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**PATHOGENESIS OF AN  
EMERGING PATHOGEN –  
CRIMEAN-CONGO  
HEMORRHAGIC FEVER  
VIRUS**

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**It is better to live your own destiny imperfectly than to  
live an imitation of someone else's life with perfection.**

- The Bhagavad Gita

*To my family*



## ABSTRACT

Epithelial cells represent the first barrier to viral infection and the site of viral entry and release is often intimately connected with viral spread and pathogenesis. The plasma membrane of epithelial cells is polarized into an apical and basolateral domain separated by tight junctions (TJs). TJs also regulate paracellular permeability and can cause leakage upon deregulation.

The key players underlying VHF pathogenesis are believed to be endothelial cells (ECs) and immune cells. Generally ECs can be targeted either directly by viral infection and/or indirectly by soluble mediators released from infected immune cells leading to the observed hemorrhage and vascular permeability.

The main aims of this thesis were to study the direct and indirect effect of viral replication on epithelial and immune cells as a possible contribution to CCHF molecular pathogenesis.

By studying the site of entry and release in polarized epithelial cells, we showed the entry and release of CCHFV to be preferentially basolateral. We studied the transepithelial electrical resistance (TER) over infected epithelial cells, and showed that CCHFV replication has no direct effect on epithelial permeability. Furthermore, we observed no effect of CCHFV on the localization of the TJ proteins occludin and ZO-1 in epithelial cells. Interestingly, CCHFV directly activates ECs upon infection as shown by upregulation of intercellular adhesion molecule 1 (ICAM-1), release of IL-6 and IL-8 and most importantly increased adhesion of leukocytes.

The finding that the increased vascular permeability and hemorrhage is most likely not caused by CCHFV induced TJ disassembly suggests that other cells are likely to be, at least partly, involved in pathogenesis and we therefore focused on the contribution of immune cells to pathogenesis. We showed that only monocyte-derived dendritic cells (moDCs) could be productively infected by CCHFV and that infection was followed by the release of tumor necrosis factor (TNF), IL-6 and IL-10. Interestingly, conditioned media from CCHFV-infected moDCs activated ECs as indicated by enhanced intercellular adhesion molecule 1 (ICAM-1) expression. This effect was shown to be dependent on TNF.

The work presented in this thesis provides an insight into the direct and indirect interplay between epithelial cells and immune cells and provides data that could be used for further studies into the underlying mechanisms of CCHF pathogenesis. Importantly understanding the mechanisms behind CCHF pathogenesis is most likely needed for the future development of specific treatments and/or vaccines against CCHFV.

## LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. **Connolly-Andersen AM**, Magnusson KE, Mirazimi A.  
Basolateral entry and release of Crimean-Congo Hemorrhagic Fever virus in polarized MDCK-1 cells  
*Journal of Virology* 2007 Mar; 81 (5): 2158-64
  
- II. **Connolly-Andersen AM**, Douagi I, Kraus AA, Mirazimi A.  
Crimean-Congo Hemorrhagic Fever virus infects human monocyte-derived dendritic cells  
*Virology* 2009 Aug 1;390(2): 157-62
  
- III. **Connolly-Andersen AM**, Moll G, Andersson C, Douagi I, Mirazimi A.  
Crimean-Congo Hemorrhagic Fever virus activates endothelial cells  
*Submitted*

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## LIST OF ABBREVIATIONS

AJs	Adherens Junctions
ALT	Alanine transferase
APC	Antigen presenting cell
AST	Aspartate transferase
CCHF	Crimean-Congo Hemorrhagic Fever
CCHFV	Crimean-Congo Hemorrhagic Fever virus
DCs	Dendritic cells
ECs	Endothelial cells
EHF	Ebola hemorrhagic fever
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IEJs	Interendothelial junctions
IFNs	Interferons
ISGs	Interferon stimulated genes
MHC	Major histocompatibility complex
NK cells	Natural killer cells
NP	Nucleocapsid protein
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I
RLRs	RIG-I like receptors
ssRNA	Single stranded RNA
TER	Transepithelial electrical resistance
TJs	Tight junctions
TLRs	Toll like receptors
TNF- $\alpha$	Tumor necrosis factor $\alpha$
VCAM-1	Vascular cell adhesion molecule 1
VHF	Viral hemorrhagic fever
ZO-1	Zonula occludens 1

## INTRODUCTION

Crimean-Congo Hemorrhagic Fever virus (CCHFV) belongs to a group of viruses that cause viral hemorrhagic fever (VHF). It is the most geographically widespread of medically important tick-borne viruses ranging from southern areas of Africa over Eastern Europe to Russia. It is not as well studied as some of its more famous VHF cousins such as Ebola and Marburg viruses; however the disease course of Crimean-Congo Hemorrhagic Fever (CCHF) is similar to Ebola hemorrhagic fever (EHF) albeit milder. The main focus of this thesis has been to assemble a picture of CCHF molecular pathogenesis.

CCHF pathogenesis is probably derived from a complex interaction between the virus and host cells. So far few studies have investigated the contribution of what seems to be the main players: endothelial cells and immune cells. Endothelial cells (ECs) are the likely focal point of pathogenesis since they not only recruit and orchestrate the immune response to infection but can also be influenced by inflammatory cells leading to the cardinal signs of VHF: hemorrhages and shock. In general, the two theories underlying the pathogenesis of CCHF and other VHFs is that the virus interacts either i) directly with the endothelial cell leading to activation and dysfunction and/or ii) indirectly via infection of neighboring endothelial cells or immune cells with subsequent release of soluble mediators which act upon the endothelial cell leading to activation and dysfunction. These two classical theories are the basis of this thesis.

## CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS

### A short history

In the 12<sup>th</sup> century a hemorrhagic disease now considered to be CCHF was described in the *Thesaurus of the Shāh of Khwarazam*. Clinical features included presence of blood in urine, rectum, gums, vomit, sputum and abdominal cavity (Hoogstraal 1979). The carrier of the disease was described as an arthropod that may well have been a species of the tick genera *Hyalomma* that are often found to parasitize blackbirds (Hoogstraal 1979). For several centuries the indigenous people of southern Uzbekistan recognized CCHF under three names all relating to the ability of the virus to cause hemorrhage:

Khungribta (blood taking); khunymuny (nose bleeding) or karakhalak (black death) (Whitehouse 2004).

The first described cases in modern medical science occurred in April-June 1942, 2 years before the Crimean epidemic was recognized (Hoogstraal 1979). During the time period of 1944-45 approximately 200 Soviet soldiers were infected with CCHFV while placed in Crimea (Whitehouse 2004). The disease course of CCHF was identified by inoculating psychiatric patients with isolates from CCHF patients (Hoogstraal 1979). The virus was initially named Crimean Hemorrhagic Fever honoring the geographic locality of its identification and was isolated by Chumakov in 1967 from inoculated newborn white mice (Hoogstraal 1979). However, less than two years later this virus was shown to be indistinguishable from “Congo virus” isolated from a boy in 1956 in what was then known as Belgian Congo and is now known as the Democratic Republic of Congo (Casals 1969). Henceforth the name Crimean-Congo Hemorrhagic Fever Virus (CCHFV) was adopted (Hoogstraal 1979). CCHFV has since then been identified in several countries ranging from the African continent to the Eurasian continent, closely following the distribution of *Hyalomma* spp, its tick vector (Whitehouse 2004).

### **Classification and structure**

CCHFV is classified within the *Nairovirus* genus in the Bunyaviridae family. Five genera are recognized in this family: *Nairovirus*, *Orthobunyavirus*, *Hantavirus*, *Phlebovirus* and *Tospovirus*. All infect animals, apart from the last mentioned genus which infects plants (Schmaljohn CS 2001). The *Nairovirus* genus consists of 34 viruses which are further divided into seven serogroups. Out of these viruses, three are known to cause disease: CCHFV, Dugbe virus and Nairobi sheep disease virus (Burt, Spencer et al. 1996; Nichol 2001).

Viruses in the Bunyaviridae family are spherical, approximately 100 nm in diameter and contain two glycoproteins embedded in the lipid bilayer (Donets, Chumakov et al. 1977; Schmaljohn CS 2001). The viral genome consists of three RNA segments which are single-stranded and of negative sense polarity. All three segments contain one open reading frame (ORF) flanked by a non-coding region with partially complementary nucleotides at the end. Due to base-pairing of the terminal sequences, a non-covalent closed circular structure is predicted for each of the viral segments. The large segment (L) encodes the viral dependent RNA polymerase while the two glycoproteins Gn, and Gc, are encoded by the medium (M) segment. The small segment (S) encodes the

nucleocapsid protein (NP), which complex with all viral genomic RNA segments to form individual ribonucleoprotein particles (Flick 2007).

Several viruses in Bunyaviridae also encode one or two nonstructural proteins (NS) from the S or the M segment, termed NSs or NSm, which is believed to function as an interferon antagonist, determine host range and possibly also serve as a regulatory function in replication (Schmaljohn CS 2001). The occurrence of such a protein has been shown for CCHFV but the function is currently unknown (Altamura, Bertolotti-Ciarlet et al. 2007).

## **Replication**

Entry for Bunyaviridae members is believed to occur by receptor-mediated endocytosis. The viral host attachment protein is unknown for most members of this family; though *Hantaviruses* are known to bind to integrins in order to enter target cells (Gavrilovskaya, Shepley et al. 1998; Gavrilovskaya, Brown et al. 1999). Viral glycoproteins mediate attachment to an as yet unknown receptor in target cells, and CCHFV entry occurs via clathrin-dependent endocytosis (Simon, Johansson et al. 2009). Acidification is necessary for CCHFV entry and replication (Simon, Johansson et al. 2009) and believed to induce conformational changes in the viral glycoproteins leading to fusion of viral and vesicle envelopes with consequent release of the viral genome and polymerase into the cytoplasm (Schmaljohn CS 2001). Once released, the viral genome is partially uncoated followed by transcription of the viral polymerase using capped oligonucleotides that might be cleaved by the L protein from host mRNAs (Schmaljohn CS 2001). Recent studies indicate another mechanism for generating the primers necessary for transcription where NP from *Hantaviruses* sequester mRNA caps stored in P bodies, as opposed to cleavage of mRNA caps by the L protein (Mir, Duran et al. 2008). The viral RNA (vRNA) serves as a template for both mRNA synthesis, enabling translation and generation of viral proteins, and also for complementary RNA (cRNA) synthesis followed by amplification of new vRNA genomes (Schmaljohn CS 2001). Viral proteins are either synthesized on free (S and L mRNA) or membrane-bound (M mRNA) ribosomes. Glycoproteins are synthesized as polyprotein precursors and post-translationally cleaved to yield the mature structural proteins Gn and Gc (Sanchez, Vincent et al. 2002). Apart from the previously mentioned NSm, the cleavage of viral glycoproteins yields the secreted forms GP38, GP85 and GP160, along with a possible mucin-like protein (Sanchez, Vincent et al.

2006). Signal peptides in the glycoproteins direct the transport from the endoplasmic reticulum to the Golgi complex. (Bertolotti-Ciarlet, Smith et al. 2005; Haferkamp, Fernando et al. 2005). The NP targets the viral ribonucleoparticle to the Golgi complex where assembly of progeny virions occurs by association with the glycoproteins in the Golgi. Actin filaments play an important role in transporting NP to the site of assembly (Andersson, Simon et al. 2004). Assembly and maturation of virus particles occurs by budding into the Golgi complex followed by transport in vesicles similar to those observed in the secretory pathway to the cell membrane. Fusion of the secretory vesicle with the plasma membrane releases the progeny viruses into the extracellular environment (Schmaljohn CS 2001). Some viruses within *Hantaviruses* and *Phleboviruses* seem to follow an alternative assembly pathway, as glycoproteins target the cell plasma membrane and initiate virus budding at this site (Anderson and Smith 1987; Goldsmith, Elliott et al. 1995; Ravkov, Nichol et al. 1997).

## Epidemiology

Transmission of CCHFV occurs in an enzootic tick-vertebrate-tick cycle in endemic areas (see figure 1). Particularly ticks of the genus *Hyalomma* are consistently susceptible to infection with CCHFV. The virus has been isolated from all stages of its life cycle suggesting that both transovarial (mother to egg) and transstadial (larvae to adult) transmission can occur (Turell 2007). Since the life cycle of hard ticks (among them *Hyalomma*) is approximately 2 years, an infected tick can serve as a disease reservoir for CCHFV by maintaining the viral infection over winter and re-introducing the virus to the ecosystem the following summer (Turell 2007).

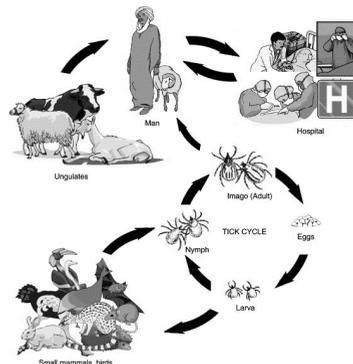


Figure 1. The transmission cycle of CCHFV. Figure from Nabeth, P. 2007 *Criean-Congo Hemorrhagic Fever virus*. Reprinted with permission from Elsevier Ltd. ©

Several vertebrates both domesticated (sheep, cattle, etc.) and sylvatic (hedgehogs, hares, etc.) can develop viremia upon infection thereby maintaining and amplifying CCHFV endemically (Hoogstraal 1979). Birds, except for ostriches, appear to be refractory to infection but can serve as carriers of disease by facilitating virus transmission over large distances (Hoogstraal 1979; Swanepoel, Leman et al. 1998).

CCHFV has one of the most extensive geographic ranges of the medically important arthropod-borne viruses, second only to Dengue virus. The distribution closely parallels that of the vector *Hyalomma* and CCHF disease has been reported from more than 30 countries in Africa, Asia and the Middle East (see table 1).

The main risk groups for acquiring CCHF are people in endemic areas exposed to infection from ticks or handling infected animals. Occupational groups include farmers who accidentally intercede in the tick-vertebrate-tick life cycle of the virus, or abattoir workers and veterinarians who come into close contact with viremic blood of infected animals or the ticks the animals carry (Ergonul 2006). Another risk includes the secondary transmission from infected patients to health care workers. Several cases of nosocomial CCHF outbreaks have been reported over the years with high mortality (Ergonul 2006; Tarantola 2007). Particularly dangerous situations occur upon exposure to infected blood as exemplified by the Tygerberg outbreak 1981-1984 in South Africa. The health care workers who handled hemorrhaging CCHF patients developed disease themselves in 8.7% of cases. There were also accidents where health care workers came into contact with contaminated needles and 33% of these cases developed disease (van de Wal, Joubert et al. 1985).

The concept of an “emerging” disease reflects either the new occurrence or increase of occurrences of a disease in a determined geographical area. CCHF is termed an emerging disease due to new and increasing outbreak reports exemplified by the outbreaks in Turkey and Greece (see table 1). Emerging diseases can occur by a variation in a pathogen by genetic mutations and/or recombination or introduction of the pathogen into an earlier non-endemic area (Pugliese, Beltramo et al. 2007; Maltezos and Papa 2010). As illustrated in the Crimea outbreak in 1944, it is also the presence of favorable environmental factors that precede a CCHF outbreak. Enemy occupation of Crimea during World War II led to neglect of farmland and abandonment

of hare hunting, which resulted in overgrown pastures and an explosion in the hare population closely paralleled by an increase in ticks. Reoccupation of this region by Soviet soldiers led to human interception in the tick-vertebrate-tick transmission cycle, resulting in an outbreak (Hoogstraal 1979). A similar explanation was hypothesized for the emergence of CCHF in Turkey in 2002 although the role of bird migration cannot be excluded (Karti, Odabasi et al. 2004). The increased awareness of a disease in a geographic region may also lead to more findings of the disease, due to the simple fact that more physicians are looking for it along with improved diagnostic facilities that enable the physical detection of virus.

Location	Years (references)	Number of cases	Case fatality rate (%)	Occupation
<b><i>Southeast Europe</i></b>				
Crimea	1944-45 (Hoogstraal 1979)	200	10	Soldiers
Bulgaria	1953-2003 (Papa, Christova et al. 2004; Lukashev 2005)	1570	11-25	Agriculture and health workers
Albania	2001 (Papa, Bino et al. 2002)	69	0	Agriculture and health workers
Kosovo	2001 (Papa, Bozovi et al. 2002)	18	33	Agricultural workers
Greece	2008 (Papa, Dalla et al. 2009)	1	100	ND
Russian Federation	2000-2009 (Maltezou, Andonova et al. 2010)	≥ 1300	3,2	ND
<b><i>Asia</i></b>				
China	1965-97 (Papa, Ma et al. 2002)	286	21-24	Agricultural workers
Kazakhstan	1948-68 (Hoogstraal 1979)	75	50	Agricultural workers
Tajikistan	1943-70 (Hoogstraal 1979)	97	23	Agricultural and laboratory workers
Pakistan	1976-2000 (Burney, Ghafoor et al. 1980; Smego, Sarwari et al. 2004; Sheikh, Sheikh et al. 2005)	26	29-55	Agricultural and health care workers
<b><i>Middle East</i></b>				
United Arab Emirates	1979-95 (Baskerville, Satti et al. 1981; Schwarz, Nsanze et al. 1997)	17	50-73	Health care and agricultural workers
Iraq	1979-80 (Tantawi, Al-Moslih et al. 1980; Al-Tikriti, Al-Ani et al. 1981)	55	64	Agricultural workers
Saudi Arabia	1990 (el-Azazy and Scrimgeour 1997)	40	30	Slaughterhouse workers
Iran	2003 (Mardani, Jahromi et al. 2003)	81	18	Agricultural workers
Turkey	2002-09 (Karti, Odabasi et al. 2004; Bakir, Ugurlu et al. 2005; Ergonul 2006; Yilmaz, Buzgan et al. 2009; Leblebicioglu 2010)	4000	5-10	Agricultural workers and health care workers
<b><i>Africa</i></b>				
Democratic Republic of the Congo	1956 (Hoogstraal 1979)	2	0	Health care workers
Uganda	1958-77 (Hoogstraal 1979)	12	8	Laboratory workers
South Africa	1981-87 (Swanepoel, Gill et al. 1989)	50	30	Agricultural and health care workers
Kenya	2000 (Dunster, Dunster et al. 2002)	1	100	Agricultural worker
Mauritania	2004 (Nabeth, Cheikh et al. 2004)	38	27	Butchers, Agricultural workers

Table 1. Examples of CCHF outbreaks. Numbers in brackets after the year refers to references.

## Prevention and control

Due to limited treatment options for CCHF, supportive therapy is often the best approach for managing CCHF patients. This includes replacement therapy with thrombocytes, fresh frozen plasma, and erythrocyte preparations.

Fatal CCHF cases rarely develop antibodies (Swanepoel, Shepherd et al. 1987; Shepherd, Swanepoel et al. 1989) and treatment with serum from recovered humans or animals has been beneficially used in treatment of CCHF (Hoogstraal 1979). The main antiviral drug used for treatment of VHF is the nucleoside analogue Ribavirin. It has a broad antiviral activity towards RNA and DNA viruses attributed to multiple mechanisms of action which function simultaneously. Some suggested mechanisms are its ability to interfere with nucleic acid synthesis, inhibition of viral RNA capping and immunomodulation (Graci and Cameron 2006). The first clinical use of ribavirin against CCHF was documented in 1995 when three infected healthcare workers received treatment with ribavirin and recovered despite a poor prognosis (Fisher-Hoch, Khan et al. 1995). For ethical reasons a randomized clinical trial examining ribavirin efficacy in CCHF patients has not been performed. However, according to the clinical observations that have since then been performed with regards to ribavirin usage, it is most efficient in the first phase of disease comprising viremia. In the second stage of disease that is more reminiscent of sepsis, ribavirin would not be so efficient and instead drugs targeting disseminated intravascular coagulopathy (DIC) or sepsis should be applied (Ergonul 2007).

Type I interferons (IFNs) have shown promising antiviral results in *in vitro* studies of VHF, including CCHFV (Frese, Kochs et al. 1996; Andersson, Lundkvist et al. 2006; Karlberg, Lindegren et al. 2010). IFNs are initially produced by infected cells and are part of the innate immune antiviral response. The antiviral effects of IFNs are mediated by transcription and protein synthesis of several interferon-stimulated genes (ISGs) (Andersson, Bladh et al. 2004; Ergonul 2007). However, the beneficial effects of IFNs were heavily questioned when administered to CCHF patients, because IFNs mimicked and exacerbated CCHF symptoms and the treatment was discontinued (van Eeden, van Eeden et al. 1985). IFNs could possibly have beneficial effects as prophylaxis upon accidental exposure to CCHFV, but when disease has progressed IFNs do not seem to be a good antiviral solution.

Two vaccine candidates against CCHFV have so far been developed but neither have undergone official randomized clinical trials. The first vaccine was developed in Bulgaria from infected suckling mice brains that were formalin inactivated and administered to volunteers who developed detectable anti-CCHFV specific antibody responses (Whitehouse 2004). The second was a DNA vaccine tested in mice. Although this vaccine elicited antibodies the efficacy could not be tested since animal models did not exist for CCHF at that time (Spik, Shurtleff et al. 2006). Due to the relatively small population at risk it is unlikely that large scale manufacture of CCHF vaccine will occur.

Avoiding or minimizing exposure to CCHFV is the most efficient way of preventing and controlling disease spread. Tick management in domestic livestock occurs by spraying or dipping animals in troughs containing chemical acaricides but this practice is impractical and costly (Whitehouse 2007). Health-care workers are advised to wear protective barrier clothing when caring for patients or handling infected patient material (Tarantola 2007). Any laboratory work with infectious CCHFV is recommended only in highly specialized biosafety level 4 (BSL-4) laboratories with a full-body protective suit (Whitehouse 2007).

## POLARIZED EPITHELIAL CELLS

Polarized epithelial cells are a broad group of cells that have diverse specialized functions and exhibit various morphologies, which also include the endothelial cells that line the blood vessel surface. They constitute a boundary between tissues and the extracellular environment. The individual epithelial cells are tightly connected by junctional complexes, which by forming a barrier to diffusion of molecules across the cell layer, divide the cell surface into two distinct plasma membrane domains called the apical and basolateral domains (see figure 2). It is also this division of the cell into morphological and functional compartments that defines a polarized cell. The apical domain faces the lumen and the basolateral surface is attached to the underlying basement membrane. Tight junctions (TJs) are one of the protein complexes that are involved in maintaining this asymmetrical distribution. The restriction of viral entry and release to a specific membrane domain reflects the course of viral dissemination and in part also pathogenesis (Tucker and Compans 1993; Compans 1995).

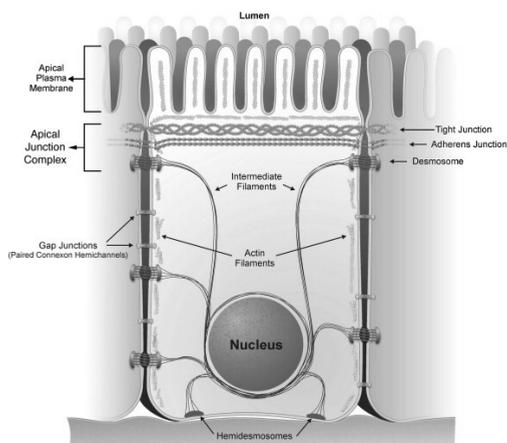


Figure 2. The prototype of a polarized epithelial cell with display of junctions. The junction complex comprises tight junctions, adherence junctions and desmosomes. Figure from Guttman and Finlay, *Biochemica et Biophysica acta* 2009, *Tight junctions as targets of infectious agents*. Reprinted with permission from Elsevier Ltd ©

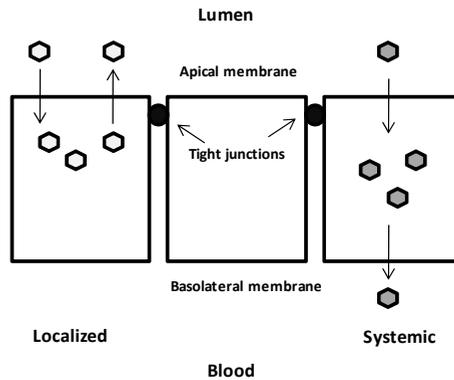
### Polarized entry and release of viruses

The distribution and access to viral receptors determines the virus tropism within the host and is often intimately connected with the pathogenesis of the viral disease. Since the epithelial cell layer is a primary boundary to infection and believed to play a pivotal

role not only in initial virus infection but also in virus transmission by releasing progeny virions, the polarity of viral entry and release has been studied for a wide range of viruses (Compans 1995). Epithelial cells have an asymmetric plasma membrane which is maintained by TJs, thereby hindering the access of virus to basolaterally placed receptors. Viruses that are transmitted by animal bites are thought to enter epithelial cell layers from the basolateral side whereas viral transmission through aerosols and surface contact with body fluids is believed to occur apically in the epithelial cell. CCHFV is spread primarily through tick bites but also by contact with infected material from humans and animals, however little else is known about dissemination within the host. Some members of Bunyaviridae such as the *Hantaviruses* Hantaan and Puumala viruses are transmitted by inhalation of aerosols contaminated with virus containing excreta of infected rodents. Even though their receptor  $\beta_3$  integrin is basolaterally localized in ECs (Schoenenberger, Zuk et al. 1994; Gavrilovskaya, Shepley et al. 1998; Gavrilovskaya, Brown et al. 1999), they have preferential apical entry. This was explained by virus attachment to decay accelerating factor (DAF), a complement receptor located in the apical membrane, which probably initiates intracellular signaling with subsequent opening of tight junctions, thereby enabling attachment to the basolateral integrin receptor (Krautkramer and Zeier 2008).

Polarized assembly of enveloped virions follows the host cells' trafficking pathway and as a result the polarized sorting plays a crucial role in determining where the virus is released. Morphogenesis at intracellular membranes probably requires either targeting signals in the glycoproteins that are recognized by the cell sorting complex or association with a targeted membrane bound factor (Compans 1995). Viral glycoproteins are believed to be the primary determinant for site of release for enveloped viruses (Compans 1995). This is exemplified by the Lassa viruses, a member of Arenaviridae, where the viral glycoproteins target the apical membrane resulting in viral release from the apical compartment (Schlie, Maisa et al. 2010). Glycoproteins, however, are not always the main determinants of the budding site. The matrix protein of Marburg virus, VP40, has been shown to redirect viral glycoproteins from the apical site to the basolateral envelope thus inducing basolateral release of virions (Sanger, Muhlberger et al. 2001; Kolesnikova, Ryabchikova et al. 2007).

The site of viral release can also have a decisive impact on pathogenesis by limiting virus dissemination to a local area or causing systemic infection (see figure 3). This is illustrated by the apical budding of Sendai viruses with concomitant localized pneumotropic infection in mice, as opposed to a Sendai virus mutant caused systemic disease, which was believed to associate with basolateral release (Tashiro, Yamakawa et al. 1990)



**Figure 3. Interaction of viruses with polarized cells.** Apical entry and release is depicted on the left with infection restricted to the epithelial surface. On the right, apical entry but basolateral viral release results in the virus crossing the epithelial barrier with the possibility of a systemic infection.

### Tight junctions

Tight junctions are a circumferential multi-protein complex that spans the space between adjacent cells, thereby maintaining the polarized distribution of proteins and lipids in the outer leaflet of the plasma membrane. One of their physiological roles is to regulate the passage of solutes between cells depending on size and charge, and this discriminatory ability depends on the epithelial type in which the TJs are placed (Aijaz, Balda et al. 2006). The proteins constituting TJs can be divided into three groups: 1) transmembrane proteins that span the intercellular space thereby forming a regulated permeability barrier; 2) cytoplasmic plaque proteins that serve as links between the integral proteins and the cytoskeleton, which also function as adapter molecules involved in cell signaling; 3) cytosolic and nuclear proteins that interact with the plaque proteins regulating permeability and cell polarity (Schneeberger and Lynch 2004). TJ integrity is analyzed by measuring the barrier to small ions across epithelial cells in an electrical field, which is referred to as transepithelial electrical resistance (TER) or by quantifying the diffusion of molecular tracers (Reuss 2001).

### *TJ Integral membrane proteins*

Occludin was the first TJ transmembrane protein to be discovered. The phosphorylation of occludin appears to determine its localization and thereby also partially the integrity of TJs (Furuse, Hirase et al. 1993; Furuse, Itoh et al. 1994; Fujimoto 1995). Phosphorylated occludin is found in the membrane attached to the cytoplasmic plaque protein zonula occludens 1 (ZO-1) via its intracellular domain (Sakakibara, Furuse et al. 1997). Only through localization with ZO-1 can occludin localize to TJs. Protein kinase C (PKC) is involved in occludin phosphorylation and distribution and is the target of the phorbol ester PMA induced disruption of TJ integrity (Andreeva, Krause et al. 2001). The extracellular loops of occludin span the paracellular space and bind to the opposite loops of the neighboring occludin, thereby mediating cell-cell adhesiveness (Balda, Whitney et al. 1996). Occludin is important for barrier function as TER increases upon occludin transfection (Van Itallie and Anderson 1997). Furthermore, when moving from the proximal to the collecting duct in the mammalian nephron, the tightness increases which corresponds with enhanced occludin expression (Gonzalez-Mariscal, Namorado et al. 2000). However, occludin-deficient embryo stem cells retained the ability to form TJs and ZO-1 was still localized to TJs, indicating that although occludin plays a role in barrier and boundary functions, there are other transmembrane proteins which are capable of upholding TJ barrier functions, as exemplified by claudins (Saitou, Fujimoto et al. 1998; Schneeberger and Lynch 2004).

### *TJ cytoplasmic proteins*

The cytoplasmic plaque consists of the cytoplasmic region underneath the TJ. This region is highly organized and contains proteins that link the transmembrane proteins of the TJ to the cytoskeleton and also act as both targets and inducers of signaling pathways (Citi 2001). ZO-1 was the first TJ associated protein that was identified (Stevenson, Siliciano et al. 1986). It is primarily restricted to tight junctions in epithelia, although there are examples where it is found in adherens junctions (Li and Poznansky 1990; Itoh, Nagafuchi et al. 1993). ZO-1 belongs to the membrane-associated guanylate kinases (MAGUK) family, which are characterized by conserved regions such as GUK (guanylate kinase) (Citi 2001). ZO-1 links both occludin and the actin cytoskeleton together (Fanning, Jameson et al. 1998).

The actin filaments of the cytoskeleton are involved in assembly, structure and regulation of TJs (Fanning 2001). They serve both structural and dynamic roles, and the overall cytoskeletal organization is crucial to TJ integrity. The perijunctional actin ring can undergo circumferential contraction in response to phosphorylation of the myosin light chain (MLC) (Fanning, Mitic et al. 1999).

$\alpha$

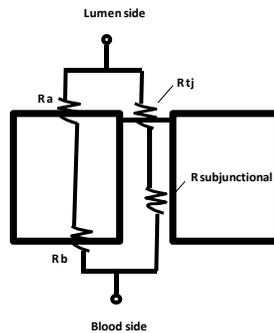
*Transepithelial electrical resistance (TER)*

TER is a measure of the barrier to ions in an experimentally applied electrical field in the media (Fanning, Mitic et al. 1999). A pulse of current is passed across a monolayer and the resulting deflection in voltage is measured, henceforth called resistance (Bazzoni and Dejana 2004). Electrical resistance methods that measure TER depict the epithelial monolayer as a circuit of resistors in parallel comprising both the transcellular and the paracellular resistance elements (see figure 4). As cytoplasmic resistance is low, the transcellular resistance is primarily composed of the resistance over the apical and the basolateral membrane in series. The paracellular pathway runs parallel with the transcellular pathway, and comprises the resistance over the TJs and the subjunctional space in a serial manner. Conductance of ions is limited by the TJs and the paracellular pathway is primarily believed to reflect the resistance over TJs (Gonzalez-Mariscal 2001).

The following formula is applied for calculating TER:

$$\mathbf{R_{cell\ monolayer} = R_{sample} - R_{blank}}$$
$$\mathbf{TER\ (\Omega * cm^2) = R_{cell\ monolayer} * area\ of\ filter\ (cm^2)}$$

$R_{blank}$  is the resistance across a cell-free filter that is bathed in cell culture medium, defined here as the background value.  $R_{sample}$  is the value that is obtained from each individual sample filter. TER is also dependent on the area over which the current passes and this is therefore included in the formula.



**Figure 4.** Electric circuit diagram of an epithelial monolayer. Two polarized cells (rectangles) are joined apically by tight junctions in a continuous manner around the cells. The circuit contains two parallel resistance arms; the paracellular ( $R_{tj}$  and  $R_{subjunctional}$ ) and the paracellular pathways ( $R_a$  and  $R_b$ ). The primary resistance across the epithelial monolayer is mainly due to the resistance across the tight junctions.

### *Pathophysiology of TJs*

Pathogens display a wide variety of mechanisms for disrupting TJ integrity, thereby gaining access to the underlying epithelial tissue (Guttman and Finlay 2009). Basically TJs can be targeted either directly by viral infection or indirectly by soluble mediators released from infected host cells followed by TJ disruption.

Rotaviruses are one of the widely used examples for direct viral disruption of TJs. They produce two enterotoxins: VP8 which is produced by cleavage of the capsid protein and NSP4 which is a non-structural protein. Both toxins lead to disruption of integrity by altering the localization of occludin and ZO-1, thereby causing severe paracellular leakage resulting in a sometimes life threatening diarrhea (Tafazoli, Zeng et al. 2001; Nava, Lopez et al. 2004; Greenberg and Estes 2009). The glycoproteins of Ebola virus are an example of a viral product with a direct effect on the cell which leads to leakage. However, there is still some debate as to the biological relevance of this observation (Yang, Duckers et al. 2000; Wahl-Jensen, Afanasieva et al. 2005).

The indirect modulation of TJs for VHF is thought to be primarily mediated by the release of soluble mediators from infected cells, such as cytokines. Generally cytokines either induce actin remodeling or induce changes in TJ structure. The activation of the inflammatory transcription factor NF- $\kappa$ B is common for the cytokines interleukin (IL) 1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and leads to downregulation of occludin transcription and increased myosin light chain kinase (MLCK) transcription, respectively. Both these transcription events lead to increased permeability (Capaldo

and Nusrat 2009). The Dengue virus-mediated internalization of occludin and ZO-1 from the cell border in ECs resulting in TJ disruption viruses was shown to be caused by release of IL-8 (Talavera, Castillo et al. 2004; Kanlaya, Pattanakitsakul et al. 2009) and Marburg virus-mediated endothelial leakage was due to release of TNF- $\alpha$  (Feldmann, Bugany et al. 1996).

## **ENDOTHELIAL CELLS**

Endothelial cells (ECs) are a form of epithelial cells that line the inner surface of blood vessels and represent a barrier between the circulating blood and the surrounding tissue. They consist of a heterogeneous population ranging from cells lining large to small vessels. ECs either form a tight continuous monolayer in organs where they are required to perform important barrier functions, as exemplified by the brain, or form a discontinuous layer with intercellular gaps or fenestrae, as in the liver (Augustin, Kozian et al. 1994). They are involved in a wide range of cardiovascular functions such as regulating permeability, coagulation and thrombosis, blood pressure and exchange of gases and metabolites (Ribatti, Nico et al. 2002).

In a resting state, ECs present an anti-inflammatory, anti-adhesive and anti-thrombotic surface. However, activation of ECs due to infection or extracellular signaling molecules such as cytokines is associated with distinct phenotype changes. The surface shows inflammatory, thrombotic and adhesive characteristics thereby recruiting leukocytes to the inflamed area.

### **Vascular permeability**

The observed changes in vascular permeability during inflammatory processes are primarily designed to enhance the movement of plasma proteins and circulating leukocytes from the circulation to sites of infection. The barrier property of the endothelium is maintained and regulated by the ECs, glycocalyx and underlying basement membrane. The primary mechanism behind increased vascular permeability is endothelial cell contraction which subsequently results in paracellular gaps (Mehta and Malik 2006).

The interendothelial junctions (IEJs) TJs and adherens junctions (AJs) mediate the barrier properties of the endothelium and they are themselves targets of a wide range of regulatory and signaling events leading to destabilization with subsequent permeability increase (Vandenbroucke, Mehta et al. 2008). AJs consist of the transmembrane vascular endothelial cadherins (VE-cadherin) that are linked through their cytoplasmic tails to the proteins p120,  $\beta$ -catenin and plakoglobin. VE-cadherin binds to opposing VE-cadherin in neighboring cells (Dejana, Orsenigo et al. 2008). IEJ disassembly can be mediated by actin cytoskeleton contraction, microtubule destabilization or

phosphorylation of IEJ proteins with subsequent internalization of proteins (Vandenbroucke, Mehta et al. 2008).

An important feature of VHF is endothelial dysfunction with increased vascular permeability with subsequent redistribution of body fluid and resulting in edema. The leakage from the vascular system contributes to hypotension and hypovolemia, which in severe cases can lead to shock and multiple organ failure (Schnittler and Feldmann 2003). Over the years several studies have concluded that increased vascular permeability is probably a response to soluble mediators released from the ECs themselves, as well as from dendritic cells (DCs), monocytes or macrophages (Feldmann, Bugany et al. 1996; Sundstrom, McMullan et al. 2001; Talavera, Castillo et al. 2004; Luplerdlop, Misse et al. 2006; Shrivastava-Ranjan, Rollin et al. 2010). This is exemplified by *Hantaviruses* that induce the release of vascular endothelial growth factor (VEGF) that binds the VEGF receptor with concomitant internalization of VE-cadherin followed by increased leakage (Shrivastava-Ranjan, Rollin et al. 2010). So far nothing is known regarding the mechanisms behind CCHFV-caused vascular leakage.

### **Endothelial adhesion molecules and leukocyte recruitment**

ECs are crucial for recruiting leukocytes to inflamed tissues and are involved in a multi-step process including rolling, adhesion and extravasation.

Selectins mediate the first step in recruitment by interacting with its ligand on the leukocyte. There are three types of selectins: E, P and L-selectin, of which E-selectin is exclusively expressed on endothelial cells (Langer and Chavakis 2009). The selectin-mediated interaction enables the leukocyte to roll along the endothelium at a reduced speed which is 100-1000 times slower than the blood flow velocity (Smith 2008).

The reduced speed mediated by rolling enables the leukocyte to bind to EC bound chemokines, such as IL-8, resulting in chemokine-triggered activation of leukocyte integrins allowing them to bind to their ligand intercellular cell adhesion molecule-1 (ICAM-1). ICAM-1 is a transmembrane glycoprotein included in the immunoglobulin (Ig) superfamily and contains five extracellular Ig domains which mediate adhesion to  $\beta_2$  integrins (Lawson and Wolf 2009). It is expressed constitutively at low levels on endothelial cells, some lymphocytes and monocytes, and expression is increased by inflammatory mediators such as cytokines or viral infection (Roebuck and Finnegan

1999). Apart from mediating leukocyte transmigration, the cross linking of ICAM-1 by integrins on leukocytes can also induce signal transduction by association with adapter proteins leading to increased expression of chemokines, cytokines and adhesion molecules (Hubbard and Rothlein 2000).

Upon leukocyte arrest on the endothelium, the leukocyte migrates to endothelial cell junctions and extravasates through the endothelial barrier facilitated by ICAM-1 and redistribution of junctional proteins (Ley, Laudanna et al. 2007). There is some debate about whether the extravasation occurs via a paracellular or a transcellular route but when the leukocyte has extravasated, it migrates along a chemotactic gradient through the extracellular matrix towards the site of inflammation (Ley, Laudanna et al. 2007).

## **THE IMMUNE SYSTEM**

### **Innate immune system**

The innate immune system ranges from the protective physical barrier of the epithelium with their corresponding cell-cell junctions (such as TJs), soluble proteins that are constitutively present in fluids (complement) or released from activated cells (cytokines) to membrane-bound and cytosolic receptors that recognize pathogen-associated molecular patterns (PAMPs) on pathogens (Chaplin 2010). Effector cells include DCs, monocytes, macrophages, natural killer (NK) cells, granulocytes and mast cells, all of which can release cytokines, reactive substances and other soluble mediators designed to inhibit or clear viral infections (Janeway and Medzhitov 2002). In this thesis the infectivity of monocytes and NK cells were studied and therefore a short background to each of these effector cells is given.

Monocytes are CD14<sup>+</sup> cells that circulate in the blood circulation and constitute about 10% of peripheral leukocytes in humans. They have a short half-life in humans and are believed to serve as a reservoir to repopulate DCs and macrophages in the periphery. Apart from this, they also have phagocytic abilities, clearing the host of apoptotic cells and toxic compounds (Ziegler-Heitbrock 2000; Auffray, Sieweke et al. 2009).

NK cells are CD56<sup>+</sup>CD3<sup>-</sup> cells and are imperative for clearing infected host cells by inducing apoptosis. The mechanisms for targeting infected cells include a complex interplay of receptors which involves sensing the absence of major histocompatibility complex (MHC) I molecules, and interaction of activating and inhibitory receptors (Caligiuri 2008).

### **Adaptive immune system**

The adaptive immune response is characterized by increased specificity to antigens due to somatic rearrangement of germline genes and selection of the lymphocytes that have the highest affinity to the presented antigen. Memory lymphocytes enable the host to launch an antimicrobial defense the next time the antigen is recognized. The immunological memory is unique to each individual and is not passed along to the next generation. The effector cells are the lymphocytes B and T cells, which comprise the humoral and the cellular response, respectively (Chaplin 2010).

B cells (CD19+) are antigen-presenting cells (APCs), cytokine producers and are defined by their production of antibodies that bind and potentially neutralize pathogens. The antibody isotype that is produced is dependent on the cytokines released in the B cell's environment and isotypes range from immunoglobulin A (Ig A), IgD, IgM, IgE to IgG (Chaplin 2010).

The cell-mediated immune response is comprised of T cells (CD3+CD56-) and the major subsets are defined by their expression of CD4 (T helper cells - Th) or CD8 (cytotoxic T cells - Tc). Tc cells recognize antigen presented on MHC I molecules and their primary role is the elimination of infected cells (Chaplin 2010). Th cells act to regulate the cell-mediated (Th1) and humoral (Th2) immune responses by secreting supportive cytokines specific for either subset. They recognize antigens loaded onto MHC II molecules bound to antigen presenting cells APCs (dendritic cells, macrophages and B cells) (Chaplin 2010).

### **Dendritic cells**

Dendritic cells (DCs) bridge the innate and the adaptive immune response and are pivotal for initiating an efficient response to viral infections. They reside in peripheral and lymphoid tissues and circulate in the blood. Upon encountering an antigen they undergo maturation, characterized by the upregulation of co-stimulatory molecules which facilitate the activation of an adaptive immune response. Maturation also leads to the upregulation of receptors that enable their migration out of the tissue and into the lymphatics followed by transport to the nearest lymph node. Their migratory abilities, their key role in eliciting an effective immune response and their pan-tropic localization make them apt target cells for viruses that thereby can exploit DCs as transport vehicles. By modulation of their intracellular response they can dampen an antiviral response allowing viral amplification.

DCs originate from hematopoietic stem cells that have developed into either myeloid or lymphoid progenitor cells. These progenitor cells can develop into multiple DC subtypes with different immune functions (Manz, Traver et al. 2001). DCs can furthermore be categorized as either conventional DCs (cDCs) or plasmacytoid DCs (pDCs). cDCs can be divided into three functional groupings: a) migratory DCs, which reside in the periphery such as the skin, b) lymphoid-tissue resident DCs that reside in

lymphoid tissues during their life span and c) monocyte-derived or inflammatory DCs that require inflammatory signals to differentiate into DCs, as described above (Belz, Mount et al. 2009). Migratory DCs are exemplified by Langerhans cells and reside in the epidermis where they collect antigen and upon inflammatory stimuli undergo a maturation change leading to an increased immunostimulatory phenotype. In steady state conditions they also migrate to lymph nodes but do not display a mature phenotype (Belz, Mount et al. 2009). Resident lymphoid tissue DCs are found in the thymus, spleen and lymph nodes, where they sample and present antigen within the lymphoid tissue displaying an immature phenotype (Wilson, El-Sukkari et al. 2004). Plasmacytoid DCs are professional interferon inducers and accumulate at inflammatory sites where they secrete IFN- $\alpha$  upon viral infection. During steady state they display an immature phenotype and require further stimuli to develop into functional DCs (Jegalian, Facchetti et al. 2009).

DCs are rare and difficult to isolate without altering their phenotype. It was therefore a breakthrough for DC research when it became possible to differentiate monocytes into immature monocyte derived DCs (moDCs) *ex vivo* using a combination cocktail of the cytokines granulocyte-macrophage stimulation factor (GM-CSF) and interleukin 4 (IL-4) (Sallusto and Lanzavecchia 1994). The experiments performed included in this thesis are based on human blood moDCs differentiated through culture with the above mentioned stimuli.

Immature DCs recognize PAMPs by the pattern recognition receptors (PRRs) which upon stimulation mediate activation of the cell subsequently leading to transformation into a mature APC. It is in this manner that the DCs act as sentinels in the periphery by sampling antigen and undergoing a maturation process enabling them to activate and regulate the adaptive immune response. The PRRs recognize multiple viral components such as single stranded RNA (ssRNA), double stranded RNA (dsRNA), RNA with 5' triphosphate ends and viral proteins. Of particular importance for viral detection are the two PRR systems Toll like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Takeuchi and Akira 2009).

Activation of TLRs and RLRs induces intracellular signaling cascades leading to secretion of IFNs, proinflammatory cytokines and chemokines, and also to the

important upregulation of MHC II complexed with the exogenous antigen and co-stimulatory molecules CD40, CD80 and CD86 required for efficient Th stimulation. DC maturation can also be stimulated by proinflammatory cytokines and chemokines secreted from surrounding cells, such as TNF- $\alpha$  (Cumberbatch and Kimber 1995). In essence, maturation leads to the shift from an antigen-capturing cell to an APC. The downregulation of C-C chemokine receptor type 5 (CCR5) and upregulation of CCR7 enables the maturing DC to migrate out of the inflamed tissues and into the draining lymph nodes via the afferent lymph vessels into the T-cell rich areas (Banchereau, Briere et al. 2000; Takeuchi and Akira 2009).

### **Cytokines and chemokines**

Cytokines are messengers alerting the host to a viral infection and are crucial for orchestrating and regulating systemic antiviral responses. They regulate and down-tune the inflammatory response when the system has cleared the viral infection and the response is no longer needed. It is the balance between proinflammatory and antiinflammatory cytokines that determine the outcome of the immune response. In this manner they are also markers of the strength of the inflammatory response. All nucleated cells have the ability to produce cytokines, but specific cytokines can be limited to subsets of cells.

IL-1 $\beta$ , TNF- $\alpha$  and IL-6 are cytokines that induce the release of acute phase proteins from the liver such as proteins involved in the complement system, coagulation and fibrinolysis (Kerr, Stirling et al. 2001; Hehlhans and Pfeffer 2005; Song and Kellum 2005; Barksby, Lea et al. 2007). TNF- $\alpha$  is released by immune and non-immune cells such as DCs, macrophages, NK cells, T cells, smooth muscle cells and keratinocytes. It can lead to fever, hypotension and shock and is a potent proinflammatory cytokine leading to production of cytokines and recruitment of leukocytes. TNF- $\alpha$  induces increased permeability by increasing transcription of myosin light chain kinase (MLCK) and inducing internalization of TJ component proteins (Capaldo and Nusrat 2009). IL-1 $\beta$  and TNF- $\alpha$  have been identified as the most important cytokines in septic shock. When either IL-1 $\beta$  or TNF- $\alpha$  were infused into animal models the animals developed a disease picture reminiscent of septic shock, and when infused at very low levels these two cytokines were shown to have synergistic effect (Okusawa, Gelfand et al. 1988; Hehlhans and Pfeffer 2005; Tracey, Klareskog et al. 2008). IL-1 $\beta$  is released

from cell types such as endothelial cells, monocytes, macrophages and many other cell types (Barksby, Lea et al. 2007). IL-6 is mainly released from endothelial cells, dendritic cells, macrophages and monocytes. IL-6 induces fever and is involved in hemostasis (Kerr, Stirling et al. 2001; Song and Kellum 2005).

IL-19 is a recently discovered new cytokine and even though it belongs to the IL-10 family of cytokines, it is a proinflammatory cytokine. It is involved in the pathogenesis of septic shock (Sabat, Wallace et al. 2007; Hsing, Chiu et al. 2008).

IL-8 is a chemokine produced in response to proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  and viral products (Hoffmann, Dittrich-Breiholz et al. 2002). IL-8 provides a chemotactic gradient in which leukocytes can migrate towards the inflamed tissue. It binds to glycosaminoglycans (GAGs) present on the EC surface and is a ligand for leukocyte G-protein coupled receptors required for integrin activation (Apostolakis, Vogiatzi et al. 2009). IL-8 also contributes to increasing endothelial permeability (Petreaca, Yao et al. 2007).

IL-10 is an important immunomodulatory cytokine and is believed to play a role in generating and activating regulatory DCs and T cells. IL-10 is important for downregulating the release of proinflammatory cytokines, adhesion molecules and antigen presentation in cell types such as macrophages and DCs. IL-10 correlates with the severity of the inflammatory response in sepsis and reflects the levels of TNF- $\alpha$  in septic shock patients (Scumpia and Moldawer 2005; Commins, Steinke et al. 2008).

## **CRIMEAN CONGO HEMORRHAGIC FEVER – THE DISEASE**

### **Clinical features**

Approximately 1 out of 5 persons who were exposed to CCHFV developed disease according to an epidemiological model developed for CCHF. This model was based on data derived from 264 CCHF patients during 1963-1976 (Goldfarb, Chumakov et al. 1980). If disease develops after exposure to virus there are four phases: Incubation, pre-hemorrhagic, hemorrhagic and convalescence (Hoogstraal 1979).

An early study performed in South Africa analyzed the correlation between the route of transmission and the incubation period and found shorter incubation times for infection by tick bite or livestock contact (3.4 days and 4.7 days, respectively) compared to nosocomial infections (5.7 days) (Swanepoel, Gill et al. 1989).

Disease is manifested by influenza-like symptoms and is characterized by a rapid onset of fever, headache, myalgia, nausea and vomiting. Neuropsychiatric changes have also been reported in some patients with displays of violent behavior (Hoogstraal 1979; van Eeden, Joubert et al. 1985; Swanepoel, Gill et al. 1989). These are the main characteristics of the pre-hemorrhagic phase and lasts 3 days on average. Not all patients enter the hemorrhagic phase, and it is not even certain that patients with a milder or asymptomatic variant of CCHF are detected.

The hemorrhagic phase has a rapid onset with a short duration. Bleeding can range from dermal petechiae to large scale gastrointestinal hemorrhaging (van Eeden, Joubert et al. 1985; Swanepoel, Shepherd et al. 1987; Ergonul, Celikbas et al. 2006) and most common bleeding sites include the nose, gastrointestinal system, urinary tract and the respiratory tract (Ergonul 2007). Gastrointestinal and pulmonary hemorrhaging can be life threatening and carry a worse clinical prognosis (van Eeden, Joubert et al. 1985; Onguru, Dagdas et al. 2010). Other features of the hemorrhagic phase are elevated liver enzymes, coagulopathy as marked by prolonged bleeding times, enlarged liver and spleen, thrombocytopenia and leucopenia (Ergonul 2007). In very severe cases, death can ensue and is marked by multiple organ failure caused by severe anemia, dehydration and shock (Swanepoel, Shepherd et al. 1987). Clinical prognosis markers for fatality are decreased platelets, elevated liver enzymes, prolonged bleeding times, decreased fibrinogen and gastrointestinal bleeding (Swanepoel, Gill et al. 1989;

Ergonul, Celikbas et al. 2006; Ergonul 2007). Deceased patients had more than  $10^9$  viral genome copies/ml serum, which indicates that virus levels are a good prognostic marker for fatality (Cevik, Erbay et al. 2007; Duh, Saksida et al. 2007). Antibody responses mark the end of viremia and are rarely detected in fatal cases (Joubert, King et al. 1985; Shepherd, Swanepoel et al. 1989). DIC and hemophagocytosis are two pathological conditions that associate with disease severity (Swanepoel, Gill et al. 1989; Karti, Odabasi et al. 2004).

Convalescence is characterized by lethargy, labile pulse and even confusion and amnesia, all of which are reversible but may persist for a year or more (Whitehouse 2004).

### **Pathology**

Immunohistochemical studies in organs of deceased CCHF patients and in a recently established mouse model for CCHF pathogenesis consistently indicate infection of Kupffer cells, hepatic endothelial cells and hepatocytes. Necrosis of hepatocytes varied in extent, but generally the necrosis existed in multiple foci with association of viral antigen and no inflammatory infiltrates (Baskerville, Satti et al. 1981; Burt, Swanepoel et al. 1997; Bente, Alimonti et al. 2010). The extent of hepatocyte necrosis corresponded well with the association of elevated liver enzymes as a prognostic marker of fatality (Hoogstraal 1979; Baskerville, Satti et al. 1981; Joubert, King et al. 1985; Burt, Swanepoel et al. 1997).

The spleen is another important target for the course of disease characterized by lymphocyte depletion, splenocyte necrosis and dilated sinusoids (Baskerville, Satti et al. 1981; Bente, Alimonti et al. 2010). In other organs, the endothelial cells were sparsely infected; however hemorrhages, edema and/or necrosis were still present without inflammatory infiltrates (Al-Tikriti, Al-Ani et al. 1981; Baskerville, Satti et al. 1981; Joubert, King et al. 1985; Burt, Swanepoel et al. 1997). The necrosis in other organs besides the liver is supported by the clinical observation of a higher ratio of aspartate transferase (AST) to alanine transferase (ALT) in serum. A high ratio indicates whether there is necrosis in other organs besides the liver, and this ratio is almost always higher in CCHF cases (Ergonul 2007).

## **Pathogenesis**

The 1944-45 Crimean epidemic of CCHF was originally named “acute infectious capillary toxicosis” indicating the important role of the endothelium in pathogenesis (Hoogstraal 1979). The endothelium can be targeted directly by virus and/or indirectly by soluble mediators and these are the two classical theories for explaining the mechanisms underlying VHF (Schnittler and Feldmann 2003). The pathogenesis of CCHF has yet to be clarified and it was only recently that two animal models were developed which enable such in-depth *in vivo* studies (Bente, Alimonti et al. 2010; Berezky, Lindegren et al. 2010). So far the pathogenesis of CCHF has been inferred from the few pathological studies performed, the newly developed *in vivo* model and extrapolation from the pathogenesis of other VHF such as Ebola virus.

### *Endothelial cells in CCHF pathogenesis*

The extent of the direct involvement of endothelial cells has been questioned in EHF since these cell types are apparently infected at late stages of disease when coagulopathy has already ensued (Geisbert, Young et al. 2003). CCHFV has the ability to activate endothelial cells with consequent cytokine release and leukocyte recruitment *in vitro* (paper III).

ICAM-1 is one of the most widely used markers for endothelial activation (Roebuck and Finnegan 1999). It has been used to analyze the impact of viruses on the activation of endothelial cells as a possible mechanism for pathogenesis. The VHF Junin viruses (Gomez, Pozner et al. 2003), Ebola viruses (Wahl-Jensen, Afanasieva et al. 2005) Dengue viruses (Peyrefitte, Pastorino et al. 2006) and Hanta viruses (Song, Min et al. 1999; Geimonen, Neff et al. 2002) all activated ECs as shown by enhanced ICAM-1 expression. ICAM-1 expression was also increased on ECs when exposed to supernatants from Dengue virus-infected monocytes and patient plasma from Dengue patients, indicating the role of cytokines in activating endothelial cells (Anderson, Wang et al. 1997; Cardier, Marino et al. 2005).

Proteolytic cleavage of the leukocyte adhesion molecules ICAM-1 and VCAM-1 results in a soluble variant that correlates with the amount of cell membrane expression and can be studied *in vivo* as markers of both activation of endothelial and leukocytes and also as an indicator of vascular damage (Leeuwenberg, Smeets et al. 1992;

Reinhart, Bayer et al. 2002; Videm and Albrigtsen 2008). Evidence for endothelial activation and damage in CCHF was demonstrated in two separate studies correlating the levels of soluble ICAM-1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1) to disease severity (Bodur, Akinci et al. 2010; Ozturk, Kuscu et al. 2010). Similar evidence for endothelial damage came from a study of Dengue patients with higher plasma levels of sVCAM-1 than the control groups (Murgue, Cassar et al. 2001; Cardier, Rivas et al. 2006). The levels of sICAM-1 were also shown to be higher in patients with hemorrhagic fever with renal syndrome (HFRS) caused by Hantaan virus (Han, Zhang et al. 2010).

The interaction between leukocytes and ECs could play a role in exacerbating CCHF pathogenesis. Addition of leukocytes to Dengue virus infected ECs induced internalization of VE-cadherin and increased permeability which could be one of the factors contributing to vascular leakage (Dewi, Takasaki et al. 2008).

#### *The immune system in CCHF pathogenesis*

The involvement of the immune system in CCHF severity has been studied with some contradicting results and immune markers are studied with the intent to find a good prognostic factor for disease severity and fatality.

One patient study found a correlation between disease severity and increased amounts of NK cells (Yilmaz, Aydin et al. 2008), but this correlation was not observed in another similar study (Akinci, Yilmaz et al. 2008). A recently developed *in vivo* pathogenesis mouse model for CCHF indicated activation of NK cells but an overall loss of NK cells with time thereby eliminating the host of antiviral mediators (Bente, Alimonti et al. 2010).

Macrophages play a crucial role in VHF as exemplified by Ebola virus, with excessive release of tissue factor from infected macrophages leading to thrombi formation in the microvasculature in primate models and also a de-regulated release of cytokines contributing to further exacerbation of disease (Gupta, Mahanty et al. 2001; Geisbert, Young et al. 2003). The specific contribution of macrophages to CCHF is not quite clear but in patient studies the correlation of the monocyte/macrophage activation marker neopterin with disease severity indicates that they do take part in CCHF disease

exacerbation (Onguru, Akgul et al. 2008). Hemophagocytosis, which is excessive phagocytosis of erythrocytes by monocytes and macrophages due to hyperactivation by cytokines, has been found in several CCHF patients, thus pointing to yet another role for macrophages in CCHF (Tasdelen Fisgin, Fisgin et al. 2008). Whether this is caused by direct infection is unknown, but macrophages are target cells of CCHFV as shown in immunohistochemistry studies (Burt, Swanepoel et al. 1997). *In vitro* studies confirmed the susceptibility of macrophages to CCHFV infection and this also resulted in increased release of pro-inflammatory cytokines and chemokines (Peyrefitte, Perret et al. 2010). However, their ability to function as an APC could be impaired upon infection as shown by a recent *in vivo* study where only one macrophage population out of the two populations that were studied in the spleen was capable of upregulating MHC II molecules. The consequence of this is likely to be a partial impairment of the ability to activate the adaptive immune system (Bente, Alimonti et al. 2010).

In conditions of sepsis where the inflammatory response is deregulated, a dysfunctional cytokine storm leads to a systemic collapse. The pathogenesis of VHFs is speculated to be similar to septic shock and one of the underlying mechanisms for VHF disease exacerbation is the deregulated release of cytokines (Bray and Mahanty 2003). CCHF disease severity has in separate patient studies been correlated with serum levels of IL-6, TNF- $\alpha$ , IFN- $\gamma$  and IL-10 (Ergonul, Tuncbilek et al. 2006; Papa, Bino et al. 2006; Saksida, Duh et al. 2010). One weakness with these studies is the inconsistency in time points when the patient samples were drawn and often only sample is drawn from each patient. It therefore becomes more difficult to compare the cytokine levels between studies and possibly even between patients in the same study. Generally, TNF-  $\alpha$  levels consistently correlated with disease severity and fatality in all three mentioned studies. IL-6 on the other hand was equally enhanced in both severe and mild CCHF cases in Albanian patients (Papa, Bino et al. 2006), whereas in Turkish patients the levels of IL-6 correlated positively with fatality (Ergonul, Tuncbilek et al. 2006). High levels of IL-10 in severe or fatal CCHF cases were found in two studies (Papa, Bino et al. 2006; Saksida, Duh et al. 2010), while serum levels of IL-10 was negatively correlated with disease severity as measured by DIC scores (Ergonul, Tuncbilek et al. 2006). However these studies do show a deregulated and excessive release of cytokines that can ultimately lead to multiple organ failure in fatal cases. This was also manifested in the *in vivo* CCHF pathogenesis model where the onset of fever and wasting coincided with

IL-1 $\beta$  and IL-6, and TNF- $\alpha$  peak levels respectively (Bente, Alimonti et al. 2010). The cumulative effect of pro-inflammatory cytokines released during EHF initiated the clinical commencement of disease and in severe cases lead to vascular permeability and systemic vasodilatation followed by hypotension, shock and eventually multiple organ failure (Villinger, Rollin et al. 1999; Leroy, Baize et al. 2000; Baize, Leroy et al. 2002; Geisbert, Young et al. 2003; Bray and Geisbert 2005). Whether a similar mechanism exists for CCHF is unknown but it seems highly plausible as shown for the CCHF patient studies and cytokine levels.

Most viruses have some form of strategy for avoiding IFNs and CCHFV is no different. CCHFV is sensitive to IFNs and to several of its induced antiviral proteins (Andersson, Bladh et al. 2004; Andersson, Lundkvist et al. 2006). CCHFV delays induction of interferons by processing the viral 5' RNA termini, thus avoiding RIG-I stimulation and IFN transcription (Habjan, Andersson et al. 2008). Another mechanism for avoiding IFN response is exemplified by the *Hantaviruses* where the glycoproteins delayed IFN induction (Spiropoulou, Albarino et al. 2007).

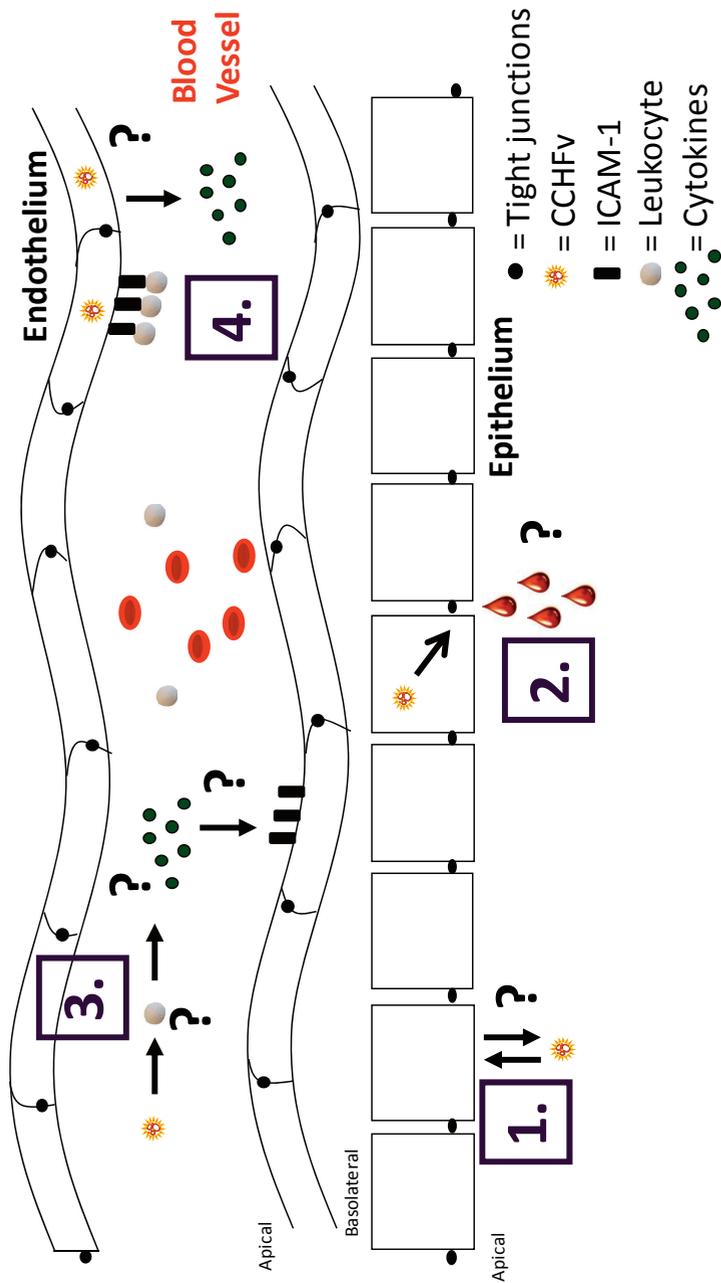
Since DCs are instrumental in initiating and controlling the adaptive immune system, they are important for understanding the disease mechanisms of CCHF. DCs are target cells of CCHFV as shown by two separate studies and they are activated to the extent that they release the cytokines TNF- $\alpha$ , IL-6, IL-10 and the chemokine IL-8 (Connolly-Andersen, Douagi et al. 2009; Peyrefitte, Perret et al. 2010). According to an *in vitro* study using human moDCs and an *in vivo* pathogenesis model, these cell types are only partially matured during infection as analyzed by co-stimulation markers CD40, CD80, CD80 and MHC II. Both studies found a lack of upregulation of MHC II for dendritic cells, however there was some discrepancy regarding the co-stimulatory molecule CD-80 which was upregulated in the *in vivo* study but not in the *in vitro* study. Regardless, adequate MHC-II upregulation is required for priming naïve T cells, and could explain the lack of adaptive immune response with no antibody production in severe and fatal cases of CCHF (Bente, Alimonti et al. 2010; Peyrefitte, Perret et al. 2010). DCs that are infected by Marburg and Ebola viruses fail to undergo the required maturation process to initiate the adaptive immune response and support T cell proliferation (Bosio, Aman et al. 2003). Whether this is also the case for CCHFV remains to be seen.

The adaptive immune system is crucial for clearing CCHFV as fatal incidents of CCHF rarely establish an antibody response, and endogenous antibody production to CCHFV is believed to represent one of the most important factors for survival (Joubert, King et al. 1985; Shepherd, Swanepoel et al. 1989). The underlying mechanisms are so far not known but it could be due to poor DC activation of the adaptive immune response. In one study, the amount of Tc cells correlated positively with disease severity and viral load, but despite the increased cytotoxic T cell response fatality could not be prevented (Akinci, Yilmaz et al. 2008). *In vivo* studies showed activation of B cells followed by a total loss in cell numbers, which is also verified by lymphocyte depletion in patients (Baskerville, Satti et al. 1981; Burt, Swanepoel et al. 1997; Bente, Alimonti et al. 2010). Uncontrolled apoptosis of lymphocytes appears to be a common feature of EHF's probably resulting in depleting the host of its antiviral properties (Geisbert, Hensley et al. 2000; Gupta, Spiropoulou et al. 2007; Wauquier, Becquart et al. 2010).

In conclusion, studies so far indicate a delay in innate immune system induction, thereby also delaying the adaptive response leading to uncontrolled viral amplification. Infected macrophages and DCs could partake in viral dissemination by travelling to lymph nodes and recruiting more target cells for infection, leading to further viral amplification and dissemination. The high rates of viral replication presumably leads to lysis and necrosis in cells of the liver, spleen and other organs but most importantly leads to reduced liver function. If similar to EHF, then the impaired ability of APCs to initiate the adaptive response concomitant with the deregulated innate immune response leads to severe deficiency in antiviral functions of the host with a cytokine storm in the host leading to subsequent hemorrhage and increased vascular permeability. The cytokine storm, organ necrosis and endothelial activation would lead to development of shock with possible multiple organ failure and death.



**Over 1000 hours in the BSL-4 laboratory later....**



## **SPECIFIC AIMS**

1. To determine the route of entry and release in polarized epithelial cells
2. To investigate if viral infection leads to increased permeability in epithelial cells with particular focus on tight junctions
3. To study target immune cells for CCHFV infection and determine possible soluble mediators for endothelial activation
4. To analyze the ability of CCHFV infection to activate endothelial cells and determine if activation is due to a direct or indirect effect



## RESULTS AND DISCUSSION

### POLARIZED ENTRY AND RELEASE OF CCHFV (PAPER I)

The distribution and access to viral receptors in the host is intimately connected with the pathogenesis of viruses. The epithelium is the first barrier that CCHFV must overcome and this is probably facilitated by the tick bite. In order to determine the site of entry for CCHFV, we used Madin-Darby Canine Kidney cells (MDCK-1) as a model cell for polarized epithelial cells. Confluent and semi-confluent MDCK-1 cell layers were infected followed by immunofluorescence (IF) detection of CCHFV NP as a marker of infection. Semi-confluent cell layers had not yet established an asymmetric membrane distribution as determined by the presence of the TJ protein occludin (figure 1A), indicating the putative viral receptor(s) were not yet segregated. Semi-confluent MDCK-1 cell layers were more susceptible to CCHFV infection than confluent cell layers, thus indicating that the viral receptor(s) is indeed segregated to the basolateral plasma membrane upon TJ establishment (figure 1B). Another study using the approach of semi-confluent cell layers was performed, this time by apical addition of virus to cells grown on a semi-permeable filter. The confluence was measured by TER. Once again, the confluent cells were not infected whereas semi-confluent cells were infected as shown by CCHFV NP by WB (figure 2B). The preference of CCHFV for basolateral entry was confirmed by infecting confluent MDCK-1 cells on a semi-permeable filter from either the top or the bottom compartment. CCHFV NP could primarily be detected in cells infected from the bottom (basolateral) compartment whereas cells infected from the apical (top) compartment showed minimal CCHFV NP (figure 2A). Segregation of viral receptor(s) seemed to be the primary limiting factor, and in order to further verify this hypothesis we disrupted TJs by addition of the chelator EGTA (ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid), which sequesters  $Ca^{2+}$ , to confluent MDCK-1 cells and inoculated cells apically with virus. Apical infection only occurred when TJs were disrupted by EGTA; otherwise the intact cell layer remained refractory to infection (figure 2C).

We concluded that intact TJs remain a barrier to infection and viral attachment proteins are localized in the basolateral membrane in MDCK-1 cells. So far the main viral receptors remain elusive however the entry of CCHFV is known to be cholesterol- and

clathrin-dependent (Simon, Johansson et al. 2009). The sequential steps in viral infection and dissemination are so far unknown for CCHFV although the early hepatic symptoms and consistent infection of hepatocytes and sinusoidal endothelial cells indicate the liver is an important target for CCHFV (Burt, Swanepoel et al. 1997; Bente, Alimonti et al. 2010). The basolateral plasma membranes of hepatocytes and ECs are easily accessible due to the fenestration of liver sinusoids and the lack of a basement membrane. It is possible that the targeting of CCHFV to the liver is due to the release of viral particles into the blood stream by the tick bite followed by transmission to the liver and infection of hepatocytes via the basolateral compartment.

The tick bite probably also facilitates viral entry to epithelial cells in the skin and tissue resident macrophages and DCs. Another possibility for viral transmission would be viral amplification in macrophages and DCs with subsequent dissemination to local lymph nodes and spleen as has been hypothesized for the viral dissemination of other VHFVs (Schnittler and Feldmann 1998). More studies are needed to uncover the viral receptors and thereby also determine the extent of target cells in the host.

Viral release often determines whether infection is systemic or localized (Compans 1995) since the basolateral compartment of epithelial cells is directed towards the blood vessels as opposed to the apical compartment that face the inner lumen of the organ. In general, basolateral virus budding is believed to cause systemic disease (Compans 1995).

In order to study the site of release we basolaterally inoculated confluent MDCK-1 cells to CCHFV, followed by extensive rinsing to remove residual virions. Supernatants from both compartments were collected daily followed by titration to determine the virus titer in the media. Basolaterally conditioned media contained increasing titers of virus whereas apical supernatants contained minimal to no infectious viral particles, indicating the preferred site of release is from the basolateral plasma membrane (figure 3). The release of virus is generally believed to be determined by the glycoproteins and so far it is known that CCHFV glycoproteins localize to the Golgi (Bertolotti-Ciarlet, Smith et al. 2005; Haferkamp, Fernando et al. 2005). Viruses within the Bunyaviridae are matured by budding into the Golgi complex and most likely follow the secretory pathway with subsequent release from the host cell.

The belief that basolateral budding causes a systemic disease can certainly be verified in the case of CCHFV. Whether the polarized release of CCHFV holds true for other

cell types in the human host is unknown. The direction of viral release can vary depending on the type of tissue involved as shown by Sindbis and Semliki Forest viruses that were released either apically or basolaterally in thyroid and intestinal epithelial cells, respectively (Zurzolo, Polistina et al. 1992).

In conclusion, the data show polarized entry and release in epithelial cells with preference to the basolateral compartment. The tick bite would probably facilitate crossing the epithelial barrier with possible basolateral infection of epithelial cells, or infection of macrophages and DCs with subsequent dissemination to local lymph nodes. The tick bite could also result in viral release into the blood stream followed by targeting to liver cells due to easy access of the basolateral compartment via fenestrated liver endothelium. Basolateral release from epithelial cells might mediate the entry of virus into the vascular system followed by systemic spread as observed in CCHF patients.

## TJ INTEGRITY IS NOT DISRUPTED BY CCHFV (I)

TJs can be targeted in one of two ways; either the virus releases viral products that directly interact with the host cell with concomitant TJ modulation or it is the response of the host cells to viral infection that leads to release of soluble mediators, such as cytokines, that influence TJs. The end result could be the hemorrhage that is observed in CCHF patients and which, in fatal cases, can lead to anemia and shock (van Eeden, Joubert et al. 1985). To study whether CCHFV modulates TJ integrity we infected confluent MDCK-1 cell layers grown on a semi-permeable filter and measured the TER over time with non-infected cells serving as a negative control. The TER of both non-infected and infected MDCK-1 cells was similar throughout the whole experiment, indicating that CCHFV during infection and replication does not induce disruption of TJs (figure 4A). PMA has been shown to cause TJ disruption and upon treatment with this chemical we observed a PMA concentration-dependent decrease in TER, which served as a positive control (figure 4B). These experiments were performed in confluent cells that had already established TJs. To investigate whether CCHFV infection could hinder TJ formation, we infected semi-confluent MDCK-1 cells and visualized the localization of the TJ proteins occludin (figure 5) and ZO-1 (figure 6) at different time points by IF. Both occludin and ZO-1 could localize to cell boundaries forming TJs in infected cells, verifying that CCHFV does not hinder formation of TJs.

The pathogenesis of CCHF does not seem to be caused by direct viral interaction with TJ proteins or any other intracellular pathway leading to TJ disassembly in this model system. Studies for other VHFs point towards the release of soluble mediators such as cytokines, chemokines and matrix metalloproteinases from infected monocytes, macrophages, dendritic and even ECs themselves that ultimately acts upon the endothelial cell with subsequent disruption of junctional complexes followed by increased permeability (Feldmann, Bugany et al. 1996; Carr, Hocking et al. 2003; Talavera, Castillo et al. 2004; Cardier, Marino et al. 2005; Luplerdlop, Misse et al. 2006; Bockeler, Stroher et al. 2007; Luplertlop and Misse 2008). Whether this is the case for CCHFV induced permeability is currently unknown and would require the development of an animal model as shown for Dengue viruses (Luplerdlop, Misse et al. 2006) or an *in vitro* endothelial model system similar to the ones applied for studies of

Marburg virus (Bockeler, Stroher et al. 2007), Dengue virus (Talavera, Castillo et al. 2004) and even the *Hantaviruses* (Gorbunova, Gavrilovskaya et al. 2010).

## **CCHFV INFECTION AND ACTIVATION OF IMMUNE CELLS (II) + (III)**

For some VHF's animal models exist which enable investigation of the sequential infection of target cells, and the determination of which cells are primarily involved in pathogenesis. This is exemplified by the extensive studies undertaken for Ebola virus in primate pathogenesis models, which determined the early roles of macrophages and DCs in viral amplification and exacerbating disease (Geisbert, Hensley et al. 2003). Endothelial cells were infected at later stages when coagulopathy had already progressed (Geisbert, Young et al. 2003). Not much was known for CCHF other than that the levels of NK cells and cytotoxic T cells correlated with disease severity in two separate studies (Akinci, Yilmaz et al. 2008; Yilmaz, Aydin et al. 2008). CCHFV had infected mononuclear phagocytes and endothelial cells at least by the end stage of disease (Burt, Swanepoel et al. 1997).

We therefore decided to study the susceptibility to infection of peripheral blood mononuclear cells (PBMCs) and leukocyte subsets to CCHFV infection to discern possible target cells in the human host. PBMCs, B cells, T cells, NK cells, monocytes and moDCs were inoculated with viral suspension and successful infection determined by harvesting cells for qPCR and IF. MoDCs were the only cell type that showed consistent presence of CCHFV NP RNA and protein (figures 1 B and C, paper II). The infection was productive, as shown by the release of infectious progeny virions from moDCs (figure 2, paper II). Another study verified the susceptibility of moDCs as well as macrophages to CCHFV infection (Peyrefitte, Perret et al. 2010).

We had determined that moDCs were susceptible to infection with CCHFV; however DCs perform a multitude of functions necessary for the initiation and regulation of the immune response. Part of these functions is the release of cytokines and our next study focused on targeting cytokines that could be released from infected moDCs. The supernatants from non-infected and infected moDCs were assayed for the levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-19, IL-10 and IL-8 (figure 3, paper II). The release of IL-1 $\beta$  could not be detected in non-infected and infected moDCs, and the levels of IL-8 and IL-19 did not differ in either treatments. However, CCHFV-infected moDCs released higher levels of the proinflammatory cytokines IL-6 and TNF- $\alpha$  and the antiinflammatory cytokine IL-10. These particular cytokines have been identified as prognostic factors for CCHF disease severity in several patient studies and it is possible

that infected DCs in the host contribute to the release of these cytokines (Ergonul, Tuncbilek et al. 2006; Papa, Bino et al. 2006; Saksida, Duh et al. 2010).

Based on our data, it appears as if CCHFV infection activates moDCs to the extent that they release cytokines; though the activation seems to be only partial as shown in both *in vitro* and *in vivo* studies (Bente, Alimonti et al. 2010; Peyrefitte, Perret et al. 2010). It would seem that CCHFV can impair the adaptive immune system, most likely permitting uncontrolled viral replication with subsequent systemic dissemination. This is supported by the impaired upregulation of co-stimulatory molecules on DCs and macrophages required for antigen-presentation to Th cells and the lack of antibodies in fatal cases. The inability of the immune system to regulate viral replication is also supported by the studies correlating viral levels with both the levels of cytokines and CCHF disease severity (Duh, Saksida et al. 2007; Saksida, Duh et al. 2010). Ebola virus also impairs the ability of DCs to act as an APC, and this impairment coupled with release of cytokines is believed to mediate bystander apoptosis of lymphocytes depleting the host of its antiviral response in some cases leading to death (Bosio, Aman et al. 2003; Mahanty, Hutchinson et al. 2003). In the Ebola patients who survive the disease, an early but transient innate immune response with subsequent activation of adaptive responses is crucial for survival. In fatal cases the inflammatory response appears to be out of control and is correlated to septic shock where a de-regulated response ultimately contributes to killing the host (Leroy, Baize et al. 2000; Baize, Leroy et al. 2002).

ECs are important for recruiting leukocytes to the site of inflammation and require activation in order to perform their roles in the inflammatory process. A marker of endothelial activation is the upregulation of the leukocyte adhesion molecule ICAM-1. Activation can occur either directly by viral infection or by virus-induced host-derived soluble factors. We decided to study the contribution of virus-induced host-derived soluble factors to endothelial activation by adding supernatant from non-infected and infected moDCs to ECs and analyzing ICAM-1 mRNA, total protein levels and cell surface levels of ICAM-1. Conditioned media that had been incubated with CCHFV infected moDCs was added to resting ECs followed by analysis for mRNA and protein levels of ICAM-1. The ICAM-1 mRNA, total protein and cell surface levels were increased indicating ECs are activated in response to soluble factors present in the

supernatant (figures 4A and B, paper II, figure 4 paper III). In patient studies there was a consistent correlation between fatality and TNF- $\alpha$  levels in serum (Ergonul, Tuncbilek et al. 2006; Saksida, Duh et al. 2010) and TNF- $\alpha$  serum levels were also associated with disease severity for other VHF $\alpha$ s (Heller, Saavedra et al. 1992; Villinger, Rollin et al. 1999). We therefore focused our attention on TNF- $\alpha$  as a possibility for being the key cytokine mediating EC activation. Media that had been conditioned by exposure to CCHFV-infected and non-infected moDCs were pre-incubated with or without a neutralizing antibody targeting TNF- $\alpha$  followed by addition to resting ECs. The cell surface ICAM-1 up-regulation on ECs was effectively prevented by the addition of anti-TNF- $\alpha$  to the supernatants from infected moDCs or recombinant TNF- $\alpha$  (figure 5, paper III). We thereby identified TNF- $\alpha$  as the key cytokine released from moDCs that mediated activation of ECs. A similar observation was made for other VHF $\alpha$ s, where neutralization of TNF- $\alpha$  released from infected monocytes or present in patient sera, inhibited endothelial activation and permeability (Feldmann, Bugany et al. 1996; Anderson, Wang et al. 1997; Cardier, Marino et al. 2005). Whether TNF- $\alpha$  causes the vascular permeability in CCHF is unknown. But it is possible since EC exposure to TNF- $\alpha$  leads to increased permeability by a mechanism involving the destabilization of microtubules (Petrache, Birukova et al. 2003).

### **THE ACTIVATION OF ENDOTHELIAL CELLS BY CCHFV INFECTION (III)**

The increased vascular permeability and hemorrhage observed in CCHF patients, and also the early terminology “Capillary toxicosis” by Russian scientists point to the crucial role of ECs in CCHF pathogenesis. Paper I indicated an indirect targeting of virus, since TJs were not affected by CCHFV infection and this hypothesis was further underscored in paper II when soluble mediators from infected moDCs activated ECs. However, the direct interaction between CCHFV and ECs had yet to be studied. In order to investigate the interactions between CCHFV and ECs, a time-line study of ICAM-1 mRNA and protein levels in infected ECs was conducted. ICAM-1 transcripts peaked at 48 hpi in infected cells with concomitant increase in protein levels (figures 1 A and B). These observations were verified by the analysis of cell surface levels of ICAM-1 at 48 hours post infection (hpi) (figure 1 C).

Several patient studies have correlated the viral load of CCHFV with disease severity (Cevik, Erbay et al. 2007; Duh, Saksida et al. 2007; Saksida, Duh et al. 2010) but the underlying molecular mechanisms were never further investigated. To determine whether the level of endothelial activation correlates with increasing amounts of viral particles, ECs were infected with increasing multiplicity of infection (MOI) of CCHFV and ICAM-1 cell surface levels with concomitant leukocyte adhesion were studied. The infection with different amounts of viral particles resulted in enhanced ICAM-1 levels which were reflected by increased adhesion of resting leukocytes to infected ECs (figures 2 A and B). It is possible the association between virus levels and disease severity could reflect an enhanced activation of ECs followed by disease exacerbation. The patient studies correlating the soluble variant of ICAM-1 with disease severity support the enhanced activation of endothelium as a possible mechanism underlying CCHF pathogenesis (Bodur, Akinci et al. 2010; Ozturk, Kusu et al. 2010).

ECs can also produce cytokines, and since our data show they are activated, we decided to study if infected cells released higher amounts of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, TNF- $\alpha$  and the chemokine IL-8. CCHFV infected ECs released higher levels of the soluble mediators IL-6 (figure 3A) and IL-8 (figure 3B) as quantified by ELISA, however neither IL-1 $\beta$  nor TNF- $\alpha$  could be detected by ELISA. Levels of IL-6 were

associated with CCHF disease fatality in one patient study and were positively correlated with DIC scores (Ergonul, Tuncbilek et al. 2006). So far *in vitro* studies have pinpointed DCs, macrophages and now also ECs as possible target cells that release IL-6 upon CCHFV infection (Connolly-Andersen, Douagi et al. 2009; Peyrefitte, Perret et al. 2010).

In order to determine whether the observed EC activation was a reaction to soluble mediators released from neighboring cells, resting ECs were exposed to conditioned media from CCHFV infected ECs and the resulting ICAM-1 response was analyzed. ICAM-1 levels did not increase beyond background levels in resting ECs exposed to supernatants from CCHFV infected ECs. It would seem that the observed ICAM-1 cell surface expression is likely due to an intracellular signaling event as a result from infection, and probably not due to soluble mediators released from neighboring infected ECs.

In conclusion, a direct activation of ECs by CCHFV cannot be excluded as a possible mechanism for pathogenesis. It is most likely a complex combination between direct and indirect effects exerted on the endothelium that is partially responsible for the mechanisms underlying CCHF pathogenesis.

## CONCLUDING REMARKS

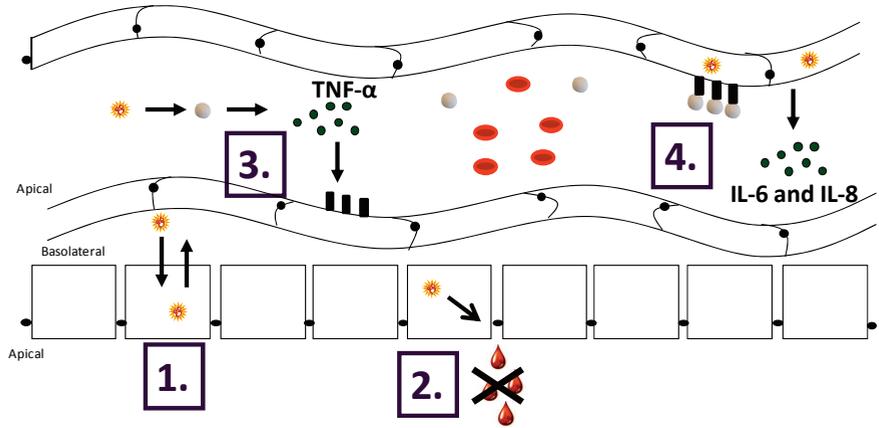
The first barrier to overcome in infection is the epithelial barrier and the site of entry and release is often intimately connected with the extent of viral spread and pathogenesis in the host. In this thesis, the preferred site of entry and release in polarized epithelial cells was studied and the basolateral plasma membrane was determined as a site for viral entry and release. This correlates with the fact that CCHFV is transmitted by tick bites thereby giving access to the basolateral plasma membranes of epithelial cells, and the systemic spread of virus probably facilitated by basolateral viral budding.

The increased vascular permeability and hemorrhages observed in CCHF are cardinal signs of VHFs. There are several possible mechanisms ultimately resulting in the increased permeability that could explain these two cardinal signs. We determined that CCHFV infection of polarized epithelial cells did not disrupt TJs, indicating that some other mechanism must be at play that could explain the hemorrhage and vascular permeability. For other VHFs the leakiness was mediated by the release of soluble mediators from neighboring infected ECs, monocytes, macrophages and dendritic cells. It is possible that the same mechanism exists for CCHFV, yet the answer to this question required the setting up of an endothelial permeability model system which was not possible during the course of this thesis.

CCHF and EHF have similar disease patterns although CCHF is a milder variant. Dendritic cells and macrophages are instrumental for the exacerbation of Ebola hemorrhagic fever and possibly also for CCHF. We analyzed possible target cells in human leukocytes and discovered dendritic cells were the only cell type susceptible to infection. DCs released higher levels of the cytokines TNF- $\alpha$ , IL-6 and IL-10 upon CCHFV infection and we identified TNF- $\alpha$  as the key cytokine mediating endothelial activation. This cytokine has also been identified for other VHFs as a cause of endothelial permeability and it is possible this also occurs for CCHFV. TNF- $\alpha$  has in several patient studies been consistently identified as the main correlate to CCHF disease severity. In another study the susceptibility of macrophages to infection was confirmed, and together with the data from our study it would seem that similar mechanisms are at play as the ones observed for Ebola hemorrhagic fever.

The two classical theories explaining the observed increased vascular permeability and hemorrhage involve either a direct or an indirect effect on ECs. The first two papers (I+II) both pointed towards an indirect effect since TJs were not disturbed by CCHFV infection and soluble mediators released from infected moDCs activated ECs with TNF- $\alpha$  as the key mediator of activation. Nonetheless, the direct effect cannot be excluded since ECs were directly activated by CCHFV infection. Whether CCHFV infection of ECs induces permeability is not known since an attempt to establish an *in vitro* system for endothelial permeability failed. The increased leukocyte recruitment by infected ECs and the impact of soluble mediators released from infected immune cells indicate that the pathogenesis of CCHF is most likely due to a complex interplay between both direct and indirect effects on ECs and probably all these mechanisms take part in exacerbating disease.

The pathogenesis of CCHF is complex and many more questions remain to be answered before the specific underlying mechanisms can be determined and possibly targeted by medical interventions. So far, the data from this thesis have clarified there are several possible routes for targeting ECs with subsequent activation. Hopefully the future will yield many more answers to the CCHF puzzle and the recent generation of two animal models (Bente, Alimonti et al. 2010; Bereczky, Lindegren et al. 2010) seems promising for the study of CCHF pathogenesis. With these animal models it will be possible to follow the sequential infection of specific target cells and will help determine when and what type of medical interventions are appropriate.



**Figure 5.** The conclusions from this thesis. 1: Basolateral entry and release of CCHFV in polarized epithelial cells. 2: The permeability of epithelial cells is not increased upon CCHFV infection. 3: CCHFV infected moDCs release TNF- $\alpha$  that activates endothelial cells. 4: ECs are activated upon infection with increased ICAM-1 levels, release of proinflammatory proteins IL-6 and IL-8, and increased recruitment of resting leukocytes.

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