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Hantaviruses – from interferons to development of an *in vitro* model

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To my family and friends up north

ABSTRACT

Hantaviruses can cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), two severe diseases that often are associated with a deadly outcome as there is no cure. The natural hosts of hantaviruses are rodents and insectivores, which are believed to harbor the virus asymptomatically. The pathogenesis in humans is unclear, but increased vascular leakage and affected endothelial cells, possibly due to rigorous and unhampered immune responses, are hallmarks of HFRS and HCPS. Interferons (IFNs) are part of the innate immunity and mediate production of antiviral molecules but can also modulate the adaptive immune responses against infections. In an attempt to increase the knowledge about pathogenesis in humans, we aimed to understand the interactions between hantaviruses and the IFN-response and provide tools to investigate the potential differences in innate immune regulation between human cells and cells from a natural hantavirus host.

Our results in paper I indicate that hantaviruses can interfere with induction of innate immune responses in patients and inhibit the antiviral effect of all types of IFNs. We observed that serum levels of IFN-α and -β were unaltered in Puumala virus (PUUV)-infected patients while the level of the more recently discovered IFN-λ was decreased during the acute phase of the disease. IFN-λ was shown to inhibit replication of the prototype hantavirus Hantaan (HTNV) but to a lesser extent than IFN-α, -β or -γ. The function of STAT1, a protein that is crucial for IFN signalling, was inhibited in HTNV infected cells.

In paper II, we observed that a hantavirus infection can induce IFN-λ specifically, without inducing IFN-α or -β. This has never been described before for any virus, and has taught us more about the complexity of IFN induction.

In paper III, we described both mutations and substantial phenotypical differences in two PUUV isolates compared to each other and to their parental strain. These results imply that we might study spontaneous mutations rather than true differences between hantaviruses when we use cell culture propagated viruses.

Due to lack of host-specific tools, not much is known about the responses to infection in natural hantavirus hosts. In paper IV, we describe the development of an *in vitro* model (vole embryonic fibroblasts, VEFs) for studies of bank vole borne viruses and induction of innate antiviral reactions in response to infection. This model will be a valuable tool for future studies of how PUUV and other zoonotic viruses harbored by bank voles affect cells from their natural host compared to human cells. Also, wild type PUUV was shown to infect VEFs, indicating that cells from natural hosts might be a way of isolation and propagation of hantaviruses.

In conclusion, the results included in this thesis contribute to an increased knowledge about hantaviruses and their interactions with human and natural host cells. This knowledge, combined with future studies will hopefully lead to a better understanding of hantavirus pathogenesis and ultimately result in a cure.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA (SUMMARY IN SWEDISH)

Virus kan orsaka en mängd sjukdomar. De är beroende av värdceller för att föröka sig, använder byggstenar från sin värd för att bygga upp nya viruspartiklar och har en anmärkningsvärd förmåga att snabbt anpassa sig till nya förutsättningar. Därför är det svårt att utveckla läkemedel mot virus.

Hantavirus

Denna avhandling handlar om en grupp virus som kallas hantavirus. Dessa virus finns i de flesta delar av världen och sprids av infekterade gnagare (t ex råttor och sorkar) via deras avföring, urin eller saliv. Människor kan bli smittade genom inandning av förorenat damm, t ex vid städning av sommarstugan eller när man hugger ved. Globalt hamnar upp till 200 000 personer på sjukhus varje år pga hantavirus och än så länge finns det inget effektivt vaccin eller botemedel.

Sveriges variant av hantavirus finns i norra Sverige samt i stora delar av övriga norra Europa och kallas för Puumala efter den by i Finland där viruset isolerades första gången. Puumala orsakar sorkfeber och sprids av sorkar. I övriga världen finns det nära besläktade hantavirus som kan orsaka mycket allvarliga infektioner med Ebolaliknande blödarfeber som följd. De allvarligaste hantavirusinfektionerna sker i Nord- och Sydamerika med upp till 40% dödlighet. I jämförelse med dessa infektioner är vår inhemska sorkfeber relativt mild med cirka 0,4% dödlighet även om man kan bli mycket sjuk. De vanligaste symptomen vid sorkfeber är hög feber, muskelvärk, allmän sjukdomskänsla och ofta svåra buk- och ryggsmärtor. Njurarna påverkas och nedsatt njurfunktion kan ses under en till två veckor. Ibland kan även blödningar i huden och synrubbningar uppträda. Efter tillfrisknandet kan man känna sig trött i flera månader, men blir oftast helt återställd inom ett år. Troligtvis får man en livslång immunitet.

Varför blir vi sjuka?

Troligtvis är det immunförsvarets reaktioner mot infektionen och inte viruset i sig som orsakar sjukdom. De specifika reaktioner som skulle kunna vara involverade i detta är än så länge i stort sett okända. Vi vet att blodkärlen börjar läcka med risk för blödningar som följd. Vad som är orsaken till detta är oklart, men förmodligen formar tidiga ospecifika reaktioner mot viruset de senare och mer specifika reaktionerna på ett sådant sätt att väggarna i blodkärlen påverkas.

Gnagarna bär på hantavirus utan att bli sjuka, men orsakerna till detta har varit svåra att kartlägga pga att det inte har funnits några specifika verktyg för att studera immunförsvaret i dessa värddjur.

Syftet med avhandlingen

I denna avhandling har vi ställt oss frågan: varför blir människor men inte gnagare sjuka av hantavirus? Specifikt ville vi studera de tidigaste svaren mot hantavirus i infekterade celler från människa och jämföra dessa svar med vad som sker i celler från den naturliga värden. Skulle vi få svar på vilka immunförsvarsreaktioner som sker i

människor men inte i gnagare är vi ett steg närmare en behandlingsmetod som skulle kunna hämma de skadliga reaktionerna som sker mot hantavirus i människor!

Interferoner

En av de tidigaste ospecifika reaktionerna mot virusinfektioner är interferoner. Dessa har antivirala egenskaper som både kan hämma virusets tillväxt men också forma de specifika immunförsvarsreaktionerna som brukar utvecklas i kroppen något senare. Hantavirus och även många andra virus har tidigare både visat sig hämma produktionen av interferoner men också deras antivirala effekter.

För att öka kunskapen om hantavirus och deras interaktioner med det tidiga ospecifika immunförsvaret försökte vi svara på nedanstående frågor:

Hur ser produktionen av interferoner ut i patienter och kan hantavirus hämma interferonernas effekt?

I artikel I visade vi att produktionen av interferoner är hämmad i sorkfeberpatienter och att en relativt nyupptäckt typ av interferon, interferon lambda, kan hämma tillväxt av hantavirus i cellkultur. Dock syns effekten endast om interferonet tillsätts innan infektion eftersom redan infekterade celler verkar vara helt motståndskraftiga mot interferonets effekt. Vi tror att avsaknaden av interferon i hantavirusinfekterade patienter kan vara förknippat med det kraftiga immunsvar som uppkommer i samband med en hantavirusinfektion.

Hur ser interferonproduktionen ut över tid i hantavirusinfekterade celler?

I artikel II redogjorde vi för upptäckten att produktionen av interferon lambda kan regleras annorlunda än övriga interferoner, vilket aldrig har visats tidigare. Vi visade vidare att de celler vi använder för att tillverka högkoncentrerade virus som vi sen använder i vår forskning, kan producera interferon lambda. Detta är en viktig upptäckt som man bör ha i åtanke vid framtida studier med virusstockar odlade i dessa celler så att man inte råkar studera effekten av interferon lambda i stället för effekten av virusinfektionen.

Finns det risk att dra felaktiga slutsatser om man studerar virus som är anpassade till en cellkultur?

Virusstockar som används i vetenskapliga experiment är egentligen en blandning av en mängd olika varianter av samma virus som är mycket nära besläktade. Detta beror på att virus förändrar sina egenskaper väldigt fort. Man kan anta att de virusstockar som vi använder oss av har förändrat sig bort från hur de beter sig i den naturliga värden till någonting annat som växer bra i cellkulturer på laboratoriet.

I artikel III kartlade vi egenskaperna hos två varianter av sorkfeber som vi hittade i en och samma virusstock. Vi upptäckte flera skillnader mellan dessa två men också jämfört med originalstocken. Tillväxthastigheten men även storleken på interferon-svaret i infekterade celler skiljde sig en hel del mellan de olika varianterna. Dessa resultat visar att risken med användandet av de virusstockar vi har på laboratoriet idag är att man studerar effekten av spontana förändringar hos viruset i stället för det man skulle sett om man använde ursprungsviruset. Problemet är dock att virus som kommer

direkt från värden eller patienter inte växer bra i cellkultur, vilket betyder att för studier på laboratoriet är man tvingad att använda virus anpassade till cellkultur.

Hur ser interferonsvaret mot virusinfektioner ut i celler isolerade från sorkar?
Hittills har det varit enormt svårt att studera sorkfeber och andra gnagarburna virus i den naturliga värden pga brist på artspecifika verktyg. Därför har man inte kunnat jämföra immunsvaren mot hantavirus i människa med vad som sker i gnagare.

I artikel IV beskriver vi utvecklandet av en metod för att studera sorkfeber och andra sorkburna virus såsom fästingburen encefalit (TBE), kokoppor och Ljunganvirus, i en cellkultur som vi isolerat från sorkembryon. Vidare har vi utvecklat flera artspecifika verktyg för att göra grundläggande studier av tidiga ospecifika immunförsvars-reaktioner i dessa celler. Våra resultat från Puumalainfekterade sorkceller tyder på att interferonsvaren i sorkar skiljer sig markant från hur det ser ut i människor. Framtida studier kommer visa mer i detalj vilka skillnaderna är och hur regleringen av interferonsvaret skiljer sig åt mellan arterna. Man kan dessutom utveckla liknande metoder för andra gnagararter som bär på andra mer dödliga hantavirus, och det vore intressant att studera skillnaderna mellan dessa och sorkar. Vad är det som gör att Puumala är mindre dödlig än andra nära besläktade hantavirus? Svaret på den frågan skulle kunna hjälpa oss att utveckla mer effektiva behandlingsmetoder än de vi har idag.

En annan viktig upptäckt var att virus som endast odlats i sorkar tidigare, och inte i cellkultur, växte bra i vår sorkcellinje. Detta bådar gott för framtida isoleringar av hantavirus i dessa celler från den naturliga värden, vilket förhoppningsvis kommer att generera virusstockar som mera liknar det virus som infekterar sorkar och patienter. Användandet av sådana virusstockar vid experiment på laboratoriet kan ge resultat som mer liknar vad som sker vid naturliga infektioner jämfört med de virus som är anpassade till cellkultur som vi använder idag.

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
ANDV	Andes virus
BSL	Biosafety level
cDC	Conventional DC
CTL	Cytotoxic T cell
CPXV	Cow pox virus
CRP	C-reactive protein
DC	Dendritic cell
DOBV	Dobrava virus
ds	Double-stranded
ER	Endoplasmic reticulum
G	Guanosine
HCPS	Hantavirus cardiopulmonary syndrome
HFRS	Hemorrhagic fever with renal syndrome
hpi	Hours post infection
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPS	IFN-β promoter stimulator protein
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
ISGF	IFN-stimulated gene factor
Jak	Janus kinases
KHAV	Khabarovsk virus
L	Large RNA segment
LV	Ljungan virus
M	Medium RNA segment
MDA	Melanoma differentiation associated gene
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MyD	Myeloid differentiation factor
N	Nucleocapsid protein
NE	Nephropathia epidemica
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
OAS	2’5’-oligoadenylate synthetase
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid DC
PHV	Prospect Hill virus
PRR	Pattern recognition receptor
PUUV	Puumala virus
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible protein-I

RSV	Respiratory syncytial virus
S	Small RNA segment
SEOV	Seoul virus
SNV	Sin Nombre virus
ss	Single-stranded
STAT	Signal transducer and activation of transcription
TBEV	Tick-borne encephalitis virus
TBK	TANK-binding kinase
Th	T helper
TLR	Toll-like receptor
TOPV	Topografov virus
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
TRIF	Toll/IL-1-resistance domain-containing adaptor inducing IFN-β
TULV	Tula virus
VE	Vascular endothelial
VEF	Vole embryonic fibroblast
VEGF	VE growth factor
vRNA	Viral RNA
wt	Wild type

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which in the text will be referred to by their roman numerals:

I. **Stoltz, M.**, Ahlm, C., Lundkvist, Å., Klingström, J. 2007. Lambda interferon (IFN-lambda) in serum is decreased in hantavirus-infected patients, and in vitro-established infection is insensitive to treatment with all IFNs and inhibits IFN-gamma-induced nitric oxide production. *J Virol*. 81(16):8685-91.

II. **Stoltz, M.**, Klingström, J. 2010. Alpha/beta interferon (IFN-alpha/beta)-independent induction of IFN-lambda1 (interleukin-29) in response to Hantaan virus infection. *J Virol*. 84(18):9140-8.

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

IV. **Stoltz, M.**, Sundström, K. B., Hidmark, Å., Tolf, C., Vene, S., Ahlm, C., Lindberg, A. M., Lundkvist, Å., Klingström, J. 2011. A model system for in vitro studies of bank vole borne viruses. *PLoS ONE, in press*.

CONTENTS

1 INTRODUCTION

Hantaviruses are rodent and insectivore borne viruses that have been identified as etiological agents of two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulomonary syndrome (HCPS). Globally, there are approximately 200 000 reported hantavirus cases annually, with a mortality rate of up to 40% depending on type of infecting hantavirus. The mechanisms causing the disease are not well known, and there is no cure or safe and reliable vaccine available.

1.1 HISTORY OF HANTAVIRUSES

Clinical syndromes that resemble HFRS have been described several times over the years, even as far back as the 10th century in China [1], and during the World War I [2]. In Sweden a mild form of HFRS was first described as early as 1934 [3, 4], a disease that later was designated nephropathia epidemia (NE) [5]. It was not until an outbreak in the early 1950s during the Korean War that HFRS was brought to the attention of the western world. Thousands of United Nations soldiers got ill with fever, renal failure and shock, which led to a long search for the disease-causing agent. Finally, the Hantaan virus (HTNV) was isolated from a striped field mouse (*Apodemus agrarius*) captured near the Hantaan river in 1978 [6], and was later also successfully propagated in A549 [7] and Vero E6 cells [8]. A few years after the isolation of HTNV, another hantavirus called Seoul virus (SEOV) was isolated from rats (*Rattus norvegicus*) in Korea [9]. The first hantavirus to be discovered in the Americas was the non-pathogenic Prospect Hill virus (PHV), which was isolated from meadow voles (*Microtus pennsylvaticus*) in the United States [10, 11]. In Europe, the etiological agent of NE was first detected with an immunofluorescence assay on tissue samples from bank voles (*Myodes glareolus*) [12]. Later, the virus was isolated from these bank voles, that were captured near the village Puumala in Finland, and subsequently named Puumala virus (PUUV) [13]. A decade later, Dobrava virus (DOBV) was isolated from a yellow-necked field mouse (*Apodemus flavicollos*) in former Yugoslavia [14], and shown to be associated with HFRS [15, 16].

In 1993, the first cases of HCPS were described in the southwestern United States [17], and soon thereafter the disease-causing Sin Nombre virus (SNV) was isolated from a deer mouse (*Peromyscus maniculatus*) and propagated in Vero E6 cells [18]. A few years later another hantavirus named Andes virus (ANDV) caused a large number of HCPS cases in South America [19].

Until quite recently, the only confirmed hantavirus with another natural host than a rodent was Thottapalyam virus, isolated from the insectivore Musk shrew (*Suncus murinus*) in India [20]. However, several other hantaviruses have now been associated with insectivore hosts [21-23].

Since the first initial discoveries the number of known hantaviruses has increased rapidly. Today, there are 23 officially recognised species (as defined by the International Committee on Taxonomy of Viruses) (Table 1).

Old World Hantaviruses

Virus	Disease	Reservoir	Distribution
Dobrava-Belgrade virus (DOBV)	HFRS	*Apodemus flavicollis*	Balkans
Hantaan (HTNV)	HFRS	*Apodemus agrarius*	China, Korea, Russia
Khabarovsk virus (KBAV)	Unknown	*Microtus fortis*	Russia
Puumala virus (PUUV)	HFRS	*Myodes glareolus*	Europe, Russia, Scandinavia
Saaremaa virus (SAAV)	HFRS	*Apodemus agrarius*	Europe
Seoul virus (SEOV)	HFRS	*Rattusnorvegicus, Rattus rattus*	Worldwide
Thailand virus (THAIV)	Unknown	*Bandicota indica*	Thailand
Tula virus (TULV)	Unknown	*Microtus arvalis*	Europe
Thottapalayam virus (TPMV)	Unknown	*Suncus murinus*	India
Topografov virus (TOPV)	Unknown	*Lemmus sibericus*	Siberia

New World Hantaviruses

Virus	Disease	Reservoir	Distribution
Andes virus (ANDV)	HCPS	*Oligoryzomys longicaudatus*	Argentina, Chile
Bayou virus (BAYV)	HCPS	*Oryzomys palustris*	USA
Black Creek Canal virus (BCCV)	HCPS	*Sigmodon hispidus*	USA
Cano Delgadito virus (CADV)	Unknown	*Sigmodon alstoni*	Venezuela
El Moro Canyon virus (ELMCV)	Unknown	*Reithrodontomys megalotis*	USA, Mexico
Isla Vista virus (ISLAV)	Unknown	*Microtus californicus*	USA
Laguna Negra virus (LANV)	HCPS	*Calomys laucha*	Argentina, Bolivia, Paraguay
Muleshoe virus (MULV)	Unknown	*Sigmodon hispidus*	USA
New York virus (NYV)	HCPS	*Peromyscus leucopus*	USA
Prospect Hill virus (PHV)	Unknown	*Microtus pennsylvanicus*	USA, Canada
Rio Mamore virus (RIOMV)	Unknown	*Oligoryzomys microtis*	Bolivia, Peru
Rio Segundo virus (RIOSV)	Unknown	*Reithrodontomys mexicanus*	Costa Rica
Sin Nombre virus (SNV)	HCPS	*Peromyscus maniculatis*	USA, Canada, Mexico

Table adapted from Schmaljohn and Hjelle 1997 and Jonsson *et al.* 2010 [24, 25].

Table 1. Hantaviruses according to the International Committee on Taxonomy of Viruses.

1.2 CLINICAL SYNDROMES CAUSED BY HANTAVIRUS INFECTIONS

Hantaviruses are found almost all over the World and are often referred to as Old World and New World hantaviruses due to their geographic distribution and the type of illness (HFRS or HCPS, respectively) they cause. The Old World HFRS-causing hantaviruses can be found in Eurasia and includes DOBV, HTNV, PUUV and SEOV while the New World HCPS-causing hantaviruses are found in the Americas with ANDV and SNV as important examples. Mortality rates vary with type of hantavirus. HCPS-causing hantaviruses are the most deadly with death rates up to 40%, while the HFRS-causing hantaviruses have mortality rates that vary between 0.4% for PUUV [26], which causes a milder form of HFRS, and up to 15% for HTNV [24]. Why some

hantaviruses cause more severe pathogenesis than others is not known. Generally, HFRS is characterised by hemorrhage and renal failure while HCPS is associated with pathology of the lung. However, there are reports of NE-patients with pulmonary dysfunction and involvement of the lung [27-29] and HCPS-cases with kidney involvement [30] or renal sequelae [31]. For most hantaviruses, it appears that the infection can be asymptomatic or cause a mild disease not possible to diagnose correctly. One feature that implies subclinical infections is that the seroprevalence can be quite high, e. g. up to 8.9% for PUUV in northern Sweden [32].

1.2.1 Transmission to humans and other hosts

Humans are infected mainly by inhalation of infected rodent excreta [33] and therefore disease caused by hantaviruses occurs within the geographically range of the natural host. Activities involving for instance farming [34, 35], forestry [36], handling of rodents [33], handling of firewood or cleaning [37] can increase the risk of being exposed. Humans are usually described as dead-end hosts, with ANDV as the exception being the only hantavirus that has been reported to be transmitted from person-to-person [38-40]. For PUUV, it has also been reported that transmission can occur via transfusion of platelets [41].

There have been human hantavirus infections associated with infected laboratory animal colonies [33, 42, 43]. The likely cause of infection here could be inhalation of contaminated air in the animal facility or wounding during animal experiments. Therefore, rodents infected with HFRS-causing hantaviruses should be handled under at least biosafety level 3 (BSL-3) conditions, and animals infected with HCPS-causing viruses must be maintained under BSL-4 conditions. Although there are no reports of illness associated with cultivating hantaviruses in cell cultures, most hantaviruses are designated as BSL-3 agents when propagated in cell culture [44].

In addition to humans, transmission of hantaviruses from rodents to domestic and wild animals seem to be possible. Antibodies directed against hantaviruses have been found in several animals including cats, dogs [45], monkeys living in outdoor enclosures [46], hares, deer, cows [47] and moose [48]. Interestingly, hantavirus antigens have been found in the lungs of birds [49] and bats [50].

1.2.2 HFRS

The up to 200 000 cases of HFRS described each year occur in Europe and Asia, especially in China [24, 51]. The onset of disease is characterised by non-specific flu-like symptoms followed by thrombocytopenia, capillary leakage with hemoconcentration, and in severe cases renal failure and shock [52]. The infection starts in the lungs after inhalation of infected aerosols with an incubation period ranging from 2-3 weeks for HTNV [53] and up to 42 days for PUUV [54]. HFRS is generally divided into five different phases: febrile, hypotensive, oliguric, polyuric and convalescent. However, all of these five stages are not always present or easy to distinguish, especially for the milder cases, and there are huge differences in manifestation and severity of symptoms between different individuals and between different types of hantaviruses. A summary of some of the clinical manifestations that can be observed during the different phases of HFRS is presented below (as reviewed in [24, 52, 55, 56]):

- The febrile phase starts with an abrupt onset of high fever and chills, followed by headache, back and abdominal pain, nausea and thirst. Flushing of the face and tissue edema are often observed for HTNV, but rarely for PUUV.
- At the end of the febrile phase, the hypotensive phase might develop which includes low blood pressure (hypotension) induced by vascular leakage and hemorrhage. Nausea and vomiting are common and this phase sometimes includes severe or fatal shock.
- The oliguric phase is caused by a transient impairment of renal function with decreased urine output that contains protein and red blood cells. High levels of nitrogen-containing compounds, such as urea and creatinine, can be found in the blood. In severe cases, haemodialysis might be required. Central nervous system involvements sometimes occur with symptoms such as restlessness, confusion and tremor. Convulsion may also develop. This phase is also characterised by continued bleedings and thrombocytopenia. Severe, life-threatening internal bleedings can occur, especially for HTNV patients. In NE patients, less severe bleedings are observed.
- As the renal function returns to normal the polyuric phase with increased urine output starts. Patients that survive until this phase will most often recover from the disease, but shock may still occur.
- The convalescent phase can last for several months before the patient is fully recovered.

1.2.3 HCPS

HCPS occurs in the Americas and is characterised by a rapid onset of respiratory failure and cardiogenic shock. Since HCPS was first described, over 500 cases have been reported in the United States with an overall mortality rate of 36% [57]. In fatal cases, a widespread presence of viral antigen has been detected in endothelial cells of the vasculature (especially in the lung), but also in dendritic cells (DCs), macrophages and lymphocytes [58]. HCPS resemble HFRS in some ways, including fever, thrombocytopenia and capillary leakage, but is characterised by severe cardiopulmonary dysfunction instead of renal failure. After an incubation period of 9 to 33 days [59], the first symptoms can be fever, muscle pain, headache, abdominal pain and nausea that last for 3-5 days. After that, the cardiopulmonary involvement begins with coughing, rapid breathing ("shortness of breath"), increased heart rate and low blood pressure followed by respiratory distress and pulmonary edema (fluid in the lungs). Patients may often need mechanical ventilation to survive, but despite hospitalisation in a well-run intensive care unit 30-40% of the patients die [25, 60]. The convalescent phase is 3-4 weeks but symptoms such as shortness of breath may persist for 1-2 years after acute infection [61].

1.3 CLASSIFICATION

After the recognition of HTNV it was included in the family *Bunyaviridae* based on the characteristics that are shared among all other members of this family, e. g.:

- A morphological investigation with electron microscopy revealed a spherical shape with a diameter of approximately 95 nm, and an envelope [8, 62].
- A single-stranded (ss) negative-sense RNA genome [63, 64].

- Three separate viral nucleocapsids, each composed of nucleocapsid protein (N) and a single RNA [63].
- Two virus-specific glycoproteins associated with the virion membrane [65].
- Replication of the virus in cytoplasm and budding from the endoplasmic reticulum (ER) [66].

Because of the absence of serological relation with any of the other viruses within the four genera of *Bunyaviridae*, a different mode of transmission and a unique terminal genome sequence [63, 67] it was proposed that HTNV should be placed in a new genus within this family. The name hantavirus was suggested, a name that later on got generally accepted.

1.4 STRUCTURE

The hantavirus particle is spherical and generally 80-120 nm in diameter [68]. The virus has an envelope, formed by membrane lipids derived from the host cell, with inserted heterodimers of the glycoproteins Gn and Gc. Inside the envelope, there are three separate nucleocapsids consisting of N and viral (v)RNA. The nucleocapsids are associated with the RNA-dependent RNA polymerase (RdRp). As for the other members of *Bunyaviridae*, each genomic RNA forms a circular structure by base pairing of the highly conserved and complementary 3´- and 5´-terminal nucleotides [69] (Figure 1).

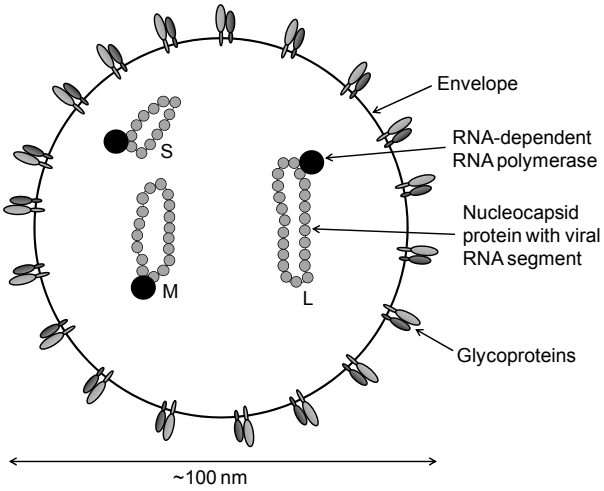

Figure 1. The hantavirus particle.

The genome is divided into three negative-sense RNA segments [63]. The S (small), M (medium) and L (large) segments encodes the N, the glycoproteins and the RdRp, respectively [70]. At least PUUV and TULV have an additional open reading frame in the S segment that encodes a possible nonstructural protein [71].

1.5 REPLICATION

The replication cycle of hantaviruses is rather slow, *in vitro* it can take more than 24 hours from infection to presence of secreted infectious virus [72]. Replication occurs in the cytoplasm of infected cells, involving the ER/Golgi system [73]. *In vitro*, hantaviruses can infect many cell types including endothelial cells, monocytes/macrophages [74, 75] and DCs [76].

1.5.1 Attachment and entry

Attachment to host cells occurs via interaction of the glycoproteins to β1 intergrin for non-pathogenic, and to β3 integrin for pathogenic hantaviruses [77-79], with decay-accelerating factor/CD55 as a critical cofactor for infection at least for HTNV and PUUV [80]. HTNV enter the cell by endocytosis via clathrin-coated vesicles and subsequent transfer to early endosomes that acidifies into lyzosomes [81]. It is believed that the low pH mediates fusion of the lyzosomal and viral membrane [81]. In this process, the three nucleocapsids are uncoated and delivered to the cytoplasm, where the viral RdRp synthesises mRNA from the S, M and L segments.

1.5.2 Transcription, replication of genome and translation

The initiation of transcription is a process where N is believed to be involved [82, 83] and the primers that are needed are cleaved from the 5' ends of host cell cytoplasmic mRNAs in a process called cap-snatching [84, 85]. During cap-snatching, there is a strong preference for the viral RdRp (that can function as an endonuclease) to cut the host mRNA after a guanosine (G). Initiation of both transcription and replication has been suggested to occur according to a prime-and-realign model [85] where the G at the 3' end of the primer aligns with the third nucleotide of the template RNA. The polymerase elongates the primer a few nucleotides, but before further elongation the produced molecule slips back (realigns) on the template such that the original 3' G of the primer ends up at position -1. Thereby, an exact copy of the 5' end of the template RNA is created [85]. Like for other RNA viruses the main source of genetic variation is genetic drift due to a polymerase without proofreading [86].

During replication, the vRNA is used as a template for synthesis of antigenomic RNA, which in turn acts as template for synthesis of new vRNA. The newly synthesised vRNA is either used as an additional template for production of more mRNA (secondary transcription) or assembly [73]. The initiation of translation is operated by the hantaviral N [87]. The S and L mRNAs are translated into N and RdRp proteins, respectively, on free ribosomes. The glycoproteins are translated from M mRNA as a glycoprotein precursor on membrane bound ribosomes, followed by a cleavage into Gn and Gc at the conserved WAASA-motif [88], and glycosylation [89, 90]. For HTNV it has been shown that the glycoproteins then dimerise in ER followed by transportation to the Golgi complex [91, 92].

1.5.3 Assembly and exit

Encapsidation and formation of nucleocapsids are suggested to be initiated after interaction between trimeric N and the panhandle structures on vRNA [93]. The panhandle structures are unique and allow N to discriminate between viral and non-

viral RNA molecules. After that, the nucleocapsids are assembled with Gn and Gc, likely via an interaction between N and the cytoplasmic tails of the glycoproteins [94, 95], but also RNA [83, 96].

The envelope for viruses within the *Bunyaviridae* is derived when budding from the Golgi, and the infectious virus particles are released by exocytosis [73]. An alternative pathway for exit by budding at the plasma membrane has been suggested for SNV and Black Creek Canal virus [97, 98].

1.6 INNATE IMMUNE RESPONSES TO VIRAL INFECTIONS

Immune responses to viral infections are shaped by early innate events. The recognition of viruses is mediated by interactions between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs). PAMPs are broadly shared among pathogens but distinguishable from self molecules. The viral PAMPs have primarily been described as viral genomes or replication intermediates, but there are examples of other parts of the virus, such as glycoproteins, that can be recognised by PRRs [99].

The innate immunity comprises non-specific barriers such as skin, mucous membranes, phagocytes, natural killer (NK) cells, inflammation and chemical mediators e. g. interferons (IFNs) and complement.

1.6.1 Cells and inflammation

The innate immunity and the phagocytic cells are crucial for development of the adaptive immunity and conversely, soluble factors produced by the specific immune response improve the function of innate immunity. Phagocytic cells such as blood monocytes, DCs, neutrophils, and tissue macrophages, phagocytose and break down foreign material. DCs and macrophages are activators of the adaptive immune system through antigen presentation where ingested antigens are presented to T cells [100].

NK cells do not attack pathogens directly but act on infected cells that in response to the infection express more activating than inhibiting ligands. NK cells are also activated by IFN-α, IFN-β, tumor necrosis factor (TNF)-α, interleukin (IL)-12 and IL-15. The killing of cells occurs in a similar manner as for cytotoxic T cells (CTLs). Granules containing perforin and granzymes can be found in the cytoplasm of NK cells. Degranulation occurs after adhesion to a target cell and perforin and granzymes are released in the immunological synapse formed between the two cells, with granzyme B inducing subsequent apoptosis of the target cell. In addition, NK cells can influence the adaptive immune response directly by secreting IFN-γ [101].

The inflammatory response is triggered by wounding or an invading pathogen. Within minutes the nearby blood vessels increase their diameter and the blood flow is decreased. This is a process called vasodilation and causes redness of the inflamed area and a local increase in tissue temperature. The capillaries also increase their permeability which results in transfer of fluid and cells into the inflamed tissue, which can induce tissue swelling (edema). Neutrophils are generally the first cell type to move from the blood to the damaged tissue. Other phagocytes, such as macrophages, also

accumulate in the inflamed area where they release lytic enzymes that kill microorganisms but also cause damage to nearby healthy cells. Another important player during inflammation is histamine, which is released by a variety of cells (e. g. mast cells) in response to tissue damage. The role of histamine is to interact with the surface of nearby blood capillaries, resulting in vasodilation and increased permeability [100]. Neutrophils and macrophages can also produce other anti-microbial substances such as reactive oxygen or nitrogen intermediates (e. g. nitric oxide (NO)) which have an important role in killing pathogens but also contribute to the tissue damage that can result from an inflammatory response [102].

Cytokines work at picomolar concentrations by binding to receptors on target cells. The production is carefully regulated, and the effects are often local, but can also be systemic. Cytokines are produced by a variety of cells and can also act on numerous types of cells. For example, IL-1 (IL-1α and IL-1β) is produced by phagocytes, endothelial cells, fibroblasts, B and T cells and stimulates T cell proliferation and histamine release from mast cells. IL-6 can be produced by a variety of cells, but the main sources are monocytes, macrophages, T cells, fibroblasts and endothelial cells. IL-6 promotes production of neutrophils in the bone marrow, T cell activation and differentiation, growth and differentiation of B cells and stimulates antibody production. TNF-α is often macrophage-derived, but may also be produced by fibroblasts, neutrophils, mast cells, T or NK cells, and is involved in increasing the vascular permeability. IL-1, IL-6 and TNF-α all affects the thermoregulatory centre in the brain, which results in fever. Both IL-1 and TNF-α induce synthesis of each other and IL-6 whereas IL-6 inhibit the synthesis of IL-1 and TNF-α in a negative feedback loop [100, 103, 104].

In response to production of IL-1, IL-6 and TNF-α, there is a release of chemical mediators such as acute-phase proteins from cells in the liver. C-reactive protein (CRP) is a major acute-phase protein that can increase in serum during an acute-phase response. Its physiological role is to bind to the surface of a wide variety of damaged dead or dying cells and thereby activate complement-mediated lysis or complement-mediated increase in phagocytosis [103, 105].

1.6.2 Toll like receptors

Of the PRRs, the Toll-like receptors (TLRs) are the most well-studied. TLRs are expressed by various immune cells including macrophages, DCs, B cells, specific types of T cells, but also by non-immune cells such as fibroblasts and epithelial cells [106]. Ligands to human TLRs include bacterial lipoproteins for TLR2 [107], double-stranded (ds)RNA for TLR3 [108], lipopolysaccharide for TLR4 [109], flagellin for TLR5 [110], ssRNA for TLR7 and TLR8 [111, 112] and unmethylated CpG DNA for TLR9 [113]. TLRs specialised in recognition of viral infections (nucleic acids of viral genomes) include TLR3, TLR7, TLR8 and TLR9 which all are located in the membranes of endosomal compartments, with the exception of TLR3 that also has been detected on the surface of epithelial cells. Stimulation of a TLR (with the exception of TLR3) recruits adaptor proteins such as myeloid differentiation factor 88 (MyD88) [114] to the cytoplasmic part of the receptor and activation of downstream signalling cascades with production of proinflammatory cytokines and IFNs. RNA viruses are normally recognised by TLR3, which is expressed in conventional (c)DCs and

epithelial cells. TLRs can also be stimulated by host-derived immune-stimulators such as heat-shock proteins and uric acid, which are present during tissue damage (e. g. caused by viral infections) [106].

1.6.3 Intracellular recognition of virus

In the cytoplasm, viral genomes in the form of dsRNA are detected by retinoic acid-inducible protein-I (RIG-I) [115] or the melanoma differentiation associated gene 5 (MDA5) [116]. Interestingly, poly(I:C), a dsRNA analogue and known inducer of IFN, has been shown to activate MDA5 but not RIG-I [117, 118]. Recognition of vRNA by RIG-I requires a 5'triphosphate group [119, 120], but RIG-I can also bind short dsRNA in a 5'triphosphate-independent manner [121]. When transfecting cells with vRNA isolated from virus particles it was indicated that some RNA viruses, including HTNV, posttranscriptionally remove the RIG-I-activating triphosphate group from its RNA during viral replication as a way of counteracting recognition by RIG-I [122]. However, studies of replicating HTNV in cells, in which RIG-I was knocked-down, demonstrated a potential role of RIG-I in the antiviral response. Transfections with *in vitro* transcribed RNA demonstrated that RNA encoding for N was responsible for stimulation of RIG-I [123].

1.6.4 Interferons

IFNs are a group of secreted cytokines that have antiviral effects. They are divided into three classes named type I, II and III IFN. Type I IFNs were discovered in the 1950s [124]. In humans the type I IFNs consist of IFN-α (at least 13 genes), -β, -ε, -κ and -ω. Induction of IFN-α/β is important in response to viral infections while IFN-ε, -κ and -ω play less defined roles [125] and will not be discussed more in detail here. Type II IFN (IFN-γ) is produced mainly by activated T and NK cells and not as a direct response to viral infections. Type III IFNs comprise IFN-λ1, -λ2 and -λ3, also known as IL-29, IL-28A and IL-28B, respectively. They were described in 2003 and were shown to have antiviral activity *in vitro* and *in vivo* [126-129]. We showed that IFN-λ can inhibit HTNV *in vitro* [paper I]. The ways of induction and biological properties for IFN-α/β and -λ have been described as overlapping [130-132]. However, we showed that the gene for IFN-λ1 can be induced without the simultaneous induction of IFN-α/β in hantavirus infected cells [paper II]. This differential regulation has now also been described for human nasal epithelial cells infected with respiratory syncytial virus (RSV) [133] and Tioman virus infected bat splenocytes [134].

1.6.4.1 Induction of type I and III IFNs by viruses

The mechanisms involved in induction of type I IFNs are well documented. Recognition of a viral infection in a cell by either a TLR or an intracellular PRR induces production of IFN and can occur in at least three ways (Figure 2):

- Recognition of dsRNA by TLR3 triggers its dimerisation and tyrosine phosphorylation [135] and recruitment of an adaptor called Toll/IL-1-resistance domain-containing adaptor inducing IFN-β (TRIF) [136]. TRIF activates two pathways that lead to transcriptional activation of IFN: the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the IFN regulatory factor (IRF) 3 [137] pathways. The NF-κB pathway involves interaction between

several different molecules in a signalling complex including TNF receptor-associated factor (TRAF) 6 [137] that ultimately leads to phosphorylation of IκB [138], its subsequent ubiquitination and degradation and eventual nuclear uptake of NF-κB [139] but also activation of MAPK and the phosphorylation and activation of ATF2/c-Jun [140]. Activation and phosphorylation of IRF-3 is mediated by TRAF3 [141, 142] that activates TANK-binding kinase (TBK-1) and the related IKKε [143].

- Activation of RIG-I or MDA5 results in recruitment and activation of a mitochondrion-associated adaptor called IFN-β promoter stimulator protein 1 (IPS-1) [144]. IPS-1 transmits the signal to both the NF-κB and the IRF-3 [145] pathways described above.
- Triggering of TLR7, TLR8 and TLR9 results in recruitment of MyD88 that in turn recruits a complex including TRAF3 and TRAF6 [141]. This leads to the activation of IRF-3, NF-κB and c-Jun in the same manner as described above for TLR3. In plasmacytoid (p)DCs, another molecule than IRF-3 is involved in IFN induction, named IRF-7, which is constitutively expressed. In pDCs, TRAF6 polyubiquitinates IRF-7, ultimately leading to its activation [146].

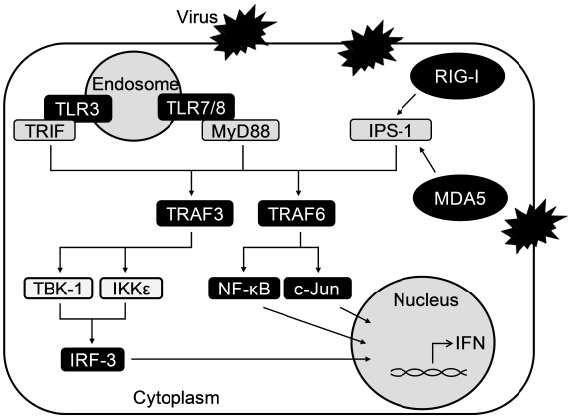

Figure 2. Induction of type I IFN by viruses. The induction of IFN-β transcription is initiated by the recognition of viral PAMPs by TLRs, RIG-I or MDA5. The recognition transmits the signal via different adaptor molecules to TRAF3 and TRAF6. TRAF3 activates the IRF-3 kinases TBK-1 and IKKε. Phosphorylated IRF-3 forms dimers that translocates to the nucleus. Activation of TRAF6 leads to translocation of NF-κB and c-Jun to the nucleus where they together with IRF-3 induce transcription of IFN-β.

IFN-α/β can be produced by any nucleated cell, with pDCs being the primary IFN-producing cells [147]. Expression of IFN-λ mRNA has been detected mainly in antigen-presenting cells such as pDCs [148, 149] but also in other types of cells [127, 150]. For herpes simplex virus it was shown that nearly all cell types have the capacity to produce IFN-λ upon infection but that pDCs and cDCs are the most effective producers [151].

TLR3, TLR4, TLR7, TLR8 and TLR9 are known to induce both IFN-α/β and IFN-λ [149]. Knockout of RIG-I and MDA5 with siRNA indicate that RIG-I, but not MDA5, is involved in induction of IFN-λ at least when it comes to a response to RSV [133].

The majority of IFN-α promoters are only regulated by IRFs and they favor IRF-7 over IRF-3 [152]. The IFN-β promoter is activated by an enhanceosome containing IRFs, NF-κB and c-Jun [153]. The IRF part in the enhanceosome might be both IRF-3 and IRF-7. With the exception of pDCs, IRF-3 is constitutively expressed while IRF-7 is an interferon stimulated gene (ISG), so generally IFN-β is induced early whereas the IFN-αs are expressed at higher levels but somewhat later [152, 154]. A similar pattern as for IFN-α was also observed for IFN-λ2/3, with a delayed kinetics compared to IFN-λ1, as a response to Sendai virus [155]. Analyses of the promoter regions for the IFN-λ genes showed that both NF-κB and IRF-3/7 activate transcription of IFN-λ1 and IFN-λ2/3. Additionally the IFN-λ1 gene, like IFN-β, depends more on IRF-3 while the IFN-λ2/3, like the IFN-α, are induced mainly by IRF-7 [155].

Previously, the expression patterns for IFN-α/β and IFN-λs have been suggested to be similar [130]. However, and in contrast to what has been observed for activation of the IFN-β promoter by the enhanceosome, the IFN-λ1 promoter may be activated by NF-κB independently of IRF-3/7 [156], indicating different modes of regulation between the IFN-β and IFN-λ genes. In line with these assumptions it was shown that inhibition of the NF-κB pathway in DCs and in animals only had a minor effect on IFN-α/β expression while IFN-λ expression was highly impaired [157]. Furthermore, we showed that IFN-λ can be induced without the simultaneous induction of IFN-α/β as a response to HTNV infection [paper II]. However, the specific mechanism underlying recognition of hantaviruses and specific induction of IFN-λ remain unclear and further experiments are needed.

1.6.4.2 Activation of signal transduction by IFN

IFN-α/β signals by interaction with its receptor IFNAR, which is composed of IFN-α1 and IFN-α2 subunits and is ubiquitously expressed. The induction of antiviral functions in the absence of immune cells is generally defined as the antiviral state, which can be achieved by stimulation of cells with IFN [125].

The interaction between IFN-α/β and its receptor activates Janus kinases (Jak). Jak activate the signal transducer and activation of transcription (STAT) 1 and STAT2 by phosphorylation. The activated STATs dimerise, associate with the DNA-binding protein IRF-9 to form the complex IFN-stimulated gene factor 3 (ISGF3) and translocate to the nucleus. The ISGF3 binds to the IFN-stimulated response element and induces transcription of large number of ISGs [158] (Figure 3).

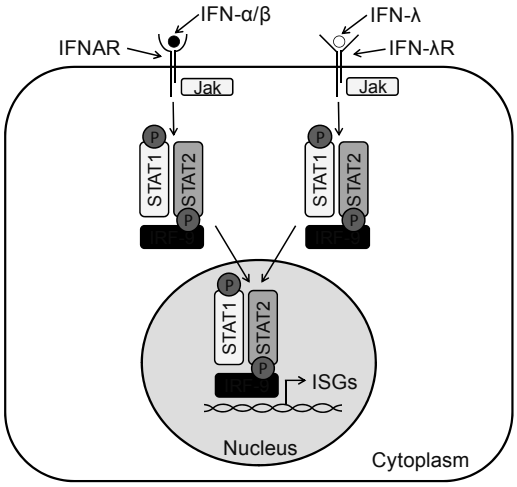

Figure 3. Interferon signalling. Secreted IFNs bind to their respective receptors and activate Jak that phosphorylates STAT1 and STAT2. The phosphorylated STATs dimerise and recruit IRF-9 to form the ISGF3 complex that translocates to the nucleus and activate ISG transcription.

The IFN-λs signal through another receptor complex than IFN-α/β. The receptor complex for IFN-λ consists of the subunits IFN-28Rα (also designated as CRF2-12) and IL-10R2 (also named CRF2-4). Sensitivity to IFN-λ is restricted to immune cells, e. g. pDCs and T cells [159, 160] and epithelial cells [161]. Neutrophils and NK cells have been shown to be important for IFN-λ-mediated inhibition of *in vivo* tumor growth [162, 163]. However, it is debated if NK cells can respond to IFN-λ or not [162, 164]. The sensitivity of epithelial cells suggests that IFN-λ prevent viral infections through skin and mucosal surfaces. This was confirmed *in vivo* using a Mx2 reporter system based on luciferase and stimulation of mice with IFN; for IFN-α/β the strongest responses were found mainly in organs intensively supplied with blood (liver and kidneys) while the most IFN-λ-responsive organs were large and small intestine, lung and salivary gland. Moderate responses were detected in lung and spleen for IFN-α/β and in stomach, kidney, spleen and heart for IFN-λ [165].

As for the IFNAR, the receptor for IFN-λ signals through the Jak-STAT pathway and activates the ISGF3 [127] and thereby also induction of ISGs and the antiviral state (Figure 3) [166]. The actions of IFN-α/β and IFN-λ are known to be negatively regulated by a group of proteins named suppressors of cytokine signalling [167, 168]. The signalling response to IFN-λ follows a similar pattern to the IFN-α/β response with induction of similar subsets of genes [169]. However, the antiviral effect of IFN-λ has been described as lower than for IFN-α/β, and IFN-λ1 is generally more potent against viruses than IFN-λ2/3 [128], as we also showed for HTNV [paper I].

Below is a summary of the functions of some of the more well-studied antiviral genes that are upregulated following IFN-α/β/λ stimulation:

- dsRNA-dependent protein kinase R is involved in the inhibition of translation and reduced protein synthesis through phosphorylation of a substrate known as the α subunit of the eukaryotic translational initiation factor 2.

- 2'5'-oligoadenylate synthetase (OAS) activates RNaseL which is a protein that degrades cellular and vRNAs. Interestingly, some of the cleaved products have been shown to activate RIG-I [170, 171]. Furthermore, OAS is upregulated in hantavirus infected cells [172].
- Mx is a family of proteins with antiviral activity against a variety of viruses including the hantaviruses PUUV, TULV [173] and HTNV [174] and influenza [175]. The exact mechanisms of their functions remains largely unknown, but they are believed to recognise nucleocapsids and restrict them to the inside of the cell, thereby restricting viral replication [176]. It has been postulated that human MxA forms oligomeric rings around the tubular structure of viral nucleocapsids, forming a complex that blocks their function [177].

1.7 ADAPTIVE IMMUNE RESPONSES TO VIRAL INFECTIONS

Unlike the innate immunity, the adaptive immunity is specific and develops an immunological memory upon recognition and response to a specific antigen, meaning that a second encounter results in a faster and more reactive response.

1.7.1 Cell-mediated response

The effector cells of cell-mediated immunity are T cells, which are produced in the bone marrow and mature in the thymus. There are two well-defined subpopulations of T cells: CD4+ T cells that are called T helper (Th) cells and CD8+ T cells that after activation gain cytotoxicity and are called CTLs. There is also a third type of T cell, regulatory T cells (Tregs), which is involved in turning off an immune response once the antigen has been eliminated from the body. Th cells recognise antigen presented on major histocompatibility complex (MHC) class II, and CD8+ T cells recognise antigen presented on MHC class I. The MHC class I molecule is expressed on all types of nucleated cells, while the MHC class II is present only on antigen-presenting cells (B-cells, DCs and macrophages) [100]. Depending on which types of cytokines that are present in the surroundings of antigen-primed Th cells, different types of subsets develop; Th1 or Th2. In general IL-12 is essential for development of Th1 cells and IL-4 is important for development of a Th2 response [178, 179].

- Th1 cells secrete IL-2, IFN-γ, and TNF-β and are responsible for many cell-mediated functions such as activation of CD8+ T cells and macrophages, production of opsonization-promoting antibodies with Fc regions that fix complement, and the promotion of excessive inflammation and tissue injury. IFN-γ inhibits the expansion of Th2 cells. Generation of a Th1 response is mediated mainly by IL-12 that is produced by activated macrophages and DCs. More IL-12 is produced if DCs/macrophages are stimulated by IFN-γ (that mainly comes from activated T and NK cells). Thereby the whole process forms a positive feedback loop [178-180]. IL-12 may also be produced by IFN-λ1-stimulated DCs [181]. Furthermore, IFN-λ1 acts to limit Th2 responses [159, 182] and the Th2 response (IL-4) also induces its inhibitor IFN-λ1 from pDCs in a feedback loop [182].
- Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 and are responsible for activation of B cells and B cell-production of large amounts of IgM, IgE and noncomplement-activating IgG subtypes. Furthermore, IL-4 and IL-10 suppress

the expansion of Th1 cells. Even small amounts of IL-4 are sufficient to direct the development of Th2 cells. Mast cells and NK cells are also known to produce IL-4, but to a lesser extent. However, it is a bit unclear which exact cell types (except from Th cells) that are involved in the initial IL-4 release that triggers a Th2 response [178, 179].

The CD8+ T cells are activated to become CTLs by cytokines from activated Th1 cells (e. g. IL-2 and IFN-γ) together with recognition of antigen-MHC class I. Activated CTLs have the potential of killing infected cells by cytotoxic activity but can also produce IFN-γ. The mechanism for killing, as for NK cells, involves degranulation and release of perforin and granzymes [100].

1.7.2 Humoral response

Humoral immunity involves B cell activation and results from the production of antibodies that circulate in the blood, plasma and lymph, fluids that a long time ago were called humors. Antibodies are often referred to as immunoglobulins (Igs). Antigen receptors on B-cells are transmembrane versions of their antibody molecules (mostly IgD or IgM). When antigen binds to the B cell membrane-bound antibody, the B cell gets activated. Some of the bound antigen is internalised and after processing, small fragments of the antigen is presented on MHC class II on the cell surface to Th cells. Activated Th cells then secrete cytokines that stimulate B cell division and differentiation into both antibody-secreting plasma cells and memory cells. Upon activation, different isotypes of antibodies can be produced: IgA, IgD, IgM, IgE or four subclasses of IgG (IgG1, IgG2, IgG3 and IgG4). Biological activities of secreted antibodies include:

- Neutralisation of infectious pathogens by block of viral binding sites and coating of bacteria.
- Opsonization, a process where IgG antibodies bind the pathogen and mediate its phagocytosis by macrophages or neutrophils. The Fc region on antibodies, bound to pathogens, can be recognised by the Fc receptor on macrophages and neutrophils. Binding to the Fc receptor enhance phagocytosis.
- Activation of complement is a process where antigen-antibody complexes bind to complement components, which then are activated and mediate lysis. IgM and most subclasses of IgG can activate complement.
- Antibody-dependent cell-mediated cytotoxicity (ADCC) is conducted by e. g. NK-cells, macrophages, monocytes, and neutrophils. Nonspecific cytotoxic cells lyse target cells specifically by binding to the Fc region of antibodies bound to surface antigens on the target cells. Various proteins that are secreted from the nonspecific cytotoxic cells then mediate the destruction of target cells by ADCC. For example macrophages and neutrophils release lytic enzymes, activated monocytes, macrophages and NK cells secrete TNF. ADCC mediated by NK cells may also involve cytotoxic granule-mediated killing [100].

1.8 HANTAVIRUS PATHOGENESIS AND IMMUNE RESPONSES IN HUMANS

The main symptoms during HFRS/HCPS are most likely caused by vascular dysfunction. Endothelial cells are infected without cell lysis or disruption of the endothelium. Hantavirus infection of endothelial cells might alter the permeability in other ways than direct killing of the cells. It is primarily the kidneys that are affected during HFRS and the lungs during HCPS. There is also a row of inflammatory responses involved, often termed as a "cytokine storm". In addition, hantavirus-specific T cells have been suggested to play an important role in the pathogenesis by attacking endothelial cells.

1.8.1 Innate and cell-mediated adaptive responses

The initial cells that are believed to be infected are β3 integrin-bearing cells in the lung [77]. After that, there is a systemic infection (maybe mediated by movement of infected cells from the lung). The main target cells have been suggested to be endothelial cells since viral antigen has been detected in the endothelium of small vessels in the brain, lungs, heart, liver and kidneys in fatal HFRS and HCPS cases [28, 58, 183]. The non-cytopathogenic infection of endothelial cells *in vitro* [184] and the intense immune activation in patients suggest that symptoms are immunomediated and not a result of the infection itself, but the mechanisms for this are largely unknown.

The function of β3 integrins on endothelial cells is to regulate endothelial cell barrier functions and activate platelets. Increases of fluid movement across the endothelial cell barriers is among other factors regulated by vascular endothelial growth factor (VEGF), a cytokine that is ~50 000 times more efficient in increasing the vascular permeability than histamine [185]. It has been shown that β3 integrins form complexes with the receptor for VEGF, and that this complex formation is involved in the negative regulation of VEGF responses [186]. Pathogenic hantaviruses attach to the inactive form of β3 integrin [187], resulting in inhibited integrin-directed endothelial cell migration [188]. In addition, and similar to the increased effect of VEGF that can be seen on β3 integrin deficient cells [186], pathogenic hantaviruses enhance the permeabilising effect of VEGF [189]. For ANDV, infected endothelial cells have been shown to secrete VEGF, and elevated levels of VEGF have been found in patients. Also, ANDV infected endothelial cells display increased permeability and downregulation of the adherence junction molecule vascular endothelial (VE) cadherin *in vitro* [190]. Taken this together, it has been suggested that one mechanism for enhanced vascular permeability during hantavirus infections could be the block of β3 integrin functions with increased sensitivity to VEGF and subsequent downregulation of VE cadherin [186].

Numerous pathogenic viruses [191, 192], including hantaviruses [71, 193-196] can inhibit IFN production. Type I IFN responses are e. g. inhibited by the cytoplasmic tail of NY-1 hantavirus Gn protein that disrupts TBK-1-TRAF3 complex formation [197] and the HTNV N that can sequester NF-κB in the cytoplasm [198]. Furthermore, we [paper I] and others [195] have shown that hantaviruses can inhibit transduction of the IFN signal and subsequent induction of the antiviral state by interfering with Jak/STAT signalling. Transient expressions of the glycoproteins for ANDV and PHV implicate

that these proteins are involved in this inhibition [195], albeit the exact mechanism for this still is unknown.

IFN-γ is a molecule that is produced by activated NK or T cells that induces the production of TNF-α in monocytes and macrophages and stimulates the release of NO. It has been suggested to play a role in hantavirus pathogenesis. Elevated levels of IFN-γ have been detected in serum from HFRS patients from the Korean war [199]. SNV-patients have SNV N-specific T cells in their blood that produce large amounts of IFN-γ but not IL-4 [200] and that there are rigorous amounts of SNV-specific T cells (up to 44.2% of CD8+ T cells during early disease) in patients with a more severe form of disease than in less severe cases [201].

Immunohistochemical staining of tissues from fatal HCPS cases revealed both cells expressing monokines (IL-1α, IL-1β, IL-6, and TNF-α) and lymphokines (IL-2, IL-4, and TNF-β) [202]. A more recent study of the cytokine profile in HCPS patients suggests that IL-6 is associated with fatal outcome, and that there is a mixed Th1/Th2 immune response during the disease. Furthermore, the magnitude of the cytokine levels associated with a Th1 response correlated with the severity of the disease [203]. This goes well in line with the assumption that IFN-γ is associated with a Th1 response and that production of IFN-γ would activate macrophages to produce TNF-α [204] which could contribute to the capillary leakage. However, in contrast to these findings for HCPS, IFN-γ-producing T cells during the acute phase of HFRS caused by HTNV have been reported to reduce the risk of progression to acute renal failure [205]. In addition, memory T cells can be found in individuals after infection with PUUV and HTNV [206, 207].

Tubular damage, accumulations of CD8+ T cells in peritubular areas and increased expression of adhesion molecules (important for immune cell migration through endothelium into tissues), such as intercellular adhesion molecule (ICAM) 1, have been found in kidney biopsies from NE patients. Interestingly, the ICAM-1 increase occured at the same locations as TNF-α could be detected [208], again indicating a role for TNF-α in the increased vascular permeability. In addition to the increased TNF-α expression in kidneys [208], elevated serum levels of IL-6, IL-10, TNF-α [209] and NO [210, 211] have been detected in PUUV-infected patients, with a correlation between levels of TNF-α and severity of disease [209]. NO is, as TNF-α, also a known inducer of vascular permeability [212, 213] and might also be involved in the pathogenesis.

Tregs suppress activation of the immune system, with involvement of cytokines such as transforming growth factor β and IL-10 [214]. In infected rodents with no signs of disease, Tregs are upregulated [215-217]. In contrast, during a longitudinal investigation of NE patients, no induction in Tregs could be observed, suggesting a possible mechanism for the rigorous and seemingly uncontrolled expansion of hantavirus-specific T cells in humans [218].

Lactate dehydrogenase is often used as a marker of tissue breakdown, and is often occurring at elevated levels in serum of hantavirus-infected patients [219]. Increased levels of serum perforin, granzyme B, and the epithelial cell apoptosis marker caspase-cleaved cytokeratin-18 have also been observed during the acute phase of PUUV

infection. The observed increase in perforin and granzyme B suggest a possible role for CTLs and/or NK cells in the tissue damage during hantavirus-caused disease [220].

Indeed, NK cells seem to somehow be involved. Normally one would expect a rapid contraction of NK cell-mediated responses after an infection. However in NE patients, proliferation of a specific subset of NK cells, $NKG2C^{+}$-NK cells, was observed, that remained functional at elevated numbers for at least 60 days after symptom debut. Interestingly, experimentally infected endothelial cells were found to express the corresponding activating NKG2C ligand HLA-E [221].

Taken together, HFRS/HCPS might be caused by infection of endothelial cells, with corresponding vascular leakage and infiltration of tissues by immune cells. Production of IFN and the antiviral state is inhibited in infected cells. Elevated levels of IFN-γ, IL-6, TNF-α, NO, VEGF and markers for apoptosis and tissue damage can be found in serum. Since infection does not induce direct cytopathogenic effects, it is likely that the pathology is mediated by other mechanisms. One suggested mechanism is that binding of pathogenic hantaviruses to β3 integrins inhibit their ability to negatively regulate the permeabilising effects of VEGF. Also, the absence of Tregs might contribute to the development of uncontrolled immune reactions. Activated NK cells and CTLs might induce tissue damage, either by direct cytotoxic effects by release of perforin and granzyme B, or indirect via release of IFN-γ and subsequent TNF-α and NO release from macrophages that in turn can affect the permeability of endothelium.

1.8.2 Humoral responses

It has been demonstrated that DCs can be productively infected with HTNV, and that infected DCs do not gain loss of function but instead mature [76]. One could speculate that infected DCs serve as a vehicle for the virus to spread systemically but also to gain access to regional lymph nodes and induction of a powerful adaptive immune response. Antibodies of the IgA and IgM subtypes develop rapidly, while IgG arise more slowly [222, 223]. Interestingly, SNV-patients with detectable SNV-specific IgG responses during the first week of disease have a higher chance of a less severe outcome than SNV-patients with undetectable IgG responses [223], indicating an important role of the humoral response in defense against hantavirus infections. For NE, elevated levels of IgE are also present [224]. The humoral response towards hantavirus infections is long lasting, e. g. IgG antibodies can be detected in individuals that were infected with PUUV up to more than 50 years ago [225-227], and infected people are believed to gain life-long immunity.

1.8.3 Animal model systems for studies of pathogenesis

Over the years, several different animal model systems have been developed, trying to evoke clinical syndromes resembling what we see in humans. Laboratory mice are not known to harbor any hantavirus, but have still been used to study hantavirus infections. Newborn suckling mice were shown susceptible to and die from intracerebral inoculations [228] and later also intraperitoneal, intramuscular and subcutaneous inoculations with HTNV [229]. In addition to HTNV, SEOV [230] and DOBV [231] have also been shown to be lethal in the suckling mice model. However, the symptoms displayed in suckling rodents does not resemble HFRS/HCPS much [232].

The first and so far only animal model resembling HCPS in humans is the Syrian hamster (*Mesocricetus auratus*) model for infections with ANDV [233]. This hamster model has been useful for studies of the antiviral effect of ribavirin against hantaviruses [234], and for vaccination studies [235]. Interestingly, using the Syrian hamster model, it was shown that depletion of T cells had no impact on clinical disease or survival, indicating that T cells might not be as important as previously believed for pathogenesis [236].

As for HCPS there is only one animal model described that resembles HFRS; cynomolgus macaques infected with PUUV. Infection of cynomolgus macaques with wild type (wt) PUUV caused NE-like symptoms with elevated levels of cytokines (IL-6, IL-10 and TNF-α), CRP, creatinine and NO [237]. Staining for N was positive in kidney, spleen and liver. In the kidneys similar findings as in NE patients could be seen with tubular damage and inflammatory cell infiltrations containing mainly CD8+ T cells [238]. Interestingly and indicating an important role for antibodies against hantavirus infections, passive immunisation using serum from previously PUUV-infected monkeys could induce sterile protection and protect against pathogenesis [239].

1.9 TREATMENT AND CONTROL

A cure against hantavirus-induced disease will most likely involve a combination of therapies. In addition to an effective antiviral, there also might need to be a component involved that inhibit the immune responses of the host. As yet, there are no effective treatments available.

1.9.1 Antiviral drugs

There is no approved antiviral compound against hantaviruses. The only drug that has been shown to possess an antiviral activity *in vitro* [240] and *in vivo* in suckling mice [241] and Syrian hamsters [234] is ribavirin. This drug has also been tested intravenously in HFRS patients in China where ribavirin-treated patients showed reduced morbidity and severity of symptoms compared to the placebo control group [242]. In HCPS patients, intravenous administration showed no positive effects on the outcome of the disease [243].

Recombinant IFN-α in combination with antiviral drugs are sometimes used against viral infections, e. g. hepatitis C [244]. IFNs have been tested against hantavirus infections, both *in vitro* and *in vivo.* IFN-α, -β -γ [245] and -λ [paper I] have been shown to inhibit HTNV *in vitro*. In addition, pretreatment with IFN-β protect against HTNV in murine macrophages [245]. When IFN-β was administered to newborn mice, both treatment before, during or up to 7 days after HTNV infection increased the survival rates [245]. In a human trial where IFN-α was administered intramuscularly in HTNV patients, no significant effect could be seen on mortality. However, severe bleedings and proteinuria were less pronounced in the treated compared to in the placebo group [246].

1.9.2 Vaccines

Hantavax™ is an inactivated vaccine produced in rodent brains. Only about 50% of the recipients develop neutralising antibodies [247], but its use has been associated with a 45% decrease in Korean HFRS-hospitalisations between 1991 and 1996 [248]. Large-scale human trials in China report that HTNV- and SEOV-inactivated vaccines produced in cell cultures induce a protective efficacy with neutralising antibodies in over 85% of the individuals enrolled in the trial [249]. However, the methods for preparation of these vaccines in rodent brains and formalin inactivation are considered unacceptable for human vaccination in most western countries. Several other approaches based on recombinant protein [250, 251], vaccinia-vectored vaccines [252, 253] and DNA plasmids [254, 255] have been tested with so far promising results.

1.9.3 Passive immunisation

The correlation that has been observed between early development of IgG antibodies and outcome of HCPS suggests an important role for antibodies in the clearance of the virus [223]. Passive immunisation has not been conducted in humans but has worked very well in several animal models. Cynomolgus macaques were protected against PUUV after being passively immunised with sera from previously infected monkeys [239] and passive transfer of immune serum (produced in rabbits by DNA vaccination) administered before and/or after intranasal challenge protected Syrian hamsters against ANDV [256].

1.10 HANTAVIRUSES AND THEIR HOSTS

Unlike viruses in all other genera within the family *Bunyaviridae*, which are transmitted by insects and other arthropods such as ticks, hantaviruses are harbored by small mammals [6, 257]. Hantaviruses are generally associated with a specific rodent or insectivore host (Table 1). The overall seropositivity among rodents in areas where hantaviruses are known to occur can be quite high, up to 9.5-11% in deer mouse [258, 259] and 15% [260] to 50% in bank voles [261, 262]. In nature, seropositivity has been shown to be higher in rodent males than females, e. g. for PUUV in bank voles [262] and for SNV in deer mice [258].

Transmission between rodents occurs through inhalation of aerosolised infected excreta in the environment, or by wounding [263, 264]. Viral spread by inhalation of aerosols demands a high capacity of keeping infectivity and being stable outside the host. PUUV can infect new bank voles up to 15 days after excretion [265] and HTNV can keep infectivity at least 96 days at 4 ·C under wet conditions in a laboratory setting [266].

The resemblance of the phylogenetic trees for hantaviruses and their rodent hosts, respectively, has for long been supporting the idea of co-evolution of hantaviruses with their carrier rodents and that they most probably share an ancient ancestor virus [257]. However, there are evidence suggesting that we should re-evaluate the co-divergence hypothesis and consider host switches to genetically related rodents and local adaption

as an explanation to the similarities between the phylogenies of hantaviruses and their mammalian hosts [267, 268].

1.10.1 Shedding and maintenance of infection

Shedding of virus is believed to occur asymptomatically during the whole life span of the infected host. Intramuscular HTNV infection of *Apodemus* results in shedding of infectious virus for at least one year [269]. Intramuscular PUUV infection of suckling bank voles leads to excreted infectious virus in urine for at least 130 days and infectious virus in lung for at least 270 days [270]. In experimentally infected deer mice, the heart, lung and brown adipose tissue have been shown to be important sites for the replication and maintenance of SNV during persistent infection [271].

Observations of infected animals in the laboratory and the ability to isolate virus from rodents even in the presence of high levels of antibodies in serum [269] led to an initial believe that hantaviruses are carried and shed by their natural hosts without a clinical picture. However, more recent reports suggest that this might not be entirely true. Morphologic examination of SNV-infected wild deer mice [272] indicated that infection does cause some pathology within its reservoir and there are also reports of decreased survival among SNV- and PUUV- infected wild rodents, especially during winter [273, 274].

1.10.2 Immune responses and mechanisms of persistence

Displaying a clinical picture or not, the mechanisms mediating persistence of zoonotic pathogens, such as hantaviruses, in their natural hosts remain unknown. Since HFRS/HCPS are believed to be immunomediated and rodent hosts are assumed to carry hantavirus infection persistently and without obvious symptoms, studies and comparisons of immune reactions in both species are of great importance to understand why we, but not the rodents, get ill. Understanding the pathogenesis would simplify the search for a cure. However, immune responses in natural rodent hosts have been hard to study due to the lack of specific reagents.

Today, new molecular methods can be used to simplify studies of the natural hosts. Development of quantitative real-time PCRs to detect gene expression require only partial sequencing of genes. This has been done for several immune-related genes in deer mouse [275-277]. It has been hypothesised that hantaviruses have evolved ways of escaping the host's immune reactions in their natural hosts. A mouse model where spleen cells from immunocompetent mice were transferred into HTNV-infected severe combined immunodeficient mice demonstrated a correlation between HTNV persistence and the lack of CTLs. It was suggested that the persistent hantavirus infection in rodent reservoirs is caused by escape from CD8+ T cell surveillance [278]. In line with this assumption, one key to persistence of SEOV in rats and SNV in deer mice has been suggested to be virally induced proliferation of Tregs, that might limit pathogenesis but also the viral clearance by suppression of proinflammatory and CTL responses. These results indicate that early innate immune response events evoked by hantavirus infections of natural hosts drive the development of Tregs [215, 217].

In addition to this, specific data for innate immune reactions in hantavirus rodent hosts is available from studies of sex differences between expression of innate antiviral genes in SEOV-infected rats. Using a microarray, about 200 immune-related genes were shown to be induced to higher levels in infected female compared to in infected male rats [279]. In addition, expression of IFN-β and Mx2 mRNA in the lungs were shown to be higher for females than males during infection [280]. Another observation, and in contrast to findings in HFRS and HCPS patients, was no induction in TNF-α gene expression in the spleen of persistently PUUV-infected bank voles [281] and no elevation of NO in response to SNV infection in deer mice [282]. Interestingly and corroborating with the results seen for SEOV-infected rats [279], female bank voles expressed higher levels of TNF-α than males [281]. These studies reporting differences in immune responses between females and males, might at least partly explain why male rodents tend to be more prone to get persistently infected by hantavirus infections [258, 262].

In conclusion, the information we have about immune responses against hantaviruses in their natural hosts is incomplete. Before we can elucidate why severe symptoms can occur in humans while the natural hosts survive hantavirus infections and appear asymptomatic, more immunological tools specific for the rodent hosts are needed. To perform studies of antiviral responses to bank vole-borne viruses in cells from their natural host, we have isolated embryonic cells from bank voles and developed tools for detection of IFN-β and Mx2 gene expression [paper IV].

1.11 *IN VITRO* CELL CULTURE SYSTEMS AND ISOLATION OF VIRUS

Due to the lack of suitable animal models and/or lack of specific immunological tools for these animal species, most studies of hantaviruses are performed in cell culture with cell culture-adapted strains. Viruses have been isolated both from human clinical samples and small mammal reservoir hosts. Generally it is much harder to isolate virus from clinical samples than from infected tissues from rodents. Starting material can be serum or whole blood from patients or serum, whole blood, saliva, urine or supernatant from suspensions of minced tissue (such as lung or kidney) from small mammal hosts. Human hantavirus isolates for example include ANDV from serum [283] and PUUV from blood leucocytes [284]. Examples of isolates from natural hosts of hantaviruses are HTNV from striped field mouse lungs [6], PUUV from lungs and spleens from bank voles [12], SEOV from lungs, spleen, feces and saliva from rats [9] and SNV from deer mouse lungs [18].

To adapt the virus to cell culture, cells to be infected are co-cultivated with the starting material and several blind passages might be needed before infectious virus can be detected in the cell culture media. If the starting material contain very little virus, an initial passage through an experimental vertebrate host (such as colonised bank voles) might be required before cell culturing to obtain a successful virus isolation. The cell line-adaption is often performed on Vero E6 cells that lack the capacity to produce IFN-α/β [285], which of course may lead to evolution of strains with phenotypes that differ from the parental wt strain. For PUUV it has been shown that adaption to cell culture can induce mutations [286], reduce the ability to infect the natural host [287]

and lead to evolution of substrains with different phenotypes [paper III]. Mutations caused by adaption to cell culture are also known for other viruses, e. g. tick borne encephalitis virus (TBEV) [288] and Ljungan virus (LV) [289-291]. Therefore, it is important to know that results observed in cell culture might not necessarily reflect infection in natural hosts or patients. Isolation and propagation of zoonotic viruses in cells from their natural hosts might be an alternative approach to avoid or at least minimise differences to the parental wt strain.

2 AIMS

The ultimate goal with this work has been to understand the immunomediated hantavirus pathogenesis better and to investigate why humans but not the rodent hosts get ill.

The specific aims have been:

1. To investigate hantavirus induction of and/or interference with innate antiviral responses and the mechanisms for this [paper I and II].
2. To study the kinetics of IFN responses evoked against PUUV in patients and HTNV in cell culture with the focus on the relatively newly discovered IFN-λ [paper I and II].
3. To study if different subspecies of PUUV show different characteristics compared to each other and the parental strain [paper III].
4. To develop an *in vitro* model and tools for studies of innate immune responses against PUUV and other bank vole borne viruses in cells from their natural host [paper IV].

3 RESULTS AND DISCUSSION

3.1 IFN-λ RESPONSIVENESS AND INHIBITION OF IFN SIGNALLING (PAPER I)

In the first paper, we reported that serum levels of IFN-λ were significantly reduced during the acute phase of NE. Furthermore, pretreatment of cells with IFN-λ inhibited replication of HTNV *in vitro* whereas an already established infection was shown to be insensitive to any type of IFN.

IFN-α, -β and -γ have for long been known to interfere with viral replication [292], including HTNV [245]. The more recently discovered IFN-λ have also been shown to inhibit viruses [127, 128]. Previously, elevated levels of IFN-α have been detected in Ebola and Junin hemorrhagic fever patients [293-295], but only a weak increase in IFN production has been detected in hantavirus infected cells *in vitro* [172, 193, 194, 296]. Here, we investigated serum levels of IFN-α, -β, and -λ in NE patients during the acute and convalescent phases of the disease. We found no alterations of IFN-α or IFN-β levels, but IFN-λ was significantly lower during the acute phase of the disease.

When conducting this study, nothing was known about the antiviral capacity of IFN-λ against hantaviruses. Therefore, we treated cells with IFN 24 hours before or 30 hours after infection with the prototype hantavirus HTNV, and determined the effect of the treatment on production of infectious viral progeny. As previously reported, pretreatment with IFN-α, -β and -γ reduced levels of progeny virus production. The novel finding was that also IFN-λ displayed antiviral effects, with IFN-λ1 acting as a stronger antiviral than IFN-λ2. Combining IFN-λ with IFN-γ resulted in an additive antiviral effect. However, treatment of an established infection did not induce reduction of the viral production, indicating that HTNV inhibit the IFN signalling pathway in infected cells. Treatment of cells with the NO-inducing cytokines IFN-γ and IL-1β resulted in less secreted NO from infected compared to noninfected control cells, confirming that HTNV could inhibit transduction of the IFN signal. Interference with the innate antiviral IFN responses had previously also been shown for ANDV, with inhibition of STAT1 and STAT2 phosphorylation as the proposed mechanism [195]. In line with this, we observed that also HTNV can inhibit STAT1 phosphorylation.

A transient decrease in amount of lymphocytes in the blood has been associated with the acute phase of a variety of diseases [297-301], and it has been suggested that systemically increased levels of type I IFNs are needed for lymphocytopenia to occur [302]. With this in mind we believe that the lack of systemic elevations of IFNs observed in this study might, at least partly, explain the rigorous expansion of lymphocytes that have been observed during hantavirus infections [56, 218, 221].

In summary, we have shown that activation of the IFN responses are inhibited in patients and that IFNs, if produced, are incapable to protect already infected cells. These results indicate that the clearance of hantaviruses is dependent on other mechanisms than IFNs, such as activated NK cells and CTLs.

3.2 IFN-α/β-INDEPENDENT INDUCTION OF IFN-λ (PAPER II)

The second paper describes, for the first time for a viral infection, the existence of differential induction pathways for type I and type III IFNs.

Since the initial discovery of the IFN-λs in 2003 [127, 128], the initiation pathway of their production has been believed to overlap with the pathway for production of IFN-α/β [132]. Induction of the antiviral molecule MxA is regulated by IFN-α/β or -λ [303]. Just before our study began, it was shown that the organisation of the IFN-λ enhancer differs significantly from the IFN-β enhanceosome [156]. Knowing this, and that the induction of the ISG MxA in response to SNV can occur without the production of IFN-α/β [304], we postulated that hantaviruses although being poor IFN-α/β inducers [193-196] might activate the production of IFN-λ.

As a response to HTNV infection of A549 cells, we observed a strong induction of IFN-λ transcription that occurred before induction of MxA and IFN-β. Furthermore, IFN-λ1 and MxA gene expression were induced by HTNV in the complete absence of IFN-α/β responses in Vero E6 cells, and pre-treatment of these cells with IFN-λ1 had an inhibitory effect on HTNV progeny virus production. However, when analysing cell culture media from infected A549, no IFN protein could be detected with ELISA. We assumed that a possible explanation for this could be that secreted IFNs directly bind to their respective receptors on the surface of the cells, thereby rendering supernatants with undetectable levels of IFN. To enable measurable amounts of IFN-λ, we instead infected MRC-5 fibroblast cells, which lack the receptor for IFN-λ [166]. Using this approach we found detectable secreted IFN-λ1 as a response to HTNV. Analysis of the gene expressions for IFN-α, -β, -λ1, -λ2 and MxA in MRC-5 showed an upregulation of MxA that coincided in time with induction of IFN-β.

When we stimulated cells with poly-I:C (a known stimulator of MDA5 and TLR3), we observed inhibited induction of both IFN-β and -λ1 gene expression in infected cells compared to in noninfected cells. Our results suggest that HTNV can block MDA5/TLR3-activated signalling pathways that induce both type I and III IFNs and that the observed type I IFN-independent IFN-λ1 induction is not caused by a specific inhibition of type I IFN. Instead, our results indicate that there might be ways of induction that are specific for IFN-λ. UV-inactivated virus did not induce any IFNs, which indicates that only replicating HTNV can be recognised by the cells to activate IFN-λ specifically.

Next, we wanted to investigate if the cellular antiviral responses evoked by HTNV were dependent on multiplicity of infection (MOI). Many studies are performed with high doses of virus, and one could speculate if this really reflects a natural infection. Interestingly, we found that in Vero E6 cells, a MOI of 0.04 resulted in higher viral titers in the cell culture medium from 7 days post infection and onwards, compared to a MOI of 40. This goes well with the observation that when growing viral stocks in Vero E6, a low initial dose of virus is preferable to harvest stocks with high viral titer (our unpublished observation). Furthermore, we observed that the higher dose induced expression of the antiviral genes MxA and IFN-λ1 at an earlier time point than what

was observed for the lower dose. This indicates that a high initial dose of virus is recognised directly, inducing a strong antiviral response that inhibit replication. It would not be surprising if the scenario in human patients resemble more of what we see when we use a lower dose of virus *in vitro*.

In addition to direct induction of antiviral molecules, IFN-λ may enhance adaptive immunity, suppress Th2 responses, reduce Treg populations and increase antigen-specific cytolytic degranulation by CTLs [159, 305, 306]. These immunomodulatory effects of IFN-λ are strikingly similar to the strong adaptive immune responses that can be observed in HFRS/HCPS patients. It would be interesting to increase the knowledge about the potential role of IFN-λ during pathogenesis and/or its role in protection against viral infections.

3.3 CHARACTERISATION OF DIFFERENT SUBSTRAINS OF PUUV (PAPER III)

In paper III, we analysed the genotype and phenotype of two different substrains of PUUV, and compared their characteristics to the parental strain.

Hantaviruses are ssRNA viruses, with an estimated mutation rate similar to other RNA viruses [307]. When growing and titrating a stock of PUUV (strain Kazan), we noted differences in foci size. This led us to the conclusion that the stock might contain a mixture of different phenotypes. We isolated two substrains from the original parental strain (PUUV-Pa); one with large (PUUV-La) and one with small (PUUV-Sm) focus size, and performed sequence analysis on the genetic material. For both substrains, we found amino acid substitutions in the S and L segments, while the M segment was unaltered compared to in the parental strain. Surprisingly, the mutations were found in well conserved regions of the S and L in residues that also are highly conserved among hantavirus species. The identical M segments indicate that a reassortment between PUUV-La and PUUV-Sm may have occurred (that one of these substrains has picked up the other's M segment) or that this M segment is so well conserved that it represents the only M-segment present in PUUV-Pa.

For PUUV-Sm, we found the amino acid substitutions D35Y in the S segment and L611F in the L segment. Interestingly, the monoclonal antibody 1C12 was unable to detect the D35Y mutant form of N. 1C12 recognises the N-terminal region of PUUV N [308]. Using this antibody, we indirectly could estimate that up to 9% of PUUV-Pa contained PUUV-Sm.

For PUUV-La, we observed the amino acid substitutions D27E in the S segment and P702S in the L segment. Furthermore, a large region consisting of 43 nucleotides was missing in the 5' untranslated region of the S segment. *In silico* predictions of the secondary structure revealed a hairpin loop in this region present in hantaviruses that are carried by *Arvicolinae* rodents (such as KHAV, PHV, PUUV, TOPV, TULV) while this region is missing in hantaviruses with *Murinae* rodents (e. g. DOBV, HTNV and SEOV) or *Sigmodontinae* rodents (e. g. ANDV and SNV) as natural hosts. It is interesting that this region is well conserved among *Arvicolinae*-borne hantaviruses,

albeit not being crucial for hantaviruses in general and that the PUUV-La substrain can replicate without it.

The secondary structure for the first N-terminal 70 amino acids of the N has been solved. In this model, the N-terminal of the protein forms two α-helixes separated by a loop in a helix-loop-helix structure [309-311]. Applying this knowledge on PUUV, D27 would be located in α-helix 1 and D35 on the tip of the loop formed between α-helix 1 and α-helix 2. The D35 residue has been indicated to be involved in N interaction and dimerisation [310], indicating an important role for this amino acid.

In Vero E6, infection with PUUV-La produced higher amounts of infectious viral progeny than PUUV-Pa, and PUUV-Pa produced more infectious progeny than PUUV-Sm. PUUV-La had the lowest ratio of vRNA to infectious virus particles, suggesting that a possible explanation for more efficient replication of PUUV-La is that PUUV-Sm and PUUV-Pa contain more defective particles per infectious virus than PUUV-La.

Vero E6 cannot produce IFN-α or IFN-β due to a chromosomal deletion [285]. In order to analyse the effectiveness of the replication for the different substrains in an IFN-competent cell line, MRC-5 fibroblasts were used. PUUV-Pa produced less viral progeny than PUUV-La and PUUV-Sm. PUUV-La and PUUV-Sm replicated to the same level and showed no difference in production of infectious virus despite the difference observed in Vero E6. We analysed the induction of gene expression for IFN-β, and the ISGs ISG56 and MxA. Generally, PUUV-Pa induced higher expression levels that might explain the inability to replicate as efficient as PUUV-La and PUUV-Sm in these cells. Knowing that PUUV-Pa most probably is constituted of a large amount of different substrains (including PUUV-La and PUUV-Sm), it is not so surprising that some of these induce cellular innate immunity.

Altogether our results from paper III show that mutations accumulate during virus propagation which can affect the overall phenotype. This is important to consider before drawing conclusions from *in vitro* experiments where cell culture-adapted viruses have been used.

3.4 *IN VITRO* STUDIES OF BANK VOLE BORNE VIRUSES (PAPER IV)

Paper IV describes a method for studies of bank vole borne viruses and cellular innate immune activation in bank vole cells *in vitro*.

Bank voles are the asymptomatic natural hosts of a variety of viruses including cow pox virus (CPXV), LV, PUUV and TBEV. In humans, PUUV and TBEV infections might be quite severe and are associated with NE and encephalitis, respectively, while CPXV causes milder symptoms and is associated with skin lesions [56, 312, 313]. Due to the lack of specific reagents it has been difficult to study if PUUV and other bank vole borne viruses interfere with bank vole cell functions. Therefore it is largely unknown why rodent hosts can carry viruses asymptomatically, while humans get ill.

We wanted to establish an *in vitro* model to enable studies of bank vole borne viruses, including PUUV, in cells from their natural host. Thereby, we isolated vole embryonic fibroblasts (VEFs) from colonised bank voles. We showed that the VEFs were susceptible to PUUV, including a wt variant previously only passaged in bank voles. In addition to PUUV, the VEFs were susceptible to CPXV, LV and TBEV. CPXV and LV caused lytic infections while PUUV and TBEV showed no signs of cytopathogenicity.

That wt PUUV could infect VEFs is promising for future studies using the wt virus. As far as we know, wt PUUV has never been studied in cell culture before. Using the wt virus would be preferable since cell line-adapted variants most probably contain mutations [paper III]. It is likely that VEFs and cell lines isolated from other natural hosts that harbour zoonotic viruses could be used for isolations and propagations of wt viruses.

To confirm functional antiviral response pathways, VEFs were transfected with poly(I:C), followed by an IFN bioassay on cell culture medium from the transfected cells [314]. IFNs were indeed present, and we also confirmed this by the observation that addition of this medium to new VEFs induced STAT1 phosphorylation and NO production. We sequenced parts of the antiviral genes IFN-β and Mx2, followed by development of real-time PCR protocols. In order to investigate gene expression in response to infection, we infected VEFs with CPXV, LV, PUUV and TBEV and collected samples at different time points after the infection.

CPXV and LV induced IFN-β but not Mx2 at 12 hours post infection (hpi) and onwards, suggesting that these viruses activate IFN-β but inhibit induction of the antiviral state in VEFs. The clear presence of cytopathogenesis at 48 hpi coincided with a dramatic increase in CPXV progeny production. For LV, lysis of cells did not induce an increase in viral titers, indicating a less efficient replication than for CPXV.

PUUV did neither induce IFN-β nor MxA gene expression, and viral titers in supernatants increased over time. In paper III, we observed that PUUV infection of human fibroblasts induced both IFN-β and MxA with less production of viral progeny in response to increased antiviral activity. In combination, our results suggest that some of the immune responses that are evoked in humans might be inhibited in rodent reservoirs.

TBEV induced IFN-β with subsequent induction of Mx2 and the amount of viral progeny did not change over time. This indicates that TBEV infections of VEFs are controlled by the cellular innate immune responses, albeit not to a level where production of new virus is inhibited completely.

In conclusion, we have established a functional *in vitro* model and developed the first tools for studies of bank vole innate immune reactions in response to viral infections. Using this model, it will hopefully be possible to better understand how zoonotic viruses interfere with host cell signalling pathways and how they affect induction of innate immune responses.

4 CONCLUDING REMARKS

Hantaviruses are zoonotic viruses that can be found in most parts of the world. They cause severe symptoms, often with haemorrhagic manifestations and high death rates, with a possible important involvement of the immune reactions against the virus in the pathogenesis. The innate immune system is involved in the early defense against virus infections. One early response is IFNs that are produced by infected cells to defend themselves and surrounding cells. The IFNs are also involved in the shaping of adaptive immune responses against the infection. In summary, several valuable conclusions can be drawn from my thesis.

The results from paper I include the findings of low levels of IFNs in serum of hantavirus-infected patients and that already infected cells can counteract the antiviral effects of all types of IFN. These observations alone indicate that induction of the antiviral state might be absent or inhibited in hantavirus-infected patients. However, in paper II increased transcription of IFN-λ and Mx2 were observed in infected cell cultures in the complete absence of IFN-α/β. This was a novel finding, being the first report of differential induction pathways for type I and III IFN in response to a viral infection. Despite a large increase in transcription, secreted IFN-λ protein could only be detected in cell culture medium from cells without the IFN-λ receptor. This might indicate that IFN-λ, if produced in patients, would be consumed too fast to be detected in serum and that the effects of produced IFN-λ is localised near the site of secretion. However, as the results from paper I describe, already infected cells are protected from the direct antiviral effects. Endothelial cells are the prime targets of hantaviruses [52]. They increase the transcription of IFN-λ in response to hantavirus infection (our unpublished results) and lack the receptor for IFN-λ [161]. Knowing this, one could speculate that infected endothelial cells in patients produce IFN-λ, but that other cells expressing the receptor bind the secreted protein, e. g. immune cells such as pDCs and T cells [159, 160]. IFN-λ has previously been associated with reduced Treg populations and increased CTL-mediated killing [306, 315], IL-12-secretion from DCs [181] and increased NK cell activity [162]. To conclude the results from paper I and II, it seems likely that IFN-λ has a role in hantavirus pathogenesis (Figure 4).

The major conclusion from paper III is that laboratorial viral stocks may contain a mixture of different substrains with different characteristics, and that we should be well aware of this when performing infectious experiments *in vitro*. The use of wt viruses would be preferable, but unfortunately stocks with high virus concentration have previously only been obtained after cell culture adaptation. We hope that our *in vitro* model, that we present in paper IV, could be useful when isolating and propagating wt viruses in the future. The results from paper IV also indicate that there are differences in innate responses to hantavirus infections between humans and rodent hosts, even on a cellular level. Further experiments are needed, and it would e. g. be interesting to investigate hantavirus infections of bank vole cells with an endothelial cell origin.

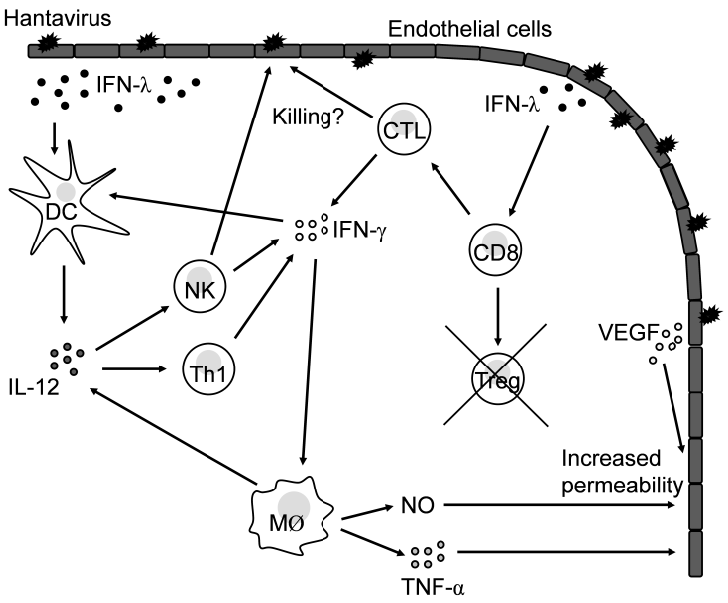

Figure 4. Hantavirus-induced IFN-λ and its possible role in increased vascular permeability. Hantavirus-infected endothelial cells secrete IFN-λ that act on CD8+ T cells, DCs and activated NK cells. T cells differentiate into IFN-γ producing CTLs, DCs secrete IL-12 that stimulates Th1 and NK cells to produce IFN-γ. IFN-γ stimulates DCs, but also macrophages to produce more IL-12 in a positive feedback loop. IFN-γ-stimulation of macrophages induce production of NO and TNF-α. Infected endothelial cells do also produce VEGF. IFN-λ does not induce Tregs. MØ, macrophage.

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