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STUDIES OF INFLAMMATORY RESPONSES IN HANTAVIRUS INFECTION

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Studies of inflammatory responses in hantavirus infection

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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"Seven human-mediated factors are most likely driving the emergence of zoonotic diseases: 1) increasing human demand for animal protein; 2) unsustainable agricultural intensification; 3) increased use and exploitation of wildlife; 4) unsustainable utilization of natural resources accelerated by urbanization, land use and extractive industries; 5) increased travel and transportation; 6) changes in food supply; and 7) climate change."

Preventing the next pandemic - Zoonotic diseases and how to break the chain of transmission
United Nations Environment Programme (2020)

POPULAR SCIENCE SUMMARY

Hantaviruses cause acute infections in humans world-wide. In nature, hantaviruses that cause disease in humans are carried by different rodent species that do not get sick themselves. The viruses are transmitted to humans through air containing dust contaminated with droppings or urine from infected rodents. Yearly, hantaviruses cause disease in around 100 000 individuals. In contrast to the coronavirus that has caught the world's attention lately, most hantaviruses do not transmit between humans. The hantavirus species' that circulate in Europe and Asia cause a disease called hemorrhagic fever with renal syndrome, HFRS. This disease is in some respects similar to the seasonal flu, with fever, headache, muscle ache, back pain, and stomachache as common symptoms. Approximately one third of the diagnosed patients require hospitalization and of these, some need dialysis due to kidney dysfunction. Hantaviruses in the Americas cause a more severe form of disease, referred to as hantavirus pulmonary syndrome, HPS. This disease often gives rise to severe lung symptoms that quickly can become life-threatening. Among individuals with HPS, up to 40% succumb to the disease.

Generally, hantavirus infection is characterized by a strong inflammatory response and leakage of fluid from the blood vessels out into the surrounding tissue. These hallmarks are not specific for diseases caused by hantaviruses, but are also seen in several other viral infections, including COVID-19. Despite the morbidity and mortality associated with hantavirus diseases, there is no treatment or approved vaccine available. Moreover, the mechanisms behind how hantaviruses cause disease in humans remain unknown.

In the work included in this doctoral thesis, my colleagues and I have studied the inflammatory response in hantavirus-infected patients, with the aim to identify specific immunological factors that can help us understand why some individuals get more ill than others. By studying blood samples from Argentinian patients infected with hantavirus, we were able to identify that increased levels of the inflammatory protein IL-6 were associated with severe HPS. Moreover, we found that fatal HPS was associated with increased levels of a protein that is released upon intestinal injury. In our subsequent experiments we wanted to investigate how IL-6 possibly could be involved in causing disease during hantavirus infection. Using blood vessel cells that we infected in the laboratory, we found that IL-6 in some contexts could drive an inflammatory response and cause a separation between blood vessel cells. These findings suggest a possible mechanism behind the leakage of blood fluid seen in patients. This thesis also includes a study of a subset of T cells named MAIT cells. We found a prominent decline in MAIT cells in the blood of Swedish hantavirus-infected patients. Further, we found that the MAIT cells were strongly activated, and that this activation was associated with increased levels of IL-6 in the patients. In the laboratory, we discovered that a subset of inflammatory mediators called type I interferons are responsible for causing hantavirus-mediated activation of MAIT cells. We also observed that these activated MAIT cells released protein-cleaving proteins called granzymes. Altogether, this study shows that type I interferons have a more important role in MAIT cell activation than what was previously known. It also suggests that MAIT cells may be important producers of inflammatory proteins such as granzymes during hantavirus infection.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hantavirus orsakar akuta infektioner hos människor över hela världen. I naturen bärs hantavirus av olika gnagararter som själva inte blir sjuka. Viruset sprids till människor via inandning av damm som kontaminerats med avföring eller urin från infekterade gnagare. Årligen insjuknar ungefär 100 000 individer i världen med hantavirusinfektion. Till skillnad från det coronavirus som senaste året har fångat världens uppmärksamhet, smittar de flesta hantavirus inte mellan människor. De hantavirusarter som cirkulerar i Europa och Asien orsakar en sjukdom som kallas för "hemorrhagic fever with renal syndrome", HFRS, eller sorkfeber som vi kallar den variant av sjukdomen som finns i Sverige. Sorkfeber påminner i vissa avseenden om säsongsinfluensan och ger upphov till symptom så som feber, huvudvärk, muskelvärk, ryggsmärta och magont. Ungefär en tredjedel av alla patienter som diagnosticeras med HFRS behöver bli inlagda på sjukhus. Av dessa patienter behöver en mindre andel dialys, till följd av njursvikt orsakat av virusinfektionen. I Amerika finns en allvarligare form av hantavirussjukdom som kallas för "hantavirus pulmonary syndrome", HPS. Denna sjukdom ger ofta upphov till allvarliga lungsymptom hos patienten, som i många fall är livshotande. Upp till 40% av de individer som insjuknar i HPS avlider till följd av infektionen. Generellt karaktäriseras hantavirusinfektion av en stark inflammatorisk respons samt ett läckage av blodplasma från blodkärlen ut i vävnaden. Dessa kännetecken är inte specifika för de sjukdomar som orsakas av hantavirus, utan ses även vid flertalet andra virussjukdomar, inklusive COVID-19. Trots den sjuklighet och dödlighet som är kopplad till hantavirussjukdomar finns det idag varken behandling eller godkänt vaccin mot hantavirusinfektion. Vidare är mekanismerna bakom hur hantavirus orsakar sjukdom okända.

I arbetet som ingår i denna doktorsavhandling har jag och mina kollegor studerat immunförsvaret vid hantavirusinfektion med syftet att försöka förstå varför vissa individer blir mer sjuka än andra. Genom att studera blodprover från argentinska HPS-patienter kunde vi se att förhöjda nivåer av ett inflammatoriskt protein som kallas för IL-6 är kopplat till svår sjukdom. Dessutom fann vi att dödlig hantavirusinfektion är associerad med ökade nivåer av en markör för tarmskada. I våra efterföljande experiment undersökte vi hur IL-6 skulle kunna bidra till symptomen vid hantavirusinfektion. Genom att på laboratoriet infektera blodkärlsceller fann vi att IL-6 i vissa sammanhang kan driva en inflammationsrespons samt orsaka en separation mellan blodkärlsceller. Dessa resultat tyder på en möjlig mekanism bakom det läckage av blodplasma som ses hos hantavirusinfekterade patienter. Avhandlingen innehåller även en studie där vi har undersökt en grupp av T-celler som kallas för MAIT-celler. Vi fann en nedgång i antalet MAIT-celler i blod hos svenska sorkfeberpatienter. Vidare observerade vi en stark aktivering hos MAIT-cellerna som var kopplad till ökade nivåer av IL-6. På laboratoriet fann vi att en typ av inflammatoriska proteiner som kallas för typ I interferoner orsakar hantavirusmedierad aktivering av MAIT-celler. Vi såg också att dessa MAIT-celler frisläppte protein-klyvande proteiner som kallas för granzymer. Sammantaget visar denna studie att typ I interferoner har en viktigare roll vid MAIT-cellsaktivering än vad som tidigare varit känt. Studien antyder också att MAIT-celler kan vara en viktig källa till inflammatoriska proteiner så som granzymer under hantavirusinfektion.

ABSTRACT

Throughout the world, orthohantaviruses cause severe, acute infections in humans. Orthohantaviruses, commonly referred to as hantaviruses, are zoonotic viruses with a single stranded RNA genome of negative sense. Hantavirus strains endemic to Europe and Asia cause a systemic infection with renal involvement referred to as hemorrhagic fever with renal syndrome (HFRS). In the Americas, hantaviruses cause hantavirus pulmonary syndrome (HPS) - a severe and highly fatal infection characterized by severe pulmonary compromise. Individuals infected with hantaviruses typically display increased levels of cytokines, decreased platelet counts, and vascular leakage. As specific treatments are lacking, the long-term goal of the studies within this thesis was to provide leads that will aid in the development of such. Specifically, this thesis aimed to characterize inflammatory responses and MAIT cell responses during hantavirus infection, as a step to increase the understanding of protective versus detrimental immune responses. Moreover, this thesis aimed to investigate the role of the cytokine interleukin-6 (IL-6) in the pathogenesis of hantavirus infection.

In both HFRS patients and HPS patients, we observed increased systemic levels of many pro-inflammatory cytokines and other inflammatory markers. In HPS patients, serum levels of IL-6 were found to be associated with increased odds of developing severe disease. On the contrary, serum levels of complement factor (C) 5/C5a and B cell activating factor were associated with decreased odds of developing severe disease. Intestinal fatty acid-binding protein (I-FABP), a systemic marker of intestinal damage, was increased during HPS and associated with increased odds of a fatal outcome. Next, we demonstrated that IL-6 trans-signaling in hantavirus-infected endothelial cells led to increased pro-inflammatory responses and increased monolayer permeability. In HFRS patients, we observed an altered balance of soluble IL-6 receptors in plasma, which may increase the likelihood of IL-6 trans-signaling in patients. The imbalance in these markers was associated with an increased need for supplemental oxygen treatment.

When investigating the phenotype of peripheral blood MAIT cells in HFRS patients, we observed a strong decline in MAIT cell numbers during the acute disease. MAIT cells remaining in the circulation were highly activated and exhibited decreased expression of mucosal tissue homing markers. *In vitro*, we were able to recapitulate these findings, and show that MAIT cell activation mediated by hantavirus was dependent on type I interferons (IFNs) produced by antigen-presenting cells or endothelial cells.

In conclusion, this thesis adds to the view that HFRS and HPS are diseases characterized by strong inflammatory responses. The identification of IL-6 and I-FABP as markers of disease severity and fatality, respectively, may help in the understanding of hantavirus pathogenesis and the development of treatment options. The demonstration of the effects of IL-6 on hantavirus-infected endothelial cells suggest a potential mechanism behind IL-6-driven pathogenesis. Finally, this thesis provides further evidence on the involvement of MAIT cells during acute viral infection, and highlights type I IFNs as important mediators in MAIT cell activation.

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CONTENTS

LIST OF ABBREVIATIONS

5-OP-RU	5-(2-oxopropylideneamino)-6-d-ribitylaminouracil
ANDV	Andes orthohantavirus
ARDS	acute respiratory distress syndrome
BAFF	B cell-activating factor
C	complement component
CCL	C-C motif chemokine ligand
CRP	C-reactive protein
CCR	C-C motif chemokine receptor
CRS	cytokine release syndrome
DOBV	Dobrava-Belgrade orthohantavirus
ECMO	extracorporeal membrane oxygenation
ELISA	enzyme-linked immunosorbent assay
FDA	The United States Food and Drug Administration
gp130	glycoprotein 130
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HFRS	hemorrhagic fever with renal syndrome
HPS	hantavirus pulmonary syndrome
HTLV-1	human T-lymphotropic virus 1
HTNV	Hantaan orthohantavirus
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
I-FABP	intestinal fatty acid-binding protein
IFN	interferon
IL	interleukin
IL-6R	IL-6 receptor
ISG	interferon-stimulated genes
JAK	Janus kinase
KHF	Korean hemorrhagic fever

LBP	LPS-binding protein
LPS	lipopolysaccharide
MAdCAM-1	mucosal vascular addressin cell adhesion molecule 1
MAIT	mucosal-associated invariant T
MDA-5	melanoma differentiation-associated protein 5
MHC	major histocompatibility complex
MR1	MHC class I-related gene protein 1
MxA	myxovirus resistance protein 1
N	nucleocapsid
NE	nephropathia epidemica
NETs	neutrophil extracellular traps
NK	natural killer
NSs	nonstructural protein
PAMPs	pathogen-associated molecular patterns
PCDH-1	protocadherin 1
PHV	Prospect Hill orthohantaviurs
PRRs	pattern-recognition receptors
PUUV	Puumala orthohantavirus
RIG-I	retinoic acid-inducible gene 1
sCD14	soluble CD14
sCD25	soluble CD25
SEOV	Seoul orthohantavirus
sgp130	soluble gp130
sIL-6R	soluble IL-6R
SNV	Sin Nombre orthohantavirus
STAT	signal transducer and activator of transcription
sTRAIL	soluble TRAIL
TCR	T cell receptor
TEER	transendothelial electrical resistance
TLRs	Toll-like receptors
TNF	tumor necrosis factor

TRAIL	TNF-related apoptosis inducing ligand
TULV	Tula orthohantavirus
VCAM-1	vascular cell adhesion protein 1
VE	vascular endothelial
VEGF	vascular endothelial growth factor
ZO	zonula occludens

1 INTRODUCTION

1.1 HANTAVIRUS

1.1.1 Brief hantavirus history

Hantaviruses are zoonotic viruses with world-wide distribution. In humans, hantaviruses cause two severe diseases; hemorrhagic fever with renal syndrome (HFRS), caused by "Old World" hantaviruses, and hantavirus pulmonary syndrome (HPS), caused by "New World" hantaviruses (1). Diseases resembling HFRS were reported in clinical records in China already 960 AD (1) and in Russia in 1913 (2). Similar syndromes were also described during World War I (3) and the Korean War in 1951, under the names "war nephritis" and "Korean hemorrhagic fever" (KHF), respectively (4).

In Sweden, HFRS-like illness was first reported in 1934 by the physicians Zetterholm and Myhrman, independent of each other (5,6). The cases were characterized by an acute onset of chills, abdominal pain, back pain, proteinuria, and kidney dysfunction (5,6). In 1945, Myhrman proposed the name nephropathia epidemica (NE) for the disease (7). Myhrman noted that many of his NE patients reported contact with mice and speculated that the agent was transmitted to humans from animals (8). In 1976, NE was found to be related to the disease KHF (9). However, the causative agents of these illnesses were unknown until 1978, when Lee *et al.* reported isolation of the causative agent of KHF - Hantaan orthohantavirus (HTNV) - from a striped field mouse captured close to the Hantaan river in South Korea (10). In the early 1980s, the causative agent of NE, Puumala orthohantavirus (PUUV), was isolated from bank voles (*Myodes glareolus)* captured in Puumala, Finland (11). Shortly after, KHF and NE were collected under the name HFRS (12). Since then, additional HFRS-causing hantaviruses, such as for example Seoul orthohantavirus (SEOV), carried by rats (*Rattus rattus, R. norvegicus*) (13) and Dobrava-Belgrade orthohantavirus (DOBV), carried by the yellow-necked mouse (*Apodemus flavicollis*) (14), have been identified in Europe and Asia.

In 1993, a cluster of cases of a highly fatal respiratory disease appeared in the Four Corner region in the United States (15,16). The index cases were two young individuals with acute onset of fever that after a couple of days rapidly progressed into severe respiratory distress with fatal outcome (17). In just over a month, the causative agent was found to be a new hantavirus, later given the name Sin Nombre orthohantavirus (SNV), carried by deer mouse (*Peromyscus maniculatus*) (18–20). The disease was named hantavirus pulmonary syndrome (HPS) (also known as hantavirus cardiopulmonary syndrome) (21). In 1996, another HPS-causing hantavirus species was identified upon an outbreak in Argentina in 1995 (22). This virus was named Andes orthohantavirus (ANDV) and is carried by the long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*) (23). Several other hantaviruses related to SNV and ANDV have been reported to cause HPS in the Americas. These include Bayou orthohantavirus, Black Creek Canal orthohantavirus and Laguna Negra orthohantavirus, among several others (24). New World hantaviruses sporadically cause outbreaks in the Americas, with the SNV-outbreak in Yosemite national park in 2012 and the ANDV-outbreak in Argentina in 2018-2019 being

some of the most recent (25,26). Also non-pathogenic or low-pathogenic hantavirus species exist, the most well-studied being Prospect Hill orthohantavirus (PHV) and Tuula orthohantavirus (TULV) (27–30).

1.1.2 Hantavirus structure

Orthohantavirus comprises a genus within the *Hantaviridae* family of viruses that belongs to the *Bunyavirales* order. To date, the *Orthohantavirus* genus consists of 38 different orthohantaviruses (31), hereinafter referred to as hantaviruses. Hantavirus virions (Figure 1) are pleiomorphic and can be either round-shaped or tubular, with an average diameter/length of around 100 nm (32,33). The virions are enveloped and carry a tri-segmented negative-sense single stranded RNA genome. The genome segments are named small, medium, and large, and encode for the nucleocapsid protein (N), the glycoprotein precursor that is cleaved into Gn and Gc, and the RNA dependent RNA polymerase, respectively (27,34). The small segment of certain hantavirus strains also encodes a non-structural protein called NSs (35,36). The hantavirus envelope is densely covered with Gn-Gc spike complex molecules, although with some empty patches (37) (Figure 1).

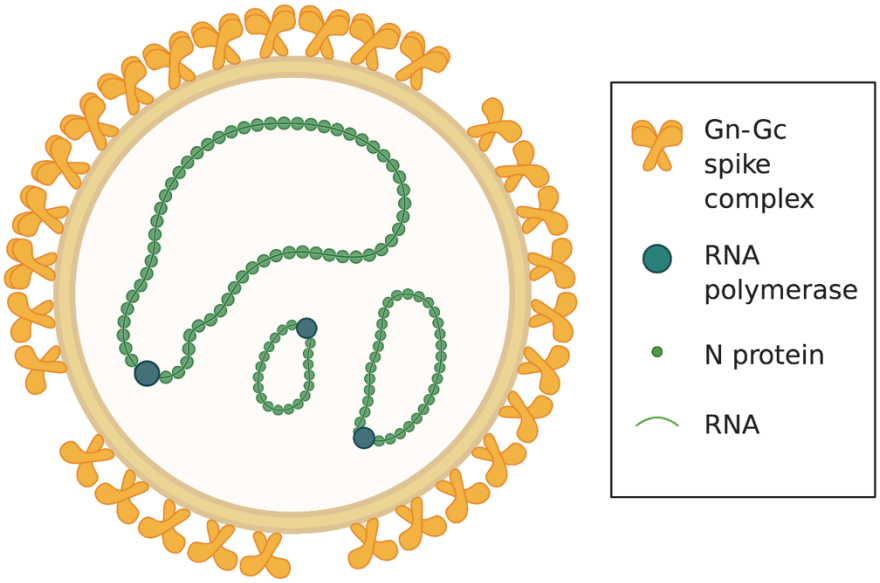

Figure 1. Hantavirus structure. Hantavirus virions are enveloped and contain a single-stranded negative sense RNA genome divided into three segments. The segments encode for the nucleocapsid protein (N), the glycoproteins Gn and Gc, and the RNA polymerase. The hantavirus envelope is densely packed with Gn-Gc spike complexes.

1.1.3 Hantavirus replication

Hantaviruses primarily replicate in endothelial cells, but have *in vitro* been shown to infect also renal, pulmonary and intestinal epithelial cells as well as monocytes and dendritic cells to some extent (38–42). There are several described hantavirus receptors, including $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins and complement decay-accelerating factor. However, it is not known if these

receptors are used *in vivo* (27). Recently, protocadherin-1 (PCDH-1) was identified as a new hantavirus receptor, essential for the establishment of ANDV and SNV infection (43). Remarkably, PCDH-1 was shown to be critical for the development of fatal ANDV infection in Syrian hamsters (43). For establishment of HTNV and SEOV infection, expression of PCDH-1 was redundant (43).

The cell entry mechanisms of hantaviruses are not fully understood, and different mechanisms have been described for different species. For example, HTNV has been described to invade cells by clathrin-mediated endocytosis (44), while both clathrin-dependent and clathrin-independent entry has been described for ANDV (45,46). As most RNA viruses, hantaviruses replicate in the cytosol by first creating a complementary RNA strand as a template. After translation, Gn and Gc proteins polymerize into heterotetramers and are glycosylated in the Golgi apparatus (47,48). Assembled virions egress infected cells through exocytosis. For some New World hantaviruses, also assembly at the plasma membrane and egress through budding has been described (32,49). Many details of the hantavirus replication machinery are still unknown, as reverse genetics systems are lacking (27).

1.1.4 Natural hosts and transmission

Hantaviruses are zoonotic viruses, meaning they are passed on to humans from natural hosts. In nature, hantaviruses are carried by rodents as well as insectivores such as moles, shrews, and bats. Each hantavirus strain is carried by its specific natural host and the geographical distribution of each hantavirus species depends on the distribution of its specific host (27). Most human-pathogenic hantaviruses are carried by rodents of the *Muridae* (mouse, rat) and *Cricetidae* (bank vole) families. However, also transmission from the *Soricidae* family (shrew) has been suggested in Africa (50). The prevalence of hantavirus infection in rodents is affected by numerous ecological factors, such as the host density, predator density, food availability, biodiversity in the habitat, and climate (51–55). As a consequence, the incidence of HFRS in human populations many times peak following rainy seasons (56,57).

In the natural hosts, hantaviruses cause an asymptomatic persistent infection. The natural hosts secrete virus through urine, feces and saliva (58–61). Humans are normally infected with hantavirus following inhalation of dust containing viruses shed from rodent excreta (Figure 2). Thus, human risk factors for contracting hantavirus infection include activities that bring humans in close proximity to rodents or rodent excreta, such as handling of firewood, forestry, farming, and military work (62–68) (Figure 2). In addition, smoking has been reported to be a risk factor for HFRS (69).

Generally, humans are dead-end hosts for hantaviruses, meaning that the infection is not further transmitted from an infected human to other humans. However, as was noted after a cluster of cases in Argentina in 1996-1997 (70), ANDV is an exception and is transmissible between humans (25,70,71). In 2018-2019, a cluster of cases with human-to-human transmitted ANDV occurred in Argentina (71). In this outbreak, a single transfer of ANDV from natural host to one individual resulted in four waves of transmission with in total 33 secondary cases and 11

deaths. The reported initial median reproductive number, commonly referred to as the R value, was 2.12 (71). After 18 confirmed cases, control measures were taken, which reduced the R value to 0.96. In total, person-to-person transmission was confirmed from 10 of the 34 cases. Interestingly, a high viral titer was associated with a higher likelihood of transmission to other persons (71).

1.1.5 Hantavirus-caused diseases

In humans, different hantavirus species give rise to different symptoms, which has led to the classification of two separate diseases; namely, hemorrhagic fever with renal syndrome - HFRS, and hantavirus pulmonary syndrome - HPS (27) (Figure 2). Yearly, 100 000 HFRS cases are reported worldwide, while HPS cases are more rare, reaching a few hundred cases per year (72).

1.1.5.1 Incubation time and diagnosis

Onset of HFRS/HPS symptoms usually start after an incubation period of two to three weeks (12,25,73,74). However, both shorter and longer incubation times of one to eight weeks have been reported (73–76). During the early acute phase, most patients display virus-specific IgM antibodies (77–81). Low titers of virus-specific IgG antibodies are also detected in most patients, already early during the acute phase (79,81). Thus, serological assays, including immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA), are commonly used for diagnosis of HFRS and HPS (81,82). In most hantavirus-infected patients, viremia can be detected upon admission to the hospital, after which the viral titers rapidly decrease (81,83,84). Therefore, also quantitative polymerase chain reaction on serum samples is commonly used in the diagnosis (85). In individuals with previous hantavirus infection, titers of neutralizing virus-specific IgG antibodies have been reported to continuously increase over two years following infection (11,79,86), and have been detected several decades after infection (86–88). A previous hantavirus infection is believed to provide life-long immunity and re-infection has never been reported.

1.1.5.2 HFRS

HFRS is primarily caused by PUUV in Europe, and HTNV in Asia, but also other hantavirus species cause HFRS, including SEOV and DOBV. Individuals with HFRS initially present with flu-like symptoms including headache, fever, and malaise as well as gastrointestinal manifestations such as diarrhea and abdominal pain (89,90). Less than half of the patients also develop renal dysfunction presented as back pain and oliguria (i.e., low urine output). Dialysis treatment is required in 5% of hospitalized PUUV-infected patients (90). Hemorrhagic symptoms, mainly presented as hematuria, petechiae, or epistaxis are displayed by 10-28% and 75% of the PUUV- and HTNV-infected patients, respectively (81,89,91). In addition, gastrointestinal bleedings are common (92–94). A few reports have described cases of pancreatitis and cholangitis during HFRS (95–99). HFRS is sometimes divided into five different clinical phases; febrile phase, hypotensive phase, oliguric phase, diuretic phase, and convalescent phase (12). However, these phases are not often evident in mild cases. The case-

fatality rate during HFRS has been reported to be 0.1-0.4% for PUUV, 1% for HTNV, and 0.3-16% for DOBV infection (27,45,100–105) (Figure 2). The case-fatality rate of HFRS increases with age, and is in Sweden 6.5% for PUUV-infected individuals above 80 years (100). Although most patients recover after two weeks, patients that have had HFRS show increased risk for getting myocardial infarction, stroke, thromboembolism, and lymphoma (106–108). Furthermore, one study reported higher hantavirus seroprevalence in patients with kidney disease compared to controls, suggesting that hantavirus infection might cause long-term kidney problems (109).

Globally, the majority of HFRS cases are documented in China (110). In Sweden, normally 50-400 HFRS cases are reported yearly (90,111). However, the seroprevalence in Northern Sweden has been shown to be 13% and increase with age, suggesting that there are many unrecorded cases of PUUV infection (67). A similar seroprevalence has been described in Finland (112). In Sweden, the incidence of HFRS peaks every third to fourth year, as it follows the size of the bank vole population (113,114). Following a remarkably mild weather in December 2006, HFRS incidence in Sweden spiked, giving rise to more than 2000 cases nationally (115).

1.1.5.3 HPS

HPS is primarily caused by ANDV in South America and SNV in the United States. As described earlier, HPS was first recognized during the outbreak in the Four Corners region in the United States, 1993 (18). Since then, HPS caused by ANDV, SNV, and related hantaviruses has been described also in South American countries such as Argentina, Chile, Brazil, and Paraguay, as well as in Canada and Panama (70,74,80,116–118). HPS can be divided into three phases; the febrile phase, the cardiopulmonary phase and the convalescent phase. The febrile phase is characterized by flu-like symptoms, similar to in HFRS. A majority of HPS patients also experience gastrointestinal symptoms (119,120). Unlike HFRS, HPS quickly develops to include pulmonary symptoms such as cough, pulmonary edema, and dyspnea. Chest X-rays of patients have revealed severe pleural effusion, referring to the accumulation of fluid between the layers of the pleura covering the lungs, and interstitial as well as alveolar infiltrates (17,121). The pulmonary dysfunction during HPS often leads to hypoxia, which in many cases leads to the need for intubation or extracorporeal membrane oxygenation (ECMO) (17,80,122). Ultimately, 21-40% of HPS patients succumb due to cardiogenic shock (17,122,123) (Figure 2).

The seroprevalence of HPS-causing hantaviruses has been reported to be 1-6.5% in the general population of Argentina, 1% in Chile, and 3.5% in Brazil (124–127). In Argentinians with agricultural occupations, 17% were reported to be seropositive (125). Higher seroprevalences of 17% and 40% has been also been reported for indigenous populations of Argentina and Paraguay, respectively (128). Thus, it is likely that New World hantaviruses, like Old World hantaviruses, can cause subclinical infections.

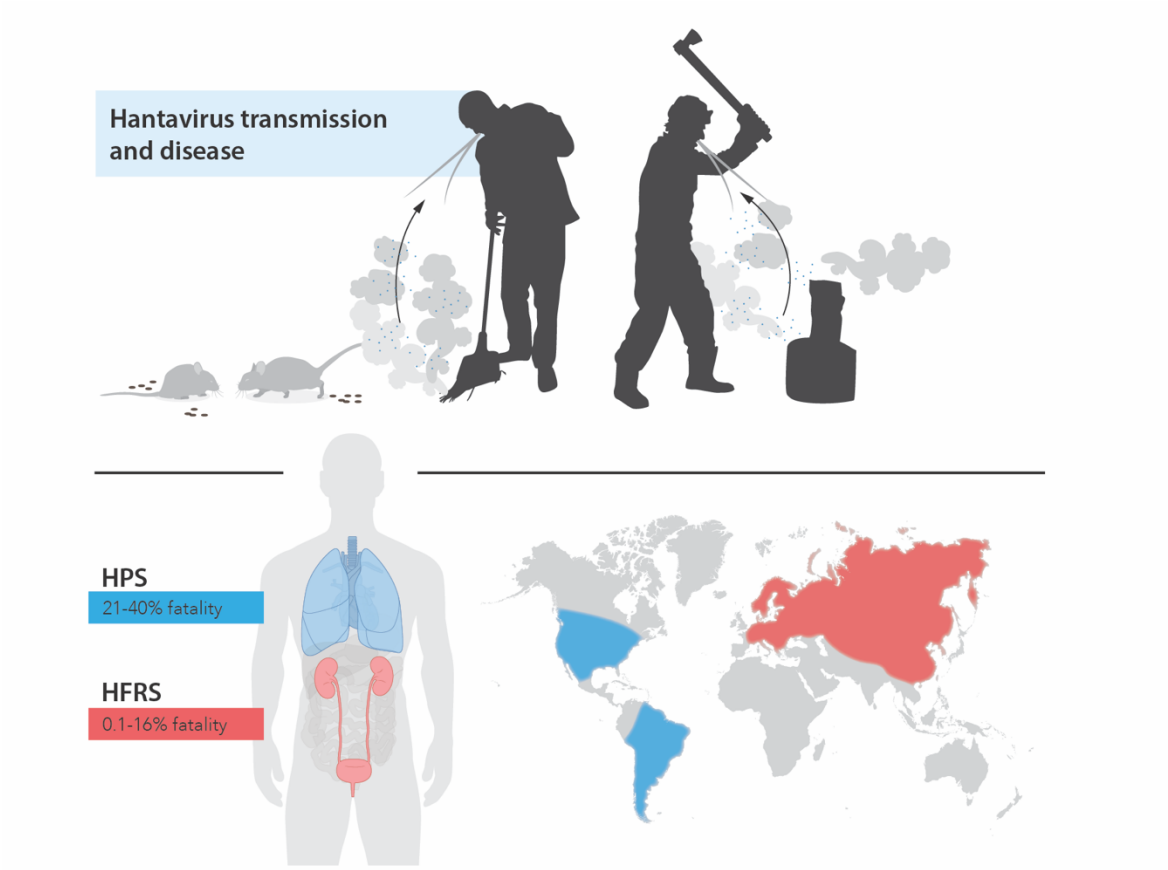

Figure 2. Hantavirus-caused diseases in humans. Hantaviruses are transmitted to humans from rodent excreta. Human activities that bring up dust allow the inhalation of virions and thus, increase the risk of infection. Depending on the virus species, hantaviruses can cause hantavirus pulmonary syndrome (HPS) or hemorrhagic fever with renal syndrome (HFRS) in humans. HPS-causing hantaviruses are found in North and South America and cause a highly fatal disease that mainly affects the lungs. HFRS-causing hantaviruses are found in Europe and Asia and cause a milder disease with lower case-fatality rate and often involve kidney dysfunction. Modified from Klingström et al., 2019 (129) under the terms of the Creative Commons CC BY license.

1.1.5.4 Clinical hallmarks of HFRS and HPS

As described above, HFRS and HPS are considered two separate diseases caused by different hantavirus species. Although the diseases differ a lot in terms of severity, with case-fatality rates being markedly higher in HPS, the diseases share many characteristics. While severe pulmonary symptoms are more pronounced in HPS, milder pulmonary involvement, including dry cough and dyspnea, has been described also in HFRS patients (38,130–133). Also common between HFRS and HPS are the gastrointestinal symptoms displayed by the majority of patients (17,89,98,101,119,120,134–136).

Vascular leakage is an important clinical hallmark shared between HFRS and HPS, and is clinically evident by hemoconcentration concurrent with hypoalbuminemia, hypotension and edema (15,17,27,122,130,137–139). Coagulopathy is another common hallmark of HFRS and HPS, and is indicated by thrombocytopenia and increased serum D-dimer concentration

(15,81,122,130). Low thrombocyte levels have been associated with a more severe disease (140,141). Kidney dysfunction in patients is assessed by increased serum creatinine levels and proteinuria (81,122,130). Further, patients usually display increased levels of CRP, which marks inflammation (130,142). In addition, HPS patients often exhibit increased heart rate and respiratory rate (17).

While a high viral titer has been associated with increased disease severity in infections caused by SNV, DOBV, and HTNV (142–145), such associations have not been observed in studies of patients infected with PUUV or ANDV (81,83,84). A low virus-specific antibody titer has during infection with PUUV and SNV been associated with a more severe disease (81,146).

1.1.5.5 Treatment options

Although hantaviruses cause severe disease in humans, no specific treatments or vaccines approved by The United States Food and Drug Administration (FDA) are available. Thus, supportive care aiming at maintaining the electrolyte balance constitutes the standard treatment. In severe HPS, ECMO treatment improves survival rates in patients with a predicted fatal outcome (147). In the past, studies have evaluated the effects of the nucleoside analogue ribavirin as a treatment for HFRS and HPS. While treatment with ribavirin in one study was suggested to be beneficial in treatment of HFRS, no effect was seen in a study of HPS patients (148,149). Moreover, the effects of the corticosteroid methylprednisolone have been evaluated in HPS, without showing any clear effects (150). Given that patients with high virus-specific antibody titers usually present with a milder disease (115,146), treatment of hantavirus-infected patients with convalescent plasma therapy has been considered a promising strategy. One study suggested that passive transfer of antibodies from convalescent HPS patients may reduce the fatality of HPS (151), although this has not yet been the subject of a randomized controlled trial.

1.2 THE IMMUNE SYSTEM

1.2.1 Brief overview of the human immune system

The human immune system is orchestrated by a variety of different immune cells and soluble mediators within the innate and adaptive immune system. The innate immune system elicits a rapid and unspecific response upon infection. The skin barrier and mucosal surfaces represent the first line of defense against pathogens. When broken, mononuclear phagocytes, including dendritic cells, monocytes and macrophages exert important functions in the innate immune response, by engulfing pathogens and presenting their antigens to cells of the adaptive immune system (152,153). Other important phagocytes of the innate immune system include the neutrophils, which rapidly respond to infection and kill pathogens using reactive oxygen species, by phagocytosis, or by releasing neutrophil extracellular traps (NETs) (154). Furthermore, natural killer (NK) cells are innate lymphocytes that kill virus infected cells. NK cell-killing is mediated either via the release of cytotoxic granules consisting of perforin and granzyme B, via antibody-dependent cellular cytotoxicity, or via the interaction between death

receptor ligands, such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and death receptors (152,153).

The adaptive immune system, which consists of B cells and T cells, is antigen-specific and provides immunological memory. B cells are responsible for the production of pathogen-specific antibodies. These antibodies neutralize pathogens and facilitate killing by phagocytes and NK cells. Conventional T cells can be divided into helper T cells (CD4 T cells) and cytotoxic T cells (CD8 T cells). CD4 T cells are activated by antigen-presenting cells presenting peptide-antigens on their major histocompatibility complex (MHC) class II molecules. CD8 T cells, on the other hand, respond to endogenous peptide-antigens presented on MHC class I molecules on any nucleated cell. Activated CD4 T cells provide activating signals to antigen-stimulated B cells and CD8 T cells. In turn, CD8 T cells kill virus-infected cells using cytotoxic granules. In addition to conventional T cells, the adaptive immune system includes unconventional T cell subsets. These cells have innate-like features and include NKT cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells. Unconventional T cell subsets respond to non-peptide antigens with low polymorphism (152,153).

The complement system belongs to the innate immunity but acts in concert with both innate and adaptive cells, facilitating their functions. The complement cascade can be initiated via three independent pathways, all which merge at the activation of complement component (C) 3 that becomes cleaved into C3a and C3b. C3b, in turn, cleaves C5 into C5a as well as C5b. Together with other complement factors, C5b creates the membrane attack complex that mediates cell lysis. C3a and C5a are pro-inflammatory mediators and are often referred to as anaphylatoxins (152,153,155).

1.2.2 Viral recognition

A virus that has entered a host cell can be sensed by pattern-recognition receptors (PRRs) that recognize pathogen-specific structures, so-called pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the most well-described PPRs and include a set of receptors, each recognizing a specific molecular pattern. For instance, TLR-4 and TLR-5 are localized on the cell surface of cells and recognize bacterial surface structures of extracellular bacteria. TLR-3, TLR-7, TLR-8, and TLR-9, on the other hand, are expressed within endosomes, and sense double stranded RNA, single-stranded RNA, and unmethylated CpG regions in DNA, respectively. Thus, viruses that are taken up into endosomes will after endosomal degradation expose their PAMPs that bind to one or several of TLR-3/-7/-8/-9, depending on the type of virus. Viruses replicating within a cells' cytosol can be detected by retinoic acid-inducible gene-1 (RIG-I)-like receptors, which include RIG-I and melanoma differentiation-associated protein 5 (MDA-5), or nucleotide-binding and oligomerization domain-like receptors, which belong to separate PRR families (156,157).

PRR-signaling leads to downstream signaling resulting in activation of specific transcription factors, in turn leading to production of pro-inflammatory cytokines and interferons (IFN). Type I IFNs, including IFN-α and IFN-β, are key mediators of the so-called antiviral state.

Secreted IFNs bind to ubiquitously expressed IFN receptors on neighboring cells and induce signaling via Janus kinases (JAK) and signal transducer and activator of transcription (STAT) proteins. This, in turn, leads to transcription of interferon stimulated genes (ISG), such as myxovirus resistance protein 1 (MxA). ISG transcription induces a cascade of effector molecules that together contribute to the antiviral state. For instance, double stranded RNA results in activation of protein kinase R that in turn leads to inhibition of all protein synthesis in the cell (156,157).

1.2.3 Endothelial cells

Endothelial cells are epithelial cells that line the inner wall of blood vessels. The primary function of endothelial cells is to maintain normal blood flow, regulate exchange of proteins between the blood and tissue, and to prevent coagulation of the blood. Inflammation requires the migration of leukocytes from blood to affected tissues. Thus, dynamic regulation of the vessel wall is essential. The integrity of the vessel wall is regulated by tight junctions and adherence junctions connecting the endothelial cells (158). Tight junctions, such as claudins and occludin, regulate the inter-cellular exchange of ions and molecules. The tight junction-associated zona occludens (ZO) proteins bind to tight junction proteins and link those to actin filaments (159). As indicated by the name, the function of adherence junctions is to mediate adhesion between cells. Vascular endothelial (VE)-cadherin is one of the most important adherence junction proteins. VE-cadherin molecules are organized in dimers and attach cells by binding to adjacent VE-cadherin molecules in a zipper-like manner. The intracellular tails of VE-cadherin molecules interact with β-catenin and plakoglobin that in turn bind to α-catenins, which interact with actin filaments (160) (Figure 3). Stimulation of endothelial cells with inflammatory mediators such as thrombin, histamine, or vascular endothelial growth factor (VEGF) causes phosphorylation of the intracellular tail of VE-cadherin and leads to its internalization, with increased permeability as a consequence (160–162).

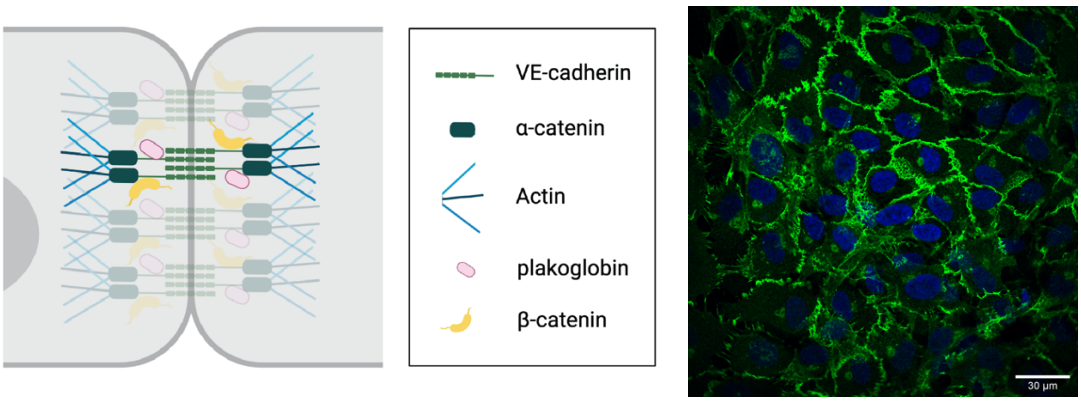

Figure 3. VE-cadherin organization. Endothelial cells are connected by VE-cadherin (green) adherence molecules. The intracellular tail of VE-cadherin binds to β-catenin and plakoglobin, which interact with actin-binding α-catenins.

Endothelial cell activation, induced by inflammatory mediators, leads to upregulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), and E-selectin on the endothelial cell surface (163,164). This allows for binding of immune cells and margination along the endothelium, which facilitates their extravasation into infected sites. Endothelial activation also causes aggregation of platelets and constriction of the blood vessels (164). Endothelial cell dysfunction refers to the inability of endothelial cells to maintain the above-mentioned functions and is often a result of continuous endothelial cell activation (158).

1.2.4 Inflammation

Inflammation is a fundamental response to any insult threatening to disturb the homeostasis. Inducers of inflammation can be of exogenous origin, as during an infection, or endogenous, as result of for example tissue injury (165). More specifically, inflammation describes a state of vasodilation and an increase in permeability between the endothelial cells lining the blood vessels. This process, commonly referred to as increased vascular permeability, causes leakage of blood plasma out from the circulation, into the surrounding tissue (165,166).

A healthy inflammatory response is well-regulated and actively resolves after some time, when the infection has been eradicated. In some cases, however, the inflammation develops into an uncontrolled process, with immunopathology as a consequence (166,167). This has for example been described in cytokine release syndrome (CRS), which is an acute inflammatory state that can develop as a side-effect of chimeric antigen receptor modified (CAR)-T cell treatment (168). An inflammatory response is mediated and regulated by a wide range of soluble mediators such as cytokines.

1.2.4.1 Cytokines and other markers of inflammation

Cytokines are signaling proteins that allow communication between cells. All nucleated cells can produce as well as respond to cytokines (169). A cytokine binds to one or several specific receptors on the target cell and initiates an intracellular signaling cascade. Cytokine signaling can be autocrine, paracrine, or endocrine. Autocrine signaling refers to when a cell secretes a cytokine that then binds to receptors on the same cell. Paracrine signaling, on the other hand, refers to cytokine signaling between neighboring cells. Endocrine signaling describes a more global form of paracrine signaling, in which a cytokine that has reached the blood stream binds to cells in a different tissue (170). The effects of a cytokine are influenced by different factors, including the kinetics, half-life and location of the cytokine as well as the expression of its receptors (170).

Cytokines can be separated into pro-inflammatory and anti-inflammatory based on their functions. Typical pro-inflammatory cytokines include interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF) (166). These cytokines can among other things elicit fever and stimulate the secretion of acute phase proteins such as C-reactive protein (CRP) and ferritin from the liver (171,172). These cytokines also have a role in activating the endothelium (164). Other pro-inflammatory cytokines exert important functions that in different ways support the

maturation, expansion, or function of T cells and NK cells. These include for example IL-2, IL-12, IL-15, and IL-18. IL-2 and IL-15 are mainly known for stimulating activation and proliferation of T cells and NK cells and IL-12 and IL-18 are strong stimulators of IFN-γ production (173–181). B cell-activating factor (BAFF) is, as the name implies, an important factor for the survival, maturation and activation of B cells (182). Moreover, IL-10 is an anti-inflammatory cytokine that suppresses the production of pro-inflammatory cytokines and chemokines in monocytes and macrophages (183).

IFNs are key cytokines in antiviral immunity and are divided into type I, type II and type III IFNs. Type I IFNs exist in many different forms, out of which IFN-α (existing in 13 different subtypes) and IFN-β are the most important (184). IFN-γ is the only type II IFN and has important roles in stimulating the effector functions of macrophages and T cells (184). Type III IFNs include three IFN-λ subtypes. IFN-λ shares many functions with the type I IFNs but particularly controls antiviral responses at mucosal surfaces (185).

With their diverse functions and regulated expression, cytokines are often useful biomarkers in different disease syndromes. However, also other inflammation markers, such as CRP, ferritin, and complement factors including C5a can be used as biomarkers in inflammatory diseases. The soluble IL-2 receptor α, also known as soluble CD25 (sCD25), is another marker that is often increased in blood during inflammation. CD25 is upregulated on activated lymphocytes, in particular T cells, and is shed into sCD25 (186,187).

1.2.4.2 IL-6: signaling and function

IL-6 was first identified as "B cell stimulating factor 2", after the discovery of a soluble factor produced by T cells that stimulated antibody production in B cells (188). IL-6 is a pro-inflammatory cytokine mainly produced by T cells, endothelial cells and antigen-presenting cells such as monocytes, dendritic cells and macrophages (189,190). IL-6 is pleiotropic, meaning it has a wide range of functions on different cells. For example, it induces the acute phase response, elicits fever, and promotes B cell differentiation and polarization of T cells (189,191). IL-6 signals via the IL-6 receptor (IL-6R) in complex with glycoprotein 130 (gp130) (189,192) (Figure 4). Upon ligation, dimerization of gp130 induces downstream signaling via the JAK family kinases, causing phosphorylation of STAT1 or STAT3 (Figure 4).

While all cells of the body express gp130, IL-6R expression is restricted only to hepatocytes and certain immune cells, including neutrophils and B cells (189,192). However, the actions of IL-6 are not limited to IL-6R-bearing cells as IL-6 also can bind to the soluble form of IL-6R (sIL-6R), shed from immune cells, and in complex with sIL-6R bind to any gp130 expressing cell. This form of IL-6 signaling, referred to as trans-signaling, allows for IL-6 to exert its biological effects also on cells lacking IL-6R (189) (Figure 4). Soluble gp130 (sgp130), created by alternative splicing or shedding, can bind to the IL-6:sIL-6R complex and inhibit its binding to membrane gp130. Thus, sgp130 is an inhibitor of trans-signaling (Figure 4). Studies showing lack of responses to IL-6 in endothelial cells have led to the view that endothelial cells do not express IL-6R (190,193). However, a few studies have demonstrated a very low IL-6R

expression on endothelial cells and some effects of classical signaling in endothelial cells (194,195). Addition of sIL-6R to endothelial cells has been shown to cause effects distinct from those of the classical signaling (195). These include increased endothelial cell secretion of IL-6 and CCL2 (196–198) and upregulation of ICAM-1 and VCAM-1 on the cell surface, which in turn stimulates adhesion of neutrophils and other immune cells to the endothelial cells (196). Thus, IL-6 trans-signaling is considered more pro-inflammatory than classical IL-6 signaling (192).

IL-6 has been implicated in the pathophysiology or severity of multiple inflammatory diseases, including rheumatoid arthritis (189,199). Tocilizumab is a therapeutic monoclonal antibody targeting membrane bound IL-6R as well as sIL-6R. In 2010, FDA approved the use of Tocilizumab in the treatment of rheumatoid arthritis, and since then, its applicability has been evaluated also for other diseases (200). Tocilizumab is currently being evaluated as a treatment option in COVID-19, with conflicting results (201–203).

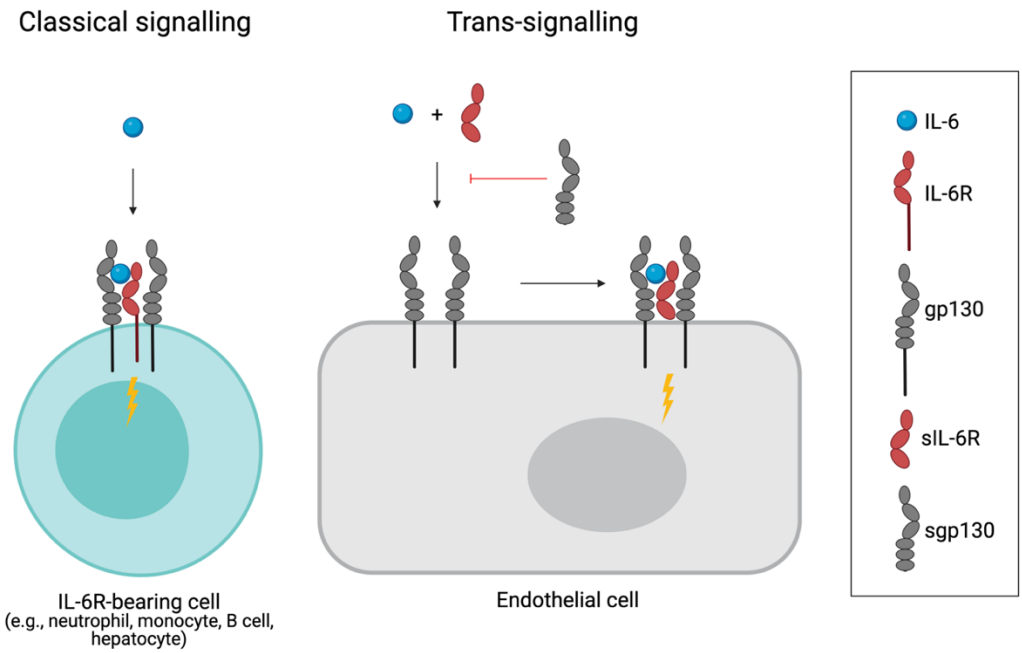

Figure 4. IL-6 signaling is mediated through classical signaling or trans-signaling. IL-6 binds to IL-6R and gp130 on cells and mediates signaling (classical signaling). IL-6 can also bind to sIL-6R and then the IL-6:IL-6R complex can bind to any cell that expresses gp130 and mediate signaling (trans-signaling). Trans-signaling is inhibited by sgp130 that binds to the IL-6:IL-6R complex and hinders its binding to membrane-bound gp130.

1.2.4.3 Chemokines and homing

Chemokines are cytokines that control the migration of cells by stimulating chemotaxis along a chemokine gradient (204). Chemokines and their cognate receptors are often described in the context of homing and trafficking of immune cells between blood and tissue (204). Chemokines related to this thesis include C-C motif chemokine ligand (CCL) 2, CCL20, and

CCL25. CCL2, also referred to as monocyte chemoattractant protein 1 (MCP-1), is produced by many different cell types, including endothelial cells and fibroblasts. CCL2 binds to C-C motif chemokine receptor (CCR) 2 and attracts monocytes and T cells to sites of infection or inflammation by mediating transmigration across the endothelium (205,206). CCL20 is produced at mucosal sites, by a wide range of cells including immune cells and endothelial cells (206). Upon binding of CCL20 to its cognate receptor CCR6, CCR6 may be down-regulated (207). CCL25 is produced by epithelial cells of the small intestine and mediates homing of lymphocytes to the gut, after binding to CCR9 (208).

Homing is not solely regulated by chemokines but is also driven by other chemotactic mediators. One such protein is mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1). MAdCAM-1 is expressed by endothelial cells of the intestine and attracts cells expressing α4β7 integrin (209,210). Another example of a chemotactic protein is C5a, which stimulates the recruitment of monocytes and neutrophils (155).

1.2.4.4 Microbial translocation

Microbial translocation is a well-recognized driver of inflammation and refers to the passive migration of bacterial products from the intestinal tract into the systemic circulation (211,212). In the circulation, these products recruit and activate antigen-presenting cells, leading to secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF. These cytokines can, when secreted in excess, cause systemic inflammation that has detrimental effects on the vasculature (213). Microbial translocation has been associated with inflammation and increased disease severity during HIV, dengue virus, hepatitis B virus (HBV), and hepatitis C virus (HCV) infection (211,213–220). While presence of lipopolysaccharide (LPS) and bacterial DNA in the blood can be used as direct markers of microbial translocation, measurement of these markers has been complicated by technical issues regarding variability, sensitivity, and specificity of the involved assays (213,221–223). There are also systemic surrogate markers of microbial translocation, including soluble CD14 (sCD14) and LPS-binding protein (LBP), both involved in the recognition of LPS by antigen-presenting cells (213,224–226).

Leakage of bacterial products from the intestine to the circulation is possible upon epithelial cell barrier disruption. Potential causes of epithelial barrier disruption include loss of tight junctions between the epithelial cells, and epithelial cell death (213). Cell death of intestinal epithelial cells may result from ischemia, bystander killing by cytotoxic cells, increased levels of TNF, or as a direct effect of virus infection (213,227). Intestinal fatty acid-binding protein (I-FABP) is a protein exclusively expressed in epithelial cells lining the intestine. Upon damage of intestinal epithelial cells, I-FABP leaks out into the circulation, making it a systemic marker for intestinal injury (217,228). Thus, I-FABP is indicative of intestinal epithelial cell damage that may be associated with microbial translocation.

1.2.5 MAIT cells

MAIT cells were identified as a new T cell subset in 1999 (229) but it was not until the beginning of 2010s that the MAIT cell research field started to grow. MAIT cells are cytotoxic innate-like T cells that respond to microbial antigens derived from the vitamin B2 (riboflavin) metabolism. MAIT cells are specifically activated by binding to the MHC class I-related protein 1 (MR1) on antigen-presenting cells, upon presentation of the specific antigens (230,231) (Figure 5). MAIT cells express a semi-invariant T cell receptor (TCR) containing Vα7.2-Jα33/12/20 paired with a limited set of Vβ segments. MAIT cells are traditionally defined by their expression of TCR Vα7.2 and high expression of the C-type lectin receptor CD161. Since 2016, availability of an MR1 tetramer loaded with the riboflavin precursor 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) has allowed for a more specific identification of MAIT cells (232).

MAIT cells develop in the thymus, during a process dependent on microbial metabolites (233,234). In the peripheral blood of healthy individuals, MAIT cells constitute 1-10% of the T cells (230), while in liver, MAIT cells are enriched and constitute 20-50% of the T cells (235,236). Upon activation, MAIT cells, similar to CD8 T cells and NK cells, can kill target cells using perforin and granzyme B. Resting MAIT cells express granzyme A and perforin constitutively, whereas granzyme B is expressed only upon activation (237). In addition to their cytotoxic function, activated MAIT cells also produce the pro-inflammatory cytokines TNF, IFN-γ, and IL-17 (Figure 5). In some contexts, MAIT cells may also produce additional cytokines such as GM-CSF and IL-22 (238–240).

While MAIT cells were initially described to respond specifically to microbial antigens, they can also become activated by cytokines, in an MR1-independent manner (230,241,242) (Figure 5). Cytokine-mediated activation of MAIT cells leads to a limited cytokine production, dominated by IFN-γ, compared to TCR-mediated MAIT cell activation which leads to a broader cytokine response (243). IL-12 and IL-18 have traditionally been considered as the main cytokines responsible for activation of MAIT cells, with IL-15 and type I IFNs having synergic effects (241,242,244). However, recent studies have highlighted an independent role for type I IFNs in MAIT cell activation (245, **Paper III**).

1.2.5.1 MAIT cells in viral infections

Given the ability of MAIT cells to respond to cytokines, MAIT cells are often activated during viral infections (238,242). A substantial number of studies have investigated how viral infections can affect MAIT cells. Early studies on this topic described depletion of peripheral blood MAIT cells in patients with HIV-infection (246–248). These reports have been followed by studies describing a similar loss of MAIT cells also during infections with HCV, influenza virus, hepatitis D virus (HDV), human T-lymphotropic virus type 1 (HTLV-1), SARS-coronavirus-2, and hantavirus (241,245,249–255, **Paper III**). While chronic viral infections seem to lead to a chronic depletion of MAIT cells (246,248,255), the loss of MAIT cells during acute viral infections has been reported to be transient (251,253, **Paper III**). During acute

dengue virus infection, blood MAIT cell frequencies were not decreased until after 10 days post symptom onset, when patients were considered to be in the convalescent phase (241). In all these viral diseases, the residual MAIT cells still remaining in the circulation displayed an activated phenotype (241,245,248,255,250,252,251,253,254, **Paper III**). Despite the increasing number of reports on the subject, the cause of this massive loss of peripheral MAIT cells during viral infections remains unknown. Migration to tissue and activation-induced cell death have been suggested as possible explanations (242,246, **Paper III**).

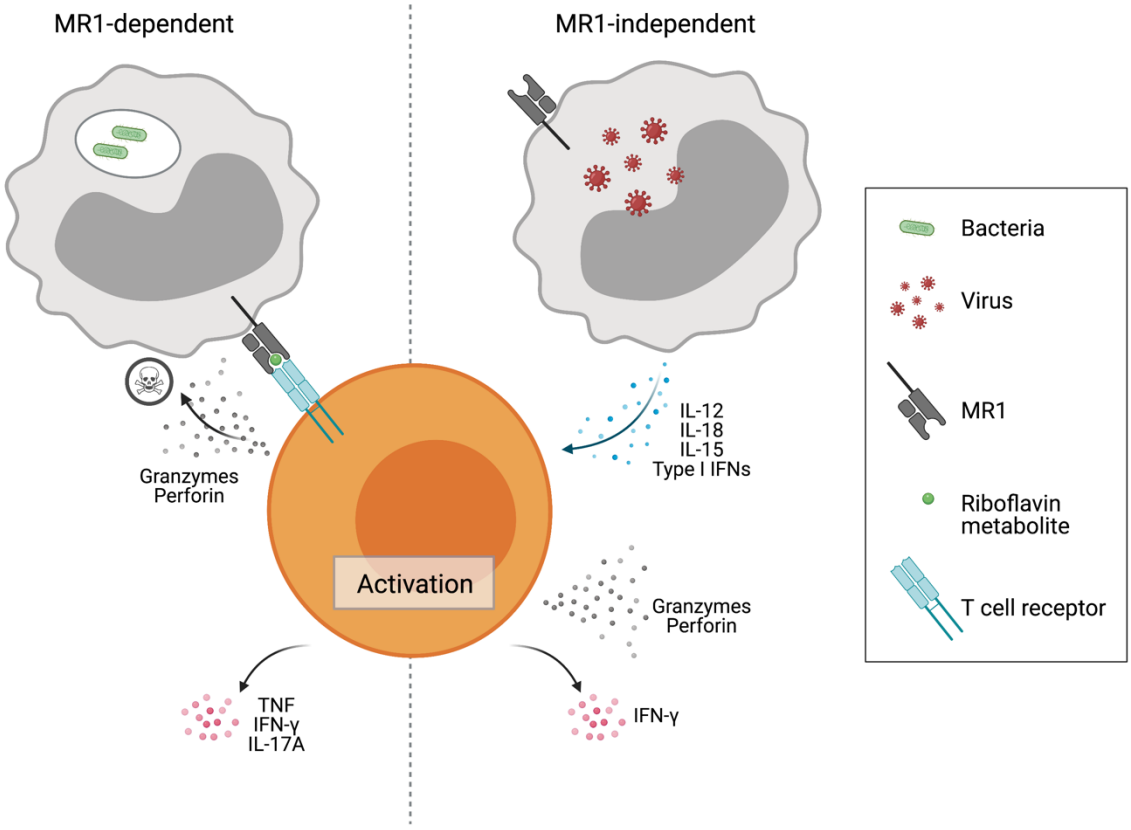

Figure 5. Two modes of MAIT cell activation: MR1-dependent and MR1-independent. Antigen-presenting cells that have phagocytosed microbes with the riboflavin biosynthesis pathway present riboflavin metabolites on MR1. Binding of the MAIT cell TCR to MR1 and the ligand, leads to MAIT cell activation, production of cytokines and killing of the infected cell. During viral infection, antigen-presenting cells do not present any MAIT cell ligand, but instead cause MAIT cell activation via the cytokines IL-12, IL-15, IL-18, and type I IFNs. Also this mode of activation can induce cytokine expression, mainly IFN-γ, and cause degranulation.

To date, the role of MAIT cells during viral infections is not well understood. As activated MAIT cells can produce pro-inflammatory cytokines including TNF, IFN-γ, and IL-17, both anti-viral effects and inflammatory effects contributing to immunopathology are possible consequences of their activation. During influenza virus infection, a decrease in peripheral blood MAIT was associated with a more severe disease (249). In line with this, influenza virus infection caused higher mortality and more weight loss in MAIT cell-deficient mice compared

to wild type mice (256). Moreover, in HIV/HCV co-infected individuals, the level of liver fibrosis was inversely correlated to the MAIT cell frequency in blood (257). These observations may indicate that MAIT cells have protective roles in some viral infections. In individuals infected with dengue virus or SARS-coronavirus-2, stronger MAIT cell activation has been observed in individuals with a more severe disease (241,245). While MAIT cells activated by IL-18 produce IFN-γ, MAIT cells activated by type I IFNs do not produce any of the MAIT cell-specific cytokines (241,244, **Paper III**). Thus, it is likely that MAIT cells may exert diverse functions depending on the cytokine milieu in different tissues and different disease contexts.

1.3 HANTAVIRUS PATHOGENESIS

Although hantavirus infection can give rise to severe, life-threatening symptoms in patients, autopsies of deceased HPS patients have revealed no obvious tissue damage, suggesting that the virus is not cytopathogenic *per se* (15,17). Supporting this, it has been shown that hantavirus inhibits both chemically induced as well as lymphocyte-mediated apoptosis in endothelial cells (258,259). These observations, together with reports showing strong immune responses in patients, have led to the view that hantavirus diseases may be partly immune-mediated.

1.3.1 Sensing of hantaviruses and inhibition of antiviral responses

To date, the PRRs that sense hantaviruses are not fully known. A recent study identified that RIG-I and MDA-5 were critical for type I IFN responses in HTNV infected endothelial cells (260). Further, one study suggested that TLR-3 is important for induction of type I IFN responses in cells infected with HTNV, but not with PHV (261). In another study, secretion of type I IFNs, IL-6, and TNF by HTNV-infected cells was suggested to be dependent on expression of TLR-4 (262).

Multiple studies have explored the capacity of hantaviruses to inhibit antiviral responses. It is generally accepted that cells pre-treated with type I IFNs, or treated with type I IFNs early during infection, are not susceptible for productive hantavirus infection (260,263,264). Likewise, MxA expression within a cell inhibits replication of HTNV, PUUV, and TULV (265,266). However, once the infection is established, treatment with IFNs does not have an effect on hantavirus replication (263,264), suggesting that hantaviruses somehow inhibit the antiviral effects of type I IFNs. The mechanisms behind this are not fully understood. The NSs proteins of PUUV, TULV, and PHV have been described to inhibit IFN-β gene expression (35,267). Other studies have suggested inhibition of RIG-I as one strategy of hantaviruses to protect against the antiviral responses (260,263,268,269). Moreover, reduced STAT-1 phosphorylation has been seen in cells infected with ANDV, PHV, or HTNV (264,270).

1.3.2 Virus-induced direct responses

Endothelial cells are the primary target cells of hantaviruses (27). Endothelial cells infected with ANDV, HTNV, or PHV display increased secretion of IL-6 and CCL5 (271) and infection with PUUV causes increased IL-6 secretion (**Paper II**). As vascular leakage is a prominent

hallmark of HFRS and HPS, several studies have focused on exploring direct and indirect effects of hantavirus infection on the endothelial cell barrier integrity. Upon hantavirus infection of primary human endothelial cells, the adhesion proteins ICAM-1 and VCAM-1 are upregulated on the surface (41,272,273, **Paper II**). In HFRS patients, increased plasma levels of soluble ICAM-1 and VCAM-1 are seen (274,275). These findings suggest that hantavirus infection directly, or indirectly by inducing secretion of cytokines, activates endothelial cells.

While a few studies have indicated that hantavirus infection *per se* does not seem to induce increased permeability of an endothelial cell monolayer (276–278), one study demonstrated increased permeability in endothelial cells infected with ANDV or SNV (279). Several studies have suggested a role for VEGF in causing vascular permeability in hantavirus-infected cells. In this context, it was suggested that VEGF causes a VEGF-receptor 2-dependent downregulation of VE-cadherin from the surface of infected endothelial cells, leading to increased permeability (278–281). In one of these studies, also downregulation of claudin-1 was observed (281). However, these findings could not be recapitulated in a capillary blood-vessel model system in which VEGF production was demonstrated (282). In this model, hantavirus-induced permeability was suggested to be a result of increased activity of the kallikrein-kinin system (282).

1.3.3 Cytokine responses upon hantavirus infection

Both HFRS patients and HPS patients exhibit elevated systemic levels of pro-inflammatory cytokines including IL-6, IL-8, IL-15, IL-18, IFN-α, and TNF as well as increased levels of IL-10 (134,283–292, **Paper I**, **II**, **and III**). In HPS patients, and sometimes in HFRS patients, also increased levels of IL-2 and IL-12 are observed (284,293, **Paper I**). Moreover, the chemokines CCL2, CCL20 and CCL25 are all increased during acute HFRS (291,294,295, **Paper III**). IL-6 is the only cytokine that has been associated to the disease severity of HFRS and HPS (283,285,292, **Paper I**). Further, IL-6 levels are higher in HPS patients with a fatal outcome, compared to in patients with a non-fatal outcome (285, **Paper I**).

Although an emerging number of reports point toward a cytokine storm during hantavirus infection, the cellular sources of these cytokines and the processes leading to their secretion remain unknown. Parts of the cytokine response during hantavirus infection seems to result from direct effects of the virus infection in endothelial cells (271, **Paper II**). The specific roles of these and other cytokines in hantavirus pathogenesis remain to be further investigated.

1.3.4 Immune cell responses upon hantavirus infection

Early during PUUV infection, NK cell numbers in circulation are decreased (273). This is then followed by a long-term NK cell expansion, characterized by activated and proliferating NK cells displaying increased expression of granzyme B and perforin (273,296). *In vitro* studies have shown that hantavirus-infected endothelial cells can directly activate NK cells via IL-15 trans-presentation (296).

Strong activation and expansion of CD8 T cells has been observed during both HFRS and HPS, (297–299). Further, infiltration of CD8 T cells into lung and kidney has been described in patients (38,42,131,300). The role of CD8 T cells in hantavirus pathogenesis is however controversial. One study suggested that a strong but transient early epitope-specific CD8 T cell response was associated with mild HFRS, while a weak CD8 T cell response that expanded over time was associated with severe HFRS (301). In another study, it was shown that epitope-specific CD8 T cell responses were stronger in patients with mild compared to severe HFRS (302). Conversely, in a study of HPS patients, epitope-specific CD8 responses were reported to be stronger in patients with severe disease (297). The conflicting findings of these studies could be due to differences in the pathogeneses of different viral species. However, more comprehensive studies are needed to clarify this. Regarding the functionality of CD8 T cells during hantavirus infection, one study showed that CD8 T cells of PUUV-infected patients exhibited poor IFN-γ responses in response to stimulation with PUUV-peptides or the T cell mitogen phytohemagglutin (298). Interestingly, increased IFN-γ expression of CD8 T cells could be observed after direct *ex vivo* staining of CD8 T cells, without prior stimulation (298).

Invariant T cell subsets have not been extensively studied in hantavirus-infected individuals. However, the MAIT cell phenotype was recently characterized in HFRS patients (**Paper III**). During acute HFRS, peripheral blood MAIT cells are highly activated and decline in numbers (**Paper III**). Residual MAIT cells show altered expression of homing receptors (**Paper III**), but whether they constitute the CD8 T cells infiltrating tissues (38,42,131,300) has not been studied.

Other immune cells that may have a role in hantavirus pathogenesis include B cells, mononuclear phagocytes, and neutrophils. In ANDV-infected HPS patients, a great expansion of plasmablasts has been observed (303). Interestingly, antibodies produced by these plasmablasts were not specific only to hantavirus proteins, but also to a wide range of other antigens, including LPS and tetanus toxin (303). Mononuclear phagocytes of HFRS patients have been studied in a few different studies. During acute HFRS, monocytes accumulate in kidney and lung (38,291). In a study of Swedish HFRS patients, monocytes and dendritic cells were shown to be massively depleted in peripheral blood (38). On the contrary, a recent study found an increase in peripheral blood monocytes in Finnish HFRS patients (291). Increased monocyte levels in blood have also been observed in HFRS patients infected with HTNV (304). The responses of neutrophils have not been comprehensively studied in patients. Neutrophil activity in patients has been indirectly indicated by the increased systemic levels of histones, histone-double stranded DNA complexes, and anti-nuclear antigen antibodies (303,305,306). Moreover, neutrophils exposed to HTNV *in vitro* have been suggested to release NETs (305). However, this could not be repeated with purified PUUV (306).

2 RESEARCH AIMS

The general aim of this thesis was to generate a better understanding of hantavirus-induced immune responses in humans, to guide the development of treatments.

The specific aims of this thesis were to:

- Identify inflammatory mediators associated with severity and fatality during hantavirus infection
- Investigate the role of IL-6 in hantavirus pathogenesis
- Characterize MAIT cell responses in hantavirus infection

3 METHODS

Below, a brief introduction is given to the main assays used in this thesis. Details regarding these assays, and other methods used in the studies, are found in the respective papers.

3.1 ANALYSIS OF SOLUBLE MARKERS

In this thesis, the concentrations of cytokines and other soluble markers in plasma, serum, and cell culture supernatants were analyzed using either sandwich ELISA or multiplex immunoassay. Which of these two methods that was used for a specific marker depended on the available sample volume, the availability of commercial kits and the possibility to perform the experiment in a biosafe manner.

3.1.1 Sandwich ELISA

In a sandwich ELISA, the so-called capture antibodies are coated onto the bottom of the wells of a 96-well plate. When the sample is added to the well, capture antibodies, which are specific against the protein of interest, bind to the analyte within the sample. Washing of the wells removes excess sample and subsequently secondary antibodies are added to the wells. Also the secondary antibody is specific against the analyte but binds to an epitope different from the epitope of the primary antibody. The secondary antibody is conjugated to biotin molecules, which in the subsequent step bind to streptavidin conjugated to an enzyme. In the final step, a substrate is added to the wells. The enzymes bound to the secondary antibodies utilize the substrate and the reaction product gives rise to a color change in the solution. The reaction is stopped by the addition of acid which gives rise to a color shift (Figure 6). The absorbance of this color is proportional to the concentration of the analyte of interest and is measured by a spectrophotometer (307).

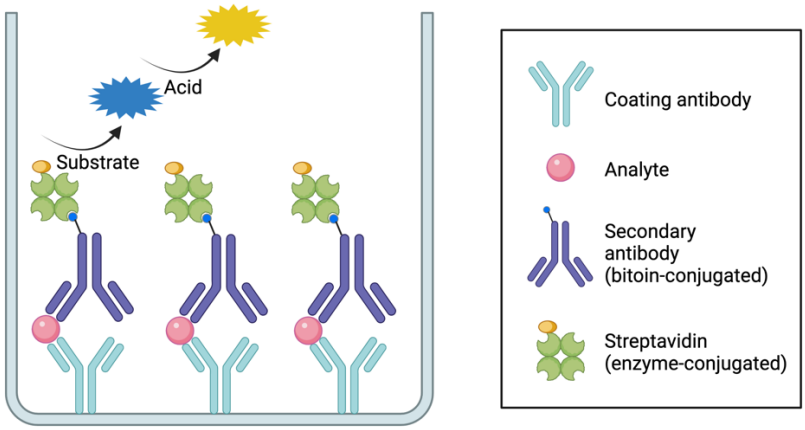

Figure 6. Principles of sandwich ELISA. Capture antibodies bind to the analytes within a sample. Secondary antibodies conjugated with biotin also bind to the analyte, creating a "sandwich". In the next step, streptavidin conjugated with enzymes bind to the biotin molecules. Addition of a substrate gives rise to a color that is proportional to the amount of bound analyte. Addition of acid then causes a change in color, which is measured in a spectrophotometer.

3.1.2 Multiplex immunoassay

Multiplex immunoassays are in principle very similar to the sandwich ELISAs, but with the difference that the capture antibodies are coupled to microbeads instead of a 96-well plate. In a multiplex immunoassay, microbeads of different fluorescent colors are paired with capture antibodies specific against different analytes. This allows the simultaneous detection of multiple analytes in the same sample, using the same assay. The amount of bound analyte per microbead is detected using biotin-labeled secondary antibodies that in the next step are attached to fluorescently labeled streptavidin molecules. The microbeads are read within a flow cytometer with dual detection; the fluorescence of the microbead specifying the analyte, and the fluorescent signal of the secondary antibody signaling the intensity (308).

3.2 ANALYSES OF MAIT CELL PHENOTYPES BY FLOW CYTOMETRY

The phenotype of peripheral blood MAIT cells was in **Paper III** analyzed using a flow cytometer equipped with five different lasers. Activation of MAIT cells can be assessed by their increased surface expression of one or several of the markers CD69, CD38, human leukocyte antigen-DR, and CD25 (241,309,310). Increased intracellular expression of granzyme B, or the cytokines IFN-γ, TNF, and IL-17A is also indicative of MAIT cell activation (241,309,310). The expression of these markers can be quantified by flow cytometry on the single-cell level, after staining with a mix of fluorescently labeled antibodies, each specific against one antigen of interest. Hence, a single cell is stained by multiple different antibodies, each with their specific fluorochrome. In the flow cytometer, the lasers excite these fluorochromes, giving rise to specific emission wavelengths that are read by detectors and converted into digital signals. These signals give information about the cell size, cell granularity, and intensities of the different fluorochromes (311).

3.3 ANALYSIS OF ENDOTHELIAL CELLS USING IMMUNOFLUORESCENCE MICROSCOPY

In **Paper II**, the expression of VE-cadherin and PUUV proteins in primary human endothelial cells was assessed using immunofluorescence microscopy. Also in this method, fluorescently labeled antibodies were used. Cells grown on glass cover slips were fixed and then stained with primary antibodies specific against the antigens of interest. In the subsequent step, fluorescently labeled secondary antibodies specific against the primary antibodies were added to the cells. The expressions of VE-cadherin and PUUV proteins could be visualized using a fluorescence microscope that excited the fluorochromes.

3.4 PERMEABILITY ASSESSMENT USING TRANSENDOTHELIAL ELECTRICAL RESISTANCE

Transendothelial electrical resistance (TEER) is used as a measure of permeability across a cell monolayer (Figure 7). In **Paper II**, the permeability across transwells with endothelial cell monolayers were assessed using a voltohmmeter. Infected or uninfected cells were cultured inside the inner chamber of a transwell and treated with sIL-6R. After 24 h of treatment, the

TEER was measured. A low TEER indicates low resistance between the two chambers and thus an increased monolayer permeability.

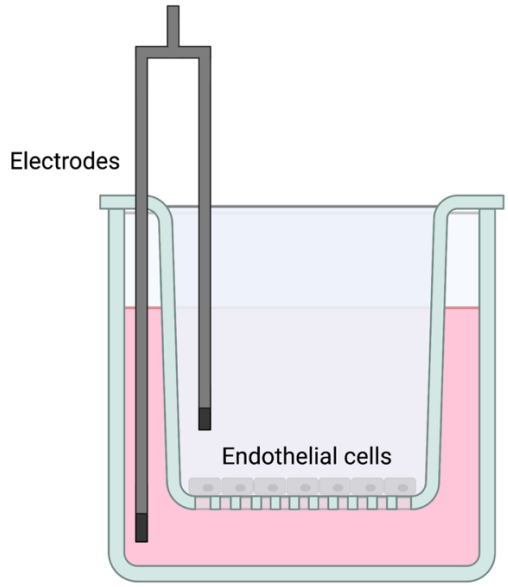

Figure 7. Illustration of TEER measurement in a transwell system. Endothelial cells are cultured in the bottom of the inner chamber. Electrodes connected to a voltohmmeter measure the electrical resistance between the inner and outer chamber of the transwell system, thereby assessing the permeability between the chambers.

3.5 ETHICAL CONSIDERATIONS

Medical research inevitably comes with various ethical challenges. In this thesis, we studied blood samples from human subjects. All studies were approved by the regional ethics committees, as indicated in the respective papers. Further, individuals donating blood specifically for the studies of **Paper II** and **Paper III** provided written consent prior to enrollment. In **Paper I** we took use of left-over diagnostic samples and thus, written consent from patients was not available. Patients and samples were coded, and sensible data were handled according to the General Data Protection Regulation.

4 RESULTS AND DISCUSSION

4.1 INFLAMMATORY RESPONSES IN HANTAVIRUS-INFECTED PATIENTS

Numerous studies have described an increase in a variety of cytokines and other inflammatory markers in individuals infected with hantavirus (134,283–292, **Paper I**, **Paper II**, **Paper III**). Yet, the lack of insights into pathological versus protective responses hampers the development of specific treatments against HFRS and HPS. The fact that HPS is more severe than HFRS and has a case-fatality rate of up to 40%, makes HPS ideal to study factors that correlate with disease progression and outcome. However, the limited access to patient samples has made it difficult to perform large studies that allow complex statistical analyses. In Argentina, ANDV causes approximately 60 HPS cases yearly (123). Through a collaboration with a group in Buenos Aires, Argentina, we had the opportunity to study the inflammatory responses in 93 HPS patients.

In **Paper I**, we analyzed the concentrations of 20 different serum markers in these 93 HPS patients, out of whom 34 had a fatal outcome. As patients were sampled at different timepoints post debut of symptoms, patients were divided into three groups; patients sampled during days 1-4, days 5-10, and days 11-23 post symptom debut. This was done to decrease the risk of bias related with the day of sampling. The patients could be divided into four different severity groups, based on the clinical information.

4.1.1 The inflammatory response in HPS patients

Out of 20 analyzed serum markers, 18 were shown to be significantly increased in HPS patients sampled at days 1-4 post symptom debut, compared to uninfected controls. These markers included IL-1β, IL-2, IL-6, IL-10, IL-12, IL-15, IL-18, TNF, IFN-γ, BAFF, C5/C5a, sCD25, ferritin, granzyme A, granzyme B, sCD14, LBP, and I-FABP (Figure 8). In patients sampled at days 5-10, the picture was similar, with the exception that only a few patients displayed detectable IL-2 levels. In patients sampled at days 11-23, most serum markers were still significantly higher compared to controls. However, at this time point, IL-2, IL-12, IL-15, and ferritin were not significantly increased compared to controls, suggesting distinct time kinetics in the regulation of these markers. IL-10 was significantly lower in patients sampled at days 11-23, compared to patients sampled earlier, suggesting that also this cytokine may have a shorter timespan during acute hantavirus infection. Altogether, these data show that a wide range of cytokines and other inflammation markers are increased during HPS. The serum concentrations of VEGF and soluble TRAIL (sTRAIL) were not altered during HPS. However, it is possible that their concentrations are altered at local sites.

Overall, these data are in line with what has previously been reported in HPS patients (284,285). Furthermore, we showed an increase in ferritin, granzyme A, granzyme B, BAFF, and markers associated with microbial translocation.

4.1.2 Serum markers associated with severity of HPS

The classification of patients into four different severity groups allowed us to investigate which serum markers that were associated with severity of disease. This was achieved using univariate and multivariate logistic regression models that were adjusted for gender, age, and day of sampling. IL-β, IL-6, IL-15, ferritin, granzyme B, and I-FABP were associated with increased odds of severe disease in the univariate analysis. C5/C5a, on the other hand, was associated with decreased odds of severe disease in both univariate and multivariate analyses (multivariate: OR=0.59; 95% CI, 0.35-0.99) (Figure 8). In the multivariate analysis, also BAFF was associated with decreased disease severity (OR=0.47; 95% CI, 0.25-0.91) (Figure 8). Among the markers associated with increased disease severity, only IL-6 was associated with increased severity in the multivariate model (Figure 8). Hence, IL-6 was the single marker that independently of the levels of other markers was associated with increased disease severity (OR=2.25; 95% CI, 1.01-5.01). These data support what was previously reported in a separate cohort of HPS patients (285). Systemic IL-6 levels have been associated with the disease severity also in other viral diseases, including Ebola virus disease, dengue fever, and recently, COVID-19 (312–315). However, IL-6 levels in COVID-19 are relatively low compared to inflammatory diseases such as hyperinflammatory acute respiratory distress syndrome (ARDS) and CRS, in which IL-6 levels are usually on the ng/ml level (316). The serum IL-6 levels in HPS patients were in median 28 pg/ml, which is comparable to the median level of 37 pg/ml reported in COVID-19 patients (316). Despite of the relatively low IL-6 levels in COVID-19, some studies have found IL-6R-targeted treatment effective in COVID-19 (201,203). This highlights the possibility that also a relatively small increase in IL-6 may be important to consider.

The notion that high virus-specific antibody titers are protective during hantavirus infection (81,146) suggest that BAFF may serve protective effects in HPS patients, by promoting the antibody production of B cells (182). Given that C5a has been associated with development of ARDS (317), we were slightly surprised to find a protective role for C5/C5a in HPS. As the assay could not separate C5 from the cleaved fragment C5a, speculations on these findings should be done with caution. One might speculate that C5a contributes to favorable immune responses by attracting neutrophils and monocytes to the lungs of patients (154,155).

4.1.3 Serum markers associated with fatality of HPS

Next, we studied associations between serum markers and fatality of HPS. Comparing serum marker concentrations in patients with fatal compared to non-fatal disease, IL-6 and I-FABP were the only two markers that were significantly increased in patients with fatal outcome during both days 1-4 and days 5-10 post symptom onset. Among patients sampled at days 1-4, median IL-6 levels were almost six times higher in patients with a fatal outcome (113 pg/ml vs 19 pg/ml, p=0.0008) (Figure 8). In support of this, higher IL-6 levels in patients with fatal compared to non-fatal HPS were previously reported (283,285). Increased IL-6 levels have also been described in fatal compared to non-fatal Ebola virus disease and COVID-19 (245,313). Levels of IL-10, IFN-γ, and sTRAIL were found to be higher in patients with fatal outcome,

during days 5-10 post symptom onset. Also IL-15 levels showed a tendency towards being higher in fatal HPS, during days 5-10 (p=0.062). In patients sampled at days 1-4, C5/C5a levels were higher in patients with a non-fatal outcome, again suggesting a possible protective effect of C5/C5a in HPS pathogenesis. The mentioned findings were to a high extent reflected in the univariate regression analysis, in which a fatal outcome was associated with increased levels of IL-6, IL-15, IFN-γ, ferritin, granzyme B, and I-FABP, and C5/C5a was associated with decreased odds of a fatal outcome. However, in the multivariate analysis, only I-FABP was independently associated with a fatal outcome (OR=1.64; 95% CI, 1.01-2.64). I-FABP is an intracellular protein expressed by intestinal epithelial cells. Upon cell damage, I-FABP leaks out into the circulation, and is thus considered as a systemic marker for intestinal damage (228). The finding that I-FABP is associated with a fatal outcome suggests that intestinal damage may be a marker of fatal HPS. During both HFRS and HPS, hemorrhage in the gastric mucosa has been reported (15,92,318). Whether also cell damage occurs in the gastrointestinal tract of patients is unknown. While the endothelial cells of the gastrointestinal tract can be infected with hantavirus (92), the infection *per se* likely does not cause cell death as hantaviruses can inhibit apoptosis (258,259). Given that hypoxia is common during severe HPS, it is possible that ischemia in the intestines may cause cell injury and subsequent leakage of I-FABP during HPS.

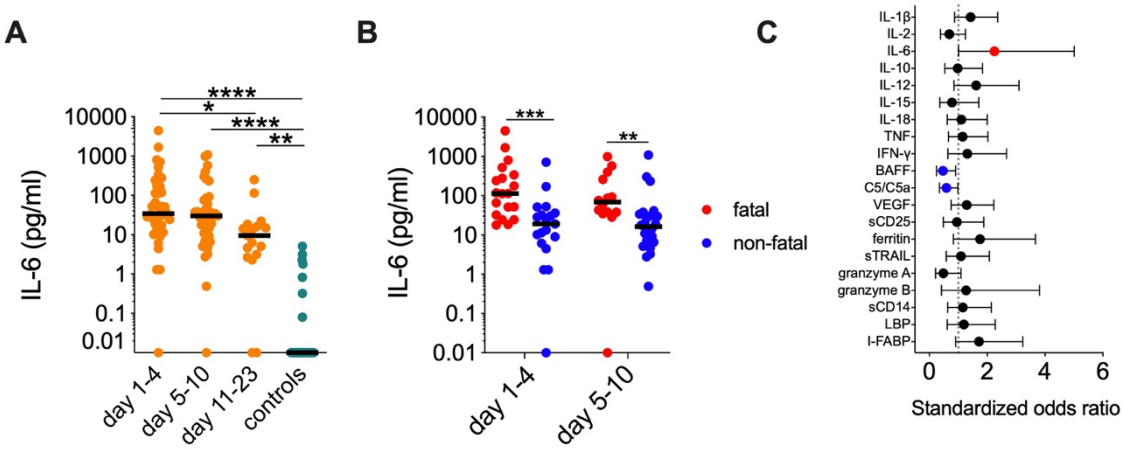

Figure 8. Serum IL-6 levels are associated with severity of HPS. (A) Serum levels of IL-6 are increased during HPS, compared to uninfected controls. (B) Serum IL-6 levels are higher in HPS patients with a fatal outcome, compared to non-fatal outcome. (C) Multivariate regression analysis adjusted for gender, age, and day of sampling highlights IL-6 as an independent factor associated with the severity of HPS. On the contrary, BAFF and C5/C5a are associated with decreased severity.

4.1.4 Conclusions and future directions on inflammatory responses in HPS patients

In conclusion, **Paper I** showed increased levels of multiple cytokines and inflammation markers in HPS patients. Multivariate regression analysis revealed that IL-6 was an independent marker associated with HPS disease severity. In support of this finding, an

association between HPS severity and IL-6 levels was previously also found in another study (285). Together, these findings highlight IL-6 as a potential therapeutic target during hantavirus infection. Moreover, I-FABP levels were associated with increased odds of a fatal outcome. This suggests the occurrence of intestinal injury in patients with fatal HPS. Future studies should evaluate the potential of IL-6 and I-FABP as predictors of severe disease and fatality in HPS patients.

It is important to consider the possibility that also additional markers that were not analyzed in this study may be associated with HPS disease severity or fatality, either in synergy with, or independently of IL-6. High-throughput methods for the quantification of serum markers would allow for unbiased and systematic studies with the possibility for unexpected findings. Moreover, comparisons to other inflammatory diseases, as was done by Thwaites *et al.* and Leisman *et al.* (314,316) with COVID-19, would be useful in the search for HPS-specific factors, or more general factors, that drive hantavirus pathogenesis.

4.2 ROLE OF IL-6 IN HANTAVIRUS PATHOGENESIS

The association between serum IL-6 and severity of HPS, prompted us to investigate possible consequences of IL-6 signaling in the context of hantavirus infection. Hantavirus infection primarily targets the endothelial cells (42) and vascular permeability is likely responsible for many of the symptoms in patients (27). However, the mechanisms underlying these symptoms are poorly understood.

4.2.1 Sources of IL-6 during hantavirus infection

To investigate possible sources of IL-6 production during PUUV infection, we first assessed IL-6 secretion from infected and uninfected human umbilical vein endothelial cells (HUVECs). Endothelial cells are known to produce IL-6 under steady state (190). We showed that PUUV infection of HUVECs led to a 5.7-fold increase in IL-6 secretion, at 48 h post infection (Figure 9). Moreover, in PUUV-exposed PBMCs, IL-6 secretion was increased 4.5 times at 48 h post exposure (Figure 9). In line with these data, endothelial cells infected with ANDV, HTNV or PHV also show increased IL-6 secretion (271). These data suggest that infected vascular endothelial cells may be an important source of IL-6 in hantavirus-infected patients.

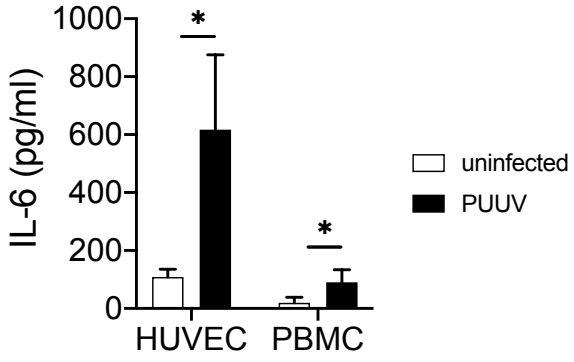

Figure 9. IL-6 secretion by PUUV-infected cells. IL-6 secretion is increased in PUUV-infected HUVECs and PUUV-exposed PBMCs at 48 h post infection.

4.2.2 Pro-inflammatory effects of IL-6 trans-signaling during hantavirus infection

Next, we were interested to study possible effects of IL-6 signaling during hantavirus infection in HUVECs. As infected endothelial cells were found to produce high levels of IL-6, this endogenously produced IL-6 was utilized to study possible autocrine effects. Endothelial cells have been described as non-responsive to IL-6 due to their absent or low IL-6R expression (190,197). Thus, we evaluated the effects of IL-6 trans-signaling upon treatment with recombinant sIL-6R. First, the pro-inflammatory effects of IL-6 trans-signaling were assessed. IL-6 trans-signaling in endothelial cells has been shown to cause increased secretion of IL-6 and CCL2 (195–198). Therefore, we assessed the concentrations of IL-6 and CCL2 secreted from infected and uninfected cells with or without sIL-6R treatment, using ELISA. In PUUV-infected HUVECs, but not in uninfected, sIL-6R treatment led to increased secretion of both IL-6 and CCL2, in a dose-dependent manner (Figure 10). A similar increase in CCL2 was also seen after addition of recombinant IL-6 and sIL-6R to uninfected HUVECs. These findings are in line with what has been shown earlier in endothelial cells treated with exogenous IL-6 and sIL-6R (195–198). Our data suggest that IL-6 trans-signaling augments the pro-inflammatory responses of PUUV-infected HUVECs by stimulating secretion of IL-6 and CCL2. Thus, endothelial cells may be a source of the increased levels of IL-6 and CCL2 in HFRS patients (291,294,295). CCL2 attracts T cells, mononuclear cells and other immune cells to sites of inflammation (205,319). Moreover, CCL2 presented on the surface of endothelial cells mediates adhesion and transmigration of immune cells across the endothelium (205,319,320). Hence, endothelial cell-derived CCL2 may be an important mediator of immune cell infiltration into tissue during acute hantavirus infection.

IL-6 is, among a few other cytokines, known to cause endothelial cell activation, which is characterized by upregulation of adhesion molecules such as ICAM-1 and VCAM-1 that facilitate the binding of immune cells to the endothelium (164,321–324). Hantavirus infection of endothelial cells leads to upregulation of ICAM-1 and VCAM-1 on the cell surface (41,272,273). To in more detail study the effects of IL-6 trans-signaling in infected cells, we next assessed the expression of ICAM-1 on sIL-6R-treated HUVECs, using flow cytometry. In infected HUVECs, sIL-6R treatment led to increased ICAM-1 expression, in a dose-dependent manner (Figure 10). In HFRS patients, plasma levels of sICAM-1 are increased (274), suggesting that endothelial cells are activated also in patients. Together, these findings indicate that IL-6 trans-signaling in PUUV-infected endothelial cells, by inducing CCL2 and ICAM-1 expression, promotes the attachment and transmigration of immune cells. In addition, IL-6 trans-signaling in infected cells leads to an autocrine loop of IL-6 production that further fuels the inflammatory responses.

4.2.3 Effects of IL-6 trans-signaling on the barrier integrity of infected cells

Having observed profound pro-inflammatory effects upon IL-6 signaling in PUUV-infected endothelial cells, we next sought to investigate possible effects of IL-6 on the endothelial monolayer barrier integrity. While previous reports have described VEGF-mediated increased

permeability in hantavirus-infected cells (278–281), the role for IL-6 has not been studied in detail in the context of hantavirus infection. In one study, IL-6 treatment of hantavirus-infected endothelial cells did not affect the permeability (277). In endothelial cells treated with IL-6 together with sIL-6R, increased permeability and altered VE-cadherin organization has been reported (325). To investigate if endogenously produced IL-6 had similar effects during hantavirus infection, we assessed the expression of VE-cadherin in infected and uninfected cells, with or without sIL-6R treatment, using immunofluorescence microscopy. In uninfected HUVECs, VE-cadherin organization was intact despite treatment with sIL-6R. However, as previously reported for ANDV infected cells (279), the morphology of infected cells was clearly affected by the infection alone. Furthermore, VE-cadherin junctions between infected cells were less clear and indicated that the VE-cadherin organization was disrupted. Downmodulation of VE-cadherin has previously been observed in endothelial cells infected with ANDV and SNV (279). Upon addition of 250 ng/ml sIL-6R to infected cells, further downmodulation of VE-cadherin was observed, and formation of inter-cellular gaps were apparent (Figure 10). With 500 ng/ml sIL-6R, even less VE-cadherin expression was observed. These observations strongly indicated that the barrier integrity was severely disrupted in infected cells treated with sIL-6R. To confirm this, TEER was performed. TEER analyses suggested that PUUV-infection alone to some extent reduced the permeability. Addition of sIL-6R to infected cells caused a strong decrease in TEER, in a dose-dependent manner.

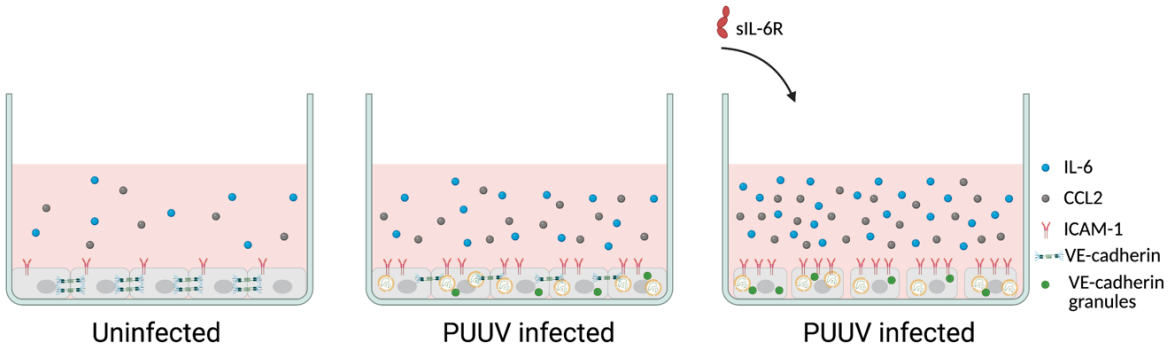

Figure 10. sIL-6R causes increased cytokine production and decreased barrier integrity. PUUV-infection of endothelial cells leads to increased secretion of IL-6 and CCL2, upregulation of ICAM-1, and VE-cadherin downmodulation. These effects are augmented by treatment with sIL-6R. In addition, sIL-6R treatment of infected cells leads to decreased barrier integrity in the cell monolayer.

4.2.4 Levels of soluble IL-6 receptors in hantavirus-infected patients

While systemic levels of IL-6 have been repeatedly measured in HFRS and HPS patients (284–286,289,292, **Paper I and Paper III**), the concentrations of IL-6 receptors have not been comprehensively studied in hantavirus-infected patients. To get an overview of the different components involved in IL-6 signaling in patients, we studied the levels of sIL-6R, sgp130, as well as the complex of IL-6:sIL-6R in plasma of HFRS patients. No significant differences were observed in the concentrations of the IL-6:sIL-6R complex. Surprisingly, we found that

sIL-6R levels were decreased during convalescent HFRS, compared to acute HFRS and controls. Moreover, sgp130 levels were decreased compared to uninfected controls, both during acute and convalescent HFRS. Decreased systemic levels of sgp130 have previously been reported in patients with coronary artery disease and type 2 diabetes (326–328).

The imbalance in the concentrations of the IL-6 receptors was well-reflected in the sIL-6R/sgp130 ratio, which was strongly increased in acute HFRS compared to convalescence and controls. With sIL-6R being an agonist of IL-6 trans-signaling, and sgp130 being the antagonist (329), the higher sIL-6R/sgp130 ratio during acute HFRS suggests that the neutralizing capacity of sgp130 may be reduced during PUUV infection. This disturbance in the IL-6 buffer system might imply that the likelihood of IL-6 trans-signaling is increased during HFRS. Given the effects of IL-6 trans-signaling in endothelial cells *in vitro*, one can speculate that such a receptor imbalance would lead to increased inflammation and vascular leakage in patients. In support of this view, we observed a positive correlation between sgp130 levels and serum albumin levels. Albumin can be used as a marker of vascular permeability (330,331), thus suggesting that patients with low plasma levels of sgp130 may also experience more vascular leakage. Moreover, we observed a negative correlation between sgp130 levels and the number of interventions given (i.e., intravenous fluid treatment, oxygen treatment, or platelet transfusion), which may suggest that patients with low serum sgp130 levels experienced more severe symptoms. In line with this, patients who received oxygen treatment exhibited a higher sIL-6R/sgp130 ratio compared to patients that did not require such treatment.

4.2.5 Conclusions and future directions on the role of IL-6 in hantavirus pathogenesis

In conclusion, **Paper II** showed that endothelial cells infected with PUUV produced large amounts of IL-6 that in combination with sIL-6R stimulated increased secretion of IL-6 and CCL2, upregulation of ICAM-1, and increased permeability. Further, we demonstrated an imbalance in the concentrations of the IL-6 receptors sIL-6R and sgp130 during HFRS.

In the continuation of this study, the effects of IL-6 trans-signaling on the secretion of a wider range of cytokines should be investigated. Moreover, future studies should aim at exploring whether also other hantaviruses give rise to similar effects. VEGF receptor 2-dependent VE-cadherin downregulation and increased permeability have previously been reported in cells infected with ANDV (332). Also thrombin and bradykinin have been shown to affect VE-cadherin expression of endothelial cells (160,161). Thus, future studies should investigate if the VE-downmodulation observed upon PUUV infection of endothelial cells is dependent on VEGF, thrombin, or any other soluble factor present in the supernatants of infected cells. Shrivastava-Ranjan *et al.* reported a strong decrease in VE-cadherin expression in ANDV-infected cells on total protein level (279). Such a decrease appeared to occur also in PUUV-infected cells, upon treatment with sIL-6R. This raises the question as to whether VE-cadherin is shed from the cell surface into the supernatants of infected endothelial cells. Finally, levels of sIL-6R and sgp130 should be analyzed also in HPS patients, to reveal possible correlations to the disease severity and outcome.

4.3 MAIT CELL RESPONSES IN HANTAVIRUS INFECTION

During HPS and HFRS, CD8 T cells infiltrate tissues such as the lungs and kidneys (38,131,300). Moreover, peripheral blood CD8 T cells are highly activated in both HFRS patients and HPS patients (297,299). Lately, MAIT cells have gained increased attention within the context of viral infections (238,242). As a step to understanding the immune responses leading to inflammation during hantavirus infections, we in **Paper III** investigated the responses of MAIT cells during PUUV infection.

4.3.1 MAIT cell responses in HFRS patients

Through a collaboration with researchers at Umeå University, we had access to PBMCs and plasma from 24 HFRS patients infected with PUUV. Samples were obtained during the acute, intermediate, and convalescent phase, as well as from 19 uninfected controls. Using an 18-color flow cytometry panel, we characterized the phenotype of peripheral blood MAIT cells in patients and controls. As previously reported in infections caused by HIV, HTLV-1, HCV, HDV, influenza virus, and SARS-coronavirus-2 (241,245,249–255), MAIT cell numbers were decreased in blood during acute HFRS. Similar to what has been reported in other acute viral infections (251,253), the drop in MAIT cells appeared to be largely transient. Decreased expression of the mucosal tissue homing markers CCR6 and α4β7 integrin showed that the MAIT cells displayed an altered homing profile. Moreover, patients displayed increased levels of CCL20 and CCL25 in plasma. Together, these findings suggested that the MAIT cells expressing mucosal tissue homing markers may have homed to mucosal sites during the acute disease. However, there is also a possibility that expression of CCR6 and α4β7 integrin was downregulated upon MAIT cell activation. Decreased CCR6 expression has also been observed on MAIT cells in HIV infected individuals (247).

Increased expression of the activation markers CD38, CD69, and granzyme B on MAIT cells remaining in the circulation suggested that MAIT cells of HFRS patients were highly activated (Figure 11). Moreover, increased expression of Ki67 on residual MAIT cells of HFRS patients suggested that the cells were proliferating (Figure 11). MAIT cell activation has previously been reported in viral infections caused by HIV, HTLV-1, HCV, HDV, influenza virus, and SARS-coronavirus-2 (241,245,248,250–255). This suggests that MAIT cells constitute a general component of the human immune response towards viral infection. The role of MAIT cells during viral infections is, however, not well understood. In patients infected with dengue virus or SARS-coronavirus-2, MAIT cell activation was higher in individuals with a more severe disease (241,245,251). In line with this, we observed a correlation between the MAIT cell activation during acute HFRS and levels of IL-6 levels and platelets, both of which have been associated with HFRS severity (140,292). While these relationships may not be causal, they could indicate that patients with more inflammation have higher MAIT cell activation. Supporting this, MAIT cell activation in COVID-19 patients positively correlated with multiple cytokines, including IL-6 (245). Interestingly, higher IFN-γ production was observed in *ex vivo*-stimulated MAIT cells of COVID-19 patients with a fatal compared to non-fatal outcome (245). A similar finding was also reported for patients with severe versus mild COVID-19

(253). In other contexts, for example during influenza virus infection in mice, a protective role for MAIT cells has been suggested (256). Given the capacity of MAIT cells to respond differently depending on the cytokine milieu, it is likely that MAIT cells may serve different functions in different tissues as well as in different virological contexts.

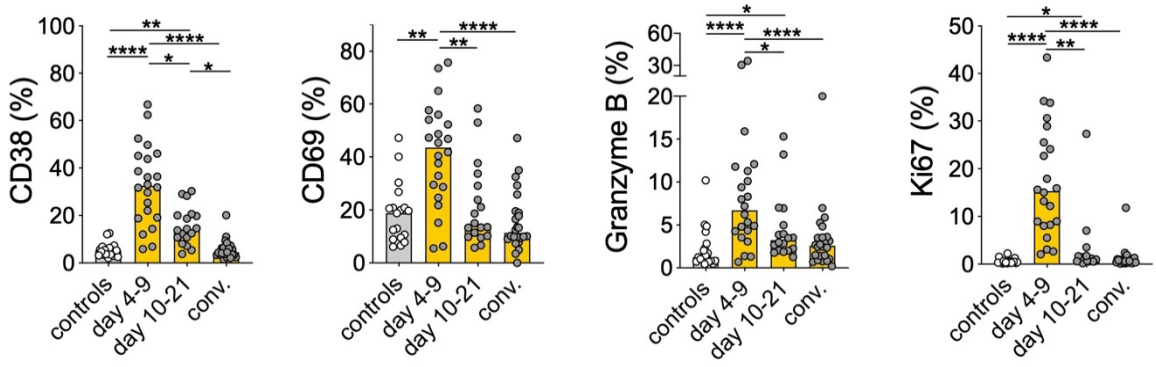

Figure 11. Peripheral blood MAIT cells are activated during acute HFRS. Using multi-color flow cytometry, we observed increased frequencies of MAIT cells expressing CD38, CD69, granzyme B, and Ki67 in HFRS patients (n=24), suggesting strong activation.

4.3.2 PUUV-mediated MAIT cell activation *in vitro*

Having observed strong activation of MAIT cells in HFRS patients, we next explored the mechanisms behind this activation. First, we observed activation of purified MAIT cells upon co-incubation with PUUV-exposed cells of the human acute monocytic leukemia cell line, THP-1. Next, we showed that this activation was dependent on replicating virus, and independent on MR1. This was in line with previous reports showing that virus-driven MAIT cell activation is independent on MR1 and dependent on live viruses (249,256). Our subsequent experiments showed that the activation was dependent on soluble factors and independent on contact between the THP-1 cells and the MAIT cells.

IL-12 and IL-18 have been suggested as key cytokines involved in MAIT cell activation driven by HCV-, dengue virus-, and influenza virus-exposed antigen-presenting cells (242,249,256). Thus, we hypothesized that blocking of these cytokines would inhibit the MAIT cell activation. To our surprise, neither blocking of IL-18 alone or blocking of both IL-18 and IL-12 inhibited the MAIT cell activation stimulated by PUUV-infected antigen-presenting cells. Knowing that Wilgenburg *et al.* had observed a synergistic effect of type I IFNs in HCV-driven MAIT cell activation (256), we next decided to investigate the concentrations of this and other cytokines in the THP-1 supernatants. While no IL-12, IL-15, TNF, or IL-6 was detected in the supernatants, the concentrations of IL-18 and IFN-α were found to be increased in PUUV-exposed cells compared to unexposed cells. Thus, we performed additional blocking experiments targeting IFN-α, using a recombinant B18R, an IFN receptor decoy protein encoded by vaccinia virus. Addition of B18R to the cultures completely abrogated activation of the MAIT cells. Finally, we confirmed these findings using primary monocytes exposed to PUUV and primary endothelial cells infected with PUUV. Supernatants from these cells also activated purified MAIT cells in a type I IFN-dependent manner (Figure 12). This effect was

different to what was shown in the context of HCV-driven MAIT cell activation, where blocking of type I IFNs only had a limited inhibitory effect (256). Recently, type I IFN-dependent MAIT cell activation was also described in MAIT cells stimulated with SARS-coronavirus-2-infected cells (245).

4.3.3 Function of PUUV-activated MAIT cells

Although PUUV-exposed cells caused strong activation of MAIT cells, the function of these MAIT cells remains unclear, as no cytokine expression could be observed. This is in contrast to previous reports showing increased expression of IFN-γ in MAIT cells activated by virus-exposed antigen-presenting cells (241,249), but in line with reports showing no IFN-γ induction in MAIT cells treated with IFN-α (241,244). As IL-18-mediated MAIT cell activation is known to stimulate IFN-γ expression (241,243), it is possible that MAIT cells do express IFN-γ *in vivo*, in hantavirus-infected patients, in which IL-18 levels are increased (293, **Paper I and Paper III**).

PUUV-exposed cells not only caused activation of MAIT cells, but also an increase in the cytotoxic proteins granzyme B and perforin (Figure 12). This was also reflected in the HFRS patients, in which an increased percentage of MAIT cells expressing granzyme B was observed, as well as increased plasma levels of granzyme A and granzyme B. Increased granzyme B expression has also been reported in MAIT cells of patients infected with influenza virus, dengue virus, and HCV, as well as in MAIT cells stimulated with antigen-presenting cells exposed to those viruses (241). In **Paper III**, we showed that MAIT cells stimulated by PUUV-exposed cells *in vitro* showed increased degranulation, as indicated by the increased expression of CD107a. In supernatants of these cells, also increased concentrations of granzyme B and perforin were observed, confirming that the MAIT cells had released their cytotoxic granule content. Together, these findings suggest that PUUV-driven MAIT cell activation leads to increased cytolytic capacity in MAIT cells, concomitant with a release of their granule content. This is in line with studies showing degranulation in MAIT cells stimulated with cytokines or SARS-coronavirus-2-infected cells (243–245). The role of granzymes within the extracellular space is not well understood. However, granzyme A has been suggested to have a pro-inflammatory function by stimulating the secretion of IL-1β, IL-6, and TNF in PBMCs (333). Granzyme B has been shown to cleave extracellular proteins and mediate detachment of endothelial cells *in vitro* (334). In addition, granzyme B has been suggested to cleave VEGF from the endothelial cell matrix, and thereby mediate vascular permeability (335).

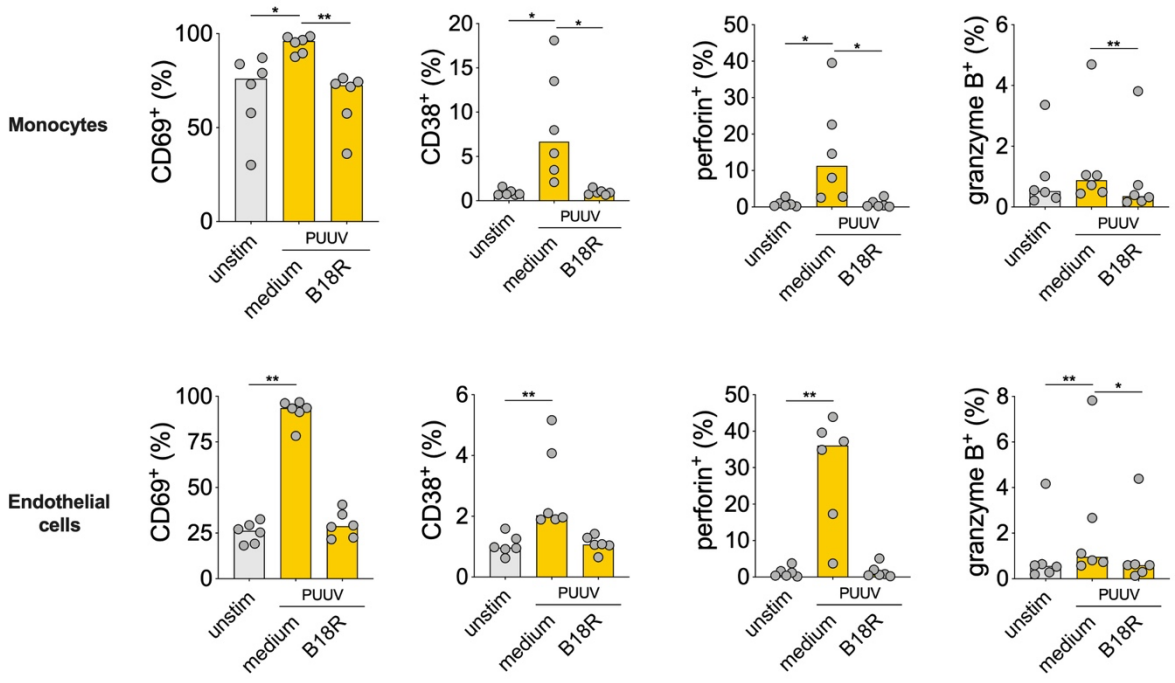

Figure 12. Cells exposed to PUUV activate MAIT cells in a type I IFN-dependent manner. Supernatants from PUUV-exposed primary human monocytes and PUUV-infected primary human endothelial cells both stimulate MAIT cell activation. This activation is inhibited by the interferon inhibitor B18R.

4.3.4 Conclusions and future directions on MAIT cells in hantavirus infection

In **Paper III**, we showed a massive decrease in peripheral blood MAIT cells in patients with HFRS. In residual MAIT cells, we observed strong activation, including increased expression of granzyme B. The MAIT cell activation was correlated with the levels of plasma IL-6 as well as the platelet counts, which are associated with the severity of HFRS (140,292). Future studies of the MAIT cell responses in HPS patients would help clarify if there is indeed an association between the disease severity and the MAIT cell activation during hantavirus infection.

The infection-driven activation of MAIT cells was re-capitulated *in vitro*, by culturing MAIT cells in supernatants from PUUV-exposed monocytes and endothelial cells. In this context, MAIT cell activation was completely dependent on type I IFNs. While activated MAIT cells did not express any of the classical MAIT cell cytokines, they did degranulate, releasing granzyme B and perforin into the supernatants. Future studies should explore the function of extracellular granzymes in the context of hantavirus infection and investigate whether they may have any effects on the integrity of the vasculature. Moreover, it would be interesting to investigate if professional antigen-presenting cells such as dendritic cells or macrophages exposed to PUUV would stimulate the expression of IFN-γ in MAIT cells. In the next step, potential antiviral effects of MAIT cells in the context of hantavirus infection should be investigated. Such studies could help delineate the role of MAIT cells in viral infections.

The majority of MAIT cells express the CCL2-receptor CCR2 and the CCL20-receptor CCR6 (336). CCR2 expression on T cells, including MAIT cells, has been described to mediate transendothelial migration (320,337). Moreover, CCR6 has been shown to be important for MAIT cell arrest along the endothelium, prior to transmigration (320). The transient decline in MAIT cells of HFRS patients, together with the altered homing profile of residual MAIT cells, suggested that MAIT cells might have homed to mucosal sites during acute HFRS. This merit further studies investigating whether the MAIT cell numbers in tissues such as the intestine and lung are altered during hantavirus infection. Such studies in combination with clinical information regarding symptoms in patients may have the potential to indicate on the role of MAIT cells during hantavirus infection.

5 CONCLUDING REMARKS

Despite a growing number of reports describing how the cytokine profile and different immune cell compartments are affected during hantavirus infection, comprehensive studies with clear associations to disease severity parameters are few. Ultimately, the pathogenesis of hantavirus-induced disease remains enigmatic, hampering the development of treatments against HFRS and HPS.

The work included in this thesis is the result of studies with the aim to find immunological factors that may drive disease progression in patients. Below, the key findings of this thesis are summarized point by point:

- HFRS patients and HPS patients display increased levels of a wide range of cytokines and other inflammation markers (**Paper I**, **Paper II**, **Paper III**)
- In HPS patients, serum levels of IL-6 are associated with disease severity and serum levels of I-FABP are associated with a fatal outcome (**Paper I**)
- Serum levels of C5/C5a and BAFF are associated with decreased disease severity in HPS patients (**Paper I**)
- PUUV-infected endothelial cells secrete increased levels of IL-6 compared to uninfected cells, which in combination with sIL-6R lead to augmented pro-inflammatory responses and increased endothelial permeability (**Paper II**)
- HFRS patients with PUUV infection exhibit an altered sIL-6R/sgp130 ratio, indicating that the potential for IL-6 trans-signaling may be increased (**Paper II**)
- HFRS patients infected with PUUV show decreased levels of MAIT cells in peripheral blood (**Paper III**)
- Circulating MAIT cells of HFRS patients are highly activated, proliferate and show an altered homing marker expression (**Paper III**)
- Type I IFNs produced by PUUV-exposed monocytes and endothelial cells activate MAIT cell *in vitro* (**Paper III**)

Lastly, I hope that future hantavirus research will focus on the endothelial cells and the vascular dysfunction described in patients. Studies providing further evidence to clarify the respective roles of the virus itself, as opposed to those of the immune system, will have the potential to provide important leads valuable in the understanding of hantavirus-induced disease. Future studies should systematically, and in a high-throughput manner, investigate how endothelial cells are affected by hantavirus infection *per se* and how soluble factors produced by these cells, immune cells, or tissues may affect endothelial cells. Further, it is my hope that lessons learned from the ongoing COVID-19 pandemic will benefit also the hantavirus field and related fields.

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