting macropinocytosis for entry into MDCK cells. The main criteria to establish a requirement for macropinocytosis include the dependence of viral entry on actin dynamics and NHE (Mercer & Helenius, 2012). Thus we tested the impact of treating MDCK cells early in infection with several drugs that target these cellular components, namely: the amiloride analog 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), an inhibitor of NHE; the anti-actin polymerizing drug cytochalasin D (Cyt-D); ML-7, a specific inhibitor of myosin light-chain kinase (MLCK); and wortmannin, a PI(3)K inhibitor. A standard plaque reduction assay was used to assess the impact of the inhibitors on CHV-1 replication when present for two hours, following attachment of the virus at 4 ˚C. We confirmed that treatment for 2 h at the highest concentration of each inhibitor used in our experiments, did not significantly affect the viability of MDCK cells (Fig. 2A-B). A resazurin viability assay was used, except for EIPA where a trypan blue exclusion assay was used because EIPA inhibits the mitochondrial reductases such as NADH oxidoreductases (Batista *et al.*, 2011), which are essential for the reduction of resazurin to the highly fluorescent resorufin. For each of the inhibitors tested, we carried out dose response curves. NHE activity is crucial for membrane ruffling and macropinosomes formation. EIPA blocks NHE activity through the reduction of submembranous pH, thus inhibiting the activation of the GTPases Rac1 and Cdc42 and actin polymerization (Koivusalo *et al.*, 2010). EIPA has little impact on other endocytic pathways, and this specificity contributes to its usefulness as a diagnostic test for macropinocytosis (West *et al.*, 1989). When EIPA was present in the media during the first 2 h of infection, the number of plaques was reduced in a dose dependent and statistically significant manner by 64% and 77% (Fig. 2C). Moreover, treatment with Cyt-D, an inhibitor of actin polymerization, also caused a statistically significant reduction in CHV-1 plaque formation (43% and 76%) (Fig. 2C). The dependence of CHV-1 infection of MDCK cells on NHE and actin dynamics demonstrates that the virus relies on the macropinocytic machinery for entry (Mercer & Helenius, 2009). Both PI(3)K (Amyere *et al.*, 2000; Levin *et al.*, 2015) and myosins (Buss *et al.*, 1998; Dowrick *et al.*, 1993) function to modulate membrane ruffling, macropinosome formation, and closure. We next tested the requirement of myosin and PI(3)K signaling early in CHV-1 infection. We found that ML-7 (a specific inhibitor of MLCK) reduced the number of plaques by 84% and 98% (Fig. 2C) at concentrations that did not perturb cell viability (Fig. 2B). Wortmannin (Wort) also caused a statistically significant reduction

in the number of CHV-1 plaques (63% and 80%) (Fig. 2C). Because virus was allowed to attach to cells in the absence of the inhibitors, we could exclude an effect on viral attachment.

To test if the reduction in the numbers of CHV-1 plaques observed with the inhibitors of NHE, actin, MLCK, and PI(3)K was the result of an inhibition of viral entry, and not due to inhibition of post entry events (*e.g.* viral gene expression, translation or viral egress), we compared the impact of adding the drugs at different time points post temperature shift (1-5 h). For each of the four drugs tested, we found that the ability to inhibit viral plaque formation was limited to when the drug was present during the first 2 h of infection, a time that corresponds to the viral entry step (Fig. 2D). Collectively, these results suggest a role for macropinocytosis in CHV-1 entry into MDCK cells.

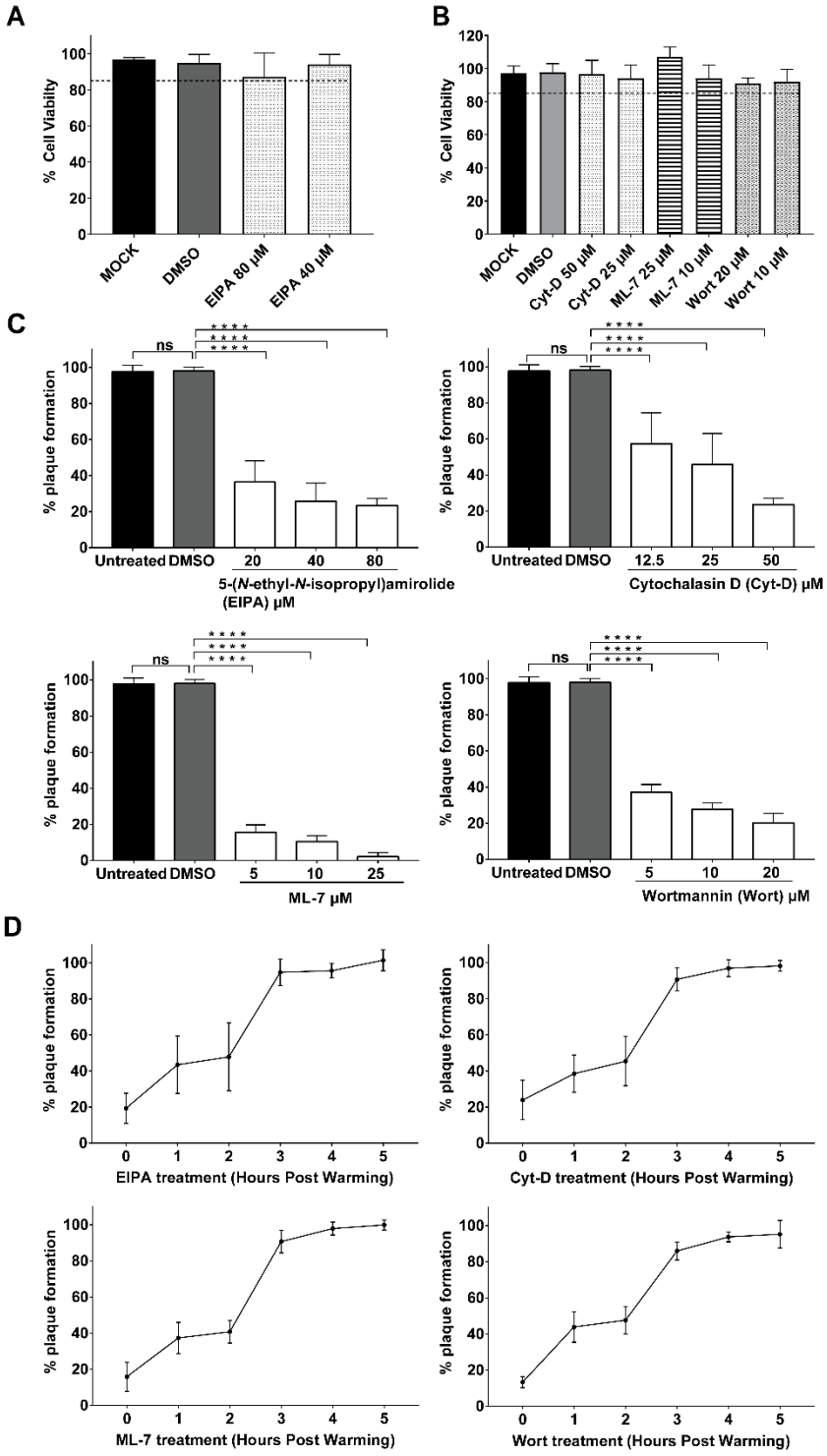


Fig 2. The presence of macropinocytosis inhibitors at early times blocks CHV-1 infection

(A) Impact of EIPA on MDCK cell viability measured by trypan blue exclusion assay. The cells were MOCK- or DMSO-treated, or treated with the indicated EIPA concentrations in duplicate for 2 h (mean ± standard deviation [SD] *n*=3).

(B) Results of resazurin viability assays for MOCK, DMSO-, Cyt-D-, ML-7-, and Wort-treated cells. The assays were performed in triplicate (mean ± standard deviation [SD] *n*=3). The dotted line indicates 85% viability. **(C)** Results of plaque reduction assays showing the impact of DMSO (vehicle alone), EIPA, Cyt-D, ML7, and Wort treatment early in CHV-1 infection. The percentage of plaque formation relative to DMSO-treated cells is shown. **(D)** Results of plaque reduction assays for time-of-addition experiments with inhibitors. CHV-1 (150 pfu) was bound to cells at 4 ˚C for 2 h, then cells were shifted to 37 ˚C. At the indicated time points, cells were treated with the inhibitors for 2 h (EIPA (80 μM), Cyt-D (50 μM), ML7 (25 μM) or Wort (20 μM)), the media with the inhibitors were then replaced by complete medium containing methylcellulose and cells were incubated for 4 days. Results represent the average of three independent experiments. Error bars represent the standard deviation from the mean. ****$P < .0001$.

3.3 Signaling via RTKs and integrins mediates the entry of CHV-1

Macropinocytosis is typically induced via activation of cell surface receptors such as RTKs (including EGFR and PDGFR) or integrins. While activation of RTKs initiates signaling through Rho GTPases (Rac1, and Cdc42) and p21-activated kinase 1 (Pak1), activation via integrins involves signaling through non-RTK focal adhesion kinase (FAK) (reviewed in (Mercer & Helenius, 2009; Mercer & Helenius, 2012)). To investigate the possible role of these cellular signaling pathways in CHV-1 entry, we tested the effect of genistein (an inhibitor of tyrosine kinases), Iressa and 324674 (inhibitors of EGFR), Gleevec (an inhibitor of PDGFR), Y11 (an inhibitor of FAK), IPA-3 (an inhibitor of Pak1), and Calphostin C (an inhibitor of PKC) early in infection in our CHV-1 plaque reduction assay. Using resazurin viability assays we established that the effective concentrations of these drugs were non-toxic to MDCK cells (Fig. 3A). In our assays, we found that CHV-1 plaque formation was significantly reduced in a dose-dependent manner by Iressa, 324674, Gleevec, Y11, IPA-3, and Calphostin C (Fig. 3B). The effect of genistein was less pronounced; nevertheless, a significant reduction in the number of plaques was observed at the highest tested non-toxic concentration of the drug (Fig. 3B). We concluded that CHV-1 entry in MDCK relies on signaling via integrins and the receptor tyrosine kinases (EGFR and PDGFR). Because infection by CHV-1 was significantly inhibited by a panel of drugs targeting signaling proteins involved in macropinocytosis, our results are further evidence in support of the macropinocytic machinery being important for CHV-1 entry into MDCK cells.

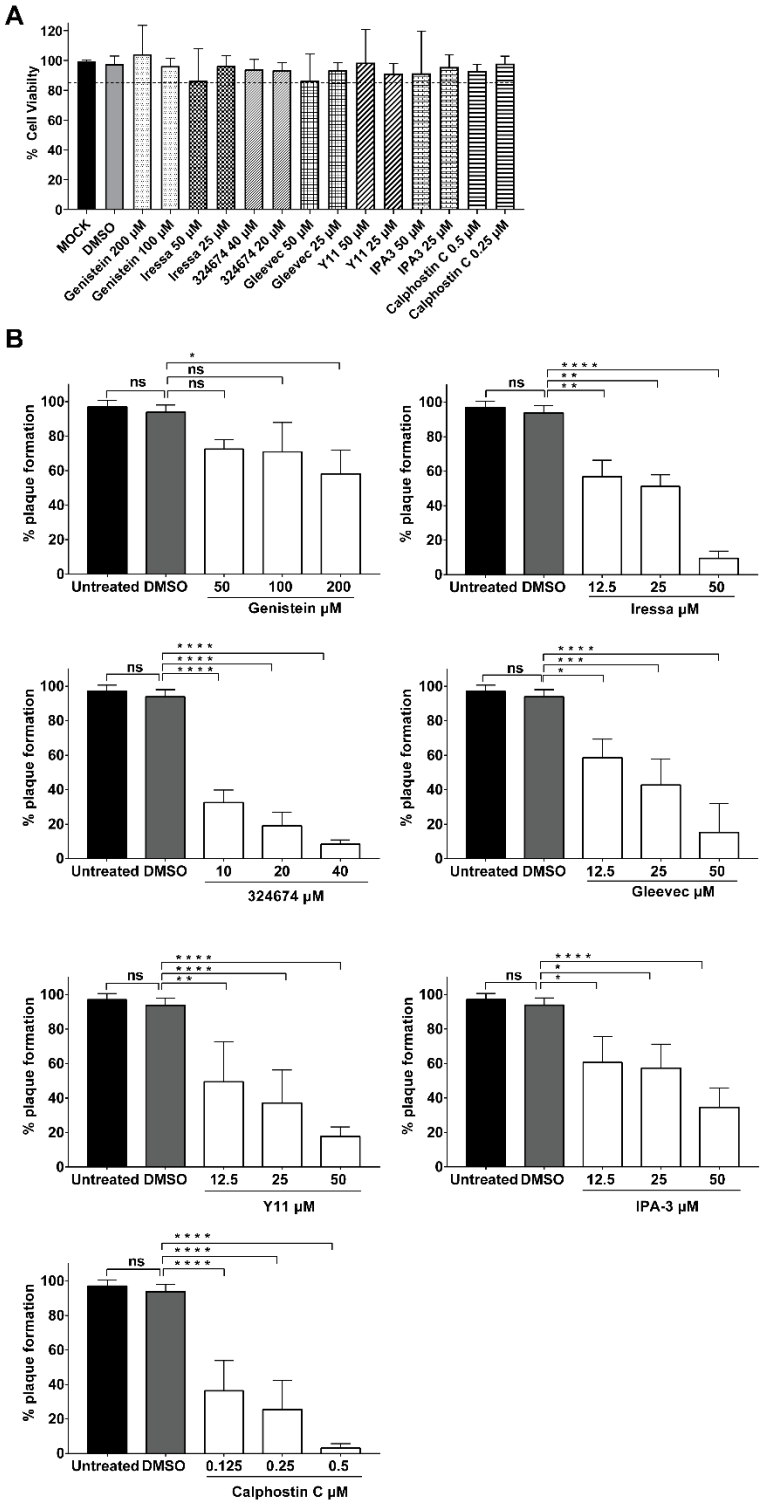


Fig 3. RTK and integrins signalling pathways mediate the entry of CHV-1

(A) Results of resazurin viability assays for MOCK, DMSO-, genistein-, Iressa-, 324674-, Gleevec-, Y11-, IPA3-, and Calphostin C-treated MDCK cells. The assays were performed in triplicate (mean ± standard deviation [SD] *n*=3). The dotted line corresponds to 85% viability. **(B)** Results of plaque reduction assays showing the impact of RTK

and integrins signaling inhibitors early in CHV-1 infection. The test was performed as described in Fig. 2C. The results represent the average of three independent experiments. The error bars represent the standard deviation from the mean. *P < .05, **P < .01, ***P < .001, ****P < .0001, ns = non-significant.

3.4 CHV-1 entry into MDCK cells is pH-independent

We extended our investigation to probe the role of other host factors that may be involved in CHV-1 endocytosis. Once again, we first confirmed that the effective concentration of the drugs used was not toxic to MDCK cells (Fig. 4A). Dynamin functions as a scission factor during vesicle fission from the plasma membrane in clathrin- and caveolin-mediated endocytosis, and in some forms of macropinocytosis (reviewed in (Ferguson & De Camilli, 2012; Mercer & Helenius, 2012; Orth & McNiven, 2003; Schmid & Frolov, 2011)). We tested the impact of treatment with dynasore (Dyn), which inhibits all dynamin isoforms (Harper *et al.*, 2013), on CHV-1 entry. The addition of Dyn at non toxic concentrations resulted in a reduction in CHV-1 plaques formation of 23.2% and 66.7% (Fig. 4B). Similar to our results for drugs tested in Fig. 2, in time of addition experiments, we found that the inhibitory effect of Dyn was limited to the first 2 h of infection, a time corresponding to viral entry (data not shown). We also tested the effect of α-Keto-γ-(methylthio) butyric acid sodium salt (MTOB), which inhibits the scission factor C-terminal binding protein (CtBP); however, treatment of cells with MTOB did not result in a significant difference in plaque formation. We expanded our panel of inhibitors and investigated the possible role of clathrin and caveolin/lipid raft-mediated endocytosis, endosomal acidification, trafficking from early to late endosomal compartments, and microtubules in CHV-1 entry. We tested the impact of chlorpromazine (CPZ) (inhibits clathrin pit formation by blocking the assembly of the clathrin adaptor protein AP2 (Wang *et al.*, 1993)), nystatin (Nyst) (complexes with cholesterol, thus inhibits the formation of lipid rafts in the plasma membrane and caveolin pit formation (Matveev *et al.*, 2001)), Ammonium chloride (a lysosomotropic weak base that raises endosomal pH), bafilomycin A (a selective inhibitor of the vacuolar H^+/ATPase pump, which increases endosomal pH (Yoshimori *et al.*, 1991)), brefeldin A (BFA) (blocks translocation to late endosomes and trafficking from the Golgi to the ER (Klausner *et al.*, 1992)), nocodazole (Noc) (a drug targeting microtubules that inhibits the microtubule-dependent trafficking of endosomal vesicles to late endosomes (Bayer *et al.*, 1998)), and paclitaxel (Tax) (inhibits microtubule dynamics by binding and stabilizing microtubule polymers (Schiff *et al.*, 1979)) on CHV-1 entry. We found that neither CPZ nor Nyst inhibited CHV-1 entry (Fig. 4D). These results are consistent with our TEM results as we observed virions within uncoated vesicles. Moreover, we found that raising endosomal pH through treatment with ammonium chloride had no effect on CHV-1 plaque formation. We further

confirmed this result through the use of bafilomycin A to reduce endosomal pH. Bafilomycin was non-toxic at 50 nM. It has been reported that the ability of bafilomycin concentrations between 10 and 100 nM to raise endosomal pH is cell line-dependent (Ohkuma *et al.*, 1993). Therefore, we first confirmed that a bafilomycin concentration of 50 nM was sufficient to raise endosomal pH in MDCK cells like that seen for a concentration of 100 nM (Fig. Suppl. 1). Then, using bafilomycin early in infection, we once again we found that raising endosomal pH had no impact on plaque formation. Thus, our results indicate that endosomal acidification is not required for CHV-1 to escape the macropinosomes and enter the cytosol. We also found that CHV-1 infection was insensitive to BFA treatment, suggesting that maturation to late endosomes is not essential for CHV-1 entry. Blocking microtubule dynamics by Noc and Tax at these early times in infection had no impact on the virus plaque numbers. In summary, these results show that CHV-1 infection is sensitive to Dynasore, and suggest that CHV-1 entry is pH-independent, and is independent of clathrin, caveolin/lipid rafts, C-terminal-binding protein (CtBP), trafficking to late endosomes and microtubules.

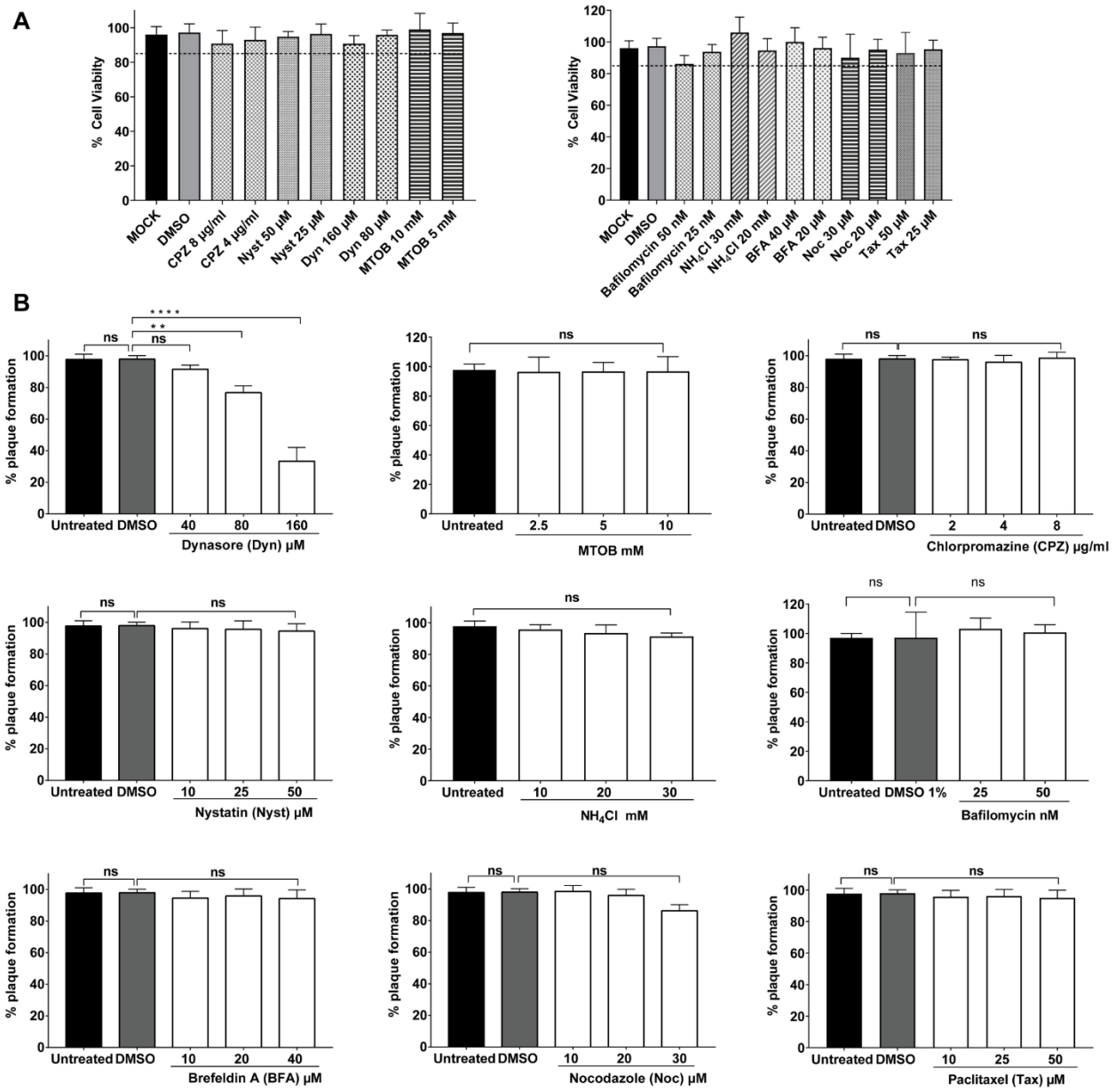


Fig 4. CHV-1 entry in MDCK cells is inhibited by the dynamin inhibitor dynasore but is independent of clathrin- and caveolin-mediated endocytosis, endosomal acidification, and microtubules

(A) Results of resazurin viability assays for DMSO-, chlorpromazine (CPZ)-, dynasore (Dyn)-, nystatin (Nyst)-, α-Keto-γ-(methylthio) butyric acid sodium salt (MTOB), bafilomycin A, ammonium chloride (NH_4Cl)-, brefeldin A (BFA)-, nocodazole (Noc)-, and paclitaxel (Tax)-treated MDCK cells. The assays were performed in triplicate (mean ± standard deviation [SD] *n*=3). The dotted line represents 85% viability. **(B)** Results of plaque reduction assays showing the impact of the dynamin inhibitor dynasore (Dyn), of the inhibitors of C-terminal binding protein (MTOB), clathrin-mediated endocytosis (CPZ), caveolin (Nyst), endosomal acidification (NH_4Cl and Bafilomycin), translocation to late endosomes (BFA), and microtubules (Noc and Tax) on CHV-1 entry. The analysis was performed as described for Fig. 2C. The

results represent the average of three independent experiments. The error bars represent the standard deviation from the mean. *P < .05, **P < .01, ***P < .001, ****P < .0001, ns = non-significant.

3.5 CHV-1 induces lamellipodial membrane ruffles at the surface of MDCK cells

One of the characteristic features of viral entry by macropinocytosis is the induction of actin-mediated, cell-wide plasma membrane ruffling such as lamellipodial, filopodial, circular ruffles, or blebs (reviewed in (Mercer & Helenius, 2009; Mercer & Helenius, 2012)). To test if CHV-1 induces changes to MDCK cell membrane morphology early in infection, we used scanning EM (SEM) to analyze the MDCK cell surface at 10 and 30 min post-viral entry. Mock-infected MDCK cells had a relatively smooth surface with few protrusions (Fig. 5, left-hand panels). In contrast, we found that the virus induced extensive lamellipodial cell membrane ruffling when MDCK cells were incubated with the virus for 2 h at 4 ˚C followed by a 10 or 30 min incubation at 37 ˚C (Fig. 5, middle and right-hand panels respectively). This result is consistent with our TEM images showing viral particles adjacent to protrusions of the plasma membrane (Fig 1C), and is also consistent with a role for macropinocytosis in CHV-1 entry.

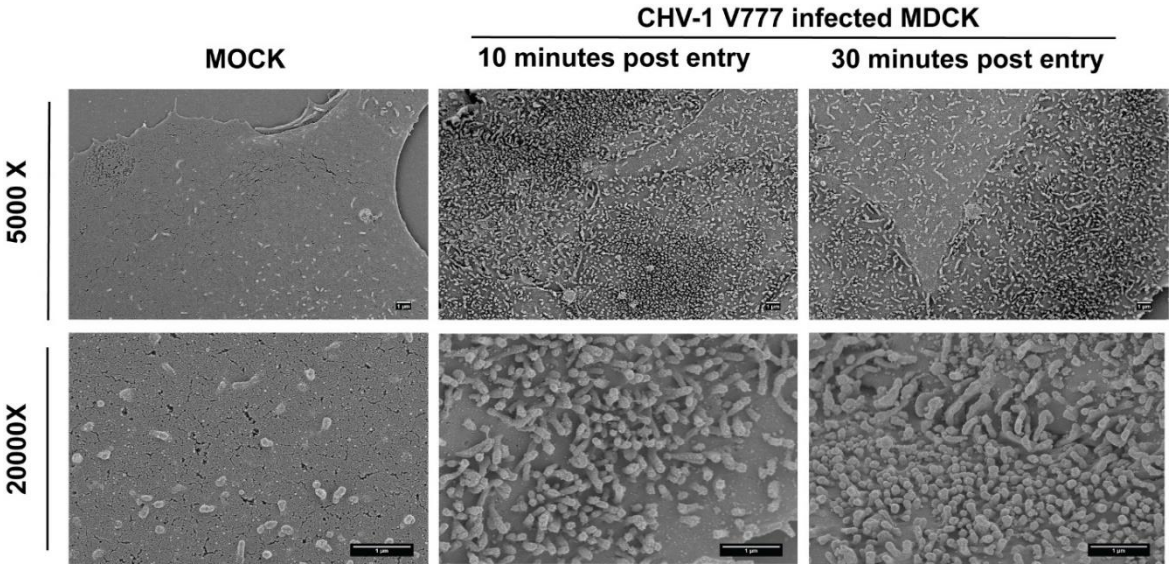


Fig 5. CHV-1 induces lamellipodial membrane ruffles during entry into MDCK cells

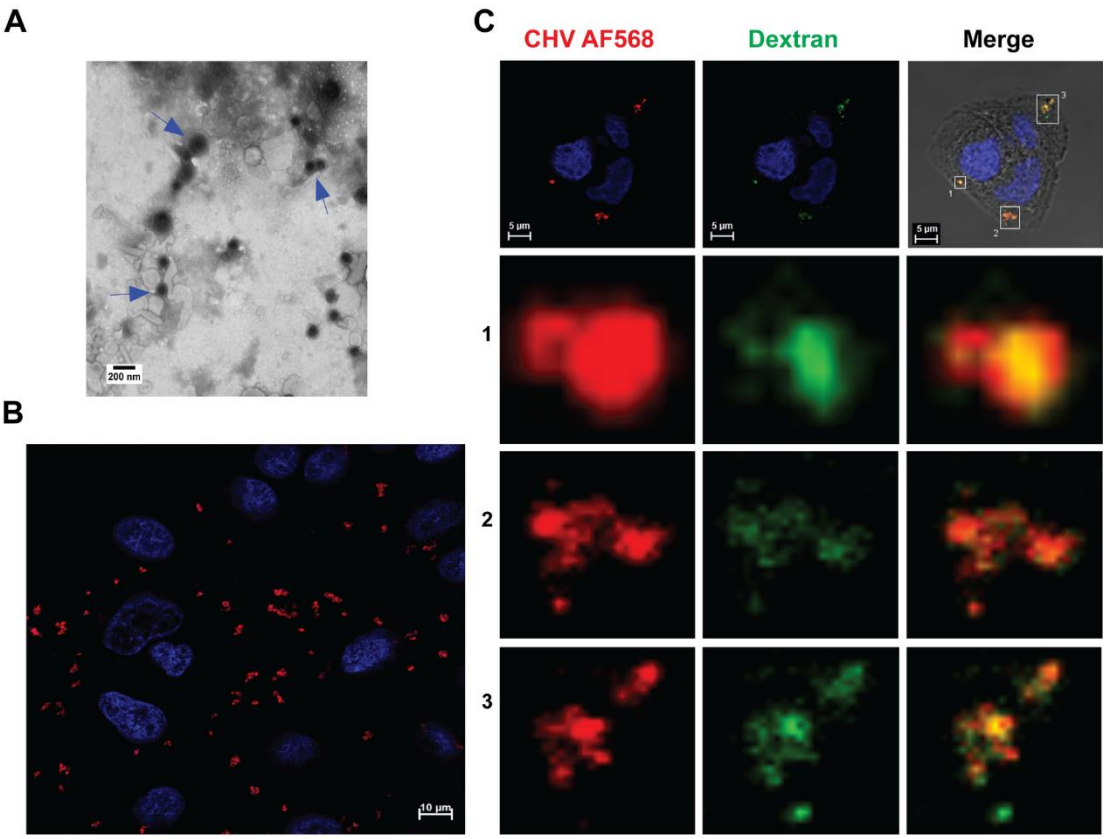
Scanning EM micrographs of mock-infected and CHV-1 V777-infected MDCK cells. CHV-1 was pre-bound to MDCK cells (MOI 2.5) at 4 ºC for 2 h. Following incubation at 37 ºC for 10 or 30 min, the cells were glutaraldehyde fixed and processed for SEM. Total magnification of images in each row is shown at the left of the figure.

3.6 CHV-1 colocalizes with the fluid phase marker dextran but does not increase its uptake

When cells are starved, macropinocytosis is induced to promote the internalization of nutrients present in the local cellular environment. Thus, induction of macropinocytosis typically leads to the transient formation of large, fluid-filled vacuoles (macropinosomes), and a transient increase in the uptake of extracellular fluids (up to 5–10 fold) (Kerr & Teasdale, 2009). To test if CHV-1 internalization is accompanied by fluid-phase pinocytosis, we studied the colocalization of fluorescently-labeled CHV-1 with a fluorescent fluid phase marker, dextran-AF647 (10 kDa). To render the virus particles fluorescent, we used an Alexa Fluor® succinimidyl esters 568 (NHS esters) dye to label the primary amines of the glycoproteins present in the envelope of CHV-1 V777. The labeled virions were then purified on a nycodenz density gradient, and the fluorescent band corresponding to the viral fraction was extracted using a needle and syringe. The presence of intact viral particles in the fraction was confirmed by TEM after negative staining (Fig. 6A). We also confirmed the ability of the labeled virus to infect MDCK cells by titrating the extracted virus in a plaque assay. We next tested the ability to visualize the fluorescently-labeled viral particles (CHV-1 AF568) bound to the surface of MDCK cells. The purified labeled virus was incubated with MDCK cells at 4 ºC for 2 h, then unbound viral inoculum was removed and the monolayer fixed with 4% paraformaldehyde. The fluorescently-labeled virus was visualized at the cell membrane by confocal microscopy (Fig. 6B). To assess if CHV-1 particles were taken up in macropinocytotic vesicles, we carried out colocalization experiments of the labeled virus and fluorescently labeled dextran, a marker for macropinosomes. MDCK cells were pre-incubated with CHV-1 AF568 (MOI 0.2) at 4 ˚C to allow attachment. Virus internalization in MDCK cells then proceeded for 10 min in the presence of 200 µg/ml dextran-AF647 following a temperature shift to 37 °C. By confocal microscopy, we observed multiple instances of co-localized signal for virions with that of dextran (Fig. 6C). We carried out Mander's overlap coefficient analysis of 42 images containing a total of 124 infected cells. We found that approximately 70% of intracellular signal for virions colocalized with the dextran signal (Fig. 6D), demonstrating uptake of the virus into macropinosomes.

Because induction of classic macropinocytosis coincides with an increase in fluid phase uptake, we next tested whether CHV-1 entry induces such an increase, once again using fluorescently-labeled dextran (FITC-Dextran) as a marker. We used multiparametric flow cytometry (FACS) analysis to quantify the uptake of FITC-Dextran in MDCK cells in serum-free medium that were either infected at an MOI 2 with CHV-1 V777 for 10, 20, 30, and 60 min, or that were mock-infected. As a positive control for the stimulation of fluid phase uptake, MDCK

cells were starved in Earl's balanced salt solution (EBSS) prior to incubation with fluorescent dextran, which led to a significant increase in dextran uptake at 60 min (Fig. 6E). Interestingly, our results showed no enhancement of dextran uptake by CHV-1-infected cells relative to mock-infected MDCK cells at any of the time points. Moreover, raising the MOI to 4.5, 9, or 18 did not lead to a statistically significant increase in fluid uptake (Fig. 6F). We obtained similar results when quantitation was carried out on confocal images (Fig. Suppl. 2A). Moreover, similar to our results for infections carried out at low MOI, we found that at a high MOI such as 9, a high percentage of the signal for the virus co-localized with the fluorescent dextran signal (Fig. Suppl. 2B-C). We concluded that the mechanism of CHV-1 entry into MDCK cells, while dependent on the macropinocytic machinery, differs somewhat from classic macropinocytosis.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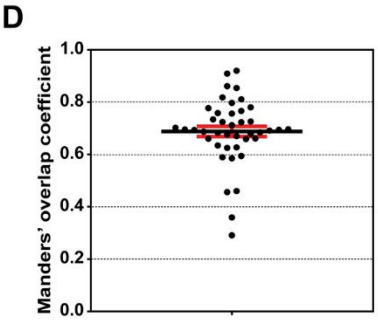
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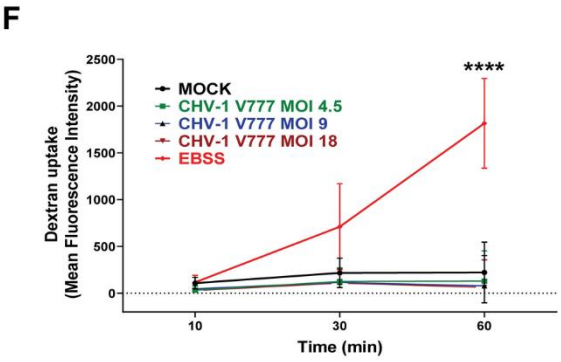
F
Dextran uptake
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1000
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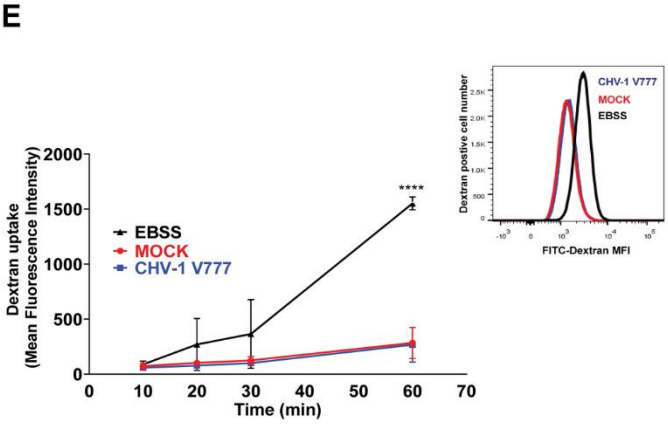
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Fig 6. CHV-1 colocalizes with the fluid-phase, macropinocytosis marker dextran

(A) Transmission electron micrograph showing nycodenz gradient-purified, fluorescently labeled CHV-1 particles after negative staining with phosphotungstic acid. **(B)** Confocal microscopy image showing gradient-purified CHV-1 virions where the glycoproteins were labeled with Alexa Fluor® 568 succinimidyl ester dye. Labeled CHV-1 (red) was bound to MDCK cells at 4 ºC for 2 h, followed by cold fixation with 4% paraformaldehyde. The nucleus was stained with DAPI (blue). **(C)** Confocal microscopy images showing CHV-1-AF568 (left-hand panels, red) colocalization with dextran-AF647 (10 kDa) (middle panels, green) 10 min post-entry. The merge images are shown in the right-hand panels. Z-stacks were recorded by confocal microscopy and a representative image is shown. Values show the portion of the virus signal that colocalized with the signal for dextran. Boxes highlight close-ups of the indicated areas of the image. **(D)** Graph showing the Mander's overlap coefficient analysis of CHV-1 colocalization with dextran as measured by Fiji's Coloc 2 plugin. The black bar represents the mean colocalization value. **(E)** Quantification of dextran uptake by FACS analysis for single, viable MDCK cells for the following conditions: starved in EBSS buffer as a positive control for stimulation of fluid uptake (black); mock-infected in serum-free medium (red), and CHV-1 V777-infected (MOI 2) (blue), at 10, 20, 30 and 60 min post entry (PE)/dextran pulse. The results represent the average of two independent experiments. One hundred thousand cells were counted per sample. The error bars represent the standard error of the mean. The inset shows a typical plot of single, viable dextran positive cell numbers versus the mean fluorescence intensity (MFI) of FITC-dextran 60 min post dextran pulse. **(F)** Quantification of dextran uptake for infection with different MOIs of CHV-1.

3.7 Macropinocytosis-like entry of CHV-1 is strain independent

To assess if the importance of the macropinocytic machinery for CHV-1 entry is strain-dependent, we tested if a different CHV-1 strain, namely 0194, would exhibit the same response to the panel of inhibitors we used for our study using strain V777. We found that similar to strain V777, infection with CHV-1 0194 was independent of clathrin, caveolin, microtubules, endosomal acidification, and trafficking to late endosomes (Fig. 7A). Moreover, infection of MDCK cells with CHV-1 0194 was inhibited by the same drugs that blocked the entry of strain V777 and targeted elements important for macropinocytosis (Fig. 7A and B). One exception was treatment with Wort, which did not block CHV-1 0194 infection, indicating that PI(3)K is not essential for the entry of this strain. Nevertheless, SEM analysis of the CHV-1 0194-infected MDCK cells showed prominent lamellipodial cell membrane protrusions at both 10 and 30 min post entry (Fig. 7C), similar to those observed with strain V777. Together, our results indicate that a macropinocytosis-like pathway represents a general entry mechanism for different CHV-1 strains into MDCK cells.

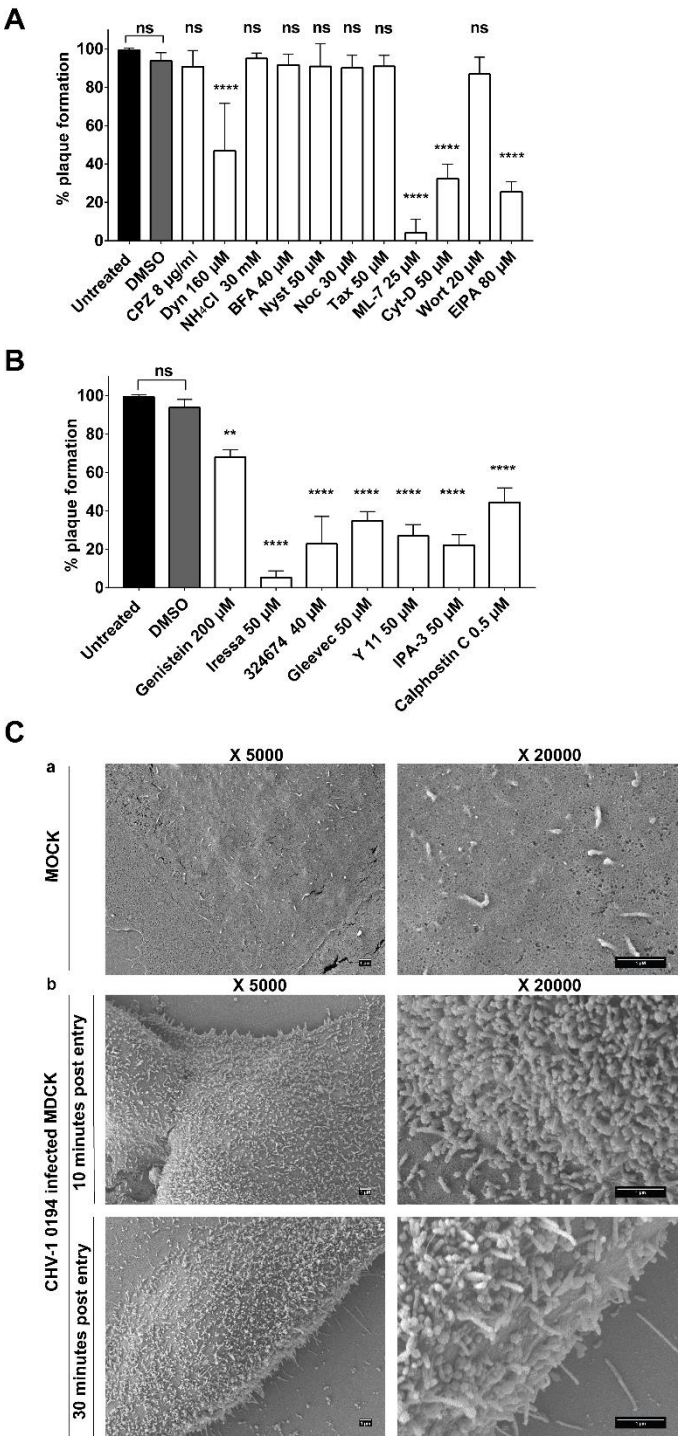

Fig 7. Macropinocytic-like entry of CHV-1 is strain independent

Results of plaque reduction assays for CHV-1 strain 0194 showing the impact of **(A)** DMSO-, chlorpromazine (CPZ)-, dynasore (Dyn)-, ammonium chloride (NH_4Cl)-, brefeldin A (BFA)-, nystatin (Nyst)-, nocodazole (Noc)-, paclitaxel (Tax)-, ML-7-, cytochalasin D (Cyt-D)-, wortmannin (Wort)-, 5-(N-ethyl-N-isopropyl) amiloride (EIPA)-, and **(B)** DMSO-, genistein-, Iressa-, 324674-, Gleevec-, Y11-, IPA3-, and Calphostin C early in infection. The experiments were performed as described for Fig. 2C. The results represent the average of three independent experiments. The error

bars represent the standard deviation from the mean. *P < .05, **P < .01, ***P < .001, ****P < .0001, ns = non-significant. **(C)** Scanning EM of noninfected (a) and CHV-1 0194-infected MDCK cells (b) at 10 and 30 min post viral entry.

4 DISCUSSION

Herpesviridae members are known to use a variety of entry pathways into host cells (Nicola, 2016). The interaction of a particular herpesvirus with the corresponding host cell is specific to each pairing. These differences are not only cell specific, but may also be influenced by specific viral gene products that trigger cell signaling pathways (Azab *et al.*, 2013). The results of our study reveal the entry mechanism of CHV-1 into epithelial cells. Our results combining imaging and chemical inhibitors showed that CHV-1 entry into MDCK cells fulfilled several major conditions defining canonical macropinocytosis in a strain-independent manner, and moreover that the virus was taken up in functional macropinosomes; however, the virus did not stimulate an increase in fluid phase uptake, indicating a role for a macropinocytosis-like pathway for CHV-1 entry.

4.1 Endocytic entry of CHV-1 into MDCK cells

We found that uptake of CHV-1 was very rapid; virions were observed in cytoplasmic vesicles at 30 sec and 2 min following a temperature shift from 4 ˚C to 37 ˚C. Although the aldehyde fixation process, which was used in our TEM experiments, can sometimes affect the apperance of structures in TEM, the effect is mainly with regard to the volume of structures not their 2D dimensions (Murk *et al.*, 2003), therefore the diameters of the vesicles we measured can be expected to accurately reflect their sizes. The large size of CHV-1 virions would initially seem inconsistent with their entry via clathrin or caveolin pits; however, clathrin-coated pits have previously been found to mediate the entry of certain relatively large viruses including vesicular stomatitis virus (Cureton *et al.*, 2010) and African swine fever virus (Hernáez *et al.*, 2016), both of which can reach 200 nm in length, as well as that of cell-free VZV into human embryonic lung fibroblasts (Hambleton *et al.*, 2007). Furthermore, EHV-4 entry into equine epithelial cells has been shown to be dependent on caveolin (Azab *et al.*, 2013). Nevertheless, we found that neither CPZ (inhibitor of clathrin coat assembly) nor Nyst (inhibitor of caveolin pit formation) affected CHV-1 entry into MDCK cells. Moreover, the absence of cytosolic coat on the virus-containing vacuoles observed by TEM further confirms that clathrin and caveolin-mediated pathways are not critical for CHV-1 entry.

4.2 Host factors essential for membrane ruffling and macropinocytosis are required for CHV-1 entry into MDCK cells

Viruses that induce macropinocytosis for entry reorganize the cell membrane by triggering rearrangements of the actin cytoskeleton that result in membrane ruffling. These ruffles can take different forms (lamellipodial, circular, filopodial, or blebs) (Mercer & Helenius, 2009; Mercer & Helenius, 2012; Swanson, 2008). HSV-1 transiently activates the Rho GTPase Cdc42 to induce actin remodeling and long filopodial ruffles that are utilized for virion surfing towards the cell body (Oh *et al.*, 2010), while Rac1 is the cellular Rho GTPase that triggers lamellipodial and blebbing membrane ruffles during viral entry (Mercer *et al.*, 2010a; Tan *et al.*, 2008; Taylor *et al.*, 2011b). TEM revealed the presence of CHV-1 virions at the plasma membrane associated with membrane protrusions that we hypothesized could fold back and engulf the virion through macropinocytosis. The requirement of specific cellular factors for macropinocytosis-based entry varies depending on the virus and host cell (de Vries *et al.*, 2011; Krzyzaniak *et al.*, 2013; Mercer *et al.*, 2010a; Mercer *et al.*, 2010b); however, NHE and actin dynamics are essential (Mercer & Helenius, 2012). By reducing submembranous pH, the NHE inhibitor EIPA blocks the activation of the GTPases Rac1 and Cdc42, thus actin polymerization and membrane ruffling are inhibited (Koivusalo *et al.*, 2010). We found that EIPA strongly reduced CHV-1 entry. These results are consistent with studies of HSV-1 entry in Vero, HeLa, HEp-2, and PtK_2 cells (Devadas *et al.*, 2014) and HCMV entry in fibroblasts (Hetzenecker *et al.*, 2016). Actin dynamics are important for multiple steps in virus replication: membrane ruffling, viral fusion with endosomal and plasma membranes, the transport of cytosolic capsids towards microtubules, and virion egress (Roberts & Baines, 2011; Taylor *et al.*, 2011b). The perturbation of the actomyosin machinery by Cyt-D and ML-7, specifically at early times in infection, was also found to reduce CHV-1 entry. In the context of classic macropinocytosis, PI(3)K is an important host factor for membrane ruffling, macropinocytic cup closure, and macropinosome maturation and fusion (Amyere *et al.*, 2000; Araki *et al.*, 2007; Levin *et al.*, 2015; Lindmo & Stenmark, 2006; Swanson, 2008). The effect of the PI(3)K inhibitor Wort on HSV-1 infection has been demonstrated to be highly cell type dependent (Clement *et al.*, 2006; Devadas *et al.*, 2014; Gianni *et al.*, 2004; Gianni *et al.*, 2013; Nicola & Straus, 2004). We observed that the impact of Wort on CHV-1 was strain dependent in that it reduced CHV-1 V777 replication significantly, but had no impact on CHV-1 0194. A similar variability in response to Wort inhibition has been reported for vaccinia virus strains IHD-J and WR, and corresponds to differences in the activation of Rho GTPases by the two strains (Mercer *et al.*, 2010a). However, in that case, unlike in our study, a difference in the response to the tyrosine kinase inhibitor genistein was also observed for the different strains. While several tyrosine kinase inhibitors

blocked CHV-1 entry, we detected only a modest effect of genistein on CHV-1 replication; genistein has also been shown to affect VZV replication, but at a late stage in infection (Hambleton *et al.*, 2007). Although Wort was shown not to have an effect on VZV early in infection (Hambleton *et al.*, 2007), the concentrations used in that report were lower than those used in our experiments. In the case of the vaccinia virus strains IHD-J and WR that exhibit different sensitivities to Wort, the strains also induced different ruffling patterns (Mercer *et al.*, 2010a); however, our SEM results showed that the CHV-1 strains V777 and 0194 both induced lamellipodial membrane ruffles similar to those described for adenovirus type 2 (Meier *et al.*, 2002b). Thus, dependency on PI(3)K is not in and of itself indicative of this specific plasma membrane morphology during macropinocytosis. Our results suggest that different CHV-1 strains may have different impacts on the activation of downstream GTPases and kinases, which nevertheless ultimately activate the macropinocytic machinery to drive entry.

IPA-3 (the inhibitor of Pak1), ML-7 (a specific inhibitor of MLCK), and Calphostin C (inhibitor of PKC) were all found to inhibit CHV-1 replication at early times. Pak1 is required for all stages of macropinocytosis, myosins are important for macropinosome closure, and PKC activation triggers plasma membrane ruffling and macropinosome formation (Mercer & Helenius, 2009). These proteins are also involved in macropinocytic uptake of other herpesviruses (Devadas *et al.*, 2014; Hetzenecker *et al.*, 2016; Lv *et al.*, 2018a). Our study excluded CtBP as a scission factor for endosome formation during CHV-1 entry. Dynamin acts as a scission factor not only in clathrin- and caveolin-mediated endocytosis but also in some forms of macropinocytosis, and is involved in the regulation of actin and microtubule dynamics (reviewed in (Ferguson & De Camilli, 2012; Orth & McNiven, 2003; Schmid & Frolov, 2011)). We found that the dynamin inhibitor dynasore blocked CHV-1 early in infection, consistent with observations for HSV-1 and HCMV (Devadas *et al.*, 2014; Hetzenecker *et al.*, 2016), although the internalization of PrV into HeLa cells is not inhibited by this drug (Lv *et al.*, 2018a). However, given that results using dynasore in dynamin knock-out cells indicates off-target inhibitory effects on macropinocytosis (Park *et al.*, 2013), further studies must be conducted to confirm the role of dynamin in CHV-1 infection. Our time-of-addition experiments showed that the drugs that inhibited CHV-1 replication when present early in infection, but following adsorption, had a significant impact when added upto 2 hours post temperature shift to trigger entry. Although the initial endocytosis of viral particals can be very fast, subsequent stages of viral entry may occur over a longer time period, which may explain the impact of some of the drugs in the second hour after entry was triggered.

4.3 Perturbing the RTK and integrin signaling pathways blocks entry of CHV-1

Macropinocytosis induction usually involves signaling through cell surface receptors like RTKs (EGFR and PDGFR) and integrins (through activation of FAK) (Mercer & Helenius, 2012). Perturbing these signaling pathways via genistein (an inhibitor of TK), Iressa and 324674 (inhibitors of EGFR), Gleevec (PDGFR inhibitor), and Y11 (an inhibitor of FAK) was found to significantly reduce CHV-1 entry; however, the infection was less sensitive to genistein than to the other inhibitors of this class. An inhibitory effect of genistein on HSV-1 entry has also been reported, although strong inhibition was only seen at concentrations higher than we used in our experiments (300-400 µM) (Clement *et al.*, 2006). These findings suggest that signaling through both RTKs and integrin pathways is important for macropinocytic-like entry of CHV-1 in MDCK cells.

4.4 Low endosomal pH and microtubules are dispensable for CHV-1 entry

Viruses that enter by endocytosis may require endosomal acidification for the induction of conformational changes in viral fusion proteins that allow for successful penetration into the cytosol, but this requirement is not absolute. HSV-1 varies in its requirement for endosomal acidification in a cell type-dependant manner (Devadas *et al.*, 2014; Milne *et al.*, 2005; Nicola *et al.*, 2003). We found that CHV-1 entry was insensitive to treatment with ammonium chloride, a weak base that elevates the pH of acidic compartments and inhibits the entry of viruses that rely on endosomal acidification. Moreover, CHV-1 entry was not blocked when endosomal pH was raised by the treatment of cells with bafilomycin A. This result contrasts findings for the entry of the varicelloviruses bovine herpesvirus 1, equine herpesvirus 1, PrV, and VZV, which have each been shown to depend on low endosomal pH (Finnen *et al.*, 2006; Hasebe *et al.*, 2009; Miller *et al.*, 2019; Pastenkos *et al.*, 2018). We cannot rule out that the differences are due to the cell type and not the virus. Regardless, our results lead us to conclude that a different mechanism must be driving fusion of the CHV-1 envelope with the endosomal membrane in MDCK cells, such as the interaction of one or more of the viral glycoproteins with different cellular receptors, or cellular or viral proteases whose actions lead to a conformation change in the viral fusion protein that activates it (discussed in (Nicola, 2016; White & Whittaker, 2016)).

Microtubules function in endosomal trafficking and also mediate HSV-1 capsids transport towards the nucleus (Apodaca, 2001; Sodeik *et al.*, 1997). Treatment of MDCK cells with the microtubule-disrupting drugs nocodazole and paclitaxel at early times in infection showed no reduction in CHV-1 entry. Nocodazole treatment has no effect on CMV binding or entry; however,

microtubules are critical for post entry capsid transport to the nucleus (Ogawa-Goto *et al.*, 2003). Paradoxically, podofilox, a microtubule polymerization inhibitor, has been found to block CMV entry. It has been proposed that this drug may have a different impact on the more dynamic microtubules at the cell surface compared to the more stable intracellular microtubules (Cohen *et al.*, 2016). It remains to be determined if the sensitivity of HCMV entry to podofilox reflect a mechanistic difference in the macropinocytic-like entry pathways of CHV-1 and HCMV.

4.5 The macropinocytosis-like entry of CHV-1 into MDCK is not associated with increased uptake of fluids

In macropinocytosis-based entry of viruses, the folding back of the membrane ruffles that ultimately fuse with the plasma membrane leads to internalization of relatively large volumes of extracellular fluids along with the membrane-associated viruses (Mercer & Helenius, 2009; Mercer & Helenius, 2012), such as what has been seen for vaccinia virus entry into HeLa cells (Schmidt *et al.*, 2011). Cells often sample their environment leading to a low basal level of macropinocytosis. Accordingly, we regularly detected a small number of vesicles containing fluorescent dextran in both infected and uninfected cells. Interestingly, although CHV-1 induced membrane ruffling and colocalized with the fluid-phase marker dextran, it did not increase dextran uptake suggesting that the virus does not enter by classic macropinocytosis, but rather uses a macropinocytosis-like entry mechanism. A similar phenomenon has been reported for *Chlamydia trachomatis* where bacteria colocalized with dextran but did not enhance its uptake (Ford *et al.*, 2018), and for Adeno-associated virus 2, where the virus triggered lamellipodial plasma membrane ruffling but did not increase fluid-phase uptake (Nonnenmacher & Weber, 2011). Because we did not detect the stimulation of fluid uptake during CHV-1 entry, we conclude that the induction of lamellipodia formation does not serve to increase the uptake of virus present in the fluid bathing the cells. Rather, a more likely model is one whereby the lamellipodia serve to increase the surface area of the plasma membrane to which the virus can adsorb.

In this study, we report the entry mechanism for CHV-1 into MDCK epithelial cells. Our TEM results showed CHV-1 virions present in large, uncoated vacuoles shortly after infection. The characteristics of this uptake fulfilled major criteria for macropinocytosis as it was blocked by inhibitors targeting NHE and actin dynamics. Moreover, the results of SEM indicated that the virus triggers extensive lamellipodial plasma membrane ruffling at early times in infection. Entry was found to be pH-independent, indicating that the viral fusion machinery must be activated in a manner other than endosomal acidification. The mechanism of CHV-1 entry was largely strain independent as all features of entry were conserved for the two different CHV-1 strains tested

except for sensitivity to Wort, which was only observed for one of the two viral strains, indicating the virus can bypass PI(3)K for entry into MDCK cells. Interestingly, although the virus colocalized with the fluid phase uptake marker (dextran) it did not induce an increase in its uptake, indicating that the entry mechanism of the virus depends on the macropinocytic machinery, but deviates from classic macropinocytosis. This entry pathway may offer novel cellular targets for developing antiviral drugs against this virus and possibly for other varicelloviruses.

5 MATERIALS AND METHODS

5.1 Cells and viruses

MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 8% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. The CHV-1 strains V777 and 0194 (Papageorgiou *et al.*, 2016) were kindly provided by Dr. Andrew Davison (University of Glasgow).

5.2 Small compound inhibitors and viability assays

The chemical inhibitors chlorpromazine, α-Keto-γ-(methylthio) butyric acid sodium salt (MTOB), dynasore, NH_4Cl, nystatin, nocodazole, cytochalasin D, ML-7, EIPA, 324674, and genistein were obtained from Sigma. The inhibitors paclitaxel, brefeldin A, bafilomycin A, wortmannin, and Iressa were obtained from LC Laboratories. Calphostin C, IPA-3, and Y11 were obtained from Tocris, and Gleevec from Selleckchem. A resazurin viability assay was used for all the inhibitors except EIPA, for which a trypan blue exclusion assay was used to assess toxicity. MDCK cells were seeded in triplicate at a density of 5×10^3 cells per well of a 96 well plate and incubated overnight at 37 °C. The next day, the media was replaced with fresh DMEM containing 2% FBS and the indicated drug concentration, and the cells were incubated at 37 °C for 2 h. The medium with the inhibitors was then removed, replaced with a serum-free medium containing 10% resazurin (R&D Systems, Inc), and incubated at 37 °C for 3 h. Cell viability relative to DMSO-treated cells was measured according to the manufacturer's instructions using a Bio-Tek Synergy™ HT multi-detection microplate reader.

Since the resazurin assay measures the activity of the mitochondrial respiratory chain reductases which are inhibited by EIPA (Batista *et al.*, 2011), we tested the viability of EIPA-treated cells using a trypan blue exclusion assay. A cell suspension (10 µl) was mixed with 0.4% trypan blue

(Bio-Rad) at a ratio of 1:1, and cell viability measured via Bio-Rad TC20^{TM} automated cell counter according to the manufacturer's instructions.

5.3 Transmission electron microscopy

To visualize CHV-1 entry events using transmission electron microscopy (TEM), 5 × 10^5 MDCK cells were cultured in 35 mm tissue culture dishes overnight. The next day, the media was replaced with fresh, cold DMEM supplemented with 2% FBS and buffered with 25 mM HEPES, and the cells were chilled at 4 °C for 20 min. CHV-1 V777 was then added to the cells at an MOI of 2.5 and incubated for 2 h at 4 °C in DMEM medium supplemented with 2% FBS and buffered with 25 mM HEPES. After 2 h, the dishes were shifted to 37 °C for 30 sec or 2 min to trigger viral entry. Cells were then processed for TEM, essentially as described previously (Döhner *et al.*, 2018), and ultrathin sections were prepared for analysis using Ultramicrotome (LKB, Sweden). Final staining was performed using 5% uranyl acetate in 50% ethanol for 15 min, followed by lead citrate for 5 min. All electron micrographs were taken with on Hitachi H-7100 transmission electron microscope at 75 kV with an AMT-XRIII camera. The size of viral particles and endocytic vacuoles were measured using Fiji software (fiji.sc) (Schindelin *et al.*, 2012).

5.4 Inhibitor treatment and plaque reduction assays

5 × 10^5 MDCK cells were seeded per well of a 6-well plate. The next day, media was replaced with fresh, cold DMEM supplemented with 2% FBS and buffered with 25 mM HEPES, and the cells were incubated at 4°C for 20 min. One hundred and fifty pfu of CHV-1 were then added to each well, and the plates were incubated for 2 h at 4 °C to allow for viral adsorption to the cells. Following this step, the inoculum was removed and replaced with a medium containing the indicated inhibitor, and cells were then placed at 37 °C for 2 h to trigger viral entry. Alternatively, for time of addition experiments, the inhibitors were added at the specified time point post warming. Next, the medium containing the inhibitors was removed and the monolayer was washed twice with DMEM supplemented with 2% FBS, following which the cells were overlaid with 2 ml of DMEM supplemented with 2% FBS and 0.4% methylcellulose. Plates were incubated at 37°C for 4 days, and then fixed and stained with crystal violet. Plaques were counted manually using a Nikon (SMZ800) stereomicroscope.

5.5 Scanning electron microscopy

5×10^4 MDCK cells were seeded on glass coverslips in 24-well tissue culture plates. The next day, the media was replaced with fresh, cold DMEM supplemented with 2% FBS and buffered with 25 mM HEPES, and incubated at 4 °C for 20 min. CHV-1 was then added to the cells at an MOI of 2.5 and incubated for 2 h at 4 °C in DMEM supplemented with 2% FBS and buffered with 25 mM HEPES. After 2 h, the plates were shifted to 37 °C for 10 or 30 min to trigger viral entry. The cells were fixed overnight in 2.5% glutaraldehyde diluted in a 0.05 M cacodylate buffer containing 3% sucrose at a pH of 7.4, followed by three washes with 0.5 ml cacodylate buffer. Samples were then processed by the INRS-Centre Armand-Frappier Santé Biotechnologie electron microscopy facility. After a postfixation step (2% osmium tetroxide diluted in a s-collidine buffer), the cells were washed three times with cacodylate buffer, dehydrated in ethanol, critical point dried, and coated with 5 nm palladium-gold in a sputter coater. Electron micrographs were acquired using a high-resolution scanning electron microscope (Hitachi SU-8230 or JEOL JSM-7400 F) with LEI detector, at 3 kV.

5.6 Fluorescent labeling and gradient purification of CHV-1

Alexa Fluor® succinimidyl esters 568 dye (NHS esters) (Thermo Fisher Scientific) was used to label the primary amines of CHV-1 V777 viral envelope glycoproteins, essentially as described previously (Hoffmann *et al.*, 2018). 8.27 $x10^6$ pfu of CHV-1 was concentrated by microcentrifugation and resuspended in MNT buffer (20 mM morpholino-ethane-sulfonic acid (MES), 100 mM NaCl, 30 mM Tris, pH 7.5). Labeling was carried out by incubating the virions with 4.5 µM of the dye at room temperature for 2 h in the dark with gentle mixing on a shaker. The labeled virion particles were banded in a density gradient of 10-40% (w/v) Nycodenz (Accurate chemical & scientific corporation) dissolved in MNT buffer. Centrifugation was performed using a SW41 rotor in a Beckman L-100 XP ultracentrifuge at 20000 rpm (acceleration and deceleration set to 5) for 2 h, at 4 °C. Unbound dye remained at the top of the gradient and the resulting viral band was extracted with a syringe and needle, aliquoted, and stored at -80 ˚C. The titer of the stock of fluorescent virions was determined via standard plaque formation assay.

5.7 Fluid phase colocalization

5×10^4 MDCK cells were seeded on glass coverslips in wells of 24-well plates for 18 h. Cells were then incubated in serum-free medium for 3 h at 37 °C and chilled at 4 °C for 20 min. AF568-labeled CHV-1 V777 was bound to the cells at an MOI of 0.2, at 4 °C for 2 h. Unbound viral

inoculum was removed, and the cells were pulsed with serum-free medium containing 200 µg/ml dextran-AF647 (10 kDa) (Invitrogen). For experiments using a higher MOI, a concentration of 500 µg/ml of dextran-AF647 was used. The cells were incubated for 10 min at 37 °C, after which the dextran-containing medium was removed, and cells were washed once with 500 µl of ice-cold PBS per well. Surface-bound but non-internalized dextran was removed by washing with 1 ml of an ice-cold solution of 0.1 M sodium acetate, 0.05 M NaCl, pH 5.5, for 10 min. The cells were washed twice with 500 µl of ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Coverslips were then washed three times in PBS, incubated for 10 min with 300 nM DAPI (Molecular Probes) followed by three washes with PBS, and then mounted onto glass slides using Prolong Gold antifade reagent (Invitrogen) or Mowiol® (Sigma). Slides were visualized using a Zeiss LSM780 laser scanning confocal microscope. The Manders' overlap coefficient analysis was performed using Fiji software (fiji.sc) (Schindelin *et al.*, 2012).

5.8 Fluid phase uptake

Fluid phase uptake was quantified using a FITC-labelled dextran, essentially as described previously (de Vries *et al.*, 2011; Meier *et al.*, 2002b). 3×10^5 MDCK cells were grown in 6-well plates overnight. The next day, cells were incubated for 2 h in serum-free DMEM at 37 °C, then chilled at 4 °C for 20 min. CHV1-V777 was bound to the cells at an MOI of 2.5, 4.5, 9 or 18 as indicated in the text for 2 h at 4 °C in serum-free DMEM. The infectious medium was then removed and replaced with 500 µl FITC-Dextran (10 kDa) (Sigma) or AF647-Dextran (10 kDa also) (Invitrogen) diluted in DMEM, and the cells were incubated at 37 °C for the indicated times. Dextran uptake was terminated by the addition of 2 ml ice-cold PBS, and cells were washed once with 500 µl ice-cold PBS. Surface-bound non-internalized dextran was washed off with 1 ml of a solution of ice-cold 0.1 M sodium acetate, 0.05 M NaCl, pH 5.5, for 10 min. The cells were then washed twice with ice-cold PBS, detached from the wells by treating with 0.25% trypsin (Wisent Inc) for 25 min, transferred into microcentrifuge tubes containing 0.5 ml DMEM with 8% FBS, pelleted for 5 min at approximately 1000x *g*, then 7-AAD (7-amino-actinomycin D) diluted in PBS was added in order to identify viable cells. The cells were incubated at 4 °C for 10 min then fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and resuspended in 350 µl PBS. Mock-infected MDCK cells in serum free-medium, or starved for 2 h in Earle's balanced salt solution (EBSS) (GIBCO®) were analyzed in parallel to the infected cells. FACS analysis was carried out at the INRS, Centre Armand-Frappier Santé Biotechnologie flow cytometry facility. For each sample within an experiment, the percentage of dextran positive, single cells was similar. Mean fluorescence intensity (MFI) was measured for single viable cells

and the background fluorescence obtained for MDCK that were not pulsed with dextran was subtracted. Data were acquired on a BD LSRFortessa™ (BD Biosciences) cytometer and analyzed by FACS Diva software (Version 8.0.1).

5.9 Statistical analysis

The results presented for the experiments comparing the effect of the various inhibitors were collected from a minimum of three independent experiments unless mentioned otherwise. Data were analyzed using GraphPad Prism software version 8 (GraphPad, La Jolla, CA). The statistical differences between drug treated samples and MOCK- or DMSO-treated samples were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. In Fig. 6E, the statistics shown are the result of a one-way ANOVA followed by Tukey's multiple-comparison test. The results presented are expressed as means ± standard deviations (SD). *P* values of $< .05$ were considered to be statistically significant.

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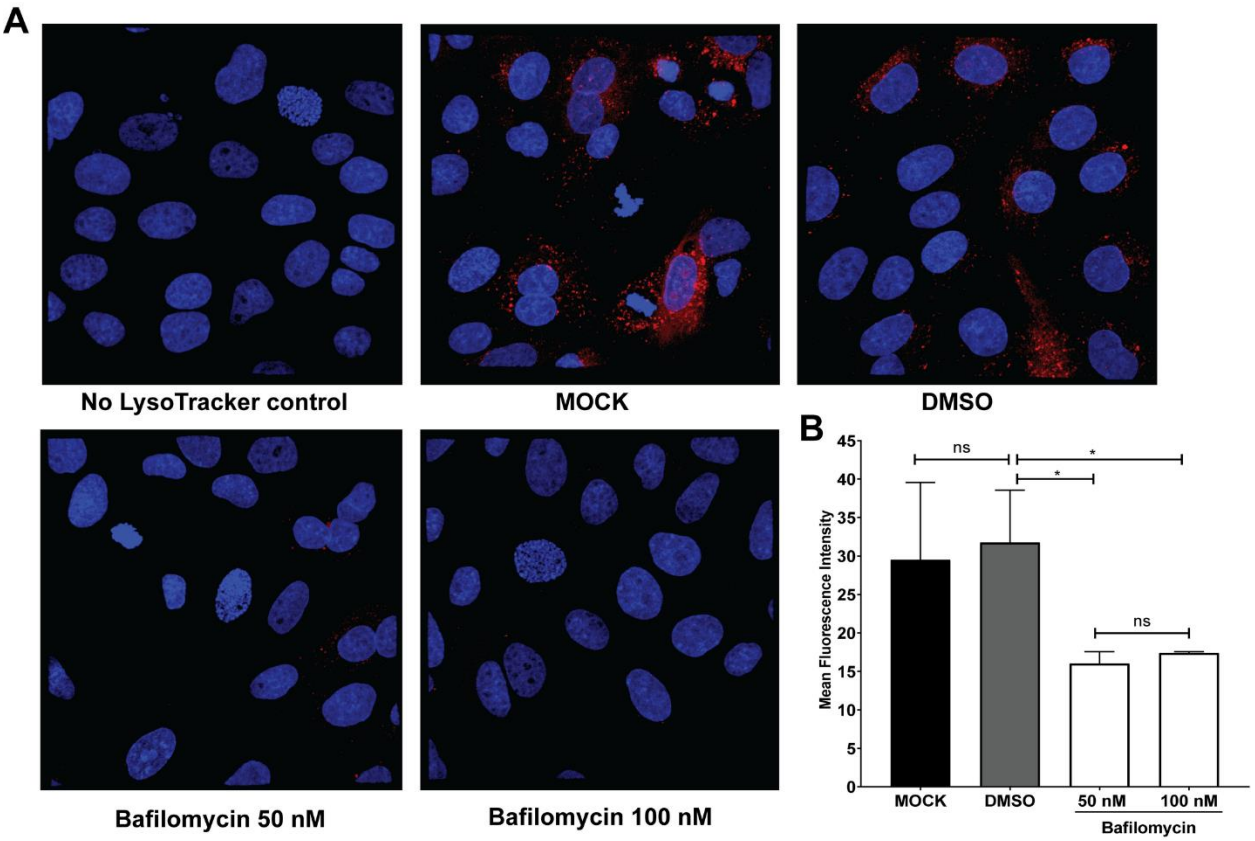
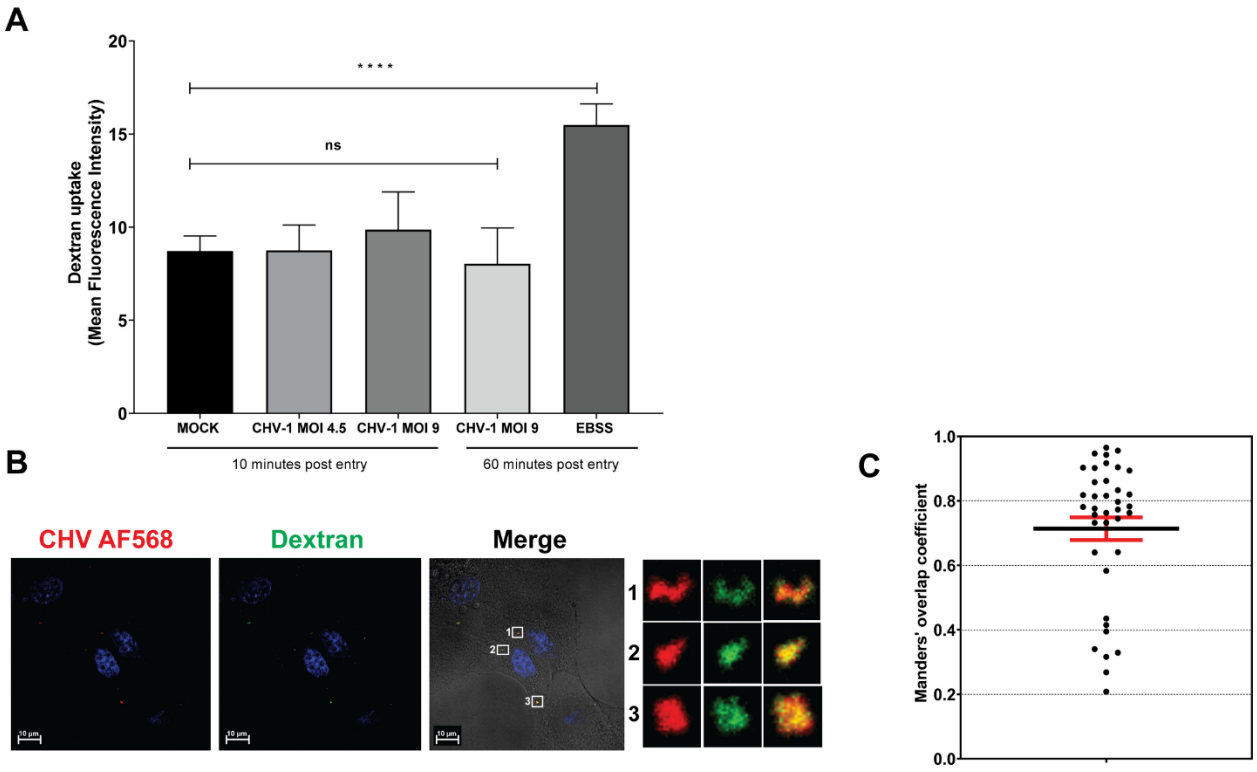
Supplementary figures:



Supplementary Fig1: Inhibition of endosomal acidification in MDCK cells by bafilomycin-A1:

MDCK cells cultured on coverslips in 24-well plates were treated with bafilomycin-A1 (50 or 100 nM) for 2 hours at 37^{o}C. Then, the inhibitor containing medium was removed, and the cells were incubated with medium containing 200

nM LysoTracker Red DND-99 for 1 hour at 37°C. The cells were then processed for confocal microscopy. The nucleus was stained with DAPI (blue). As controls, cells that were untreated or treated only with the vehicle DMSO, were included in the experiment as well as untreated cells that were not incubated with LysoTracker Red. **(A)** Confocal images showing LysoTracker Red DND-99 staining of MOCK, DMSO- or bafilomycin-A1-treated (50 nM and 100 nM) MDCK cells. **(B)** Histogram showing the quantification of mean fluorescence intensity (MFI) for the signal of LysoTracker Red DND-99 in MDCK cells under the specified treatment conditions. MFI was determined using Icy bioimage analysis software from Z-stacks containing from 33 to 80 cells in total for each condition. MFI values are expressed as mean ± standard deviation. *P < 0.05, and ns = non-significant.



Supplementary Fig 2: Colocalization of CHV-1 with fluorescent dextran without induction of fluids uptake for infection at higher MOIs:

(A) Quantification of dextran uptake (expressed as mean fluorescence intensity MFI) at the indicated time points post entry in MOCK, CHV-1 V777 (MOI 4.5 or 9) infected MDCK cells, and EBSS starved cells (as a positive control for stimulation of fluid-phase uptake). The MFI was calculated using Icy software (http://icy.bioimageanalysis.org/) from projected confocal microscopy Z-stacks containing a total of 156-243 MDCK cells per condition. **(B)** Confocal microscopy images showing CHV-1-AF568 (MOI 9) (first panel, red) colocalization with dextran-AF647 (10 kDa, 500 µg/ml) (second panel, green) at 10 min post-entry. The merge image is shown in the third panel. Z-stacks were recorded by confocal microscopy and a representative image is shown. Boxes highlight close-ups of the indicated areas of the image. **(C)** Quantifation of the portion of the virus signal that colocalizes with the signal for dextran. Graph shows the

Mander's overlap coefficient analysis of CHV-1 colocalization with dextran as measured by Fiji's Coloc 2 plugin. The black bar represents the mean colocalization value (0.74). (data was collected from 38 images from 5 Z-stacks containing a total of 319 cells).

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CHAPITRE TROIS: DISCUSSION

1 CHV-1 ENTERS MDCK CELLS VIA AN ENDOCYTOSIS-MEDIATED PATHWAY.

Our TEM analysis revealed the uptake of CHV-1 within large (264 to 1943 nm), uncoated, irregularly shaped cytosolic vacuoles. Fixation techniques can sometimes affect cell and tissue morphology. Importantly, aldehyde fixation used in the processing of our TEM samples, is reported to not affect the average membrane length of endosomes, however, it may cause a significant reduction of endosomal volume compared to that of obtained with cryofixation (Murk *et al.*, 2003). The estimated reduction in the diameter of endocytic vesicles in aldehyde fixed samples is about 29.7% for early endosomes and 17.7% for late endosomes. However, the diameter size of CHV-1 containing vesicles is still within the predicted size range for macropinosomes (0.2–10 µm). The morphology of CHV-1 containing vacuoles was different from those driven from clathrin or caveolin-mediated endocytosis which usually have an electron-dense coating protein on their cytosolic surface. In addition, the size of CHV-1 containing vacuoles is larger than that reported for the clathrin-coated vesicles (60–120 nm) (Kaksonen & Roux, 2018) and the caveolin coated vesicles (50–100 nm) (Doherty & McMahon, 2009; Li *et al.*, 2005; Ni *et al.*, 2020). The size and morphology of CHV-1- containing vacuoles was similar to those described for the endocytosis of several other herpesviruses such as HCMV (Bodaghi *et al.*, 1999; Duyckinck & De Harven, 1974; Haspot *et al.*, 2012; Hetzenecker *et al.*, 2016; Sinzger, 2008; Wang *et al.*, 2007), HSV-1 (Clement *et al.*, 2006; Duyckinck & De Harven, 1974; Mockel *et al.*, 2019; Nicola *et al.*, 2003), and PRV (Lv *et al.*, 2018b). Moreover, TEM micrographs showed CHV-1 virions in association with plasma membrane protrusions, while in contrast, viruses that enter via CME or caveolae-mediated endocytosis are usually detected at membrane invagination of either clathrin or caveolin coated pits. The large size of the vacuoles containing CHV-1 is within the size range (0.2–10 µm) of that reported for macropinosomes (Chang *et al.*, 2021; Mercer & Helenius, 2009; Mercer & Helenius, 2012). These observations support a hypothesis that entry of CHV-1 may be mediated by macropinocytosis, an actin-dependent, non-specific form of endocytosis used by the cells for the uptake of nutrient-rich extracellular fluids (Mercer & Helenius, 2009; Mercer & Helenius, 2012).

Caveolin-mediated endocytosis is commonly observed for the entry of small viruses such as HPV (52–55 nm) (Humans, 2007; Smith *et al.*, 2007), Japanese encephalitis virus (40–50 nm) (Lannes *et al.*, 2017; Xu *et al.*, 2016), human adenovirus species D (65–80 nm) (Ghebremedhin, 2014; Yousuf *et al.*, 2013) and SV40 (45 nm) (Engel *et al.*, 2011; Toscano & de Haan, 2018). Also, clathrin-mediated endocytosis is known to mediate the entry of relatively small and medium-sized viruses like SFV (50–70 nm) (Garoff et al., 1994; Leung et al., 2011), HCV (40–100 nm)

(Catanese et al., 2013; Meertens et al., 2006), HBV (42 nm) (Herrscher et al., 2020; Venkatakrishnan & Zlotnick, 2016) and SARS-CoV-2 (60–140 nm) (Bayati et al., 2021; Zhu et al., 2020). The relatively large size of CHV-1, which can reach up to 200-280 nm (Lara *et al.*, 2016) would theoretically hinder its uptake by the small-sized clathrin or caveolin-coated pits and vesicles. However, clathrin-coated vesicles have been reported to deform to accommodate the entry of relatively large viruses including the herpesviruses HSV-1 (155–240 nm) (Laine *et al.*, 2015; Praena *et al.*, 2020) and VZV (150–200 nm) (Hambleton *et al.*, 2007; Mueller *et al.*, 2008), as well as VSV (can reach 200 nm) (Cureton *et al.*, 2010) and ASVF (can reach 200 nm) (Hernáez *et al.*, 2016). Moreover, caveolae-mediated endocytosis has been described for the entry of EHV-4 into equine epithelial cells (Azab *et al.*, 2013). In our TEM study, attempts to detect events reflecting the fusion of the viral envelope with the plasma membrane resulted in a single observation of what appeared to be a naked capsid in the cytoplasm early in infection, suggesting that direct fusion is not a major entry route for CHV-1.

The uptake of CHV-1 was very fast as the virus could be detected within the endocytic vacuoles at 30 seconds and two minutes post the temperature shift of the infected culture from 4^{O}C to 37^{O}C. A rapid entry process has been also reported for HSV-1 and HCMV (2-10 minutes) (Duyckinck & De Harven, 1974; Maurer *et al.*, 2008; Milne *et al.*, 2005; Nicola & Straus, 2004). Further studies are needed to determine the time required for the penetration of the CHV-1 capsid from the endocytic vacuoles into the cytosol. Moreover, future work should be conducted to identify the profile of CHV-1 containing vacuoles with regard to the markers of early endosomes, late endosomes, and lysosomes.

2 SMALL COMPOUND INHIBITORS TARGETING KEY FACTORS OF MACROPINOCYTOSIS BLOCK CHV-1 ENTRY.

CHV-1 entry was blocked early in infection by inhibitors targeting the key factors involved in macropinocytosis. We found that inhibitors of actin dynamics (Cyt.D), NHE (EIPA), MLCK (ML-7), and PI(3)K (Wort) significantly inhibited CHV-1 plaque formation when added during the first two hours of the infection. Blocking viral entry through inhibition of NHE by EIPA and actin dynamics by Cyt.D is usually used as a diagnostic test for the dependency of viral entry on macropinocytosis (Mercer & Helenius, 2009; Mercer & Helenius, 2012). Moreover, MLCK is required for myosin activity which is essential for actin-based contractile activity, membrane ruffling, and macropinosomes closure (Buss *et al.*, 1998; Chew *et al.*, 1998; Mercer & Helenius, 2009). By reducing submembranous pH, NHE inhibitor EIPA blocks the activation of the Rho GTPases Rac1 and Cdc42, thus preventing actin polymerization and membrane ruffling which

are essential for macropinocytosis. These results support the hypothesis for the role of the macropinocytic machinery in CHV-1 entry.

PI(3)K is an important factor for membrane ruffling, macropinocytic cup closure, and macropinosome maturation and fusion (Amyere *et al.*, 2000; Araki *et al.*, 2007; Di Paolo & De Camilli, 2006; Levin *et al.*, 2015; Lindmo & Stenmark, 2006; Swanson, 2008). Although Wort treatment significantly inhibited the entry of CHV-1 strain V777, it did not affect the entry of the CHV-1 strain 0194. A similar strain-dependent variability to the response of Wort has been reported for the entry of the vaccinia virus (VV) strains, IHD-J and WR. The two VV strains additionally showed differences in the activation of, and the response to the inhibition of, the Rho GTPases, the response to the tyrosine kinase inhibitor genistein, and the dependency on endosomal acidification. Moreover, the two VV strains have been found to induce different patterns of membrane protrusions, where the WR strain induces membrane blebbing, but IHD-J strain induces filopodial membrane protrusion (Mercer *et al.*, 2010a). In contrast to the differences between the two VV strains, our results showed that the entry of both CHV-1 strains is independent of endosomal acidification, blocked by the tyrosine kinase inhibitor genistein, and the two strains induced the same lamellipodial membrane ruffling. Although the entry of the two CHV-1 strains is sensitive to the inhibitors of the RTKs EGFR and PDGFR, the differences in sensitivity to Wort suggests that the pathway downstream of the RTKs may diverge between the two strains. However, this difference in response to Wort does not seem to impact the type of membrane protrusion induced by the two strains, indicating that PI(3)K is not the determinant factor for membrane ruffling triggered by CHV-1 in MDCK. Furthermore, the impact of Wort on HSV-1 entry is highly cell type-dependent in that HSV-1 infection is reduced by Wort in HeLa and CHO-nectin1 cells, while it does not affect HSV-1 infection in Vero, HeLaS3, HEp-2, or PtK2 cells (Clement *et al.*, 2006; Devadas *et al.*, 2014; Gianni *et al.*, 2004; Gianni *et al.*, 2013; Nicola & Straus, 2004). Moreover, Wort does not affect the entry of VZV in human embryonic lung fibroblasts (Hambleton *et al.*, 2007) however, the concentration used in this study was lower than that used in our experiments.

Macropinocytosis can be triggered by the activation of RTKs like EGFR and PDGFR. Alternatively, it can be induced via activation of integrins that involves signaling through the non-RTK focal adhesion kinase (FAK). Using small compound inhibitors that target tyrosine kinases (genistein), EGFR (Iressa and 324674), PDGFR (Gleevec), FAK (Y11), Pak1 (IPA-3), PKC (Calphostin C), our results showed that the cell entry of CHV-1 strain V777 and 0194 is blocked by these inhibitors when they are present in the media during the first two hours of infection.

These findings suggest that signaling via RTKs and integrins may be required for CHV-1 to induce actin remodeling and membrane ruffling that are essential for viral entry by macropinocytosis. HCMV stimulates its macropinocytic entry by signaling through integrins and PDGFR but not EGFR (Cobbs *et al.*, 2007; Hetzenecker *et al.*, 2016; Soroceanu *et al.*, 2008; Streblow *et al.*, 2003; Wang *et al.*, 2003). In addition, the entry of BHV-1 in human lung carcinoma cells A549 and bovine kidney (MDBK) cells is blocked by the EGFR inhibitor Iressa (Gefitinib), and the productive infection by BHV-1 activated the phosphorylation of EGFR (Qiu *et al.*, 2020). It will be interesting to study if CHV-1 triggers the activation of RTKs to facilitate its macropinocytic entry.

Although our results showed that CHV-1 entry was blocked by several tyrosine kinases inhibitors, the impact of the tyrosine kinase inhibitor genistein on CHV-1 infection was less pronounced and a significant reduction in viral plaque formation was only observed at the highest tested non-toxic concentration of the drug. Genistein has also been shown to block the entry of EHV-1 in equine monocytic cells (Laval *et al.*, 2016) and the post-entry stages of infection of VZV (Hambleton *et al.*, 2007). Furthermore, genistein was shown to strongly block the entry of HSV-1 in keratinocytes but not neurons (Nicola *et al.*, 2005) suggesting that genistein could affect different stages of viral infection in virus and cell type-dependent manners.

Future work is required to study the role of cellular signaling through the Rho GTPases Rho A, Rac1, and Cdc42 in CHV-1 macropinocytosis entry. These small GTPases are downstream signaling molecules of the RTKs and regulate actin dynamics that are important for membrane ruffling and macropinocytosis. EHV-1 has been shown to activates Rac1 and Cdc42 to induce tubulin acetylation, viral transport towards the nucleus, and cell-to-cell viral spread (Kolyvushko *et al.*, 2020). Moreover, Rac1 and Cdc42 are essential for the macropinocytic entry of PRV in HeLa cells (Lv *et al.*, 2018b) and the entry of dengue virus type-2 (DENV-2) in the endothelial cells, HMEC-1 (Zamudio-Meza *et al.*, 2009). Although HSV-1 transiently activates the phosphorylation of Rac1 and Cdc42 during its entry, the expression of constitutively active mutants of Rac1/Cdc42 reduces HSV-1 infection but with no impact on viral binding, internalization, or transport (Hoppe *et al.*, 2006).

3 ENTRY OF CHV-1 IS INDEPENDENT OF CLATHRIN- AND CAVEOLIN-MEDIATED ENDOCYTOSIS, MICROTUBULES, AND ENDOSOMAL ACIDIFICATION.

Although our TEM results did not show viral particles in small coated vesicles, we tested the possible implication of clathrin and caveolin endocytosis in the entry of CHV-1 fraction that was not blocked by the inhibitors of macropinocytosis in MDCK cells. Our results showed that

inhibition of clathrin by chlorpromazine and caveolin/lipid raft-mediated endocytosis by nystatin did not affect the entry of CHV-1. These results confirm that CME and caveolin-mediated endocytosis are not required for the uptake of CHV-1 into MDCK cells. In contrast to our results, CME endocytosis has been described for the entry of certain herpesviruses in some cell types, namely HSV-1 in human oligodendroglial cells (Praena *et al.*, 2020), the entry of VZV in human embryonic lung fibroblasts (Hambleton *et al.*, 2007), and KSHV entry in human foreskin fibroblasts (Akula *et al.*, 2003). Moreover, caveolin-mediated endocytosis was reported for the entry of EHV-1 into equine brain microvascular endothelial cells (Hasebe *et al.*, 2009), and EHV-4 entry in epithelial cells (Azab *et al.*, 2013). Thus, herpesviruses exploit different endocytic pathways to enter host cells in a cell-type and virus dependent manner.

We also tested the impact of dynasore, an inhibitor of the small GTPase dynamin, which we hypothesized plays a role in the fission of CHV-1 containing vacuoles from the plasma membrane. Dynamin act as a scission factor in CME and caveolin-mediated endocytosis as well as some forms of macropinocytosis, and is important for the regulation of actin and microtubules dynamics (Ferguson & De Camilli, 2012; Mercer & Helenius, 2012; Orth & McNiven, 2003; Schmid & Frolov, 2011). Our results showed that inhibition of dynamin by dynasore at the first two hours of infection blocks CHV-1 viral entry, which is consistent with the fission of the macropinosomes containing CHV-1 from the plasma membrane requiring the activity of dynamin. However, off-target inhibitory effects of dynasore on macropinocytosis and peripheral membrane ruffling have been reported in dynamin triple knock-out cells (Park *et al.*, 2013). For example, dynasore treatment has been shown to reduce HSV-1 gene expression in the context of macropinocytosis entry in Vero, HeLa, HEp-2, and PtK2 cells, however, down regulation of dynamin using RNAi or DN dynamin had no effect in HSV-1 gene expression or nuclear targeting (Devadas *et al.*, 2014) suggesting that the reduction of HSV-1 gene expression by dynasore may be related to its off-target effects. Thus, further studies using RNAi or DN dynamin perturbations are required to confirm the requirement of dynamin in CHV-1 entry.

As a fundamental component of the cytoskeleton, microtubules are important for the trafficking of endosomes within the cytosol and have been shown to play a role in the entry of several viruses. Microtubules are involved in the transport of HSV-1 capsids towards the nucleus (Apodaca, 2001; Sodeik *et al.*, 1997; Yamauchi & Helenius, 2013). Moreover, rhinoviruses have been reported to rapidly trigger microtubule-dependent translocation of the intracellular sphingomyelinase enzyme to the extracellular leaflet of the cell membrane to facilitate their entry (Grassmé *et al.*, 2005). Using nocodazole and paclitaxel to disrupt microtubule dynamics at early

times in infection, we did not detect an inhibition of CHV-1 plaque formation. Although, there is evidence for the existence of microtubule-dependent and -independent macropinocytic pathways (Kruth *et al.*, 2005; Mercer & Helenius, 2009), our results suggest that microtubules are not essential for early events of macropinocytosis entry of CHV-1, in agreement with similar results for the macropinocytosis of vaccinia virus (Rizopoulos *et al.*, 2015) and respiratory syncytial virus (Krzyzaniak *et al.*, 2013), where nocodazole and paclitaxel did not affect viral entry. In contrast to our results, nocodazole has been reported to inhibit rabies virus intracellular transport in Vero cells during the first four hours of the infection (Xu *et al.*, 2015). Although nocodazole treatment has no impact on the binding or internalization of HCMV into human embryonic lung fibroblasts, it blocks post-entry nuclear targeting of viral capsids (Ogawa-Goto *et al.*, 2003). Paradoxically, inhibition of microtubule polymerization by podofilox has been reported to block HCMV entry into human fibroblasts (Cohen *et al.*, 2016), which is mediated via macropinocytosis (Hetzenecker *et al.*, 2016). The authors suggested that podofilox may have a different impact on the more dynamic microtubules at the cell surface compared to the more stable intracellular microtubules. Further studies are required to reveal if there are mechanistic differences in the dependency of CHV-1 and HCMV on microtubules for their macropinocytosis-based entry. In addition, the impact of nocodazole and paclitaxel on post-entry trafficking of CHV-1 could be investigated by TEM to study the impact of these drugs on capsid transport to the nuclear membrane.

Following their endocytosis, enveloped animal viruses penetrate into the cytosol to deliver their capsid by fusion of the viral envelope with the luminal limiting membrane of the endocytic vacuoles. Conformational changes in viral fusogens are necessary for the fusion process that results in capsid penetration into the cytosol. These conformational changes may be triggered by different cellular cues such as exposure to low endosomal pH, proteolytic cleavage by proteases present in endocytic vacuoles, redox reactions, interaction between viral proteins and receptors in the plasma membrane, or by binding to a receptor followed by exposure to low pH or followed by proteolytic cleavage (Staring *et al.*, 2018; White & Whittaker, 2016; Yamauchi & Helenius, 2013). For the prototypic alphaherpesvirus HSV-1, the requirement of endosomal acidification for capsid penetration is cell type-dependent (Devadas *et al.*, 2014; Milne *et al.*, 2005; Nicola, 2016; Nicola *et al.*, 2003). Our results showed that CHV-1 entry into MDCK cells is not blocked by inhibition of endosomal acidification using the lysosomotropic weak base, ammonium chloride. This finding suggests that endosomal acidification is not necessary for CHV-1 penetration from the macropinosomes. Therefore, other factors like the binding of viral glycoproteins to specific cellular receptors or proteolytic processing of the fusogenic viral glycoproteins may be required to induce conformational changes in CHV-1 fusion proteins. Our result contrast the reported low-

pH dependent endocytic entry of several varicelloviruses including BHV-1, EHV-1, PRV, and VZV (Bloom, 2016; Finnen *et al.*, 2006; Hasebe *et al.*, 2009; Miller *et al.*, 2019; Pastenkos *et al.*, 2018). However, Epstein-Barr virus (EBV) has not been reported to enter any cell type in a manner dependent on low-pH (Nicola, 2016). The entry of EBV into B-cells involves pH-independent endocytosis in which binding of gp42 in the gH/gL-gp42 complex to the B cell-specific human leukocyte antigen (HLA) class II receptor induces widening of the hydrophobic pocket within gp42. This conformational change is critical to trigger the fusion process (Miller & Hutt-Fletcher, 1992; Möhl *et al.*, 2019). Future studies are needed to identify the cellular and viral factors that are essential to trigger CHV-1 fusion with the macropinosomal membrane, and capsid release into the cytosol.

To investigate the requirement of trafficking to late endosomes or from the endoplasmic reticulum to Golgi for the entry of CHV-1, we tested the impact of brefeldin A (BFA) on CHV-1 entry. BFA is a fungal metabolite that inhibits guanine nucleotide exchange factors that mediate GTP binding of several ADP-ribosylation factors which regulate vesicular trafficking to endosomes and the Golgi complexes including trafficking from the endoplasmic reticulum to Golgi (Chai *et al.*, 2014; D'Souza-Schorey & Chavrier, 2006; Deng *et al.*, 2017; Klausner *et al.*, 1992). BFA-sensitive trafficking to the endoplasmic reticulum is critical for the entry of several viruses including HPV16, SV40, BK virus, and JC virus (Jiang *et al.*, 2009; Laniosz *et al.*, 2009; Norkin *et al.*, 2002; Querbes *et al.*, 2006). Moreover, BFA blocks the trafficking of Adeno-associated virus 2 to the Golgi apparatus (Nonnenmacher & Weber, 2011) and blocks translocation of VSV and porcine parvovirus to late endosome (Boisvert *et al.*, 2010; Johannsdottir *et al.*, 2009). In contrast to these viruses, our results show that CHV-1 entry was not affected by BFA treatment, suggesting that maturation to late endosomes and trafficking to Golgi are not essential for CHV-1 entry. Consistent with our finding, BFA does not affect HSV-1 infection when added during the first four hours of infection, however, when added at later time points (4-12 hours) it blocks the maturation and egress of HSV-1 virions and leads to the accumulation of the virions at the inner nuclear membrane(Cheung *et al.*, 1991). Future studies using TEM are required to investigate the possible impact of BFA on CHV-1 egress, by adding the drug at 2 hours post entry and maintain it for different time points (8-, 12-, and 18-hours post-infection) to investigate if it results in entrapment of the virions at perinuclear space of infected cells as has been reported for the impact of BFA on HSV-1 egress.

4 CHV-1 INTERNALIZATION IS MEDIATED VIA A MACROPINOCYTOSIS-LIKE PATHWAY IN WHICH THE VIRUS INDUCES CELL-WIDE PLASMA MEMBRANE RUFFLING AND COLOCALIZES WITH FLUID-PHASE MARKERS BUT DOES NOT INDUCE AN INCREASE IN FLUID UPTAKE.

One important characteristic of viral entry by macropinocytosis is the induction of actin-dependent plasma membrane protrusions. The protrusions may take different forms such as lamellipodial ruffles, circular ruffles, filopodial protrusions, or blebs depending on the cell type, receptors, signaling pathway, and virus involved (Mercer & Helenius, 2009; Mercer & Helenius, 2012). These membrane extensions fold back towards the plasma membrane to internalize extracellular fluids and membrane-associated particles and cargoes including viruses. Using SEM, our results showed that CHV-1 entry induces cell-wide plasma membrane lamellipodial ruffles. These images were consistent with the protusions we observed in our TEM micrographs (Fig 1C), to which CHV-1 was associated. This ruffling is similar to lamellipodial ruffles induced during macropinocytosis entry of adenovirus type 2 (Meier *et al.*, 2002a). These results further confirm the implication of macropinocytosis in CHV-1 entry. Consistent with our results, HSV-1 is reported to preferentially enter MDCK at lamellipodial protrusion sites (Hoppe *et al.*, 2006), however, it induces filopodial protrusion in HeLa, HEK-293, nectin-1-CHO, and Vero cells (Clement *et al.*, 2006; Oh *et al.*, 2010), and both filopodia and lamellipodia during its entry into neuronal SK–N–SH cells (Zheng *et al.*, 2014b). In contrast, HCMV has been shown to induce circular dorsal ruffles for its macropinocytosis entry into primary human fibroblasts (Hetzenecker *et al.*, 2016). The Rho GTPase Cdc42 is transiently activated by HSV-1 to induce long filopodial ruffles (Oh *et al.*, 2010), while Rac1 is the Rho GTPase that triggers viral-induced lamellipodial and blebbing membrane protrusions (Mercer *et al.*, 2010b; Tan *et al.*, 2008; Taylor *et al.*, 2011b). Further studies are required to investigate the requirement of these signaling GTPases in CHV-1 induced lamellipodial ruffles. These studies should investigate if CHV-1 entry triggers activation of the Rho GTPases; Rho A, Rac1, and Cdc42, as well as studying the impact of perturbing these proteins by pharmacological inhibitors (using Clostridium difficile toxin B (inhibits all Rho GTPases), NSC23766 (Rac1), or ML 141(Cdc42)) or by expressing constitutive-active or dominant-negative forms of the protein, on CHV-1 entry.

In macropinocytosis-mediated viral entry, some of the membrane ruffles fold back to internalize a relatively large volume of the surrounding fluids along with the membrane-associated viruses in large, fluid-filled vacuoles (macropinosomes). This is accompanied by a transient elevation in cellular fluid uptake (5–10-fold over baseline) (Kerr & Teasdale, 2009; Mercer &

Helenius, 2009). Our results showed colocalization of fluorescently-labeled CHV-1 with the fluid phase marker fluorescent dextran, indicating that CHV-1 uptake occurs within functional macropinosomes that were filled with fluids. However, surprisingly, using FACS analysis, we did not detect an increase in fluid phase uptake during the first hour of CHV-1 infection. These findings suggest that the entry of CHV-1 into MDCK is mediated by a non-classical macropinocytosis-like pathway. Our results regarding induction of the fluid phase uptake are in contrast with the reported classical macropinocytic entry of vaccinia virus into HeLa cells that was associated with increased dextran uptake (Schmidt *et al.*, 2011). However, our results are similar to other studies in which deviations from the classic macropinocytosis entry pathway have been reported. These include the entry of *Chlamydia trachomatis*, in which the bacteria colocalize with dextran but do not induce its uptake (Ford *et al.*, 2018), and the EIPA-sensitive entry of Adeno-associated virus 2, in which, the virus induces extensive membrane ruffling but does not increase fluid-phase uptake (Nonnenmacher & Weber, 2011). Moreover, the entry of Lassa virus into human epithelial cells involves an unusual macropinocytosis pathway that requires sodium hydrogen exchangers, actin, and the GTPase Cdc42, but the virus does not induce cell membrane ruffling or fluid uptake (Oppliger *et al.*, 2016). Our results suggest that the lamellipodial ruffles that are induced by CHV-1 may not in most cases fold back to engulf extracellular fluids but may simply melt back into the plasma membrane, an activity that has been documented (Mercer & Helenius, 2009; Swanson & Watts, 1995). Thus, the role of the ruffles would be to increase the exposed surface area of the plasma membrane to provide additional attachment sites for the virions.

Finally, our results comparing the entry of CHV-1 strains 0194 and V777 indicate that macropinocytosis could serve as a general mechanism for the entry of CHV-1 into epithelial cells. The inhibitor profile showed that entry of the CHV-1 0194 is similar to that of CHV-1 V777, and is mediated via macropinocytosis. Moreover, SEM analysis of MDCK cell surface infected by CHV-0194 showed extensive lamellipodial ruffling similar to that induced by CHV-1 V777. However, entry of CHV-0194 is not blocked by wortmannin the inhibitor of PI(3)K, indicating that this kinase is dispensable for the entry of CHV-1 0194, and suggesting that the signaling downstream of RTKs may bypass the need for PI(3)K for the entry of CHV-1 0194.

5 CONCLUSION AND IMPORTANCE

Our study provides a model for the mechanism of CHV-1 entry into epithelial cells (**Fig 3.1**). The virus induces extensive cell-wide lamellipodial membrane ruffles as visualized by SEM. CHV-1 virions are associated with a lamellipodial-type membrane ruffles and then are rapidly

internalized within large, uncoated vesicles as detected by TEM. Studies with a panel of pharmacological inhibitors showed that the entry pathway of CHV-1 fulfills major criteria for the uptake by macropinocytosis. CHV-1 entry is blocked by inhibitors targeting cellular factors required for viral entry by macropinocytosis, including Na^+/H^+ exchangers, F-actin, myosin light-chain kinase, protein kinase C, p21-activated kinase, phosphatidylinositol-3-kinase, focal adhesion kinase, and possibly dynamin. The inhibitor profile also suggests a role for signaling via both integrins and receptor tyrosine kinases in CHV-1 entry. The entry of CHV-1 is independent of clathrin and caveolin/lipid rafts mediated endocytosis, microtubules, and endosomal acidification. Although CHV-1 entry involved extensive lamellipodial membrane ruffling and colocalization with the fluid phase uptake marker, the entry did not induce fluid uptake, suggesting that the virus uses a macropinocytosis-like pathway that deviates from classic macropinocytosis.

Endosomal viral entry pathways such as macropinocytic entry of viruses provides many advantages to the virus by allowing it to hide from the host immune surveillance. Moreover, by gaining access to cytoplasmic compartments from the endosomal system, the virus avoids exposing its capsid protein at the plasma membrane, which could then be detected by the host immune system. Furthermore, endosomal transport of the virions deeper into the cytosol overcomes the barriers represented by cortical actin cytoskeleton.

The dependency of CHV-1 on macropinocytosis machinery may be good news for the design of new antiviral strategies against this important veterinary pathogen because macropinocytosis is a transient process that could be blocked by several nontoxic drugs without significant effects on the host cells. Major open questions that remain to be answered about CHV-1 entry include identification of the cellular and viral factors that trigger fusion of the viral envelope with macropinosomal membrane. In addition, host cellular receptors that are responsible for CHV-1 entry into, and restriction to canine cells are not known. There is presently an ongoing project in our laboratory that is dedicated to identifying CHV-1 functional receptors in MDCK epithelial cells. Moreover, studies should be conducted *in vivo* to evaluate if the inhibitors used in our study could prevent or reduce CHV-1 infection in newborn pups, the most vulnerable host for CHV-1 infection. For example, both Gleevec and Iressa are FDA-approved drugs to treat certain types of human cancer, it would be interesting to study if these drugs can be repurposed to inhibit/reduce CHV-1 infection in dogs.

Of particular interest, the mechanism of CHV-1 entry into canine neuronal and hematopoietic cells should be a subject of future studies. Following its replication at the mucosal sites, CHV-1 is likely to infect hematopoietic cells (monocytes or T cells) that mediate viremia and

viral spread to the latency sites in the neurons of the trigeminal or lumbosacral ganglia (Evermann et al., 2011). EHV-1 enters into CD172a^{+} monocytic cells via clathrin, caveolae, and macropinocytosis independent endocytic pathway that requires tyrosine kinase, actin, dynamin, endosomal acidification, αVβ3 integrins, and cholesterol (Laval *et al.*, 2016). On the other hand, entry of HSV-1 into neurons is mediated by pH-independent fusion of viral envelope with the neuronal plasma membrane (Miranda-Saksena *et al.*, 2018; Nicola *et al.*, 2005). Understanding how CHV-1 enters hematopoietic cells would contribute to a better understanding of the pathogenesis of the virus.

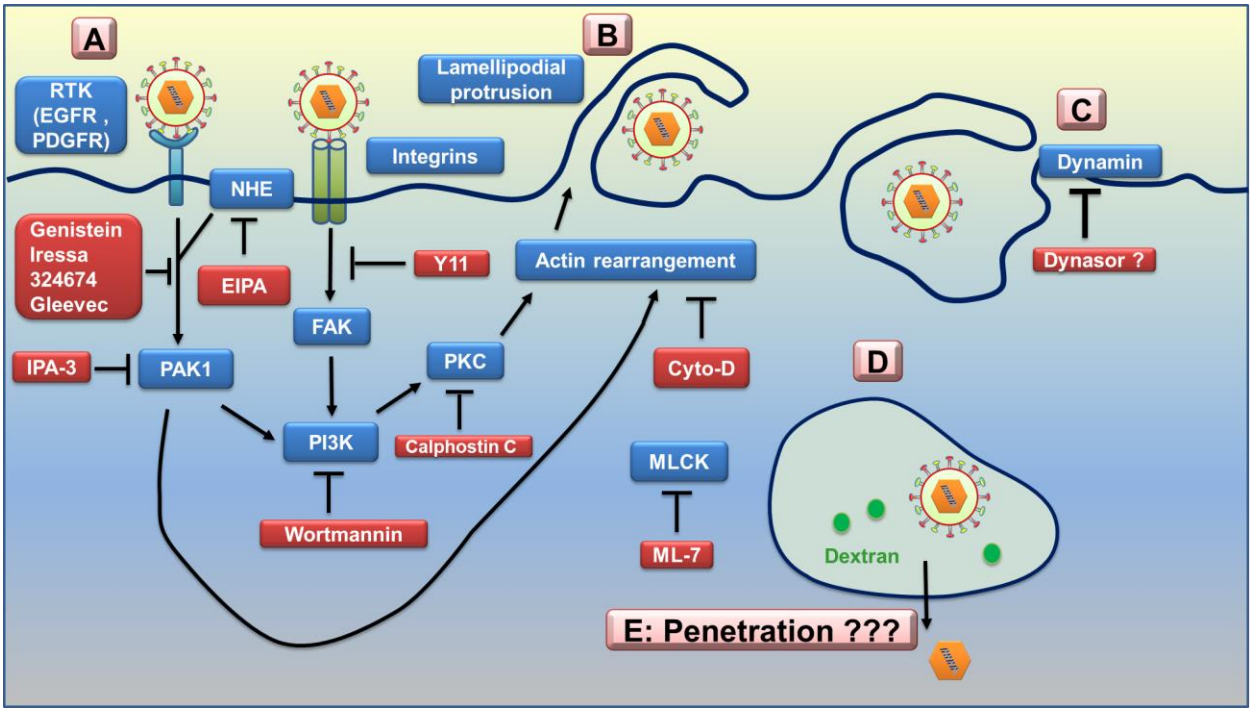

Figure 3.1 Proposed model of CHV-1 entry into epithelial cells.

Schematic diagram representing the main characteristics of the CHV-1 entry pathway: **(A)** Entry of CHV-1 is blocked by small compound inhibitors targeting the main cellular machinery of macropinocytosis including NHE, actin, MLCK, PAK1, PI(3)K, PKC, signaling via integrins (FAK) and RTKs (EGFR and PDGFR). **(B)** CHV-1 induces extensive lamellipodial membrane ruffles. **(C)** Closure of the ruffles to which CHV-1 particles are bound forms large endocytic vacuoles (macropinosomes) that then pinch off the plasma membrane. **(D)** Large uncoated macropinosomes containing CHV-1 colocalized with the fluorescent fluid-phase uptake marker dextran. **(E)** Release of CHV-1 from the macropinosome is pH-independent, the cellular cues that trigger virus penetration remain to be identified. The red boxes indicate the small compound inhibitor(s), and the blue boxes represent the corresponding cellular target. The figure is not to scale.

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