

## Theme Issue Article

## Hantavirus regulation of endothelial cell functions

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## Summary

Hantaviruses cause two vascular permeability-based diseases and primarily infect endothelial cells which form the primary fluid barrier of the vasculature. Since hantavirus infections are not lytic, the mechanisms by which hantaviruses cause haemorrhagic fever with renal syndrome (HFRS) or Hantavirus Pulmonary Syndrome (HPS) are indeterminate. HPS is associated with acute pulmonary oedema and HFRS with moderate haemorrhage and renal sequelae, perhaps reflecting the location of vast microvascular beds and endothelial cell reservoirs available for hantavirus infection. Endothelial cells regulate capillary integrity, and hantavirus infection provides a primary means for alter-

ing vascular permeability that contributes to pathogenesis. The central importance of endothelial cells in regulating oedema, vascular repair, angiogenesis, immune cell recruitment, platelet deposition as well as gas exchange and solute delivery suggest that a multitude of inputs and cellular responses may be influenced by hantavirus infection and contribute to pathogenic changes in vascular permeability. Here we focus on understanding hantavirus interactions with endothelial cells which are linked to vascular permeability, and provide insight into the contribution of endothelial cell responses in hantavirus pathogenesis.

## Keywords

Virology, endothelial cells, integrins, hypoxia, chemokines

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## Hantavirus genetics and life cycle

Hantaviruses predominantly infect the endothelial cell lining of capillaries and cause two vascular permeability-based diseases in the absence of cell lysis. Relative to many RNA viruses, the hantavirus replication cycle is slow, resulting in viraemia 5–10 days post-infection (204, 229). Hantaviruses are enveloped viruses (100 nm) with a tripartite negative-stranded RNA genome and are members of the Bunyaviridae family (175). Hantavirus S, M and L gene segments encode four viral proteins and hantavirus replication is cytoplasmic (175). The 6.6 kb L segment encodes a single 220 kDa polymerase which directs the transcription and replication of viral RNAs. The S segment (1.7–2.1 kb) encodes a 428 amino acid nucleocapsid or N-protein which is abundantly expressed in the cytoplasm. The N-protein is the predominant viral antigen synthesised during infection and has recently been associated with cellular processing bodies (P-bodies) (137). The N-protein is highly conserved across hantaviruses and contains cross-reactive epitopes that permit the identification of hantavirus infected cells (175, 177).

The M genomic segment (3.6 kb) encodes a polyprotein that is cleaved co-translationally into two glycoproteins, the 70 kDa

Gn (G1) and the 58 kDa Gc (G2) (175, 178). Gn and Gc proteins are trafficked to the cis-Golgi (152, 153) and form heterodimers which oligomerise and form the highly structured virion surface (175, 178). Hantaviruses are formed intracellularly, acquiring GnGc on their surface through the budding of particles into the lumen of the cis-Golgi and exiting cells through a mechanism consistent with aberrant vesicular transport (69, 153, 175). Although virion surface glycoproteins are presumed to mediate interactions with cellular receptors, domains of Gn or Gc involved in viral attachment have yet to be resolved. Hantaviruses lack matrix proteins; however, the Gn protein contains a 142 residue cytoplasmic tail that is suggested to play additional roles in viral assembly and the regulation of cellular responses during infection (2, 3, 67, 180).

Although hantaviruses are segmented, gene reassortment between pathogenic and non-pathogenic hantaviruses has not been demonstrated. Even between very similar pathogenic hantaviruses only one gene segment has been experimentally reassorted (132). Reverse genetic systems have been used successfully to genetically modify some bunyaviruses, and hantavirus mini-genome reporter systems have been presented (16, 54, 121, 239). However, hantavirus reverse genetics resulting in the generation

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of genetically modified hantaviruses have yet to be reported (54, 239).

## Hantaviruses, hosts and human infection

Hantaviruses are a genetically and antigenically diverse group of viruses that are present worldwide. In contrast to other members of the Bunyaviridae which are arthropod borne, each hantavirus persistently infects a primary small mammal host (175, 176) and virus is spread to humans primarily from the inhalation of excreted virus. Hantaviruses appear to have co-evolved with their hosts and human infections are geographically delimited by the range of their primary hosts. In humans, hantaviruses cause one of two vascular leak syndromes: Haemorrhagic Fever with Renal Syndrome (HFRS) or Hantavirus Pulmonary Syndrome (HPS) (also referred to as Hantavirus CardioPulmonary Syndrome; HCPS) (25, 41, 107, 113, 114, 118, 119, 148, 175, 176, 237). In all cases, hantaviruses predominantly infect endothelial cells and fundamentally impact vascular barrier and platelet functions which maintain haemostasis (41, 113, 114, 118, 119, 148, 175, 176, 237).

HFRS-causing hantaviruses are primarily present in Eurasia and include Hantaan (HTNV), Seoul (SEOV), Puumala (PUUV) and Dobrava (DOBV) viruses which have a 0.1–5% mortality rate (113, 119, 175, 176). HPS-causing hantaviruses are present throughout the Americas and cause an acute respiratory distress syndrome characterised by pulmonary edema and a high mortality rate (35–40%) (18, 41, 45, 148, 175, 176, 237). There are a large number of HPS-causing hantaviruses including the prototypic Sin Nombre virus (SNV) and NY-1V found in North America and the South American Andes virus (ANDV) (48, 60, 79, 146, 174, 175, 184). Thus far, only the South American ANDV has been reported to be spread from person to person (48).

There are two notable hantaviruses which are not associated with any human disease, Prospect Hill virus (PHV, America) and Tula virus (TULV, Europe) (110, 158, 215, 233, 234). PHV enters but fails to replicate in human ECs and this reflects PHVs failure to regulate early innate IFN responses in human ECs (2, 3, 234). PHV and TULV also use discrete integrins from those used by pathogenic hantaviruses and unique integrin usage is consistent with the inability of PHV and TULV to enhance endothelial cell permeability at late times post-infection (61, 63–65).

## Hantavirus disease

A complete presentation of hantavirus disease is provided by several reviews (25, 41, 76, 113, 119, 148, 216, 237). Hantaviruses infect patient endothelial cells and cause acute disease after a one- to two-week incubation period (25, 41, 76, 113, 119, 148, 216, 237). Hallmarks of hantavirus disease include increased vascular permeability (haemorrhage or oedema) and acute thrombocytopenia with marked permeability of microvascular beds (25, 41, 113, 119, 148, 176, 237). Both HFRS and HPS patients may have pulmonary or renal symptoms and the localisation of hantavirus disease may reflect hantavirus infection of endothelial cells within expansive alveolar and glomerular capillary beds (175, 176).

## HFRS

Disease includes microvascular haemorrhage, acute thrombocytopenia, hypotension, shock, and in some cases renal failure (113, 114, 175, 176). The mechanism by which HFRS viruses cause vascular haemorrhage and glomerular dysfunction is unclear. However, circulating immune complexes, which are evident in HFRS patients and deposited in the kidney may contribute to renal and haemorrhagic disease (26, 62, 96, 113, 114, 155).

## HPS

The main clinical manifestations of HPS include severe pulmonary oedema, hypoxia and acute thrombocytopenia (18, 41, 148, 237). With the onset of symptoms, acute pulmonary oedema rapidly progresses into acute respiratory distress (18, 41, 148, 237). Viraemia and low platelet counts have been associated with severe disease and poor outcomes (18, 25, 34, 41, 97, 107, 148, 237). The role of hypoxia in the disease process is unclear; however, extracorporeal membrane oxygenation (ECMO) has been shown to substantially reduce the mortality of HPS patients by correcting hypoxia and shock (25, 39, 97).

## Syrian hamster model of hantavirus disease

ANDV infection of Syrian hamsters is currently the only animal model of hantavirus pathogenesis that closely mimics human disease (84, 136, 220). ANDV infection of Syrian hamsters mimics the long onset, course and acute respiratory distress symptoms of HPS patients resulting in a fatal HPS-like disease (84, 220). The onset of focal pulmonary oedema occurs concomitant with viremia six days post-infection (p.i.) and both oedema and viraemia increase with time (84, 220). Endothelial cell inclusions are detected eight days p.i. and death occurs 10–14 days p.i. Leukocytosis and lymphopenia were observed 11–14 days p.i. along with severe pulmonary oedema, shock and death (22, 84, 220). Curiously, neither SNV nor HTNV, which cause HPS and HFRS in humans, respectively, cause disease in Syrian hamsters suggesting that species-specific determinants permit ANDV pathogenesis in Syrian hamsters (84, 220). Reassorting the ANDV M segment into SNV did not alleviate the block to SNV pathogenesis in Syrian hamsters suggesting that this SNV host restriction is multigenic or dependent on S or L segments (132).

## Endothelial cells are targeted by hantaviruses

Clinical and pathological findings demonstrate that pathogenic hantaviruses specifically target endothelial cells for infection (113, 148, 234, 237). Hantavirus antigens are present in capillary endothelial cells of all HFRS and HPS patient tissues and prominently demonstrated in pulmonary, splenic and kidney endothelial cells (26, 148, 222, 237). *In vitro* infection of primary human endothelial cells results in the high level synthesis of viral antigens as well as viral replication, and demonstrates endothelial cell sensitivity to pathogenic hantavirus infection (154, 175, 234). Electron microscopy of lung autopsy materials reveals typical perinuclear hantavirus inclusions within the cytoplasm of infected endothelial cells and a slightly swollen but intact cellular morphology (148, 222). Hantavirus particles are also visible

in pulmonary microvascular endothelial cells by immunoelectron microscopy (IEM) (148, 237). Hantavirus antigens are associated with perinuclear aggresome-like structures and P-bodies which could reflect the location of viral factories and permit hantavirus regulation of cellular functions (137, 187). Experimental infection of tissue culture cells reveals that at late times p.i. virions cover the surface of infected cells (69) suggesting a potential role for adherent extracellular virions in altering endothelial cell functions as well as immune responses and platelet recruitment to the infected endothelium.

## Hantavirus pathogenesis

The means by which hantaviruses cause pulmonary oedema or haemorrhagic disease have been widely conjectured but have yet to be defined. Given the plethora of regulatory responsibilities of the vascular endothelium, hantavirus pathogenesis is likely to be a complex multifactorial process that includes contributions from immune responses, platelet dysfunction and the dysregulation of endothelial cell barrier functions. Immune complex deposition clearly contributes to HFRS patient disease and renal sequelae but is rare in HPS (26, 62, 96, 113, 114, 155). Oedema appears to precede leukocytosis and lymphopenia in Syrian hamsters which develop HPS-like disease suggesting that immune cell recruitment may be a secondary rather than primary contributor to permeability (84, 220). However, what triggers vascular permeability following hantavirus infections remains to be resolved and it remains an enigma as to why Eurasian and American hantaviruses cause discrete haemorrhagic or oedematous diseases (33, 41, 148, 237). It is clear that vascular permeability and acute thrombocytopenia are hallmarks of both hantavirus diseases, and this suggests that there are common underlying mechanisms of capillary permeability which are overlaid with HPS- and HFRS-specific parameters of disease (25, 33–35, 41, 107, 113, 148, 237).

Several mechanisms of hantavirus pathogenesis have been suggested based primarily on the analysis of specific autopsy samples or clinical observation. Suggested disease mechanisms include roles for CD8<sup>+</sup>T-cells (47, 104, 203, 209), tumour necrosis factor (TNF) $\alpha$  (102, 108, 120, 126, 139, 147, 194, 201), the “cytokine storm” (108, 109, 120, 126, 139, 182, 201), additional immune responses (51, 52, 120, 125, 126, 182, 201, 237),  $\beta_3$  integrin regulation (61, 63–65, 117, 124, 163), viraemia (99, 202, 204, 220), complement activation and platelet dysfunction (25, 33–35, 52, 99, 113, 114, 201). It is likely that several mechanisms actually contribute to hantavirus diseases and that different components may contribute dynamically to specific aspects of pathogenesis (25, 33–35, 99, 113, 114, 201).

Findings from hantavirus patients reflect a disease state resulting from acute thrombocytopenia and the infection of endothelial cells in the absence of cell lysis or disruption of the endothelium (25, 41, 76, 148, 237). A lack of endothelial cell disruption is clear in HPS patients who lack haemorrhagic sequelae and instead have acute pulmonary oedema (25, 41, 76, 148, 237). Pleural effusions from HPS patients are primarily transudative in nature and contain little or no inflammatory cells (18). In fatal HFRS and HPS, analysis of pulmonary autopsy samples identifies hantavirus-infected endothelial cells, hyaline membranes, and little to moderate mono-

cyte/macrophage interstitial infiltrates with a CD4<sup>+</sup> to CD8<sup>+</sup> T-cell ratio of 1.2 to 1.8 (41, 148, 237).

It was reported that a higher frequency of CD8<sup>+</sup> T-cell responses were present in samples from patients with severe HPS (104). However, this is not clear since there was little overlap in specific T-cell numbers between severe and moderate infections, and the conclusions are limited by small numbers and the capability of measuring only epitopes restricted to one MHC Class I allele (104). A CD8<sup>+</sup> T-cell line derived from an HFRS patient reportedly targeted and permeabilised monolayers of SNV infected EA.hy926 cells (73). Yet, the T-cell lysis observed does not reflect the intact endothelium of HPS patients or the absence of haemorrhagic disease (73).

Several studies have evaluated TNF $\alpha$  in patients or the effects of TNF $\alpha$  on hantavirus-infected endothelial cells but with unclear associations with viral pathogenesis. One study indicated that 14 of 276 HFRS sera had elevated TNF $\alpha$ , IL-1 and IL-6 levels, and that these cytokines did not correlate with HFRS disease (109). In contrast, low TNF $\alpha$  levels were associated with more severe clinical disease in 36 HFRS patients (126). A separate study indicated that TNF $\alpha$  levels were elevated in 15 HFRS sera, but also demonstrated that this coincided with elevated levels of soluble TNF receptors which prevent cellular responses to TNF $\alpha$  (120). Increased TNF $\alpha$  was observed in the glomeruli of HFRS patients (201) and may explain responses to immune complex deposition within the kidneys of HFRS patients (33). HPS pathogenesis is also reportedly associated with high levels of cytokine producing cells in the lung tissues of 2/6 HPS autopsy samples (139). However, in 4/6 HPS patient lung samples, TNF $\alpha$ -positive cells were present at low levels and there were no TNF $\alpha$ -positive cells present in lung samples from one fatal HPS case (139). In support of reduced pathogenesis caused by TNF $\alpha$ , a recent report indicates that a 40-fold induction of TNF $\alpha$  in alveolar fluid was associated with a 90% reduction in lethal influenza virus infection, reducing rather than enhancing pulmonary disease (210).

Two studies evaluated TNF $\alpha$ -directed permeability of hantavirus-infected endothelial cells. The first indicates that TNF $\alpha$  induced a <2-fold change in permeability, albeit at only one timepoint (147), while a second study indicated that TNF $\alpha$  levels do not change in SNV-infected cells and that the permeability of SNV-infected endothelial cells was not increased by the addition of TNF $\alpha$  (102). This finding is supported by a recent paper indicating that the hantavirus N-protein blocks TNF $\alpha$ -directed endothelial cell responses (200), and suggests that hantaviruses may have a mechanism for regulating the effects of secreted cytokines.

Hantaviruses bind human  $\beta_3$  integrins (63, 65, 163) which have primary roles in regulating vascular integrity, endothelial cell permeability and platelet functions (14, 15, 19, 28, 30, 55, 80, 88, 90, 101, 164, 167); however, the role of  $\beta_3$  integrins in hantavirus patients has not been evaluated. Pathogenic hantaviruses bind plexin, semaphorin and integrin (PSI) domains on inactive conformations of  $\beta_3$  integrin subunits (163, 230), block endothelial cell migration (61) and enhance the permeability of endothelial cells in response to VEGF days after infection (64). This mirrors the hyperpermeability of  $\beta_3$  integrin knockout cells and mice in response to VEGF and provides a mechanism for hantavirus directed endothelial cell permeability (14, 15, 19, 30,

42, 80, 128, 223, 228, 232). In fact, hantavirus inhibition of  $\beta_3$  integrin functions is consistent with the presence of cell associated hantaviruses covering the surface of infected cells at late times post-infection (69) and the role of  $\beta_3$  integrins in maintaining endothelial cell fluid barrier functions. These and other findings add to rationales for hantavirus- $\beta_3$  interactions to contribute to vascular permeability (14, 19, 38, 42–44, 58, 77, 116, 138, 150, 156, 167, 170, 171, 205, 206, 219, 227, 240).

Viraemia is associated with increased hantavirus pathogenesis in humans and Syrian hamsters (26, 204, 220, 229). Although it is unclear what role viraemia plays in hantavirus pathogenesis, increased viraemia provides a means for circulating hantaviruses to interact with platelets, endothelial cells and immune cells throughout the body. Viraemia could contribute to defective platelet aggregation of HFRS patients (34), through interactions with inactive platelet  $\beta_3$  integrins ( $\alpha_{IIb}\beta_3$ ), and contribute to thrombocytopenia common to hantavirus diseases. However, the role of platelets in hantavirus pathogenesis is complicated by the plethora of factors that impact platelet functions and vascular integrity (20, 29, 30, 55, 57, 88, 90, 105, 111, 133, 162, 168, 179, 217, 218, 226).

As a result, there are still many questions that remain to be addressed in order to fully understand vascular permeability changes occurring during hantavirus infections that contribute to pathogenesis. One underlying focus of all systems that alter vascular permeability is the endothelium which forms the primary fluid barrier that maintains and regulates capillary integrity.

## Endothelial cell functions

A single layer of approximately  $10^{11}$  endothelial cells lines the vasculature and collectively forms one of the largest organs of the body with an enormous surface area (9, 212). The endothelium is regulated by both systemic and local tissue-specific responses and responds to a large array of secreted or circulating compounds and conditions as well as the physical adherence of platelets and immune cells that dynamically alter localised endothelial cell functions (9, 10, 29, 30, 90, 150, 212). The endothelium also produces a variety of physiologically active substances that regulate immune cell and platelet responses and barrier functions of the endothelium (8, 44, 49, 70, 77, 91, 101, 116, 133, 183, 191, 212, 214, 221). The endothelium serves a critical regulatory role in angiogenesis, vascular permeability, haemostasis, cell trafficking, thrombosis, anti-thrombosis and inflammation (9, 212).

Endothelial cells maintain vascular integrity while still permitting cell mobility required for angiogenesis and vascular repair (1, 7, 8, 11, 19, 43, 44, 49, 56, 89, 105, 115, 127, 128, 151, 165, 166, 206, 208, 221). Endothelial cells also facilitate and regulate the translocation of fluid, nutrients, oxygen and cells into tissues (9, 212). This complex organisation and the unique regulation of endothelial responses is demonstrated by the presence of receptors, growth factors and junctional proteins that are exclusively used by endothelial cells (31, 38, 58, 75, 115, 116, 144, 221, 223). Vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 are endothelial cell-specific growth factors that, along with integrins and platelet-released factors, serve to regulate a unique endothelial cell adherence junction assembly and disassembly process (5, 27, 38, 42, 43, 56, 58, 59, 75, 93, 94, 115, 116, 140, 141, 170, 171, 181, 205, 221, 223, 224, 231). These en-

dothelial cell-specific regulatory factors coordinately regulate endothelial cell movement and microvascular permeability. Endothelial cells are further impacted by localised immunological or physiological conditions that either facilitate permeability or enhance barrier functions (5, 27, 38, 42, 43, 56, 58, 59, 75, 93, 94, 115, 116, 140, 141, 170, 171, 181, 205, 221, 223, 224, 231).

## Hantaviruses infect endothelial cells

Interestingly, the majority of the endothelium is located in vast microvascular beds which are central to pulmonary and kidney functions (9, 212) and also the focus of hantavirus infection and disease. Hantaviruses are one of the few viruses that predominantly infect and replicate within host and human endothelial cells (41, 113, 148, 175, 212, 234, 237). This requires successful interactions with cellular receptors that permit viral entry as well as successfully engaging intracellular responses that might otherwise limit viral replication (2, 68). Interestingly, pathogenic and non-pathogenic hantaviruses use discrete integrins for viral entry (60, 65) and at one level this may result in unique endothelial cell regulation that contributes to disease. However, successful replication following entry is also required in order to be pathogenic and at least one non-pathogenic hantavirus is replication incompetent following entry of human endothelial cells (234).

## Hantaviruses regulate early interferon responses

There is little information about the replication of non-pathogenic hantaviruses within human endothelial cells (215, 233, 234). PHV is not associated with any human disease, yet, as measured by the synthesis of nucleocapsid protein, PHV infects human endothelial cells (233, 234). However, there is little if any replication of PHV within human endothelial cells (68). In contrast, PHV replicates successfully in VeroE6 cells which lack a type-I IFN genetic locus (46, 225) suggesting that PHV fails to negotiate intracellular responses of human endothelial cells (2, 68, 234). PHV induces the transcription of high level IFN stimulated gene (ISG) responses within endothelial cells one day post-infection which are absent following infection by HTNV or NY-1V (68). This finding is consistent with the regulation of PHV replication by innate cellular IFN responses of human endothelial cells and provides at least one explanation for why PHV is not a human pathogen (2, 3, 68, 110). Since all hantaviruses must bypass innate cellular responses within their hosts, this likely reflects a species-specific difference in PHV protein interactions with human IFN regulatory proteins (3). However, differences in human and host IFN pathway proteins that might differentiate these regulatory responses are currently unknown.

## Hantavirus proteins regulate IFN transcription

Pathogenic hantaviruses prevent the early induction of cellular IFN responses that are likely to block viral replication. Expression of the cytoplasmic tail of the NY-1V Gn protein (Gn-tail) has been shown to inhibit RIG-I- and TBK1-directed transcriptional responses from promoters containing interferon stimulated response elements (ISREs) (2, 3). In contrast the PHV Gn-tail and the nucleocapsid protein from all hantaviruses tested

were unable to block RIG-I and TBK1 directed ISRE transcriptional responses (2, 3). RIG-I is an upstream activator of TBK1 and TBK1 phosphorylates IRF-3 which is a transcription factor required for the induction of interferon- $\beta$  (IFN- $\beta$ ) (78, 86, 129, 235, 236). Similar to ISRE regulation, the NY-1V Gn-tail blocks TBK1 and TRAF2 directed nuclear factor (NF)- $\kappa$ B activation which is also required for IFN- $\beta$  induction (3, 129). Collectively these findings suggest that the Gn-tails of pathogenic hantaviruses regulate interferon signalling responses at the level of the TBK1 complex (3).

Components of TBK1 complexes are compelling targets for IFN regulation since TBK1 directs the activation of both ISRE and NF- $\kappa$ B transcriptional responses required for IFN induction (129, 159). TBK1 forms a complex with TRAF3 that is required for IRF-3 activation (71, 74, 149). TRAF3 appears to be indispensable for IFN- $\beta$  transcription since TRAF3 knockout cells fail to induce type-I interferon responses directed by virtually all upstream pathway activators (149). One report indicates that the Gn-tail of pathogenic but not non-pathogenic hantaviruses binds an N-terminal domain of TRAF3 and provides a means for hantavirus protein interactions to directly engage and regulate the IFN induction pathway within cells (3). TBK1-TRAF3 complex formation is also regulated by the state of TRAF3 ubiquitination (74, 100), and the Gn-tail of pathogenic hantaviruses is ubiquitinated and degraded while the Gn-tails of non-pathogenic PHV are stably expressed (66, 180). This suggests that Gn-tail ubiquitination and TRAF3 interactions may alter TBK1 complex formation and regulate IFN induction (3, 180). However the specific interactions of Gn-tails with IFN pathway proteins remain to be defined. Nonetheless, these findings define fundamental differences between pathogenic and non-pathogenic hantaviruses and suggest that at one level the Gn-tail is likely to be a determinant of viral virulence since it regulates early IFN transcriptional responses and thereby permits hantavirus replication within human endothelial cells.

Some members of the *Bunyaviridae* family have been shown to encode a non-structural NSs protein, which inhibits IFN- $\beta$  transcription 50-fold (12, 13). A recent report suggests that Tula and Puumala hantaviruses encode an NSs protein that also reduces IFN- $\beta$  transcription (92). Yet, Tula and Puumala NSs proteins reduced IFN- $\beta$  transcriptional responses by only 30%, and it is unclear whether this level of inhibition significantly contributes to the regulation of cellular IFN responses during viral infection (92). NSs proteins have not been identified during HTNV, ANDV, SNV or NY-1V infection, and the ORF corresponding to the Tula NSs protein is disrupted by termination codons in NY-1V, HTNV and ANDV. However, it is possible that hantaviruses have evolved redundant mechanisms for inhibiting IFN- $\beta$  induction since preventing early IFN inhibition is essential to successful viral replication.

## Replication in the presence of ISGs late in infection

All hantaviruses highly induce ISGs at late times post-infection (68, 103, 106), indicating hantavirus-infected cells develop resistance to the effects of IFN over time. Reports indicate that

neutralising IFN- $\beta$  antibodies enhance HTNV replication but only when applied early after infection (97, 197), and this is consistent with clinical data indicating that hantaviruses are only sensitive to early IFN administration (97, 197). This indicates that pathogenic hantaviruses are sensitive to early interferon responses and rationalises the need for pathogenic hantaviruses to regulate IFN responses at early times post-infection.

The *in vitro* addition of type-I interferon is also effective at inhibiting NY-1V and HTNV infection up to 12 hours post-infection; however, the addition of IFN 15–24 hours post-infection has little effect on NY-1V or HTNV replication (2). These findings suggest that a viral product may be synthesised 12–24 hours post-infection which compensates for IFN induction and ISG responses at later times (2). However, the means by which pathogenic hantaviruses replicate in the presence of high levels of cellular ISGs at late times post-infection remains to be investigated. One report suggests that hantaviruses regulate IFN receptor directed STAT phosphorylation at later times post-infection (186); however, this contradicts the high level induction of STAT-dependent ISGs 2–4 days after hantavirus infection (68). HTNV also reportedly triggers TLR-3/4-induced IFN responses differentially from those of PHV (68, 72, 95) and innate immune responses to SNV are reported to occur independent of viral entry, IRF-3 or characterised pattern recognition receptors (160). Collectively, these findings suggest that additional studies are required to define activation signals and pathways that are induced and regulated by hantaviruses, and that pathogenic hantaviruses have at least two means for bypassing innate cellular responses (2, 3, 68, 186). The combination of the early regulation of IFN induction and resistance to the late induction of ISGs provides a mechanism for hantaviruses to evade innate cellular responses and permits hantaviruses to effect pathogenic programs.

## Pathogenic hantaviruses induce endothelial cell transcriptional responses

Viruses engage and regulate cellular signalling pathways and elicit specific transcriptional responses in order to successfully infect cells. Approximately five times more genes are upregulated than downregulated following hantavirus infection with noted changes in important chemokines and cytokine responses (68). HFRS has been described as at least a partially immune-mediated disease, and endothelial cell responses involved in immune cell recruitment and regulated by chemokines are likely to play specific roles in HTNV directed pathogenesis (33–35, 176). In fact, HTNV induced a fundamentally different proinflammatory chemokine response from infected endothelial cells than NY-1V and PHV (68). Complement components and a unique set of immune cell recruiting and attachment responses were elicited by HTNV (68), and as a result endothelial cell transcriptional events may direct the deposition and clearance of immune complexes which contribute to HFRS disease.

## Hantavirus receptors

Interestingly, both pathogenic and non-pathogenic hantaviruses infect human endothelial cells (234) suggesting that viral entry is

required but insufficient for pathogenesis. In contrast to the use of  $\alpha_5\beta_1$  integrins for the entry of non-pathogenic hantaviruses, all pathogenic hantaviruses tested thus far use  $\alpha_v\beta_3$  integrins as receptors regardless of whether they cause HPS or HFRS diseases (HTNV, PUUV, SEOV, SNV, ANDV and NY-1V) (63–65, 163). This is rather remarkable given the diversity of hantaviruses, their unique small mammal hosts and their disparate worldwide geographic origins. However, the use of  $\beta_3$  integrins by pathogenic hantaviruses is also consistent with the role of  $\beta_3$  integrins as primary regulators of endothelial cell barrier function and platelet activation.

Differences in integrin use between pathogenic and non-pathogenic hantaviruses was first demonstrated by experiments where vitronectin, the high-affinity ligand for  $\alpha_v\beta_3$ , inhibited infection by pathogenic HTNV and NY-1V but not PHV (65). Antibodies to  $\alpha_v\beta_3$  also inhibited infection of human endothelial cells and VeroE6 cells by pathogenic hantaviruses but not PHV or TULV, further suggesting that differential receptor usage could be responsible for differences in hantavirus pathogenesis. CHO cells transfected with plasmids expressing human  $\beta_3$  integrins ( $\alpha_v\beta_3$  or  $\alpha_{11b}\beta_3$ ) permitted pathogenic hantavirus infection (65). However, hantaviruses lack RGD sequences known to bind integrins, and this raised the question as to how hantaviruses interact with  $\alpha_v\beta_3$ . The ability of pathogenic hantaviruses to enter cells using a mutant  $\beta_3$  integrin incapable of binding RGD motifs, demonstrated that hantavirus interactions with  $\beta_3$  integrins were RGD-independent and that hantavirus integrin interactions were unique from those of known integrin ligands (63, 65).

Hantavirus use of  $\beta_3$  integrins was found to be species-specific since murine  $\beta_3$  integrin recombinants failed to confer hantavirus infection (163). This may explain why *Mus musculus* is not a hantavirus host or model of hantavirus disease, but also permitted the use recombinant integrins to define human  $\beta_3$  domains that are required for hantavirus infection. The N-terminal 43 residues of human  $\beta_3$  integrin subunits were found to be recognised by pathogenic hantaviruses and to serve as inhibitors of hantavirus infection when expressed and purified (163). The polypeptide recognised by pathogenic hantaviruses is a component of the plexin, semaphorin integrin (PSI) domain and antibodies to the PSI domain also block hantavirus infection (163).

### $\beta_3$ integrin sequence requirements for infection of human endothelial cells

PSI domain residues required for hantavirus binding and infectivity have been defined for NY-1V and ANDV and suggest that individual hantaviruses may have unique  $\beta_3$  receptor binding requirements (Matthys, in press). Changing specific residues within the PSI domain between species has provided an initial understanding of hantavirus  $\beta_3$  integrin specificity. NY-1V infectivity is altered by aspartic acid to asparagine changes in residue 39 of the PSI domain (163). However, for ANDV a L33P mutation within human PSI domains blocks the use of  $\beta_3$  integrins and P33 is present in murine and bovine  $\beta_3$  subunits which are unable to confer hantavirus infection. In contrast a L33Q present in Syrian hamster  $\beta_3$  PSI domains permits ANDV infectivity (Matthys, in press). Thus even pathogenic hantaviruses interact with discrete

PSI domain residues that determine their ability to infect cells. Whether this is important for the person to person transmission of ANDV (48) is unclear. However, these findings are also of interest since human platelet antigen (HPA) 1A and HPA1B are specifically differentiated by the L33P polymorphism (98, 168, 188, 226) within the  $\beta_3$  PSI domain and thus HPA-1 residues may be a determinant of hantavirus infection. Interestingly, the L33P substitution is sufficient to direct immune responses against  $\beta_3$  integrins that contain a different HPA type and result in two auto-immune diseases, Fetomaternal Allo-immune Thrombocytopenia (FMAIT) and Post Transfusion Purpura (PTP) (98, 213, 226). FMAIT and PTP patients display vascular permeability and acute thrombocytopenia similar to symptoms of hantavirus-infected HFRS and HPS patients (98, 207, 213).

Crystal and solution structures revealed that  $\beta_3$  integrins exist in two conformations: a bent, inactive  $\alpha_v\beta_3$  structure which serves as the basal form of the integrin, and an extended ligand binding conformer (122, 123, 195). These observations are consistent with PSI domain binding by pathogenic hantaviruses since PSI domains are present at the apex of the bent  $\alpha_v\beta_3$  integrin conformers (163). Hantavirus binding to bent, inactive  $\alpha_v\beta_3$  integrins also explained why hantavirus infectivity is inhibited, instead of enhanced by  $Mn^{+2}$  which activates  $\alpha_v\beta_3$  (65, 124, 163). These findings also reveal the potential importance of electron microscopy studies indicating that hantaviruses coat the surface of cells days after infection (69). Thus hantavirus binding to bent inactive  $\alpha_v\beta_3$  integrin conformers provides a mechanism by which pathogenic hantaviruses block  $\beta_3$  integrin activation (61) and the integrins function in regulating vascular permeability.

### Pathogenic hantaviruses enhance endothelial cell permeability in response to VEGF

$\beta_3$  integrins are prominent cell surface receptors on endothelial cells, and regulate vascular integrity, permeability and haemostasis (4, 6, 29, 30, 55, 88–90, 193). VEGF was originally identified as a vascular permeability factor which was very effective at directing localised tissue oedema (42–44). In fact, VEGF is a cytokine that is ~50,000 times more effective at directing fluid across vascular barriers than histamine (42). Interestingly, the ecta-domains of  $\beta_3$  integrins form complexes with VEGF receptor 2 (VEGFR2) and  $\alpha_v\beta_3$  normally regulates VEGFR2-directed endothelial cell permeability (14, 42, 43, 80, 88, 164, 167).  $\beta_3$  integrin knockout mice and cells are hyperpermeabilised by VEGF and this demonstrates the role of  $\beta_3$  integrins in regulating VEGF responses and vascular permeability (14, 164, 167).

Endothelial cells are neither permeabilised by hantavirus infection alone nor by the addition of TNF $\alpha$  to hantavirus-infected endothelial cells (64, 102, 194). However, similar to  $\beta_3$  knockouts, pathogenic but not non-pathogenic, hantaviruses enhance endothelial cell permeability in response to VEGF (18– to 23-fold) (64). This occurs days after infection (64) and is consistent with the inactivation of  $\beta_3$  integrin functions (61, 64) and the appearance of hantaviruses coating the cell surface (69). These findings suggest that pathogenic hantavirus interactions with inactive  $\beta_3$  integrins has the potential to disrupt VEGFR2- $\beta_3$  integrin complexes and result in endothelial cells which are pheno-

typically similar to  $\beta_3$  integrin knockouts (14, 42, 43, 80, 88, 164, 167). These results are significant since even a two-fold difference in permeability is a dramatic change in fluid flux across a pressurised vessel (167). Thus viral interaction with inactive  $\beta_3$  integrins provides at least one means for hantaviruses to increase vascular permeability by altering normal endothelial cell functions (8, 10, 31, 38, 58, 61, 64, 80, 164, 167).

Hypoxia is a hallmark of HPS patients (25, 148, 237) and hypoxic conditions induce the synthesis and release of VEGF from endothelial cells (10, 37, 140, 156, 189, 198). Hypoxia transcriptionally induces VEGF and in turn VEGF signaling responses enhance the stability of the hypoxia-inducible transcription factor, HIF-1 $\alpha$ , forming an autocrine loop that further amplifies hypoxia induced VEGF transcriptional responses (10, 37, 156, 189, 198). In fact hypoxia is a fundamental mechanism for permeabilising the pulmonary vascular endothelium and is responsible for high altitude induced pulmonary oedema which results from localised VEGF induction (10, 85, 140, 141, 198). In addition to low ambient oxygen levels, hypoxic vascular conditions can result from a number of events including platelet deposition, thrombosis, and inflammation (24, 140, 141, 156, 198), and this may have an especially significant effect on gas exchange within the pulmonary microvasculature (189). As a result, it is possible that HPS is in part contributed to by hypoxia, VEGF induction and the enhanced permeability of hantavirus infected endothelial cells resulting from  $\beta_3$  integrin dysregulation.

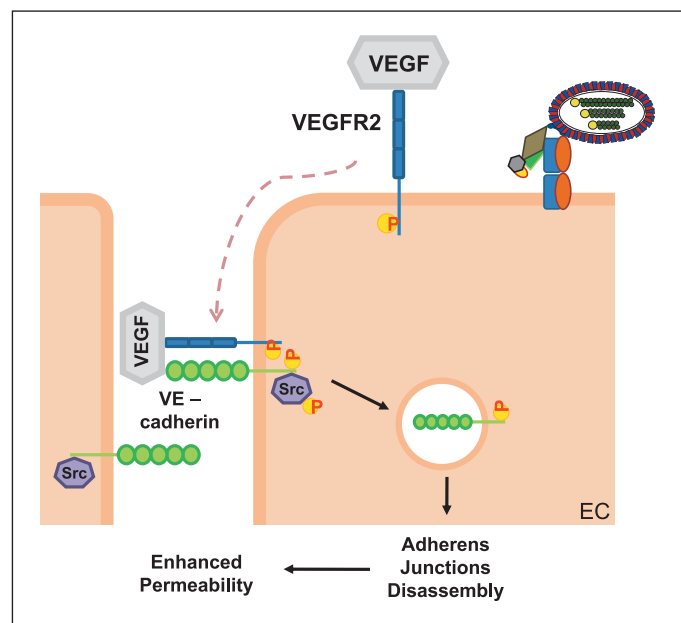
VEGF-directed permeability is further impacted by endogenous and exogenous cellular factors including angiopoietin-1 (Ang1) and sphingosine-1-phosphate (S1P) which regulate endothelial cell permeability through effects on cellular VEGF responses (5, 27, 59, 91, 93, 130, 133, 134, 157, 170, 171, 173, 199, 205, 206, 224). Ang1 is also an endothelial cell-specific growth

factor; however, Ang1 counters the permeabilising effects of VEGF and stabilises the vasculature (5, 93, 94, 130, 157, 171, 205, 206). S1P is a lipid mediator released by platelets that reduces vascular permeability by binding to Edg-1 receptors on endothelial cells (91, 133, 134, 173, 199). S1P enhances the accumulation of vascular-endothelial cadherin (VE-cadherin) within adherens junctions and thereby increases the fluid barrier between endothelial cells (115, 116, 172, 221, 231). Interestingly, addition of Ang1 or S1P to hantavirus infected cells blocked the hyperpermeability of endothelial cell monolayers in response to VEGF and suggests their potential therapeutic importance for hantavirus disease.

## Role of VE-cadherin

Endothelial cell barrier functions are mainly provided by adherens junctions which are composed of the endothelial cell-specific protein, vascular endothelial (VE)-cadherin (115, 116). Genetic deletion of VE-cadherin or mutations which decrease VE-cadherin binding result in increased capillary permeability (38, 58, 115, 116, 221, 223). VEGFR2 activation directs VE-cadherin phosphorylation, dissociation and internalisation and increases endothelial permeability by directing the disassembly of adherens junctions (31, 32, 38, 58, 101, 116, 144, 150, 221, 238).

Recent findings indicate that HTNV and ANDV infection of ECs increased the internalisation of VE-cadherin by approximately two- to seven-fold in response to VEGF. In contrast, non-pathogenic TULV did not enhance VE-cadherin internalisation in the presence or absence of VEGF and hantavirus-enhanced VE-cadherin internalisation was nearly completely abolished by the addition of Ang1 or S1P. These findings suggest that pathogenic hantaviruses enhance the internalisation of VE-cadherin in response to VEGF and thereby dissociate endothelial cell adherence junctions which regulate vascular permeability. A potential model of enhanced hantavirus-enhanced endothelial cell permeability is presented in Figure 1.



**Figure 1: Schematic representation of hantavirus binding to a bent inactive  $\alpha_v\beta_3$  integrin resulting in enhanced VEGF directed VE-Cadherin internalisation, adherens junction disassembly and endothelial cell permeability.**

## Hantavirus infection of endothelial cells recruits quiescent platelets

Hantavirus patients develop severe disease days to weeks after their initial hantavirus exposure, and acute thrombocytopenia is a common element of HPS and HFRS disease (25, 107, 113, 119, 148, 237). Cosgriff et al. demonstrated that platelets from HFRS patients are defective in platelet activation (34), and this implies that thrombocytopenia in HFRS patients results from a block in platelet activation. This could also account for the absence of microthrombosis in lung capillaries of HPS patients (148).  $\alpha_{IIb}\beta_3$  integrins are the most abundant receptors on platelets (80,000 copies/platelet) and direct platelet activation (30). Like  $\alpha_v\beta_3$ , recombinant  $\alpha_{IIb}\beta_3$  integrins confer cellular permissivity to pathogenic hantavirus infection (63, 124) through binding interactions of  $\beta_3$  integrin subunits (163). In fact, platelet inactivation during HFRS is consistent with the effect of hantavirus binding to inactive  $\beta_3$  integrins on platelets (34). As a result pathogenic hantavirus regulation of  $\beta_3$  integrins may impact platelet functions and thereby contribute to thrombocytopenia and enhanced

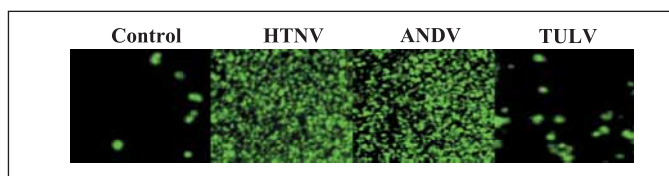
vascular permeability following hantavirus infection (33–35, 41, 64, 148, 163, 237).

## Hantavirus interactions with platelets

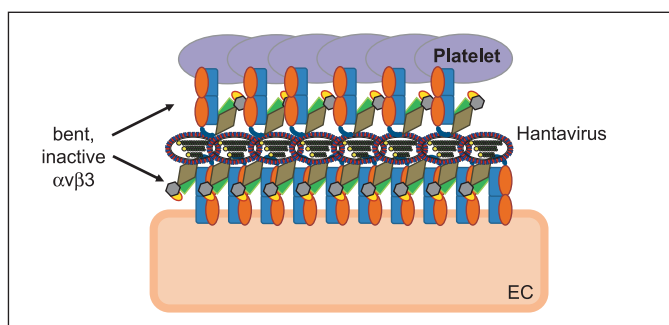
Platelets are normally quiescent and poorly adherent to endothelial cells, but following activation by thrombin, ADP or other factors, platelets are highly adherent to each other, additional ligands and activated endothelium (7, 20, 21, 30, 111, 217). Recent studies demonstrate that pathogenic hantaviruses bind quiescent platelets and that platelet bound hantaviruses remain infectious. Further, quiescent platelets bind specifically to endothelial cells infected with pathogenic HTNV or ANDV, but not to TULV-infected endothelial cells (unpublished observations). Quiescent platelets were bound by infected endothelial cells three days p.i. (Fig. 2) and binding was inhibited by pretreating infected endothelial cells with hantavirus specific neutralising antibody or FAb fragments to  $\beta_3$ . This indicates that platelet adherence is dependent on the presence of hantaviruses on the surface of endothelial cells and is consistent with the presence of cell-associated hantaviruses coating the surface of cells at late times post-infection (69). As schematically suggested by Figure 3, this provides a compelling rationale for quiescent platelets to form a covering layer on the surface of endothelial cells and dramatically change the adherence properties of the endothelium. Within the pulmonary microvasculature platelet covered endothelial cells might also alter oxygen exchange and contribute to hypoxia and HIF-1 $\alpha$ -directed VEGF induction which causes pulmonary oedema (10, 37, 145, 156, 189, 198). As a result hypoxia and thrombocytopenia may both be linked to pathogenic hantavirus dysregulation of  $\beta_3$  integrin functions and contribute to endothelial cell responses that permeabilise the vasculature.

## Potential therapeutics which target endothelial cells

A great deal of progress has been made in the development of hantavirus vaccines (36, 82, 83, 131, 185), and ribovirin and interferon can potentially be used prophylactically against some hantavirus exposures (97). However, there are currently no FDA-approved therapeutics for use against hantaviruses (97). Reports suggesting the effect of S1P, Ang1 and antibodies to VEGFR2 or VEGF on hantavirus-induced permeability suggest that there are potential hantavirus therapeutics that need to be evaluated *in vivo* (8, 17, 28, 64, 81, 87, 133–135, 190). Studies further suggest that pathways downstream of Edg-1, Tie-2 and VEGFR2 receptors or which direct the assembly of adherens junctions may be additional targets for regulating hantavirus-directed permeability (38, 57, 91, 116, 128, 138, 150, 170, 173, 196, 231). It also remains to be determined whether platelet antagonists will have a role in ameliorating hantavirus disease or exacerbate the acute thrombocytopenia and permeability deficits of patients (30). Endothelial cell microRNAs are alluring new therapeutic targets that have the potential to enhance endothelial cell permeability and regulate hantavirus effects on P-bodies, but have yet to be investigated for use against hantaviruses (23, 40, 50, 53, 142, 143, 192, 211).



**Figure 2: Calcein-labelled platelets binding to the surface of mock, HTNV, ANDV or TULV infected ECs, three days p.i.** Gel-filtered platelets were rendered quiescent by PGE2 treatment, calcein AM labelled and bound to cells for 30 minutes. After five washes monolayers were analysed on a fluorescence microscope.



**Figure 3: Model depicting cell-associated hantavirus directing the adherence of quiescent platelets to surface of infected endothelial cells.**

## Future directions

An understanding of hantavirus interactions with endothelial cells and the contribution of endothelial cell responses to pathogenesis are impacted by many factors that still need to be investigated. Studies of hantavirus interaction with platelets will provide insight into the role of platelets in the hantavirus life cycle and potentially reveal the mechanism of hantavirus-directed thrombocytopenia. The role of platelets in altering endothelial cell functions following adherence and transporting hantaviruses systemically are of fundamental importance since both are linked to the regulation of platelet deposition and vascular integrity. Further, platelet endothelial cell interactions following hantavirus infection require investigation since these interactions could be fundamental to localised hypoxia and VEGF induction within the alveolar microvasculature that contributes to pulmonary oedema (198). Additional studies are needed to define viral regulation of signalling pathways that direct IFN induction and endothelial cell permeability as well as cell surface changes in endothelial cell receptors that mediate vascular endothelial cell interactions. The development of reverse genetics systems for modifying hantaviruses is essential for understanding hantavirus interactions with intracellular pathways as well as for defining attachment protein interactions, IFN regulation and the role of specific integrin receptors in pathogenesis.

An understanding of how hantavirus proteins and RNAs interact with endothelial cell P-bodies (137) is just beginning to unfold. However, P-bodies regulate the production of cellular miRNAs and miRNAs 126, let-7a-e and 210 are uniquely linked to vascular integrity, VEGF permeability and hypoxia-induced

responses of endothelial cells (23, 40, 50, 53, 112, 142, 161, 211). As a result, hantavirus P-body interactions have the potential for hantaviruses to alter the unique constellation of endothelial cell miRNAs that regulate vascular integrity (169). It remains to be investigated whether hantaviruses alter endothelial cell miRNAs or whether specific miRNAs can be targeted in order to regulate hantavirus-induced endothelial cell permeability.

## Conclusions and perspective

Hantaviruses are one of only a few viruses that specifically infect the vascular endothelium and correspondingly cause diseases with hallmark vascular barrier deficits. However, the increased vascular permeability caused by hantaviruses is quite distinct from the haemorrhagic disease caused by Ebola and other lytic haemorrhagic viruses. From a viral perspective, hantaviruses infect endothelial cells in order to establish persistence, but instead induce an acute infection which results in endothelial cell dys-

function and vascular permeability. How hantaviruses alter endothelial cell responses, receptor functions, signalling pathways, interactions with additional immune and platelet components remains to be investigated but appears to be fundamental to an understanding of hantavirus-induced endothelial cell permeability that contributes to pathogenesis. Since all factors that increase vascular permeability have an effect on the endothelium, responses and interactions of hantavirus-infected endothelial cells are at the center of hantavirus disease and remain a primary target for therapeutic intervention.

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