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IL-6 trans-signaling causes endothelial cell activation and barrier dysfunction during hantavirus infection

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Abstract

Hantaviruses are of major global health concern, causing over 150.000 infections annually. They cause two distinct acute inflammatory diseases, hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Impaired endothelial barrier function is an important hallmark of both diseases, leading to vascular leakage and formation of oedema in major organs. What causes vascular leakage and determines the disease outcome remains unknown. Recent studies have identified IL-6 as a marker for disease severity and suggested an impact of IL-6 in hantavirus pathogenesis. IL-6 signaling occurs via the membrane-bound IL-6 receptor (IL-6R), known as classical signaling, or via the soluble IL-6R, known as trans-signaling. As IL-6 trans-signaling has been shown to mediate mainly proinflammatory effects, we sought to investigate the role of IL-6 trans-signaling on vascular endothelial cells in hantavirus infection. In the present study, we examined the plasma concentration of sIL-6R in acute and convalescent Puumala virus (PUUV) infection. Further we examined IL-6 and CCL2 secretion, ICAM-1 and VE-cadherin expression and the barrier function upon IL-6 trans-signaling in PUUV infected endothelial cells. For the first time we showed increased levels of sIL-6R in acute HFRS and proved endothelial cell activation and barrier dysfunction upon PUUV infection and IL-6 trans-signaling. IL-6 and CCL2 secretion as well as ICAM-1 expression were increased, and VE-cadherin structures and barrier functions were disrupted. Our findings suggest an impact of IL-6 trans-signaling on vascular leakage and reveal new possible treatment targets for hantavirus infections.

Key words:
Hantavirus, HFRS, IL-6, IL-6 receptor, IL-6 trans-signaling, Puumala virus, vascular leakage

A step closer to combating hantavirus infections

IL-6 – a new possible treatment target for hantavirus infections

Hantavirus infections are a major global health issue, affecting over 150.000 people annually. These viruses, which naturally infect different rodent species worldwide can be transmitted to human by inhalation of aerosols from rodent excreta. Humans come in contact with these microparticles in the air for example when cleaning a summer house after a long winter or camping in the nearby forest. While hantaviruses do not cause any symptoms in rodents, they cause two distinct diseases in human – hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Both diseases are characterized by unspecific symptoms such as headache, fever and gastrointestinal symptoms but sometimes they can proceed to formation of oedema in major organs and can even lead to death. HPS is generally more severe and leads to death more frequently than HFRS. However, HPS is much less common than HFRS. To date, no treatment or vaccine is available for hantavirus infections.

What determines whether a person develops mild or severe symptoms is unknown. But we know, that an important complication in hantavirus infection is the formation of small holes in the walls of blood vessels, which allow fluid and proteins to leak out from the blood stream into the tissue, leading to formation of oedema. This is called vascular leakage and is seen in many diseases, but which mechanisms create the holes is still unknown. In our study we aimed to identify how these holes in the blood vessels are created in hantavirus infection. Recent studies described that the patients infected with hantavirus have high concentrations of interleukin-6 in blood plasma. Interleukin-6 is an important molecule of the immune system. It can bind to its soluble interleukin-6 receptor in blood plasma and then bind to vascular cells which build the inner wall of blood vessels. Then interleukin-6 creates a signal in these cells and changes the cell structure. We suspected that this effect plays a role in hantavirus infection and creates the holes in the blood vessels.

To investigate this, we measured the blood plasma concentration of the receptor and found that it is increased in acute hantavirus infection. Next, we investigated in more detail how hantavirus infected vascular cells change when they receive the interleukin-6 signal. For that we infected vascular cells with hantavirus, they produce interleukin-6 upon infection and then we added the receptor to allow the signaling. We measured inflammatory markers and all of those markers were increased. Additionally, we stained the cells with immunofluorescence to visualize the cell-cell connections. Under the microscope we saw, that hantavirus infected vascular cells that receive the interleukin-6 signal lose their connection to each other. With this and further experiments we proved, that interleukin-6 affects hantavirus infected vascular cells. Thus we concluded, that interleukin-6 may be involved in the mechanism that creates holes in blood vessels and suggest interleukin-6 and its signal as a new treatment target for hantavirus infections.

Abbreviations

(s)gp130	(soluble) glycoprotein 130
(s)IL-6R	(soluble) interleukin-6 receptor
ADAM	A desintegrin and metalloprotease
ANDV	Andes virus
CRP	C-reactive protein
DOBV	Dobrava virus
eNOS	Endothelial nitric oxide synthase
HFRS	Hemorrhagic fever with renal syndrome
HIV	Human immunodeficiency virus
HPS	Hantavirus pulmonary syndrome
HTNV	Hantaan virus
HUVEC	Human umbilical vein endothelial cells
IAV	Influenza A virus
ICAM-1	Intercellular adhesion molecule 1
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-6/IL-6R complex	Interleukin-6/Interleukin-6 receptor complex
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MOI	Multiplicity of infection
NE	Nephropathia epidemica
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
PP2A	Protein phosphatase 2A
PUUV	Puumala virus
rIL-6	Recombinant interleukin-6
TNF	Tumor necrosis factor
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor

Introduction

Hantaviruses are prevalent worldwide and have influenced human health for over a century.[1,2] Hantaviruses are enveloped, single stranded, negative-sense RNA viruses. The family of Hantaviridae, belonging to the order of *Bunyavirales*, comprise 24 hantavirus species, which form three clusters. While the first cluster includes ancestral hantavirus species with unknown pathogenicity, the second and third cluster distinguish old world hantaviruses from new world hantaviruses. Rodents, shrews, moles and bats can serve as natural hosts, which carry hantaviruses asymptomatically. The geographic distribution of different hantaviruses is determined by the distribution of each strains specific host. Transmission of hantaviruses to humans occurs via inhalation of aerosols from rodent excreta. Thus, risk factors for hantavirus infections are any activities that bring humans and natural hosts in close proximity, such as forestry work, farming and camping.[3]

In humans, hantaviruses cause two distinct hyperinflammatory diseases with over 150.000 cases annually.[4] Hemorrhagic fever with renal syndrome (HFRS) is caused by old world hantaviruses, which include Hantaan virus (HTNV) in Asia, Puumala virus (PUUV) and Dobrava virus (DOBV) in Europe, and Seoul virus distributed worldwide. New world hantaviruses, including Sin Nombre virus in North America and Andes virus (ANDV) in South America, cause hantavirus pulmonary syndrome (HPS).[5] ANDV is the only hantavirus species for which person-to-person transmission has been reported.[6] Both HFRS and HPS are acute, systemic infections.[5]

Common symptoms of HFRS are fever, headache, abdominal pain, backache, nausea and vomiting. Renal involvement and hemorrhagic complications are indicated by proteinuria, haematuria and kidney dysfunction.[3,5] The case fatality rate of HFRS ranges from 0.08-0.4% for PUUV to up to 10% for HTNV and DOBV.[2,3]

PUUV is the most common hantavirus species in Europe, causing between 1000 and 4000 reported cases annually, which mostly occur in Finland and Sweden.[7] PUUV causes a relatively mild form of HFRS, also called nephropathia epidemica (NE). Due to its relatively mild disease progression it is assumed that many NE cases remain undetected.[2] This is supported by a study that detected a comparatively high PUUV seroprevalence of 13% in northern Sweden.[8] While most PUUV infections are mild, some patients experience severe symptoms, proceeding to hypotension, renal failure, hemorrhagic manifestations and death.[2] Interestingly, one study detected mild mucosal bleeding in the gastrointestinal tract in all NE patients.[9]

HPS is less prevalent than HFRS, but more severe, and has a case fatality rate of up to 40%.[4,10] It is characterized by rapidly progressing pulmonary dysfunction due to pulmonary oedema and cardiogenic shock. Myalgia, cough and diarrhea are symptoms distinguishing HPS from HFRS.[3] No approved treatment or vaccine is currently available for hantavirus infections.[11]

In both HFRS and HPS, increased permeability of capillary endothelia, also known as vascular leakage, is the most important pathophysiological hallmark.[12,13] In general, the barrier function of vascular endothelial monolayers is most importantly maintained by vascular endothelial-cadherin (VE-cadherin) binding to catenins of the adjacent cell, stabilizing intercellular junctions. VE-cadherin-catenin complexes can be targeted by many substances including vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), platelet activation factor, thrombin and histamine which induce phosphorylation of VE-cadherin to increase vascular permeability. Furthermore, tight junction proteins, including

junction adhesion molecule, occludin, claudin, zona occludens and cingulin, are involved in barrier functions of endothelial cells. While a regulated increase in vascular permeability is necessary for several physiological processes like embryonic vasculogenesis, menstrual cycle and wound-healing, pathological disruption of endothelial barrier function leads to extravasation of fluid and protein, resulting in hypotension and oedema in major organs.[14] Therefore, vascular leakage is associated with many diseases including diabetes[15], cardiovascular diseases and cancer[16] and viral hemorrhagic fevers[17] as among others caused by hantaviruses.[18]

The mechanisms behind hantavirus pathogenesis are poorly understood, but likely depend both on the virus itself as well as the immune response.[3] While virus replication occurs mainly in vascular endothelial cells it is associated with only little, if any, cytopathic effect.[19] However, previous studies have indicated a virus driven increase of vascular permeability by VEGF-induced downregulation of VE-cadherin in ANDV infected endothelial cells.[20] Furthermore, hantaviruses can infect tubular epithelial cells, glomerular endothelial cells and podocytes of human kidneys supporting renal manifestations of HFRS.[21] A common characteristic of hantavirus infection is thrombocytopenia, which is associated with the degree of inflammation and other variables reflecting vascular leakage.[22] Hantavirus infection is associated with an excessive immune activation and heavy cytokine responses.[23–25] High levels of proinflammatory cytokines such as TNF, interleukin-1 (IL-1) and interleukin-6 (IL-6) have been linked to common symptoms including fever, shock and induced acute-phase proteins.[26] Monocytes and dendritic cells have been shown to infiltrate into lung tissue upon hantavirus infection with a parallel depletion in peripheral blood.[27] Natural killer cells have been shown to expand upon hantavirus infection and numbers remain high long after initial infection.[28] Complement activation is common in hantavirus infections[29] and is associated with hantavirus pathogenesis and disease severity.[30]

Despite growing understanding of hantavirus pathogenesis, factors determining the disease outcome for HRFS and HPS remain poorly understood. Nevertheless, a few studies have identified serum markers associated with severity and outcome of hantavirus infections. IL-6 has been identified as a significant marker for severity of HPS[25] and HFRS[31–34].

The role of IL-6 in diverse viral infections and its exacerbating influence on disease outcome gained awareness over the last years.[35] IL-6 is a proinflammatory cytokine with a vast number of effects on immunological and physiological processes. It is mainly produced by monocytes and macrophages, but also T cells, B cells, hepatocytes, endothelial cells, fibroblasts, keratinocytes, mesanglial cells and adipocytes.[36] Endothelial cells have been shown to release IL-6 upon diverse stimuli, including lipopolysaccharide, IL-1 and TNFα.[37] IL-6 is involved in the generation of acute phase proteins such as C-reactive protein (CRP), drives antigen-specific immune responses and inflammation, and influences hematopoiesis and apoptosis.[36,38] Besides its activating effects, IL-6 also regulates metabolism, maintains bone homeostasis and influences neural functions in an anti-inflammatory manner.[38]

IL-6 signaling is mediated by a receptor complex consistent of the membrane bound IL-6 receptor (IL-6R) and its co-receptor glycoprotein 130 (gp130). IL-6 first forms a complex with IL-6R which then binds to two molecules of gp130, leading to downstream signal transduction by activating JAK/STAT, ERK and PI3K signaling pathways. This signaling, known as classical signaling, is limited to cells expressing the transmembrane IL-6R subunit, i.e. T cells, B cells, myeloid cells and hepatocytes.[38,39] However, IL-6R can be shed from cells via proteolytic cleavage mediated by a desintegrin and metalloproteinase (ADAM) 10 and ADAM17, giving rise to soluble IL-6R (sIL-6R).[40] sIL-6R can also be produced by

alternative splicing.[41,42] sIL-6R binds IL-6 and is able to mediate signaling in all cells, as the co-receptor gp130 is ubiquitously expressed.[38,43,44] This signaling, known as trans-signaling, illuminates the pleiotropic effect of IL-6. However, IL-6 trans-signaling has been shown to exert mainly proinflammatory effects.[44]

Endothelial cells have been reported to lack IL-6R[45], but previous studies have highlighted the impact of IL-6 on endothelial cells via trans-signaling. Several studies have shown that IL-6 trans-signaling upregulates intercellular adhesion molecule 1 (ICAM-1) on the cell surface of endothelial cells.[46–49] ICAM-1 is constitutively expressed at low levels on vascular endothelial cells as well as lymphocytes and monocytes. Besides other functions, ICAM-1 participates in leukocyte adhesion and subsequent transendothelial migration into tissue, highlighting its importance in inflammation.[50] Another main player in inflammation is the chemokine CCL2, also referred to as monocyte chemoatractant protein-1. CCL2 regulates the migration and infiltration of monocytes, T cells and NK cells and is produced by many cell types including, monocytes, macrophages, endothelial cells, fibroblasts and epithelial cells. The secretion of CCL2 is induced by oxidative stress, growth factors or proinflammatory cytokines.[51] Following this, IL-6 trans-signaling has been shown to induce CCL2 secretion by endothelial cells.[48,45] Further studies have illustrated an impact of IL-6 trans-signaling on VE-cadherin followed by impaired endothelial cell barrier function. VE-cadherin is downregulated and internalized upon phosphorylation, resulting in increased permeability of endothelial monolayers.[47,52–55] Additionally, IL-6 trans-signaling has been shown to induce IL-6 secretion in epithelial cells.[56]

Interestingly, also gp130 is present as a soluble form in the blood, acting as natural inhibitor of IL-6 trans-signaling[57] and creating a buffer system for IL-6 signaling.[38,44,58] While free IL-6 is only present at very low concentrations, between 1 and 14 pg/ml in serum of healthy individuals[59], levels can increase to up to 100 ng/ml during disease conditions.[60] By contrast, sIL-6R and sgp130 are continuously present in high concentrations of 25 to 75 ng/ml[61–64] and 150 to 400 ng/ml[62,64], respectively. Several studies have reported increased plasma/serum levels of sIL-6R in different diseases, including asthma[65], multiple sclerosis[64], human T-lymphotropic virus 1 associated myelopathy[66] and human immunodeficiency virus (HIV) infections[67]. This reflects the complex nature of the IL-6/sIL-6R/sgp130 buffer system and the possible role of IL-6 trans-signaling in different pathophysiological processes.

Despite increasing understanding of hantavirus pathogenesis, the importance of vascular leakage in disease progression, the increased levels of IL-6 in hantavirus infection and the impact of IL-6 on endothelial cells and their barrier function via trans-signaling, no connection has been made about the role of IL-6 trans-signaling in hantavirus pathogenesis. Thus, this study aims to identify the importance of IL-6 trans-signaling in hantavirus infection. First, to study the physiological relevance of IL-6 trans-signaling in hantavirus infection, levels of IL-6, sIL-6R, IL-6/sIL-6R complex and sgp130 were examined in plasma of PUUV infected patients. Further, the impact of IL-6 on hantavirus infected endothelial cells was investigated in vitro. For that, primary human umbilical vein endothelial cells (HUVEC) were infected with PUUV and treated with recombinant sIL-6R. Additionally, peripheral blood mononuclear cells (PBMCs) were stimulated with PUUV to study their impact on IL-6 levels and trans-signaling in PUUV infection. The identification of IL-6 trans-signaling as a possible key player in hantavirus pathogenesis, may be useful in the establishment of new life-saving treatment strategies for hantavirus infections.

Aims

- To examine the plasma levels of IL-6, sIL-6R, IL-6/sIL-6R complex and sgp130 in PUUV infected patients
- To investigate the effects of IL-6 on PUUV infected endothelial cells treated with sIL-6R
- To investigate the role of PBMCs in IL-6 trans-signaling, upon PUUV exposure

Materials and Methods

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were maintained in endothelial growth medium (EGM-2) supplemented with growth factors according to manufacturer's instructions (Lonza), in 5% CO_2 at 37°C. For infection experiments, hydrocortisone was excluded from medium. Stem Pro Accutase cell dissociation reagent (Thermo Fisher Scientific) was used to detach the cells prior to flow cytometric analyses. HUVECs were used for experiments until a maximum passage number of 6.

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Ficoll-Hypaque) from buffy coats from healthy blood donors. Buffy coats were obtained from the Blood Transfusion Clinic at the Karolinska University Hospital Huddinge, Stockholm, Sweden. PBMCs were cultured in complete RPMI 1640 medium supplemented with 10 % FCS and L-glutamine in 5% CO_2 at 37°C.

Patient samples

Plasma samples of PUUV infected patients in acute and convalescent phase were kindly provided by Clas Ahlm, Umeå University. The study was approved by the Regional Ethics Committee of Umeå University (application number 04-133M).

Infection and treatment of cells

HUVECs (passage 4 or 5) were seeded in appropriate cell density (24-well plate $1x10^5$ cells/ml in 1 ml medium; 6-well plate $1.6x10^5$ cells/ml in 3 ml medium) over night and infected with PUUV (strain CG1820) the next morning. Infection was performed by adding virus (MOI=1) in medium (200 µl per 24-well, 1 ml per 6-well) to each well and incubate for 1 h in 5% CO_2 at 37°C with gentle shaking every 10 min. After infection, cells were provided with fresh medium. 48 h post infection cells were washed with PBS and treated with different concentrations (0, 31.25, 62.5, 125, 250 and 500 ng/ml) of recombinant human IL-6R alpha protein (sIL-6R) (R&D systems, 227-SR-025) in fresh medium for 24 h. Additionally, some cells were treated with 10 ng/ml recombinant IL-6 (rIL-6) (R&D systems, 206-IL) simultaneously to sIL-6R treatment for 24 h. Then, cells or supernatants were used for further analysis.

PBMCs were cultured in U-bottomed 96-well plates, 1 million cells in 200 µl medium per well. Cells were exposed to PUUV (MOI=10) for 2 h and then maintained in fresh (virus free) medium. Supernatants were collected after 8, 24, 48 and 72 h by centrifugation (500g, 5 min). Supernatants were stored at -80°C until analysis.

ELISA

Cell culture supernatants were diluted in HUVEC medium prior to analysis. Patient plasma samples were diluted in Ready-to-use ELISA diluent (Mabtech, 3652-D2). Levels of IL-6, CCL2, sIL-6R, IL-6/sIL-6R complex and sgp130 in cell culture supernatants and/or plasma samples were determined using commercially available duoset ELISA kits. Kits and the sample dilutions used for different assays are summarized in Table 1. Procedures followed manufacturer's protocols with minor adjustments. In brief, 50 µl antibodies, samples, standards, TMB substrate (Abcam) and stop solution (Abcam) were used. Blocking was performed using 100 µl 1% BSA in PBS. Incubation times were adjusted to over night at 4°C for capture antibody, 1 h blocking, 2 h sample/standard, 1 h detection antibody and 30 min streptavidin conjugated to horseradish-peroxidase. Optical density was measured at 450 nm (with substraction correction at 620 nm) with xMark Microplate Spectrophotometer (Bio-Rad).

Table 1. ELISA kits summary

substance of interest	ELISA kit	standard conc. range pg/ml	dilution factor for supernatants		dilution factor for patient sample
			uninfected *	PUUV infected*†	
IL-6	Mabtech, Human IL-6 ELISA development kit, 3460-1H-6	7-5000	1[a,b]	5[a,b]	2
sIL-6R	R&D systems, DuoSet ELISA development system, Human IL-6 Rα, DY227	7-5000	1[b]	1[b]	400
IL-6/sIL-6R complex	R&D systems, DuoSet ELISA development system, Human IL-6/IL-6 Rα Complex, DY8139-05	34-25000	--	--	2
sgp130	R&D systems, DuoSet ELISA development system, Human gp130, DY228	156-10000	--	--	400
CCL2	R&D systems, DuoSet ELISA development system, Human CCL2/MCP-1, DY279	7-5000	5[a]	5[a]	--

*or unexposed/exposed supernatants, †or rIL-6 treated cells, [a]HUVEC supernatants, [b]PBMC supernatants

qPCR IL-6 pathway array

HUVECs were cultured in 6-well plates and infected and treated with sIL-6R as described above. Cells were harvested and lysed using 900 µl Tri-pure isolation reagent (Roche) and stored at -80°C until analysis. RNA was extracted from cell lysates by chloroform-isopropanol extraction as described in the manufacturer's protocol.[68] RNA was reverse transcribed to cDNA using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's protocol. Finally, cDNA was used to determine the mRNA abundance of selected genes using the commercially available TaqMan Gene Expression Assay – TaqMan Array plates (Applied Biosystems, 6391016), according to manufacturers protocol. In brief, 10 ng of cDNA was mixed with master mix and 10 µl of reaction mix was applied to each well of the 96 well plate. qPCR was performed with a

QuantStudio 5 System. CT values were normalized towards the internal control gene "GUSB" and fold-change values were calculated as described elsewhere.[69]

Flow cytometry

HUVECs were cultured in 24-well plates and infected and treated with sIL-6R as described above. Cells were then harvested and transferred to a V-bottomed 96-well plate. Plate was centrifuged 3 min at 1700 rpm and supernatant discarded. Cells were stained with 50 µl antibody cocktail containing anti-ICAM-1 antibody (color: PE-Vio770, dilution factor 10) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) diluted in FACS buffer (PBS with 4% EDTA, 20% FCS) for 20 min at RT. After washing, cells were fixed with fix/perm solution (BD biosciences, 562725) for 30 min at RT. Cells were then washed prior to flow cytometric analysis using a BD LSR Fortessa instrument (BD Biosciences), FlowJo version 10.4 was used for data analysis.

Immunofluorescence assay

HUVECs were cultured on glass cover slips in 24-well plates and infected and treated as described above. 24 h post treatment, cells were fixed with 1 ml pre-warmed 4% paraformaldehyde for 15 min at RT. Cells were permeabilized using 0.5% Triton-X in PBS for 5 min at RT, washed three times with PBS and then blocked with 0.5% BSA in PBS for 30 min at RT. Cells are stained with antibodies against VE-cadherin (Cell Signaling Technology, VE-cadherin XP (R), Rabbit mAb, D87F2) and PUUV protein (polyclonal antibodies isolated from a convalescent PUUV infected patient) 1:400 diluted in 0.5% BSA in PBS. 25 µl of antibody mix per cover slip was incubated for 1 h at RT. Cells were washed three times 5 min with 0.5% BSA in PBS. Secondary antibodies (goat anti-human, color: AF 647 and goat anti-rabbit, color: AF 488) and DAPI were diluted 1:1000 in 0.5% BSA in PBS. 25µl per cover slip was incubated in the dark for 1 h at RT. Washing was repeated and cover slips mounted onto glass slides. Cells were examined by immunofluorescence confocal microscopy at X60 magnification. Images were analyzed using imageJ.

Transendothelial electrical resistance (TEER)

HUVECs, uninfected or infected with PUUV for 24 h, were cultured in Corning HTS transwell 24-well plates (Corning, CLS3396) with $1x10^5$ cells in 100 µl medium per transwell. 600µl medium was added to the lower compartment. 24 h post seeding, cells were treated with sIL-6R as described above. At 24 h post sIL-6R treatment, the transendothelial electrical resistance (TEER) was measured using an EVOM2 epithelial voltohmmeter (World precision instruments) according to the instrument manufacturer's protocol. In brief, the electrode was held still into wells for 10 to 30 s until measurement was steady. Between wells, electrode was washed with ethanol, distilled water and medium. Blank value was substracted from each measurement.

Statistical analysis

Statistical analyses were performed using Excel and Graph Pad prism version 7. Standard curve interpolations were used to determine concentrations from optical density values obtained by ELISA. Two-way ANOVA test was used to compare two groups (infected to uninfected) at different treatment concentrations. Wilcoxon test was used to compare two groups of patient samples (acute phase and convalescence). Spearman's rank correlation coefficient was used to examine associations between serum markers and clinical parameters.

Results

Plasma levels of IL-6 and sIL-6R are elevated in acute HFRS

IL-6 is increased in acute HFRS[31–33] and HPS[25]. IL-6 signaling occurs via the membrane-bound IL-6 receptor (IL-6R) or its soluble form (sIL-6R).[36] Several inflammatory diseases are characterized by increased levels of sIL-6R in serum and plasma[65,64,66,67], indicating the role of IL-6 trans-signaling in pathophysiological processes. To date, plasma levels of sIL-6R have not been examined in hantavirus infections. Same applies for sgp130, which serves as a natural inhibitor of IL-6 trans-signaling.[57] To investigate the role of IL-6 trans-signaling in hantavirus infection, plasma samples from 28 PUUV infected patients were kindly provided by Clas Ahlm, Umeå University. Samples were collected during acute (day 2-7 post symptom debut) and convalescent phase (day 42-494 post symptom debut). Patient characteristics are summarized in Table 1. The cohort consisted of 13 women and 15 men with a mean age of 49 years suffering from mild to moderate HFRS. Clinical data including lymphocyte count, platelet count and creatinine were available for all, CRP for most patients. In general, CRP and creatinine were increased during acute HFRS, while platelet count was reduced. Some patients required medical interventions including application of intravenous fluid, platelet transfusion and oxygen treatment. Four patients suffered from thrombosis whereof one also had severe bleeding. In total, four patients suffered from severe bleeding. (Table 2)

Table 2. PUUV infected (HFRS) patient characteristics

Characteristic	Acute phase	Convalescence
No. of patients	28	
Sex, female/male, No.	13/15	
Age, years, mean ± SD	49 ± 18	
Days post symptoms debut, median (range)	5 (2-7)	63 (42-494)
CRP, mg/L, mean ± SD[a]	74.8 ± 46.5[b]	4 ± 3[c]
Lymphocyte count x 10^9/L, mean ± SD[d]	8.9 ± 5.27	7 ± 2
Platelet count x 10^9/L, mean ± SD[e]	90.96 ± 48.86	276 ± 46
Creatinine, µmol/L, mean ± SD[f]	203.75 ± 149.83	87 ± 41
Intravenous fluid, no. of patients	19	
Platelet transfusion, no. of patients	3	
Oxygen treatment, no. of patients	8	
Severe bleeding, no. of patients	4	
Thrombosis, no. of patients	4	

SD, standard deviation
[a]CRP, C-reative protein: normal range, 1-10 mg/L
[b]n=26
[c]n=25
[d]Lymphocyte count: normal range, 4.5-10.5 x 10^9/L
ePlatelet count: normal range, 150-400 x 10^9/L
[f]Creatinine: normal range, 50-110 µmol/L

Levels of IL-6, sIL-6R, IL-6/sIL-6R complex and sgp130 were measured in plasma of PUUV infected patients during acute and convalescent HFRS, using ELISA. In line with previous reports, IL-6 was significantly increased during acute HFRS with values ranging from 3 to 217 pg/ml with a median of 8.5 pg/ml (Figure 1a). In 11/28 patients, no plasma IL-6 was detected. In the convalescent phase, most patients had undetectable levels of IL-6 in plasma. sIL-6R plasma levels were significantly increased during acute HFRS with values ranging between 20 ng/ml and 71 ng/ml with a median of 39.5 ng/ml (Figure 1b). Only one patient had undetectable levels of sIL-6R. Looking at the individual change of sIL-6R in plasma in

acute compared to convalescent phase, 26/28 patients had increased sIL-6R in acute HFRS (Figure 1e). The increase ranged from 1% to 130% with a median of 37% and was not associated with age (Figure 1e), IL-6 levels, platelet count or CRP levels (data not shown). Levels of IL-6/sIL-6R complex showed no significant difference between acute and convalescent phase (Figure 1c). Levels ranged from 10 pg/ml to 381 pg/ml, but several patients had undetectable concentrations of the complex. sgp130 was detected in high concentrations ranging from 78 ng/ml up to 315 ng/ml with a median of approximately 170 ng/ml in acute as well as convalescent phase (Figure 1d). On an individual level, sgp130 was decreased by 2-26% in acute phase compared to convalescence in 17/27 patients (63%). 9/27 patients (33%) showed an increase in sgp130 by 9-59% in acute HFRS. Only one patient had no change in sgp130 (Figure 1e). Changes in sIL-6R positively correlated with changes in sgp130 (Figure 1e).

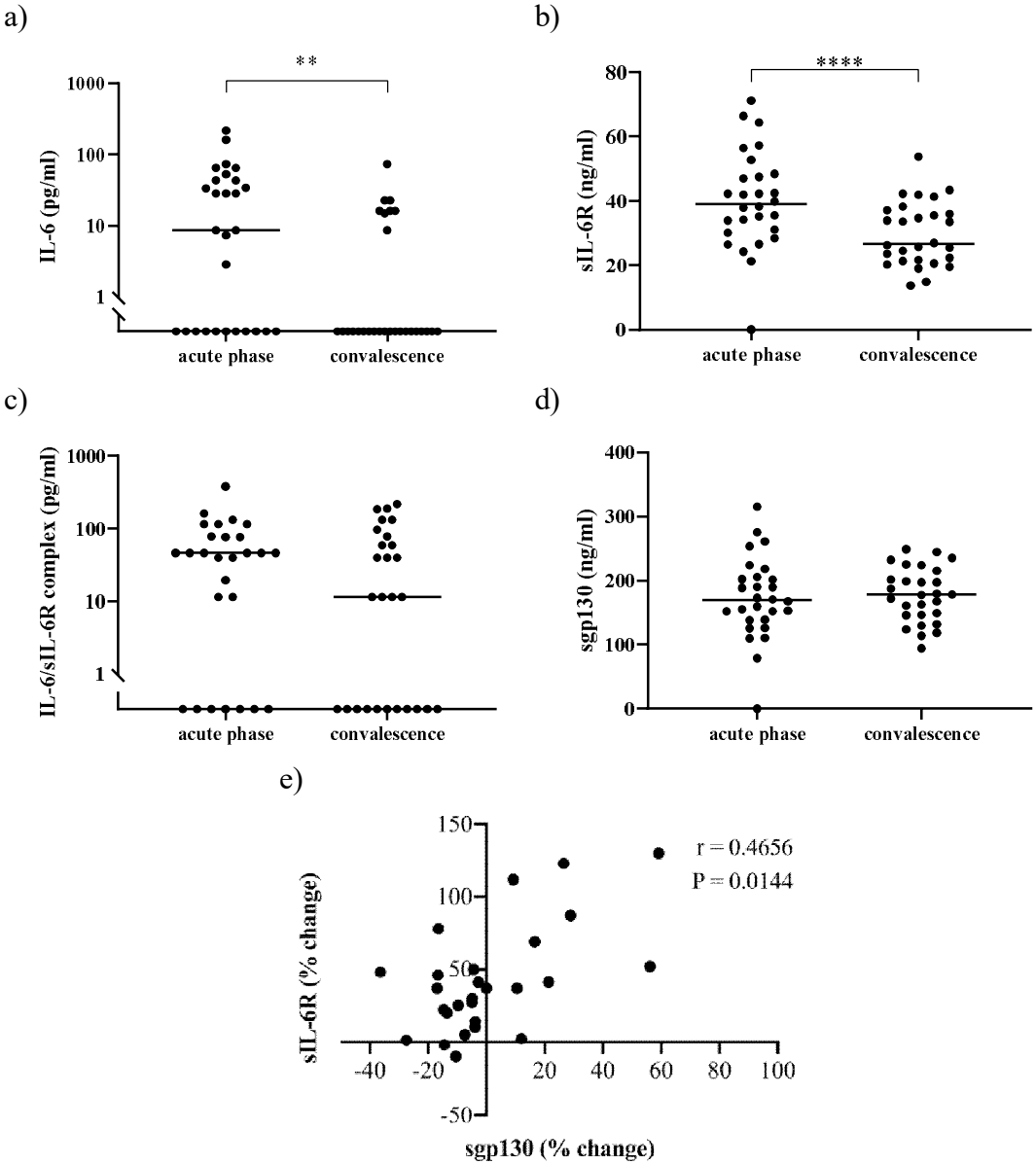


Figure 1. Increased plasma levels of IL-6 and sIL-6R during acute HFRS. Plasma levels of a) IL-6, b) sIL-6R, c) IL-6/sIL-6R complex and d) sgp130 in patients with acute and convalescent HFRS (n=28). Wilcoxon test, median. **P<0.001; ****P<0.0001. e) % change of plasma level of sIL-6R and sgp130 (n=27). Spearman's rank correlation. r = correlation coefficient

HUVECs and PBMCs secrete IL-6 upon PUUV infection

Monocytes, macrophages and other lymphocytes are known to be a major source of IL-6.[36] To investigate the contribution of PBMCs to high IL-6 levels in hantavirus infected patients, PBMCs isolated from healthy blood donors were exposed to PUUV for 8, 24 and 48 h. IL-6 levels were then detected in cell culture supernatants by ELISA. IL-6 levels in supernatants of PBMCs exposed to PUUV were increased compared to levels in unexposed controls. The highest IL-6 secretion was detected 48 h post PUUV exposure (Figure 2a).

Endothelial cells are the main targets of hantavirus replication[19] and are known to release IL-6 upon diverse stimuli.[37] To investigate whether endothelial cells secrete IL-6 upon hantavirus infection and thus may contribute to the high IL-6 levels in hantavirus infected patients, HUVECs were infected with PUUV for 8, 24, 48 and 72 h. IL-6 concentrations in cell culture supernatants were determined by ELISA. IL-6 secretion by HUVECs was increased upon PUUV infection, with a clear peak at 48 h post infection, subsiding after 72 h (Figure 2b). IL-6 concentrations in HUVEC supernatants were higher than in PBMC supernatants. (Figure 2)

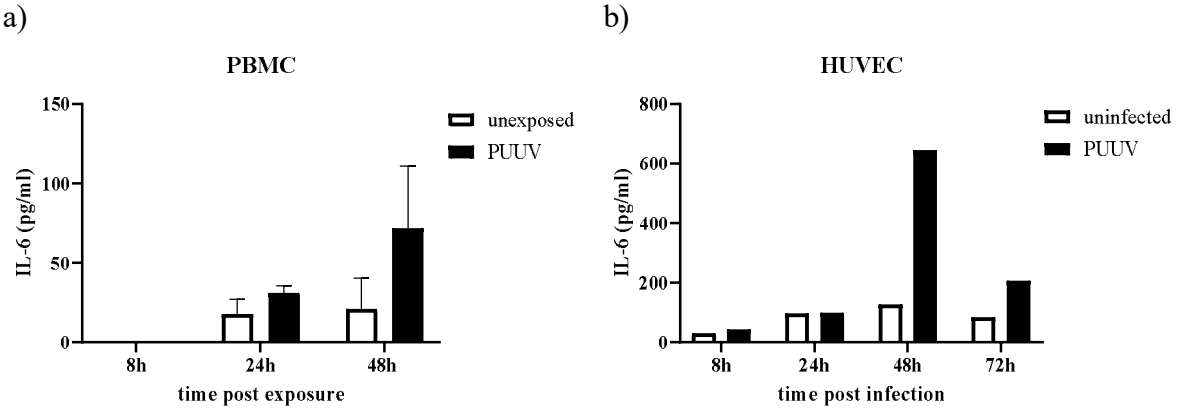


Figure 2. IL-6 secretion by PBMCs and HUVECs is increased upon PUUV infection. IL-6 levels in supernatants of a) PUUV exposed (black) and unexposed (white) PBMCs (n=4) at 8, 24, 48 h post virus exposure and b) PUUV infected (black) and uninfected (white) HUVECs 8, 24, 48, 72 h post infection. Data shows mean with standard deviation from two independent experiments.

No increased IL-6R shedding by PBMCs upon PUUV exposure

IL-6R is expressed on the surface of only a few cell types, including T cells, B cells, monocytes, macrophages, neutrophils and hepatocytes.[38,39] As, IL-6R can be shed by proteolytic cleavage or alternative splicing[40–42], PBMCs are a possible source for sIL-6R in peripheral blood. To investigate if PBMCs may shed sIL-6R upon hantavirus infection, PBMCs were exposed to PUUV *ex vivo*. The concentration of sIL-6R in the cell culture supernatant was then analyzed after 8, 24, 48 and 72 h. PUUV exposure did not increase shedding of IL-6R by PBMCs compared to unexposed controls. (Figure 3)

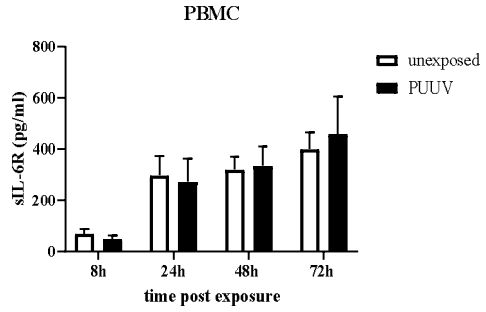


Figure 3. Shedding of sIL-6R by PBMCs is not increased upon PUUV exposure. PBMCs (n=6) were exposed to PUUV for 2 h or left unexposed. sIL-6R levels in supernatants of PBMCs unexposed (white) or exposed to PUUV (black) 8, 24, 48 and 72 h post exposure. Data shows mean with standard deviation for three independent experiments.

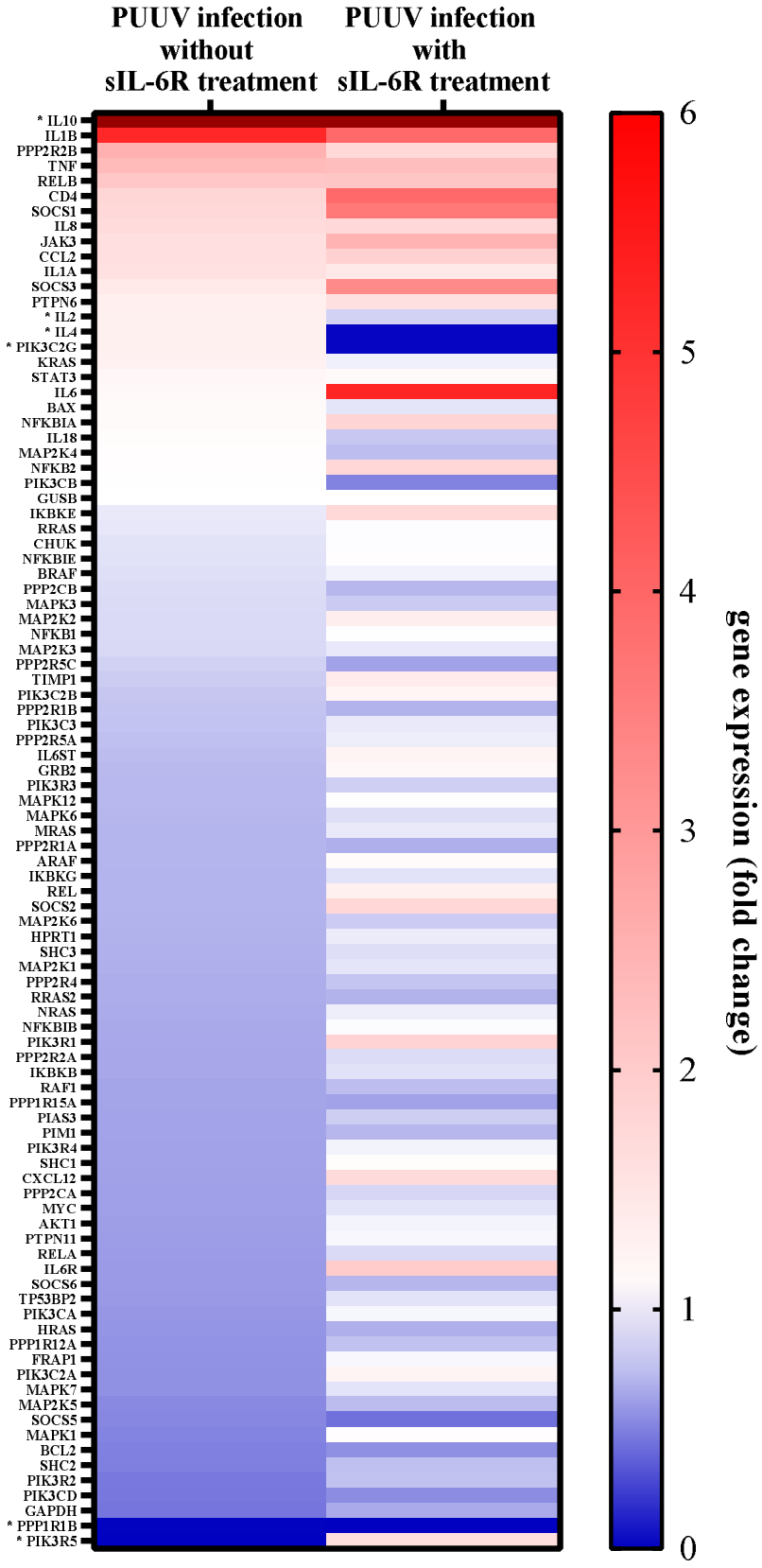


Figure 4. Expression of genes downstream of IL-6 is changed upon PUUV infection and sIL-6R treatment. HUVECs were infected with PUUV for 48 h and treated with 250 ng/ml recombinant sIL-6R for 24 h. Heatmap displays the fold change gene expression of 95 selected genes downstream of IL-6 signaling in infected compared to uninfected cells without (first column) and with (second column) sIL-6R treatment. Colors depict increased (red) and decreased (blue) gene expression. *, at least one sample under detection level/ not expressed, but was given CT of 45 to enable calculations.

IL-6 trans-signaling alters gene expression in PUUV infected HUVEC

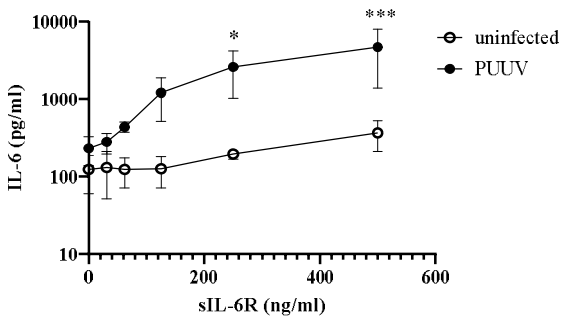
Although endothelial cells have been reported to lack IL-6R expression, IL-6 can affect endothelial cells via trans-signaling[45]. Whether IL-6 produced by HUVECs upon PUUV infection affects the cells via trans-signaling is unknown. Thus, we aimed to investigate which genes downstream of IL-6 are induced or downregulated upon IL-6 trans-signaling in PUUV infected endothelial cell. PUUV infected and uninfected HUVECs were treated with 250 ng/ml sIL-6R or left untreated, before mRNA abundance of 95 genes were determined by qPCR using a commercially available IL-6 pathway array. Gene expressions of cytokines, including IL-6 and IL-10 were upregulated upon infection and further induced upon sIL-6R treatment (Figure 3). A similar induction was seen for *CCL2* and *Jak3*. *IL1B* is highly induced upon infection without sIL-6R treatment. The transcription activation factor *RELB* and *PPP2R2B* were upregulated upon infection and suppressor of cytokine signaling 1 and 3 (*SOCS1* and *SOCS3*) were highly induced by IL-6 trans-signaling. Many genes were slightly downregulated upon infection but induced upon sIL-6R treatment including *SOCS2, CXCL12, IL6R, IL6ST* (gp130) and others. *PPP2CA* was downregulated upon infection. (Figure 4).

IL-6 trans-signaling induces IL-6, CCL2 and ICAM-1 in PUUV infected HUVEC

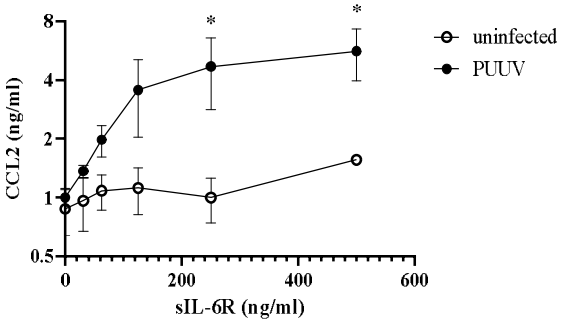
To confirm activation of PUUV infected endothelial cells upon IL-6 trans-signaling on the protein level, HUVECs were infected with PUUV for 48 h and then treated with different concentrations of recombinant sIL-6R for 24 h. Levels of IL-6 and CCL2 were determined in cell culture supernatants by ELISA and the surface expression of ICAM-1 was examined by flow cytometry. IL-6 secretion was induced upon PUUV infection compared to uninfected controls (Figure 5a). IL-6 secretion was clearly enhanced after addition of increased concentrations of sIL-6R in PUUV infected HUVECs. Notably, this did not apply for uninfected controls. Similar patterns were seen for the secretion of CCL2 and the surface expression of ICAM-1 (Figure 5b-c).

To investigate whether CCL2 release can be equally induced by trans-signaling in uninfected cells, uninfected HUVECs were treated with recombinant IL-6 (rIL-6) and sIL-6R. CCL2 secretion of rIL-6 treated cells reached similar levels as of PUUV infected cells. rIL-6 without sIL-6R did not induce CCL2 release (Figure 5d).

a)



b)



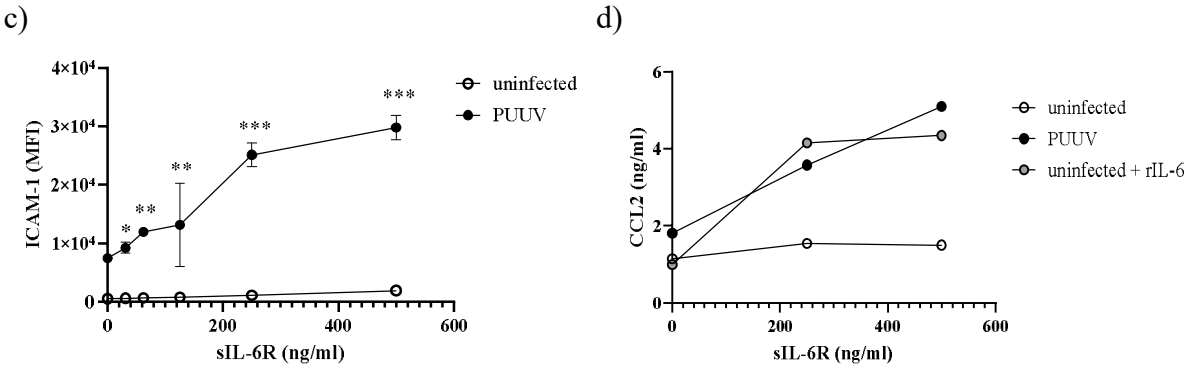


Figure 5. IL-6 and CCL2 secretion and ICAM-1 surface expression are increased in HUVEC upon PUUV infection and sIL-6R treatment. HUVEC infected with PUUV for 48h were treated with increased concentrations of recombinant sIL-6R for 24h. Levels of a) IL-6 and b) CCL2 in supernatants of infected (black) and uninfected cells (white) treated with sIL-6R. c) Expression (MFI) of ICAM-1 on the cell surface of infected and uninfected cells treated with sIL-6R. d) Levels of CCL2 (n=1) in supernatants of HUVECs left uninfected, infected with PUUV or uninfected and treated with 10 ng/ml rIL-6 (grey) after sIL-6R treatment. Symbols depict mean with standard deviation of three independent experiments for IL-6 and two independent experiments for CCL2 and ICAM-1. Two-way ANOVA test *P<0.05 **P<0.01; ***P<0.001.; ****P<0.0001

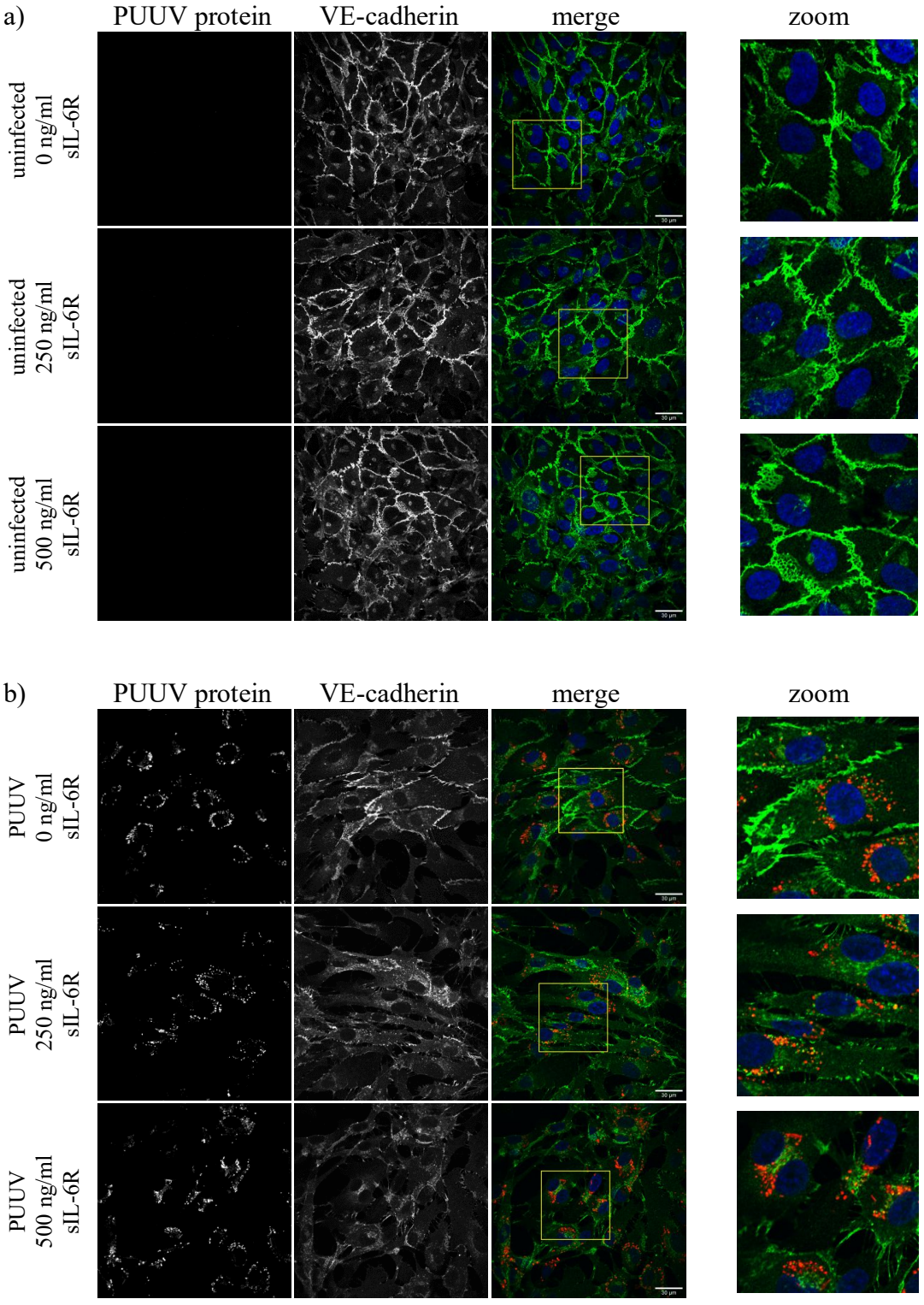
IL-6 trans-signaling disrupts barrier functions of HUVEC monolayers

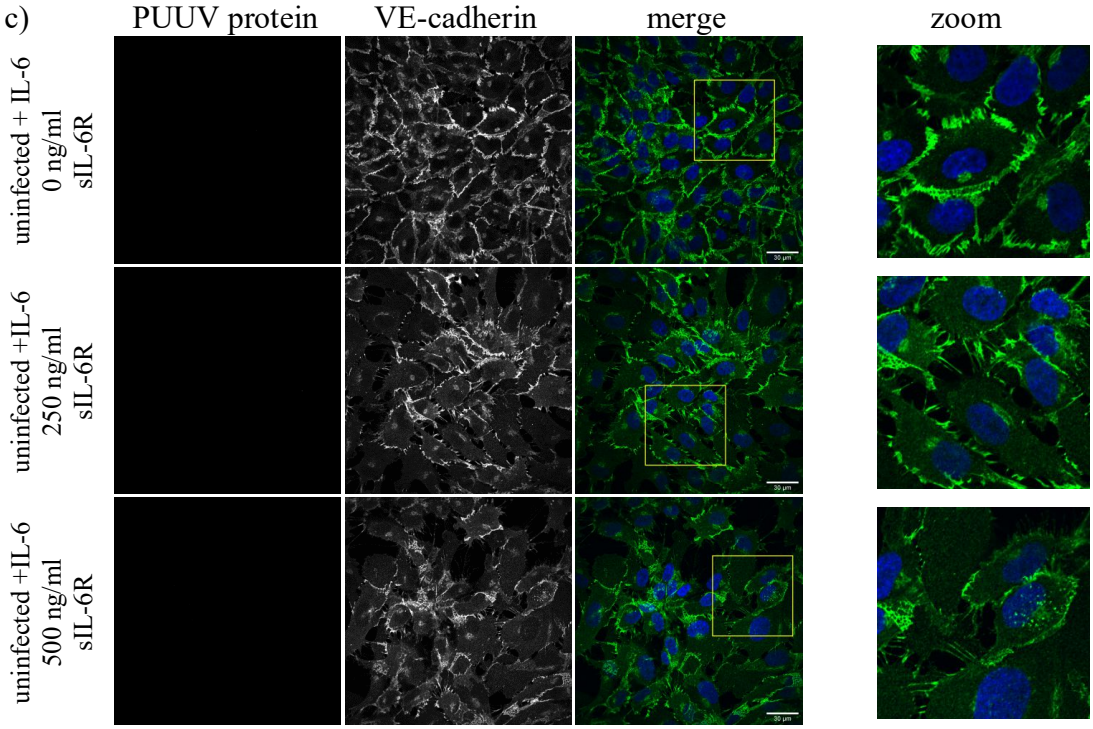
A hallmark of hantavirus pathogenesis is increased vascular permeability leading to extravasation of fluids and formation of oedema in major organs.[12,13] IL-6 trans-signaling has previously been shown to disrupt endothelial barrier functions by downregulating VE-cadherin.[47,52–55] However, no studies have addressed the impact of IL-6 trans-signaling on VE-cadherin and endothelial barrier function in the context of hantavirus infection. To investigate this, HUVECs were infected with PUUV for 48 h and then treated with 250 or 500 ng/ml sIL-6R for 24 h or left untreated. PUUV protein and VE-cadherin was visualized by immunofluorescence microscopy. Uninfected cells were not affected by sIL-6R treatment as shown by round or rectangular shaped cells forming a confluent monolayer with solid VE-cadherin junctions (Figure 6a). In contrast, PUUV infection without sIL-6R treatment caused some internalization of VE-cadherin, as indicated by intracellular vesicles in all infected cells. However, the monoloayer integrity of PUUV infected cells appeared intact in general, with only a few gaps found between cells. Interestingly, treatment with sIL-6R clearly disrupted the monolayer integrity, creating many gaps between the infected cells (Figure 6b). An increase in sIL-6R concentration from 250 to 500 ng/ml did not significantly increase the endothelial cell barrier disruption, as visualized by VE-cadherin staining (Figure 6b). However, observations of cell monolayers before fixation presented a difference in cell monolayer integrity (data not shown).

To investigate whether similar changes in monolayer integrity could be induced only by trans-signaling without PUUV infection, uninfected cells were treated with 10 ng/ml rIL-6 and sIL-6R as described before. While rIL-6 without sIL-6R had no effect on the cells, addition of sIL-6R created gaps between the cells with enhanced effects upon increasing concentration of sIL-6R. Also, some cells showed intracellular vesicles of VE-cadherin upon treatment with rIL-6 and 500 ng/ml sIL-6R (Figure 6c).

To investigate whether the changes in VE-cadherin structures affect the barrier function of endothelial monolayers, uninfected and PUUV infected HUVECs were seeded on semipermeable membranes in transwell inserts and treated with different concentrations of sIL-6R for 24h. To examine the barrier function of the monolayers, the transendothelial

electrical resistance (TEER) was measured. Interestingly, PUUV infection together with 250 and 500 ng/ml sIL-6R decreased the TEER of HUVEC monolayers by 55% and 40%, respectively, indicating impaired barrier function (Figure 6d). A slight reduction in TEER was also seen for PUUV alone (Figure 6d).





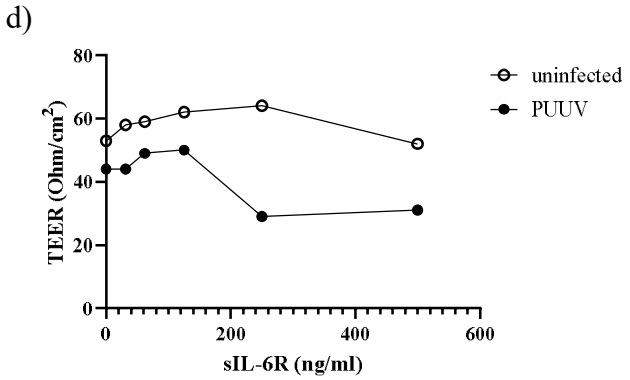


Figure 6. IL-6 trans-signaling disrupts the barrier function of PUUV infected endothelial monolayers. HUVECs were infected with PUUV for 48 h or left uninfected and then treated with different concentrations of sIL-6R for 24 h. Immunofluorescence images of a) uninfected, b) PUUV infected and c) uninfected, rIL-6 treated HUVEC after treatment with 0, 250 or 500 ng/ml sIL-6R. PUUV protein (red), VE-cadherin (green), DAPI (blue). scale bar = 30µm, yellow rectangle marks area of zoom image. d) Transendothelial electrical resistance (TEER) of uninfected (white) and PUUV infected (black) HUVEC monolayers after treatment with different concentrations of sIL-6R.

Discussion

Hantaviruses are prevalent worldwide and have been recognized as an important global health issue since decades ago.[70] While most patients with hantavirus infections develop only mild symptoms, some show a severe disease progression which may even lead to death.[3] This partly depends on the causative hantavirus species, as HPS, caused by new world hantaviruses, is generally associated with a higher case fatality rate[71] than HFRS, caused by old world hantaviruses.[2] What determines the disease outcome is still unclear. Several studies have aimed to identify markers of disease severity for HPS[25] and HFRS[31–34]. In these studies, IL-6 was shown to be significantly increased in hantavirus infection[25,31–33] and associated with disease severity.[25,31] However, through which mechanisms IL-6 affects hantavirus pathogenesis is unknown.

In the present study, we confirmed increased plasma levels of IL-6 in acute HFRS and concentrations coincide with previous findings.[31–34] Some HFRS patients had no detectable IL-6 in plasma which can be explained by a relatively poor sensitivity of the ELISA assay used in this study. Other methods, such as multiplex assays used in other studies[25] are more sensitive[72] and may be more appropriate for the detection of cytokines like IL-6 that occur in very low concentrations.[59] However, multiplex assay has been criticized for its poor reproducibility.[72] Here, some patients showed increased IL-6 levels in convalescence, but this is not likely to be linked to PUUV infection as patients develop life-long immunity after acute PUUV infection.[73,74] Also, many more physiological and pathological processes associate with increased IL-6 levels.[75] The cohort examined in this study only comprised patients with mild to moderate HFRS. Thus, no conclusions could be drawn about the association of increased IL-6 with disease severity.

The effects of IL-6 are diverse[36] and are mediated by classical signaling via membrane-bound IL-6R or by trans-signaling via sIL-6R.[38] Trans-signaling has been reported to mainly exert proinflammatory effects of IL-6.[44] Thus, we suspected a contribution of IL-6 trans-signaling in immune-mediated pathogenesis of HPS and HFRS. In general, IL-6 trans-signaling has been recognized as an important player in diverse diseases as reflected by increased serum or plasma levels of sIL-6R.[64–67] Following this approach, we examined sIL-6R levels in plasma of 28 PUUV infected patients in acute and convalescent phase and found increased levels of sIL-6R during acute HFRS. However, sIL-6R levels widely overlapped with the levels measured in convalescent phase and levels measured in healthy individuals in other studies.[61–64] However, all studies reporting increased levels of sIL-6R in diverse diseases have so far showed only a small increase.[62–67] This highlights the importance to consider changes in sIL-6R concentrations on an individual level in healthy and diseased condition. In this study, we found that all, except two patients, had increased sIL-6R levels in acute HFRS compared to convalescence. These findings suggest a contribution of IL-6 trans-signaling in hantavirus infection that should not be neglected in future hantavirus research.

To further investigate the interplay of IL-6 and sIL-6R in PUUV infected patients we measured the plasma levels of the IL-6/sIL-6R complex. We found no significant difference in plasma concentrations of the complex between acute phase and convalescence and no correlation between complex, IL-6 and sIL-6R. These findings do not allow a deeper insight into the relation of IL-6 and sIL-6R in the studied cohort. However, the complex may have bound to gp130 (trans-signaling) or sgp130 (inhibition) and is therefore not detected in significantly higher levels in plasma. This highlights, that constant levels of the complex do not exclude increased IL-6 trans-signaling. Notably, the assay used to measure the IL-6/sIL-6R complex concentrations has been criticized for its poor sensitivity.[58] Another important

player in IL-6 trans-signaling is sgp130, which acts as a natural inhibitor of IL-6 signaling.[57] In our study, we found no significant difference in sgp130 levels in acute HFRS compared to convalescence. The sgp130 concentrations measured in this study support previous studies, which report concentrations ranging between 150 to 400 ng/ml in healthy individuals.[62,64] However, changes in sgp130 concentrations on an individual level showed a decrease in 65% of the patients in the acute phase. These changes ranged only between 2-26%, but one study reported that IL-6 trans-signaling is not fully inhibited by those physiologically high concentrations of sgp130, suggesting a sensitive buffer system in which small changes may have larger consequences than earlier expected.[58] Furthermore, we could show a positive correlation between changes in sIL-6R and sgp130. Reduction of sgp130 was associated with a smaller increase in IL-6R, possibly suggesting that sIL-6R first had bound to IL-6 and then sgp130 bound to the IL-6/IL-6R complex and inhibited trans-signaling. Notably, sgp130 has been shown to bind only to the IL-6/sIL-6R complex and not to IL-6 or sIL-6R alone.[57] Interestingly, some patients showed an increase in sgp130 which was associated with a higher increase in sIL-6R. This finding may suggest that sgp130 binds to IL-6/sIL-6R complex in different affinities. However, what determines the relations of IL-6, sIL-6R and sgp130 is to be further explored. As there are no commercial assays available to detect the IL-6/sIL-6R/sgp130 complex conclusions about the buffer system are rather complicated. Important to note is that we only examined systemic plasma concentrations, local concentrations of IL-6, sIL-6R and sgp130 may differ at site of infection and inflammation.

After finding increased levels of IL-6 and sIL-6R in HFRS patients, we aimed to identify the source of these molecules. IL-6 is produced by various cell types[36] and we suspected PBMCs as one source of IL-6 in hantavirus infection. PUUV exposed PBMCs showed increased IL-6 secretion compared to unexposed controls, supporting our hypothesis. As hantavirus replication mainly occurs in vascular endothelial cells[19], we examined whether the infection triggers IL-6 release. We found increased IL-6 levels in supernatants of PUUV infected HUVECs compared to uninfected controls and IL-6 concentrations were higher than in supernatants of exposed PBMCs. This indicated that vascular endothelial cells are a major source of IL-6 in hantavirus infection. However, other cells, including granulocytes, fibroblasts or adipocytes are also able to secrete IL-6[36] and may also contribute to the increase in plasma IL-6 *in vivo*.

In contrast, sIL-6R can only derive from a limited number of cell types[38,39] by proteolytic cleavage[40] or alternative splicing[41,42]. We suspected PBMCs as a possible source but found no increased IL-6R shedding upon PUUV exposure. However, mechanisms of IL-6R shedding are rather complex and may require the interplay with other enzymes or immune cells, or infection instead of only direct exposure to PUUV. To date, a low-grade PUUV infection of monocytes has been reported[27], but infection of other PBMCs is unknown. Also there may be unknown inhibitors or activators of IL-6R shedding in cell culture medium, which may explain the generally high concentrations of sIL-6R in PBMC supernatants. Studies about the origin of physiological sIL-6R are rather inconclusive, while some suggest proteolytic cleavage as a major source[76] others support alternative splicing as an important mechanism of sIL-6R shedding.[61,77] Notably, one study showed decreased levels of alternative spliced sIL-6R with increasing age, while total sIL-6R levels remained constant, suggesting a shift over life time.[61] This needs to be considered when examining different cohorts of various age groups and may support individual variations in sIL-6R levels. Interestingly, infections, such as HTLV-1 and influenza have been linked to increased sIL-6R levels mediated by alternative splicing[56,66]. Additionally, extracellular microvesicles, such as exosomes, have been shown to express IL-6R and give rise to up to 36% of sIL-6R in plasma[40]. However, these findings require further research before significant conclusions can be drawn. Understanding the

mechanism of origin of increased sIL-6R levels in acute HFRS may help to identify the cell type participating in IL-6R shedding in HFRS and whether the virus infection directly or the activated immune system induces sIL-6R shedding. Deeper insights into these mechanisms may facilitate treatment development.

An important hallmark of hantavirus infections is increased capillary permeability[12,13], allowing fluid and protein to leak out from the blood vessels into the tissue, causing severe disease progression and the formation of oedema in major organs that can result in death.[14] Increased capillary permeability, also referred to as vascular leakage, has been appreciated as an important complication in many diseases[15–18], but the mechanisms remain poorly understood. Previous studies have linked increased vascular permeability to the proinflammatory effects of IL-6 on vascular endothelial cells.[47,52–55] As endothelial cells lack the membrane-bound IL-6R, IL-6 can affect endothelial cells only by trans-signaling via sIL-6R.[45] However, if this applies to IL-6 released by endothelial cells upon PUUV infection is unknown. Thus, we focused on the effects of IL-6 trans-signaling on hantavirus infected endothelial cells.

We showed an increase in IL-6 gene expression upon PUUV infection which was further induced by IL-6 trans-signaling. These findings were validated on protein levels. To our knowledge, an IL-6 release upon IL-6 trans-signaling has not been shown for endothelial cells, but has been reported for IL-6 trans-signaling in epithelial cells.[56] Interestingly, increased IL-6 secretion upon IL-6 trans-signaling has also been shown for PBMCs[56] and should be tested for PUUV exposed PBMCs in the future. Furthermore, we showed similar patterns for CCL2 and ICAM-1, supporting previous studies showing an increase in CCL2 secretion by endothelial cells[45,48] and upregulation of ICAM-1 on the cell surface[46–49] upon IL-6 trans-signaling. Additionally, we reported an upregulation of IL-1β gene expression upon PUUV infection. IL-1β has been shown to upregulate ICAM-1 on endothelial cells[78], which could explain upregulation of ICAM-1 upon PUUV infection, but a previous study did not find induced IL-1β secretion upon PUUV infection in HUVEC (unpublished data). However, this study measured IL-1β concentrations starting 24 h post infection and an earlier secretion may be possible as seen for VEGF in ANDV infected cells.[20] Interestingly, we could reproduce the increase in CCL2 secretion by IL-6 trans-signaling in uninfected cells by treatment with rIL-6. Upon IL-6 trans-signaling, levels of CCL2 in supernatants of uninfected cells reached the same concentrations as in supernatants of PUUV infected cells, indicating that also uninfected adjacent cells can be highly activated by IL-6 trans-signaling in hantavirus infections. Even though activation was more prominent upon IL-6 trans-signaling, we saw an increase in IL-6, CCL2 and ICAM-1 already upon PUUV infection without sIL-6R stimulation, suggesting that PUUV infection itself also activates endothelial cells to a certain level.

IL-6, CCL2, ICAM-1 and IL-1β, are all proinflammatory molecules[38,50,51,79], thus, our findings support the hypothesis the IL-6 trans-signaling has proinflammatory effects in hantavirus infection *in vitro*. However, how PUUV and IL-6 trans-signaling induce endothelial cell activation is unknown. One study investigating influenza A virus infection (IAV) in lung epithelial cells showed increased IL-6 secretion upon infection and IL-6 trans-signaling in an IL-32 dependent manner.[56] They showed that knock out of IL-32 inhibited IL-6 secretion upon IL-6 trans-signaling.[56] Additionally, other studies report an IL-32 dependent upregulation of IL-1β-induced ICAM-1 on the endothelial cell surface[80], and IL-32 dependent secretion of CCL2 by THP-1 cells.[81] Furthermore, IL-32 has been reported to downregulate IL-6R[56] which was also shown on mRNA level upon PUUV infection in our study. Thus, we suspect a contribution of IL-32 in the activation of endothelial cells upon PUUV infection and

IL-6 trans-signaling seen in this study. We encourage the investigation of the role of IL-32 in hantavirus infection as IL-32 has gained awareness in studies of other viral infections caused by HIV[82], IAV[56], human papilloma virus[83], hepatits B virus[84], human cytomegalovirus[85] and Epstein Barr virus[86].

After showing activation of endothelial cells by PUUV infection and IL-6 trans-signaling, we further wanted to examine the consequences of this, i.e. whether IL-6 trans-signaling disrupts the barrier function of PUUV infected endothelial cells. We observed an internalization of VE-cadherin and a decreased endothelial barrier function upon PUUV infection without IL-6R treatment which support previous findings in ANDV infected endothelial cells.[20] Whether these effects upon PUUV infection occur early after infection and in a VEGF dependent manner as previously shown for ANDV[20] should be part of future studies. As we hypothesized, IL-6 trans-signaling induces downregulation of VE-cadherin and disruption of the barrier function of PUUV infected endothelial cells. However, IL-6 induced disruption of endothelial barrier function in PUUV infected HUVEC was only observed upon treatment with sIL-6R above 250 ng/ml. No additional effects were seen with 500 ng/ml sIL-6R. In further studies, changes in VE-cadherin expression should be studied also after treatment with lower doses of sIL-6R. Additionally, we could reproduce the downregulation of VE-cadherin by IL-6 trans-signaling in uninfected cells by treatment with rIL-6 as reported previously.[47,52–55] This suggests that also uninfected cells within hantavirus infected cultures can be affected by IL-6 trans-signaling. Whether endothelial permeability can be increased by IL-6 trans-signaling to a similar extent in uninfected cells as in PUUV infected cell should be investigated in future studies. Notably, the measurement of TEER is very sensitive to temperature changes, cell passage number, cell culture medium composition and other variable conditions.[87] Thus, repetition of this experiment is necessary for significant conclusions. However, TEER values measured for HUVEC monolayers in this study are in line with previous reports.[88] Altogether, our findings highlight the importance of IL-6 trans-signaling in hantavirus infection and suggest a possible contribution of IL-6 in vascular leakage.

Moreover, we found an upregulation in *PPP2R2B* gene expression upon PUUV infection without sIL-6R treatment. *PPP2R2B* encodes for the serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta, also referred to as B55β.[89] It is a natural inhibitor of the protein phosphatase 2A (PP2A).[90] Additionally, *PPP2CA* gene, encoding for PP2A, was downregulated upon PUUV infection. Activation of PP2A was previously shown to inhibit IL-6 secretion.[91] Thus, its inhibition by PUUV, if confirmed on protein level, could have proinflammatory effects. Interestingly, one study reported that PP2A activity is required for functional adherens junctions in endothelial cells.[92] The same study also showed internalization of VE-cadherin and changes in cytoskeleton structure upon inhibition of PP2A.[92] Furthermore, PP2A has been identified as an important inhibitor of endothelial nitric oxide synthase (eNOS)[93] and increased levels of B55β in endothelial cells have been associated with increased phosphorylation and activation of eNOS.[94] eNOS derived NO has been reported to regulate the rearrangement of the actin cytoskeleton and is required for VEGF-induced phosphorylation and downregulation of VE-cadherin, resulting in endothelial barrier disruption.[95] Thus, it is possible that PUUV-induced upregulation of B55β inhibits PP2A inactivation of eNOS, resulting in higher NO levels leading to phosphorylation and internalization of VE-cadherin. Inhibition of PP2A has also been reported to downregulate gp130 on protein level, but not on transcriptional level.[96] We saw a downregulation of gp130 gene expression in the current study. This suggests an anti-inflammatory feedback in PUUV infection but may be time-dependent and has to be investigated in further studies.

To date, there is no vaccine or treatment available for hantavirus infections. As our findings suggest an impact of IL-6 trans-signaling in hantavirus pathogenesis, treatments targeting IL-6-signaling may be considered. Monoclonal antibodies (mAb) blocking IL-6 (i.a. siltuximab) or IL-6R (i.a. tocilizumab) have been approved for different inflammatory diseases and are under constant development.[36] However, those mAb inhibit both, classical and trans-signaling of IL-6 and more specific targeting of IL-6 trans-signaling may be desired for treating hantavirus infections. A possible candidate is anti-sgp130 mAb (olamkizept)[36] or even newer approaches targeting vascular leakage directly. Newly characterized strategies decrease VE-cadherin internalization[97,98], prevent stress fiber formations[99–101] or enhance cell adhesion[102,103] specifically, thus, allowing treatment of vascular leakage without affecting the inflammatory response which is desirable for pathogen clearance.[104] However, those recently characterized agents are partly still under development, but should be followed as possible future treatment strategies for hantavirus infections.

In conclusion, we reported an increase of sIL-6R in acute HFRS for the first time. Furthermore, we observed activation of PUUV infected endothelial cells upon IL-6 trans-signaling which induced inflammatory markers and reduced barrier function of endothelial cells. These findings suggest a relevant impact of IL-6 trans-signaling on hantavirus pathogenesis and vascular leakage and reveal new possible treatment targets for hantavirus infections. Finally, we suggest further investigation of the role of IL-1β, IL-32, PP2A and eNOS in IL-6 trans-signaling and encourage future studies on IL-6 trans-signaling in hantavirus infection.

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