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VIRUS-HOST INTERACTIONS: ENTRY AND REPLICATION OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS

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Virus-Host interactions: Entry and replication of Crimean-Congo hemorrhagic fever virus

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"When I was 5years old, my mother always told me that happiness was the key to life. When I went to school, they asked me what I wanted to be when I grew up. I wrote 'happy'. They told me I didn't understand the assignment, and I told them they didn't understand life"

John Lennon

To my family

ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is a severe acute human disease with potential lethal outcome caused by a virus, Crimean-Congo hemorrhagic fever virus (CCHFV). Not much is known regarding how CCHFV infects cells, replicates and why it cause vascular dysfunction. To better understand CCHFV-pathogenesis increased knowledge regarding these issues is needed.

Viruses have to enter a host cell in order to replicate its genome and here we show that CCHFV entry occur by clathrin-mediated endocytosis and is pH-dependent.

A new *in situ* detection technique was establihed to visualize individual CCHFV cRNA and vRNA transcripts. Potential colocalization with the viral nucleocapsid protein (NP) was also investigated. cRNA was found to be more concentrated to particular regions within the cytoplasm and co-localized with CCHFV NP. While vRNA was detected throughout the cytoplasma not colocalizating with CCHFV NP.

It is not known if the vascular leakage observed in CCHF is due to direct virus infection or is immune-mediated. A new *in vitro* model system was therefore established and it was found that CCHFV has a preference for basolateral entry. However and surprisingly, using CCHFV-infected monocyte-derived dendritic cells (moDCs) or their supernatant, a preference for apical entry was observed. This indicate that the change in entry site could be due to soluble factors from the moDCs. Neither direct infection nor addition of CCHFV-infected moDCs affected the cellular permeability of the human polarized epithelial cell layer, indicating that other factors are most likely are causing the vascular leakage.

Taken together, we established several new *in vitro* model systems to study CCHFV's interaction with host cells. We also demonstrated the entry pathway for CCHFV into cells. These data and tools will hopefully facilitate and promote research on virus-host interactions which in turn may result in the development of new antivirals and/or vaccines against CCHFV.

LIST OF SCIENTIFIC PAPERS

I. Simon M, Johansson C, Mirazimi A.
Crimean-Congo hemorrhagic fever virus entry and replication is clathrin, pH and cholesterol dependent.
Journal of General Virology. 2009 Jan; 90 (Pt 1):210-5

II. Andersson C*, Henriksson S*, Magnusson KE, Nilsson M, Mirazimi A.
***In situ* rolling circle amplification detection of Crimean Congo hemorrhagic fever virus (CCHFV) complementary and viral RNA.**
Virology. 2012. May 10; 426 (2): 87-92

III. Andersson C, Mirazimi A.
An *in vitro* assay to study the molecular pathogenesis of Crimean-Congo hemorrhagic fever virus
Manuscript

*contributed equally

RELATED SCIENTIFIC PAPERS

Simon M, Johansson C, Lundkvist A, Mirazimi A.
Microtubule-dependent and microtubule-independent steps in Crimean-Congo hemorrhagic fever virus replication cycle.
Virology, 2009 Mar 15; 385 (2): 313-22

Connolly-Andersen AM, Moll G, Andersson C, Åkerstöm S, Karlberg H, Douagi I, Mirazimi A.
Crimean-Congo hemorrhagic fever virus activates endothelial cells.
Journal of Virology. 2011 Aug; 85(15): 7766-74

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
BSL-4	Biosafety level 4
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
CME	Clathrin-mediated endocytosis
cRNA	Complementary ribonucleic acid
DC	Dendritic cell
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FISH	Fluorescence in situ hybridization
IFN	Interferon
ISH	In situ hybridization
LNA	Locked nucleic acid
moDC	Monocyte-derived dendritic cell
MHC	Major histocompatibility complex
mRNA	Messanger ribonucleic acid
NP	Nucleocapsid protein
NK	Natural killer
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
RCA	Rolling circle amplification
RCP	Rolling circle product

RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
vRNA	Viral ribonucleic acid

1 INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is exclusively a human disease with a high case fatality rate, of up to 30%. The virus causing the disease, Crimean-Congo hemorrhagic fever virus (CCHFV), is spread by ticks and humans contract the virus following tick bites, the handling of infected livestock or caring for a patient in the acute phase of the disease. There is currently no commercially available vaccine and no specific treatment.

1.1 HISTORY

A human disease with bleeding from numerous sites and where the causing agent was believed to be a small tough tick or louse was first described in 1100 ad [1]. A similar disease has also been known in the Termez region of Uzbekistan under 3 names indicating blood taking, nose bleeding and black death [1]. But the first clinical description of CCHF was made during an outbreak in Crimea in 1944, when over 200 military personnel developed severe disease with bleeding from various sites while helping restore abandoned farmland [1]. The following years there were other outbreaks of what was then referred to as Crimean hemorrhagic fever in other parts of Eastern Europe [2]. The viral nature of the disease was determined by Chumakov, who gave serum from hemorrhagic patients to psychiatric patients. He also determined ticks as the vector by giving a solution of crushed Hyalomma ticks to healthy volunteers and in both studies disease was manifested [1].

In 1969, the Soviet reference strain for Crimean hemorrhagic fever was found to be antigenically identical to several strains isolated in Congo where it was known to cause a similar disease [3]. After some dispute over the name, Crimean-Congo hemorrhagic fever was finally accepted [1].

2 CCHF-VIRUS

CCHFV belongs to the *Bunyaviridae* family and is classified within the *Nairovirus* genus. The *Bunyaviridae* family is a large family containing more than 350 viruses divided into 5 genera, *Orthobunyavirus, Nairovirus, Phlebovirus, Hantavirus and Tospovirus* [4]. With the exception of Tospoviruses all other members are transmitted by animals, rodents for *Hantavirus* and arthropods (mosquitos, tick or sandfly) for the others. *Bunyaviridae* viruses are enveloped and circular. All members have a genome consisting of 3 single stranded RNA segments of negative sense, the large (L), medium (M) and the small (S) segment, encoding 4 structural proteins [5], several members also encode nonstructural proteins.

Nairoviruses are spread by ticks and have a very large L segment compared to other *Bunyaviridae* members, nearly twice the size [6]. The genus consists of 35 viruses, but only 5 viruses are known to cause human disease. Apart from CCHFV, Farallon and Erve virus may cause human disease with headache, fever and neurological disorder while Dugbe and Nairo sheep disease virus can cause human disease but mainly cause disease in sheep and goats [7].

2.1 MOLECULAR CHARACTERISTICS

The CCHF virus particle is spherical and between 90-100nm in diameter [8, 9]. It has an outer cell-derived lipid envelope membrane, through which the glycoproteins, Gn and Gc, protrude. Like the other *Bunyaviridae* members, CCHFV has a negative single strand genome divided on three segments, the large (L), medium (M) and small (S). Together with the nucleocapsid protein (NP) each genome strand form individual ribonucleocapsids [5, 10]. Each viral particle also contains RNA-dependent RNA polymerase (RdRp), necessary to initiate transcription and genome replication.

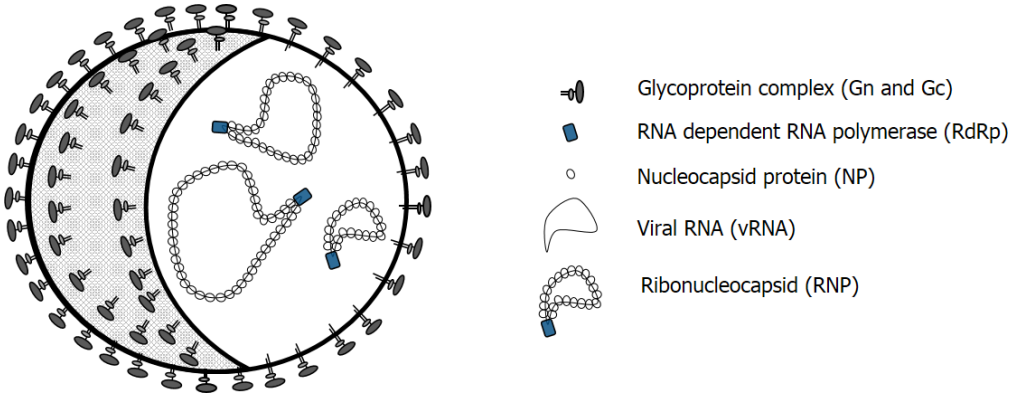

Figure 1: Schematic representation of CCHFV

The terminal sequences on each strand is complementary and conserved in all *Nairoviruses* [11, 12]. From other *Bunyaviridae* it is predicted that the terminal sequences bind to each other forming stable panhandle structures making the genome segments into closed circular RNA molecules [13]. It has been suggested that the terminal base-pairing provide the functional promoter region for the viral polymerase [12]. CCHFV's genome, encodes 4 structural proteins. The S-segment encodes the NP, which coats and protects the genome from degradation. It has a large globular domain that can bind RNA and a protruding arm, with a caspase-3 cleavage site [14, 15]. Structural alignment with other RNA virus NPs showed it to be most similar to Lassa virus (*Arenaviridae*) [15]. The M-segment encodes a polyprotein, that through complex post-translational cleavage by several proteases produce the two transmembrane glycoproteins, Gn and Gc, named in accordance with their relative proximity to the respective ends of the polyprotein. The mRNA is first cleaved to preGn and preGc in the ER and are then transported to the Golgi, where they are further cleaved, glycosylated, folded and integrated into virions [16, 17]. Gn has a chaperone-like function for Gc and must be present for correct folding and transportation to the Golgi complex to occur [18, 19], while Gc has been suggested to be more important during infection [19]. A nonstructural protein [20] as well as three other domains, GP38, GP85 and GP160 with unknown functions has also been detected on the polyprotein [17]. The L-segment encodes the viral polymerase,

RdRp, necessary for viral replication and mRNA synthesis. It has an ovarian tumor protease domain near its N-terminus that has been shown to remove ubiquitin from cellular proteins [21].

2.2 REPLICATION

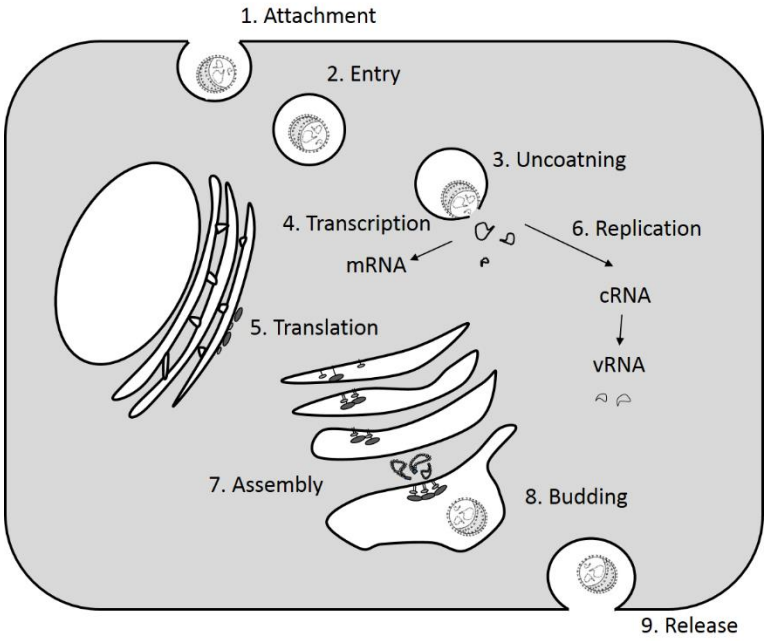

Figure 2: Schematic representation of CCHFV's replication

The two glycoproteins, Gn and Gc, are believed to determine cell and tissue tropism and the ability of the viruses to infect susceptible cells via recognition and binding of one or more cellular receptors. Neutralizing antibodies towards both glycoproteins are produced during infection, but *in vitro* and *in vivo* studies showed that only antibodies towards Gc protected both cells and mice from infection [19, 22]. The exact receptor for CCHFV is not known, but one study using the ectodomain of Gn and Gc found nucleolin to be important for entry

[23]. Following attachment, CCHFV enters through clathrin-mediated endocytosis (CME) in a pH-dependent manner with fusion likely to occur in the early endosomes [24, Paper I]. Viral replication occurs in the cytoplasm where the negative stranded genome interacts with the viral RdRp for the synthesis of the positive stranded messaganger RNA (mRNA) and full-length complementary RNA (cRNA). The mRNA is used for transcription of the viral proteins and the cRNA is used as template for the synthesis of new genomic viral RNA (vRNA). Newly synthesized vRNA binds to the NP and is then incorporated with the glycoproteins in the Golgi complex (Donets Chumakov 1977), after which the virus is released by exocytosis. Host-cell microtubule are needed during replication, assembly and egress [25] while actin is important for transporting NP to the site of assembly [26].

2.3 OCCURRENCE

The occurrence of CCHFV closely coincides with that of its vector, ticks primarily of the *Hyalomma* species [1, 27, 28]. It can be found in western, central and southern Africa, the Balkans, the Middle East, southern Russia and south-western Asia [1, 27, 29].

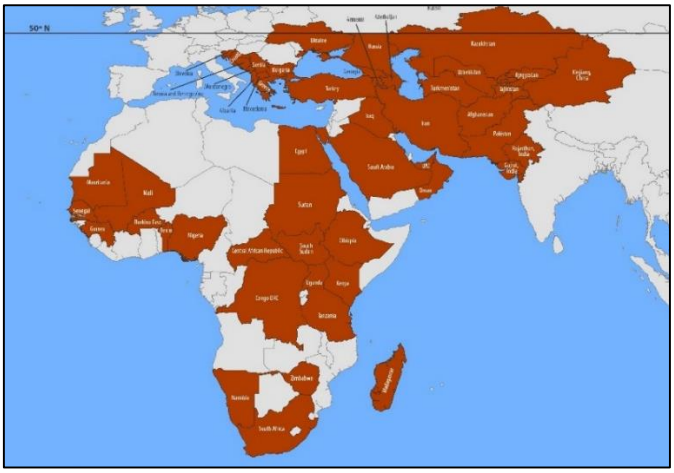

Figure 3: Occurrence of CCHFV, reprinted with permission from CDC

In Europe it is mainly found in the Balkans, Russia and countries of the former Soviet Union. Previously there had only been two antibody based reports of CCHFV from Western Europe, both on bats, one from the border of France and Spain and one from Portugal [1, 30]. But a recent report found CCHFV positive ticks in Spain [31]. Another recent report found CCHFV positive ticks on a migratory bird travelling from Africa to southern Europe [32]. Imported cases of human CCHF have occurred to France [33] and the United Kingdom [34], but without further transmission. During the last few year most cases of CCHF have been reported from Turkey. Although the virus was known to circulate there before, the first clinical cases of CCHF in Turkey didn't occur until 2002 [35]. However, since then numerous cases have been confirmed, between 2002 and 2012, 6864 cases were reported [36].

The increase in cases is most likely a combination of better awareness within the health care system, effective molecular methods for virus detection and virus spread. The spread can both be explained by natural bird migration, where birds are carrying infected ticks [32], and by the trade of virus infected and/or tick-infested livestock to previously unaffected areas where permissive ticks are available [1, 37, 38]. The number of human cases also increase when previously abandoned farm land is recultivated in areas where the virus is already circulating [2].

2.4 TRANSMISSION

CCHFV is spread and maintained by ticks. Human are exposed to the virus following tick bites, the handling of viremic or tick-infected livestock or through person-to-person (nosocomial) transmission. Humans who are mainly at risk of contracting the virus therefore include agricultural and slaughterhouse workers as well as hospital staff caring for infected patients.

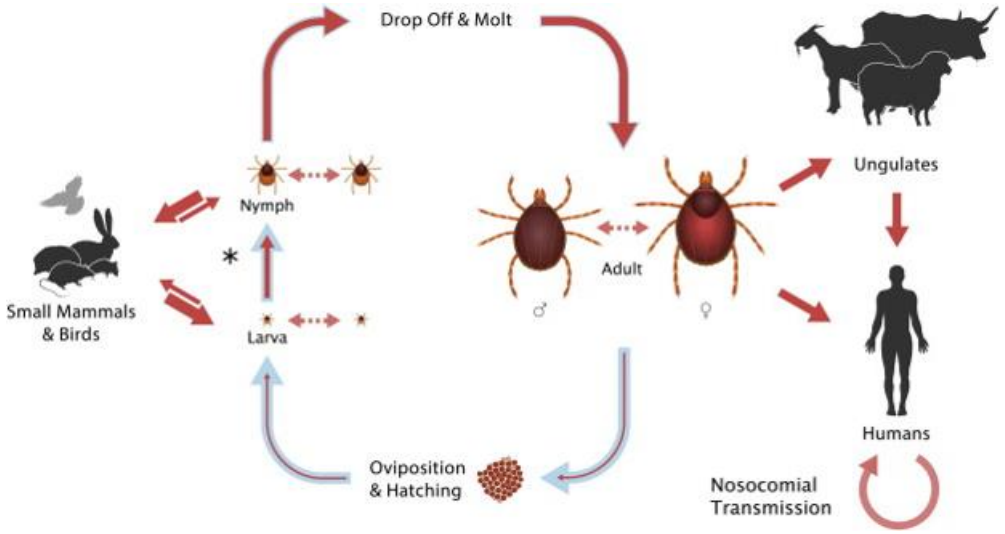

Figure 4: Schematic depiction of the different transmission routes for CCHFV. Reprinted with permission from Elsevier.

Ticks

The virus circulates unnoticed in nature in a tick–vertebrate-tick cycle where humans are accidental hosts. The role of ticks in the maintenance of the virus has been well established both through field studies and experimental assessments of vector competence in the laboratory, and while some *Nairoviruses* infect argasid (soft) ticks, CCHFV are maintained exclusively in ixodid (hard) ticks [1, 6, 39]. The primary vector and reservoir for CCHFV is ticks of the *Hyalomma* species, particular *H. marginatum*, and the ticks can remain infected throughout their several year life-time [6]. The virus can be maintained in the tick through all its life stages from egg via larvae and nymph to adult (transstadial transmission), as well as being spread from male to female (venereal transmission) and mother to egg (transovarial transmission) [6, 40, 41]. Additionally, CCHFV has been shown to spread by "non-viremic" transmission so called cofeeding, when virus present in one ticks saliva is spread directly to nearby feeding ticks without causing viremia in the animal that they feed on [42,

43]. *Hyalomma* ticks are generally 2-host ticks where the first host usually is ground-feeding birds or small mammals such as hares or hedgehogs, while the second host is a larger animal such as sheep or cattle [1]. Some tick species wait passively to encounter a vertebrate ("ambush ticks"), but *Hyalomma* ticks are "hunting" ticks, which can quest up to 400m to find their hosts [6].

Wild and domestic animals

Most mammals appear to be susceptible to infection with CCHFV, but only a few develop a sufficiently high viremia to efficiently infect ticks [44]. But in some vertebrates the bite of an infected tick causes viral replication and viremia, providing a source of infection for additional ticks as well as the risk of spreading the virus to humans. Experimental infections of wild and domestic animals have found that sheep, calves, scrub hares and ostriches develop a short viremia and in some cases were able to transmit the virus to feeding ticks [6, 45]. Because most vertebrates infected with CCHFV develop only a transient viremia without apparent illness, the identification of animal hosts of CCHFV has largely been done on the detection of virus-specific antibodies in collected serum from livestock or occasionally wild animals. The occurrence of antibody positive livestock has been found to correlate with the occurrence of human cases [6]. Even though birds can carry a large number of virus infected ticks, most birds appear to be refractory to the virus [45, 46].

Nosocomial transmission

The virus has been shown to spread from person-to person and a number of nosocomial cases have been reported where hospital staff, laboratory personnel and/or relatives have contracted the virus from a CCHFV infected patient [1, 47-50]. Fortunately, increased information regarding protective nursing has reduced the number of nosocomial cases and showed that standard barrier nursing methods are efficient to prevent further transmission of CCHFV [47, 50, 51].

3 CCHF- THE DISEASE

CCHFV infection is only known to cause disease in humans. It has been calculated that approximately 1 out of 5 persons exposed to CCHFV develop symptoms [52, 53]. Although the duration and symptoms vary among affected individuals, most patients only develop a mild or subclinical infection. Some patients do however, progress into the more severe symptoms.

The disease is divided into 4 phases: incubation, prehemorrhagic, hemorrhagic and convalesce [1]. The incubation time can vary from a few days up to a week and has been suggested to differ depending on transmission route, with shorter incubation times for tick bite or livestock handling compared to nosocomial [54]. Viral load has also been suggested to affect incubation time [29].

Following incubation, the patients enter the prehemorrhagic phase. This is characterized by a rapid onset of fever, headache, myalgia, nausea and vomiting [1, 49, 54] which usually last approximately 3 days after which some patients enter the hemorrhagic phase. The hemorrhagic phase is characterized by bleeding, ranging from dermal petechiae to gastrointestinal hemorrhage [29, 49, 54, 55]. The most common bleeding sites includes the nose, gastrointestinal system, urinary tract and the respiratory tract [56]. Other symptoms include enlarged liver and spleen, elevated liver enzymes, prolonged bleeding times and in severe cases disseminated intravascular coagulation (DIC) [1, 29, 54]. The severity of the disease has been correlated to high viral load and low antibody response [55, 57-61]. Other severity markers include thrombocytopenia, leucopenia, elevated liver enzymes, prolonged bleeding times and bleeding [55, 60, 62, 63]. Death usually occur between days 6-10 after disease onset and is due to multiple organ failure caused by severe anemia, dehydration and shock [60]. For patients that recover, the convalescence can be long and include symptoms like weakness, labile pulse and confusion, all of which are temporary [1, 54]. There have not been any reports of relapse of the infection [29].

3.1 TREATMENT

Most human CCHFV-infections appear to be asymptomatic or only cause a mild febrile illness [6, 53, 64]. But patients that develop the severe form of the disease require extensive hospital stay with special care and protective nursing in order to limit the spread of the disease. As there currently is no commercial vaccine or specific treatment available, patients are usually given a combination of supportive treatments. This includes giving volume replacement, to treat the fall in blood pressure and diminishing organ perfusion, giving fresh frozen plasma and platelets for the coagulation abnormalities as well as blood transfusions for significant hemorrhage [6].

It has been suggested that the different phases of the disease should be treated differently with an antiviral such as ribavirin, given during the first clinical phase when viremia and virus replication is high and drugs targeted at DIC or sepsis during the second phase when the viral load declines and patients enter the hemorrhagic phase [65]. In line with this a recent study found corticosteroids to be effective, particularly for patients with severe CCHF [62].

Studies of the efficiency of giving immunoglobulin is lacking, although immunoglobulin derived from plasma of CCHF survivor donors is used as treatment in Bulgaria [66, 67] and was recently used in Turkey [68]. But no case-control studies have been published on the efficiency in CCHF patients [69].

Ribavirin

Ribavirin (Virazole®) is a synthetic guanosine analogue that is used to treat a number of RNA and DNA viral infections. Its full mechanism is not yet known, although both indirect and direct functions have been proposed to explain its antiviral activity [70]. Most clinical comparative CCHF studies has found that ribavirin is beneficial, as long as it is initiated early in the course of the disease [62, 65]. However only one randomized clinical trial has been conducted and they found no significant difference with regard to disease outcome [71].

Vaccines

There is no FDA approved vaccine for CCHFV. A vaccine against CCHFV using formaldehyde-inactivated mouse brain tissue was developed in the Soviet Union in the early 1970ies [69] and a similar vaccine is still being used in Bulgaria and given to soldiers, medical personnel and other high risk groups in endemic areas [72]. However, no case-controlled studies of its efficiency has been conducted and a recent study found that the Bulgarian vaccine only elicited a low neutralizing antibody response, even in persons that had received it 4 times [73]. Two studies where vaccination with CCHFV Gn and Gc either delivered as a DNA vaccine [74] or as purified proteins from transgenic plants [75], induced neutralizing antibodies in mice. Yet, at the time there was no animal model available for CCHF and so the actual response could not be assessed in a challenge model. However, a promising new CCHFV vaccine candidate based on a poxvirus vector expressing the CCHFV glycoproteins, was found to elicit both a cellular and humoral response and protect mice challenged with CCHFV [76]. But whether this could be used as a human vaccine against CCHFV remains to be investigated.

3.2 ANIMAL MODELS

CCHF symptoms has so far only been reported in humans and this in combination with the requirement for BSL-4 containment, has made it difficult to develop an animal model to study CCHF pathogenesis. Attempts to study the disease in adult mice, rats, hamsters, guinea pigs, rabbits, sheep, calves, donkeys and non-human primates have all proven unsuccessful [1, 6]. Although a short viremia could be detected in several animals this was not sufficient to cause CCHF symptoms [6]. The virus does however replicate to high titers in newborn mice [77, 78], but as they have an immature immune response they are not a good model to study the pathogenesis of the disease. But recently, two different knock-out mice models were presented which revealed the importance of the IFN type I response in controlling the disease. The mice lacked either the cell-surface IFN- αβ receptor [79] or the intracellular signal transducer and activator

of transcription (STAT)-1 protein [80] and developed CCHF symptoms following infection. Hopefully, the introduction of these new animal models can provide more knowledge regarding CCHF's pathogenesis.

3.3 PATHOLOGY

Due to the safety regulations and the sporadic occurrence of the disease, there are only a few reported necropsies on CCHF patients [57, 81, 82]. Further information comes the new animal models [79, 80]. The main finding from human necropsies included necrosis of the liver, which varied in extent but generally existed in multiple foci associated with viral antigen and no inflammatory infiltrates [54, 57, 81, 82]. The liver dysfunction is also reflected by the elevated liver enzymes detected during CCHF which can be used as a prognostic marker [56, 60, 63]. Damage to the spleen was also noted with marked lymphocyte depletion as well as hemorrhage and infection of endothelial cells of other organs [57, 81, 82]. The two new animal models developed similar symptoms with necrosis of the liver as well as prominent lymphocyte depletion and debris in the spleen, consistent with lymphocyte apoptosis, and gastrointestinal bleeding [80, 83].

4 IMMUNE RESPONSES TOWARDS CCHFV INFECTION

The host response is made up of the innate and adaptive immune systems, which usually act together in synergy. With the innate response representing the first line of defense and the adaptive becoming prominent after several days as antigen-specific T and B cells have undergone clonal expansion. They support each other with components of the innate immune system contributing to the activation of the antigen-specific cells and the antigen-specific cells amplifying the innate response [84].

4.1 INNATE IMMUNE SYSTEM

The innate immune system broadly includes all aspects encoded in their mature form by the germline genes of the host, this includes physical barriers like epithelial cell layers that express tight cell-cell contacts, soluble proteins as well as membrane-bound receptors and proteins that binds to the surface of invading microbes [84].

Interferons

Interferons (IFNs) are a group of secreted cytokines that have antiviral effects. They recognize viruses through toll-like receptors or pathogen recognition receptors. There are 3 different types, type I, II and III. *In vitro* studies has shown that CCHFV is sensitive to type I IFNs and several of its antiviral proteins [85-87]. However, CCHFV is able to avoid recognition by RIG-I by processing the 5'RNA termini of the genome, thereby delaying initial induction of IFNs [88]. The new animal models also showed the importance of a functional IFN response in controlling CCHFV replication with more severe symptoms and higher levels of viral replication in IFN type I knock-out mice compared to wild type mice [79, 80].

Nitric oxide

Nitric oxide, NO, is another mediator of the innate response, which can be induced either directly by virus or through cytokine dependent activation [89]. *In vitro* experiments have shown that NO can reduce CCHFV replication [90].

Natural killer cells

Natural killer (NK) cells play an important role in the early anti-viral defense. They have a complex regulation and do not act on pathogen-specific antigens but rather on the NK cell activation and inhibitory receptors expressed on cells. If activated they can induce apoptosis in the target cell [91]. In CCHF patients, one study found greater number of circulating NK cells in fatal cases [92] while another found no correlation between mild and severe CCHF [93]. *In vivo* experiments have shown activation of NK cells but an overall loss over time [80].

4.2 MACROPHAGES AND DENDRITIC CELLS

Antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs), are important members of the immune system as they present processed antigens on their surface to T cells. They express both class I and class II major histocompatibility complex (MHC) molecules.

For Ebola it has been hypothesized that infection of macrophages and dendritic cells is crucial to pathogenesis, as this leads to the release of proinflammatory cytokines and other mediators, causing impairment of the vascular and coagulation systems that ultimately lead to multiple organ failure and possibly death [94].

Macrophages

Macrophages are phagocytosing cells that in addition to their role as APC also can employ a battery of innate immune mechanisms for initial anti-viral defense [95]. An *in vitro* study have shown that they produce cytokines upon CCHFV infection [96] and a CCHF patient study found a correlation between elevated levels of neopterin, a monocyte/macrophage activation marker, and disease severity, indicating that macrophages could have a role in CCHF disease exacerbation [97]. Recent work using the new animal model found that only one macrophage population out of the two studied in the spleen was capable of upregulating MHC class II molecules, indicating a possible partial impairment of the ability to activate the adaptive immune system [80].

Dendritic cells

Dendritic cells have a crucial role as a bridge between the innate and adaptive immune response. They normally reside in peripheral and lymphoid tissues in an immature form where they acts as sentinels sensing the antigenic microenvironment and capture antigens. When they encounter an antigen they undergo maturation, release cytokines as well as migrate to regional lymph nodes where they present the antigen to and thereby activate naïve T cells [98]. They can also become activated by proinflammatory cytokines such as TNFα [99].

In vivo experiments showed no increase of MHC class II molecules in DCs during CCHFV infection [80] while *in vitro* studies of CCHFV-infected monocyte-derived DCs (moDCs) have shown that they are activated to the extent that they release cytokines (TNFα, IL-6, IL-10 and IL-8) and become partially matured [96, 100]. For Ebola and Marburg it has been shown that infected DCs aren't able to initiate an effective adaptive immune response and support T cell proliferation [101-103]. But whether something similar is true for CCHFV remains to be investigated. An active adaptive immune system is thought to be important for survival as fatal cases of CCHF rarely develop an antibody response [57, 61].

4.3 ADAPTIVE IMMUNE SYSTEM

The adaptive immune system manifests high specificity for its target antigens. It is primarily based on the antigen specific receptors expressed on the surfaces of T and B lymphocytes [84].

The humoral response is accomplished by B cells. They are APCs that produce cytokines and are defined by their production of antibodies that bind and potentially neutralize pathogens [84]. *In vivo* studies have shown activation of B cells during infection, followed by decrease in B cell numbers, which is in accordance with the lymphocyte depletion observed in patients [80-82].

The cell mediated response consists of T cells, defined by their cell-surface expression of receptors that bind processed antigens displayed by APCs [84]. They have several subtypes, including cytotoxic T cells and helper T cells. An investigation of lymphocyte levels in CCHF patients found increased levels of cytotoxic T cells among fatal compared to nonfatal cases of CCHF but no change in the helper T cells [93].

4.4 CYTOKINES

Cytokines are cell-signaling proteins that mediate and modulate immunity, such as interleukins, IFNs, TNFα, chemokines, migration inhibitory factor, and transforming growth factor β [104]. For Ebola, it has been found that proinflammatory cytokine release could lead to vascular permeability and ultimately hypotension, shock and organ failure [94, 95, 105]. The new CCHF animal models have showed increased levels of proinflammatory cytokines, TNFα, IL-6, IFN-γ and IL-10 [80, 83]. Several studies of CCHF patients have also found elevated levels of proinflammatory cytokines, which was correlated to disease severity and death [106-108]. All patient studies found increased levels of TNFα, two found higher levels of IL-6 and IL-10 and one found higher IFN-γ. Unfortunately, the studies had different sampling times and often only one sample could be taken from each patient making comparison difficult. *In vitro* experiments show that moDCs and macrophages release proinflammatory

cytokines upon infection [96, 100] and supernatant from infected moDCs could activate endothelial cells mainly by released TNFα [109].

4.5 PROPOSED PATHOGENESIS

The pathogenesis of CCHFV remains poorly understood. As previously stated most of the cases occur in remote regions and the high virulence of the virus limits laboratories studies. Most of the knowledge therefore comes from a few pathological studies, the relatively new animal models, and similarities to other viral hemorrhagic fever viruses and their pathogenesis.

The primary targets for CCHFV during infection has been suggested to be endothelial cells, macrophages, DCs and hepatocytes [81, 83]. As with other viral hemorrhagic fevers, the endothelium plays an important role and vascular dysfunction would account for the characteristic rash seen in CCHF as well as contribute to platelet aggregation and activation of the coagulation cascade [20, 110]. The effect on the endothelial cells that lead to the vascular permeability could either be a direct result of viral infection or an indirect effect of the host's immune response through soluble factors [111].

Direct effects

There is evidence of endothelial activation and damage in CCHF patients where levels of sICAM-1 and sVCAM-1 were correlated to disease severity [112, 113] and *in vitro* CCHFV infection has been found to activate endothelial cells causing cytokine release and leukocyte adhesion [109]. Viral RNA and antigens have also been detected in endothelial cells of the liver, spleen, heart and intestinal tissues during necropsy and in the new animal models [81, 83]. However the presence of virus in the endothelium at time of death does not mean that it was the cause of the vascular permeability, as the dysfunction is apparent during early part of the disease [6].

Enlarged liver has been reported as one of the symptoms of CCHF [1, 54] with necrosis of liver found at autopsy [81]. It was suggested to be due to high viral replication, which is supported by results found with the new animal model where enlarged livers was only found in animals with high viral load in the liver [79, 83]. Further support comes from recent *in vitro* experiments on hepatocytes showing that CCHFV infection could induce apoptosis and replicate to high titers [114].

In vitro studies of direct CCHFV infection found no effect on permeability in epithelial cells [115, Paper III]. Indicating that other factors most likely cause the vascular leakage. This is in line with what has been observed for Ebola virus where the vascular leakage is not caused by direct infection but rather due to the effects of soluble mediators [104].

The viral replication does not appear to cause vascular leakage but lead to activation, release of cytokines and the recruitment of leukocytes in endothelial cells. In contrast, high viral replication in the lymphoid organs could be an explanation for the observed lysis and necrosis of the liver and spleen.

Indirect effects

Indirect effects of CCHFV infection is not fully understood although the release of proinflammatory cytokines have been well documented both in patients, animal models and *in vitro* [6, 83, 96, 100, 108, 109, 114]. Low levels of cytokines work locally, while excess amounts can have a systemic effect, a so called cytokine storm that subsequently could lead to vascular leakage and hemorrhage and ultimately and organ failure. Macrophages and moDCs have been shown to be productively infected and release cytokines [96, 100]. Cytokines-hyperactivated monocytes and macrophages could be the cause of the hemophagocytosis that has been demonstrated in a number of CCHF patients [116-118].

At present it is not known if the APCs are able to initiate an effective T cell response but fatal CCHF patients rarely develop or show a late antibody response indicating that the adaptive response is either suppressed or not fully activated. The combination of high viral replication, cytokine storm, endothelial activation and organ necrosis could be what ultimately leads to the shock and multiple organ failure observed in severe CCHF.

5 POLARIZED CELLS

Polarized cells are found throughout the human body and act as a boundary between different environments, for example epithelial cells lie between tissues and the extracellular environment and endothelial cells line the inner surface of blood vessels and acts as a barrier between blood and tissue. They often constitute the first line of defense against the environment and regulate the passage of substances into and out of organs such as the gut, lung, liver and brain.

Polarized cells have an asymmetric plasma membrane with an apical and a basolateral side [119]. They are organized as selectively permeable continuous sheets that separate the two different sides, where the apical side face the lumen i.e. the outside for the epithelial cells and the blood for the endothelial cells while the basolateral side face the tissues and is in contact with the neighboring cells and the underlying extracellular matrix. The two domains also have distinct lipid and protein compositions which is generated and maintained by a specific sorting machinery [119, 120].

The space between the neighboring cells is sealed by junctions which restrict the paracellular transit of ions and macromolecules and separate the two sides of the cells [121]. There are different junctions between the cells, the tight junctions are the most apical of them, located at the boundary between the apical and the basolateral domain of the cells [121]. They tightly seal the space between neighboring cells, preventing the passage of fluids, electrolytes and macromolecules between the cells [121]. Adherens junctions are located on the basolateral side of the cells. They mediate cellular polarization and organogenesis. There are also gap junctions that form channels between the neighboring cells, allowing intercellular dissemination of small molecules [121].

Transepithelial resistance (TER)

To study polarized cells *in vitro*, the cells can be grown on permeable support, where the cells have free access to medium from both sides and samples can be taken from both sides. Since electrical conductivity is almost limited to the paracellular ion flux, the transepithelial resistance (TER) across the monolayer is a good marker of the development of tight junctions and can therefore be used to describe cell monolayer integrity [122]. The "chopstick system", consisting of 2 electrodes stuck together, one for each side, is easy to use for routine determination of TER in filter-grown monolayers.

5.1 POLARIZED CELLS AND VIRUS INFECTION

As cellular proteins and receptors could be differently distributed on the different sides of a polarized cells [120], it could be a limitation for viral entry and release. While some viruses are restricted to entry from a specific side, others viruses like influenza and poliovirus, have little or no preference [119]. Many virus receptors are not located on the apical side, but at the basolateral side, rendering intact cells layers resistant against many viral infections, such as HSV-1, poliovirus, reovirus and human adenovirus [123].

Viruses have therefore been forced to come up with ways to overcome this. Some viruses use more than one receptor, this appear to be the case for herpes simplex virus [124]. While others, like rotavirus, disrupts the tight junctions between the cells to get access to the basolateral side [125]. It has also be shown that the same virus using the same receptors can enter from different sides in different epithelial cells demonstrating cell type specificity [126]. The junctions may also be remodeled by cytokines to allow the passage of activated infected immune cells across the epithelial border [123]. Recently another way was shown, where adenovirus infected macrophages enabled the translocation of the viral receptor from the basolateral to the apical side, thereby enabling entry of a virus that normally requires basolateral access [127].

CCHFV has been found to primarily enter from the basolateral side [115, Paper III] although the addition of infected moDCs lead to more virus entry from the apical side, the reason for this is at present not known [Paper III].

Viral release can occur from both sides but can have an effect on whether the infection becomes localized (apical release) or systemic (basolateral release) [119]. CCHFV has been found to be released from the basolateral side further aiding systemic spread [115].

6 ENDOCYTOSIS

Viruses are obligate intracellular organisms and as such they must enter target cells in order for successful replication to occur. Different viruses have therefore developed different ways of entering and the route of entry can also vary depending on cell type. Most viruses do however exploit the cell's preexisting endocytic routes in order to gain entry into their target cells.

Virus endocytosis begins with the virus attaching to cell attachment factors and/or virus receptors that in turn induces a conformational change of the virus and/or signal cascade within the cell which promotes the internalization of the virus. Most endocytosis routes transports the encapsulated virion to the early endosome, through the multivesicular body to the late endosome and further on to the lysosome. In order for the virus to escape degradation in the lysosome it therefore has to penetrate the endosomal membrane at some point and deliver its genome to the appropriate replication site. Enveloped viruses fuse the viral and cellular membranes to release their genome into the cytosol while non-enveloped viruses use membrane-lysis or pore-formation [128]. The catalytic event that determines when release occurs is usually pH, where the acidic environment inside the endosome or lysosome induces conformational changes in the virion structure that enables fusion [128].

The most common viral entry route is clathrin mediated endocytosis (CME), followed by caveolae and a number of clathrin and caveolae independent routes. The virus could also be engulfed and enter through macropinocytosis or phagocytosis. A few enveloped viruses have also been shown to enter by fusion with the plasma membrane for example herpes simplex-1 and HIV [128]. But as the same viruses are also endocytosed at the same time it is difficult to determine if plasma membrane fusion lead to productive infection. Using the cells own machinery when entering can have many advantages for the virus, it not only offers a protected environment for the virus to pass through the actin cortex and the dense cytoplasm while transporting it to necessary sites and organelles where virus penetration can occur but it also helps the virus to escape recognition by the immune system as no virus specific parts are left at the plasma membrane.

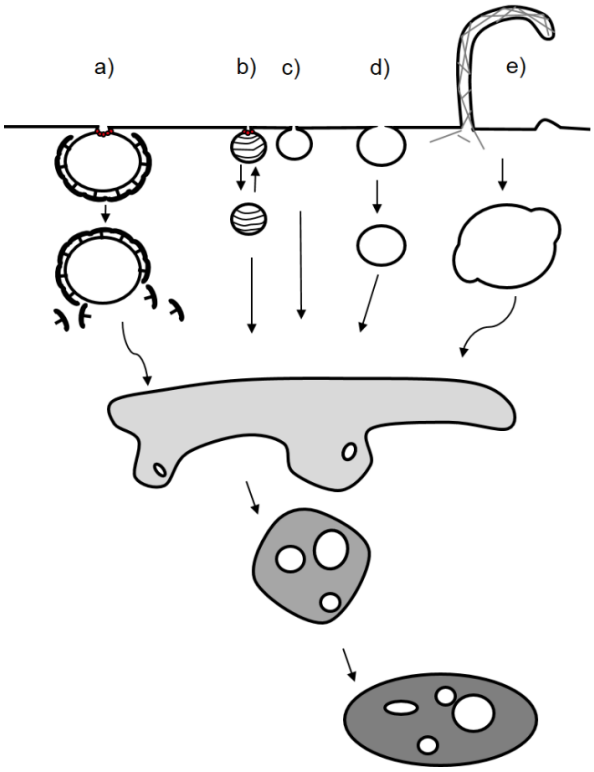

Figure 5: Schematic depiction of different endocytosis routes used by viruses. a) Clathrin mediated endocytosis, b) Caveolae, c) Lipid raft, d) Clathrin- and Caveolae-independent endocytosis and e) Macropinocytosis

6.1 CLATHRIN MEDIATED ENDOCYTOSIS

CME is a constitutive event that is found in all eukaryotic cells and normally used to take up cargo and nutrients, control many plasma membrane activities and is fundamental in neurotransmission. Clathrin is a self-polymerizing protein that is composed of three heavy and three light chains that together form a triskelion shape consisting of three bent limbs radiating from a centre [129]. It can polymerize into either flat lattices or cages and is not only involved in endocytosis from the plasma membrane but is also involved in the endosomal

sorting complex required for transport (ESCRT)- dependent cargo sorting at the endosomes, protein secretion from the trans-Golgi network and mitosis [129]. Clathrin can be found in stable islands in the plasma membrane or become recruited through receptor mediated signaling. Some viruses like Reovirus, Influenza A and Vesicular stomatitis virus (VSV) have for example been shown to induce *de novo* formation at the site of binding [130]. The clathrin triskelions form a lattice-like coat on the cytoplasmic surface of the plasma membrane. Binding of clathrin to the membrane is mediated by adaptor protein 2 (AP2) that links the membrane cargo to clathrin and accessory proteins like epsin and AP180 [129]. AP2 is considered to be an absolute requirement for CME [129], some are however controversially suggesting that for example VSV can enter by CME without AP2 [130]. Yet this is still very controversial and needs to be further analyzed.

The process of clathrin coated invagination continues, leading to the formation of deeply invaginated pits and the formation of a vesicle neck. Clathrin polymerization helps in the formation and constriction of the vesicle neck, helping to bring the membranes surrounding the neck into close apposition [131]. The fission of the vesicle is then made by the membrane scission protein dynamin, a large GTPase that form a helical polymer around the vesicle neck [130]. After internalization the clathrin coat is removed by uncoating proteins, Hsc70 and auxilin to form a naked vesicle [132]. The vesicle and its cargo is then transported to Rab5 positive early endosomes while the clathrin triskelia and accessory proteins are recycled to perform more CME. Some virus like VSV exit in the early endosome while others like Influenza A continues through the multivesicular body to fuse with the late endosome [130]. It is thought that the later stages of endocytosis (formation of curved coats and vesicle budding) can occur independently of what cargo is present in the coated pit. Thus, cargo recruitment to the clathrin-containing lattice structure is the key sorting step defining the specificity of the internalization process [132].

The first virus that was shown to enter by CME was Semliki Forest virus (SFV) [133] but today CME is well established as the main entry route for viruses [130]. Yet in some cases like for Influenza A and lymphocytic choriomeningitis virus, CME is only one of several entry pathways that the virus can use [130].

6.2 CAVEOLAE AND LIPID RAFT ENTRY

Caveolae and lipid rafts are found in cholesterol rich domains of the plasma membrane and are involved in cellular endocytic processes and signaling. Lipid rafts is a specialized cholesterol- and sphingolipid-enriched membrane microdomain that can influence membrane fluidity, receptor clustering and assembly of signaling molecules [130]. Caveolae are seen in electron microscope as small regular shaped membrane invaginations [134] and can exist either as single caveolae or clusters of multiple caveolae [135]. They are important in the regulation of various signaling processes, such as nitric oxide activity, and in cholesterol uptake and trafficking [134]. The major structural protein, caveolin-1 forms a coat-like surface around the vesicle and is necessary for the formation of invaginated caveolae [135]. Caveolin is present in the plasma membrane as well as on intracellular structures [135]. It is found in nonmuscle cells with the exception of neurons and leukocytes [131]. Expression of caveolin-1 alone is sufficient to cause the formation of caveolae [135]. Under normal conditions caveolae do not appear to be involved in endocytosis and are kept at the plasma membrane by actin filaments [134]. They do however perform short range "kiss and run" movements, but whether this is in fact is endocytosis is not yet clear [134]. Although caveolae are not ordinarily internalized they can be if stimulated by for example Simian virus 40 (SV40) that initiate a signal cascade that leads to the depolymerization of the actin cytoskeleton [135]. The fission protein is as for CME, dynamin [135].

Caveolae has mainly been suggested to be involved in viral endocytosis for members of the polynoma family i.e. SV40, BK and JC virus [130]. Where the endocytosed polynoma viruses pass through the early and late endosome and are released in the ER. The role of caveolar as a primary route of viral entry has however come in question in recent years and it has now been suggested that only a fraction of SV40 and probably other polynomaviruses enter via caveolae and that the fraction may vary with cell type and virus [130]. The rest use a related caveolin-1 independent mechanism. Both routes appear to be sensitive to cholesterol depletion, and require Arf1 and dynamic actin [130]. Internalization through caveolae also appears to be slower, dynamin-2 dependent and more dependent on actin dynamics than the noncaveolar pathway [130].

6.3 CLATHRIN AND CAVEOLAE INDEPENDENT ENDOCYTOSIS

As already stated, some viruses have been shown to become endocytosed in a clathrin and caveolae/caveolin-1 independent way. The number of viruses that can use these routes are expanding but at the moment very little specific information exists regarding these routes. For Influenza A it has been shown that 1/3 of the virus particles are endocytosed in a clathrin and caveolin-1 independent way and that this infection is just as effective and that trafficking through early to late endosomes appear to similar to CME [136]. An IL-2 tentative route of entry has been suggested for Rhesus rota virus and possibly also for SARS virus [130]. In both cases it was shown to be clathrin and caveolin-1 independent and cholesterol and dynamin dependent. Adeno-associated virus 2 was recently suggested to enter through the GEEC/CLIC pathway [137], which is another clathrin and cavoelin-1 independent route. Not much is known regarding this route and so far this is only virus that has been proposed to use this route.

6.4 MACROPINOCYTOSIS AND PHAGOCYTOSIS

Macropinocytosis is a cargo triggered endocytosis route which normally is activated by growth factor. This triggers the activation of a complex signal cascade that induces changes in the actin filaments dynamics and triggers plasma membrane ruffling [138]. These ruffles will eventually collapse back towards the plasma membrane creating an uncoated irregularly shaped vacuole. It is mostly a transient process responsible for the internalization of fluid, solutes and sometimes particles into large vacuoles [139]. It is strictly dependent on cortical actin but independent of dynamin and does not require binding to a specific receptor [140]. The vacuole can become acidified and intersect with endocytic vesicles, making it a possible entry pathway [128]. Macropinocytosis is the main route of entry for Ebola virus, although a fraction of the virus also uses CME [141]. Human adenovirus, Influenza A and Vaccinia virus has also been suggested to use this route for part or all of their entry [138].

Unlike the other entry routes, phagocytosis is only found in specialized cells such as macrophages and amoeba, where it is used for uptake of large particles [130]. Mimivirus has been suggested to use phagocytosis [142] while herpes simplex virus 1 has been suggested to use phagocytosis-like uptake [143].

6.5 *BUNYAVIRIDAE* ENTRY AND RECEPTORS

Bunyaviridae entry has been shown to be pH dependent with some virus, like CCHFV, Hantavirus and Oropouche virus, entering by CME [24, 144, 145, Paper I]. While others, like Uukuniemi virus (*Phlebovirus*) has been shown to enter cells mainly through clathrin-independent endocytosis [146] while Rift valley fever virus (*Orthobunyavirus*) was shown to be using caveolae [147].

Viruses express glycoproteins on their surface, by which they can attach to cellular receptors or attachment factors. Although not all viruses require specific receptors for attachment and internalization most virus attach to some cellular receptor or attachment factor. There are numerous viral receptor expressed on

cell surfaces and not all receptors are expressed on all cells. Some viruses have therefore evolved to use different receptors depending on the cell their infecting [124]. The availability and position of a specific receptor can determine whether a virus is able to infect that particular cell [123]. For *Bunyaviridae* viruses, DC-SIGN and β3 integrin have been demonstrated as receptors [148, 149]. A recent report suggested nucleolin to be essential for CCHFV entry [23], but whether it acts as a receptor or an attachment factor aiding in the entry of the virus remains to be investigated.

7 *IN SITU* DETECTION OF NUCLEIC ACID

In situ detection has the advantage of giving the precise and spatial localization of specific nucleic acid sequences in their natural setting. This gives the possibility to correlate the single cell results to that of the surrounding cells or tissue. There are a number of ways to detect nucleic acids *in situ*.

7.1 ISH AND FISH

In situ hybridization (ISH) is a technique where labelled DNA probes are hybridized to specific target sequence in fixed samples giving a localized detection of nucleic acids. It was first performed using radio-labelled probes [150] but had limitations in resolution and probe instability, therefore the technique was further developed. With the addition of fluorescence (FISH, fluorescent in situ hybridization), the method became more applicable and more than one target could be detected simultaneously since different fluorophores could be used for different targets [151, 152]. The detection can be done either directly, so that the fluorescence could be analyzed immediately after hybridization, or indirectly, where probes are labeled, for example with hapten, and then detected with fluorescent-labeled antibodies against hapten. The technique has primarily been used for detection of chromosome abnormalities. During the last few years several modifications of FISH have been presented. Among them adding locked nucleic acids (LNAs) to improve resolution and sensitivity [153, 154]. LNAs are a class of RNA analogs with exceptionally high affinity towards complementary DNA and RNA [153].

Both ISH and FISH have been used for the detection of mRNA *in situ*, both individually and as multiplexed analysis [150, 155-157]. Hybridization-based methods are however usually semiquantitative and do not allow precise digital quantification of the signals. They can also not discriminate between highly similar sequences, making them unsuitable for studying cell-specific allelic expression or expression of splice variants [158].

7.2 PADLOCK PROBES

In oligonucleotide ligation assay (OLA), two oligonucleotides bind next to each other and are ligated by a DNA ligase if they form a perfect match [159]. This allows for the distinction of single nucleotide variants at the ligation junction. Padlock probe is a further development of OLA. Instead of having two different oligonucleotides, a padlock probe is one long linear oligonucleotide where the 3'- and 5' ends bind juxaposed to each other on the target sequence. The ends of the padlock probe are joined together by a target-non-complementary DNA backbone that is later used as detection site. If the two ends are correctly hybridized to the target sequence a nick is formed between them. The nick can then be closed by a DNA ligase, creating a closed "locked" circular DNA molecule [160]. A locked probe remains attached to its target sequence and can withstand highly stringent wash conditions, further reducing the amount of non-specific signals [160]. The ligated probe can then be detected by labeled oligonucleotides towards the target-non-complementary DNA backbone of the padlock probe.

Padlock probe detection is highly specific as two target complementary sequences at the correct position are required for ligation and the DNA ligase has a strong preference for a perfect match, particular at the 3'-end [161]. Mismatched padlock probes will therefore not be circularized. Accordingly, padlock probes can therefore be used for detection of single nucleotide differences [162, 163] and can easily be utilized against multiple targets with limited cross-reactivity between probes [163]. However, to effectively detect the padlock probes signal *in situ*, amplification is needed.

7.2.1 Rolling circle amplification

Rolling circle amplification (RCA), creates a linear single stranded DNA molecule (RCP, rolling circle product) consisting of tandem repeats of the complementary sequence of a single stranded circle [164]. If correctly ligated, padlock probes can be used as templates for RCA, thereby increasing detection

sensitivity. In order for the RCA to proceed more than 1 turn around the template, a polymerase with strand displacement activity is needed, Phi29 polymerase has this. It also has two other important activities for RCA, 3'-5' exonuclease activity on single stranded DNA as well as polymerase activity [165, 166]. This means that it can remove the non-base-paired nucleotides downstream of the padlock probe and when reaching the probe bound to the target, it can perform polymerization using the target strand as a primer. RCA of a ligated padlock probe can also be initiated by an external primer. Alternatively, the DNA can be cut site-specifically before amplification by introducing an G:A mismatch, where the padlock probe contains a G which is mismatched with an A in the target molecule [167]. The padlock probe should then be designed so that the mismatch is located at the end of the padlock probe binding site on the target sequence. MutY enzyme will then remove the mismatched A in target molecule, leaving an abasic site in the target molecule. This site is then recognized and cleaved by Endo IV, enabling free access for the Phi29 polymerase to start the RCA [167].

RCA is a very specific way to detect padlock probes since only ligated probes can serve as templates and the produced RCPs will still remain attached to the target sequence [168]. The long RCP spontaneously collapses into a micron-sized object that can be visualized by hybridization with fluorescence-labelled oligonucleotides [169] , which have the same sequence as the backbone of the padlock probe. As the RCP consists of repeated sequences, this will create a local enrichments of fluorophores over background, visual as a single dot in the microscope [169, 170]. The fluorescent dots can then be quantitatively counted [171]. Due to the specificity of the padlock probe, RCA can be performed against multiple nucleic acid targets. Padlock probes and RCA was first used to detect mitochondrial DNA *in situ*, where the DNA first was made single stranded to enable hybridization and ligation of the padlock probe [172].

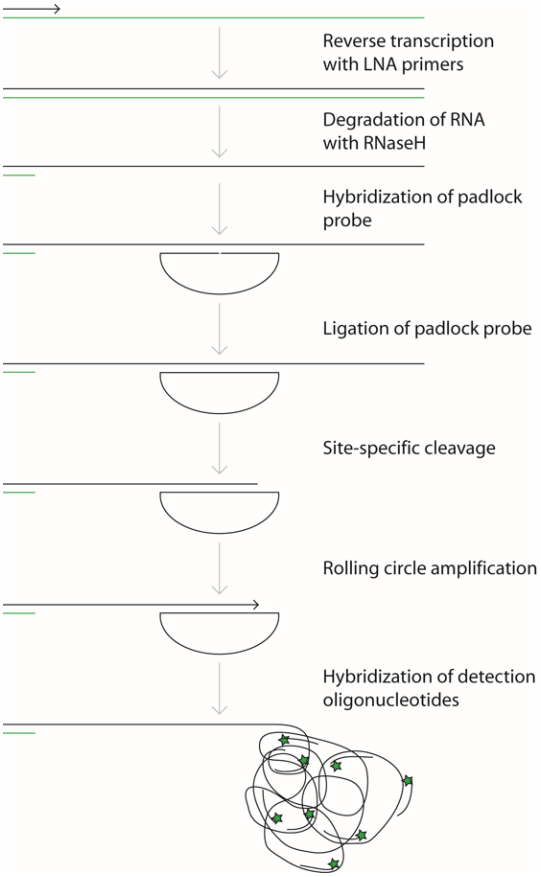

Figure 6: Schematic representation of RNA detection using LNA-primer, padlock probe and target-primed RCA.

7.2.2 RNA detection *in situ* using padlock probes and RCA

RNA can serve as template for the ligation of padlock probes but it has less sensitivity and specificity compared to DNA template ligation [165, 173]. A way to overcome the problems with low sensitivity and specificity of RNA template ligation *in situ*, is to first perform a reverse transcription step where a primer containing LNA modified bases is used to first convert the RNA into complementary (c) DNA. The LNA primer then ensures that the cDNA remains attached to the target RNA even after RNaseH digestion as the LNA primer

protects bound RNA from being degraded [174]. The cDNA can then be detected by padlock probes and RCA. This method has been used to distinguish single nucleotide differences of actin in cultured cells and tissue, and had a detection efficiency of approximately 30% of available transcripts as determined by qPCR [174]. With the addition of RCA and LNA primers, RNA viruses could effectively be detected with padlock probes both in solution [175] and *in situ* [Paper II].

8 PRELIMININARY RESULTS

CCHFV entry is independent of human αV, α5, β3 or β5 integrins.

We have previously shown that CCHFV enters by clathrin-mediated endocytosis [Paper I], but there is, however, no clear data on which receptor CCHFV use. A number of viruses have been shown to use integrins as cellular receptors including another *Bunyaviridae* member, Hantavirus, that was shown to use β3 integrin during entry [148]. Although not the only receptor for Hantavirus, it has been suggested that the binding of hantavirus to β3 integrins could have an effect on cellular integrity by relocation of VE-cadherin and thereby be a cause for the vascular permeability observed in patients [176, 177]. Integrins are transmembrane glycoproteins that consist of an α and β subunit and mediate cell-matrix and cell-cell adhesions [178]. The number of varieties of α and β subunits produce ligand selectivity to extracellular matrix. Integrins transmit signals, outside-in and inside-out and regulate cell survival and migration [123].

To investigate if CCHFV could be using human αV, α5, β3 or β5 integrins during their entry, we used cells that were manipulated to express human integrins to see if this increases internalization as compared to the control cell line. We used Chinese Hamster ovary (CHO-B2) cells, transfected with cDNA to continuously express either human αV integrin (CHO-B2-αV) or α5 integrin (CHO-B2-α5) as well as Chinese hamster melanoma (CS1) cells that had been transfected with cDNA encoding either human β3 (CS1-β3) or β5 (CS1-β5) integrin protein. The CHO-B2 cells were grown in Dulbecco's modified Eagle´s minimal essential medium (DMEM) with 10% FBS, 2mM L-glutamine, 1% NEAA, 100units/ml penicillin, 100ug/ml streptomycin, and 10mM hepes with addition of 700µg/ml of geneticin (all from Gibco, Life technologies) for the CHO-B2-αV and CHO-B2-α5 cells. The CS1 cells were grown in RPMI medium with 10% FBS, 2mM L-glutamine, 100units/ml penicillin, 100ug/ml streptomycin, and 10mM hepes and with the addition of 500µg/ml of geneticin (all from Gibco, Life technologies) for the CS1-β3 and CS1-β5 cells. Cells were seeded on 24 well plates and grown to 80% confluence. The same number of cells was always used for all compared cell lines, as CS1 cells grow as suspension cells due to the lack of anchoring integrins.

Cells were infected with CCHFV Ibar 10200 (moi 1, as determined by Vero titration) for 1h at 37°C in a 5% CO_2 humidified atmosphere. The adherent cells were rinsed twice in PBS and the nonadherent cells were centrifuged and rinsed twice with PBS before replenished in fresh medium, added to the original well and incubated for 24h at 37°C in a 5% CO_2 humidified atmosphere. At which point the nonadherent cells were centrifuged and cell pellet added TRIzol, the lysed cells were then combined with lysed attached cells in the original well. All handling of the virus was carried out at the BSL-4 facility at the Public Health Agency of Sweden, Solna, Sweden. Total RNA extraction, cDNA synthesis and quantitative real-time PCR was performed as previously described [25]. Amplifications was always performed in triplicates and relative amount of CCHFV S segment transcripts were calculated with the $2^{-(\Delta\Delta Ct)}$ method in relation to GAPDH (and with the reference Ct set to 1). All assays included noninfected cells and were always negative for CCHFV RNA.

Surprisingly, we found that cells not expressing human αV, α5, β3 or β5 integrins had the highest level of infection. These data suggest that these integrins are not essential for CCHFV entry. In paper III in this thesis, we hypothesize that the shift towards apical entry that occur when infected dendritic cells or their supernatant is added could be explained by a translocation of an viral receptor from the basolateral side to the apical side. This has previously been shown for adenovirus infected macrophages that release soluble factors, including IL-8, causing a translocation of the viral receptor αvβ3 from the basolateral to the apical side [127]. We can here show that CCHFV infection is not dependent on either human αV, α5, β3 or β5 integrin. The reason for the change in entry side in polarized cell is therefore not due to translocation of these integrins and therefore remains to be further investigated.

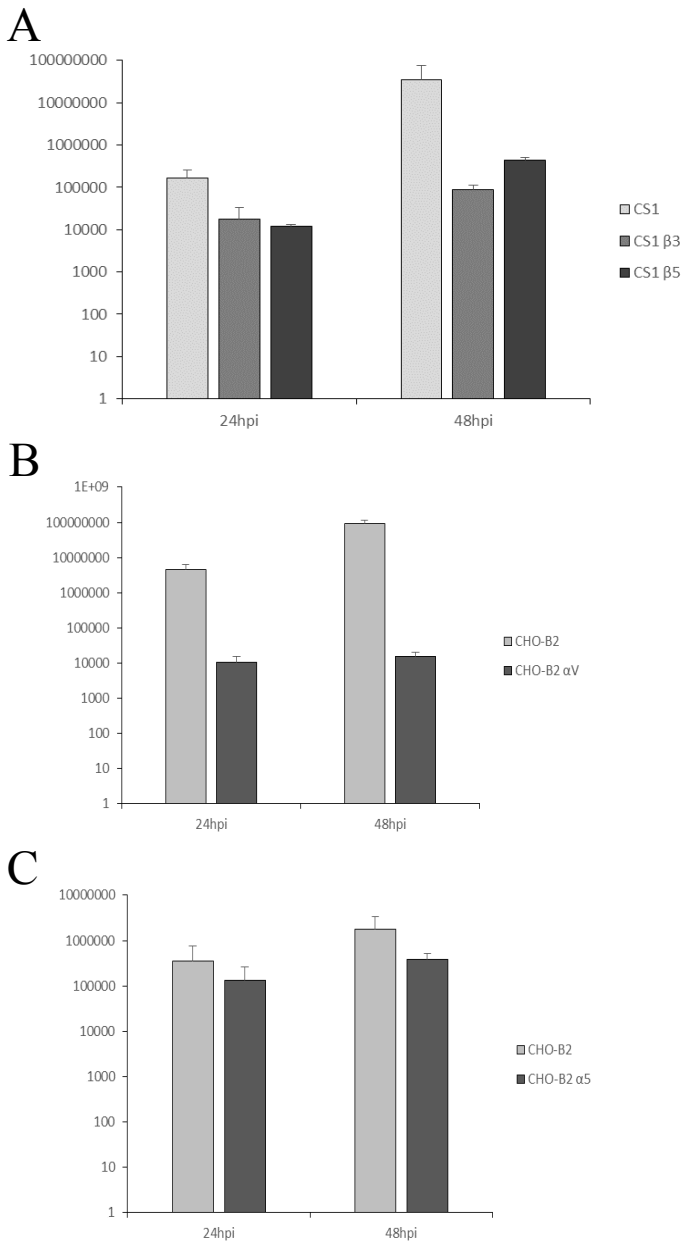

Figure: Cells expressing or lacking human integrins were infected with CCHFV for 24h and then analyzed by RT-PCR for CCHFV RNA levels. Results are shown as fold change to noninfected cells. A) CS1 cells lacking or expressing human integrins β3 or β5. CHO cells expressing or lacking (B) human integrin αV or (C) human integrin α5.

9 RESULTS AND DISCUSSION

9.1 CCHFV ENTRY (PAPER I)

In order for virus to replicate, they have to enter a target cell. Viruses have therefore evolved a of numbers ways to do this. Hantavirus, another *Bunyaviridae* member, was known to enter cells though clathrin-mediated endocytosis in a pH-dependent manner [144] but nothing was known regarding CCHFV's entry.

We therefore sought to investigate two common viral entry routes; the clathrin-mediated and caveolae endocytosis by reducing the expression of the main proteins of each pathway, clathrin and caveolin-1 respectivly. The siRNA knockdown of caveolin-1, as confirmed by qPCR and western blot, had no effect on the level of CCHFV infection, suggesting that caveolae is not necessary for CCHFV infection. This was further confirmed by the fact that Vero E6 cells, one of the most commonly used laboratory cell line for CCHFV infection was discovered to have no or very low levels of caveolin-1, yet it had the highest level of CCHFV infection of the investigated cell lines in this study.

On the contrary, by treating cells with sucrose or CPZ, we found an indication that CCHFV could use clathrin-mediated endocytosis. This was confirmed by a drastic reduction of infection when clathrin protein and mRNA levels were reduced. We therefore concluded that CCHFV most likely enters through clathrin-mediated endocytosis. However, we could not completely knock-down clathrin, and in accordance with this, some CCHFV infection could still be detected in the clathrin knock-down treated cells. Thus, we could not exclude that CCHFV could also use a different clathrin-independent route for entry.

As clathrin-mediated endocytosis transports virus to the early endosomes and many viruses require a drop in pH to induce a conformational change in their glycoproteins and thereby escape from the endosome, we also wanted to investigate pH-dependency. By treating cells with chemicals known to disrupt

the acidification of the endosome, we could determine that CCHFV entry is pH-dependent.

Furthermore, in this paper we also investigated whether membrane-bound cholesterol was needed for CCHFV entry by treating the cells with a cholesterol-depletion drug. Caveolae and lipid raft endocytosis are located in cholesterol-rich parts of the membrane and are therefore highly dependent on cholesterol but CME also requires cholesterol. By treating the cells at various time-points before, during or after infection we concluded that CCHFV binding was unaffected by cholesterol depletion but that cholesterol was needed during or soon after entry and possibly also during later event, such as transcription or replication.

Since this paper was published another study has confirmed our results showing that CCHFV enters by clathrin-mediated endocytosis and that CCHFV escapes from the early and not the late endosome [24].

9.2 *IN SITU* DETECTION OF CCHFV RNA (PAPER II)

Single cell detection of RNA has several advantages compared to other molecular methods as it will give the expression level for each cell, which can vary significantly from the mean expression detected in a cell population [179, 180]. Visualization of single RNA molecules *in situ* will give the spatial location as well as reveal potential interactions within the cell, differences that would not be detected by for example PCR. We therefore wanted to develop an *in situ* based method where we could detect individual CCHFV RNA transcripts using a fluorescent microscope.

CCHFV is a negative single stranded virus, meaning that during its replication it must produce a positive RNA strand for transcription and replication. We therefore wanted to target the different strands and be able to differentially detect them to investigate their cellular location during infection. Reverse transcription together with specific detection by padlock probes and target-primed RCA had

previously been used to detect individual RNA molecules *in situ* [174]. Yet it had never been done on negative stranded RNA viruses.

Therefore, we investigated if this technique could be used to detect and differentiate between CCHFV vRNA and cRNA in fixed cells as well as combining this with detection of CCHFV proteins. Cell were infected for various times and fixed. vRNA or cRNA was then reverse-transcribed into cDNA by using individual LNA-containing primers. The LNA primer protects the target from being degraded during RNaseH treatment, leaving the cDNA still attached to the target. Each cDNA is then detected by a padlock probe which if correctly ligated becomes circularized. The target is then site specifically cleaved to produce a starting point for the RCA. The resulting RCP is detected by hybridization of fluorescently labelled oligonucleotides, making them visual as single dots that then can be digitally counted.

The method worked well, however some false-positive signals could still be detected in the technical controls, but they were always significantly lower than signals observed in infected cells. Both vRNA and cRNA could be detected individually and together. vRNA was found located throughout the cytoplasm while cRNA was more concentrated to specific parts of the cell. The assay could also be used to investigate co-localization between viral RNA and viral proteins. cRNA was found to co-localize with CCHFV NP within the same cellular structures while vRNA showed no intra-cellular co-localization with CCHFV NP.

By combining *in situ* reverse transcriptase by LNA-primer with detection using padlock probes and RCA, we were able to detect the different strands as individual dots when looking in a fluorescence microscope. Unfortunately due the positioning of the primer and probe, we could not discriminate between cRNA and mRNA. While these techniques has previously been used for the detection of positive stranded RNA viruses [181], this was the first time it was used to detect a negative stranded RNA virus *in situ* and distinguish between different RNA strands.

9.3 *IN VITRO* PATHOGENESIS MODEL (PAPER III)

To understand the molecular mechanism behind CCHFV's pathogenesis, there is a need to investigate virus-host interaction. To date, there are no good *in vivo* or *in vitro* models available for this. A transwell assay has previously been developed and used to study the direct effect of CCHFV infection on cell integrity. It was then found that infection was not sufficient to cause increased permeability and it was suggested that other factors therefore most likely were involved. However this assay was based on canine cells. Therefore in order to confirm these data, we have here established an *in vitro* assay based on human epithelial cells. Moreover, this assay was used to study cell-cell interaction, to further assess other reasons behind the increased vascular permeability observed in CCHFV.

We infected the human polarized cells and could confirm that direct infection and replication is not sufficient to cause disruption in cellular integrity. Further indicating that other elements most likely are important in the development of the vascular leakage observed during CCHF. Elevated levels of cytokines has been detected in CCHF patients and in the new animal models [80, 83, 107, 108]. In addition, *in vitro* experiments have shown that infected moDCs release the same cytokines that has been found in clinical and *in vivo* studies [96, 100]. DC has also been suggested to be one of the primary target cells for CCHFV infection [81, 83] and to play a role in spreading the virus.

Infected moDCs was therefore added to our polarized human epithelial cells to study if they affected the permeability of these cells. No change in permeability could be detected regardless of the side the infected moDCs were added to. Though we could detect CCHFV RNA in the epithelial cells confirming that DCs could have a role in the systemic spread of the virus. However, quite surprisingly we could detect a higher level of infection in the epithelial cells when the infected moDCs were added on the apical side of the epithelial cells. This is in contrast to the primarily basolateral viral entry observed when only virus was added. To determine if the moDCs had an active role in the apical entry we added supernatant from infected moDCs to either side of the polarized epithelial cells.

Again, a primarily apical entry for CCHFV was observed, suggesting that released soluble factors from infected moDCs might affect CCHFV entry.

A recent report on adenovirus, which exclusively enters from the basolateral side, found entry to occur from the apical side if infected macrophages were added on that side. This was mainly due to released IL-8 causing a translocation of the adenovirus receptor human integrin $\alpha_v\beta_3$ from the basolateral side to the apical side [127]. Elevated levels of IL-8 has been shown for CCHFV both in patients and *in vitro* [96, 109, 114, 182]. It is currently not clear what receptor CCHFV is using and in the preliminary results section of this thesis we show that CCHFV entry is independent of human α_v or β_3 integrin. The observed shift towards apical entry must therefore be due to something else. It can however not be ruled out that CCHFV-infected moDCs can cause translocation of another yet unknown receptor from the basolateral side to the apical, thereby assisting viral entry from the apical side.

More experiments are however needed in order to determine the reason for the change towards apical entry for CCHFV. As well as more research on possible CCHFV receptors and the role of macrophages and dendritic cells in disseminating and aiding CCHFV entry. Knowledge regarding the exact mechanism behind CCHFV's pathogenesis would greatly improve research on specific treatments.

10 CONCLUDING REMARKS

Crimean-Congo hemorrhagic fever virus can cause severe human disease and unfortunately not much is known about this virus. Work in this thesis has contributed to new knowledge regarding the entry route into cells and established a new technique to visualization and discriminate between vRNA and cRNA in a single cell. Within this thesis I also established an *in vitro* model to study cell-cell interactions that be used to study molecular mechanisms behind CCHFV's pathogenesis.

Viruses have to enter a host cell in order to replicate its genome, in paper I, we found a strong dependency for clathrin protein for CCHFV's entry. We could also demonstrate a dependence for a lower pH during CCHFV entry. We therefore concluded, and this has since been confirmed by another group, that CCHFV enters through clathrin-mediated endocytosis in a pH-dependent manner. However, more research is needed to characterize more specific details regarding CCHFV's entry and uncoating process, work that potentially could contribute to the development of new antivirals.

In paper II, a new technique for the visualization of individual RNA molecules for CCHFV was established. We used this technique to detect the different strands of RNA that is produced during CCHFV's replication as well as to investigate their potential co-localization with the viral nucleocapsid protein. vRNA was detected throughout the cytoplasm and did not co-localize with CCHFV NP while cRNA was found to be more concentrated to particular regions within the cell. cRNA was also found to co-localize with the viral nucleocapsid protein within these regions. This new technique enables visualization of vRNA and cRNA transcripts and their interaction with viral protein, which could be used to characterize different steps of the viral replication cycle.

In paper III, a new *in vitro* model system is presented where moDCs were added to polarized human epithelial cells to study the effect on the epithelial cell integrity. This was done as a model system of the vascular permeability that is observed in CCHF patients. While neither direct infection with virus nor the addition of moDCs had an effect on the integrity of the epithelial cell layer in our model. Surprisingly, we observed an entry shift from a primarily basolateral entry when only virus was added, to a primarily apical entry when CCHFV-infected moDCs were added. The shift in entry for CCHFV was also observed when supernatant from infected moDCs were added apically. The reason for this remains unclear but we speculate that it could be due to released soluble factors from the dendritic cells that possibly could cause a translocation of a yet unknown viral receptor from the basolateral to the apical side. However this needs to be further investigated.

Taken together, we established several new *in vitro* model systems to study CCHFV's interaction with host cells. We also show data demonstrating the entry pathway for CCHFV into mammalian cells. These data and tools will hopefully facilitate and promote research on virus-host interactions which in turn may result in the development of new anti-virals.

11 POPULÄRVETENSKAPLIG SAMMANFATTNING

Krim-Kongo blödarfeber (CCHF) är en allvarlig sjukdom som drabbar människor. Den kan i allvarligast fall ge mycket svåra symtom med blödningar och organ kollaps. Sjukdomen orsakas av viruset Krim-Kongo blödarfebervirus (CCHFV), som i naturen sprids av fästingar. Människor smittas genom fästingbett, hantering av smittade eller fästingbärande djur eller vård av andra CCHF sjuka människor. Det finns idag ingen specifik behandling eller vaccin utan man kan bara behandla symtomen. Viruset finns idag i delar av Afrika, sydöstra Europa och sydvästra Asien. Flest fall förekommer för tillfället i Turkiet som sedan 2002 har haft över 6000 fall. CCHFV är ett negativt enkelsträngat RNA virus, där RNA:t eller arvsmassan som det också kallas är uppdelat på tre olika segment som tillsammans kodar för virusets 4 strukturella proteiner. Att arvsmassan är av negativ polaritet gör att viruset först måste tillverka en likadan positiv mall innan det kan utnyttja cellens maskineri för att tillverka sina egna proteiner och nytt virus RNA. Arbetet i den här avhandlingen har gjorts för att öka kunskapen om hur CCHFV tar sig in i celler, dess replikation och hur det eventuellt kan orsaka sjukdom. Kunskap som förhoppningsvis kan leda till bättre behandlingsmetoder.

Virus kan inte replikera sin arvsmassa själv, utan är beroende av värdceller för att kunna föröka sig. För att kunna göra det måste viruset ta sig in i cellen. Det kan virus göra på många olika sätt och i denna avhandling undersöker vi två av de vanligaste sätten för virus att ta sig in i celler för att se om CCHFV använder dessa. Det gjorde vi bland annat genom att nedreglera två cellproteiner som är nödvändiga för de olika vägarna. På så vis kunde vi komma fram till att CCHFV behöver cellproteinet clatrin, för att kunna ta sig in i cellen. Vi kom också fram till att viruset behöver en pH-sänkning för att kunna ta sig in i cellen. I andra virus brukar en pH-sänkning medföra en förändring i ytproteinerna hos viruset, så att det kan ta sig ur den vesikel som det transporterats in i och börja replikera sin arvsmassa. Exakt hur pH värdet påverkar CCHFVs ytprotein är dock än så länge oklart.

Vi har också använt en ny teknik för att kunna studera CCHFVs replikation, där vi visualiserar och kan skilja positivt och negativt strängat virus RNA åt, inne i fixerade celler. Teknik som denna är viktiga verktyg för att lära oss mer om hur viruset replikerar i cellen. Med denna metod kan man se varje enskilt RNA som en lysande punkt inne i cellen när man studerar dem i fluorescence mikroskop. Vi kunde även färga för virus proteiner för att studera om de befann sig på samma ställe som virus RNA:t i cellen.

Vi har även studerat hur viruset skulle kunna påverka stabiliteten mellan celler, vilket skulle kunna orsaka t.ex de inre blödningar som ses i patienter. Det har vi gjort genom att odla ut epitelceller på ett membran tills de var täta och polariserade, dvs. har en övre och en undre sida som delvis är olika. Sedan infekterade vi epitelcellerna med virus för att se om det räckte med bara virus infektion för att cellerna skulle förlora sin täthet. Vi kom fram till bara virus infektion inte var tillräckligt utan troligen måste människans immunförsvar också vara med och påverka. Därför infekterade vi även en speciell typ av immunceller, dendritiska celler, med CCHFV. Dendritiska celler har en viktig roll i immunförsvaret och kan både utsöndra cytokiner, vilket kan påverka andra celler att gå i försvarsställning och även presentera en bit av viruset för en T cell så att den aktiveras, och tillverkar antikroppar mot just det viruset. När vi tillsatte CCHFV infekterade dendritiska celler till våra polariserade epitelceller så påverkade det inte heller stabiliteten mellan cellerna. Vi såg dock att viruset nu hade mycket lättare att ta sig in från den övre sidan än den undre. Det är motsatsen till det vi såg när vi bara tillsatte virus och vi spekulerar om den denritiska cellen utsöndrar något som hjälper viruset att ta sig in från den övre sidan av epitelcellerna. Exakt vad det skulle kunna vara är i dagsläget oklart.

Antalet fall av Krim-Kongo blödarfeber har de senaste åren ökat och viruset har även hittats på nya geografiska plaster. Med tanke på de svåra symtom som sjukdomen kan ge och att det idag inte finns någon specifik behandling är det därför viktigt att öka kunskapen om detta virus. Något som denna avhandling har gjort.

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"It's a trap" –Admiral Ackbar, Star Wars