From the Department of Microbiology, Tumor and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

OF VOLES AND MEN: NOVEL HANTAVIRUS *IN VITRO* MODELS

Karin Sundström

Karolinska Institutet

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"Would you tell me, please, which way I ought to go from here?"
"That depends a good deal on where you want to get to", said the Cat.
"I don't much care where - -"said Alice.
"Then it doesn't matter which way you go," said the Cat.
"- - so long as I get SOMEWHERE," Alice added as an explanation.
"Oh, you're sure to do that," said the Cat, "if you only walk long enough."

- Lewis Carrol, Alice's Adventures in Wonderland

ABSTRACT

Hantavirus-infection can cause severe disease in humans, with up to 40% case fatalities. Presently, no therapeutics or prophylaxis against hantaviral illness exists. The mechanisms underlying the onset of symptoms and the following pathogenesis are not fully known. *In vitro* research conducted on mono-layered human cell cultures with cell line adapted hantaviruses provides important knowledge for understanding the virus and the innate immune responses induced, but the results might not always correspond to infection in humans. Furthermore, neither the interactions between the virus and its natural animal host, nor why infections in them do not cause disease are well understood. To better understand hantavirus pathogenesis, new models and tools are needed.

In **paper** I, genetic properties of hantavirus were investigated by analyzing two substrains of Puumala hantavirus (PUUV) derived from virus propagated on cells lacking parts of the innate antiviral response. From this study we could conclude that the differences in phenotype and replication were caused without mutations in the viral glycoproteins. Mutations were however observed in the nucleocapsid protein and in the RNA dependent RNA polymerase. We also observed that the phenotypic differences between the substrains and the parental strain were cell line specific.

To be able to analyze hantavirus infection of cells from the natural host *in vitro*, vole embryonic fibroblasts derived from bank voles, the host for PUUV, were isolated in **paper II**. These cells were susceptible and permissive not only to PUUV, but to a range of other bank vole-borne viruses, indicating that these cells can be an important tool for studies of several zoonotic viruses. Regarding IFN-β and Mx, two important antiviral proteins, infection of the vole fibroblasts with PUUV induced a different response compared to that observed in human fibroblasts, indicating a possible species difference in innate immune response against hantavirus infection.

To better mirror the situation in human organs, complex *in vitro* models resembling human tissue might be valuable. In **paper III** we took the advantage of using a 3-dimensional organotypic model of human lung tissue to study early and long term infections of the highly pathogenic Andes virus (ANDV). With this model we could show that a peak in progeny virus production occurs more than a week after initial infection. We also observed increased extracellular levels of the pro-inflammatory cytokines IL-6 and IL-8, and of IP-10 and eotaxin-1 upon ANDV-infection, as well as suppression of RANTES-responses. These ANDV-induced effects were observed late after infection. VEGF-A are suggested to be involved in the pathogenesis of hantaviral illness, as it might be responsible for the increased vascular permeability observed in patients. We observed higher levels of VEGF-A after ANDV-infection, suggesting that infection of lung tissue *per se* might be responsible for the increased VEGF-A levels observed in patients.

The potential role of dendritic cells (DCs) during hantaviral illness is not known. In **paper IV**, we studied the effect DCs have on hantavirus-infection, by adding these cells to the organotypic human lung tissue model. We showed that DCs had an antiviral effect against hantavirus-infection, suggesting that DCs might be involved in limiting the infection.

In this thesis, establishment of novel *in vitro* models, and studies of different aspects of hantavirus-infection were performed. Genetic properties of PUUV were investigated and by using different *in vitro* models, the cellular responses of voles and humans during hantavirus-infection were analyzed.

LIST OF PUBLICATIONS

I. **Sundström KB**, Stoltz M, Lagerqvist N, Lundkvist Å, Nemirov K, Klingström J.
Characterization of two substrains of Puumala virus that show phenotypes that are different from each other and from the original strain.
J Virol. 2011;85(4):1747-56

II. Stoltz M, **Sundström KB**, Hidmark Å, Tolf C, Vene S, Ahlm C, Lindberg AM, Lundkvist Å, Klingström J.
A model system for in vitro studies of bank vole borne viruses.
PLoS One. 2011;6(12):e28992

III. **Sundström KB**, Nguyen Hoang AT, Gupta S, Ahlm C, Svensson M, Klingström J.
Andes virus (ANDV) infection of an *in vitro* organotypic human lung model: ANDV causes a late peak in virus-production followed by elevated levels of pro-inflammatory cytokines, eotaxin-1 and VEGF-A and decreased levels of RANTES.
Manuscript

IV. **Sundström KB**, Chen P, Nguyen Hoang AT, Svensson M, Klingström J.
Hantavirus-infection can be inhibited by dendritic cells and cause elevated levels of eotaxin-1.
Submitted

CONTENTS

LIST OF ABBREVATIONS

2D	two-dimensional
3D	three-dimensional
aa	amino acid(s)
ANDV	Andes virus
BSL-3	biosafety level 3
CK18	cytokeratin 18
CPXV	cowpox virus
cRNA	complementary RNA
CTL	cytotoxic T lymphocyte
DC	dendritic cell
Gc/Gn	hantaviral glycoproteins
HFRS	hemorrhagic fever with renal syndrome
HLA	human leukocyte antigen
HPS	hantavirus pulmonary syndrome
HTNV	Hantaan virus
IFN	interferon
Ig	immunoglobulin
ISG	interferon stimulated genes
LV	Ljungan virus
MHC	major histocompability complex
MMP	matrix metalloprotease
mRNA	messenger RNA
N	hantaviral nucleocapsid protein
NCR	non coding region
NDV-EGFP	Newcastle disease virus expressing enhanced green fluorescence protein
NE	nephropathia epidemica
NK cell	natural killer cell
NSs	hantaviral non-structural protein, from S-segment
ORF	open reading frame
PRR	pattern recognition receptor
PUUV	Puumala virus
RdRp	RNA-dependent RNA-polymerase
RNA	ribonucleic acid
RNP	ribonucleoproteins
S, M, L-segment	small, medium, large RNA segment
SEOV	Seoul virus
SNV	Sin Nombre virus
TBEV	tick borne encephalitis virus
Th cell	T helper lymphocyte
TULV	Tuula virus
VE-cadherin	vascular endothelial cadherin
VEFs	vole embryonic fibroblasts
VEGF-A	vascular endothelial growth factor A
vRNA	viral RNA

INTRODUCTION

Hantaviruses exist worldwide and are carried by different rodents, insectivores and bat species. These viruses can cause diseases in humans, and the clinical symptoms and severity of human diseases varies depending on hantavirus species. The most common route of human infection is through inhalation of aerosolized virus-contaminated rodent excreta. To date, neither any post-exposure prophylactics or specific therapeutics, nor any FDA-approved vaccines are available.

HISTORY

Disease of war

The first descriptions of hantaviral illness are found in China and date back to year 960 A.D. [1]. However, this disease was not given much attention until the 20ieth century.

During World War I, British troops fighting in the Belgian trenches were plagued by symptoms of acute nephritis. What caused this so-called "trench-fever" was unknown [1-2]. In the following years, similar symptoms were reported from Japan, following the Japanese occupation of Manchuria [1] and from the Soviet Union [3]. In 1934, two independent reports describing symptoms of an acute nephritis in Sweden were published [4]. This disease was named nephropathia epidemica (NE) [4]. Then, when another great war swept the world in the 1940ies, field nephritis was once again reported, this time from troops fighting in Finnish Lapland [1, 4]. The cause for this disease, now recognised all over the Eurasian continent, was still not discovered. However, people had a hunch; in most reports a possible rodent origin of the infection was discussed [1, 3-5]. To investigate what could be the causative agent for the disease and how infection spread, several approaches were performed in the late 1930ies – 1940ies. The Japanese Germ-Warfare Unit, and Soviet scientists, conducted unethical experiments [1, 5, 6], while Gustav Myhrman, a Swedish physician, had a different approach:

> *"The author tried to transfer the disease to himself by ingesting 15 ml of urine and by intramuscular injection of 5 ml of blood. The results were negative."* [5]

In 1951, the disease again drew the world's attention, striking soldiers in yet another war. During the Korean War in 1950-1953, around 3000 soldiers of the United Nation troops succumbed with high fever, developing into acute nephritis, hemorrhagic manifestations and shock. The overall case fatality rate was 7% [1, 6]. Once more a connection to rodents was made, but what actually caused the "Korean hemorrhagic fever" was still a mystery [1].

The cause

After years of intense search, Lee and co-workers were in 1978 able to isolate what proved to be the causative agent of the Korean hemorrhagic fever, from the lungs of a field mouse [7]. The virus found was named Hantaan virus (HTNV), after the river close to the rodent capturing location [1]. In just a few years, the HTNV-related cause to NE was found in the lungs of a bank vole in Finland [8]. This virus was also named after the place where the rodent host was caught; Puumala virus (PUUV). Lee and co-workers found in 1982 another disease-causing hantavirus, this time carried by rats in the middle of the capital of South Korea; hence called Seoul virus (SEOV) [9]. During the Bosnian War 1992-1995, when 300 Bosnian soldiers fell ill with acute nephritis [10], the hantavirus isolated 1992 in former Yugoslavia (Dobrava-Belgrade virus) [11], proved to be pathogenic to humans. A common name for the diseases caused by hantaviruses were agreed upon to be hemorrhagic fever with renal syndrome (HFRS), however the disease caused by PUUV is still often termed NE [1].

New players came around

In the Americas, only one hantavirus was found before the 1990ies [12]. That was the Prospect Hill virus and this virus is not pathogenic to humans. Then suddenly in 1993, an outbreak of a disease with very severe respiratory distress symptoms in young, otherwise healthy persons, emerged in the Four Corner Region (Arizona, Colorado, New Mexico and Utah) of the United States [13]. The case fatality of this disease was high; around half of the patients died just a couple days after onset of symptoms [14]. With modern molecular methods it did not take long until the cause was detected; a novel hantavirus [15-16]. This virus was named Sin Nombre virus (SNV), and the disease it caused was named hantavirus pulmonary syndrome (HPS) [1]. According to CJ Peters in New Scientist 2012, SNV was named after a small creek in the area of where the first case was described [16b].
Since the discovery of SNV, yet more species of rodent-borne hantaviruses were to be found all over the Americas; from Canada in the north to Argentina in the south [17]. Almost all caused a highly pathogenic form of hantavirus disease. One of these new viruses was isolated after an outbreak of HPS in Argentina 1995 [18]. Not only was the case fatality very high: two out of the three infected individuals died [18], but this Andes virus (ANDV) was later the first, and so far the only, hantavirus reported to spread from human-to-human [19].

The story continues

In addition to the rodent-borne hantaviruses, the first hantavirus carried by a shrew, the Thottapalayam virus, was identified to be a hantavirus in 1989 [20]. This non-pathogenic hantavirus was originally isolated in India 1964, but was then not classified into any virus family [21]. Since 1989, more than 22 different insectivore-borne hantaviruses have been found [22]. The most recent addition to the hantavirus genus are viruses carried by a range of bats, in different locations such as Côte d'Ivoire [23], Brazil [24] and China [22].

HANTAVIRUS

The hantaviruses were described as a new genus in the *Bunyaviridae* family in 1982-1983 [25-27]. Viruses from the other genera of *Bunyaviridae* are strictly arthropod-borne, making hantavirus genus unique in this context, as it is not transmitted by mosquitoes, ticks or other insects [1].

Viruses

Hantaviruses are classified by the International Committee on Taxonomy of Viruses upon their sequence differences in the S- and M-segments, and cross-reactivity towards hantavirus-specific polyclonal sera by a two way cross-neutralization test [28]. In nature, specific hantaviruses are predominately associated with one specific host species [28]. The pathogenic rodent-borne hantavirus can be divided under which rodent host subfamily they are associated with: *Murinae*, *Arvicolinae* or *Sigmodontinae*; or in the sense of geographic distribution: Eurasia or Americas [29]. Earlier the belief was that the Eurasian hantaviruses mainly cause renal symptoms in man and the American ones predominately cause cardiopulmonary symptoms, but recent data indicate a larger similarity in symptoms induced by the different hantavirus-infections [30-31].

Rodent subfamily	Virus	Abbrevation	Disease	Reservoir species	Common name	Geographic distribution
Murinae	Amur	AMVR	HFRS	*Apodemus peninsulae*	Korean field mouse	Asia
	Dobrava-Belgrade	DOBV	HFRS	*Apodemus flavicollis*	Yellow-necked field mouse	Europe
	Hantaan	HTNV	HFRS	*Apodemus agrarius*	Striped field mouse	Asia
	Sangassou	SANGV	-	*Hylomyscus simus*	African wood mouse	Africa
	Seoul	SEOV	HFRS	*Rattus Norvegicus*	Rat	World wide
	Thailand	THAIV	HFRS	*Bandicota indica*	Great bandicoot rat	Asia
Arvicolinae	Hokkaido	HOKV	-	*Myodes rufocanus*	Grey red-backed vole	Japan
	Isla Vista	ISLAV	-	*Microtus californicus*	California vole	North America
	Prospect Hill	PHV	-	*Microtus pennsylvanicus*	Meadow vole	North America
	Puumala	PUUV	HFRS	*Myodes glareolus*	Bank vole	Europe
	Topografov	TOPV	-	*Lemmus sibericus*	Lemmings	Asia
	Tuula	TULV	-	*Microtus arvalis*	European common vole	Europe
Sigmodontinae	Andes	ANDV	HPS	*Oligoryzomys longicaudatus*	Long-tailed pygmy rice rat	South America
	Black Creek Canal	BBCV	HPS	*Sigmondon hispidus*	Hispid cotton rat	North America
	Choclo		HPS	*Oligoryzomys fulvescens*	Fulvous pygmy rice rat	South America
	El Moro Canyon	ELMCV	-	*Reithrodontomys megalotis*	Western harvest mouse	North America
	Laguna Negra	LANV	HPS	*Calomys laucha*	Vesper mouse	South America
	New York	NYV	HPS	*Peromyscus leucopus*	White-footed mouse	North America
	Rio Mamore	RIOMV	-	*Oligoryzomys microtis*	Small-eared pygmy rice rat	South America
	Sin Nombre	SNV	HPS	*Peromyscus maniculatus*	Deer mouse	North America

Table 1. Selected hantavirus species and their rodent hosts, geographical distribution, and human disease associated with the virus; – no known human disease associated with the virus.

Genome and structure

Hantaviruses have a single stranded RNA genome of negative polarity. The genome consists of three segments; the large (L, 6.5-6.6 kb), medium (M, 3.7-3.8 kb) and small (S, 1.8-2.1 kb) segments, encoding the RNA dependent RNA polymerase (RdRp), the glycoprotein precursor and the nucleocapsid protein (N), respectively [28]. Some hantaviruses also have an open reading frame (ORF) for a potential non-structural protein (NSs), inside the coding region of the N protein [32].

The 3' and 5' termini of the hantavirus RNA segments are highly conserved and capable of forming a panhandle structure [33]. This structure is believed to have a role in regulation of viral transcription and replication, but the exact mechanism(s) is still unknown. The coding regions of the viral RNAs (vRNA) are flanked by noncoding regions (NCR). Particularly, the 3'NCR of the S-segment is large, and the lengths of this region differ between hantavirus species but not much within the subspecies, indicating that this region might have a functional role [34].

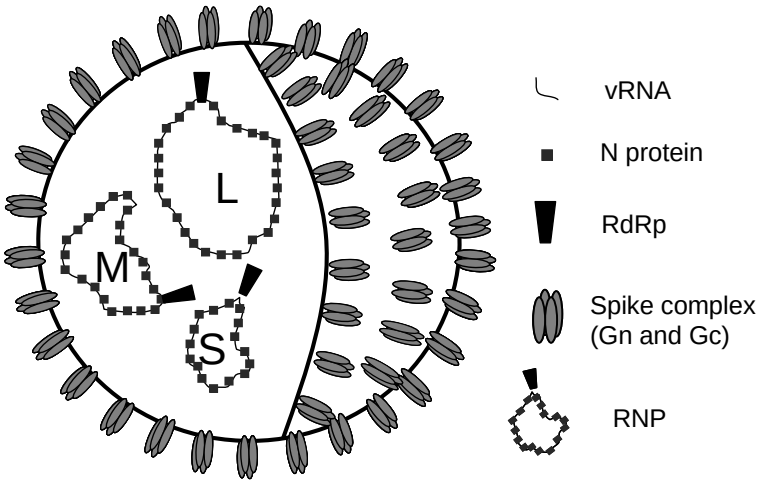


Fig 1.
Schematic representation of a hantavirus particle.

The hantavirus particle is spherical, sometimes elongated, with a size of approximately 80-120 nm [29, 35-38]. The vRNA is encapsidated by N proteins, forming the ribonucleoproteins (RNP) [33]. The RNP together with at least one RdRp per segment is enclosed by a lipid membrane. The membrane is covered with spikes formed by the glycoproteins, extending approximately 10 nm from the membrane surface [33, 36-37]. Hantaviruses lacks matrix proteins, instead the RNP is suggested to interact with the cytoplasmic tail of the glycoproteins [33, 36, 39, 40-42]. A single hantavirus particle is believed to carry only one copy of each segment, but diploid viruses may exist [43-44].

Structural and non-structural proteins

The RdRp is approximately 240 kDa and is required for viral replication. The structure of the hantavirus RdRp is predicted to be similar to the structure of other negative stranded viruses polymerases, and at least five motifs of the amino acid (aa) sequence are highly conserved among bunyaviruses [45]. The RdRp is not very well studied, but is predicted to have at least endonuclease, transcriptase and replicase activity [43, 45].

The glycoprotein precursor encoded by the M-segment is cleaved directly after translation at the conserved WAASA motif [46] into two glycoproteins; the C-terminus Gc protein (earlier called G1) and N-terminus Gn protein (earlier called G2). The glycoproteins are glycosylated after transport to the ER-Golgi intermediate compartment or *cis*-Golgi compartment [33]. The Gc/Gn proteins form spikes, suggested to be tetrameric in structure [33, 36-37]. The glycoproteins are class II fusion proteins [47] and responsible for viral binding to cellular receptors [33]. The cytoplasmic tails of the glycoproteins, which is approximately 110 aa for Gn and 10 aa residues for Gc, are suggested to interact with the RNP to facilitate assembly of the virus particle [33, 36, 39, 40-42]. The Gn cytoplasmic tail is also involved in down-regulation of cellular responses in infected cells, by inhibiting interferon (IFN) -induction and signalling [48-52]. The cytoplasmic tail of Gn is targeted for ubiquitination in both pathogenic and non-pathogenic rodent borne hantaviruses [53-54].

The N protein of hantaviruses is a multifunctional protein that is involved in viral encapsidation and assembly, in transcription and translation, and in interactions with cellular proteins [55]. The N protein is around 50 kDa in size (429-433 aa) [56] and the predicted secondary structure of the first 75 aa is a coiled-coil domain [57], later confirmed by NMR for ANDV N protein [58]. The N protein is suggested to function as an RNA chaperone [59]. The protein is also suggested to interact with Daxx [60] and SUMO-1 pathways [61-62], as well as with actin filaments and microtubulies [63-65]. Also, the N protein is suggested to modulate cellular antiviral responses by interacting with importin-α to inhibit TNF-induced activation of NFκB [66-67] and by direct binding to NFκB [68]. Recently, the N protein was also shown to carry cleavage sites for both granzyme B and caspase 3 and to inhibit apoptosis [69].

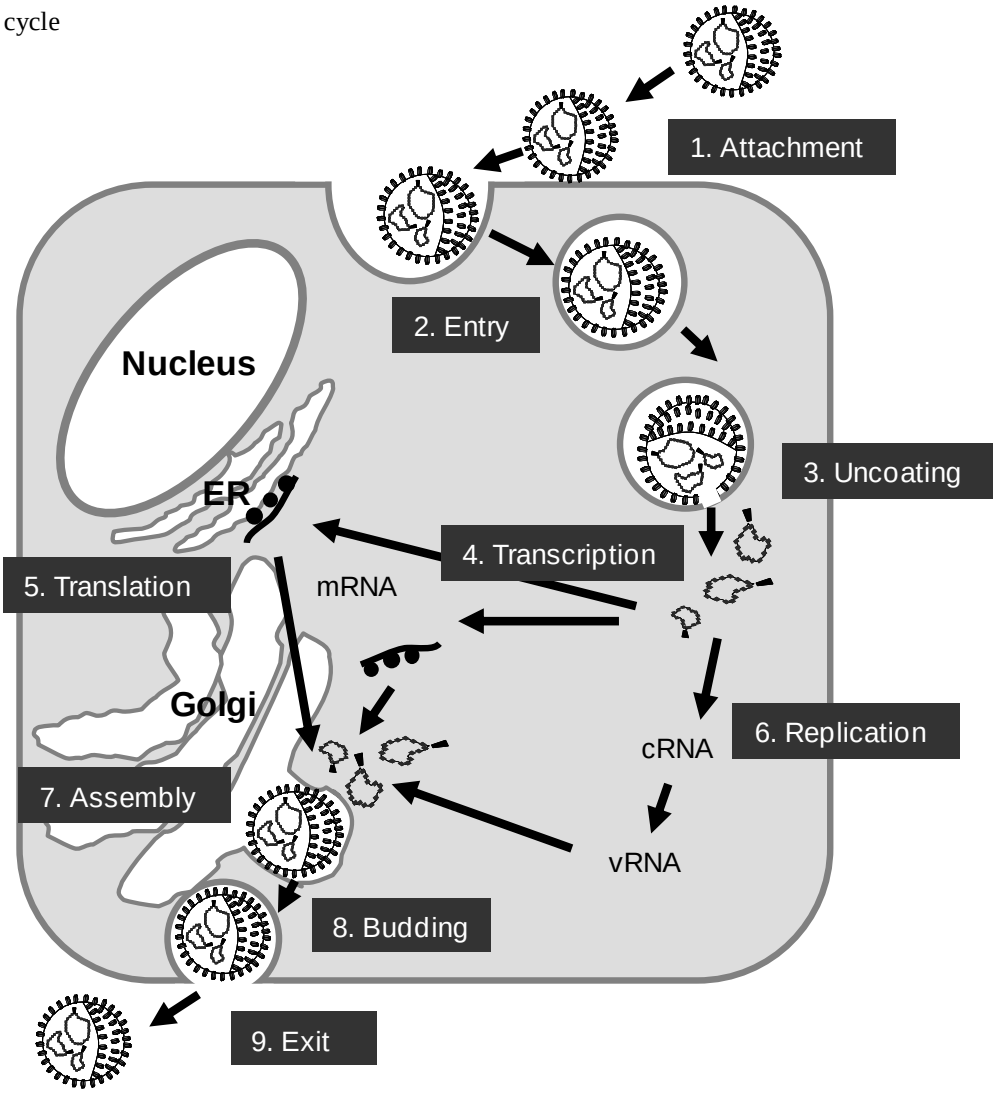
A potential NSs protein is expressed *in vitro* in Tuula virus (TULV) -, PUUV- [70] and most recently also in ANDV-infected cells [71]. The ANDV NSs protein is suggested to be translated by a leaky scanning mechanism by the cellular translation machinery [71]. The function of the TULV and PUUV NSs proteins are suggested to be down-regulation of IFN responses [70], while the function of ANDV NSs protein remains to be further investigated.

Replication

The life cycle of a hantavirus starts with the binding of the glycoproteins to a cellular receptor on a susceptible cell. The main receptor for entry of pathogenic and non-pathogenic hantaviruses are the β3-intergrin and the β1-intergrin, respectively [72-75]. Hantaviruses can infect a cell after blockage of β3-intergrins, [76-77] and ANDV-pseudovirions were observed to infect cells deficient of β3-integrins [78], indicating that other receptors for cell entry exists. DAF/CD55 [79-80], an unknown 70 kDa protein [76] and gC1qR/p32 [80-81] are suggested to be alternative receptors or co-receptors for hantaviruses. After attachment to the receptor, the virus is internalized by either clathrin coated vesicles [83], or in a clathrin independent manner [64], depending on virus and perhaps also on receptor used. The fusion is pH-dependent and uncoating is believed to take place in the early or late endosome [83]. Similar to other Bunyaviruses, the replication of hantaviruses is believed to occur in the cytoplasm and the RdRp is responsible for transcription of viral messenger RNA (mRNA), positive sense complementary RNA (cRNA) and vRNA [43]. The N protein is suggested to co-localize with cellular processing bodies and bind to host mRNA caps to protect these from degradation [84]. Following entry into cells, the RdRp starts primary transcription, and the synthesis of translation-competent capped mRNA are governed by a "prime-and-realign" mechanism, where the RdRp are suggested to cleave the N protein-protected caps of cellular mRNA, and use these as primers [85-89]. The transcription of mRNA for the *Bunyaviridae* family prototype virus Bunyamwera virus is suggested to be dependent on immediate translation; otherwise the transcription is prematurely ended [90]. If this happens in the case for hantaviruses remains to be

elucidated. The N protein is also suggested to play a role in binding viral mRNA to the ribosomes, by substituting the entire host eukaryotic translation initiation factor 4F, and by interacting directly with the ribosomal protein S19 in the 40S ribosomal subunit [89, 91-93]. The translation of N protein and RdRp is suggested to occur on free ribosomes, while translation of the glycoproteins occurs on membrane bound ribosomes [29]. The translation of N protein starts approximately 2h after infection, followed by translation of the glycoproteins and of the RdRp [94-95].

Fig 2. The hantavirus life cycle



After the primary transcription step, the virus shifts to replication of vRNA [43]. The signal for this shift is unknown, but an accumulation of N protein in the cell has been proposed as a mechanism [43]. During replicating, first a full-length positive sense RNA copy is made by the RdRp, where the transcriptional termination signals used for transcription of mRNA are ignored. Then, the cRNA is used as template for the RdRp to transcribe new negative sense vRNA [43]. The mechanisms for full-length cRNA and vRNA transcription are also "prime-and-realign" [86]. Homotrimers of N protein bind to the newly synthesised vRNA and oligomerize around it, forming the nucleocapsid [96-98] and then bind to membrane bound glycoproteins. The viral maturation and assembly takes place at the Golgi complex for most of the hantaviruses [33], while Black Creek Canal virus and SNV maturation and assembly has been described to occur at the plasma membrane [99-100]. The virus exits the cell by exocytosis [29].

Polarized entry and release

The human body consists of several groups of polarized cells, including the epithelial cells that constitute a boundary between tissues and the extracellular environment, and the endothelial cells that form the inner surface of blood vessels and acts as a barrier between the blood and the tissues. Polarized cells show an asymmetry for molecules expressed on their different sides. The apical side faces the lumen, i.e. the outside for epithelial cells and the circulating blood for endothelial cells. The other, basolateral side, faces the tissue and the underlying basement membrane. As receptors are often expressed unequally on polarized cells, viruses may have a preference for apical or basolateral entry into such a cell [101-102]. The release of newly produced virus particles can occur either apical or basolateral, which in turn governs if the virus spread is local or systemic [101-103].

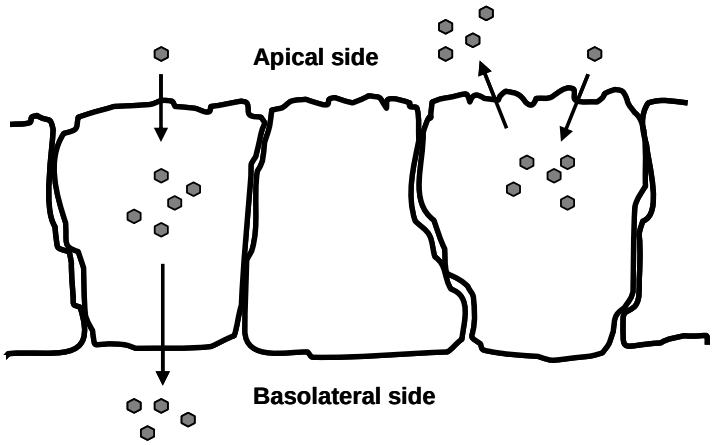


Fig 3. Schematic representation of polarized cells with either basolateral or apical virus release.

In polarized human endothelial cells and monkey epithelial cells, HTNV and PUUV-infection were only successful from the apical side [80], and only apical release of hantavirus were observed from polarized monkey and dog renal tubuli cells [104]. ANDV successfully infected both the apical and the basolateral side of polarized hamster tracheal epithelial cells, with bidirectional secretion of virus, even though apical infection and release were preferred [105]. Studies of Black Creek Canal virus infection of polarized monkey kidney epithelial cells revealed a strong preference for apical infection, and the release of virus from the apical membrane were about 1,200-fold greater than observed from the basolateral side [100]. We observed apical infection and basolateral release of viruses, when infecting *in vitro* model of human lung tissue with ANDV and HTNV [**III, IV**]. This indicates that HTNV-infection of polarized cells can lead to basolateral release of viruses, similar to that seen in ANDV-infection [105].

Isolation and adaptation

Hantaviruses are known to be notoriously difficult to isolate [106]. Most commonly, tissue-samples from infected animal hosts are added to cells, subsequently followed by blind passage several times on the highly hantavirus-susceptible cell line Vero E6 before high enough virus titres to continue with are detected [106]. Other ways of hantavirus-isolation is to passage the virus in colonies of natural rodent hosts [106], in Syrian golden hamsters [107], or through intracranial injection into

suckling mice [108]. We showed successful infection of wild-type PUUV in bank vole embryonic fibroblasts [**II**] suggesting that cells derived from a natural host might also be of use for isolation of hantaviruses. Indeed, Sanada and co-workers reported successful propagation of Hokkaido virus in kidney cells derived from grey red-backed vole, the natural host of this virus [109].

The prototype hantavirus, HTNV strain 76-118, originating from the first hantavirus isolated [7] were initially passaged in wild caught rodent hosts [9] then propagated in the human lung epithelial cell line A549 [110] before finally being passaged in Vero E6 cells [27]. If these cell line adaptations and propagations have caused mutations in this strain, making the virus less resembling the wild-type HTNV remains to be investigated.

The mutation rate for hantaviruses is estimated to be around 10^{-2} to 10^{-4} substitutions/site/year [111]. During propagation of viruses in cell lines, adaptation is accompanied by point mutations in both the coding regions and in the NCR [112-113]. Important mutations for Vero E6 cell line adaptation of wild-type PUUV strain Kazan were G5498A and G357A substitutions in the L-segment NCR, altering vRNA folding [113]. Several mutations of the S-segment were also observed in the cell line adapted virus [112]. There seems to be a "hot-spot" for mutations especially around position 1570 in the PUUV S-segment 5' NCR, [**I**, 111, 113]. As Vero E6 cells are deficient in IFN-α/β responses [114], the lack of antiviral pressure when propagating hantaviruses in these cells, may give rise to virus-substrains that would not survive in nature. One clear example is that Vero E6 cell line adapted PUUV (PUUV-Kazan-E6) has lost its potential to re-infect the natural bank vole host [112]. Other examples of mutations derived from Vero E6 adaptation and propagation are truncated variants of the potential NSs protein in both TULV and PUUV, making these viruses less able to replicate in IFN-α/β competent cells [115-116] and a single aa substitution in the HTNV Gn protein that altered virulence *in vivo* when infecting suckling mice [117]. In paper I, we characterized two substrains derived from cell line propagation of PUUV-Kazan-E6. These substrains showed different phenotypes when infecting IFN-α/β deficient cells, but interestingly not in IFN-α/β competent cells. Compared to the parental strain, which probably consists of several virus-substrains with a range of mutations accumulating during cell line propagation, the substrains also induced less antiviral response in IFN-competent cells [**I**]. Some discrepancy in the reports of antiviral responses induced by hantavirus infection exists, which is usually suspected to be due to differences between different hantavirus strains [51-52, 118-125]. However, one should be careful with interpretations of *in vitro* studies conducted with cell line adapted viruses, as these viruses might not fully mirror the viruses responsible for human infection *in vivo*.

Persistence in cell culture

Hantavirus-infection in humans is believed to be transient, but the virus cause persistent infection in cell lines and in the natural hosts [126-127]. In cell cultures, shortening of the NCR on the vRNA and the cRNA, especially in the L-segment, have been suggested to give rise to a cyclic virus production and this NCR alteration is suggested to be the mechanism behind the persistence *in vitro*, even though the regulation of this mechanism is not fully understood [128].

HOST

Rodents, insectivores and bats

Hantaviruses are carried by rodents, insectivores (e.g. shrews and moles) and bats [22]. So far, the hantaviruses known to be pathogenic to humans are only harboured by rodents [22]. If the insectivore and bat-borne hantaviruses can cause disease in humans are unknown [129]. Whether the different hantaviruses have co-evolved with their hosts, or if host switching and local adaptation during the course of evolution have occurred is a matter of controversy [130-134].

Geographic distribution

The prevalence of hantaviruses is restricted to the geographical distribution of the natural hosts. In Eurasia the most common disease-causing hantaviruses are HTNV, PUUV, SEOV, Amur virus and Dobrava-Belgrade virus. In the Americas, SNV and ANDV are the most common causes of disease [22, 29, 129, 135]. SEOV that is carried by rats probably exists worldwide [129, 136].

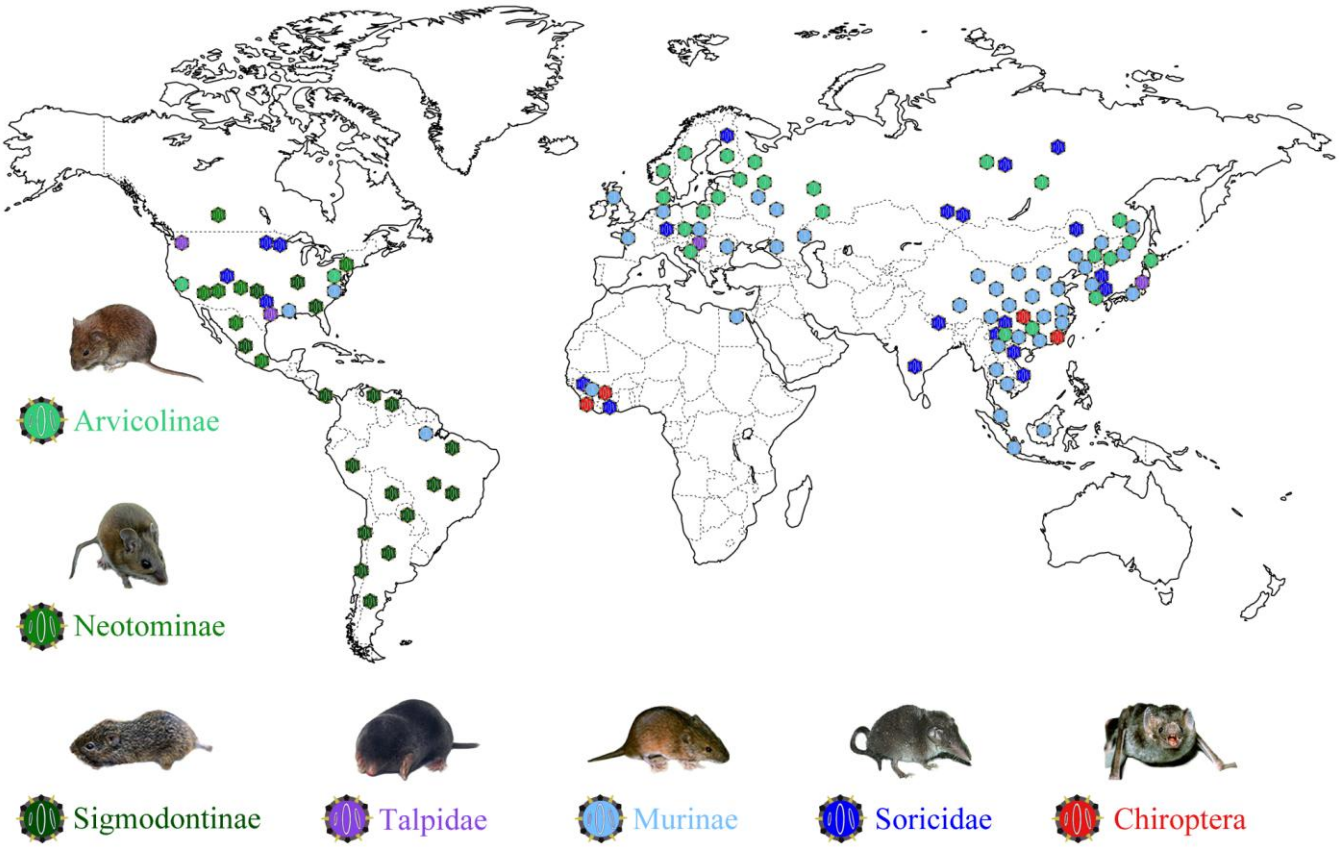


Fig 4. Geographic distribution of hantaviruses and their primary host species family. Hantavirus carried by chiropotera (bats) is also found in Brazil [24]. Reprinted from Guo WP et al, 2013. Phylogeny and origins of hantaviruses harboured by bats, insectivores, and rodents. PLoS Pathog. 2013 Feb;9(2):e1003159 [22] under the terms of the Creative Commons Attribution License.

Infection of rodent hosts

The infection of the animal hosts is believed to be lifelong and asymptomatic [127, 137]. However, some recent studies have shown decreased survival among male deer mouse infected with SNV [138], impaired winter survival of PUUV-infected bank voles [139] and higher morbidity in juvenile and sub-adult PUUV-infected bank vole males [140]. Further, pathological changes in the lungs from SNV-infected deer mouse have been reported [141]. Taken together, this suggests that natural hosts may occasionally suffer from hantavirus-mediated damages.

A few days to weeks after initial hantavirus-infection of the rodent host, the infection gets viremic, during which the virus-shedding peaks [127]. Hantavirus is shed mostly via excretion of virus in urine, faeces and saliva [127, 142-143]; though not all rodent species seem to shed high doses of virus. In deer mice infected with SNV, viruses were not at all observed in urine and faeces, and only for a little extent in saliva [144]. Virus distribution during the viremic phase in the hosts is believed to differ between the rodent species, from only a few to virtually all organs infected [127]. The viral load in tissues and the virus shedding decrease over time, when the infection progress into a chronic/persistent phase [127]. It is not clear why the infection becomes chronic in the host, and the mechanisms for persistence may involve virus evasion/suppression of host immunity [127, 137]. Activation of T regulatory cells to suppress pro-inflammatory responses to the hantavirus-infection is suggested to be a part of the persistence mechanism in the natural hosts [127, 137].

Transmission between rodents

Vertical transmission *in utero* has been suggested as a possibility for Black Creek Canal virus-infection among cotton rats [145], otherwise only horizontal transmission of hantavirus in the rodent populations are believed to occur [127, 142]. Hantavirus-infected dams protect their pups from infection via antibody-containing milk, and this protection persists months after weaning [142, 144, 146-147]. Hantaviruses are rather stable ex-vivo [148-149], for example are both PUUV and TULV infectious in cell culture after 5-11 days at room temperature [149]. Hantaviruses can therefore be transmitted through contaminated beddings [143, 150]. Another way of transmission between rodents is through wounding [142, 151-153].

HUMAN

Transmission to humans

Humans are mainly infected with hantaviruses through inhalation of virus-contaminated rodent excreta [29, 154]. Humans often get in contact with the virus when cleaning rodent-infested under-ventilated housings, like a cabin or a shed, or through outdoor activities [29, 154]. In endemic areas, hantavirus-infection is regarded as an occupational risk for farmers and forest workers [155-156]. The epidemiology of hantaviruses is closely linked to the ecology of its natural host [29, 129, 135]. The climate can have an effect on hantavirus transmission as host population may increase during years with high abundance of food. The weather may also affect the habitual patterns of the rodents [29, 154, 157-158]. For example, cold winters without protective snow cause forest dwelling rodents to seek shelter in human housings [158]. As the natural host of Seoul virus is rats, several reports of hantavirus transmission from laboratory rats, as well as transmissions from pet rats, have been published [159-162]. Other hantavirus strains may also occasionally be transmitted to humans in laboratory settings, through aerosolized droplets of viruses [163]. There is one report of hantavirus transmission suspected to have occurred through transfusion of blood platelets [164], raising the question if blood should be tested for hantavirus RNA, at least in hantavirus-endemic areas.

The lungs are lined with epithelial cells creating a barrier to the outside. The mechanism for hantavirus entry through this barrier is not yet known. Dendritic cells (DC), monocytes and macrophages can be infected by hantaviruses *in vitro* [120, 123, 165-168] and ANDV-infected intrapulmonary macrophages have been detected in the lungs of an HPS-patient [169]. This indicates that hantavirus might use immune cells residing in the lungs as vehicles to get across the epithelial barrier [127]. Polarized lung epithelial cells are susceptible *in vitro* for apical hantavirus-infection, resulting in basolateral release of virus [105, **III, IV**], indicating that vehicles for lung epithelial barrier passage might not be needed for hantaviruses.

The only hantavirus known to cause person-to-person transmission is ANDV [19, 170-173]. For this transmission to occur, close contact is required e.g. between spouses, suggesting that the virus is not easily transmitted between humans [170-171]. ANDV is believed to be spread through saliva or coughing, as viral RNA has been detected in the salivary glands [174] and in the intrapulmonary macrophages of patients [169]. In addition, infectious ANDV particles can be shed in human urine [175], suggesting that other routes of transmission might exist. In PUUV-infected patients, viral RNA have also been detected in the saliva [176], but as salivary components have proved to have strong antiviral effect against hantaviruses except ANDV [177-178], PUUV-infection is at present not believed to cause transmission between humans.

Tropism

The access a virus has to its host receptor determines what cells the virus can infect. During the acute phase of human hantaviral illness, hantaviruses are usually found in most organs of the body. Viruses have been found in human kidney, heart, spleen, lung, lymph nodes, adrenal glands, pancreas, adipose tissues, urinary bladder, skeletal muscles, salivary glands and intestine [179-184]. Infection of the

hypophysis has also been reported [185] and viral RNA in cerebrospinal fluid has been observed [186]. The main cell type infected is the endothelial cells of the capillaries and small vessels, but hantavirus has also been detected in interstitial macrophages [184], pulmonary macrophages [169, 180], renal tubular epithelial cells [179-181], pneumocytes [181] and follicular dendritic cells [180]. Hantavirus infection of cells and organs most likely do not cause cytopathology *per se* [179-183, 187], but the infection of the endothelial cells are believed to be involved in the pathogenesis [188].

Disease

The classic division of hantavirus-caused diseases is that the viruses endemic in Euroasia cause HFRS, while the hantaviruses of the Americas cause HPS. As both diseases are associated with changes in vascular permeability, drop of blood platelet number (thrombocytopenia), and both diseases may have renal or pulmonary symptoms [29, 55, 189], they have recently been suggested to be referred to as hantavirus disease/ hantaviral illness [30-31].

Around 50 000 cases of hantavirus-caused diseases are reported annually, most of the cases are in China, Russia, Fennoscandia and South America [129]. Seroprevalence studies have revealed that around 80-85% of PUUV-infections are not diagnosed; hence hantavirus-infection might lead to subclinical symptoms or a very mild disease [55].

Hemorrhagic fever with renal syndrome

The disease caused mainly by HTNV, SEOV, Dobrava-Belgrade virus and Amur virus are HFRS, and consists of five more or less distinct phases. The incubation period for HFRS is between ten days to six weeks [29, 154]. Abrupt onset of high fever, headache and malaise followed by gastrointestinal symptoms like vomiting and abdominal pain is the first phase of disease. This febrile phase usually lasts between 3-7 days, before developing into the hypotensive phase with thrombocytopenia and risk for shock. After a few hours up to some days, the oliguric phase starts. In this phase renal impairment and internal bleeding are common. Recovery begins with the onset of the polyuric phase, hallmarked by a high output of urine, sometimes requiring adequate isotonic replacement. Finally, the patients enter a long convalescence period, with slow recovery and symptoms like fatigue and back pain. It can take months before full recovery is reached [29, 154, 189].
PUUV is considered to cause a milder variant of HFRS, sometimes called NE [154]. The symptoms of PUUV-infection normally have less distinct phases compared to more severe HFRS, but also start with a sudden onset of fever, headache, back pains and gastrointestinal symptoms [154, 189]. Blurred vision is a quite common symptom during PUUV-infection [190-191]. For the severely ill, hypotension and shock might develop rapidly. Many patients experience hemorrhagic manifestations [154]. In PUUV-infected patients, pulmonary involvement with respiratory distress [30-31, 192-193] and abnormal cardiac findings [194] is common. Neurological manifestations like encephalitis have also been reported [191, 195-198]. After about two weeks, symptoms of acute disease disappear after onset of polyuria, followed by a long convalescence period. For PUUV-induced HFRS there are some reports of long-lasting complications, e.g. hypertensive disease and kidney malfunctions [199-201]. However the severity of acute disease seems not to correlate with possible long-term consequences [202].

Hantavirus pulmonary syndrome

HPS is caused by the American hantaviruses SNV, ANDV and related viruses. Similar to HFRS, HPS also has a long incubation period [17, 29], and a febrile prodrome phase manifested by headache, muscle pains and fever. Even though there are reports of renal involvement in HPS-patients [17, 203-206], most of the HPS-patients show pulmonary symptoms in the next phase of disease, starting with shortness of breath and tachycardia, often developing into respiratory failure and cardiogenic shock [17, 29]. Due to the strong involvement of myocardial depression, HPS are also referred to as hantavirus cardiopulmonary syndrome (HCPS) [184]. For patients surviving the week long cardiopulmonary phase, a polyuric and long convalescence phase await [17, 29, 198]. HPS can give long-lasting complications, like impaired renal function [207], pulmonary dysfunction [208] and central nervous disorders [209].

Mortality

The highest risk of fatality of hantavirus-infection is during the hypotensive and oliguric phases. Cause of death may be due to shock, hemorrhages, pulmonary oedema or complications from renal insufficiencies [29]. Case fatality rates have been reported to be 0.1-1% for PUUV-caused HFRS and 5-10% for HTNV-caused HFRS [55]. The cardiopulmonary involvement of HPS makes the mortality for HPS higher than for HFRS, around 40% [17].

Prophylaxis and treatment

Often, hantavirus-infections require hospitalization. Presently, the only therapeutics available for HFRS/HPS-patients is supportive care, e.g. dialysis, management of electrolytes, hydration or respiratory support [29, 189, 210]. Ribavirin, a nucleoside analogue, has antiviral activity against hantaviruses both *in vitro* [211-215] and in animal models [214-217]. From clinical trials, Ribavirin seems to have a reducing effect on HFRS-symptoms if administered early upon hospitalization [218], while the drug had less effect on SNV-infected HPS-patients [219-220]. High levels of neutralizing antibodies are correlated with a positive disease prognosis and milder disease [221-222] and passive immunization with neutralizing antibodies has been shown to be beneficial in several animal studies [223-227]. A small-scale open label trial where HPS-patients were treated with convalescent plasma is currently under evaluation, with promising preliminary results [228].

Vaccines for HFRS exist in Korea and China, but the effects of these vaccines are controversial, and the vaccines are not approved by US Food and Drug Administration [229] or the European Medicines Agency [230]. Extensive research on vaccines for different hantaviruses are conducted [129, 231].

Avoiding or minimizing exposure to hantaviruses is the most effective way for preventing infection. Rodent management by rodent-proofing buildings is one way [232-234]. Wet-wiping while cleaning houses that are at risk for rodent infestation, is to prefer rather than cleaning in ways that produces aerosols [233-234]. In the case of visible rodent droppings, treatment with chlorine or other detergents are recommended [233], as hantaviruses are sensitive to those disinfectants [235]. Protective gears are recommended for activities were encountering rodent droppings might be inevitable [234].

Immune responses to hantavirus-infections

Interferons and interferon- induced antiviral responses

Interferons serve to limit virus infection before adaptive pathogen-specific responses are mounted. IFNs are divided into type I (IFN-α/β), type II (IFN-γ) and type III (IFN-λ). IFN-γ is predominately expressed by activated effector cells and is an important activator of macrophages [236]. Type I and III IFNs are upregulated in response to virus-infections after virus-detection by pattern recognition receptors (PRRs). PRRs exist both on the extracellular surface and in the endosomal compartments of a cell. Activated PRRs initiate downstream signalling cascades leading to transcription of IFNs. Transcription of IFN-β requires activation of NFκb and IRF-3, while IFN-α requires IRF-3 and IRF-7 [236]. Once induced, IFN-α/β act both in a paracrine and in an autocrine manner, and induces an antiviral state in infected as well as in uninfected neighbouring cells. IFNs bind to IFN-receptors and trigger activation of the JAK-STAT-pathway, a signalling event finally leading to induction of more IFNs and transcription of over 300 interferon-stimulated genes (ISG). The products of these ISGs act in several different ways to directly or indirectly hinder virus-infection and replication, e.g. by interfering with viral proteins, induce translational arrest or by upregulation of major histocompability complex (MHC, also named human leucocyte antigen, HLA) class I molecules. IFNs also are able to activate immune cells, for example by promoting maturation of these cells [236].

Most viruses have developed some strategy for evading the IFN-response and thereby successfully establish infection and continued replication [236], and hantaviruses are no different. After infection, hantaviruses can escape recognition of the PRR RIG-I [48, 237], interfere with IFN-induction downstream of the PRR signalling cascade [49, 51, 238] and potentially inhibit activation of NFκB [66-68]. Hantaviruses also interacts with the JAK-STAT pathway to evade induction of ISGs, by hindering phosphorylation of STAT1 and STAT2 [52, 239]. Overall, hantaviruses are poor inducers of IFN type I responses, and elevated levels of type I IFNs are usually not observed in patients [239]. Interestingly, hantaviruses induce type III IFNs in a type I IFN-independent manner [238, 240]. In PUUV-patients, decreased levels of IFN type III have been observed in sera from the acute phase compared to from the convalescence phase [239]. If hantaviruses upregulate IFN-λ also *in vivo*, these findings suggests a possible increase of IFN-λ receptor expression during the disease. Hantaviruses are sensitive to pre-treatment with IFNs [239, 241-242], showing that IFN-responses can prevent the virus from further spreading if the neighbouring cells are in an antiviral state.

Dendritic cells

Dendritic cells are key players in the immune system. They produce pro-inflammatory cytokines and can regulate immune responses. As DCs are professional antigen presenting cells and are crucial for the initiation of antigen-specific immune responses they bridge innate and adaptive immunity. DCs constantly survey tissues, sampling antigens and then migrate to afferent lymph nodes to present the antigens to, and subsequently activate, naïve T lymphocytes [243]. The DCs that resides in the lungs are heavily influenced by the nearby epithelial cells, and this interaction partly determines the outcome of the pulmonary immune response [244].

The exact role of DCs in hantavirus infection is not known. *In vitro* studies have confirmed susceptibility of DCs to HTNV and ANDV [123, 165-166]. HTNV-infection of immature DCs activates maturation and upregulation of HLA, co-stimulatory and adhesion molecules [123, 165-166]. DCs infected with ANDV *in vitro* increased their expression of TNF and could indirectly affect the permeability of endothelial cells [166]. It has been suggested that infected DCs could facilitate dissemination of hantavirus and that a different DC response in natural hosts compared to in humans could explain some parts of the different responses toward hantavirus infection between the species [127, 154]. In a setting resembling the lung microenvironment, DCs do not clearly upregulate activation molecules when challenged with HTNV and DCs had an antiviral effect against hantavirus-infection [**IV**].

Humoral responses

B lymphocytes are part of the adaptive immunity and antibodies produced by the B lymphocytes acts against viral infections in several ways. Antibodies recognise intact viral proteins as antigens. Neutralizing antibodies protect against virus-infection by blocking the virus from binding to its cellular receptor, thereby preventing infection. Antibodies can also act via other effectors resulting in lysis of the infected cell [245]. Neutralizing antibodies against hantaviruses are detected early upon disease, sometimes preceding the symptoms. A connection between high levels of neutralizing antibodies and good prognosis has been observed. After recovery, neutralizing antibodies persist for many years [221-222].

At the onset of clinical symptoms, high levels of hantavirus specific IgM are normally present in sera [221- 222]. Usually IgM is used as a diagnostic tool, but as IgM-response sometimes is delayed, especially in PUUV-infected patients [222], additional means of diagnosis is necessary. During the acute phase of the disease, the IgM response is followed by the production of IgG. The major parts of IgG subclasses detected in HFRS/HPS are IgG1 and IgG3. Whereas IgG3 decline during the convalescence phase, IgG1 persists for years [221-222]. For example, one of the first Swedish HFRS-patients described in the 1930ies were still positive for anti-hantavirus antibodies 50 years after infection [246].
Interestingly, an IgG4 response, normally associated with repeated antigen stimulation, is observed years after HFRS [221-222]. Neutralizing IgA responses develop upon infection, with titres of IgA1 still detectable in PUUV-patients 2-10 years after infection [221-222].

In addition, significantly increased levels of anti-hantavirus IgE have been detected in PUUV-patients [247-248]. This response most often peaks early, at the onset of symptoms [247], hence it has been suggested that IgE might be involved in the pathogenesis of PUUV-infection, even though no correlation between IgE levels and clinical symptoms has been detected [247]. IgE responses is an unusual feature of viral diseases [247], but elevated levels of IgE has been observed in re-occurring varicella zoster virus-infection (shingles) [249], in chronic hepatitis C virus-infection [250], in respiratory syncytical virus-infection [251], in severe dengue virus-infection [252], as well as in parvovirus B19-infection [253].

Cellular responses

The second arm of the adaptive immunity is the T lymphocytes. Simplified, these cells are divided into CD8+ cytotoxic T lymphocytes (CTL), CD4+ T helper (Th) lymphocytes, T regulatory lymphocytes and memory T lymphocytes. T cells recognise foreign peptides presented by the MHC molecules on the surface of either professional antigen presenting cells or of infected cells. All nucleated cells have surface MHC class I molecules, which can be recognised by CTLs. Antigen presenting cells have additionally surface MHC class II molecules which are recognised by Th cells. CTLs are important for control of intracellular infections, especially virus infections, and can kill infected cells by inducing apoptosis in them [245]. One way for viruses to evade CTL-responses is to down-regulate the expression of cell surface MHC class I molecules, resulting in reduced risk of CTL-recognition [245]. CTLs also have a function in terminating the immune response by inducing anergy of activated lymphocytes [245]. Th cells have a role in driving and regulating the overall immune response by producing cytokines that acts upon other cells e.g. B lymphocytes and macrophages. Subtypes of Th cells are defined by the type of cytokines they produce [245].

During hantavirus-infection, the T cell mediated immunity is suggested to be mostly directed against the N protein. Infection with hantaviruses changes the Th cell/CTL balance in favour for CTLs [221-222, 254-255]. Increased infiltration of CTLs is found in the lungs, kidneys and hearts of HFRS/HPS-patients [184, 221-222, 256]. In PUUV-infected patients, elevated levels of mediators of apoptosis used by cytotoxic cells; granzyme B and perforin, have been detected [257]. There are also reports of high levels of hantavirus-specific memory T cells in patients, and these levels are stable even years after infection [221, 258-259]. Manigold and co-workers reported that around 17% of the total circulating memory CTLs in a recovered HPS-patient were directed against one single hantavirus epitope [258].

Cytokine responses

Cytokines are mediators, regulators and attractants of the immune system. The cytokine milieu orchestrates what type of immune response that will be directed against an intruding pathogen. While some cytokines promote inflammation (e.g. TNF and IL-6), other cytokines acts by dampening the inflammatory responses (e.g. IL-10 and IL-4), and some cytokines mainly recruit immune cells to the site of action (e.g. RANTES and IP-10) [260-261].

Imbalance in cytokine responses to infection might be a part of pathogenesis during HFRS/HPS. Elevated levels of the pro-inflammatory cytokines IL-6 and TNF are usually detected in plasma from HFRS/HPS-patients during the acute phase of disease [221-222, 262-264], and infiltration of cytokine-producing cells have been observed in patient tissues [184, 192, 265-266]. Mixed Th1 and Th2 serum cytokines and increased levels of IL-10 are sometimes reported [29, 264]. There is a discrepancy regarding cytokine responses observed in HFRS/HPS patients. This could be due to inconsistency in time points when the patient-samples were drawn. Also, confounding factors as sex is seldom correlated for, which could be of importance as a potential sex-dependent difference in cytokine responses in PUUV-infected patients have been reported [267].

Hantavirus-infection *in vitro* induces a strong upregulation of the immune cell attracting chemokine IP-10 [119, 268]. In line with this, ANDV-infection of Syrian golden hamsters resulted in elevated plasma-levels of IP-10 [269]. There are conflicting reports regarding the effects of hantavirus-infection *in vitro* on the immune cell attracting chemokine RANTES [119-121, 124, 211]. HTNV-infection and ANDV-infection were observed to upregulate RANTES mRNA expression in endothelial cells [119-120, 124, 211], while New York-1, an HPS-causing hantavirus, did not [119]. The non-pathogenic Prospect Hill virus induced an earlier RANTES response than HTNV in both lung epithelial cells and differentiated mast cells [121] while non-pathogenic shrew-borne hantaviruses down-regulated RANTES in endothelial cells after infection [120]. Interestingly, SEOV-infection of lung endothelial cells and alveolar macrophages derived from rats, the natural host of this virus, did not induce IP-10 or RANTES expression [270]. When we infected an *in vitro* model of human lung tissue with ANDV, we did not observe upregulation of RANTES protein or mRNA, instead we observed a decrease over time [**III**]. Taken together, these results indicate a virus-strain specific and/or cell-type specific regulation of RANTES expression during hantavirus-infection.

In our studies, we also observed an increased response of the chemokine eotaxin-1 from infected lung models compared to from uninfected models [**III, IV**]. Eotaxin-1 binds selectively to the chemokine receptor CCR3, expressed on a range of immune cells, especially on eosinophils [271]. Interestingly, the "typical" Th2 cytokine IL-4 is usually not observed in HFRS and HPS patients, while elevated levels of IL-5 have been reported [264]. IL-5 has a central role in eosinophil development, activation and survival [272]. The findings of elevated levels of eotaxin-1 *in vitro* and IL-5 *in vivo* might suggest a possible role for eosinophils in hantavirus-infection. Eosinophils were observed to be significantly decreased in lung biopsies from PUUV-patients [256], but as those biopsies were sampled median 8 days after onset of symptoms, and that the staining was for eosinophil cationic protein [256], which is released upon activation [272], these findings do not rule out an eventual involvement of eosinophils in hantavirus pathogenesis. The possible role of eosinophils in hantaviral illness needs to be further investigated.

The activity and the availability of chemokines in the body are regulated at many levels, e.g. availability of receptors, receptor affinity and presence of decoy receptors [273-275]. Recently, attention has been drawn to the fact that chemokines often are posttranslational modified e.g. citrullination of amino acids or truncation of the protein [276]. These changes can lead to differences in the functions of the chemokine [276]. An example is a truncated variant of IP-10 that recently was observed in chronic hepatitis C virus-infected patients. This variant of the chemokine have an antagonistic function and do not attract immune cells and are correlated with an inability of the patients to clear the virus [277]. Many of the posttranslational modifications of chemokines are performed by matrix metalloproteases (MMP) [276]. Interestingly, increased expression of MMP-9 and MMP-2 from ANDV-infected DCs *in vitro* has been reported [166]. Even if no elevated levels of MMP-9 were detected in Dobrava-Belgrade virus-infected HFRS patients [278], the question if chemokines are modified in hantavirus-infected patients should be considered, as the consequences of these modifications can be of importance for understanding the HFRS/HPS-pathogenesis.

Other innate responses

Another important cell-type involved in innate antiviral defence is the natural killer (NK) cells. NK cells are regulated in a complex manner. A very simplistic description is that they do not act against a pathogen-specific antigen, instead they respond to what type of NK cell activating and inhibitory receptors that are expressed on a cell surface [279]. When activated, NK cells are cytotoxic in the same manner as CTLs; by inducing apoptosis in the target cell they eradicate them. NK cells are not capable of controlling all viral infections, and several viruses have developed different evasion mechanisms targeting NK cell mediated responses [69, 279-280]. At the onset of HFRS, NK cells have been reported to migrate into infected tissues [221]. In PUUV-infected patients, NK cells are observed to rapidly expand and then persists at highly elevated levels throughout the convalescence phase [256, 280], implying a potential role of dysregulated NK cell responses in HFRS/HPS pathogenesis.

The complement system is another arm of the innate immune system and consists of several plasma proteins that can be induced by pathogens and subsequently induce inflammatory responses [245]. Activation of complement has been observed in PUUV-infected patients, and the level of complement-activation correlates with severity of symptoms [281-282].

Nitric oxide is involved in several cellular processes and has antiviral effect against some viruses, including hantaviruses [283]. Nitric oxide can be induced by virus infection, either directly by the virus or via cytokine dependent activation [284-285]. In hantavirus-infection, nitric oxide has been detected at elevated levels, both in patients as well as in cynomolgus macaque infected with wild-type PUUV [264, 286-289]. *In vitro,* hantavirus-infection inhibits IFN-γ induced induction of nitric oxide [239].

Proposed pathogenesis

The pathogenesis of hantavirus-mediated diseases are not fully understood. Most likely HFRS and HPS are caused by a complex multifactorial process including dysregulated immune responses, platelet dysfunction and altered endothelial cell functions. Vascular leakage is a hallmark of hantaviral illness and the main target of hantavirus infection is endothelial cells. The vascular leakage is suggested to be caused by immune responses and/or by direct effects of the virus on infected endothelial cells [188].

Vascular permeability is the term describing the way endothelial cells regulates the capacity of plasma proteins, fluid and leucocytes to cross the blood-tissue barrier. A consequence of increased vascular permeability is redistribution of body fluids, leading to oedema. Failure in regulation of vascular permeability with resulting fluid accumulation in tissue, might lead to hypotension, shock and severe multiorgan failure [188]. The vessels in the body are lined by endothelial cells that are strongly interconnected by multiprotein-complexes, like vascular endothelial cadherins (VE-cadherin), to form a tight barrier. The junctions between the cells can be altered to let through larger proteins and cells upon stimulation, allowing immune cells to migrate to the site of infection. The endothelium is regulated by both systemic and local tissue-specific responses, e.g. cytokines, growth factors, nitric oxide, hypoxia as well as adherence of immune cells and platelets. Endothelial cells themselves

produce several substances that act upon endothelial barrier functions and can regulate platelets and immune cell responses. Vascular endothelial growth factor A (VEGF-A) is a mediator that binds to its receptors on endothelial cells (VEGFR2) and can activate internalisation of VE-cadherins, potentially leading to capillary leakage [188].

Direct effects of the virus on the endothelium

Infection of endothelial cells *in vitro* does not cause any apparent damage to the cells and permeability is not increased by infection alone [268, 290-292]. In patients, infected endothelial cells seem to have intact cellular morphology [180, 184, 266].

ANDV-infection of endothelial cells *in vitro* cause increased expression of several microRNAs involved in regulating vascular permeability [293], indicating that it might exist direct ways for the virus to affect the integrity of the endothelium. The main receptor for pathogenic hantaviruses is β3-integrins [72-75]. β3-integrins normally regulate endothelial cell permeability by binding to VEGFR2, thereby altering cell responsiveness to VEGF-A [188]. VEGF-A-stimulation of hantavirus-infected endothelial cells *in vitro* increases the barrier permeability [290, 294-296], indicating that elevated levels of VEGF-A, if produced during infection, can affect the infected endothelium. Indeed, in bronchial alveolar lavage fluid from patients as well from hantavirus infection *in vitro*, increased levels of VEGF-A have been detected [297, **III**], suggesting a role for VEGF-A in deregulation of the vascular permeability.

At late time-points after infection, hantaviruses have been observed to cover the surface of the endothelium, bound to β3-integrins [298-299]. The endothelial cells use the β3-integrins during migration and binding to this receptor by hantaviruses block movement of the cells [299]. Hantavirus on the surface of endothelial cells also interacts with β3-integrins on platelets, resulting in platelet binding to the endothelium [298]. This might be involved in the thrombocytopenia seen in patients, and the platelets might also mask the virus from being recognised by immune cells and antibodies. Taken together, these results suggest a direct effect of the virus on the endothelium during disease.

Indirect effects of the virus on the endothelium

Indirect effects of hantavirus-infection on the endothelium have also been proposed as an explanation to the increased vascular permeability observed in patients [17, 29, 55, 127, 129, 300-301]. High levels of pro-inflammatory cytokines as well as infiltration of immune cells in tissues have been observed in patients and these might have an effect on endothelium permeability [17, 29, 221-222, 256].

CTLs directed against hantavirus-epitopes and highly activated NK cell are observed in patients [254-257, 259, 280, 301-302]. In line with this, high levels of granzyme B and perforin were found in plasma of PUUV-infected patients, together with increased amounts of caspase cleaved cytokeratin 18 (CK18), a marker for epithelial cell apoptosis, indicating cytotoxic cell-mediated damage on the epithelium [257]. Interestingly, ANDV N protein protects infected cells from apoptosis [69]. In addition, we observed that ANDV-infection *in vitro* resulted in lower levels of extracellular total CK18 from infected compared to uninfected lung models [**III**]. Together this indicates that hantavirus-

infected cells might not be affected by CTLs and NK cells, and that the increased death of uninfected bystander cells might explain the finding of increased apoptosis in epithelial cells. Importantly, the strong CTL response seen upon hantavirus-infection might not be involved in the pathogenesis as depletion of T lymphocytes in an HPS animal model, Syrian golden hamsters infected with ANDV, did not alter the course of disease [303].

T regulatory lymphocytes are suggested to be important for controlling infection in the natural host by turning the initial viremia into a low level persistent infection [137]. T regulatory cells suppress pro-inflammatory and CTL-responses, allowing pathogens to persist [137]. Induction of a T regulatory response in humans upon infection is suggested to be delayed or suppressed [29, 127]. Indeed, induction of TGFβ, a determinant for T regulatory cell development, is delayed in HPS-patients [264] and changes in the levels of T regulatory lymphocytes have not been observed during the acute stage of HFRS [254, 304].

AIMS

The general aim of this thesis was to gain further knowledge about how hantavirus can cause disease by investigating genetic properties of the virus and by using different *in vitro* model-systems to analyze cellular responses of voles and humans during hantavirus infection.

SPECIFIC AIMS

1. To investigate different phenotypes and genotypes of PUUV substrains evolving during *in vitro* propagation of the virus.

2. To develop an *in vitro* model for studies of PUUV-infection in cells derived from its natural host, the bank vole.

3. To study long-term infection with the HPS-causing ANDV in a complex *in vitro* 3-dimensional model of human lung tissue.

4. To study the impact of DCs on ANDV and HTNV-infection in an *in vitro* 3-dimensional model of human lung tissue.

METHODOLOGY

A range of common molecular methods were performed, for a detailed description I refer to the material and method section of each individual publication.

SPECIFIC METHODS

Focus assay (Papers I, II, III and IV)

To investigate the amount of replication-competent virus particles present in a sample, the following method was performed: Ten-fold dilution series of the sample were applied onto confluent Vero E6 (African green monkey kidney epithelial cell-line, IFN type I deficient and highly susceptible for hantavirus infection, ATCC: CRL-1586) cell culture monolayers in 24-well plates. After adsorption, the cells were overlaid with basal 0.5% agarose media and incubated for 6-10 days depending on the specific virus (six days for HTNV and PUUV-La; seven days for PUUV-Pa; eight days for PUUV-Sm and ANDV; and ten days for PUUV-Umeå). The agarose layers were then removed and cells were fixated with methanol. To visualize infection, cells were stained with either monkey PUUV-convalescence sera or mouse anti-nucleocapsid monoclonal antibody 1C12, followed by horseradish-conjugated goat anti-human IgG or goat-anti mouse IgG respectively. Then tetramethylbenzidine substrate was added to the wells, and staining was stopped by H_2SO_4. To quantify titres, single foci (representing one infectious virus that has spread to neighbouring cells), were counted and presented as focus forming units (FFU) per mL.

Focus-purification (Paper I)

To be able to investigate one single clone of a virus in detail, the parental stock of PUUV (PUUV Kazan-E6/PUUV-Pa) was diluted to approximately 5 FFU/mL, before adsorption onto confluent monolayers of Vero E6 cells as described above. After agarose removal, one week post infection, the cells were incubated with dilution media. The supernatants were collected after one hour, and cells were fixed and stained as described above. From the wells with only one single foci showing either a smaller or a larger focus phenotype, the supernatant collected were propagated on VeroE6 cells, and the purification procedure was repeated one more time for each for the two substrains isolated.

Vole embryonic fibroblasts (Paper II)

To develop vole embryonic fibroblasts (VEFs), foetuses from around day 14 of gestation were obtained from bank vole (*Myodes glareolus*). After carefully discarding head and liver, the specimens were minced to small pieces, and treated with trypsin to digest the intercellular bounds and dissolve the tissues to a single-cell suspension. Cells from four foetuses were pooled and expanded for three passages before the cells were frozen until usage. The VEFs used in studies were expanded and experiments were conducted on cells in passage four to eight.

Bio-assay of interferon responses (Paper II)

Poly(I:C), a synthetic analogue of double stranded RNA, is known to initiate IFN-responses in cells through interaction with PRRs [236]. VEFs were transfected with poly(I:C) according to the manufacturers' protocol, and 17 hours after transfection, supernatants were collected. To investigate if bioactive IFN could be secreted from bank vole cells, other VEFs were treated for 24 hours with either the poly(I:C)-conditioned supernatant or supernatant from un-stimulated cells. Then recombinant IFN-sensitive green fluorescence protein-expressing Newcastle disease virus (NDV-EGFP), were applied to the pre-treated cells. If a cell is in an IFN-induced antiviral state, the cell is resistant to NDV-infection. After 17 to 20 hours of NDV-EGFP-infection, the number of cells expressing GFP was determined by fluorescence microscopy.

3-dimensional organotypic human lung model (Papers III and IV)

To set up human organotypic lung models, MRC-5 cells (human fetal lung fibroblasts, ATCC CCL-171) were cultured in a collagen-medium suspension on transwell inserts, with pore sizes of 3.0 µm. The fibroblasts-suspension was covered with medium for eight days, allowing the fibroblast to grow and remodel into a stroma/matrix layer. Subsequently, SV-40 transformed human bronchial epithelial cells (16HBE), were seeded on top of the fibroblast-collagen layer. After three days, allowing the epithelial cells to form a confluent monolayer, the apical side was air-exposed by removing the culture media in the outer chambers. The air-exposure allowed the epithelial cells to differentiate and form tight and adherence junctions as well as a mucus layer. Seven days after air-exposure the models were ready for infection. For models containing DCs, the same protocol as above was applied, with the exception that blood-derived monocytes were added to the fibroblast-collagen gel 24 hours prior to the seeding of epithelial cells. Supernatants were subsequently collected from the medium on the basolateral side of the model.

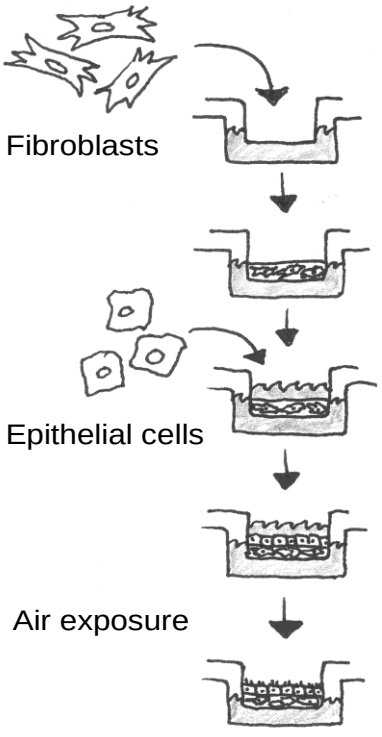


Fig 5. Set up of lung model

For RNA-analysis, models were carefully removed from the transwell-inserts. The tissue was cut into pieces and then homogenised with the use of disposable mortars, after a cycle of freeze-thawing. The total RNA was then purified.

For immunoblot and FACS analysis, the cells in the model needed to be in a single-cell format, not interconnected with the collagen matrix. To obtain this, the models were carefully removed from the transwell-insert and cut into 1-2 mm^3 pieces before treatment with 1 mg/ml collagenase A in culture medium for 45 min on rotation in room temperature. The collagenase was then inactivated by PBS-EDTA, and remaining collagen or undigested tissue-pieces were removed by filtering trough a 70 µM cell-strainer. The cell suspension was then washed with medium and number of cells was determined before cells were either treated with lysis buffer for immunoblot analysis or stained for flow cytometry analysis.

VIRUSES

The following viruses were used in these studies;

Hantaan virus, strain 76-118
Puumala virus, strain Kazan-E6 (PUUV-Pa)
Substrains PUUV-Sm
PUUV-La
Puumala virus, strain Umeå/305/human/95
Puumala virus, strain Kazan-wt
Andes virus, strain Chile 9717869
Cowpox virus, strain ATCC VR 302
Tick borne encephalitis virus, strain 93-783
Ljungan virus, strain 145SLG
GFP-expressing Newcastle disease virus

BIOSAFETY

Most hantaviruses are regarded as Biosafety Level-3 (BSL-3) agents when used in cell culture. Consequently the work for this thesis was conducted in a BSL-3 laboratory with the necessary protective gear. All infectious material was inactivated according to biosafety protocols before leaving the BSL-3 facilities.

RESULTS AND DISCUSSION

Below follows a summary of the key findings in each scientific paper included in this thesis. For details on complete results including table and figures, see each publication.

CHANGES OF PUUV DURING *IN VITRO* PROPAGATION (PAPER I)

In this study we isolated two substrains of PUUV Kazan-E6 that showed different phenotypes *in vitro*. We named them PUUV-Small (PUUV-Sm) and PUUV-Large (PUUV-La); PUUV-Sm replicated slower and caused smaller foci, while PUUV-La replicated faster and caused larger foci, than the parental strain (PUUV-Pa) in Vero E6 cells. By analyzing the ratio between viral RNA and viral titres, we found that PUUV-La produced a higher ratio of infectious replication-competent particles, which could explain the different foci size in Vero E6 cells.

When we infected the IFN-α/β competent human fibroblast cell line MRC5 with the substrains and the parental strain, we observed that PUUV-La and PUUV-Sm replicated to similar levels, with PUUV-Sm initially being slightly faster in replication than PUUV-La. PUUV-Pa replication was inhibited in these cells. We then investigated innate immune responses elicited by infection and found that PUUV-Pa induced a much stronger upregulation of IFN-β mRNA than the substrains, and a stronger and faster induction of MxA and ISG56 mRNA. Further, PUUV-Sm induced a slight stronger MxA and ISG56 mRNA response than PUUV-La, but this response did not show any impact on replication, indicating that MxA and ISG56 might not have a major role in controlling hantavirus infection. We also observed that PUUV-Pa infection of the human fibroblasts induced higher level of the pro-inflammatory cytokine IL-6, compared to the two substrains.

The PUUV-Pa stock consists of a mixture of PUUV-La and PUUV-Sm and possibly also of other uncharacterised substrains that have evolved during cell culture propagation in IFN deficient cells. Therefore, we used the sequence previously reported for PUUV Kazan adapted to Vero E6 cells [112-113] when comparing genotypes. From the sequence analysis we observed no differences in the M-segment when comparing the substrains, indicating that the phenotypic features were not due to mechanism solely including the glycoproteins. From the analysis of S- and L-segment, we detected a variety of mutations in the substrains, both compared to each other, and to PUUV-Pa.

Fig 6. Amino acid substitutions observed in PUUV-Sm and PUUV-La.

N protein	**PUUV-Sm**	**D35Y**
	PUUV-La	**D27E**
RdRp	**PUUV-Sm**	**L611F**
	PUUV-La	**P702S**
NSs protein	**PUUV-Sm**	**W21C**
	PUUV-La	**M14R**
		C55Y

The mutations observed in the coding region of the L-segment were in a highly conserved region of the RdRp, but not in any of the proposed functional domains of the protein. Whether these mutations affected the phenotype or not, remains to be investigated. Interestingly, when it comes to the mutations observed in the coding regions of the S-segment, both were on aspartic acid residues in a conserved region of the N protein, the N-terminal coiled-coil domain. It has been reported that the aa residue D35 is important for TULV N protein-interaction and dimerisation [305], however our data indicate that aa residue D27 and aa residue D35 are not required for PUUV replication.

The S-segment of PUUV has an additional ORF for a putative NSs protein, overlapping the N protein ORF. The same mutations that gave rise to the D27E and D35Y substitutions, also gave rise to M14R and W21C substitutions in the NSs protein. For PUUV-La there was an additional substitution in the NSs protein; C55Y. This mutation was silent in the N protein of PUUV-La. Our results indicate that these residues of the NSs protein are not of great importance for the proposed function of the protein in inhibition of IFN-responses [70], as both PUUV-Sm and PUUV-La replicated well in IFN-α/β competent cells.

There were several mutations in the NCR of the S-segment in both PUUV-La and PUUV-Sm. The most striking difference detected was a 43-nucleotide long deletion in the 5'NCR of PUUV-La. When analysing potential secondary structures *in silico* of this missing sequence, we discovered a predicted hairpin loop structure. More intriguing, this structure seems to be conserved among the *arvicolinae*-borne hantaviruses, indicating a possible unknown function of this RNA-structure**.** However, as PUUV-La successfully replicated in both Vero E6 cells and in MRC5 cells, whatever possible function this hairpin loop might have, it is not essential for virus replication *in vitro*.

Taken together, this study characterized two different substrains of PUUV that has evolved during propagation in the IFN-α/β deficient cell line Vero E6. As hantaviruses have an estimated mutation rate of 10^{-2} to 10^{-4} substitutions/site/year [111], mutated variants of the virus will emerge with time. Without the antiviral pressure of IFN-α/β, substrains that would not survive *in vivo* might arise as an artefact of propagation. These substrains might have an impact on the cellular responses that differs from the responses wild-type viruses would induce. Consequently, a possible risk emerging from cell line adaptation and propagation of hantaviruses, is that experiments conducted with these viruses *in vitro* might not fully mirror the *in vivo* situation of human infection with the original wild-type virus. However, a potential use of such isolated substrain is the possibility to pin-point the exact aa residue responsible for certain responses in infected cells.

DEVELOPMENT OF A MODEL SYSTEM FOR STUDIES OF BANK VOLE-BORNE VIRUSES (PAPER II)

Bank voles are important reservoirs for several viruses. In addition to PUUV, they also can harbour viruses like the flavivirus tick-borne encephalitis virus (TBEV) [306] and the orthopoxvirus cowpox virus (CPXV) [307], both pathogenic to humans, and the parechovirus Ljungan virus (LV) [308], with unknown pathogenesis [309]. Infections of bank voles with these viruses are believed to be mainly asymptomatic. Development of an *in vitro* system based on bank vole cells might serve as a good tool for studying differences in cellular responses of voles and men in vole-borne virus-infections, helping us to understand the pathogenesis of these virus-infections in humans.

In this study we isolated bank vole embryonic fibroblasts (VEFs) and showed that these cells were susceptible and permissive for several bank vole-borne viruses, including three strains of PUUV, one being a wild-type variant, never propagated in a cell line. CPXV and LV were cytopathic for VEFs, while neither PUUV nor TBEV showed any cytopathogenicity upon infection.

The α/β/λ-IFNs are the first line of defence against viral infections. As a response to infection, infected cells synthesizes and secretes IFNs to warn surrounding cells of the intruders, and IFNs also act in an autocrine way on the virus-infected cell itself. IFNs induce expression of more than 300 proteins, activating an antiviral state [236]. By stimulating the VEFs with poly(I:C), a synthetic analogue to double-stranded RNA, known to be a key activator of innate immune responses [236], and then use this supernatant in a bio-assay, we could conclude that the VEFs were able to produce and respond with bioactive IFNs.

To assay the levels of IFN-responses upon infection with the different vole-borne viruses, we first set out to sequence parts of the bank vole genome involved in antiviral responses. From these sequences, primers and probe to quantify IFN-β gene expression were designed. A protein that is specifically induced in response to IFN stimulation is the MxA protein in human (Mx2 in mouse), which has antiviral effects against several viruses [236], including hantaviruses [310-312]. Primers and probe were also designed to quantify bank vole Mx2 expression.

Upon infection of VEFs with different bank vole-borne viruses, we observed virus strain-specific expression of Mx2 and IFN-β. TBEV-infection induced strong response of IFN-β and Mx2, while LV-infection only responded with strong IFN-β induction, not Mx2 expression. CXPV-infection only induced a moderate IFN-β response. PUUV-E6 (PUUV-Pa) was a poor inducer of both IFN-β and Mx2 in the VEFs.

The results from infection of the bank vole cells with PUUV-Pa can be compared to infection of the same virus strain in human lung fibroblast. Infection of the human cells induced a strong upregulation of both IFN-β and MxA mRNA [I], which is not the case in the vole cells. Also the replication capability of PUUV-Pa differed between human fibroblasts and bank vole fibroblasts. In the human cells the virus-titres declined, while they increased into a plateau in the bank vole cells. These results indicate a difference between the species in response to hantavirus-infection. It is a possibility that this difference has an impact on the clinical outcome of the infection.

In summary, we showed that VEFs can be used as a tool to study bank vole-borne viruses and that the vole cells responded in a different manner compared to human cells during hantavirus-infection.

HANTAVIRUS-INFECTION OF A 3-DIMENSIONAL ORGANOTYPIC HUMAN LUNG TISSUE MODEL (PAPERS III and IV)

As a first step in a productive hantavirus infection in human, the virus must enter the body. Transmission of hantaviruses occurs mainly through inhalation of aerosolized virus-contaminated rodent excreta, thus the respiratory tract is believed to be the site of entry [29, 154]. In HPS, but also in HFRS, pulmonary involvement is a major part of the disease, i.e. affected lung functions are an important part of the pathogenesis [29, 154, 30-31]. The exact mechanism for how the virus

disseminates in the human body after inhalation, the early events of hantavirus-infection in the lungs, and what causes the transition from asymptomatic infection to severe disease in humans, is currently not known.

The epithelial cells of the lungs form a physical barrier to the outside. In response to pathogenic stimulation, these cells can release cytokines, recruit and activate immune cells [314]. Adjacent fibroblasts actively interact with the epithelial layer, and are involved in inflammatory responses [313]. DCs are constantly patrolling in the lungs, strategically located to detect inhaled potential threats. Immature DCs get activated either by direct recognition of a pathogen or by cytokines produced by epithelial or other cells that have encountered a pathogen. The activated DC then migrates to afferent lymph nodes to activate naïve T lymphocytes [243, 314]. In respiratory virus-infections, DCs play important roles by controlling the type and degree of inflammation, as well as mounting antiviral responses [243, 314].

In papers III and IV we used a 3-dimensional (3D) organotypic model of the human lung tissue to study hantavirus infection of the respiratory tract. This model consists of a two-cell layer-based system with polarized epithelial cells and a fibroblast matrix layer, allowing interactions between the cell types, between the cells and the extracellular matrix, and providing good conditions for growth and differentiation of the epithelial cell layer [315].

In paper III we showed that this 3D-lung model is permissive and susceptible to infection with the HPS-causing ANDV. Models were exposed apically to a high dose of ANDV and supernatants were subsequently analyzed for virus-titres over time. New viruses were produced and released to the basolateral medium-exposed side 48 hours after infection. Only low to moderate levels of progeny viruses were produced initially. Then, after ten to fifteen days, the level of virus detected in the supernatant unexpectedly increased almost tenfold, interestingly coinciding with the median length of ANDV incubation time in patients [316]. Progeny virus production continued at a high level for some days, before a sudden decrease in virus-titre occurred. For the remaining of the experiment (up to 40 days in total) low to moderate levels of virus were continuously produced. This kind of delay in progeny virus production has to our knowledge not previously been reported for hantavirus-infections *in vitro*. Interestingly, ANDV-infection of differentiated hamster tracheal epithelial cells resulted in low titres of virus secreted basolaterally, but at day 11 after infection, the latest time-point analyzed in that experiment, the virus-titres had increased [105], indicating a potential beginning of a peak. In deer mice experimentally infected with SNV, virus-RNA was observed in the lungs, but not in the heart, starting from day ten after infection [317], and in deer mice infected with ANDV a virus-RNA peak was seen in the lungs at day 14 after infection [318]. Taken together, these findings indicate that a sudden rise in virus production approximately 1-2 weeks after infection might be a feature of HPS-causing hantavirus-infection of the lung, regardless if the infection occurs in the natural host or in humans.

Further, we investigated potential causes to the sudden drop in virus production seen after the peak. One plausible explanation is induction of antiviral responses that could inhibit ANDV-replication. Indeed, IFN-β, IFN-λ1, IFN-λ2 and ISG56, were all upregulated in infected models, compared to uninfected models, especially at the peak of progeny virus production. However, we only observed a slight increase of these genes, suggesting that IFNs might not fully explain the sharp decrease in virus titres observed.

Hantaviruses do not induce apoptosis in infected cells [187]. Nevertheless, they might cause cell death in uninfected neighbouring cells [319]. To study if the abrupt decrease in titres were due to ANDV-induced apoptosis, we first measured presence of activated caspase 3 in the models. Neither infected nor uninfected models were positive for active caspase 3 at the time-points around the progeny virus peak, implying that ANDV-infection does not induce apoptosis. Further, we measured the levels of extracellular CK18, a marker for epithelial cell death. The levels of CK18 did not increase at the time of the progeny peak, indicating that elevated ANDV progeny virus production did not cause extensive damage to the epithelial cells. Surprisingly, less CK18 were detected in infected models, compared to in uninfected models, after the peak in virus production, suggesting that ANDV might have a general effect on cell survival.

In line with patient data and data from other *in vitro* studies [119, 123, 262-264, 268, 320-321], we observed elevated levels of the pro-inflammatory cytokines IP-10, IL-6 and IL-8 in supernatant from models late after ANDV-infection. In contrast to earlier reports for ANDV-infection [211], slightly lower levels of the T-cell recruiting chemokine RANTES, were observed in infected models compared to in uninfected models. Contradicting reports on RANTES-responses during hantavirus infection *in vitro* have been published [119-121, 124, 211], suggesting possible cell-type and/or virus-type specific regulations of this chemokine during hantavirus-infection.

In lung dysfunctional disorders (e.g. asthma) as well as in virus-infections of this organ, eosinophils are suggested to play a pivotal role [272]. We therefore set out to investigate if we could detect eotaxin-1, a mainly eosinophil-attracting chemokine, in the supernatant from the lung models. Indeed, from day 15 after infection, until the end of experiment, we observed eotaxin-1 from infected models, but not from uninfected controls. This finding, together with the observation of elevated levels of the eosinophil-promoting cytokine IL-5 in patients [264], makes it tempting to speculate about a possible role for eosinophils in hantavirus pathogenesis. If this potential role would be protective or harmful for the infected individual also remains to be investigated. To date, the only publication regarding eosinophils and hantavirus reported a significantly decrease of eosinophil cationic protein-containing eosinophils in lung biopsies from patients infected with PUUV [256].

In addition, we observed induction of VEGF-A over time in supernatant from infected models, indicating that the elevated VEGF-A levels seen in pulmonary oedema fluid in HPS-patients [297] is not only produced by endothelial cells but can also originate from epithelial cells or fibroblasts.

The 3D-lung model provides a local tissue microenvironment that enables *in vitro* studies of functional properties associated with pathogen-encountering by human DCs [315]. In paper IV we added DCs to the model prior to infection, to investigate if the presence of these cells had any effect on hantavirus-infection or not. In this study we used both the HPS-causing ANDV and the HFRS-causing HTNV.

Hantaviruses are able to infect immature DCs *in vitro* [123, 165-166], and HTNV-infection increases HLA class I and II as well as CD86 expression on DCs [123, 165-166]. We analyzed the DC cell surface receptor expression by flow cytometry and observed a decrease in the overall number of DCs over time, both in uninfected and HTNV-infected models. ANDV-infected models were not analyzed by flow cytometry due to biosafety regulations. The infection with HTNV did not affect DC-activation at day 3 and day 6 after infection, but at day 12 a slight possible activation was observed.

When investigating the cytokine profile in hantavirus-infected DC-containing models, clearly elevated levels of IP-10 in supernatant at day 2 after ANDV-infection was observed. ANDV-infection of models not containing DCs did not induce IP-10 at that time-point, indicating that the presence of DCs might alter the inflammatory milieu during ANDV-infection. We further observed increased levels of IL-8 at day 2 and of IL-6 at day 12 in HTNV-infected DC-models. Higher eotaxin-1 levels in both HTNV-infected and ANDV-infected models compared to uninfected models, were detected at day 12 after infection.

Finally we investigated if the presence of DCs could affect the hantavirus progeny virus production. Indeed, for ANDV the virus-titres were lower until day 10 after infection in DC-models compared to models without DCs. For HTNV, lower titres in DC-containing models were only observed at day 4 after infection. Taken together, in the premises given by this 3D-model, DCs seem to have an antiviral effect against ANDV, but not clearly against HTNV.

In summary, by using an *in vitro* organotypic model of the human lung tissue, we showed that ANDV-infection can cause a late peak in virus-production followed by elevated levels of pro-inflammatory cytokines, eotaxin-1 and VEGF-A and decreased levels of RANTES. We also showed that the presence of DCs in the lung model had an antiviral effect against hantaviruses. This might have implications for better understanding of HPS pathogenesis.

GENERAL CONCLUSIONS

This thesis provides novel knowledge regarding effects of cell line propagation on PUUV, of infection with bank vole-borne viruses in natural host cells and of hantavirus-infection in human lung tissue. Furthermore, the data suggest that the use of new *in vitro* models is beneficial for an increased understanding of hantavirus-infections, both in animals and in the natural host.

In this thesis we

- Characterized the genotypic and phenotypic properties of two substrains of PUUV that evolved during propagation after cell line adaptation [**paper I**].
- Showed that embryonic fibroblasts from bank voles (*Myodes glareolus*) can be used as an *in vitro* model to study bank vole-borne viruses [**paper II**].
- Showed that IFN-β and Mx responses induced by PUUV differ in human cells and in cells from the natural host [**papers I and II**].
- Explored the susceptibility and permissiveness of a 3D *in vitro* model of human lung to ANDV and HTNV infection [**papers III and IV**].
- Showed that infection with ANDV in the 3D-lung model produces a late peak in progeny virus production [**paper III**].
- Showed that pro-inflammatory cytokines, VEGF-A, IP-10 and eotaxin-1 are upregulated at late time-points after ANDV-infection of the 3D-lung model and that expression of RANTES are suppressed over time [**paper III**].
- Showed that DCs have antiviral effects against hantaviruses in a human lung tissue-like environment [**paper IV**].

FUTURE PERSPECTIVES

The mechanism behind hantavirus pathogenesis in humans and why infection in the animal hosts is asymptomatic is not fully understood. To better understand these issues, we need more knowledge about the virus and about what happens during infection.

Substrains of PUUV replicated differently on IFN-α/β deficient cells, but replicated to the same magnitude in IFN-α/β competent cells. Further investigations of what effect the genotypes of the substrains have on the phenotypes could be performed by infecting different cell-types with the substrains. Even though Vero E6 cells are unable to produce type I IFNs, they are capable of producing type III IFNs, and do so upon hantavirus infection [238, 240]. MRC5 cells are IFN competent but lack the receptor for IFN-λ [322], suggesting that IFN-λ could be involved in creating the PUUV-Sm phenotype in Vero E6 cells.

By making reassortments of the two PUUV subtypes, it might be possible to get indications regarding which of the mutated segments that is most important for the observed phenotypes. Quantifying innate responses during infection of MRC5 cells with PUUV-Sm together with PUUV-La might reveal if the difference in MxA, IFN-β and ISG56 mRNA expression observed after infection with PUUV-Pa, depends on interaction between PUUV-Sm and PUUV-La or by other so far uncharacterized substrains in the PUUV-Pa stock. By further investigating the gene expression upon infection with the substrains and PUUV-Pa, it might be possible to identify what gene products that might be important for limiting PUUV-Pa replication in MRC5 cells.

A possible function of the proposed hairpin loop formed by the 43 nucleotides in the NCR of S-segment 5'end of PUUV vRNA remains to be investigated. Conservation of structure is seldom a coincidence, but as PUUV-La could propagate well in human cells without these nucleotides, perhaps this structure is only of importance for the virus when infecting the natural host, or alternatively it might be involved in functions not needed for replication *in vitro*. A possible way to test this hypothesis is by infecting the bank vole embryonic fibroblasts and/or bank voles.

Isolation of hantaviruses is complicated, often requiring blind passaging on IFN-α/β deficient cells before titres high enough for use in experiments is obtained [106]. Further, cell line adaptation and propagation produce mutations that may alter cellular responses against the virus, as well as the infectivity of the virus [I, 112-113, 115-117]. The concept of using cells derived from the natural host for virus isolation has been shown to be successful by Sanada and co-workers that used cells derived from gray red-backed vole, the natural host for Hokkaido virus, to isolate wild-type Hokkaido virus [109]. We showed that infection with wild-type PUUV were possible on the VEFs, perhaps can these cells be used to produce high titres of this virus and cause less mutations than cell lines currently used.

To date, most of the reagents developed for mice were not applicable on the vole cells, making advanced studies of the effects PUUV have on its natural host cells impossible. By deep-sequencing the vole genome, it will be possible to develop more tools for studying altered gene expression upon virus infection. Also, in line with the three Rs (Replacement, Reduction and Refinement) of animal ethics, immortalisation of the vole cells would perhaps provide a tool to study hantavirus-infection of natural hosts with less need for animal experiments.

For detailed studies of the antiviral effect DCs have on hantavirus infection in the 3D-lung model setting, a live imaging approach could be applied, where immune cell movements upon infection could be studied. The DCs in the model are not long-lived, so a possibility to study DC-mediated effect on hantavirus infection over time, could be to apply new dendritic cells to the model later after infection. It would also be of interest to study the effects supernatants from infected models might have on endothelium, as Marsac and co-workers observed that supernatants from DCs infected with ANDV *in vitro* had a permeabilizing effect on endothelial cells [166].

There is a lack of animal models for studying HFRS/HPS, and a lack of functional reagents for those that exist; hence there is a need for more complex models to study hantavirus infections. More complex models can bridge the gap between cellular monolayers, animal experiments and studies of patient material. Traditional 2-dimensional (2D) cell systems have limitations and overlook factors important for tissue physiology; e.g. interplay between different cell types, communication both between cells and between cells and its matrix, as well as mechanical cues [323-324]. The environment in a 2D-culture often provides low cell-to-cell contact and a morphology that isn't fully reflecting the natural spatial organisation of a cell [323-324]. *In vivo*, tissues and organs exist in 3D, and cells *in vivo* are connected to an extracellular matrix. This extracellular matrix provides the cells with growth substances and a physiological environment that supports and promotes key cell functions. 3D-cultures give an environment more mimicking the native tissue, which can create differences in cellular behaviour and characteristics, perhaps leading to more relevant research [323-324]. When it comes to virus infections of 3D-cultures, it has been observed that in a 3D-connective tissue model, human herpes cytomegalovirus-infection had impact on the mechanical properties of the cells [325], and infection with hepatitis E virus of a 3D-culture gave successful virus replication, which was not obtained in the same cells in a 2D-culture [326]. In the future, a greater use of different 3D-models for hantavirus infections, will no doubt deepen the knowledge of the pathogenesis, and by that give clues to potential therapeutics or prophylaxis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vad är ett hantavirus och hur smittar det?
Hantavirus är en grupp av virus som finns över nästan hela världen. De hantavirus som är farliga för människan bärs av små gnagare. Även näbbmöss, mullvadar och fladdermöss kan bära hantavirus, men dessa orsakar vad man vet inte sjukdom hos människa. Symptomen på sjukdom orsakad av hantavirus varierar beroende på vilket typ av hantavirus som man smittats med. I Sverige är skogssork bärare av ett hantavirus som heter Puumalavirus. Sjukdomen Puumalaviruset orsakar kallas ibland för ”sorkfeber” och finns troligtvis bara i norra Sverige.

Puumalaviruset smittar människor genom indirekt kontakt med smittad sork. Denna indirekta kontakt sker oftast genom att en infekterad sork har tagit sig in i hus, lador eller vedbodar och utsöndrar viruset i urin, spillning och saliv. Viruset är smittsamt ungefär två veckor efter det har lämnat sorken. Människor smittas av Puumalaviruset genom att viruspartiklar inandas. Detta sker när den virusinnehållande sorkspillningen virvlar upp som damm, exempelvis när man dammtorkar eller plockar ved. Därför bör de som bor i de områden där sorkfeber är vanligt tänka på att hålla gnagare borta från husen och städa med hjälp av vatten och desinfektionsmedel, istället för att dammsuga. Hantavirus smittar inte mellan människor, men ett hantavirus som finns i Sydamerika, Andesviruset, kan i vissa fall göra detta. Det krävs dock mycket närkontakt för att Andesviruset ska smitta till en annan människa. Vad det är som gör att just detta virus och inte de andra hantavirusen smittar på detta sätt vet man inte riktigt.

Vad orsakar hantaviruset för sjukdom?
När en människa infekterats av ett hantavirus, insjuknar denne två till sex veckor efter smittotillfället med plötslig hög feber, muskel- och huvudvärk. Andra vanliga symptom är synrubbningar, illamående och yrsel. Eftersom hantavirus är så kallade blödarfebervirus uppstår ofta näsblod och hudblödningar. I allvarliga fall utvecklas sjukdomen till att påverka njurar och/eller lungor och ibland hjärtat, vilket kräver sjukhusvård. De som drabbas av svåra symptom beskriver sjukdomen som något av det värsta de har gått igenom, medan andra bara upplever lindriga influensalika symptom. Det finns inget specifikt botemedel eller vaccin mot hantavirus, vilket gör att man endast kan ge understödjande behandling. Sjukdomen pågår i en till två veckor innan tillfrisknandet påbörjas. Återhämtningen sker långsamt och det kan ta månader innan trötthet och smärtsymptom försvinner helt.

Puumalaviruset ger sällan dödlig sjukdom, färre är en på hundra av de insjuknade avlider. Tyvärr är andra hantavirus skadligare. De hantavirus som finns i Nord- och Sydamerika är särskilt farliga, eftersom dessa virus nästan alltid ger mycket svåra hjärt- och lungsymptom, med andnöd, hjärtsvikt och till och med koma som följd. Upp till fyra av tio drabbade dör.

Varför blir människan sjuk av hantaviruset?
Ett virus kan inte föröka sig av sig själv, utan måste använda sig av en cell tillhörande en annan organism för detta. Cellen som infekteras blir påverkad av viruset. Exempelvis gör viruset så att cellen producerar nya virus istället för att utföra sina normala uppgifter. Vissa virus dödar även cellen de har infekterat när de nya virusen lämnar cellen. Därför sätter en cell som infekterats av ett virus igång olika

försvarsmekanismer för att slå tillbaka mot inkräktaren. Cellen börjar också producera olika ämnen som varnar andra intilliggande celler om hotet. Dessa ämnen kan även tillkalla immunceller, kroppens ordningspolis. Beroende på vilka ämnen den attackerade cellen skickar ut så vet immuncellerna på vilket sätt de ska slå tillbaka mot inkräktaren. Virusen har i sin tur utvecklat flera olika mekanismer för att inte bli upptäckta, för att stoppa cellens varningssignaler och för att undkomma cellernas och immuncellernas vapen. Det pågår hela tiden en kapplöpning mellan kroppen och inkräktaren, där viruset vill vara kvar och föröka sig medan organismen som är infekterad vill bli av med viruset. Mycket utav det vi upplever som sjukdom är egentligen orsakat av kroppens försvar mot inkräktaren. Ett exempel på detta är feber.

Det är inte helt klarlagt varför människor blir så sjuka av hantavirusinfektionen. Gnagarna som bär på hantavirusen antas själva inte må dåligt av infektionen. Skillnaden beror troligen på hur immunförsvaret hos gnagare respektive människa reagerar på infektionen. Ett alltför stort försvarspådrag mot en infektion kan tyvärr ibland skada kroppen, vilket till viss del är det som händer under en hantavirusinfektion. Vid hantavirusinfektionen tror forskarna även att viruset påverkar vissa celler att bli mer igenomsläppliga. Då hamnar kroppens vätskor på fel ställen, vilket är det som bland annat leder till de blödningar som uppkommer hos hantavirusinfekterade patienter.

Hur studerar forskare hantavirusinfektionen?
När man studerar hantavirus i laboratorium försöker forskare förstå vilka signaler som skickas ut av den infekterade cellen, vad det är för del av viruset som är ansvarig för detta och hur dessa signaler kan påverka sjukdomssvaret i resten av kroppen.

Vad handlar denna avhandling om?
I det stora hela handlar denna avhandling om vilka delar av Puumalaviruset som kan vara viktiga för att orsaka sjukdom. Den handlar även om sorkar och människor och om hur skillnader i svar mot hantavirus kan studeras genom att använda nya modeller för infektion. I slutändan är målet att en ökad förståelse för hur vi blir sjuka av hantavirus ska leda till utveckling av mediciner som kan bota eller lindra sjukdomen.

I den första delen av denna avhandling har vi undersökt hur varianter av Puumalaviruset beter sig när de infekterar en cell. Det som skiljer dessa virusvarianter åt är enbart några förändringar i deras genetiska kod, vilket leder till att virusens byggstenar, proteinerna, ändras lite. Nu är det så att virusets hela genetiska kod inte översätts till att bli protein, utan kan användas som den är genom att bilda olika strukturer som även dessa kan ha en funktion. I våra varianter av Puumalaviruset fanns även förändringar i dessa delar. Vad vi kom fram till i detta delarbete var att små förändringar i Puumalaviruset leder till att viruset får det svårare eller lättare att överleva i en cell och att cellens försvarsmekanismer blir annorlunda. En ökad förståelse för hantavirusets generella funktion och påverkan på cellen den infekterar är väsentlig för att hitta sätt att bota eller lindra sjukdomen hantaviruset orsakar.

Eftersom hantavirusets värddjur troligtvis inte blir sjuka av viruset, ville vi studera vad som sker inuti en värddjurscell efter att den har infekterats . För att göra detta framställde vi i det andra delarbetet celler från skogssork. Som vi hade anat svarade sorkcellen annorlunda än människocellen efter att den infekterats med Puumalaviruset. Där människocellen satte igång ett starkt försvar mot viruset, reagerade sorkcellen inte särskilt mycket på viruset. Att förstå immunsvaret mot hantaviruset i värddjuret är ett viktigt steg för att förstå vad det är som går fel och orsakar sjukdom i människa.

I det tredje och det fjärde delarbetet, studerade vi vad som händer när en människa smittas av hantavirus. Till vår hjälp hade vi en avancerad modell av mänsklig lunga, som vi odlade upp i laboratoriet. I dessa experiment använde vi oss av det farligare Andesviruset som orsakar sjukdom med mycket lungpåverkan. Det vi upptäckte i dessa studier var att bildandet av nya virus i denna modell var fördröjt. Intressant nog var tiden för denna fördröjning ungefär lika lång som den tid det tar innan en människa blir sjuk av just Andesviruset. Vi såg även att Andesviruset i sig inte skadade modellen, istället verkade de infekterade lungmodellerna överleva bättre än de oinfekterade lungmodellerna som vi jämförde med. Under experimenten mätte vi vilka olika ämnen som lungmodellen producerade som svar på Andesvirusinfektionen. Ett ämne vi såg öka var en signalsubstans som gör celler mer genomsläppliga. Vi upptäckte även att lungmodellen efter Andesvirusinfektionen gav ifrån sig ett ämne som tillkallar en speciell typ av immunceller, så kallade eosinofiler. Ingen har studerat hur just denna typ av immuncell är involverad i sjukdom orsakad av hantavirus. Förhoppningsvis leder våra resultat till att eosinofilers roll vid hantavirusinfektion kommer att studeras närmare.

En immuncell som är viktig för att styra vilket försvar som skapas mot en inkräktare är de dendritiska cellerna. Dendritiska celler finns överallt i kroppen, men speciellt i de delar som ofta utsätts för intrång av bakterier och virus, till exempel lungorna. De dendritiska cellerna övervakar omgivningarna och tar stickprov då och då för att se om de hittar någonting farligt. Stöter de på ett virus alarmerar de andra immunceller och ser till att de andra immuncellerna tar med sig rätt vapen för striden. Hur de dendritiska cellerna beter sig under en hantavirusinfektion är inte känt. För att ta reda på detta lade vi i det fjärde och sista delarbetet till dendritiska celler till lungmodellen. Det vi kunde se när vi infekterade dessa lungmodeller med hantavirus var att de dendritiska cellerna hämmade virustillväxten. Vi såg också att modellerna med dendritiska celler satte igång ett tidigt försvar mot hantaviruset. Sammantaget kunde vi visa att dendritiska celler, när de befann sig i lungmodellen, hade så kallad antiviral verkan.

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" I only want to live in peace and plant potatoes and dream!"

-Tove Jansson, Moomin Vol 1