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# **HANTAVIRUSES, ESCAPEES FROM THE DEATH ROW – VIRAL MECHANISMS TOWARDS APOPTOSIS RESISTANCE**

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Front cover: “The anti-apoptotic engine of hantaviruses”

A graphical representation of the strategies by which hantaviruses hinder the cellular signalling towards apoptosis: downregulation of death receptor 5 from the cell surface, interference with mitochondrial membrane permeabilization, and direct inhibition of caspase-3 activity.

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# Hantaviruses, escapees from the death row – Viral mechanisms towards apoptosis resistance

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my family*



*“Life is best filled by learning as much as you can  
about as much as you can, taking pride in what you are doing,  
having compassion, sharing ideas, being enthusiastic.  
Rejoice in what you learn and spray it.  
Live to learn.”*

Tim Minchin



## ABSTRACT

Over the past decades, humanity has witnessed a constant stream of emerging pathogenic RNA viruses. The recurrent outbreaks of Ebola virus in Africa, the emergence of West Nile virus in North America and the sporadic but constant outbreaks of hantaviruses and arenaviruses are some examples of current public health concerns. Unravelling the strategies that these viruses use for immune evasion and for interference with the normal function of host cells remains of paramount importance in order to comprehend viral pathogenesis and ease the development of preventive measures and treatments.

Hantaviruses are worldwide-distributed zoonotic viruses that can cause spillover infections from rodents to humans. Human infection may develop into severe disease with high morbidity and case fatality rates of up to 40%. Despite continuous efforts, neither a vaccine nor specific treatments have been developed yet. Hantavirus-associated diseases appear to be immune-mediated and clinical manifestations relate to strong inflammation and vascular leakage. Although the precise mechanism of viral entry is still not well understood, endothelial cells represent the main target of hantaviruses. Despite the strong cytotoxic lymphocyte responses during acute disease, infected endothelial cells remain undamaged in hantavirus-infected patients. This puzzling observation has been partly explained by the capacity of pathogenic hantaviruses to inhibit the activity of caspase-3 and granzyme B, thereby blocking apoptosis (i.e. controlled cell suicide) in infected cells. The cellular pathways of apoptosis are multiple and represent highly complex and dynamic processes leading to heterogeneous outcomes. Such complexity echoes of course in the entangled mechanisms that some viruses use to sabotage apoptosis induction.

This thesis intends to recapitulate the current knowledge on how hantaviruses may cause pathogenesis in humans and further provides novel results on the capacity of hantaviruses to inhibit apoptosis, and on the interplay between these viruses and the host factor PCDH-1. In **Paper I**, we investigated if the anti-apoptotic characteristics previously reported for the pathogenic Andes and Hantaan hantaviruses were shared by other pathogenic and non-pathogenic hantaviruses. Second, since direct inhibition of caspase-3 and granzyme B by hantaviruses can only partly explain the strong viral-mediated inhibition of apoptosis, in **Paper II** and **Paper III** we sought to further unravel the mechanisms behind viral resistance to chemically-induced apoptosis (**Paper II**), and to TRAIL-mediated killing of infected cells (**Paper III**). PCDH-1 is an important cell entry factor for American hantaviruses and PCDH-1 knock out significantly reduced hantavirus infectivity and pathogenesis in an animal model, showing that targeting of PCDH-1 could provide strategies to hamper infection and disease caused by some hantaviruses. In **Paper IV**, we examined the role of PCDH-1 in entry and replication of the European Puumala hantavirus. As a whole, the work here presented aims at better understanding the mechanisms of hantavirus replication and pathogenesis in humans.

## LIST OF SCIENTIFIC PAPERS

- I. **Carles Solà-Riera**, Shawon Gupta, Hans-Gustaf Ljunggren, and Jonas Klingström. Orthohantaviruses belonging to three distinct phylogroups all inhibit apoptosis in infected target cells. *Sci Rep.* 2019. 9(1):834.
- II. **Carles Solà-Riera** and Jonas Klingström. Hantavirus inhibits apoptosis by preventing mitochondrial membrane potential loss through up-regulation of the pro-survival factor BCL-2. *Manuscript under review.*
- III. **Carles Solà-Riera**, Shawon Gupta, Kimia T. Maleki, Patricia González-Rodríguez, Dallel Saidi, Christine L. Zimmer, Sindhu Vangeti, Laura Rivino, Yee-Sin Leo, David Chien Lye, Paul A. MacAry, Clas Ahlm, Anna Smed-Sörensen, Bertrand Joseph, Niklas K. Björkström, Hans-Gustaf Ljunggren, and Jonas Klingström. Hantavirus Inhibits TRAIL-Mediated Killing of Infected Cells by Downregulating Death Receptor 5. *Cell Rep.* 2019. 28(8):2124-2139.
- IV. **Carles Solà-Riera**, Mirte N. Pascha, Marina Garcia, Rohit K. Jangra, Eva Mittler, Hans-Gustaf Ljunggren, Kartik Chandran, and Jonas Klingström. Possible dual role for PCDH-1 in the replication cycle of Puumala virus. *Manuscript.*

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- SI. Jonas Klingström, Anna Smed-Sörensen, Kimia T. Maleki, **Carles Solà-Riera**, Clas Ahlm, Niklas K. Björkström, and Hans-Gustaf Ljunggren. Innate and adaptive immune responses against human Puumala virus infection: immunopathogenesis and suggestions for novel treatment strategies for severe hantavirus-associated syndromes. *J Intern Med*. 2019. 285(5):510-523. Review.
- SII. Christine L. Zimmer, Martin Cornillet, **Carles Solà-Riera**, Cheung Ka Wai, Martin A. Ivarsson, Lim Mei Qiu, Nicole Marquardt, Yee-Sin Leo, David Chie Lye, Jonas Klingström, Paul A. MacAry, Hans-Gustaf Ljunggren, Laura Rivino, and Niklas K. Björkström. NK cells are robustly activated and primed for skin-homing during acute dengue virus infection in humans. *Nat Commun*. 2019. 10(1):3897.



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## LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
AmEPV	<i>Amsacta moorei</i> entomopoxvirus
ANDV	Andes virus
ATP	Adenosine triphosphate
dATP	Deoxyadenosine triphosphate
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist killer
BALF-1	Bam H1 fragment A leftward open reading frame 1
BAX	BCL-2-associated X protein
BCCV	Black Creek Canal virus
BCL-2	B cell lymphoma 2
BCL-X <sub>L</sub>	B cell lymphoma extra large
BHRF-1	Bam H1 fragment H rightward open reading frame 1
BH1	BCL-2 homology region 1
BH2	BCL-2 homology region 2
BH3	BCL-2 homology region 3
BH4	BCL-2 homology region 4
BID	BCL-2 homology region 3-interacting domain death antagonist
BIM (BCL-2L11)	BCL-2-like protein 11
BIR	Baculovirus inhibitor of apoptosis protein repeat
BIR2	Baculovirus inhibitor of apoptosis protein repeat 2
BIR3	Baculovirus inhibitor of apoptosis protein repeat 3
CAR	Chimeric antigen receptor
CARD	Caspase recruitment domain
CCL5	C-C motif ligand 5
CD	Cluster of differentiation
CIAP	Cellular inhibitor of apoptosis protein
CRMA	Cytokine response modifier A
CRMB	Cytokine response modifier B
CXCL10	C-X-C motif chemokine 10
Cyt C	Cytochrome C
DAMP(s)	Danger-associated molecular pattern(s)

DD	Death domain
DED	Death effector domain
DIABLO	Direct inhibitor of apoptosis protein-binding protein with low pI
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DOBV	Dobrava virus
DR4	Death receptor 4 (alternatively TNFRSF10A and TRAIL-R1)
DR5	Death receptor 5 (alternatively TNFRSF10B and TRAIL-R2)
DR5 <sub>s</sub>	Death receptor 5 short
EBV	Epstein Barr virus
ECMO	Extracorporeal membrane oxygenation
ER	Endoplasmic reticulum
ETAR	1- $\beta$ -D-ribofuranosyl-3-ethynyl-[1,2,4]triazole
FADD	Fas-associated death domain
FHL	Familial haemophagocytic lymphohistiocytosis
FLICE	FADD-like interleukin-1 $\beta$ converting enzyme
FLIP	FLICE-like inhibitory protein
GTP	Guanosine-5'-triphosphate
HCV	Hepatitis C virus
HeLa	Henrietta Lacks
HIV	Human immunodeficiency virus
HMVEC-L	Human lung microvascular endothelial cells
HTLV	Human T cell leukaemia virus
HTNV	Hantaan virus
HPV	Human papillomavirus
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis protein
ICTV	International committee on taxonomy of viruses
IFN	Interferon
IFN- $\beta$	Interferon beta
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IRF-1	Interferon regulatory factor 1

IRF-3	Interferon regulatory factor 3
MAPV	Maporal virus
MAVS	Mitochondrial antiviral signalling protein
MCV	Molluscum contagiosum virus
MHC	Major histocompatibility complex
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
MxA	Antiviral myxovirus resistance A
NCR	Non-coding region
NF- $\kappa$ B	Nuclear factor kappa light chain enhancer of activated B cells
NK cell	Natural killer cell
NSs	Non-structural protein
ORF	Open reading frame
PARP	Poly adenosine diphosphate-ribose polymerase
PCDH-1	Protocadherin-1
PHV	Prospect Hill virus
PKR	Protein kinase R
PLA	Proximity ligation assay
PUUV	Puumala virus
RANTES	Regulated on activation, normal T cell expressed and secreted
RER	Rough endoplasmic reticulum
RIG-I	Retinoic acid-inducible gene 1
RIP-1	Receptor-interacting serine/threonine protein kinase 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAMHD-1	Sterile alpha motif domain and histidine-aspartate domain-containing protein 1
SEOV	Seoul virus
SERP-2	Serine proteinase inhibitor 2
SMAC	Second mitochondria-derived activator of caspases
SNV	Sin Nombre virus
SPI-1	Serine protease inhibitor 1
SPI-2	Serine protease inhibitor 2
STAT-1	Signal transducer and activator of transcription 1

STS	Staurosporine
TANK	TRAF family member-associated NF-kB activator
TBK-1	TANK-binding kinase 1
TNF	Tumour necrosis factor
TNFR-1	TNF receptor-1
TNFR-2	TNF receptor-2
TNFRSF10A	TNF receptor superfamily member 10A (alternatively DR4 and TRAIL-R1)
TNFRSF10B	TNF receptor superfamily member 10B (alternatively DR5 and TRAIL-R2)
TRADD	TNF receptor-associated protein with death domain
TRAF	TNF receptor-associated factor
TRAF3	TNF receptor-associated factor 3
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R1	TNF-related apoptosis-inducing ligand receptor 1
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
TULV	Tula virus
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
Ub	Ubiquitin
UL37	Long unique region 37
UPS	Ubiquitin-proteasome system
vBCL-2	Viral B cell lymphoma 2
VEGF	Vascular endothelial growth factor
VE-cadherin	Vascular endothelial cadherin
vFLIP	Viral FLICE-like inhibitory protein
vIAP	Viral inhibitor of apoptosis protein
XIAP	X-linked inhibitor of apoptosis protein

# 1 INTRODUCTION

## 1.1 HANTAVIRUS – OF MICE AND MEN, AND A VIRUS

The history of orthohantaviruses – henceforth referred to as hantaviruses – and the diseases they cause in humans together represent a tapestry being woven at an incessant pace. No other group of zoonotic viruses spread by wild animals and causing disease in humans is as widely distributed on the planet Earth as the hantaviruses are. The diseases caused by these viruses in humans range from complete asymptomatic infections to sudden death within hours after appearance of severe symptoms; this being partly dependent on which hantavirus causes the infection.

### 1.1.1 A new zoonotic agent – unravelling a mystery

Hantavirus-associated diseases have been recognised for hundreds of years, but not until some decades ago was a particular infectious agent associated to the disease now recognised as haemorrhagic fever with renal syndrome (HFRS). Already more than 1,000 years ago, physicians in China had encountered a disease characterised by a triad of fever, haemorrhagic manifestations and renal impairment (1). The English sweating disease, dating back to 1485, might have also been caused by a hantavirus. It developed into five devastating epidemics in England and mainland Europe, with no respect for wealth or rank, killing major personalities at the time such as Henry Brandon, 2<sup>nd</sup> Duke of Suffolk, and his brother Charles Brandon (2, 3). Through years of trench warfare humans have encountered infectious diseases clinically compatible with the above-mentioned Chinese haemorrhagic syndrome and the English sweating disease. Although backed by poorly substantiated records, there exist reports of trench nephritis during the American Civil War (1861 to 1865)(4, 5), and through the First World War (1914 to 1918) cases of haemorrhagic fevers, also defined as trench nephritis, were reported (6, 7). Songo fever was described during the invasion of Manchuria (1931) and the Second Sino-Japanese war (1937 to 1945)(8, 9). The term nephropathia epidemica was coined in Sweden in the 1930s after describing a mild form of haemorrhagic fever with renal involvement (10, 11), and ‘feldnephritis’ was reported in more than 10,000 Finnish and German soldiers in Russia and Finland in the years 1941 and 1942 during the Second World War (1939 to 1945)(12, 13). Later on, during the Korean war (1951 to 1953), international concern increased when more than 3,000 Korean and North American soldiers fell severely ill from Korean haemorrhagic fever; a severe infectious disease characterised by fever, renal failure, haemorrhage and shock, and with a lethality of more than 10% (14-16).

Many hypotheses emerged regarding the cause of these haemorrhagic syndromes, until in 1976 Dr. Ho-Wang Lee and collaborators isolated the etiologic agent of the Korean haemorrhagic fever, which they named Hantaan virus (HTNV)(17, 18). The first confirmed isolate was obtained from lung tissue from a striped field mouse of the species *Apodemus agrarius* captured in the village of Songnaeri, located in close proximity to the river Hantaan in South Korea (18, 19). In 1982 the newly discovered HTNV was successfully propagated in the human carcinogenic lung epithelial cell line A549, and electron microscopy studies and

nucleic acid analyses revealed that it represented a new genus within the *Bunyaviridae* family (20-22). Subsequently, other viruses antigenically related to HTNV were described and classified into the *Bunyaviridae* (23-25). Parallely have rodents been defined as the main, although not exclusive, natural reservoirs of hantaviruses. This represented quite a revelation at the time, since all other genera of viruses within the *Bunyaviridae* family were strictly carried by arthropods.

Recent taxonomical shifts established by the International Committee on Taxonomy of Viruses (ICTV) in 2017 have resulted in the reclassification of hantaviruses into the *Orthohantavirus* genus, within the newly created *Hantaviridae* family and under the *Bunyavirales* order (earlier known as the *Bunyaviridae* family)(26, 27).

### **1.1.2 The hantaviruses – an emerging concern**

Shortly after the identification of HTNV, Lee and collaborators identified another HFRS-causing hantavirus in South Korea. The newly discovered virus, the Seoul virus (SEOV), was in this case carried by the common brown rat (*Rattus norvegicus*)(28). Early in 1980 the first European hantavirus was isolated from bank voles trapped nearby the village of Puumala in Finland (29). Puumala virus (PUUV) was found to be the causative agent of nephropathia epidemica, the milder form of HFRS that had been described in 1934 by two Swedish physicians independently (30, 31). In 1992, a hantavirus with a mortality rate of up to 20% and with high genetic similarity to HTNV was described as a new hantavirus serotype (32). The pathogenic virus was named Dobrava virus (DOBV) after isolation from its rodent vector, a yellow-necked field mouse (*Apodemus flavicollis*) trapped in Dobrava, Slovenia (33). However, it was not until the military conflict in Bosnia (1995), when more than 300 individuals contracted acute hantavirus symptoms, that DOBV was proven as being pathogenic for humans (34, 35).

The non-pathogenic Prospect Hill virus (PHV), discovered in 1982, represented the first hantavirus to be identified in the Americas (36, 37). The fact that it was a non-pathogenic virus nurtured the incorrect thought that American hantaviruses, unlike many of the hantaviruses until then found in Eurasia, were harmless to humans. But just a decade after the discovery of PHV, in 1993, a sudden outbreak of a mysterious influenza-like disease characterised by fever, myalgia, and rapidly evolving into often fatal outcomes, occurred in the southwestern region of Four Corners in the United States (defined by the shared borders between the states of Arizona, Colorado, New Mexico, and Utah). Patients fell rapidly ill and succumbed shortly after as a result of septic shock or pulmonary oedema; the epidemic resulted in mortality rates of 50% (38, 39). These symptoms had not previously been associated with hantavirus-caused disease, but extensive laboratory efforts revealed that a hantavirus was indeed the culprit and the causative agent of the outbreak. Unlike with HFRS, where the kidneys represent main target organs, the lungs were largely affected in what would become later on known as hantavirus pulmonary syndrome (HPS). The epidemic, caused by Sin Nombre virus (SNV) carried by the deer mouse (*Peromyscus maniculatus*) (40, 41), raised the alarm globally and resulted in public health concerns regarding hantaviruses,

their natural hosts and their transmissibility to humans (42). Another outbreak of HPS occurred soon after in Argentina, starting in 1995 (43), and further developing during 1996 with high mortality rates (44, 45). The epidemic had been caused by Andes virus (ANDV), carried by the long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*) (46, 47). ANDV remains as the main causative agent of HPS in South America and, interestingly, it is the only hantavirus for which human-to-human transmission has been confirmed (44, 45, 48). Additional outbreaks of HPS have been occurring both in North and South America with case fatality rates of 30 to 40% (49-53). Such epidemics have kindled the interest in hantavirus-associated diseases and increased the surveillance for similar illnesses, resulting in the discovery of novel hantaviruses, and highlighting the importance of continuously researching these zoonotic viruses and the concomitant diseases further.

#### 1.1.2.1 *Hantavirus variants and their natural hosts*

Hantaviruses, and their corresponding natural hosts, are distributed worldwide. Up to the present time, more than 60 different hantaviruses have been identified whereof 22 are pathogenic to humans (54-57). Although a few of these represent new strains of viruses already known, many are officially recognised hantaviruses. Each hantavirus is associated with a particular natural host, or a few closely related species. Most hantaviruses have been identified in cricetid and murid rodents of the subfamilies *Arvicolinae*, *Murinae*, *Neotominae* and *Sigmodontinae* (Table 1). Insectivores such as moles (*Talpidae* and *Scalopinae*), shrews (*Soricinae*, *Crocidurinae* and *Myosoricinae*) and bats (*Chiroptera*), act also as natural reservoirs of hantaviruses (Figure 1) (58-60). Additionally, new reports have demonstrated the presence of hantavirus in a plethora of other small mammals, reptiles and fish, showing that the genetic diversity of hantaviruses is much greater than previously estimated (61, 62). The rodent-borne hantaviruses are, to date, the only hantaviruses known to be pathogenic to humans.

**Table 1.** Representative rodent-borne hantaviruses.

<b>Virus</b>	<b>Disease</b>	<b>Rodent host</b>	<b>Location</b>	<b>Reference</b>
<u><i>Murinae</i> subfamily-associated viruses</u>				
Da Bie Shan	-	Chinese white-bellied rat ( <i>Niviventer confucianus</i> )	China	(63)
Dobrava	HFRS	Yellow-necked field mouse ( <i>Apodemus flavicollis</i> )	Slovenia	(33)
Hantaan	HFRS	Striped field mouse ( <i>Apodemus agrarius</i> )	South Korea	(17)
Saaremaa	HFRS	Striped field mouse ( <i>Apodemus agrarius</i> )	Estonia	(64)
Sangassou	-	African wood mouse ( <i>Hylomyscus agrarius</i> )	Guinea	(65)

Seoul	HFRS	Black rat ( <i>Rattus rattus</i> ), Brown rat ( <i>Rattus norvegicus</i> )	South Korea	(28)
Soochong / Amur	HFRS	Korean field mouse ( <i>Apodemus peninsulae</i> )	South Korea / Russia	(66-68)
Thailand	-	Bandicoot rat ( <i>Bandicota indica</i> )	Thailand	(69)
Tigray	-	Ethiopian white-footed mouse ( <i>Stenocephalemys albipes</i> )	Ethiopia	(70)

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Arvicolinae subfamily-associated viruses

Bloodland Lake	-	Prairie vole ( <i>Microtus ochrogaster</i> )	USA / Canada	(71)
Hokkaido	-	Red bank vole ( <i>Myodes rufocanus</i> )	Japan	(72)
Isla Vista	-	California vole ( <i>Microtus californicus</i> )	USA	(73)
Khabarovsk	-	Reed vole ( <i>Microtus fortis</i> )	Russia	(74)
Muju	HFRS	Royal vole ( <i>Myodes regulus</i> )	South Korea	(75)
Prospect Hill	-	Meadow vole ( <i>Microtus pennsylvanicus</i> )	USA / Canada	(36)
Puumala	HFRS	Bank vole ( <i>Myodes glareolus</i> )	Finland	(29)
Tatenale	-	Field vole ( <i>Microtus agrestis</i> )	United Kingdom	(76, 77)
Topografov	-	Lemming ( <i>Lemmus sibericus</i> )	Russia	(78)
Tula	HFRS	European common vole ( <i>Microtus arvalis</i> )	Russia	(79)
Vladivostok	-	Reed vole ( <i>Microtus fortis</i> )	Russia	(80)

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Sigmodontinae subfamily-associated viruses

Andes	HPS	Long-tailed pygmy rice rat ( <i>Oligoryzomys longicaudatus</i> )	Argentina	(43)
Araraquara	HPS	Hairy-tailed bolo mouse ( <i>Bolomys lasiurus</i> )	Brazil	(81, 82)
Bayou	HPS	Rice rat ( <i>Oryzomys palustris</i> )	USA	(83)
Bermejo	HPS	Chacoan pygmy rice rat ( <i>Oryzomys chacoensis</i> )	Argentina	(84)

Black Creek Canal	HPS	Cotton rat ( <i>Sigmodon hispidus</i> )	USA	(85)
Calabazo	HPS	Cane mice ( <i>Zygodontomys brevicauda</i> )	Panama	(86)
Caño Delgadito	-	Alston's cotton rat ( <i>Sigmodon alstoni</i> )	Venezuela	(87)
Castelo Dos Sonhos	HPS	Pygmy rice rat ( <i>Oligoryzomys utiaritensis</i> )	Brazil	(81, 88)
Catacamas	-	Coues's rice rat ( <i>Oryzomys couesi</i> )	Honduras	(89)
Choclo	-	Pygmy rice rat ( <i>Oligoryzomys fulvescens</i> )	Panama	(86)
Hu39694	HPS	Long-tailed mouse ( <i>Oligoryzomys flavescens</i> )	Argentina	(84, 90)
IP37/38	-	Black-footed pygmy rice rat ( <i>Oligoryzomys nigripes</i> )	Paraguay	(91)
Juquitiba	HPS	Black-footed pygmy rice rat ( <i>Oligoryzomys nigripes</i> )	Brazil	(82, 92)
Laguna Negra	HPS	Veser mouse ( <i>Calomys laucha</i> )	Paraguay	(93)
Lechiguanas	HPS	Rice rat ( <i>Oligoryzomys flavescens</i> )	Argentina	(84)
Maciel	-	Dark field mouse ( <i>Necromys benefactus</i> )	Argentina	(84)
Maporal	-	Pygmy rice rat ( <i>Oligoryzomys fulvescens</i> )	Venezuela	(94, 95)
Muleshoe	-	Hispid cotton rat ( <i>Sigmodon hispidus</i> )	USA	(96)
Orán	HPS	Long-tailed pygmy rice rat ( <i>Oligoryzomys longicaudatus</i> )	Argentina	(46)
Pergamino	-	Grass field mouse ( <i>Akodon azarae</i> )	Argentina	(84)
Playa De Oro	-	Coues's rice rat ( <i>Oryzomys couesi</i> ), Jaliscan cotton rat ( <i>Sigmodon mascotensis</i> )	Mexico	(97)
Rio Mamoré	-	Small-eared pygmy rice rat ( <i>Oligoryzomys microtis</i> )	Bolivia	(98, 99)

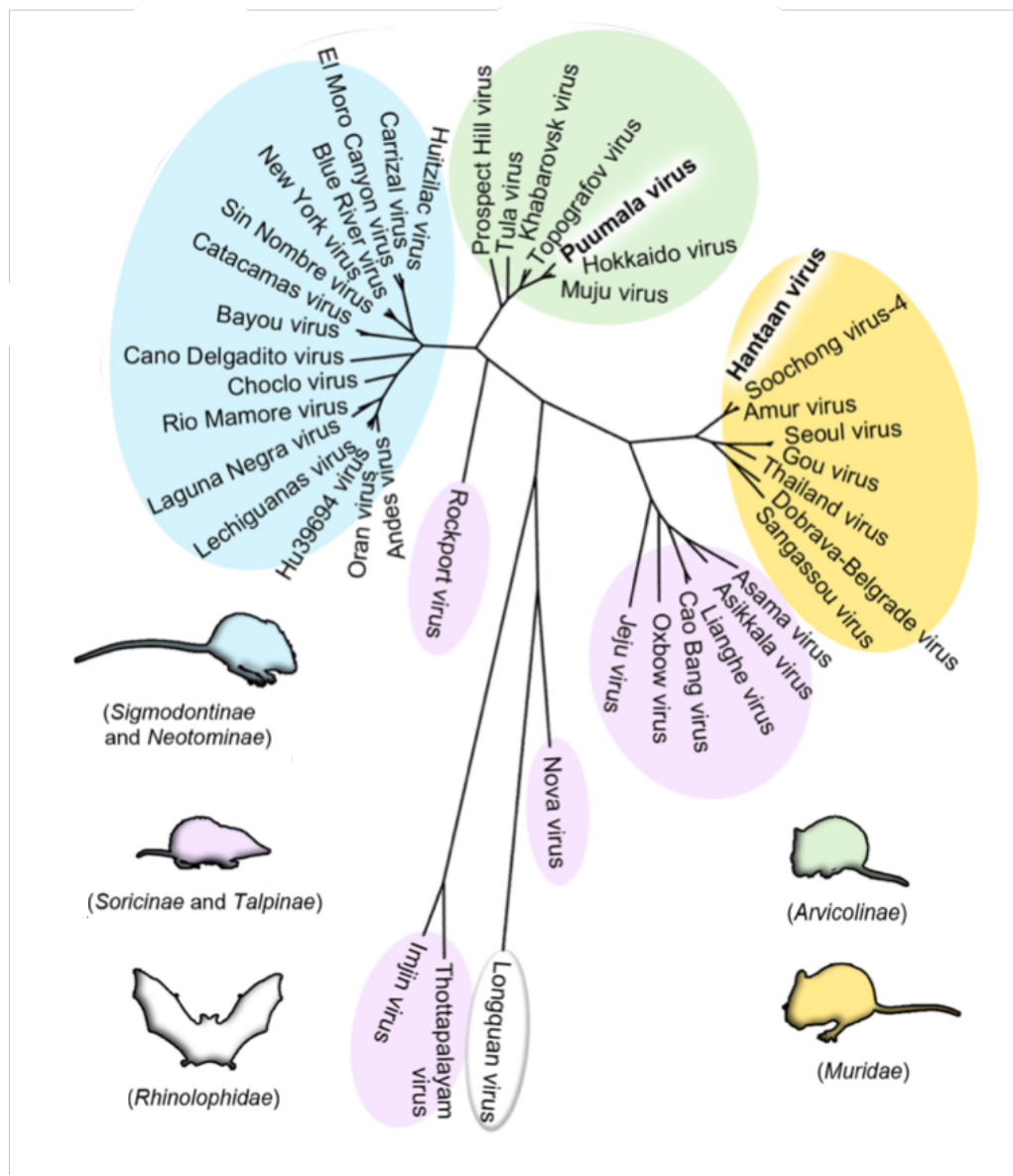
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Neotominae subfamily-associated viruses

Blue River	-	White-footed mouse ( <i>Peromyscus leucopus</i> )	USA	(100)
El Moro Canyon	-	Western harvest mouse ( <i>Reithrodontomys megalotis</i> )	USA	(101)
Limestone Canyon	-	Brush mouse ( <i>Peromyscus boylii</i> )	USA	(102)
Monongahela	HPS	Deer mouse ( <i>Peromyscus maniculatis</i> )	USA	(103)
New York	HPS	White-footed mouse ( <i>Peromyscus leucopus</i> )	USA	(104, 105)
Rio Segundo	-	Mexican harvest mouse ( <i>Reithrodontomys mexicanus</i> )	Costa Rica	(106)
Sin Nombre	HPS	Deer mouse ( <i>Peromyscus maniculatis</i> )	USA	(107, 108)

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Hantaviruses with almost identical RNA and protein sequences are carried by genetically-related rodents; in fact, analyses of mitochondrial genes from rodents and viral genetic sequences result in phylogenetic trees of high similarities (109). Although recent investigations on the evolution of hantaviruses have provided evidence for cross-species transmission and host-switching (58, 109, 110), other phylogenetic analyses indicate trends of co-divergence between hantaviruses and their respective natural hosts over a scale of time of millions of years (111-113). Rodent-borne hantaviruses tend to cluster according to whether their specific natural hosts belong to the *Cricetidae* family or to the *Muridae* family; and phylogenetically related hantaviruses are at times harboured by phylogenetically related hosts that are distributed in separate geographic regions (58). That is for instance the case of PHV isolated from meadow voles (*Microtus pennsylvanicus*) in the United States, Tula virus (TULV) isolated from the European common vole (*Microtus arvalis*), and Khabarovsk virus circulating in reed voles (*Microtus fortis*) in Russia (36, 74, 79). Another perhaps more surprising example is that of the first ever discovered hantavirus, the Thottapalayam virus (TOTV) isolated in India in 1964 from a musk shrew (*Suncus murinus*) (114), and the other newly isolated hantaviruses carried also by different species of shrews trapped in the United States, Switzerland and Vietnam (115-118). Although host-switching between different species could explain the occurrence of distinct hantaviruses in shrews which are distributed in far-distanced geographical locations, there is reason to believe that co-evolution between the viruses and the shrews has occurred since the divergence of *Soricidae* and *Rodentia*, around 100 million years ago (111, 119). All in all, the evolutionary history of hantaviruses probably results from the combination of co-divergence as well as cross-species transmission (112, 120).



**Figure 1.** A phylogeny reconstruction based on Gn glycoprotein sequences separating a total of 42 hantavirus species according to host reservoir. The clades of rodent-borne hantaviruses are annotated as blue (*Sigmodontinae* and *Neotominae*), green (*Arvicolinae*) and yellow (*Muridae*). Shrew- and mole-borne hantaviruses are coloured in light purple (*Soricinae* and *Talpinae*). The bat-borne Longquan virus is annotated in white (*Rhinolophidae*). Adapted from Rissanen, I. *et al.*, 2017 (59) under the terms of the Creative Commons CC BY license.

### 1.1.3 Transmission – the unseen enemy

Species-specific hantaviruses are transmitted horizontally within natural host populations(121). The hosts remain persistently and asymptotically infected. Shedding of virus occurs through saliva, urine and faeces (122-124). Transmission within rodent populations usually happens when vegetation is abundant and reproductive activities are high (125, 126). Increased social interactions and aggressive encounters (involving biting and increased urine production), especially during reproductive season, have been reported as behavioural aspects facilitating infection and viral spread (127-129). Also, environmental factors such as precipitation, temperature, habitat quality, food availability, food source and

predation, cause fluctuations in the populations of rodents and may thus affect the prevalence of hantaviruses (130-132).

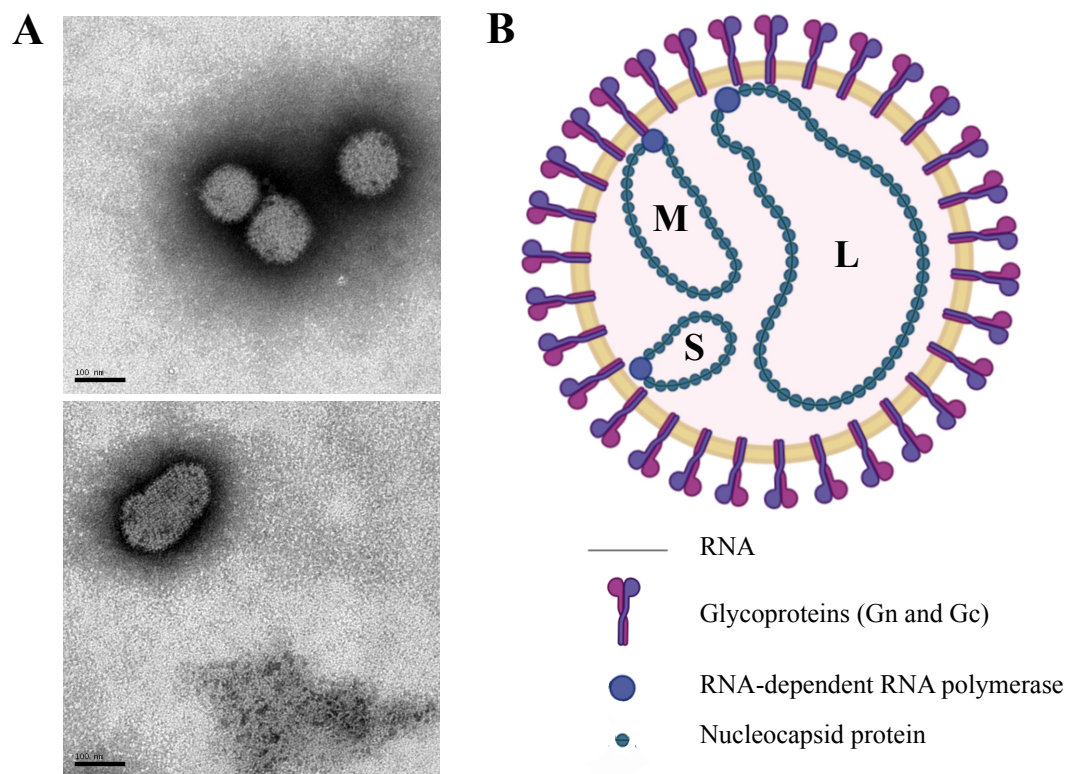
The emergence of hantavirus infections in humans is correlated with increased rodent population densities as well as human behaviour (132, 133). Transmission of rodent-borne hantaviruses to humans normally occurs when people come in contact with aerosolized secretions such as urine and faeces from infected rodents. Activities in the nature that make probable the contact of humans with the habitats of hantavirus natural reservoirs (camping, military exercises, hunting, farming, rodent trapping) increase the risk for infection. The renovation of buildings and the cleaning of, for example, summer cabins and attics represent as well anthropogenic risk factors, since humans may be encountering rodent nests and rodent-infested areas (134-138). In addition, a report documented the transmission of PUUV through transfusion of platelets and other blood products (139), and cases of laboratory-acquired HFRS have been also described (140, 141), although not related to hantavirus research but rather to the unknown usage of hantavirus-infected laboratory animals.

Cases of human-to-human transmission have remained so far exclusive to the South American ANDV (44, 45, 48). Although transmission was verified by both epidemiological analyses and molecular methods, the exact mechanism is still elusive. Family members and sexual partners have been identified as clusters at greater risk for interpersonal transmission, but direct sexual transmission *per se* still remains uncertain (48, 142). Notably, Godoy and collaborators reported the shedding of both ANDV antigens and infectious viral particles in urine of HPS patients (143). Despite the fact that human-to-human transmission seems to be unique for ANDV, viral RNA has been detected in tracheal secretions from HPS patients with SNV infection (144), as well as found in saliva from HFRS patients with PUUV infection (145).

#### **1.1.4 Structure and replication – the clockwork of a dreaded entity**

As the other members of the order *Bunyavirales*, hantaviruses are spherical enveloped viral entities (146). Hantavirus particles can display certain pleomorphism (i.e. shape and size vary) giving them a diameter ranging from 100 to 160 nm (Figure 2A)(147). The genome of hantaviruses is composed of three negative-sense single-stranded RNA segments: the small (S, 1.8-2.1 kb), the medium (M, 3.7-3.8 kb) and the large (L, 6.5-6.6 kb) segments, respectively encoding for the nucleocapsid protein (N), the glycoprotein pre-cursor (GPC) of the glycoproteins Gn and Gc (previously known as G1 and G2, respectively), and the RNA-dependent RNA polymerase (RdRp)(Figure 2B). The 3'- and 5'- terminal ends of each genome segment are characterised by their capacity to form a panhandle structure through the interaction of complementary nucleotides. Terminal deletions in the hantavirus genome segments correlate with decreased viral replication in cultures of infected cells, suggesting that the panhandle structure acts as the viral promoter (148, 149). The 3' non-coding region (NCR) of the S segment is perhaps the most puzzling component of the hantaviral genome since it presents a variable length of 229 nucleotides (PHV) to 728 nucleotides (SNV), and considerable differences in nucleotide sequence composition (111). Investigations with other

RNA viruses have unveiled crucial roles for the 3'-NCR in viral replication and infectivity (150). Interestingly a recently published study identified significantly longer 3'-NCR in HPS-causing hantaviruses when compared to HFRS-causing hantaviruses (151). The RNA genome segments are encapsidated by trimers of the nucleocapsid protein, forming ribonucleoprotein, that together with the RdRp remain enclosed inside the virus particle. The virions present a host-derived lipid envelope of about 5 nm in thickness decorated with spike-shaped structures protruding about 10 nm from the membrane (152). These spikes are formed by four units of each of the two glycoproteins, Gn and Gc, and have four-fold rotational symmetry (147, 152).



**Figure 2.** Hantavirus virion structure. The morphology of hantavirus particles can be spherical (A, up) but virions can also display certain pleomorphism, as observed by electron microscopy (A, down). The enveloped hantavirus virion has a tri-segmented RNA genome consisting of the segments S, M and L which are coiled around trimers of nucleocapsid protein. The viral genome is replicated and transcribed by the RNA-dependent RNA polymerase, which associates to each one of the three RNA segments inside the virus particle. The spike-like structures on the outer part of the virion comprise four units of each glycoprotein, Gn and Gc (B). Scale bar, 100 nm. The electron microscopy images were kindly provided by Dr Jonas Klingström.

#### 1.1.4.1 The structural proteins

As mentioned above, the hantavirus particles represent rather simplistic entities carrying only four structural proteins. Having such a limited repertoire of proteins must mean that one, and probably more, of the encoded proteins displays multifunctionality in order to ensure viral infection, replication and propagation.

The N protein of hantaviruses is encoded by the S segment and has a molecular mass of approximately 50 kDa (153). This non-glycosylated protein has some conserved

regions among hantaviruses and represents the most abundant of the viral proteins found both in virions and in the cytoplasm of infected cells, where it appears to be distributed forming filamentous and granular patterns (154). Some reports indicate the accumulation of N protein in cellular processing (P-)bodies (155) and also its aggregation in inclusion bodies (156), which could explain the observed granular distribution by immunofluorescence and histology (121, 157). Alternatively, despite not having a typical transmembrane region, the N protein appears to be also membrane-associated and localizes to the perinuclear region in infected cells (158). The reported inclusion of the N protein in P-bodies would strongly suggest the involvement of N in virus replication.

The hantaviral N protein tends to oligomerise in trimers and plays an important role in RNA packaging and in protection of the RNA genome (159-162). It is also involved in replication and assembly of the virus (154). Through years of research studies, the N protein has also been shown to specifically interact with cellular components of the cytoskeleton and many other host proteins. For instance, the N protein of Black Creek Canal hantavirus (BCCV) binds monomeric actin and colocalises with actin filaments (163). Otherwise the N protein of HTNV, ANDV, SEOV and also BCCV interact with the microtubule network (164). Viruses use cytoskeletal proteins during the budding step through cellular membranes (165). Not surprisingly, disruption of the host cytoskeleton has been shown to impair virus propagation in the abovementioned hantavirus settings (164). Recently the N protein of ANDV was shown to hamper the autophosphorylation of TANK-binding kinase 1 (TBK1), resulting in no phosphorylation of interferon regulatory factor 3 (IRF-3) and subsequent inhibition of type 1 interferon (IFN) antiviral response (166). ANDV N protein also inhibits the IFN response and the establishment of the cell's antiviral state by preventing protein kinase R (PKR) phosphorylation and dimerization, needed for PKR's enzymatic activity (167). Active PKR stimulates IFNs via nuclear factor  $\kappa$ B (NF- $\kappa$ B) and interferon regulatory factor 1 (IRF-1) up-regulation, thus hindering viral replication and promoting the establishment of an antiviral state in a cell (168, 169). Additionally, PKR mediates apoptosis in other viral settings such as influenza and picornavirus infections (170-173); therefore, inhibition of PKR activity by the N protein may contribute to protecting infected cells also from programmed cell death.

Recent publications have strengthened the belief that the hantaviral N protein plays a role in apoptosis regulation. On the one hand, pull down assays have indicated the interaction of the N protein of PUUV with the apoptosis enhancer Death-associated protein 6 (DAXX)(174). On the other hand, the N protein of different hantaviruses has been shown to interact with both granzyme B and caspase-3, and to directly inhibit the enzymatic activity of the latter two in infected cells (175)(**Paper I**). Further reports support the inhibitory effect of the N protein on caspases' activation in cells (176, 177). Other studies have documented the interaction of hantavirus N protein with importin- $\alpha$  (178), which is involved in NF- $\kappa$ B nuclear transport, and with components of the sumoylation pathway (i.e. a posttranslational modification implicated in cell proliferation, differentiation and senescence), such as SUMO-

1 and Ubc9 (179, 180), involved in nuclear trafficking and transcriptional regulation of proteins (181).

Hantavirus glycoproteins are coded by the M segment as a polyprotein precursor (GPC). The mature glycoproteins Gn and Gc result from the co-translational cleavage of GPC at the conserved WAASA motif (182). Glycosylation of both glycoproteins occurs during the maturation process in the Golgi complex (183-186). Both mature glycoproteins contain a cytoplasmic tail (CT). While the Gc-CT is a minute structure of only about 10 amino acid residues, the Gn-CT represents a construct of approximately 110 amino acid residues (147). Hantaviruses do not express any matrix proteins but it has been postulated that the Gn-CT could act as a surrogate matrix protein by interacting with the ribonucleoprotein complexes (187, 188).

The heterodimers of Gn and Gc that decorate the surface of hantavirus particles allow for host receptor recognition and may interact with  $\beta 3$  integrins expressed on the surface of target cells (189, 190). In addition, some reports have suggested an additional role for Gn-CT as modulator of host immune responses to infection. The Gn-CT of the pathogenic New York hantavirus has been shown to interact with retinoic acid inducible gene I (RIG-I) and to disrupt TNF receptor-associated factor 3 (TRAF3)-TBK1 complexes, in both instances resulting in inhibition of cellular IFN responses (191, 192). Similar findings have been documented during TULV infection of endothelial cells (193, 194). Finally, the glycoproteins of the hantaviruses ANDV and PHV have been implicated in the downregulation of IFN signalling by hampering nuclear translocation of the signal transducer and activator of transcription 1 (STAT-1)(195).

Lastly, the RdRp of hantaviruses represents the largest of the encoded proteins and derives from the L segment (196). It possesses transcriptase, replicase and endonuclease activity; thus it allows both viral transcription as well as replication (197). Since hantaviruses are negative-stranded RNA viruses, the RdRp is carried within the viral particles; it associates with the ribonucleoprotein complexes, composed of N protein and viral RNA, so that viral RNA synthesis can be initiated as soon as the virus has entered the host cell (159, 198).

#### ***1.1.4.2 Non-structural proteins***

In addition to the structural proteins, the S segment of some hantaviruses carried by rodents of the *Cricetidae* family (such as PUUV, TULV and ANDV) contains an alternative open reading frame (ORF) encoding for a non-structural protein (NSs)(199, 200). The NSs protein has been reported to have immunomodulatory functions, such as acting as a weak inhibitor of beta interferon (IFN- $\beta$ ) responses and hindering the activity of NF-kB and IRF-3 (200). The functionality of NSs as an immune modulator is further supported by investigations reporting that TULV expressing the ORF for NSs can be consequently passaged in IFN-competent cells for longer than TULV expressing a non-coding, truncated ORF for NSs (201). The NSs localises transiently in the perinuclear area in infected cells and its interaction with proteins of

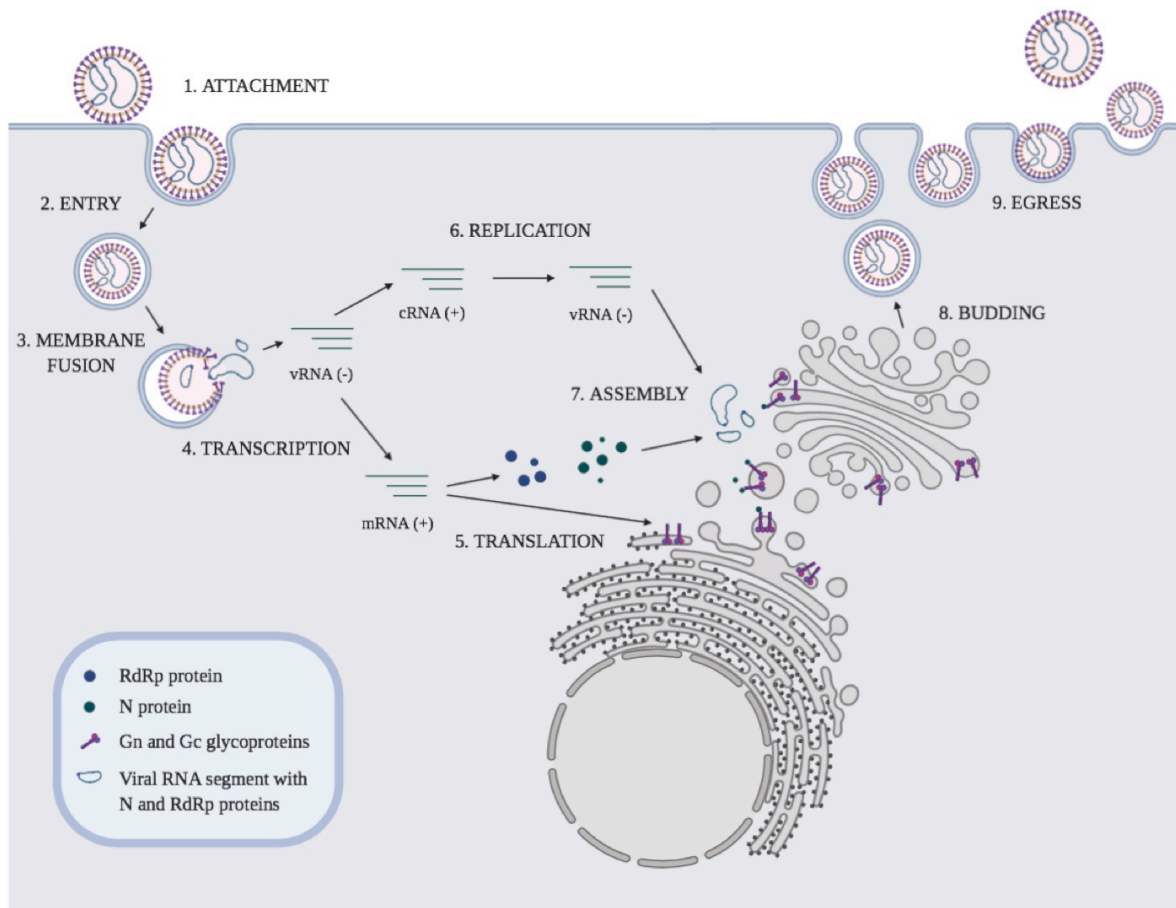
the Golgi apparatus indicates that it may play other yet unidentified roles during infection (202, 203).

#### **1.1.4.3 Viral entry, replication and egress**

Endothelial cells represent the main targets of hantaviruses (204). After initial infection of the lung endothelium, the virus spreads systemically through the human body (54, 204). Hantaviruses are also capable of infecting human epithelial, dendritic, macrophage and lymphocyte cells through the attachment of the hantaviral glycoproteins to the surface receptors of the host's cells (204-211). Although it has been reported that  $\alpha V\beta 3$  and  $\alpha 5\beta 1$  integrins act as the entry receptors for hantavirus glycoproteins *in vitro*, there is little support for such results *in vivo* (204, 212). Additionally, cells that do not express the above-mentioned integrins can be infected, demonstrating that hantaviruses do not solely rely on integrins for its successful entry into the target host's cells (213). Moreover, *in vitro* studies have shown that HTNV, PUUV and BCCV infect polarised epithelial cells mostly through the apical surface (214, 215), while ANDV can infect both via apical and basolateral surfaces (216). Notably, it is unclear whether or not  $\alpha V\beta 3$  integrins are expressed at the apical surface of cells (217-219). Other candidate receptors for hantaviruses are the decay-accelerating factor (DAF) and C1qR complement factor, both representing proteins mediating entry of hantaviruses at least in *in vitro* settings (214, 220-223). Recently, through genome wide genetic screening in human haploid cells, another factor, the protocadherin-1 (PCDH-1) was reported as having a crucial role in infectivity of the American hantaiviruses ANDV, SNV, PHV and Maporal virus (MAPV) both *in vitro* in PCDH-1 knock-out human cells and *in vivo* in PCDH-1 knock-out Syrian hamsters (224). These hantaviruses are found in the Americas; interestingly, knocking out PCDH-1 had no effect in HTNV and SEOV infectivity, both hantaviruses found mainly in Asia (222, 224). Additionally, in two different variants of the European PUUV, PCDH-1 also seems to be redundant for infection of human target cells (**Paper IV**).

As earlier described, entry of hantaviruses into target cells can occur both via the apical and the basolateral membrane surfaces (215, 216). While entry of HTNV has been reported as being clathrin-dependent (225), ANDV internalization has been shown as dependent on micropinocytosis and host factors such as cholesterol and Rho GTPase Rac1 (226, 227). Attachment of the viral particle to the cell's surface is followed by endocytosis, involving the movement of the virus from early endosomes subsequently into late endosomes. This vesicular trafficking is accompanied by a pH decrease or acidification within the vesicles, which results in pH-mediated membrane fusion and virus uncoating, liberating the three viral segments into the cytoplasm as ribonucleoprotein complexes with N protein (225, 228). The RdRp initiates then the transcription process of the RNA genome in order to produce mRNA from the S, M and L segments. The translation of the mRNA transcripts from both S and L segments occurs on free ribosomes, whereas the M segment's mRNA is believed to be translated on membrane-bound ribosomes on the rough endoplasmic reticulum (RER)(148). The GPC is proteolytically processed into Gn and Gc while in the ER

(184, 186). As mentioned in section 1.1.4.1, for many hantaviruses the proteolytic cleavage site in the GPC is presumed to be the WAASA motif, a conserved amino acid sequence located at the end of Gn (182). After glycosylation in the ER, the mature Gn and Gc dimerize, which seems to be a crucial interaction process in order for the glycoproteins to be transported to the Golgi complex, where Gn and Gc accumulate (163, 185, 229, 230). The key roles of N protein in translation, trafficking and assembly have been documented in several reports (155, 160, 161, 164, 231-233). It has been postulated that the cytoplasmic tails of the glycoproteins interact directly with the N protein during assembly in the Golgi complex (148).



**Figure 3.** Hantavirus replication cycle. After attachment of the virion to the host's cell surface receptors (1), the viral particle is incorporated through receptor-mediated endocytosis (2). The uncoating and release of the viral genome segments occurs in a pH-dependent manner (3). The complementary RNA is then obtained from the viral RNA through transcription (4). Each one of the three mRNA segments (corresponding to viral S, M or L genome segments) will be translated into the viral proteins (N, Gn, Gc and RdRp) using the host's machinery; the N and RdRp proteins are obtained in free ribosomes, and the glycoproteins in ribosomes located in the RER (5). After replication and amplification (6), the newly produced viral RNA will assemble with the N protein and be transported to the Golgi complex. Assembly of all components, including Gn and Gc, continues at the ERGIC and Golgi compartments (7). Finally, the newly produced virions bud in vesicles from the trans-Golgi network (8), thereafter being released into the extracellular space by exocytosis (9).

Successful packaging requires interactions between RdRp and the RNP, binding of the RNP complex to the glycoproteins, and further interactions between the spikes resulting from the tetrameric association between units of Gn and Gc (148). Like other members of the

order *Bunyavirales*, the assembly process of hantaviruses occurs at the ER-Golgi Intermediate Complex (ERGIC) and at the Golgi, from where these viruses obtain their membrane (56, 234). Thereafter the newly-produced virions are transported in vesicles and released through exocytosis at the plasma membrane (Figure 3)(148, 235). Alternatively, investigations based on electron microscopy suggest that for the American hantaviruses SNV and BCCV the maturation and budding can also occur directly at the infected cell's surface (156, 158, 215).

### **1.1.5 Hantavirus-associated diseases**

Rodent-borne hantaviruses can cause two severe diseases in humans; haemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and hantavirus pulmonary syndrome (HPS; also known as hantavirus cardiopulmonary syndrome), in the Americas. Hantavirus-associated diseases represent acute febrile infections that spread systemically, affecting the pulmonary, cardiac and renal systems. Vascular permeability, strong immune activation and hyperinflammatory reactions are common hallmarks observed both in HFRS- and HPS-patients (54, 56, 236, 237). On top of the acute-phase complications commonly reported in patients, long-term consequences such as chronic fatigue, shortness of breath, myalgias, proteinuria and hormonal dysregulation have been reported in patients up to several years after infection (55, 238-240).

#### ***1.1.5.1 Haemorrhagic Fever with Renal Syndrome (HFRS)***

Approximately 100,000 cases of HFRS are recognised annually. The great majority of these occur in China, accounting for over 90% of the reported cases, followed by Korea and Russia. In Europe, around 10,000 cases are diagnosed per year (55, 56). The course of the disease is surprisingly variable, ranging from asymptomatic infections to lethal outcomes, and depends upon the hantavirus causing the infection. The symptoms usually develop within three weeks after exposure to the virus, although in some reported cases it took up to eight weeks to develop the initial symptomatology after exposure (241-245). The initial symptoms may include fever, headaches, back and abdominal pain, chills, nausea, vomiting and blurred vision. Vascular leakage, acute shock and renal involvement (i.e. proteinuria, haematuria and acute kidney injury) represent later symptoms reported in HFRS patients (54-56).

HFRS progresses through five different stages: febrile, hypotensive, oliguric, polyuric and convalescent. While in some mild cases one or two of the above-mentioned may be absent, in severe cases all phases are present and frequently overlapping. Up to 40% of febrile patients develop hypotension, and oliguria (i.e. less than 400mL of daily urine output) is reported in approximately 40 to 60% of HFRS patients. Hypotensive patients tend to develop thirstiness, nausea and vomiting. These symptoms can last from hours to days. Further disease development into shock and mental confusion occurs in 30% of HFRS patients, together with vascular leakage, muscle aches and tachycardia. During the oliguric phase, which may last from one to sixteen days, at least a third of the patients experience internal haemorrhages. Moreover, it is at the oliguric phase when patients are at risk of

developing renal insufficiency and pulmonary oedema. The need for dialysis is reported in up to 60% of the patients in some cases and mortality associates with shock and/or haemorrhage, particularly during the oliguric phase (54-56).

Nephropathia epidemica (NE) represents a term used to describe HFRS caused by PUUV in Scandinavia (246). The proportion of subclinical cases is considerable and the seroprevalence is reasonably high – up to 8.9% in Northern Sweden (247, 248). NE is commonly characterised by a less severe febrile phase and occasionally presence of shock and haemorrhage. The renal involvement, although present, tends to be of less severity than in HFRS. The vagueness of symptoms documented during NE often results in misdiagnosis of the disease (249). With a case fatality rate of 0.1 to 1%, deaths by NE seldom occur (236, 250).

#### ***1.1.5.2 Hantavirus Pulmonary Syndrome (HPS)***

Between 700 and 800 cases of HPS have been reported in the United States since surveillance began in 1993 (251). In South America, more than 3000 cases have been reported since 1993, with the majority of the HPS patients being described in Argentina and Chile (252, 253). The early symptoms during HPS may develop between the first and the eighth week after viral exposure, although these data are based on limited documentation due to a much lower case incidence than HFRS (56, 254). Renal involvement reported during HFRS has been documented in some HPS cases as well (255). The severity and mortality rates associated with HPS are dependent upon the hantavirus infecting the human host (256-259). Severe cases of HPS have been reported in the United States, Canada, Panama and in numerous countries in South America, such as Chile, Argentina, Brazil and Paraguay. Interestingly, the presence of several cases of hantavirus infections without HPS would explain the considerable number of individuals with antibodies against hantaviruses but without recognizable HPS symptoms (260-263).

The first respiratory signs of disease appear after a long incubation period ranging from one to five weeks (254, 264). The abrupt onset of severe symptoms in HPS patients often occurs within one day after initial signs of respiratory problems. HPS produces lung capillary leakage rapidly escalating into respiratory failure and cardiogenic shock. The severest HPS patients are in need of mechanical ventilation and extracorporeal membrane oxygenation (ECMO)(50, 265). Renal involvement, as above mentioned, is sometimes reported (255, 266). HPS disease progression typically involves three phases: febrile, cardiopulmonary and convalescent. Patients develop a febrile prodrome similar in many ways to that described for HFRS and other viral diseases. Initial symptoms of fever and myalgia rapidly progress into thrombocytopenia, muscle pain, headache and diarrhoea. The cardiopulmonary phase is characterised by severe pulmonary oedema, as well as hypotension, oliguria and shock. The accumulation of fluid in the lungs represents a critical phase for HPS patients, since at this stage individuals may die within twenty-four to forty-eight hours (267). Survivors proceed through the cardiopulmonary phase and in general recover after a convalescent phase of more than two months (50, 268-272).

### **1.1.6 Treatment, prevention and control – an unfulfilled need**

The outbreaks of hantaviruses in human populations as well as the pathogenicity associated with such infectious agents represent a considerable public concern because currently there are neither effective treatment options nor FDA-approved vaccines. Therapy remains based on supportive care including electrolyte balance management, haemodialysis, hemofiltration, mechanical ventilation, and/or ECMO (50, 265, 273-275).

Although hantaviruses cause severe human diseases all around the globe, there is still a considerable lack of knowledge regarding both the entry mechanisms used by this group of zoonotic viruses and the pathogenicity triggered in humans upon infection. This poor understanding remains as one of the major hurdles towards the development of effective new therapies for treatment of hantavirus-associated diseases.

#### ***1.1.6.1 Prevention of transmission and awareness***

Hantaviruses are easily inactivated by detergents, general disinfectants and chlorine solutions (276). Avoiding the production of aerosols is crucial when removing potentially-infected rodent excreta, therefore wet wiping is preferred over the use of a broom, a duster or vacuum cleaning (277). In order to avoid transmission of hantaviruses from natural reservoirs to humans, prevention should be based on measures independent of the rodent population dynamics. Efforts should be made to raise awareness about hantaviruses, educate physicians and those populations potentially at risk and reduce the contact between humans and rodents. Since many HFRS/HPS-symptoms are unspecific or ‘flu-like’, knowledge on the patients’ travel history, early diagnostic testing and the physician’s awareness on hantavirus-associated diseases may be of importance for early implementation of supportive treatment and patient survival.

#### ***1.1.6.2 Antiviral drugs***

Ribavirin and 1- $\beta$ -D-ribofuranosyl-3-ethynyl-(1,2,4) triazole (ETAR) represent antiviral drugs with demonstrated *in vitro* and *in vivo* effects on hantavirus replication (278-281). Many DNA and RNA viruses succumb to the antiviral activity of this broad-spectrum chemical compound (282, 283). Ribavirin targets the *de novo* synthesis of GTP, affects capping and translation of mRNA, and can inhibit the activity of viral polymerases (282). As such, the effect on hantavirus replication is rather virus-unspecific.

Although treatment with ribavirin has shown some promising effects, administration of ribavirin or any other antiviral drug may prove inefficient for treatment of HFRS/HPS-patients, since when the symptoms start appearing, viremia is already decreasing in patients. On the one hand, clinical studies involving testing of ribavirin intravenous therapy on HFRS-patients have yielded encouraging results showing significant reduction in morbidity among ribavirin-treated patients (284, 285). On the other hand, intravenous ribavirin has been reported as ineffective for the treatment of HPS-patients (286, 287). Other antiviral drugs

reported to successfully inhibit hantavirus infection *in vitro* and *in vivo* in suckling mice and hamsters are lactoferrin (288, 289) and favipiravir (290-292).

#### **1.1.6.3 Vaccines**

In order to induce a robust antiviral state, vaccination stands as the most successful approach offering protection against viral infections. To date, no FDA-approved vaccine against hantaviruses is available, although some virus-inactivated vaccines have been licensed in some countries in East Asia (293-295). Hantavax™ has been in use in China and South Korea already for a number of years (293). The vaccine is well-tolerated in humans, and its implementation in South Korea translated into a reduction in the total number of hospitalised HFRS-patients from 1234 to 687 cases between in 1991 and 1996 (296, 297). However in a recent phase III clinical trial, poor neutralizing antibody generation was reported despite the fact that seroconversion rates were high (298). Another case-control study reported moderate protective efficacy generated by the vaccine (299). Furthermore, the vaccine is derived from formalin-inactivated HTNV purified from infected suckling mouse brain. Producing the vaccine by formalin inactivation of virus grown in rodent brain tissue represents a controversial method which has prevented its implementation in many countries (300).

Alternatively, live-attenuated vaccines and DNA-based, subunit vaccines have been developed and investigated. Although live-attenuated vaccines using recombinant vesicular-stomatitis virus (VSV) or adenovirus vectors expressing Gn and Gc have resulted in promising results in animal models, implementation in humans has remained unsuccessful up to now (301-306). Secondly, vaccines comprising cDNA vectors which encode for Gn and Gc of HTNV or PUUV have been successful in animal models but delivered low immunogenicity in phase I clinical studies (307-309). Vaccination via cDNA electroporation resulted in improved neutralizing antibody responses in 78% of the participants (307, 308).

#### **1.1.6.4 Passive immunization**

Passive immunization by administration of antibodies has been reported as effective against a variety of DNA and RNA virus-induced diseases. For instance, in patients suffering from Argentine haemorrhagic fever (caused by Junin arenavirus), administration of immune serum resulted in a decrease in mortality rates when administered up to 8 days after symptoms onset (310). Immunoglobulin treatment has also been documented to be beneficial for patients with antibody immunodeficiencies when suffering from enterovirus infections (311).

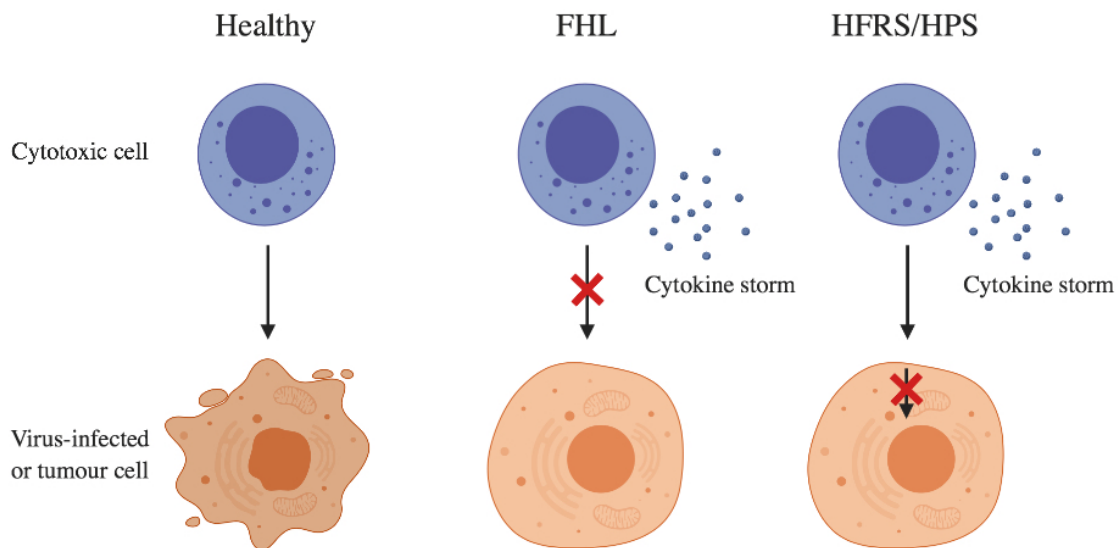
A controlled clinical trial exploring the efficacy of passive transfer of antibodies during the course of HPS showed a borderline but significant decrease in case fatality rate (312). Moreover no serious adverse effects were associated to plasma infusion (312). High titres of neutralizing antibodies during the acute phase of HPS correlate with a positive outcome for the patients, suggesting that strong IgG responses to hantavirus infection may be protective (313, 314). Additionally, during the course of PUUV-caused HFRS a low specific IgG response is associated with increase in disease severity (315). Neutralizing antibodies against ANDV obtained from vaccinated rhesus macaques or from vaccinated rabbits both

protect hamsters from developing lethal disease (316, 317); and immune plasma obtained from SNV-infected HPS patients has been reported to protect deer mice from SNV infection (318). Interestingly, the passive immunization of cynomolgus macaques with sera from previously infected macaques results in protection against PUUV infection (319). These results suggest that neutralizing antibodies could represent a potential therapeutic strategy protecting against HFRS and HPS or to be used as prophylactic.

#### ***1.1.6.5 Novel treatment strategies for severe hantavirus-associated disease***

Among the common hallmarks that define hantavirus-associated diseases, hyperinflammation stands out as one of the major challenges during patient care. Both in HPS and in some severe HFRS cases, high mortality rates associate to respiratory failure and cardiogenic shock, which result from pulmonary vascular leakage and associated inflammatory responses (54-56). The cytokine IL-15 drives Natural Killer (NK) cell and T cell activation and is involved in driving immunopathology in diverse conditions (320-322). IL-15 is up-regulated in HPS patients, and hantavirus infection of endothelial cells leads to increased surface expression of IL-15, which contributes to the massive NK cell activation observed after interaction with hantavirus-infected cells (323, 324). It is still unclear whether the robust NK cell responses in hantavirus-infected patients contribute or not to disease pathogenesis (325, 326); however, targeting of IL-15 with anti-IL-15 antibodies might reverse or palliate immune-driven pathogenesis. Increased levels of another pro-inflammatory cytokine, IL-6, have been also reported in severe cases of disease caused by either ANDV or PUUV infection (324, 327, 328). Anti-IL-6 receptor therapy has been successfully implemented for treating the adverse ‘cytokine storm syndrome’ resulting from chimeric antigen receptor (CAR) T cell therapy (329, 330). Thus, the implementation of anti-IL-6 receptor treatment in severe cases of hantavirus-associated disease could perhaps prove beneficial.

Finally, another strategy to improve the clinical outcomes of the most severe cases of hantavirus-associated disease may be aggressive immunochemotherapy. Familial haemophagocytic lymphohistiocytosis (FHL) is a life-threatening syndrome caused by genetic defects leading to impaired function of cytotoxic lymphocytes. Mutations in the perforin gene or in genes involved in cytotoxic granule release result in the impossibility to eliminate tumour and virus-infected cells, consequently leading to massive immune activation, cytokine production and hyperinflammation in patients (331). Immunochemotherapy (i.e. dexamethasone and etoposide) has been successfully implemented in FHL patients in order to control the excessive immune activation and palliate hyperinflammation (332). Since FHL and severe hantavirus-associated diseases share similar pathogenic denominators, the use of immunochemotherapy may be of benefit in the most severe cases of HFRS and HPS when involving hyperinflammatory conditions (Figure 4).



**Figure 4.** Cytotoxic lymphocytes, such as NK cells and cytotoxic T cells, represent the arm of the immune system capable of killing virus-infected cells as well as tumour cells. In rare occasions, such as familial lymphohistiocytosis (FHL) or hantavirus-associated diseases (HFRS/HPS), killing of target cells does not occur and a cytokine storm and hyperinflammation are induced. In FHL, mutations in the perforin gene or in other genes affecting degranulation result in target cell killing impairment. Implementation of immunochemotherapy as a treatment for FHL has been successful. The question remains as whether immunotherapy could represent a viable and successful strategy for severe hantavirus-associated diseases. Adapted from Klingström, J. *et al.* 2019 (333) under the terms of the Creative Commons CC BY license. FHL: familial lymphohistiocytosis; HFRS: haemorrhagic fever with renal syndrome; HPS: hantavirus pulmonary syndrome.

## 1.2 IMMUNE RESPONSES TO HANTAVIRUSES – FIGHTING BACK THE INTRUDER

The *Hantaviridae* family comprises a cluster of viruses causing very diverse pathogenicity in humans. Some hantaviruses are associated with 40% mortality rates while others are reported as causative agents of mild disease or completely non-pathogenic to humans. These facts indicate that viral pathogenicity is in part dependent on viral factors. Then, the fascinating fact that hantaviruses persist in their rodent reservoirs without causing any disease but induce severe illness in humans resulting in death or viral clearance, points towards the direct involvement of host factors in how different species cope with a specific hantavirus infection. The documented pathogenicity in humans most likely arises due to a blend of both host and viral factors.

Generally speaking, the endpoint goal of a particular virus is to inadvertently replicate in a host while maximising its' transmissibility and spread to new host individuals. Unless being part of the viral transmission mechanism, causing excessive immune reactions and death does not generally benefit the virus. The long-term co-evolution between hantaviruses and their rodent reservoir species explains the inexistent pathogenesis in the natural hosts. The accidental transmission from rodents to humans sets a new host environment for a virus which is not specialised or adapted to the human system.

The specific mechanisms of pathogenicity triggered by hantaviruses in humans are only partly understood. One of the main hurdles slowing down the comprehension of the pathogenesis of hantavirus-associated diseases is the lack of suitable and affordable animal models. The development of symptoms during HFRS has been investigated with a monkey model based on disease induced in cynomolgus macaques after infection with PUUV (319, 334, 335). A non-human primate model, this time using rhesus macaques, has been also used to understand the kinetics of infection and pathophysiology of HPS during infection with SNV (336). Such laboratory experiments represent very costly endeavours requiring advanced animal facilities. Other efforts to understand the pathogenesis of HPS through animal models have resulted in the development of a lethal model using Syrian hamsters. After infection with ANDV, hamsters develop an HPS-like disease with respect to incubation time and pathophysiology (337-339).

### **1.2.1 The role of endothelial cells – puppets in the virus' show**

The main hallmarks of hantavirus-associated diseases are vascular leakage, thrombocytopenia, strong immune activation, and hyperinflammation (54, 56). Hantaviruses replicate predominantly in endothelial cells, that is the epithelial cells that line the interior surface of blood vessels. Infection *per se* does not cause any cytopathic effects on the infected endothelium (149, 209, 340-344). Moreover, immunohistochemical analysis of patient tissue samples revealed abundant and widespread distribution of virus in lungs, heart, kidneys, pancreas, adrenal glands and skeletal muscle (270, 343). Despite hantaviruses not being cytopathic, increased capillary permeability is common during infection in humans, causing the reported vascular leakage and haemorrhagic manifestations.

*In vitro*, pathogenic hantaviruses have been reported to use  $\beta 3$  integrins for cell entry, whereas the non-pathogenic PHV was reported to bind instead to  $\beta 1$  integrins (189, 190). It has been proposed that pathogenic hantaviruses inhibit  $\beta 3$  integrins functionality and sensitize endothelial cells to vascular endothelial growth factor (VEGF), a vascular permeability factor (345). The induction of VEGF together with the downregulation of vascular endothelial (VE)-cadherin (a major component of adherens junctions), has been reported to result in endothelial cell barrier disruption during ANDV infection (204, 346). VEGF up-regulation occurs as well in a 3D lung tissue infection model, although at fifteen to twenty days post-infection with ANDV (347). On the other hand, in another cell model based on co-cultured endothelial and vascular smooth muscle cells, neither ANDV nor HTNV infections induced changes in VEGF levels, VE-cadherin degradation or cell permeability (348). Interestingly, the addition of plasma proteins to the system did result in increased permeability and secretion of the inflammatory mediator bradykinin. Furthermore, blocking the binding of secreted bradykinin prevented the reported alterations in permeability (348). In another study, no increased permeability or cytopathic effects were reported in human lung endothelial cells infected with HTNV or SNV; although the authors did observe an increased expression of the chemokines C-X-C motif chemokine 10 (CXCL10 or IP-10) and C-C motif ligand 5 (CCL5 or RANTES)(349). CXCL10 and CCL5 are chemotactic factors for

mononuclear leukocytes, suggesting that cellular immune responses may represent contributing elements to the vascular permeability during hantavirus-associated diseases (350).

### **1.2.2 Innate immunity – sensing trouble and causing trouble**

Since hantavirus infection does not associate with direct damage of endothelial cells, the increased permeability documented in patients is most probably caused by released cytokines and immune cells. Extensive evidence suggests that the pathogenicity of hantaviruses observed in humans is predominantly mediated by the immune system (56, 236, 351, 352).

#### **1.2.2.1 Cytokines**

When a virus has surpassed the skin or the mucous membranes of the host, the innate immune response acts as the initial line of defence for counteracting a viral infection, before a more specific adaptive immune response is mounted. Pathogen-associated molecular patterns (PAMPs) are recognised by a diverse group of cell receptors known as pattern recognition receptors (PRRs)(353, 354). The RNA of a virus can be recognised by PRRs on various types of cells, subsequently inducing the production of type I IFN, which enhance the innate immune response and signal the activation of adaptive immune cells. IFNs stimulate cells to produce antiviral compounds and proteins, such as nitric oxide or the MxA protein, both of which have been shown to inhibit hantavirus replication *in vitro* (355-357). Hantaviruses prevent the early induction of IFN responses in order to successfully replicate in endothelial cells (358). The Gn-CT of pathogenic hantaviruses interferes with the activation of IRF3 and the induction of IFN (191-194). Interestingly, the Gn-CT of the non-pathogenic PHV does not hinder the activation of IRF3 nor regulates early IFN induction (192). As earlier mentioned in section 1.1.4.2, the NSs protein of PUUV and TULV can act as a weak IFN inhibitor (200). The delayed type I IFN-induced antiviral state may contribute to HFRS severity in DOBV- and PUUV-infected patients according to a recently published report (359).

Many cytokines play important roles in inflammation and in immune response modulation. Inflammatory cytokines directly assist during the elimination of pathogens or indirectly induce innate and adaptive effector functions. However, the cascade of activation events associated with cytokines can enhance the immunopathogenesis reported during virus-induced diseases. The overproduction of inflammatory cytokines, causing the so-called “cytokine storm”, is generally reported in HFRS and HPS patients as a common denominator contributing to disease severity (54, 55). Increased levels of cytokine-secreting cells in lung tissue samples have been documented in fatal cases of HPS (360). Tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 are elevated in blood samples, urine and tissues of HFRS and HPS patients (324, 327, 361-364). These inflammatory markers have been associated with the induction of fever, septic shock and acute-phase proteins (365). IL-6 and IL-15 have been linked to disease severity in ANDV-infected HPS patients (324, 328). High levels of IL-6 associate to severe renal failure and thrombocytopenia in HFRS patients

infected with PUUV (327). Additionally, other cytokines increased during HFRS and HPS are IL-8, IL-10, IL-18, IFN- $\gamma$ , VEGF, IP-10, and RANTES (328, 361, 366).

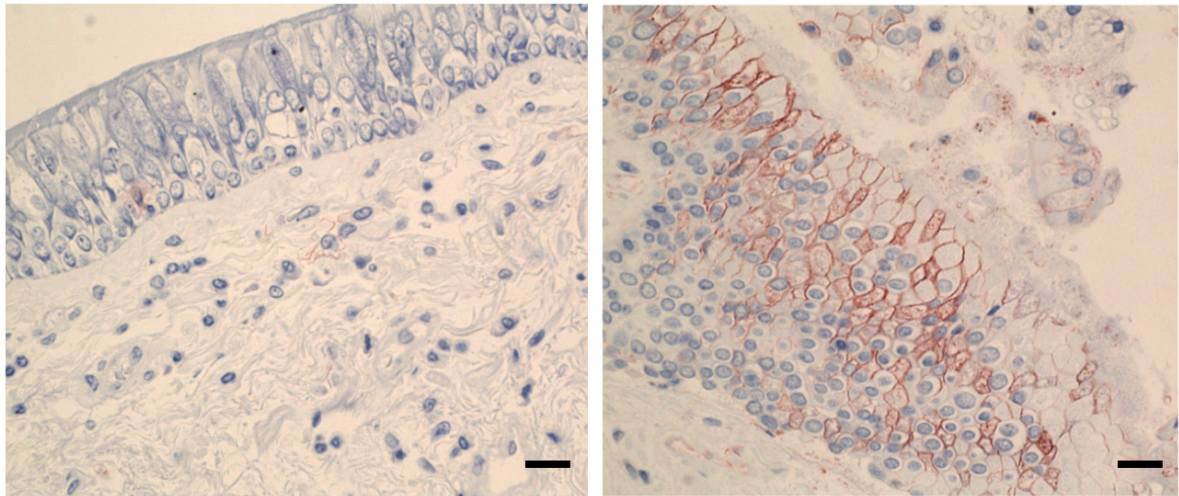
#### **1.2.2.2 Cellular components**

Thrombocytopenia is a common hallmark of hantavirus-associated diseases and represents an important characteristic for the diagnosis of HFRS and HPS (342, 343, 367). It also correlates to severe acute kidney injury in patients with acute HTNV or PUUV infections (367, 368). The interaction of platelets with endothelial cells during hantavirus infection has been postulated as mechanistically important for the development of thrombocytopenia (54). Platelets express integrin  $\alpha\text{IIb}\beta 3$  with which hantavirus glycoproteins interact, further directing the adherence of quiescent platelets to infected endothelial cells *in vitro* (369). Increased consumption of platelets could also be due to coagulation, since hantavirus infection leads to increased thrombin formation and fibrinolysis (370).

Through antigen presentation mononuclear phagocytes, such as dendritic cells and macrophages, contribute to the immune responses developing during a range of inflammatory, infectious, autoimmune and metabolic diseases. Although at lower efficiencies, monocytes, alveolar macrophages and dendritic cells are permissive to hantavirus infection (206, 208, 371, 372). Infection of dendritic cells with HTNV induces the release of the immune-modulating proinflammatory cytokines TNF and IFN- $\alpha$  (371). In PUUV-infected HFRS patients, a marked redistribution of mononuclear phagocytes to the airways was observed during the acute stage, which may relate to the local immune activation and contribute to the immune-mediated pathogenesis (206)(Figure 5). On the other hand, the infection of macrophages with hantaviruses does not seem to induce a marked cytokine release from these phagocytic cells, nor does it have a clear effect on endothelial cell permeability *in vitro* (208). Finally, depletion of alveolar macrophages in Syrian hamsters does not prevent disease pathogenesis and indicates a limited contribution of macrophages during hantavirus disease in the hamster model (339).

NK cells, which play a major role as innate killers of virus-infected cells, are rapidly expanding and remain in high levels for a long period of time (i.e. >60 days) in HFRS patients (373). The activation of NK cells during HTNV infection involves the up-regulation of TRAIL (374), and is partially dependent on virus-induced IL-15 expression, which is produced by endothelial cells after infection (323). Moreover, hantaviruses induce increased levels of intercellular adhesion molecule 1 (ICAM-1) and HLA-E in infected endothelial cells (323, 373, 374). ICAM-1 represents an important factor for endothelial cell adhesion, but it also facilitates the polarization of NK cells during cytotoxic granule content release to target cells (375). HLA-E represents a ligand for the activating NK cell receptor NKG2C (376). During HFRS, a large number of the rapidly expanding NK cells express the activating receptor NKG2C and are responsive to target cell stimulation (373). Despite the robust activation of NK cells and their up-regulation of surface TRAIL, hantavirus-infected endothelial cells remain protected from cytotoxic cell-mediated killing as well as from TRAIL-mediated killing (175)(**Paper I**; **Paper III**). Markers for cell apoptosis, as well as

granzyme B and perforin, are detected in serum from PUUV-infected HFRS patients (377, 378). Surprisingly though, despite the strong immune responses and hyperinflammation, infected endothelial cells appear intact and undamaged in hantavirus-infected patients (379).



**Figure 5.** The infiltration of antigen-presenting cells occurring in the airways of acute HFRS patients. Representative HLA-DR staining (red) in endobronchial biopsies from a healthy control (left) and an HFRS patient (right). Hematoxylin (blue) was used for counterstaining of the cells nuclei. Scale bar, 100  $\mu$ m. Adapted from Klingström, J. *et al.* 2019 (333) under the terms of the Creative Commons CC BY license.

### 1.2.2.3 Complement system activation

The complement system represents a group of plasma proteins interacting with one another that enhance the capacity of antibodies and phagocytes to detect and eliminate pathogens, thus promoting the opsonization of these pathogens and the induction of inflammatory responses during infections. During the acute phase in PUUV-infected HFRS patients, the complement system becomes activated and the levels of complement system activation correlate with disease severity (380, 381). In line with this, pentraxin-related protein 3, an acute phase protein mediating complement activation, is elevated during the acute phase of HFRS caused by PUUV infection (382). Galectin-3-binding protein, which also contributes to the activation of the complement system, is also high in plasma from PUUV-infected HFRS patients and correlates with disease severity (383).

Antibodies and their corresponding antigens can form immunocomplexes which can activate the complement system. The presence of immunocomplexes has been considered a cause of the pathogenicity reported during infectious diseases such as hepatitis (384), mononucleosis (385) and dengue virus associated disease (386, 387), but whether immunocomplexes are formed during the course of HFRS and/or HPS is currently unknown.

## 1.2.3 Adaptive immune responses – friends or foes?

### 1.2.3.1 Adaptive cellular response

The immunopathogenesis driven during hantavirus infections likely stands as a complex multifactorial reaction involving immune cells from both the innate and the adaptive immune

systems (208, 323, 373, 378, 388-391). The pro-inflammatory cytokines, such as TNF and IL-6, produced by infected and immune cells have been implicated in causing capillary leakage and hyperinflammation (54, 55). Virus-specific cytotoxic T lymphocytes are crucial for an effective clearance of viral infections; nevertheless, they are as well implicated in severe tissue damage (392, 393). During HFRS and HPS, strong cytotoxic T lymphocyte responses are associated with severity of disease and death (315, 378, 391, 394, 395). Moreover, the expression of the apoptosis-inducing ligands FasL, TNF and TRAIL is increased in CD8<sup>+</sup> T lymphocytes during the acute phase of HFRS (377). On the other hand, in a clinical study of HTNV-infected HFRS patients by Wang *et al.*, disease severity had an inverse correlation to the frequencies of virus-specific T cells (396).

Regulatory T cells play an important role in maintenance of homeostasis by limiting the inflammatory reactions and decreasing immunopathogenesis (397). Regulatory T cell-mediated suppression of the host antiviral immune response may however result in viral persistence in the host (398). In Chinese HFRS patients, decreased levels of regulatory T cells were reported, and inversely correlated with disease severity (399). Another study found increased levels of CD4<sup>+</sup>, CD8<sup>+</sup> and regulatory T cells in HFRS patients but no correlation with disease severity; however, elevated levels of FOXP3, a transcription factor involved in maintenance of regulatory T cells, did correlate with disease severity (400). Interestingly regulatory T cells contribute to the asymptomatic persistence of hantaviruses in their rodent reservoirs (401, 402).

### **1.2.3.2 Humoral response**

A study performed by Xu, *et al.* demonstrated that monoclonal antibodies against Gn and Gc display neutralizing activity, whereas antibodies directed against the N protein do not (403). Hantavirus infections trigger strong humoral immune responses including high titres of antibodies directed to the N protein and neutralizing antibodies (229, 404, 405). A vigorous production of both hantavirus-specific and unspecific plasmablasts occurs during the acute phase of HPS (389). Plasmablasts secrete great amounts of antibodies and high titres of hantavirus-specific IgG and IgM antibodies in serum correlate with patient recovery and survival (266, 314, 406, 407). The appearance of virus-specific IgM correlates with the onset of clinical symptoms. Serum levels of IgM usually peak at 7 to 11 days after initial symptoms and the decrease coincides with the rise of IgG levels (250, 408). IgA and IgE responses are detected during the acute phase of HFRS and gradually decline as the disease progresses (409-412). The levels of IgE do not correlate with disease severity in HFRS patients (352); further investigations are required in order to elucidate the implications of IgA and IgE during hantavirus-associated diseases.

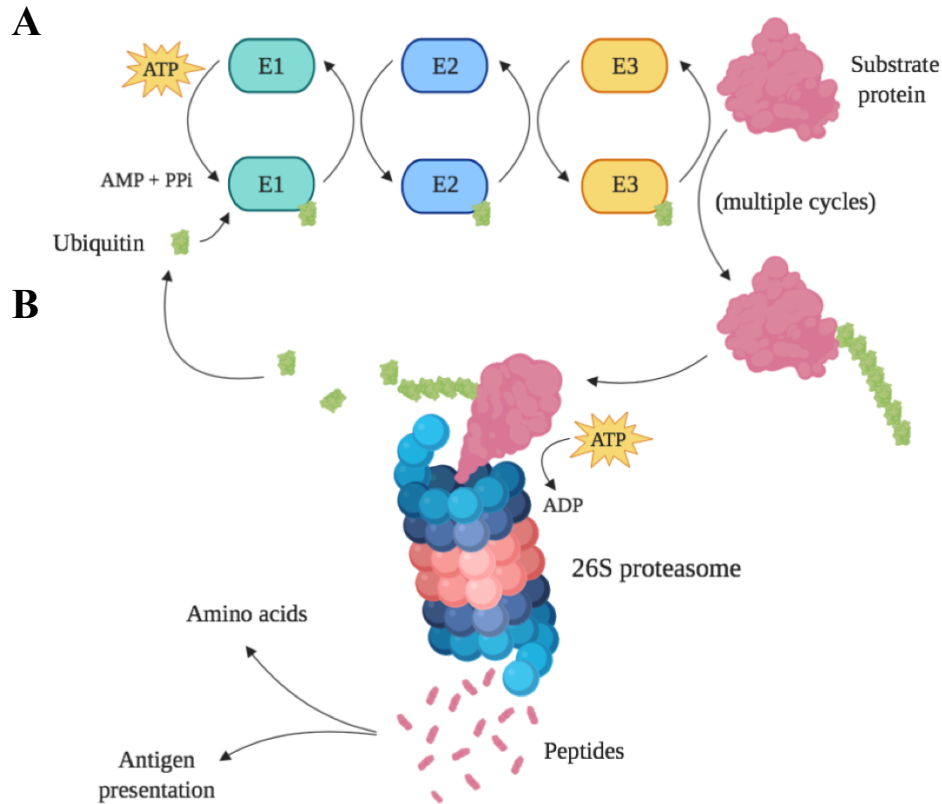
Although evidence of hantavirus persistence in humans is still absent, PUUV-specific IgG against N protein and neutralizing antibodies against Gn and Gc are still detectable in late convalescent samples and even decades after infection (409, 413, 414).

### 1.3 THE UBIQUITIN-PROTEASOME SYSTEM – DESTRUCTION FOR THE SAKE OF CONSTRUCTION

Intracellular and extracellular proteins are continuously hydrolysed to their constituent amino acids and replaced by newly synthesised proteins. This constant turnover of proteins is of extreme importance for the maintenance of homeostasis in a cell. The ubiquitin-proteasome system (UPS) acts as a quality control facility for proteins, ensures maintenance of important regulatory proteins and regulates the functional modification of specific proteins (415). Most intracellular proteins are regulated by the UPS, but the majority of extracellular proteins and some cell surface proteins are targeted for degradation in lysosomes (416). Lysosomes are organelles containing several pH-dependent proteases which perform their digestive functions in acidic conditions. During autophagy, cytosolic proteins are engulfed in autophagic vacuoles and degraded after fusion with lysosomes (417, 418).

The UPS involves the rigorous actions of specific enzymes that link chains of ubiquitin (Ub) to proteins, in order to flag the latter ones for degradation. The polyubiquitin chain resulting from this tagging process allows for protein recognition and degradation through the 26S proteasome (415, 419). The 26S proteasome, a multicatalytic protease facility, digests ubiquitinated proteins into small peptides (420). The three biochemical steps that result in the conjugation of ubiquitin to a specific protein are orchestrated by three enzymatic units: the Ub-activating enzymes (E1), the Ub-conjugating proteins (E2), and the Ub-protein ligases (E3). The component E3 determines the substrate specificity to the ubiquitin conjugating complex and catalyses the reaction of Ub transfer (Figure 6).

Increasing evidence supports a dual role of the UPS during viral pathogenesis. Cells display proteins that act as restriction factors limiting replication and propagation of viruses. The UPS is directly involved in innate immunity and host anti-viral defence. Meanwhile, viruses have evolved to interact with the cell's UPS in order to successfully replicate and avoid recognition by the immune system. Many DNA viruses, such human papillomavirus (HPV), adenoviruses, Epstein-Barr virus and human immunodeficiency virus (HIV), recruit cellular E3 ligases or express viral factors with their own E3 ligase activity to target and degrade specific cellular proteins that limit viral replication (e.g. SAMHD-1, MHC class I, p53)(421-426). The small RNA virus coxsackievirus B3 promotes degradation of p53 through activation of the proteasome activator complex subunit 3 (427). Recently, we showed that HTNV manipulates the host's UPS in order to induce ubiquitination of death receptor 5 (DR5) and degradation via the 26S proteasome, consequently protecting infected cells from DR5-initiated apoptosis (**Paper III**). Other viruses encode instead for proteins with de-ubiquitinating activity, such being the case of herpes simplex virus 1, Kaposi's sarcoma-associated herpesvirus and SARS coronavirus (428-430). In order to ensure efficient virion production, viruses cleverly use the UPS to maintain the amount of expressed viral proteins to optimal levels (431-434). Otherwise, the degradation of viral proteins represents a reflection of the cell's UPS-mediated viral clearance (435).



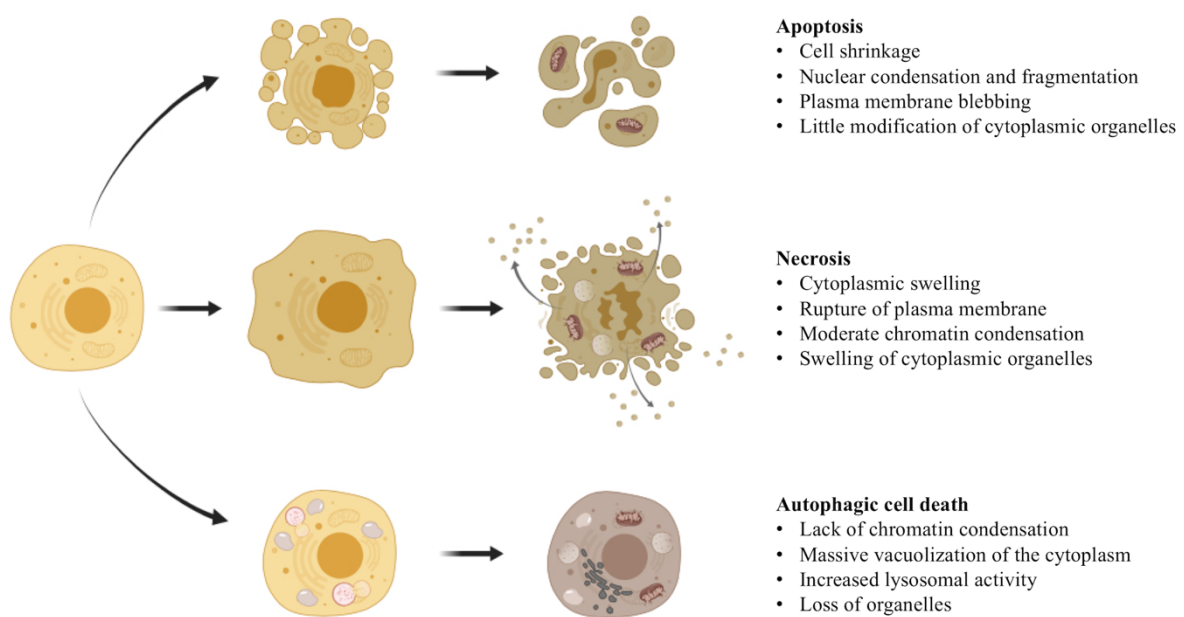
**Figure 6.** The ubiquitin-proteasome pathway. In this proteolytic process, ubiquitin conjugation to a substrate protein follows a three-step biochemical process (A). The successive repetition of this ATP-dependent ubiquitination process results in a chain of ubiquitin molecules being attached to the substrate protein, which in turn allows for the subsequent degradation of the polyubiquitinated protein through the 26S proteasome. The resulting peptides will be further digested by cytosolic peptidases into new ‘building blocks’ (amino acids) and antigenic peptides imported and processed in the endoplasmic reticulum (B). ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; PPi: pyrophosphate.

## 1.4 APOPTOSIS – A SOPHISTICATED PLAN TO KILL

In multicellular organisms, programmed cell death represents an elemental response to intracellular pathogens such as viruses. The sacrifice of ‘a few for the many’ in order to secure host survival stands as a trait shared from plants and fungi to mammals. There are different types of programmed cell death classified according to morphology, involvement of particular types of effector proteases, and immunogenicity (436). Three major types of cell death can be defined according to the appearance of a dying cell: apoptotic, necrotic and autophagic cell death (Figure 7).

Apoptosis represents the archetypical program for the controlled demolition of a cell. It allows for the elimination of damaged or infected cells in a regulated, implosive, non-inflammatory manner. During developmental stages and morphogenesis apoptosis also plays a central role, allowing for instance the moulding of tissues. Dysfunctions in cell execution

during embryogenesis have been implicated in a broad variety of developmental abnormalities and syndromes (437). Second, necrosis represents another way to die described as accidental cell death by injury, leading to abrupt cell rupture and inflammation (436, 438). Autophagic cell death is the third main type of cell death and it occurs with autophagy (that is the regulated process of degradation and recycling of cellular components), including massive vacuolization of the cytoplasm (i.e. double-membraned autophagic vacuoles), and lacking chromatin condensation (438). There exist other types of cell death which, like apoptosis, are considered as programmed cell death. Necroptosis is defined as a hybrid of apoptosis and necrosis; this signalling process of cellular killing presents with the characteristic necrotic cell death phenotype but is triggered by a receptor interacting serine/threonine-protein kinase 1 (RIP-1)-initiated signalling pathway (439-441). Also, the inflammatory caspase-1 has been given a role in triggering yet another regulated cell death pathway baptized as pyroptosis (442, 443). Unlike classical apoptosis, pyroptotic signalling is triggered by the caspase-1-activating inflammasomes upon recognition of pathogens, microbial products, or damage-associated molecular patterns (DAMPs)(444, 445). Lastly, in 2012 Dixon SJ *et al.* coined the term ‘ferroptosis’, describing an iron-dependent form of non-apoptotic killing induced by excessive production of reactive oxygen species (ROS)(446).



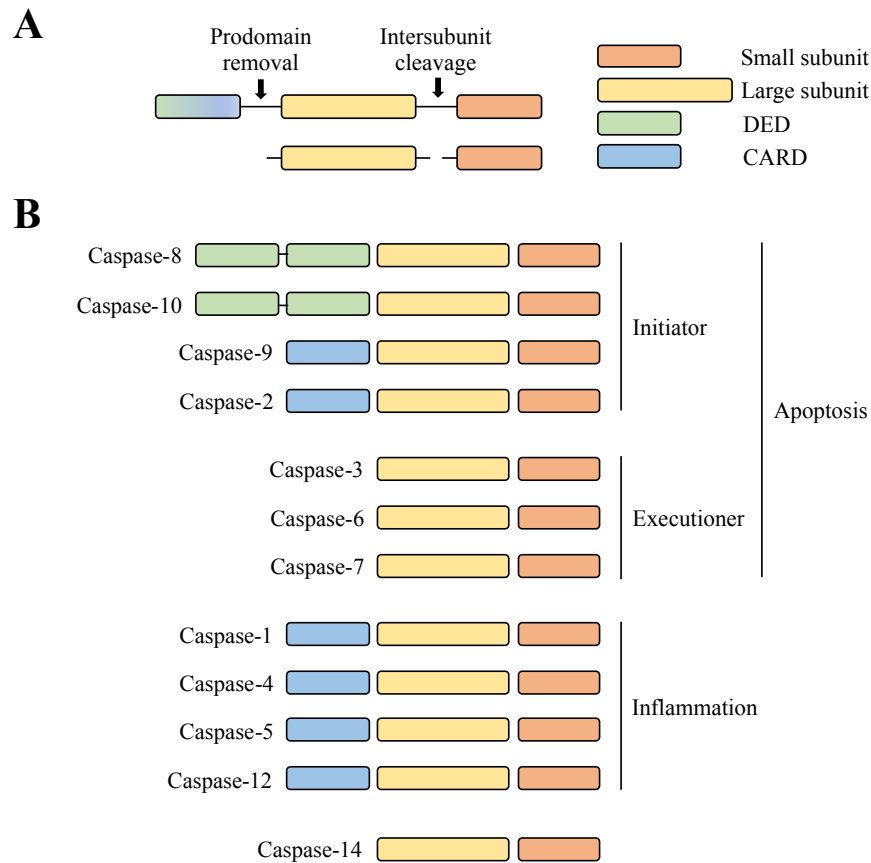
**Figure 7.** The main modalities of cell death: apoptosis, necrosis and autophagic cell death.

Apoptotic cell death is an immunologically silent process aiming at the elimination of single cells in a controlled manner. It does not result in the release of cytoplasmic content to the extracellular space, keeping from causing inflammation and damage to surrounding cells. Morphologically speaking, apoptosis shows in a cell as chromatin condensation,

shrinkage of the cytoplasm, membrane blebbing and formation of apoptotic bodies (Figure 7) (447, 448). Internucleosomal DNA fragmentation and redistribution of phosphatidyl-serine to the outer leaflet of the plasma membrane represent molecular alterations further reported (449, 450). During the intricate process of apoptosis, a plethora of initiator, adaptor and executioner proteins are recruited. Depending on which cellular factors are enlisted, different signal cascades are induced. The caspase-dependent signalling pathways described below represent the dominant *modus operandi* to cause apoptotic cell death (451, 452).

#### **1.4.1 Caspases – main operators turning the wheel of death**

Caspases (cysteine-dependent, aspartate-specific proteases) are a household of enzymes providing critical control during cell death and/or inflammatory processes (453). Throughout apoptosis, caspases orchestrate the dismantling of diverse cellular compounds as well as regulate the activation of other enzymes (454-456). Apoptotic caspases are categorized as initiators (caspase-8, -10, -9 and -2) and executioners (caspase-3, -6 and -7) (Figure 8). Caspase-2 is linked to apoptotic cell death, but its physiological roles, and even its function as an executioner caspase, are still debated (457-459). Caspases are synthesized as catalytically inactive monomeric zymogens (procaspases) which in order to become active follow a process of oligomerization and cleavage (Figure 8)(460-464). The aid of adaptor proteins, forming multicomponent scaffolding units, facilitates the aggregation of initiator procaspases for further autoproteolysis and activation as obligate homodimers (465-467). Executioner caspases exist as preformed dimers and act as cellular downstream targets for initiator caspases. Upon cleavage, the executioner caspases experiment conformational changes and become fully active (468, 469), subsequently targeting and cleaving cellular proteins such as poly ADP-ribose polymerase (PARP), lamins and fodrin among others, resulting in the above mentioned morphological alterations observed during apoptosis (470-473).



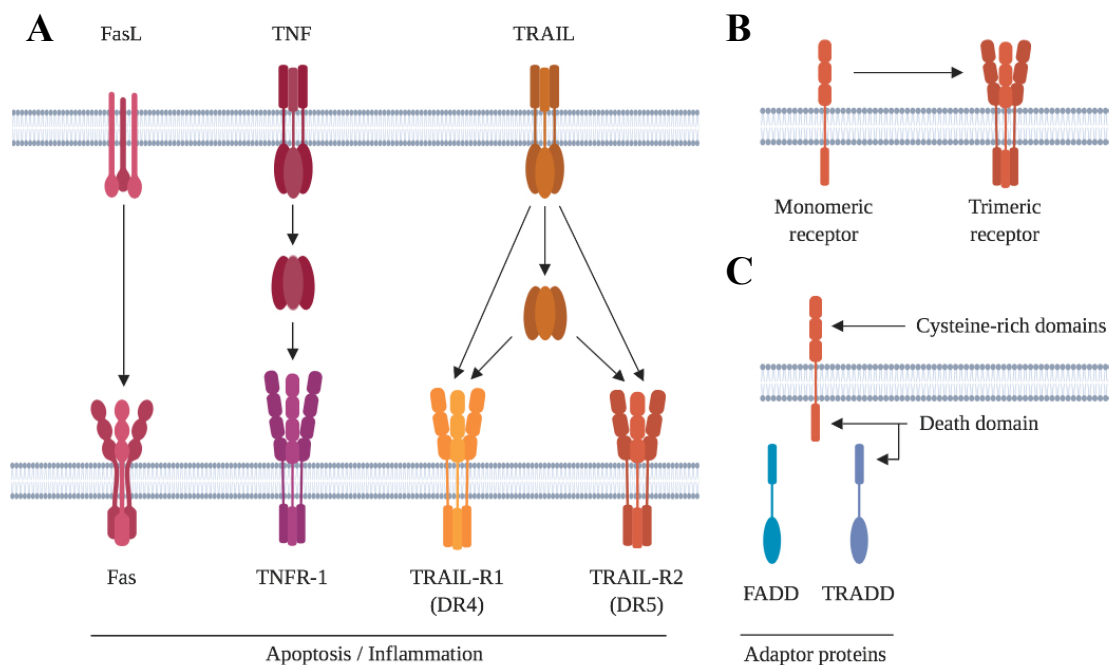
**Figure 8.** Activation (A) and structural features (B) of human caspases. CARD: caspase recruitment domain; DED: death effector domain.

## 1.4.2 Apoptotic signalling pathways – a hundred bullets to choose from

### 1.4.2.1 *Extrinsic apoptosis pathway*

The apoptotic machinery of a cell can be invoked both by extrinsic and intrinsic stimuli. The extrinsic pathway is activated after oligomerization of cell surface receptors upon contact with their particular ligands. These receptors, commonly known as the ‘death receptors’, represent a selection of members of the tumour necrosis factor receptor (TNFR) superfamily (474-476). Signalling through specific death receptors initiates an intricate communication network that can trigger not only apoptosis, but inflammation, cell survival and cell differentiation (476, 477). The most broadly investigated death receptors are Fas, TNFR-1, TNF-related apoptosis inducing ligand receptor 1 or death receptor 4 (TRAIL-R1/DR4), and TNF-related apoptosis inducing ligand receptor 2 or death receptor 5 (TRAIL-R2/DR5)(Figure 9). Death receptors are equipped with a cytoplasmic section known as the death domain (DD). Upon ligand binding and oligomerization of the receptor (often in trimeric complexes), a conformational change allows for the recruitment of adaptor proteins, such as FADD (Fas-associating protein with death domain) or TRADD (TNF receptor-

associated protein with death domain) via their corresponding DD (478, 479). These proteins act as linkers, allowing for the subsequent coupling of the cell death orchestrators, i.e. initiator caspase-8 or -10. Anchorage of initiator caspases to the system occurs through another homotypic interaction module known as death effector domain (DED). The resulting multi-protein structure is generally referred to as the death-inducing signalling complex (DISC) (465, 480). Alternatively, the caspase-8 homologue FLICE-like inhibitory protein (cFLIP) containing a DED, can bind to FADD and inhibit death receptor mediated apoptosis (480, 481). Not all initiator caspases are equipped with a DED. Caspase-9, as well as caspase-2, contains instead a caspase recruitment domain (CARD) allowing for its' activation upon interaction with yet other multi-protein platforms (Figure 8)(482, 483). The formation of DISC leads to apoptosis via direct processing and activation of executioner caspases; otherwise, activated caspase-8 can cleave BID (a pro-apoptotic BCL-2 family member; see section 1.3.3), which causes eventual caspase-9 activation through the engagement of the mitochondrial cell death pathway (described in 1.4.2.3 and graphically represented in Figure 11) (484, 485). Interestingly, recent studies have demonstrated that efficient apoptosis requires apoptotic signal amplification through a 'feedback-loop' of signaling by executioner caspase-3 or -7 to initiator caspases (486, 487).

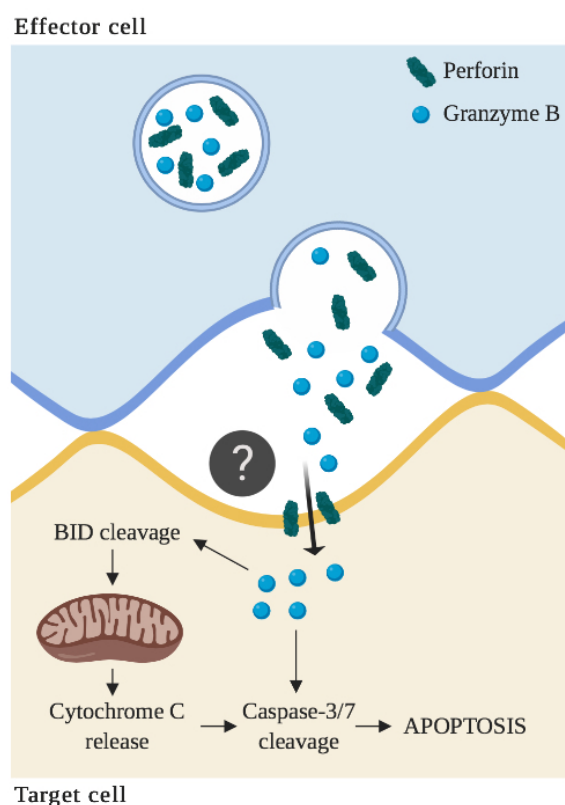


**Figure 9.** The main death receptors of the TNF receptor superfamily and their respective ligands (A). Upon ligand binding the receptors oligomerize, usually in trimeric complexes (B). Intracellular signalling is induced through the interaction of the death domains present both in the specific death receptor and the respective adaptor proteins, FADD or TRADD (C). FADD: Fas-associating protein with death domain; TNF: tumour necrosis factor; TNFR-1: tumour necrosis factor receptor 1; TRADD: TNF receptor-associated protein with death domain; TRAIL: TNF-related apoptosis inducing ligand; TRAIL-R1/2: TNF-related apoptosis inducing ligand receptor 1/2; DR4/5: death receptor 4/5 (alternative name for TRAIL-R1/2, respectively).

#### 1.4.2.2 Cytotoxic granule-mediated apoptosis

Cytotoxic T lymphocytes and NK cells have the capacity to trigger apoptosis through death receptors, but also by releasing granzymes into the target cell (488). Both processes are dependent of contact between the effector and the target cell. During cytotoxic granule-mediated apoptosis, cytoplasmic granules traffic to the immunological synapse formed between the effector and target cells. The cargo released into the synaptic cleft include perforin, granulysin and granzymes – a family of structurally-related serine proteases with diverse substrate specificities (489). There are five human granzymes (A, B, H, K, M) with various cytotoxic and non-cytotoxic mechanisms of action and with roles in antiviral defence, cancer and inflammation (490, 491). How granzymes are exactly delivered by effector cells is still fiercely debated. A commonly accepted explanation is that granzymes diffuse through perforin-formed pores on the plasma membrane of the target cell. Alternatively, it is thought that the internalization of perforin and granzymes occurs via cell membrane receptor-mediated endocytosis, for instance through mannose 6-phosphate receptors (492). Once internalised, granzymes trigger several apoptotic pathways; some of these are caspase-dependent pathways whereas others can kill the target cell when caspases are inhibited, for instance by viral proteins blocking apoptosis execution (491).

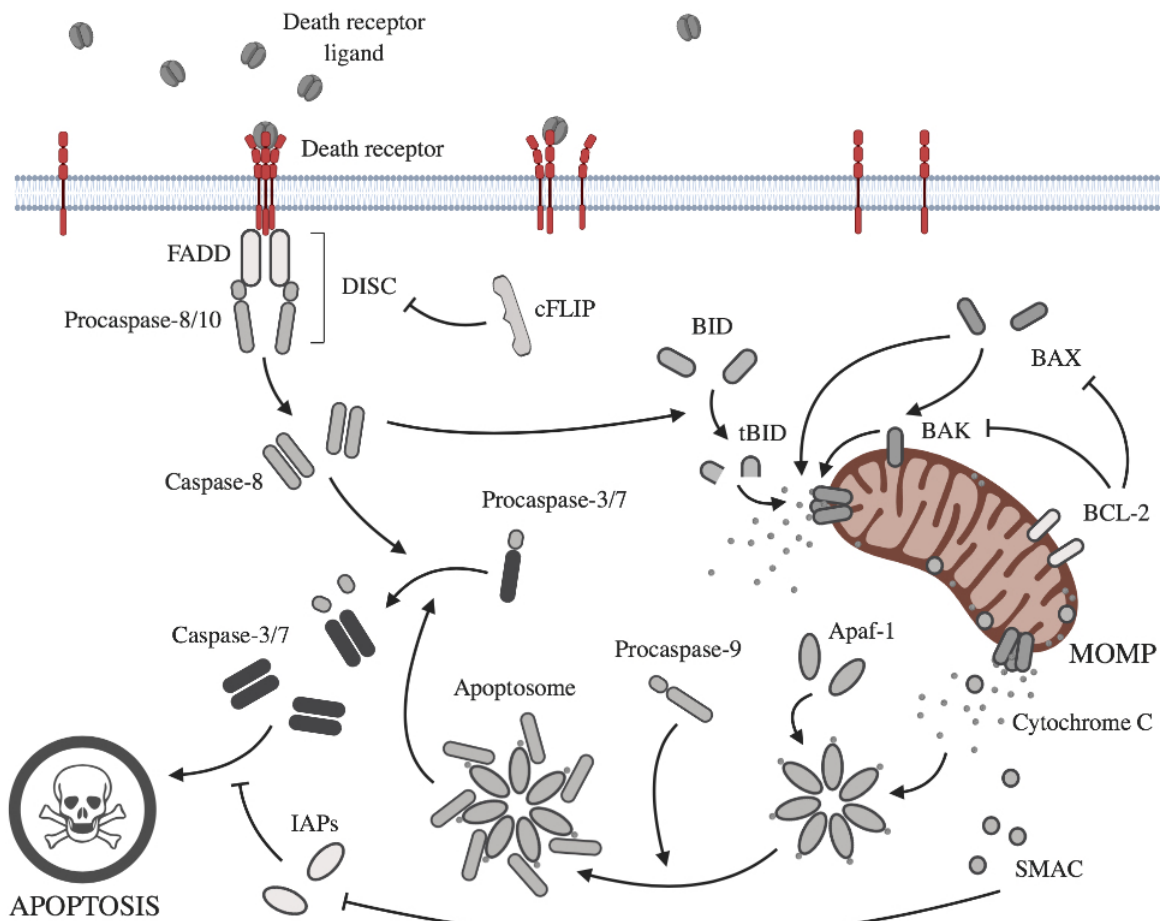
Granzyme B represents the most studied of the human granzymes. Once released into a target cell, this serine protease can induce apoptosis by direct cleavage and activation of caspase-3 (493). Alternatively, granzyme B also cleaves the pro-apoptotic BID into its truncated version (tBID), directly or through cleavage of caspase-8 (494, 495). Translocation of tBID to the mitochondria triggers activation of BAX and BAK (see section 1.4.3), leading to cytochrome C release and apoptosis induction (Figure 10).



**Figure 10.** The cytotoxic granule-dependent, perforin and granzyme B-mediated, mechanisms of apoptotic cell death. BID: BCL-2 homology region 3-interacting domain death antagonist.

### 1.4.2.3 Intrinsic apoptosis pathway

The intrinsically-initiated apoptotic process is triggered by mitochondrial outer membrane permeabilization (MOMP) and implies the activation of caspases from inside a cell. Typically, the caspase-9 activating platform known as apoptosome is formed upon cytochrome C release from mitochondria (496). Together with the apoptotic protease activating factor 1 (Apaf-1), cytochrome C recruits procaspase-9 to the apoptosome platform in an ATP or dATP dependent manner, leading to allosteric activation upon oligomerization (497, 498). Then, caspase-9 can catalytically process and activate the executioner caspases, enhancing the avalanche of proteolytic events leading to the elimination of a cell by apoptosis (Figure 11).



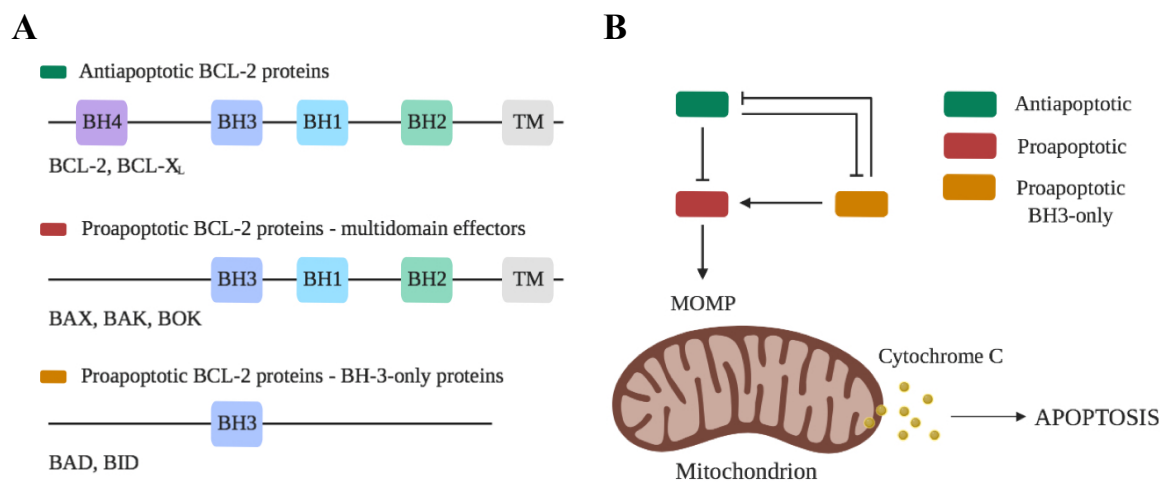
**Figure 11.** Extrinsic and intrinsic apoptosis signalling pathways. Apaf-1: apoptotic protease activating factor-1; BAK: BCL-2 homologous antagonist killer; BAX: BCL-2-associated death promoter; BCL-2: B cell lymphoma 2; BID: BCL-2 homology region 3-interacting domain death antagonist; cFLIP: cellular FLICE-like inhibitory protein; DISC: death-inducing signalling complex; FADD: Fas-associated death domain; MOMP: mitochondrial outer membrane permeabilization; SMAC: second mitochondrial activator of proteins tBID: truncated BCL-2 homology region 3-interacting domain death antagonist.

### 1.4.3 BCL-2 family – arbiters on a deadly game

Several cellular insults such as growth factor deprivation, DNA damage, ion imbalance or detachment of adherent cells (anoikis) can trigger intrinsic apoptosis (499-501). Mitochondria are central for intrinsic induction of apoptosis. These organelles integrate and process pro- and anti-apoptotic signals from the surrounding intracellular environment. Loss of mitochondrial membrane potential (MMP) represents a central event during apoptosis when induced by any of the above-mentioned stimuli. The integrity of the mitochondrial membrane is controlled by proteins of the BCL-2 (B cell lymphoma-2) family, which operate as apoptosis master regulators, delivering pro- and anti-apoptotic signals to the mitochondrion (502). Several of the BCL-2 family members share a C-terminal domain that allows for insertion into the membranes of mitochondria, endoplasmic reticulum (ER) and nucleus (503-505). The members of this protein family display extensive similarity in amino acid sequence with BCL-2, the classical inhibitor of apoptosis (506-508). The **BCL-2** homology regions (named BH1 to 4) allow for the classification of BCL-2 family proteins according to domain architecture and functionality (Figure 12)(496). Anti-apoptotic members express at least BH1 and BH2, but most contain all four BH domains (e.g. BCL-2, BCL-X<sub>L</sub>)(509, 510). Secondly, the pro-apoptotic multi-domain members (e.g. BAK, BAX, BOK) contain 2 or 3 of the homology regions (511-514). And finally, even more distantly related to BCL-2, are the pro-apoptotic BH3-only proteins (e.g. BAD, BID)(515, 516); the latter ones remain as inactive sentinels which upon activation stimulate the pro-apoptotic, multi-domain BCL-2 family members. On the one hand, it has been reported that BH3-only proteins require the expression of BAX and BAK in order to deliver their ‘death sentence’ (517, 518). On the other hand, the anti-apoptotic BCL-2 and BCL-X<sub>L</sub> sequester BH3-only molecules, thus hampering BAX- and BAD-induced mitochondrial-mediated cell death (519). These interactions depend on the affinity and the relative abundance of the different pro- and anti-apoptotic BCL-2 family members. Upon a particular cell stressor, the composition and activation state of both pro- and anti-apoptotic BCL2 family proteins change, tipping the delicate balance towards survival or death (Figure 12).

Essentially, anti-apoptotic BCL-2 family members (survival factors) play the role of gatekeepers, whereas the pro-apoptotic molecules (death factors) will try to ‘breach the gates’ of mitochondria upon cell stress, causing pore formation, MOMP, and liberating cytochrome C and other mitochondrial proteins (518, 520-523). In this manner, the released factors can trigger caspase-activation and further cell death (for instance cytochrome C triggers caspase-activation via the apoptosome platform). Alternatively, some of the released pro-apoptotic

factors can induce caspase-independent cell death. Endonuclease G as well as the apoptosis-inducing factor (AIF) have been linked to the execution of intrinsic cell death independently of caspases (524, 525).



**Figure 12.** The BCL-2 family of proteins is divided into three groups based on the number of BCL-2 homology (BH) domains (A) and their regulation of apoptosis (B). BH1-4: BCL-2 homology region 1 to 4; MOMP: mitochondrial outer membrane permeabilization; TM: transmembrane domain.

## 1.5 VIRAL INTERFERENCE WITH APOPTOSIS – OF MIMES, CAMOUFLAGE AND HIDING TRICKS

Viruses have been shaped by evolution, being constantly exposed to the pressure exerted by the host's countermeasures to infection. These structurally simple entities have been moulded as highly specialized units able to exploit the cell's machinery for their own benefit. Regulating cell viability represents one crucial strategy for viruses to multiply and spread (526-528). Viruses have developed strategies to both promote and hamper the apoptotic process of a cell. Some use the triggering of cell death as a mechanism to cripple the immune system of the host, as well as a method of rapid dissemination (529, 530). Contrariwise, many viruses act as master puppeteers of host cells by inhibiting cell suicide while promoting viral production.

### 1.5.1 Targeting the death receptors – cutting off the signal

Some viruses can affect the cell surface expression of death receptors (531, 532) or induce the production of proteins resembling cellular receptors, with which the viral fraudulent products compete for binding of apoptosis-inducing ligands. Adenoviruses for instance down-regulate the cell surface expression of Fas and DR4 molecules by means of a two-protein complex, E3-10.4kDa/14.5kDa (533, 534). These two viral proteins protect infected

cells from Fas- and TRAIL-mediated apoptosis; moreover, the E3-10.4kDa/14.5kDa complex has been shown to block TNF-induced apoptosis although not at a surface receptor level (535). Some poxviruses encode for viral factors able to bind to TNF with high affinity. The virus proteins negate surface receptor signal transduction, impeding the extrinsic initiation of apoptosis. The first of these viral soluble TNF receptor homologues to be described was T2, a protein encoded by the Shope fibroma virus. T2 lacks a transmembrane domain, thus representing a soluble cytokine receptor secreted from infected cells and able to neutralize the destructive effects of TNF (531). Another leporipoxvirus, the closely related myxoma virus, encodes for M-T2. This virulence factor is secreted as monomeric and dimeric species also acting as viral mimics of TNF receptors. Additionally, an intracellular form of M-T2 has been shown to block intrinsic apoptosis in CD4<sup>+</sup> T lymphocytes (536, 537). Also, the DNA of cowpox virus contains a gene coding for the cytokine response modifier B (CrmB), another secreted TNF receptor homologue. CrmB presents considerable similarities with the T2 proteins encoded by leporipoxviruses (48% identity) (532, 538). Yet another example is the vaccinia virus A53R, a viral product also acting as a soluble TNF-binding protein (539).

Formation of the DISC allows for the transduction of a signalling cascade leading to apoptosis. Through evolution, some viruses have acquired strategies to block components of the DISC other than the death receptors. Viral homologues of cFLIP (vFLIPs), contain two DEDs, and interact with the adaptor proteins FADD and TRADD, in a similar way as the cellular counterpart cFLIP would (481). Following this tactic, some  $\gamma$ -herpesviruses and poxviruses hamper the recruitment of pro-caspase-8 and -10 to the DISC, thus blocking the activation of the initiator caspases and apoptotic signal transduction triggered by different death receptors (481, 540, 541). While vFLIPs encoded by  $\gamma$ -herpesviruses compete with initiator caspases for binding to adaptor molecules, the poxvirus molluscum contagiosum (MCV) vFLIPs bind at least to two FADD molecules at a time, disrupting FADD oligomerization (542). MCV vFLIPs also neutralize Fas-induced cell death by means of sabotaging the association of TNF receptor-associated factor-2 and -3 (TRAF-2/-3) and the internalization of the Fas DISC, a crucial step occurring after the DISC formation (543). Although not having a distinguishable DED, the adenovirus protein E3-14.7kDa targets caspase-8 resulting in the blockage of death receptor induced apoptosis (544, 545).

In addition, despite not being a vFLIP, the human papillomavirus E6 oncoprotein of HPV-16 acts as a versatile protein targeting the DISC formation at various levels. E6 has been shown to interact with the DD of TNFR-1, subsequently impeding TRADD binding and eventual DISC formation (546). Alternatively, E6 anchors to the DED of both procaspase-8

and FADD, hampering the interaction between these proteins as well as promoting their degradation (547, 548).

Cytotoxic cell-mediated killing of virus-infected cells is executed by the engagement of cell surface death receptors, alternatively through the release of granzymes into target cells mediated by perforin-dependent pore formation. To evade recognition of infected cells, some viruses trigger surface down-regulation of antigen presenting molecules (549). As explained above, other viral entities target the death receptors and the formation of the DISC to avoid programmed killing. However, viruses can tackle the cytotoxic cell response at yet other different levels. Granzymes are serine proteases targeting multiple cellular pro-apoptotic substrates. Upon release in a target cell, these efficient proteases will start the apoptotic ball rolling (488). The human adenovirus type 5 presents with an assembly protein, namely Ad5-100kDa, able to specifically hamper the activity of human granzyme B (550, 551). The CrmA protein from poxvirus represents a polyvalent viral tool able to firmly block several proteases, among them granzyme B (552).

### **1.5.2 Cellular and viral inhibitors of caspases – putting a spoke in the wheel**

Virus proteins can effectively inhibit both the extrinsic and intrinsic apoptotic pathways at other stages. Regardless of specific stimuli, the culminant phase of apoptosis is mostly dependent on caspases. If figuratively caspases are the operators turning the wheel of death, viruses are sometimes the ones throwing a spanner in the works. The already mentioned cowpox virus CrmA protein was the first inhibitor of caspases to be discovered. It blocks the active site of caspases by conformational trapping, acting as an irreversible inhibitor of caspase-1 and -8 (553-555). To certain extent, CrmA hampers the activity of other caspases too (556). Other members of the *Poxviridae* family encode for CrmA homologues. For example, the serine protease inhibitors (SPI)-1 and -2 are produced by smallpox (557, 558), rabbitpox (559), and mousepox (560) viruses. On yet another instance, protein SERP-2 – a virulence factor from myxoma virus –, can inhibit caspase-8 and -10, as well as the inflammatory caspase-1 (561, 562). Insect baculoviruses have been shown to encode for potent caspase silencers as well. The viral product p35 (563, 564), and the related p49 (565, 566), represent broad-spectrum inhibitors of metazoan caspases. p35 contains a cleavage sequence for many caspases; upon proteolytic processing, the cleaved viral polypeptides remain in inhibitory association with the proteases. p35 has been reported to act as bait for mammalian caspase-1, -3, -6, -7, -8 and -10 (567-569), being less effective against caspase-2 and -9. The inhibitory effect that these viral proteins exert over caspases translates into apoptosis resistance when initiated by death receptors (556, 570).

The activity of caspases can also be hindered by the action of inhibitor of apoptosis (IAP) proteins. Cellular IAPs (cIAPs) act as endogenous caspase inhibitors as well as modulators of receptor-mediated signal transduction (571). Interestingly, the first IAP to be discovered (namely Op-IAP) was a baculoviral gene product that complements the action of p35 by presumably preventing the activation of initiator caspases in the insect host (572, 573). The IAP-family members contain from one to three baculovirus IAP repeats (BIRs), each of these representing a zinc binding fold of approximately 70 amino acid residues (574). Additionally, some IAPs display another zinc-binding sequence, the RING domain, for inhibition of apoptosis (575). The human X-linked IAP (XIAP) can directly inhibit the activity of caspase-3 and -7 (via a flanking region N-terminal of BIR2) and is thought to be the main acting cIAP *in vivo* (576-578). XIAP also inhibits the dimerization of initiator caspase-9 (via BIR3 binding the N-terminus of monomeric caspase-9), thus blocking its action as an active protease (579, 580). The function of XIAP is blocked by IAP antagonists such as the second mitochondrial activator of proteins (SMAC) (581) – or its equivalent in mouse: direct IAP binding protein with low pI (DIABLO) (582). Upon apoptotic stimuli, SMAC/DIABLO is released from mitochondria along with cyt C, at this point being able to interact with XIAP, thus causing the liberation of caspases to promote apoptosis (583, 584). Other cIAPs suppressing apoptosis are cIAP-1 and cIAP-2; although these inhibitory proteins do bind caspases *in vitro*, they are unable to directly silence the enzymatic activity of the latter ones (585, 586). Potentially, cIAP-1 and cIAP-2 could bind to IAP antagonists, in this manner freeing XIAP molecules that could thereafter inhibit caspases; otherwise it has been speculated that cIAPs may be inducing ubiquitination of caspases, thus flagging these for degradation by the proteasome (575).

Although it remains to be fully defined, the viral IAPs (vIAPs) reported to date seem to hamper caspase activity in an indirect fashion, as argued by the failure of baculovirus CpGV-IAP3, HcNPV-IAP3 and Op-IAP3 to bind caspases and directly inhibit their activity (587, 588). It rather seems that vIAPs generally act as decoys for cellular IAP antagonists, such as SMAC/DIABLO, hence antagonizing the antagonizers and allowing cIAPs to negatively regulate caspases (589, 590). Besides baculoviruses, other insect viruses known to encode for functional vIAPs are the entomopoxvirus AmEPV (591) and the Chilo iridescent virus, an iridovirus (592). Lastly, the African swine fever virus gene designated A224L, encodes for an IAP homologue reported to safeguard infected cells from at least chemically induced apoptosis (593).

### 1.5.3 Viral BCL-2 homologues – the imitation game continues

The cellular BCL-2 family members are also mimicked by viral homologues (vBCL-2). Viruses escape from the death row by directly interacting and regulating the above-mentioned BCL-2 family proteins, consequently promoting apoptosis resistance by impeding caspase activation in an indirect fashion (594, 595). Adenoviruses display a battery of anti-apoptotic proteins upon infection. Aiming at sustaining the viral replication for long time, the adenoviral BCL-2 homologue E1B-19kDa blocks host cell apoptosis induced by a variety of stimuli (596-599). E1B-19kDa locates to the mitochondria, where it has been shown to sequester the pro-apoptotic BCL-2 family members BAX (600, 601) and BAK (602). BCL-2 can functionally be substituted by E1B-19kDa, and vice versa – i.e. the overexpression of BCL-2 overcomes the absence of E1B-19K, allowing for productive adenovirus infection in HeLa cells as well as inhibition of Fas- and TNFR-induced apoptosis (603). The human cytomegalovirus gene UL37 encodes the viral product baptized as viral mitochondria-localized inhibitor of apoptosis (vMIA) (604). This virulence factor exerts its anti-apoptotic features by physically interacting with BAX, neutralizing it, and as a result impeding MMP loss (605, 606). vMIA has also been suggested to act as a modulator of mitochondrial fission (607) and  $\text{Ca}^{2+}$ -dependent pro-apoptotic signals (608). Unlike adenoviruses, herpesviruses have acquired and modified many host genes. Lymphotropic  $\gamma$ -herpesviruses express vBCL-2s as well as other viral proteins resembling cellular products (594, 609). Epstein Barr virus (EBV) expresses two apoptotic modulators sharing sequence and structure homology with Bcl-2 (610, 611). BHRF-1 co-localizes with BCL-2 at the outer membrane of mitochondria (612), and efficiently impedes apoptosis driven by diverse stimuli (613-615). The second EBV vBcl-2, BALF-1, is able to interact with BAX and BAK, and has been suggested to represent an antagonist of BHFR-1 (610, 616). Other herpesviruses encode for vBCL-2, e.g. human herpesvirus 8 (617), murine  $\gamma$ -herpesvirus (618), and primate herpesviruses analogues to EBV (619). The structurally complex poxviruses also encode for BCL-2 homologues that despite not presenting sequence similarities with BCL-2 do fold like their cellular counterpart. Consequently, these vBCL-2s counteract the action of pro-apoptotic BCL-2 family members such as BAK, BAD, or BID, thereby hampering the release of mitochondrial pro-apoptotic factors, such as cyt C, and caspase activation. Some of the poxvirus vBCL-2s are F1L and N1L proteins from vaccinia virus (620-622), M11L from myxoma virus (623-625), and the more recently enlisted FPV039 from fowlpox virus (626) and ORFV125, encoded by the Orf parapoxvirus (627).

vBCL-2 proteins are conserved across multiple viral strains of the families *Adenoviridae*, *Herpesviridae* and *Poxviridae*, exhibiting substantial degree of functional and structural redundancy. This reflects the fact that BCL-2 family proteins are crucial regulators of intrinsic apoptosis as well as immune cell-mediated cell death (595, 628). Some vBCL-2 differ significantly in sequence with BCL-2 family members. Still the viral proteins fold in a surprisingly similar way as their cellular equivalents do, resulting in alike three-dimensional structures (595, 603, 629, 630). Interestingly, like most members of the BCL-2 family, vBCLs present an inserting domain allowing for their location at the mitochondrial membrane (631). To date, all the described viral proteins containing a mitochondrial outer membrane-anchoring domain are anti-apoptotic.

#### 1.5.4 Hantaviruses and apoptosis – sabotaging the execution

Hantaviruses do not cause direct cytopathic effects in infected cells (340, 341, 631). Some studies have pointed towards hantaviruses being pro-apoptotic in Vero E6 cells (632, 633). However, these investigations failed to clearly show that apoptosis was specifically occurring in infected cells rather than bystander, uninfected cells. Along the same lines, another report stated that hantaviruses cause apoptosis in Human Embryonic Kidney 293 (HEK293) cells (634). The authors stressed out though that apoptosis was observed mostly in uninfected, bystander HEK293 cells and rarely in infected cells. Moreover, HEK293 cells are known to be sensitive to treatment with  $\alpha V\beta 3$ -integrin antagonist, resulting in the induction of programmed cell death (635).  $\alpha V\beta 3$ -integrin is one of the receptors that hantaviruses use for attachment and entry into human cells (189, 190). It could well be that the stress of infection *per se* caused apoptosis-induction on HEK293. Several other reports speak against the above-mentioned (175-177, 326, 340, 341, 344, 371). Hantavirus infection is described as a rather slow, non-apoptotic process in both human and non-human cell cultures (340, 341, 344, 371). The non-cytopathogenicity of hantaviruses has been further confirmed in rhesus macaques infected with either PUUV or SNV (336, 636). In the last decade, it has been demonstrated that hantaviruses induce strong and robust cytotoxic lymphocyte responses in humans (325, 388, 637, 638), however infected endothelial cells remain intact and undamaged (343). These observations suggest that hantaviruses can inhibit cytotoxic-mediated killing of infected cells. Interestingly recent publications have demonstrated that hantaviruses inhibit chemically-induced apoptosis (175)(**Paper I**; **Paper II**) as well as TRAIL-mediated killing of infected endothelial cells (**Paper III**). Other investigations have also reported the interaction of the N protein with caspases and its capacity to inhibit caspases' activation (176, 177); which have strengthened the hypothesis that hantaviruses display an anti-apoptotic engine.

Many large DNA viruses encoding hundred or more proteins harbour genes devoted to the sabotage of the host cells' defences. RNA viruses such as hantaviruses are deprived of such a luxury. Instead these simpler viral entities display a scarce repertoire of multi-functional proteins. Hantaviruses, although displaying a very simple configuration comprising only four structural proteins, have optimized their minute machinery to function as an efficient versatile engine. As earlier explained, the capacity of several viral proteins to inhibit caspases is well founded. Interestingly the N protein of hantaviruses hampers the activity of caspases, thus inhibiting apoptosis in infected cells (175, 176). The enzymatic activity of the executioner caspase-3 is significantly lessened by direct interaction with the N protein of the HPS-causing ANDV (ANDV N). Caspase-3 cleaves the ANDV N protein at the DIIDLID<sub>285</sub> site. Mutating the specific cleavage site silences the proteolytic process as well as restores the activity of caspase-3 (175). Intriguingly, the DIIDLID<sub>285</sub> site is not conserved among other hantaviruses, but the interaction and cleavage of the N protein by active caspase-3 has been reported for other hantaviruses as well (**Paper I**).

Human granzymes are strong apoptosis and inflammation modulators during viral infections (491). Different granzymes have been shown to activate specific cell death pathways upon delivery into target cells. The poxvirus CrmA protein and the adenoviral Ad5-100kDa are well-defined granzyme B inhibitors (550-552). The proteolytic activity of granzyme B is also directly hindered by ANDV, PUUV and DOBV N protein (i.e. 80% inhibition of granzyme-B activity)(175)(**Paper I**). During hantavirus-induced pathogenesis, patients present with high levels of perforin, granzyme A and B in serum and BAL fluid, illustrating the strong cytotoxic cell-mediated response occurring in patients (378, 639). Nevertheless, as earlier mentioned, infected endothelial cells remain undamaged in histological sections from HPS-patients (343). While incapable of eliminating the apoptosis-resistant infected cells, the robust lymphocyte activation leads to aberrant killing of uninfected, bystander cells (323). This has been partially explained by the ability of hantaviruses to antagonize the deadly functions of granzyme B and caspase-3, as well as the up-regulated HLA class I expression on the surface of infected cells (175, 323). However cytotoxic cells not only kill virus-infected cells by degranulation of serine proteases, but also by engagement of the death receptor pathway. Interestingly, we have recently reported that HTNV induces downregulation of DR5 from the surface of infected cells, protecting the latter ones from TRAIL-mediated killing (**Paper III**).

## 2 AIMS

This thesis aimed at contributing to the knowledge on how hantaviruses cause pathogenesis in humans following two lines of investigation: first, by gaining insights into the fundamental mechanisms behind hantavirus-mediated inhibition of the apoptotic cell death programme, and second, by further investigating the differences between phylogenetically distinct hantaviruses in terms of viral entry and receptor usage.

The specific aim of each particular study included in this thesis were:

- **Paper I.** The human pathogenic hantaviruses ANDV and HTNV had been previously reported to inhibit intrinsic apoptosis and to protect infected cells from cytotoxic lymphocyte-mediated killing. In **Paper I**, our efforts focused on describing the similarities and differences between pathogenic and non-pathogenic hantaviruses in terms of apoptosis resistance.
- **Paper II.** The nucleocapsid (N) protein of hantaviruses represents an inhibitor of active caspase-3. However, as reported previously and in **Paper I**, during apoptosis induction hantaviruses inhibit the cleavage of caspase-3 into active caspase-3, indicating an inhibitory effect upstream of caspase-3 activation. In **Paper II** we set out to uncover the specific mechanism employed by hantaviruses in order to block caspase-3 activation and apoptosis execution in infected cells.
- **Paper III.** Hantaviruses inhibit granzyme B activity, partially explaining how hantaviruses protect infected cells from natural killer cell-mediated killing. Nevertheless, cytotoxic lymphocytes can eliminate target cells also through the extrinsic apoptotic pathway. **Paper III** aimed at determining whether hantavirus-infected cells were protected from death receptor-mediated apoptosis and, if so, at defining the mechanistic insights behind such phenomenon.
- **Paper IV.** Recently the host cell factor PCDH-1 was reported as a new receptor modulating the entry of the HPS-causing ANDV and SNV. Intriguingly the HFRS-causing HTNV and SEOV were not dependent on PCDH-1 for entry. In **Paper IV**, we sought to investigate the importance of PCDH-1 in infection of human cells by PUUV, the principal causative agent of HFRS in Europe.



### 3 RESULTS AND DISCUSSION

#### 3.1 PAPER I: DISTINCT HANTAVIRUSES INHIBIT APOPTOSIS IN INFECTED CELLS – EVIDENCE OF A SHARED MECHANISM TO ESCAPE DEATH

Hantaviruses are non-cytopathogenic viruses primarily infecting endothelial cells. In hantavirus-infected humans, vascular leakage, strong cytotoxic lymphocyte responses and hyperinflammation represent common hallmarks of disease. Despite the robust immune activation reported, infection is widely spread throughout the endothelium of patients, resulting in large amounts of infected endothelial cells, the majority of which appear undamaged (270, 343). These rather puzzling pieces of evidence encouraged further investigations that have lead into the recent discovery that two pathogenic hantaviruses, ANDV and HTNV, inhibit programmed cell death – i.e. apoptosis – in infected endothelial cells (175).

##### 3.1.1 Inhibition of staurosporine-induced apoptosis is a feature displayed by different hantaviruses during infection

In order to further understand the implications of the capacity of hantaviruses to hamper apoptosis in infected cells, we investigated whether the anti-apoptotic mechanisms displayed by ANDV and HTNV represented a common attribute shared by other hantaviruses.

Human umbilical vein endothelial cells (HUVEC) were infected with different pathogenic and non-pathogenic hantaviruses belonging to three phylogroups: the *Arvicolinae*-borne PUUV, TULV and PHV; the *Murinae*-borne HTNV and SEOV, and the *Sigmodontinae*-borne ANDV (56, 58). At three days post-infection, apoptosis was induced by exposure of infected and uninfected endothelial cells to the chemical staurosporine, a kinase inhibitor that triggers caspase-3 activation and cell death (640). As in previous publications, levels of chemically-induced apoptosis were assessed by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (BOX 1), which allows for detection of DNA fragmentation, and by cleavage of PARP, a substrate of active caspase-3 (175, 344). Interestingly, all tested hantaviruses, including HPS-causing (ANDV), HFRS-causing (HTNV, SEOV and PUUV), and putative non-pathogenic (TULV and PHV) hantaviruses, inhibited staurosporine-mediated DNA fragmentation and PARP cleavage in endothelial cells. Furthermore, a 40% inhibition (on average) of the enzymatic activity of the executioner caspase-3 was reported in infected cells, compared to uninfected cells after apoptosis induction. The reduction in caspase-3 activity was significant for all tested hantaviruses. Caspase-3 is activated and functional when cleaved by an initiator caspase, such as caspase-8 or caspase-10 (454-456). Interestingly, the amount of active, cleaved, caspase-3 was significantly less in hantavirus-infected cells compared to uninfected cells, after

staurosporine treatment. These results suggested a potential inhibition upstream of the process of caspase-3 activation. This question was addressed in **Paper II** as further discussed in section 3.2.

#### **BOX 1**

Highlighted method: TUNEL (terminal deoxynucleotidyl dUTP nick end labelling)

Assay allowing for the detection of DNA breaks that occur during the last phase of apoptosis. This method relies on the enzyme terminal deoxynucleotide transferase (TdT) which incorporates deoxynucleotides to the 3'-terminus of fragmented DNA. TdT-incorporated nucleotides are tagged with a fluorescent label or a chemical label instead, which can be linked indirectly to either a fluorescent label or an enzyme.

As a whole, these results were in line with previous observations in ANDV- and HTNV-infected cells, and strongly suggested that both pathogenic and non-pathogenic hantaviruses share the capacity to inhibit intrinsic apoptosis (175).

### **3.1.2 Pathogenic as well as non-pathogenic hantaviruses inhibit cytotoxic lymphocyte-mediated killing of infected cells**

Next, we sought to discern the relevance of apoptosis inhibition in relation to cell-mediated killing and the significance of this in terms of pathogenesis of hantavirus-associated diseases. ANDV- and HTNV-infected endothelial cells are resistant to NK cell-mediated killing (175), but whether infection with non-pathogenic hantaviruses protects endothelial cells from cytotoxic lymphocyte-mediated killing had not been investigated. Since the non-pathogenic TULV and PHV displayed similar anti-apoptotic features as the pathogenic ANDV, HTNV, SEOV and PUUV, we hypothesised that infection of HUVEC with non-pathogenic hantaviruses would protect targeted infected cells from killing by cytotoxic lymphocytes.

NK cells were obtained from healthy donors and used in order to study the effects of cytotoxic lymphocyte-mediated killing in targeted hantavirus-infected endothelial cells. The isolated NK cells were pre-stimulated with IL-15 and the HLA class I on the surface of endothelial cells was blocked in order to allow for recognition and degranulation by the NK cells. As shown in **Paper I**, NK cells efficiently killed uninfected cells while hantavirus-infected cells remained resilient to cytotoxic lymphocyte-mediated killing. Regardless of the level of pathogenicity in humans, all tested hantaviruses protected infected cells from NK cell-mediated killing.

Hantaviruses replication rate is much slower than that reported for many other RNA viruses (144). In order to succeed in producing new viral particles, many RNA viruses hijack the host cell's machinery in order to inhibit antiviral defences and/or apoptosis (641). The fact that hantaviruses belonging to three distinct phylogroups inhibit apoptosis and hamper cell-mediated killing of infected target cells, suggests that apoptosis resistance represents an

important strategy for hantaviruses to persist and replicate within the host's cells. These data are also in line with previous publications showing no cytopathogenicity induced by hantaviruses in infected cells (340, 341). Importantly, these results contribute to a potential explanation for the fact that infected endothelial cells remain undamaged in patients despite the strong cytotoxic lymphocyte responses occurring in HFRS and HPS patients (270, 342, 343, 373, 390, 394).

### **3.1.3 The nucleocapsid protein of hantaviruses is an inhibitor of granzyme B and caspase-3**

Knowing that the N protein of ANDV inhibits the enzymatic activity of caspase-3 and granzyme B (175), our next step was to determine whether the N protein of other hantaviruses also inhibited the activity of these two cellular proteases. Co-incubation of recombinant active human caspase-3 with recombinant N protein from ANDV (*Sigmodontinae*-borne hantavirus), DOBV (*Murinae*-borne hantavirus) or PUUV (*Arvicolinae*-borne hantavirus) resulted in a slight inhibition of caspase-3 activity (approximately 20-25% inhibition). Moreover, active caspase-3 cleaved the N protein of different hantaviruses into two fragments of approximately 35 kDa and 10 kDa. In contrast, co-incubation of recombinant active human granzyme B with the recombinant N protein of ANDV, DOBV and PUUV resulted in a strong inhibition of granzyme B activity (>75% inhibition) lasting for more than 24 hours. As earlier reported for ANDV (175), granzyme B cleaved the N protein of other pathogenic and non-pathogenic hantaviruses, resulting however in unique cleavage patterns for each of the specific N proteins. As such, these results showed that although the N protein of hantaviruses represents a modest inhibitor of caspase-3 activity, it strongly inhibits active granzyme B.

Seeking additional knowledge on biochemical interactions, we have found out that not only granzyme B but also granzyme A is capable of cleaving the nucleocapsid protein of hantaviruses [our unpublished results]. High levels of granzyme A, as well as granzyme B and perforin, are detected in HFRS patients (378). Granzyme A is in fact the most common of the five granzymes expressed in killer cell cytotoxic granules in humans (491). This serine protease has apoptotic functions, including the degradation of histones and generation of ROS species, as well as proinflammatory effects (642). Whether the interaction between the N protein of hantaviruses and granzyme A leads to inhibition of the latter, and if this effect has some physiological repercussions during viral pathogenesis, remains to be investigated.

Poxviruses also express a viral protein, the serpin CrmA, that inhibits the activity of caspase-3 and granzyme B, thereby inhibiting cytotoxic lymphocyte-mediated apoptosis (553, 556, 558, 643). Other examples are p35 expressed by baculoviruses, which inhibits various initiator and executioner caspases, and L4-100K protein encoded by adenoviruses, which is a granzyme B substrate hampering granzyme B-mediated cell death (644, 645). Interestingly though, these are DNA viruses encoding for more than two-hundred proteins, while hantaviruses encode a total of four to five proteins.

The nucleocapsid protein of another negative-sense single-stranded RNA virus, the Junin arenavirus, represents in fact a decoy substrate hindering apoptosis execution by sequestering caspase-3 and diverting it from cleaving its natural targets (646). In line with our results showing the interaction of N protein with caspase-3, a recent publication showed that the N protein of TULV colocalizes with cleaved caspase-3 in infected cells (177). Davies *et al.* reported that TULV induced apoptosis in approximately 60% of the bystander, uninfected cells, but only around 4% of the infected cells were apoptotic. Colocalization of the N protein of TULV with cleaved caspase-3 was observed at late stages during infection (>7 days post-infection). Moreover, TULV persisted in infected cells for at least 30 days. The authors speculated that TULV N protein inhibits apoptosis in infected cells by spatially sequestering cleaved caspase-3 in the cytosol in order to avoid targeting of downstream substrates.

Caspases are also important during cell proliferation and differentiation (647). It is known that cleavage of virus proteins by caspases occurs in several settings – e.g. the capsid precursor VP90 of human astroviruses (648), the nucleocapsid protein of influenza virus (649), NS5A of hepatitis C virus (650, 651), and ICP22 of herpes simplex virus (652) –, possibly facilitating viral protein maturation and functionality (648). The N protein is highly abundant in hantavirus-infected endothelial cells (204, 229), and different smaller-in-size versions of the hantavirus N protein are formed at late stages after infection [our unpublished results]. It is thus tempting to speculate that the processing of the hantaviral N protein not only hampers the apoptotic machinery of the cell but may also be necessary for still unknown functions important for hantavirus replication and/or pathogenesis.

### 3.1.4 Conclusions and future perspectives of Paper I

As a whole, in **Paper I** we investigated the similarities in apoptosis inhibition displayed by six hantaviruses belonging to three different phylogroups and causing different grades of pathogenicity in humans, ranging from highly pathogenic to non-pathogenic. We found that ANDV, HTNV, SEOV, PUUV, TULV and PHV, all hamper chemically-induced apoptosis as well as cell death triggered by highly cytotoxic NK cells in primary endothelial cells. Furthermore, active caspase-3 and granzyme B, two cellular proteases playing key roles during apoptosis, were inhibited by the N protein of different hantaviruses and resulted in cleavage of the viral proteins in different fragments. Taken together, our results demonstrate that apoptosis resilience is a common trait shared by different hantaviruses regardless of their level of pathogenicity in humans. As such, apoptosis inhibition may represent an important factor for hantaviruses when establishing persistent infections in their hosts and may be implicated in the pathogenesis reported in humans. The specific mechanism by which hantaviruses hinder apoptosis remained elusive; in **Paper II**, we aimed at identifying other factors contributing to the described resistance to apoptosis.

### **3.2 PAPER II: HANTAVIRUSES HAMPER MITOCHONDRIAL MEMBRANE PERMEABILIZATION AND INDUCE BCL-2 UP-REGULATION – FURTHER INSIGHTS INTO THE MECHANISM**

The cellular pathways towards apoptosis are highly dynamic and complex processes. Such complexity echoes of course in the entangled mechanisms that viruses use to subvert apoptosis. Although co-incubation of active caspase-3 with hantavirus N protein results in a slight inhibition of the enzyme's activity (i.e. approximately 20 to 25% inhibition), this seems hardly enough to explain the strong anti-apoptotic features observed during chemical-induction of apoptosis in hantavirus-infected cells (175, 323)(**Paper I**). During hantavirus-mediated resistance to apoptosis, caspase-3 cleavage into its active form is hampered. This interesting result pointed towards an inhibition of the apoptotic process upstream of the executioner caspase-3. In **Paper II**, our efforts aimed at shedding some light on the mechanistic process leading to the hampered caspase-3 activation by cleavage and subsequent apoptosis in hantavirus-infected cells.

#### **3.2.1 Chemically-induced mitochondrial membrane permeabilization is hindered in hantavirus-infected cells**

Active caspase-3 coordinates the demolition of cellular structures such as proteins from the cytoskeleton and the DNA-repairing machinery, consequently triggering a point of no return leading to controlled cell destruction (456, 458, 459). Caspase-3 is produced as a zymogen and its activation is tightly regulated. Cleavage and activation of caspase-3 is mediated by initiator caspases such as caspase-8 and caspase-9, as well as by granzyme B (456, 458, 459). To further understand how hantaviruses hamper caspase-3 activation, we analysed for activity and cleavage of the initiator caspases upstream of caspase-3. Confirming our previous results, chemical induction of apoptosis with staurosporine resulted in less cleaved caspase-3 and PARP in ANDV- and HTNV-infected A549 cells. Furthermore, the enzymatic activity of caspase-8 and caspase-9 was decreased in hantavirus-infected cells as compared to uninfected cells. Western blotting revealed that, as observed for caspase-3, there was less active, cleaved caspase-8 and caspase-9 present in infected cells after chemical induction of apoptosis. Taken together, these data represented an explanation to the reported decreased activation of caspase-3 and indicated that hantavirus infection affects chemical induction of apoptosis upstream of the initiator caspase-8 and -9.

Mitochondria mediate cell growth and death, and deregulation of mitochondrial function is associated to cell stress, disease and aging (523, 653). Exogenous and endogenous stimuli such as growth factors, oxidative stress and DNA damage, initiate the intrinsic apoptosis pathway. Perturbation of the mitochondrial membrane represents the functional consequence of pro-apoptotic stimuli in a cell, leading to cyt C release from mitochondria, formation of the apoptosome and activation of caspase-9 (499, 653, 654). Subsequently, active caspase-9 cleaves and activates caspase-3. Through a 'feedback-loop', caspase-3 amplifies the apoptotic signalling through further activation of apical initiator caspases (486, 487). The interesting observation that hantaviruses hampered the cleavage and activation of both initiator and executioner caspases, led us to investigate whether or not the

permeabilization of mitochondria was implicated in the viral-mediated resilience to apoptotic cell death.

We analysed if hantaviruses could inhibit the permeabilization of mitochondria by monitoring mitochondrial membrane potential loss (BOX 2) and release of cyt C. Immunofluorescence studies revealed a significant release of cyt C from mitochondria in uninfected A549 cells after exposure to staurosporine. Conversely the cyt C release was clearly hampered in ANDV- and HTNV-infected A549 cells after chemical induction of apoptosis at 3 days post-infection. Further supporting these results, mitochondrial membrane potential loss was significantly greater in uninfected cells compared to hantavirus-infected cells after exposure to staurosporine. Overall, an average drop of 45% in mitochondrial viability (determined as membrane potential loss) was detected in uninfected cells compared to an average 7.0% and 7.8% decrease in ANDV- and HTNV-infected cells, respectively, after chemical induction of apoptosis. Altogether these results pointed towards an anti-apoptotic effect orchestrated by hantaviruses on the process of mitochondrial permeabilization and release of pro-apoptotic factors, thus preventing apoptosis induction.

#### **BOX 2**

Highlighted method: TMRE (tetramethylrhodamine, ethyl ester)

Assay used for the quantification of changes in mitochondrial membrane potential allowing for monitoring in live cells. Fluorescent measurements can be performed by flow cytometry, fluorescent microscopy or spectrophotometry. TMRE is a positively-charged cell permeant dye of red/orange fluorescence. The dye passively accumulates in active mitochondria because of the relatively negative charge of these organelles. Inactive or depolarised mitochondria have a permeabilised membrane and fail to sequester TMRE due to a decreased membrane potential.

### **3.2.2 Hantaviruses induce BCL-2 up-regulation thereby protecting infected cells from intrinsic apoptosis**

Hepatitis C virus (HCV) and human T-cell leukaemia virus (HTLV) are two different RNA viruses that exhibit anti-apoptotic effects in infected cells by inducing the cellular expression of pro-survival BCL-2 family members (655-657). As explained in section 1.4.3, the BCL-2 family of proteins control cell fate through interactions between pro-survival and pro-apoptotic family members that depend on affinity and protein abundance (658, 659). Anti-apoptotic proteins such as BCL-2 or BCL-X<sub>L</sub> impede the formation of pores in the outer membrane of mitochondria and the release of pro-apoptotic factors, such as cyt C (660). After having described that hantaviruses delay mitochondrial potential loss in infected cells, we performed a transcriptomic screening in order to identify potential alterations in the expression of any BCL-2 family members that could explain the results obtained previously. mRNA levels of BCL-2 were significantly up-regulated in ANDV- and HTNV-infected A549 cells at 3 days post-infection. Additionally, BCL-2 protein expression was also significantly increased in infected cells. The expression of the pro-apoptotic BCL-2 family members included in the screening did not differ from that reported in uninfected cells.

Moreover, flow cytometry analysis including specific gating on HTNV-infected cells (i.e. HTNV N protein positive cells) revealed that only infected cells over-expressed BCL-2 and that bystander uninfected cells had similar BCL-2 expression levels as reported in control uninfected cells. Finally, we also detected increased NF- $\kappa$ B activation in ANDV- and HTNV-infected cells. Many viral infections induce NF- $\kappa$ B activation, which in turn stimulates the expression of anti-apoptotic genes, including BCL-2 (655-657). Previous reports have shown that single expression of HTNV N protein inhibits TNF-mediated activation of NF- $\kappa$ B by sequestering NF- $\kappa$ B to the cytoplasm, but expression of ANDV, SNV or PUUV N proteins does not inhibit nuclear translocation of NF- $\kappa$ B (176, 661). On the other hand, another study showed that NF- $\kappa$ B was in fact translocated from the cytoplasm to the nucleus in HTNV-infected cells (662). Taken together, these results indicate that although the N protein may have the capacity to block nuclear translocation of NF- $\kappa$ B when solely expressed, active viral infection and replication triggers NF- $\kappa$ B activation and nuclear translocation in infected cells.

In an effort to further explain the specific up-regulation of BCL-2 in hantavirus-infected cells, A549 cells were transfected with HTNV N protein or HTNV glycoproteins to determine whether or not these viral proteins were directly involved in BCL-2 induction. However, single expression of the N protein or the glycoproteins of HTNV did not result in a significant increase in BCL-2 protein levels.

Next, we sought to confirm that the increased BCL-2 levels were key in protecting hantavirus-infected A549 cells from chemically-induced apoptosis. Inhibition of BCL-2 by the small molecule inhibitor ABT-737 followed by exposure to staurosporine restored apoptosis in hantavirus-infected cells. ABT-737-treated hantavirus-infected cells were equally susceptible to chemical-induction of apoptosis as ABT-737-treated uninfected cells and displayed similar levels of caspase-3 activity. In line with these results, siRNA knock down of BCL-2 followed by chemical induction of apoptosis resulted in increased levels of cleaved caspase-3 and PARP in hantavirus-infected cells. BCL-2 knock down also translated into restored caspase-3 activity in ANDV- and HTNV-infected cells to the same extent as that observed in treated uninfected cells.

### **3.2.3 Conclusions and future perspectives of Paper II**

In **Paper II**, we report further mechanistic insights into how hantaviruses inhibit chemically-induced apoptosis in infected cells. Infection with ANDV or HTNV caused an increase in BCL-2 expression as well as nuclear translocation of NF- $\kappa$ B. The BCL-2 increase was detected only in hantavirus-infected cells and not in bystander uninfected cells; however, single expression of viral proteins did not result in BCL-2 up-regulation and the observed phenomenon may be dependent on active viral replication. Silencing of BCL-2 in hantavirus-infected cells restored caspase-3 activity as well as PARP cleavage after chemical induction of apoptosis, demonstrating the importance of BCL-2 up-regulation in protecting ANDV- and

HTNV-infected cells from apoptosis. As such, we propose that the increased expression of BCL-2 in infected cells would suffice to hamper the permeabilization of mitochondria by counteracting the effects of pro-apoptotic BCL-2 family members, consequently delaying the release of cyt C and the activation of caspases leading to apoptosis.

Given the relevance of endothelial cells as primary targets of hantaviruses, our findings on BCL-2 up-regulation should be further confirmed in primary endothelial cells. Hantaviruses are also capable of infecting and replicating in B lymphocytes [our unpublished results]. Interestingly, HFRS-patients have an increased risk for lymphoma (663). Within the different viruses associated with lymphoma, EBV can cause infectious mononucleosis which, like hantaviruses-associated diseases, has an acute onset (664, 665). EBV also inhibits apoptosis by expressing viral homologues to BCL-2, which block the effect of the pro-apoptotic BCL-2 family members BAX and BAK (610, 611). In **Paper II**, we show that hantaviruses inhibit apoptosis by up-regulating the pro-survival factor BCL-2. Furthermore, the hantavirus N protein has been shown to stimulate degradation of p53 in a MDM-2-dependent manner (666). Apoptosis resistance, BCL-2 up-regulation and p53 loss of function all represent attributes associated to cancer and the development of lymphoma (667, 668). The effects of hantaviruses on B lymphocytes and the potential transformation of these cells remains as an interesting topic to be further investigated.

### **3.3 PAPER III: HANTAVIRUS-INFECTION PROTECTS FROM TRAIL-MEDIATED KILLING BY DOWN-REGULATING DR5 – REMOVING THE FUSE FROM THE BOMB**

Cytotoxic lymphocytes are equipped with diverse tools in order to trigger target cell death. On the one hand, cytotoxic granule content release engages the intrinsic apoptotic pathway mediated by perforin and granzymes. On the other hand, the elimination of target cells can be mediated via death receptors which trigger the extrinsic apoptotic pathway. Although hantaviruses efficiently block granzyme B and intrinsic apoptosis, whether this resilience to die encompassed as well inhibition of the extrinsic pathway towards apoptosis had not been investigated. In **Paper III**, we described the intricate process by which HTNV infection causes surface down-regulation of DR5 in a ubiquitin-dependent manner, resulting in DR5 degradation via the 26S proteasome, and protecting infected cells from extrinsic apoptosis initiated via the ligand TRAIL.

#### **3.3.1 Hantavirus causes TRAIL production *in vitro* and in HFRS patients**

Increased levels of TRAIL have been documented during the course of HFRS in HTNV-infected Chinese patients (377). In order to determine if hantaviruses can directly induce the production of TRAIL by infected cells, HUVECs were infected with HTNV and the expression of TRAIL was monitored over time. HTNV induced TRAIL on the surface of infected cells as well as the secretion of soluble TRAIL. A significant increase in TRAIL expression was detected from 72 hours after infection. NK cells are activated by contact with

HTNV-infected cells in an IL-15 dependent manner (323). In our investigations, we next sought to clarify if hantaviruses could indirectly stimulate TRAIL production by NK cells. Analysis of supernatants from co-incubated NK cells revealed an increased secretion of sTRAIL by NK cells in contact with HTNV-infected cells. Further, both subsets of NK cells, CD56dim and CD56bright, were activated and expressed significantly higher levels of TRAIL on their cell surface. Interestingly, surface up-regulation of TRAIL was not dependent on cell contact, as demonstrated by co-incubation of cells with a transwell system. Taken together, these data indicated that HTNV *per se* triggers TRAIL production in infected endothelial cells and that infection of endothelial cells induces co-incubated NK cells to express TRAIL and to secrete soluble TRAIL, which has pro-inflammatory effects (669). The general up-regulation of TRAIL expression could potentially contribute to the activation of cytotoxic lymphocytes in patients and accentuate the NK cell-mediated killing of bystander, uninfected cells that occurs at least *in vitro* (323).

Finally, we analysed if increased TRAIL production was a common feature during human hantavirus infections. As previously observed in HTNV-infected HFRS patients (377), significantly higher levels of soluble TRAIL were also detected in plasma from PUUV-infected HFRS patients.

### **3.3.2 Infection with HTNV protects from TRAIL-mediated killing**

Hantavirus-infected cells induce NK cell activation partially through IL-15 expressed on the surface of infected cells (323). Furthermore, IL-15 stimulated NK cells are incapable of killing hantavirus-infected endothelial cells (**Paper I**). NK cells are specialised killers able to destroy infected and tumorigenic cells through membrane-bound TRAIL (670, 671). Since we observed a considerable induction of TRAIL expression on the surface of NK cells, we next explored the susceptibility of HTNV-infected cells to TRAIL-mediated killing.

A549 cells, which are known to be sensitive to TRAIL-induced apoptosis, were infected with HTNV and exposed to increasing concentrations of TRAIL. Apoptosis was significantly hindered in HTNV-infected A549 cells as determined by less TUNEL positive infected cells and significantly decreased caspase activity in HTNV-infected cells compared to uninfected cells, after exposure to TRAIL. Similarly, HTNV-infected HUVECs were also protected from apoptosis. The amount of TUNEL positive uninfected HUVECs augmented with increasing concentrations of TRAIL, while the number of TUNEL positive HTNV-infected HUVECs remained significantly lower. In line with these data, the activity of caspase-8 and caspase-3 was significantly lower in HTNV-infected cells compared to uninfected cells. Overall, these results clearly showed that HTNV protects infected cells from apoptosis triggered by TRAIL.

The amount of detected cleaved caspase-8 was less in infected cells compared to uninfected cells after exposure to the apoptotic ligand TRAIL. Since caspase-8 is cleaved after interaction of TRAIL with its receptor and formation of the DISC, our results indicated that HTNV inhibited apoptosis in an initial step of the extrinsic death signalling pathway.

### **3.3.3 HTNV induces 26S proteasome-dependent depletion of DR5 in infected endothelial cells**

Certain viruses avoid being killed through extrinsic apoptosis by targeting the surface expression of death receptors (672, 673). Flow cytometry analysis of surface expression of DR4 and DR5 (the receptors of TRAIL) in HTNV-infected HUVECs and A549 cells revealed the down-regulation of DR5 from the surface of infected cells. DR5 down-regulation started already at 24 hours post-infection and still remained after 4 days post-infection. DR4 was not expressed on the surface of uninfected HUVECs and only weakly detected in A549 cells. mRNA and protein analysis indicated that the mRNA levels of DR5 were not changed over time, but that the amount of DR5 protein significantly decreased at 48 hours post-infection matching the absence of cell surface DR5. The remarkable phenomenon of DR5 down-regulation was not accompanied by degradation of other components of the DISC, such as caspase-8 or FADD, indicating that DR5 was specifically targeted for elimination. It is noteworthy that TRAIL binds with greater affinity to DR5 than to its other receptor, DR4, and that endothelial cells express low levels of DR4 (674, 675). Interestingly, rodents, the natural reservoirs of hantaviruses, express only DR5 as TRAIL receptor variant (676). Although certainly speculative, the specific downregulation of DR5 observed in human endothelial cells could represent an unexpected advantage fruit of the particular interplay between hantaviruses and DR5 in their natural reservoirs.

Studies on the acquisition of TRAIL resistance have demonstrated that DR5 is targeted for degradation by the ubiquitin proteasome system (677, 678). Treatment of HTNV-infected A549 cells with the proteasome inhibitor MG132 or the ubiquitin-ligase inhibitor SMER-3 blocked DR5 degradation and rescued it to the surface of infected cells. Importantly, HTNV-infected cells expressing MG132-rescued DR5 on their surface were susceptible to TRAIL-mediated killing. These results were further complemented with the findings that DR5 is directly ubiquitinated early after HTNV infection and targeted for 26S proteasome-dependent degradation. The *in situ* proximity ligation assay (PLA) represents a novel method allowing for the detection of protein-protein interactions with a theoretical maximal distance of 40 nm between the two protein components (BOX 3). Implementing the PLA, we could demonstrate a transitional ubiquitination of DR5 starting around 24 hours after infection and peaking at 36-42 hours post-infection. Furthermore, the decrease in total

DR5 protein levels correlated with the observed ubiquitination of DR5 in HTNV-infected cells.

### BOX 3

Highlighted method: PLA (*in situ* proximity ligation assay)

Method allowing for *in situ* detection of protein interactions as well as protein modifications with high specificity and sensitivity. The assay relies on the usage of two primary antibodies targeting specific epitopes of a single protein or targeting two different proteins of interest in a cell. Secondary antibodies carrying oligonucleotides (probes) bind to the primary antibodies. Only within close proximity will the probes become ligated (maximum interacting distance of 40 nm), resulting in the formation of closed, circular DNA templates that will be amplified by DNA polymerase. Finally, complementary detection oligos carrying fluorochromes are added to the system. The complementary detection oligos hybridize to specific sequence repeats in the amplicons. The resulting signal is detected by fluorescent microscopy as small fluorescent punctae.

#### 3.3.4 HTNV induces altered DR5 subcellular localization at late stages after infection

At late stages after HTNV infection and despite a significant increase in DR5 protein expression, no DR5 was detected on the surface of infected cells. This interesting observation pointed towards an intracellular accumulation of the death receptor in HTNV-infected cells. While DR5 presented a scattered intracellular distribution at all timepoints in uninfected HUVECs, perinuclear accumulation of DR5 was noticed in HTNV-infected HUVECs at 72 hours post-infection and onwards. DR5 co-localised with LAMP-1, a lysosome marker, in vesicle-like structures in 68% of the infected cells on average, while co-localization was reported in only 15% of the uninfected cells on average, at the same timepoint. Of note, HTNV glycoproteins were found to co-localize with DR5 and LAMP-1 in some of the infected cells. DR5 also co-localized with Rab7a, a late endosome marker, and partially with the autophagosomal marker LC3 in HTNV-infected cells. As a whole, these investigations indicated that HTNV infection alters the transport of newly produced DR5 to the cell surface, resulting in intracellular accumulation of DR5 in LAMP-1 positive compartments.

The location of HTNV in LAMP-1 positive vesicles has previously been described, although in this particular study viral proteins co-localised with LAMP-1 already at 24 hours post-infection (225). In another report, McNulty *et al.* demonstrated that in primary human lung microvascular endothelial cells (HMVEC-L) the glycoproteins of ANDV accumulated at 3 days post-infection in the lysosomes of infected cells, co-localising as well with mTOR (679). It has been suggested that autophagosomal degradation of hantavirus glycoproteins is necessary for productive viral replication of SNV, however autophagy was not clearly reported in that study (680). Several reports have highlighted the importance of autophagy as a protective mechanism against different viral pathogens such as HIV-1, chikungunya virus or human simplex virus 1 (681-684). Otherwise, some viruses target Beclin-1 or the

processing of LC3 in order to block autophagy and counteract viral clearance through autophagosomes (685-687). Then some RNA viruses, such as polioviruses or the flaviviruses dengue virus and West Nile virus, cleverly hijack the autophagic machinery in order to form vesicular clusters thought to act as sites of viral replication and virion assembly (688-690). Finally, influenza A virus and HCV have been reported to induce autophagosome formation but to block fusion with lysosomes, again resulting in beneficial effects for these viruses in terms of replication and virion production (691-693). Viruses are constantly under high pressure exerted by the host cell; how a virus interacts with the host autophagic machinery may depend on the particular replication requirements of each virus. Whether hantaviruses make use of the autophagic machinery for their own viral replication or if accumulation of hantaviral proteins in lysosomes represents the host's mechanism for viral clearance remains an enigma to be elucidated.

### **3.3.5 HTNV triggers the appearance of DR5 short in infected primary endothelial cells**

In conjunction with the increased intracellular accumulation of DR5, the short isoform of DR5 (DR5<sub>s</sub>) was also detectable by western blot in HTNV-infected cells at late timepoints. DR5<sub>s</sub> represents a differential DR5 product resulting from alternative splicing (694). Currently the regulation of DR5<sub>s</sub> expression as well as the implications of its presence in cells is not well understood. DR5<sub>s</sub> is not generally expressed in primary cells, but increased levels are generally detected in cancer cells (695-697). It is difficult to theorise on the repercussions of the presence of DR5<sub>s</sub> in hantavirus-infected cells since not much is known about DR5<sub>s</sub> itself. Alternative splicing of RNA represents a new paradigm in host-pathogen interactions, and it is well established that alternate spliced variants associate with cancer and other chronic pathologies (698, 699). Viral infections can cause changes in the usual patterns of alternative splicing in a host cell, and some viral proteins are known to directly interact with the host's RNA splicing machinery (700). Upon Sendai virus infection TBK1<sub>s</sub>, a spliced version of TBK1, is produced. This spliced variant binds to RIG-I and inhibits the IFN- $\beta$  signalling pathway by hampering the interaction between RIG-I and the mitochondrial antiviral signalling protein (MAVS) (701). Dengue virus NS5 protein has been reported to interfere with cellular splicing in the nucleus of infected cells, modulating the splicing patterns of certain antiviral factors such as MxA (702). Zika virus, influenza virus, HIV and papillomavirus are well-known to interact with and modulate the spliceosome complex (703-707).

### **3.3.6 Conclusions and future perspectives of Paper III**

Hantaviruses are not cytopathogenic and despite the strong cytotoxic lymphocyte responses observed during HFRS and HPS, infected endothelial cells remain undamaged in patients (340-344). Infection with hantaviruses confers resistance to cytotoxic lymphocyte-mediated killing by inhibiting caspase-3 and granzyme B activity, partially explaining the above-mentioned phenomenon (175). In **Paper III**, we demonstrated that HTNV silences the

expression of DR5 in infected cells, protecting these from TRAIL-mediated killing. Infection induced a ubiquitin-dependent targeting of DR5 for 26S proteasome degradation early after infection. Interestingly, DR5 remained absent from the surface of HTNV-infected cells also late after infection, accumulating in LAMP-1 positive vesicles. Noteworthy, HTNV infection induced the appearance of the short isoform of DR5, DR5<sub>s</sub>, which represents an alternative spliced version of DR5 often expressed in different cancer cell types (695-697).

Seeking further knowledge, we also tested if other hantaviruses would induce DR5 surface down-regulation in infected cells. Both PUUV and PHV caused a rapid decrease in expression of the death receptor from the surface of infected HUVECs. As such, these results indicate that not only pathogenic hantaviruses affect the surface expression of DR5 in infected cells. Further studies should however address whether these hantaviruses are also capable of inhibiting TRAIL-mediated killing. Then, since rodents only express DR5 as TRAIL receptor variant, it would be of interest to investigate if hantaviruses induce DR5 down-regulation also in their natural hosts and the significance in terms of viral persistence. Finally, further studies should aim at understanding how newly produced DR5 accumulates in LAMP-1 compartments instead of reaching the surface of the cell. It is also tempting to speculate that the presence of DR5<sub>s</sub> in HTNV-infected cells may indicate that hantaviruses have strategies by which they interact with the host splicing machinery.

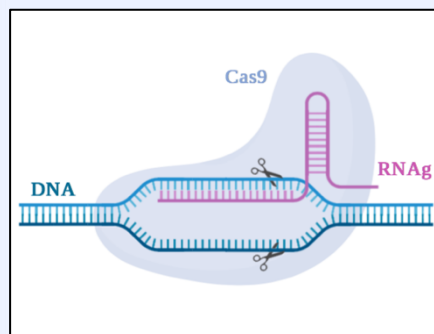
### **3.4 PAPER IV: THE ROLE OF PCDH-1 IN PUUV INFECTION AND REPLICATION**

The primary targets of hantaviruses are endothelial cells (204). Attachment to the host cell membrane receptors is mediated by the glycoproteins Gn and Gc expressed as spike-shaped complexes on the surface of the viral particles (152). Important roles in hantavirus entry have been previously reported for the integrins  $\alpha$ V $\beta$ 3 and  $\alpha$ 5 $\beta$ 1, DAF and C1qR (212, 214, 220-223). Nevertheless, the precise mechanism(s) of viral entry are still not fully understood. Protocadherin-1 (PCDH-1) is a cell adhesion factor primarily expressed in human airway epithelium and endothelium (708). PCDH-1 was recently described as an essential entry factor for the HPS-causing ANDV, SNV and MAPV, as well as for the non-pathogenic PHV, in human lung endothelial cells (224). Further, PCDH-1 knock out (KO) Syrian hamsters generated via CRISPR-Cas9 largely survived ANDV challenge and exhibited only limited pathology (224). Infection with the HFRS-causing HTNV and SEOV did not depend on PCDH-1 *in vitro* (224). PUUV also causes HFRS in humans but does not belong to the same phylogenetic group as HTNV and SEOV; in fact, it is closely related to the American PHV (59, 404). In **Paper IV**, our efforts aimed at revealing the role of PCDH-1 in PUUV entry and replication *in vitro*.

#### BOX 4

Highlighted method: CRISPR-Cas9 genome engineering

Genome editing tool adapted from a naturally occurring gene editing system in bacteria. It represents a versatile and precise method for genetic manipulation relying on two key molecules that allow the introduction of a mutation into the DNA. The guide RNA (RNAg) is a pre-designed RNA sequence forming part of a longer RNA scaffold. It has an RNA sequence that is complementary to the target DNA sequence, to which it will bind. The second component, the enzyme Cas9, binds to the RNAg and 'cuts' both strands of DNA in order to remove existing genes or add new ones.



#### 3.4.1 PUUV replication is enhanced in PCDH-1 knock out cells

In order to determine the importance of PCDH-1 for PUUV entry into host cells, wild type (WT) and KO U2OS osteosarcoma cells were exposed to two different PUUV strains: the Russian isolate Kazan and the Finnish isolate Sotkamo (709). HTNV and ANDV were also included in our investigations.

The infectivity in WT and PCDH-1 KO U2OS cells was assessed by immunofluorescence, after exposure to the distinct hantaviruses. In line with a recent report, infection with ANDV was strongly inhibited in PCDH-1 KO cells (224). Although a significant difference was observed between WT and PCDH1 KO cells after infection with HTNV, the frequency of HTNV infected PCDH-1 KO cells increased almost 4-fold between 48 hours and 72 hours after infection, indicating that HTNV can efficiently replicate and spread in PCDH-1 deficient cells. Regarding both PUUV strains, a tendency towards lower levels of PUUV-infected cells was observed in PCDH-1 KO cells, although significance was not reached. However, no differences in infection rate were observed in PUUV-infected PCDH-1 KO cells between 48 and 72 hours post-infection, potentially indicating restricted spread of PUUV in PCDH-1 KO cells. Surprisingly, higher levels of viral RNA and increased amounts of viral N protein were detected in PUUV-infected PCDH-1 KO cells than in WT cells. In contrast, lower levels of both viral RNA and N protein were reported in HTNV-infected and ANDV-infected PCDH-1 KO cells, compared to control WT cells. In line with the results of RNA and protein levels, analyses of viral titres in supernatants indicated increased production of PUUV progeny viruses in PCDH-1 KO cells. No difference was detected for HTNV and, as expected, supernatants from ANDV-infected PCDH-1 KO cells had clearly lower levels of progeny virus than supernatants from WT cells (Table 2).

**Table 2.** Infected cells, S segment, N protein and viral titres in PCDH-1 KO cells relative to WT cells.

	Infected cells		RNA		Protein		Viral titre	
	48h	72h	48h	72h	48h	72h	48h	72h
PUUV Kazan	=	-	++	++	+	++	+	+
PUUV Sotkamo	-	-	+	+	++	++	=	+
HTNV	-	--	-	--	-	--	=	=
ANDV	--	--	--	--	-	--	=	--

+ + : significant increase; + : non-significant increase; - - : significant decrease; - : non-significant decrease; = : no change.

Despite detecting less PUUV-infected PCDH-1 KO cells as compared to WT cells, viral titres as well as RNA and protein levels were clearly higher in PUUV-infected PCDH-1 KO cells (Table 2). These findings indicate that while PUUV infection is hampered, viral replication is increased in PCDH-1 KO cells compared to control cells. As such, these observations suggest that PCDH-1 may have different roles during PUUV replication. Further, re-introduction of PCDH-1 in KO cells resulted in increased production of PUUV N protein, when compared to PCDH-1 KO cells with empty vector as negative control (224). Re-introduction of PCDH-1 back in KO cells did not induce increased HTNV N protein expression but, as previously published, the PCDH-1 re-introduction did have a significant positive effect on ANDV N protein production (224).

### 3.4.2 Pre-incubation of PUUV particles with sEC1-2 does not inhibit infection

Hantaviruses infect scant numbers of U2OS cells, indicating that the majority of these cells express very low levels of entry receptors for hantaviruses and/or that these cells barely support the replication of hantaviruses. Next, we sought to identify differences in receptor expression between the U2OS cells and two other cell types known to be infected to high levels by hantaviruses: A549 cells, a human lung epithelial cell line, and primary human lung microvascular endothelial cells (HMVEC-L). Flow cytometry analyses of PCDH-1 surface expression revealed very low levels of PCDH-1 expressed on the surface of U2OS and A549 cells, while high levels were detected in HMVEC-L. The levels of DAF and integrin  $\beta 3$  were also very low in U2OS and A549 cells, compared to high levels expressed on the surface of HMVEC-L. These results were in line with previous observations documenting low levels of PCDH1, DAF and  $\beta 3$  mRNA in U2OS and A549 cells, and high mRNA levels of these cellular factors in endothelial cells (710).

Having found highly expressed PCDH-1 on the surface of HMVEC-L, we aimed at defining the importance of PCDH-1 during PUUV infection and replication in HMVEC-L. ANDV binds to the outermost region of PCDH-1 and infection of endothelial cells is inhibited by pre-incubation of ANDV particles with sEC1-2 (i.e. a recombinant truncated form of the outer part of PCDH-1). Pre-incubation of PUUV Kazan or PUUV Sotkamo with sEC1-2 had no clear inhibitory effect on the infectivity of PUUV in HMVEC-L, although slightly lower levels of PUUV RNA were detected in cells infected with sEC1-2 pre-incubated virus. Contrarily to what had been previously published, we did detect a slight inhibitory effect in HTNV infectivity when pre-incubated with the sEC1-2 compound (224).

### **3.4.3 Conclusions and future perspectives of Paper IV**

Several reports have shown that hantaviruses may use diverse cellular factors for entry (204, 212, 214, 220-223). Jangra *et al.* elegantly showed the relevance of PCDH-1 for infection and replication of the American hantaviruses ANDV, SNV, MAPV and PHV, and that PCDH-1 KO Syrian hamsters survive when challenged with ANDV (224). However, the genetic disruption of the interaction between PCDH-1 and hantavirus glycoproteins did not entirely inhibit the entry of the above-mentioned hantaviruses *in vitro*, and in a low number of the PCDH-1 KO hamsters, ANDV replication was still occurring despite the lack of PCDH-1 (222, 224). These results indicated that hantaviruses can infect host cells through PCDH-1-independent routes.

In **Paper IV**, we investigated if PUUV relied on PCDH-1 in terms of infection and replication in host cells. Our data showed slightly higher levels of PUUV infection in WT cells compared to PCDH-1 KO cells, suggesting that although PCDH-1 may play a role in PUUV infection, it is not crucial. PCDH-1 can be expressed in the cell surface as well as intracellularly (710). Interestingly, PUUV replication was altered in infected PCDH-1 KO cells as shown by increased viral RNA, N protein and progeny virus production when compared to WT cells, perhaps indicating a role for PCDH-1 during PUUV replication in infected cells. Further, pre-incubation of PUUV with sEC1-2 (a recombinant soluble truncated form of the outer part of PCDH-1) did not affect infectivity in HMVEC-L. As a whole, these results indicate that PCDH-1 may play a dual role in PUUV infection and replication.

Many studies have reported phenotypic differences when knocking out specific genes in different model systems such as zebrafish and mouse (711). PCDH-1 is a cell-adhesion protein playing important roles in epithelial cell barrier formation and repair (712). PCDH-1 KO U2OS cells may have induced expression of other cell factors in order to compensate for the lack of PCDH-1. As such, genetic compensation may have played a role in the effects that we reported during PUUV infection in genetically modified U2OS cells.

**Paper IV** mirrors the complexities of hantavirus entry and replication processes and highlights the importance, and the need, to further evaluate the interplay between hantaviruses and other proposed candidate receptors.

## 4 CONCLUDING REMARKS

The viral regulation of apoptotic cell death as well as of other cell signalling pathways represents an extremely developed process moulded through the endless clash between viruses and their hosts. This thesis provides new insights into how hantaviruses modulate the apoptotic machinery of a cell for their own benefit. Additionally, we provide further information on the PCDH-1-dependency of hantaviruses during entry and replication in human target cells. Listed below is a summary of the key findings from the four papers included in the thesis:

- Pathogenic and non-pathogenic hantaviruses belonging to three distinct phylogroups share the capacity to inhibit chemically-induced apoptosis as well as cytotoxic lymphocyte-mediated killing of infected cells (**Paper I**)
- The nucleocapsid protein of phylogenetically distinct hantaviruses strongly inhibits the enzymatic activity of granzyme B and, to some extent, that of caspase-3 (**Paper I**)
- The pathogenic ANDV and HTNV both induce up-regulation of the pro-survival factor BCL-2, thereby protecting infected cells from chemically-induced apoptosis (**Paper II**)
- HTNV stimulates the production of TRAIL by endothelial cells and by NK cells co-incubated with infected endothelial cells (**Paper III**)
- Infection with HTNV protects infected cells from TRAIL-mediated killing through specifically down-regulating DR5 (**Paper III**)
- HTNV induces DR5 ubiquitination and degradation by the 26S proteasome system early after infection; and at late stages post-infection, DR5 accumulates intracellularly in LAMP-1 positive vesicles (**Paper III**)
- DR5s, an alternate spliced variant of DR5, is produced in primary endothelial cells after HTNV infection (**Paper III**)
- PUUV entry into target cells does not depend on the host factor PCDH-1 (**Paper IV**)



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