

Tula hantavirus infection of Vero E6 cells induces apoptosis involving caspase 8 activation

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Hantaviruses are known to cause two severe human diseases: haemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. The mechanisms of pathogenesis of these two diseases are progressively becoming understood. Recently, two hantaviruses, Hantaan and Prospect Hill were reported to cause programmed cell death of Vero E6 cells. This study shows that Tula hantavirus (TULV) infection efficiently triggers an apoptotic programme in infected Vero E6 cells, and that the replication of TULV is required for the activation of caspase 3 and the cleavage of poly (ADP-ribose) polymerase, two molecular hallmarks of apoptosis. The enforced treatment of infected Vero E6 cells with tumour necrosis factor alpha (TNF- α), but not interferon alpha (IFN- α), advanced the time course of apoptosis. Furthermore, caspase 8 was activated on day 4 post-infection, the same day when caspase 3 was activated. TNF receptor 1 was induced during a late stage of TULV infection. These data suggest that, unlike during influenza A virus infection, TNF- α , but not type I IFN- α/β , may contribute significantly to apoptosis in a synergistic manner with TULV propagation. Interestingly, pretreatment with a broad-spectrum caspase inhibitor, z-VAD-fmk, efficiently inhibited apoptosis of TULV-infected Vero E6 cells. Taken together, these results suggest that TULV replication initiates a typical apoptotic programme involving caspase 8 activation.

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INTRODUCTION

Apoptosis or programmed cell death, is a genetically controlled cell death process and plays an indispensable role not only in physiological conditions in regulation of homeostasis of the multicellular organism development and renewal of tissue, but also in some pathological conditions including neurodegenerative, autoimmune and infectious diseases, such as AIDS. Cell death by apoptosis, distinguished from necrotic cell death, is characterized by chromatin condensation, DNA fragmentation, membrane blebbing, cell shrinkage and compartmentalization of the dead cells into membrane-enclosed apoptotic bodies (Nijhawan *et al.*, 2000; Strasser *et al.*, 2000).

Apoptosis is mainly executed by a distinct group of cysteine proteases termed caspases that cleave substrate proteins after aspartic acid residues (Budihardjo *et al.*, 1999). Caspase activation results in destruction of the whole cell infrastructure leading to characteristic apoptotic morphology. Due to the serious consequences of caspase activation, eukaryotic cells have evolved a number of strategies to control their activation and function. Depending on the type of death insults, apoptosis can be initiated from either extrinsic sources by ligands that bind to the death receptors on the plasma membrane or intrinsic sources like the damage of DNA, endoplasmic reticulum stress and other

serious impairment to cell division or surveillance. In addition, Bcl-2 family members, known as the 'cellular life-or-death switch' and 'mitochondrial guardians', protect cells against both external and internal death insults. They are classified into two groups: pro-survival members like Bcl-2 and Bcl-X_L and pro-apoptotic members like Bax, Bak and others. The relative ratio of pro-survival and pro-apoptotic Bcl-2 family members determines the ultimate sensitivity of cells to a wide variety of stimuli (Chao & Korsmeyer, 1998; Cory & Adams, 2002).

Type I (IFN- α/β) and type II (IFN- γ) interferons (IFNs) are known as antiviral agents that are rapidly produced by different cell types in response to viral infection. IFNs have recently been shown to act as important regulators of virus-induced apoptosis (Barber, 2001; Chawla-Sarkar *et al.*, 2003). IFNs elicit an antiviral state in uninfected cells through the transcriptional activation of antiviral proteins, while in virus-infected cells IFNs induce apoptosis (Tanaka *et al.*, 1998; Balachandran *et al.*, 2000).

Hantaviruses are normally maintained in nature in persistently infected rodent hosts, in which they do not cause disease (Meyer & Schmaljohn, 2000; Plyusnin & Morzunov, 2001). When transmitted to humans, some hantaviruses are known to cause two severe diseases: haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus

pulmonary syndrome (HPS) in the Americas (Vapalahti *et al.*, 2003). Hantaviruses belong to the *Hantavirus* genus of the large *Bunyaviridae* family and are enveloped viruses with a negative-strand RNA genome, tri-segmented as S, M and L, encoding the nucleocapsid protein (N), glycoprotein (Gn and Gc) and polymerase/transcriptase (L), respectively (Schmaljohn *et al.*, 1985, 1987; Kolakofsky & Hacker, 1991; Antic *et al.*, 1992; Plyusnin *et al.*, 1996). The molecular mechanisms of pathogenesis of HFRS and HPS are not understood (Kanerva *et al.*, 1998; Khaiboullina & St Jeor, 2002). Although, global cDNA array analyses (Geimonen *et al.*, 2002) and virus receptor studies (Gavrilovskaya *et al.*, 1998, 1999) have suggested that cell-mediated immunopathogenesis might play a central role. Differences in the regulation of cellular immune responses may reflect the characteristics of hantaviruses. A notable difference is the delayed expression of IFN-inducible genes in endothelial cells infected by pathogenic hantaviruses, but not by apathogenic ones (Geimonen *et al.*, 2002).

It has been reported that hantavirus infection could trigger programmed cell death directly or indirectly in Vero E6 (green monkey kidney) or HEK293 (human embryonic kidney) and lymphopoietic cells (Kang *et al.*, 1999; Akhmatova *et al.*, 2003; Markotic *et al.*, 2003). These studies suggested that apoptosis might play a role in the pathogenesis of hantavirus infection. We have previously shown that hantavirus N protein is able to bind to cellular Daxx, an adaptor molecule of Fas-death-domain (Li *et al.*, 2002). This indicated that during the course of host infection the N protein might interfere with the Fas apoptosis signal pathway.

Yet the molecular details of how hantavirus replication leads to activation of the apoptotic programme have not been defined. Here, we show that Tula hantavirus (TULV; Moravia strain 5302) (Vapalahti *et al.*, 1996) infection efficiently triggers an apoptotic programme in Vero E6 cells, a commonly used cell line for all hantavirus infections in cell culture.

METHODS

Antibodies and other reagents. Rabbit polyclonal antibodies against Bcl-2, Bax and tumour necrosis factor receptor 1 (TNF-R1), and goat polyclonal antibody against Bcl-X_L were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against cleaved poly (ADP-ribose) polymerase (PARP) and caspase 3, and anti-caspase 8 mouse monoclonal antibody were from Cell Signalling Biotechnology. Rabbit polyclonal antibodies against Puumala hantavirus N and G1 and G2 have been described previously (Vapalahti *et al.*, 1995). Recombinant IFN- α and rabbit polyclonal antibody against MxB (Melen *et al.*, 1996) were generous gifts from I. Julkunen (National Public Health Institute, Helsinki, Finland). Mouse monoclonal antibody against actin and tubulin, recombinant human TNF- α and caspase inhibitor z-VAD-fmk were from Sigma.

Virus and cell cultures. TULV Moravia strain 5302 (Vapalahti *et al.*, 1996) was propagated in Vero E6 cells. The Tula virus preparation used to infect cells had a titre of 4.0×10^5 focus-forming units per ml. Vero E6 cells were grown in MEM with 10%

heat-inactivated FCS, 2 mM glutamine, 100 IU penicillin ml⁻¹ and 100 μ g streptomycin ml⁻¹ at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium of Tula virus-infected cells was found to be mycoplasma-free when tested with a highly sensitive PCR ELISA-based mycoplasma detection kit (Roche).

UV irradiation of virus. A stock of virus in a lid-less 3 cm diameter culture dish was irradiated at 254 nm, using a 30 W UV lamp at room temperature at the distance of 10 cm. Exposure time was about 30 min. Then UV-treated and untreated virus stocks were used to infect cell monolayers.

Virus release protocol. Vero E6 cells were infected with Tula virus (m.o.i. 0.1). The growth media of the cells were collected 1–8 days post-infection (p.i.). Dead cells floating in the media were pelleted and the supernatants were transferred to new tubes. Sucrose [30% (w/w)] was pipetted into centrifuge tubes and the virus-containing cell growth media were carefully layered on top of the sucrose. The centrifuge tubes were filled with MEM. Viruses were pelleted through the sucrose cushion (85 000 g, 16 h, 4 °C). Supernatants were discarded and the concentrated virus pellets were dissolved in Laemmli sample buffer. The samples were run in a 10% SDS-PAGE gel, transferred to nitrocellulose filters, blotted with anti-PUUV-N antibodies and the N protein bands were visualized with enhanced chemiluminescence.

DNA laddering protocol. Vero E6 cells were infected with Tula virus (m.o.i. 0.1) or mock infected with growth medium. Following infection (1–8 days), cells were trypsinized, resuspended in MEM with 10% FCS, pelleted and washed with PBS. Cells were resuspended in cell lysis buffer (50 mM Tris/HCl pH 7.5, 0.5% SDS, 20 mM EDTA, 0.5 mg proteinase K ml⁻¹) and incubated for 20 min at 60 °C. Cell debris was removed by pelleting at 15 000 g for 10 min. DNA in the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1; pH 7.4). The DNA was then precipitated on ice for 10 min by adding 3 M sodium acetate pH 5.2, nuclease-free glycogen as a carrier and 2-propanol. DNA was pelleted at 15 000 g for 20 min at 4 °C, washed with 70% ethanol, dried and resuspended in RNaseOne (Ambion) buffer. RNA was degraded with RNaseOne at 37 °C for 30 min. Apoptotic DNA fragments were resolved in a 1.8% TAE agarose gel.

Propidium iodide staining protocol. Vero E6 cells were infected with Tula virus (m.o.i. 0.1) or mock infected with conditional growth medium. Cells were trypsinized 1–8 days p.i., resuspended in MEM with 10% FCS, pelleted and resuspended in PBS. The cells were then fixed by 2.5 times volume of ethanol with constant shaking at –20 °C. To stain the DNA, the cells were pelleted and resuspended in PBS containing 5 μ g propidium iodide ml⁻¹ and 10 μ g RNaseA ml⁻¹. Following an incubation of 1 h, the DNA contents of the cells were analysed at the Flow Cytometry Facility of the Haartman Institute.

Immunoblotting. Briefly, transfected cells (about 1×10^7 cells) were harvested by scraping with a rubber policeman and washed with 10 ml ice-cold PBS twice by centrifugation at 200 g. Next, cells were resuspended in 500–700 μ l buffer A (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM NaCl, 1.5 mM EGTA, 1.5 mM EDTA, 1 mM MgCl₂, 1 mM DTT and a cocktail of protease inhibitors from Roche; sterilized by filtration and stored at 4 °C). The preparations were incubated on ice for 20–30 min and the tubes were tapped from time to time to ensure that the cells remained in suspension. Cells were disrupted by 20–30 strokes with a glass Dounce homogenizer with a tight pestle (B-type). The cell homogenates were centrifuged at 800 g for 10 min at 4 °C. The supernatant containing mitochondria was further centrifuged at 22 000 g for 15 min at 4 °C and the resulting supernatant was stored as cytosolic extract at –70 °C until analysed. The mitochondria-containing pellets were

lysed with 100 µl buffer B [50 mM HEPES pH 7.4, 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM EDTA, 2 mM DTT, cocktail of protease inhibitors from Roche; sterilized by filtration and stored at 4 °C]. Total protein concentration was determined using BCA protein assay kit from Pierce. Finally, about 40 µg protein was analysed by 10% SDS-PAGE and immunoblotted according to standard protocols. Protein concentration was estimated by BCA.

Indirect immunofluorescence. Cells were grown on coverslips in 24-well plates and transfected with Fugene 6 (Roche). Later, the cells were fixed with 3.8% (w/v) paraformaldehyde in PBS for 10 min at room temperature, permeabilized and blocked with 3% BSA, 0.1% Triton X-100 in PBS. Binding of primary antibodies was followed by staining with the secondary antibodies (all from donkey) labelled with fluorescein, Texas red or aminomethylcoumarin acetate (Jackson ImmunoResearch Laboratories). DNA was stained with Hoechst 33342. The patterns of immunostaining were visualized and recorded using Zeiss Axioplan 2 and Axiophot 2 microscopy with a Hamamatsu CCD digital camera.

RESULTS

TULV infection causes characteristic apoptosis of Vero E6 cells

We observed that TULV infection of Vero E6 cells caused detachment of cells within 7 days p.i. indicating decreased cell viability, which did not occur in mock-infected cells. The progression of virus infection was followed 1–7 days p.i. by monitoring N protein released into the culture medium (Fig. 1a) and N protein found in cell lysates (Fig. 1b). The progression of cell death was followed by flow cytometry, and the percentage of cells in the monolayer having a degraded genome increased from 1% 1 day p.i. to 55% 7 days p.i. (Fig. 2b).

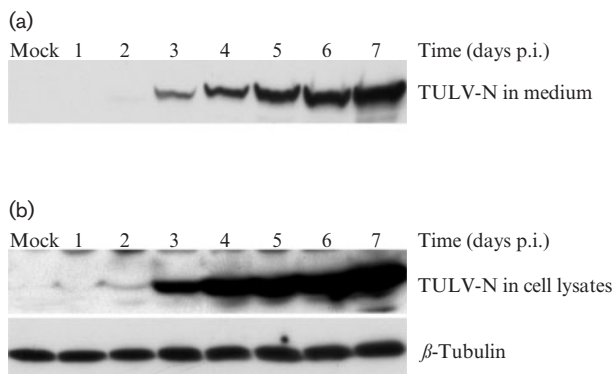


Fig. 1. Immunoblot analysis of N protein synthesis following TULV infection of Vero E6 cells at the indicated days p.i. (a) N protein release into culture medium. Virus particles from culture medium were concentrated by ultra-centrifugation and analysed by immunoblotting with monoclonal antibodies against Tula virus N protein (TULV-N). One third of total viral pellets dissolved in sample buffer was loaded into each lane. (b) N protein accumulation within the cells. Total cell lysates containing 25 µg protein per sample were loaded per lane and β -tubulin was used as an internal quantity marker.

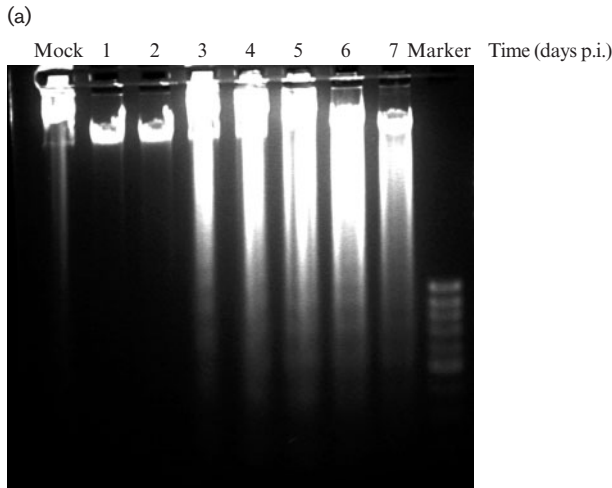
The dramatically decreasing viability of Vero E6 cells during Tula virus propagation, prompted us to determine whether the observed cell death was necrotic or apoptotic. In DNA laddering experiments, as early as 3 days p.i. with a m.o.i. of 0.1, we observed DNA fragmentation characteristic of apoptosis (Fig. 2a). We also employed TUNEL staining of DNA fragmentation (data not shown) and flow cytometry with propidium iodide staining of DNA (Fig. 2b) to confirm this observation. A sub G1 cell fraction, which indicates the existence of apoptotic bodies, was clearly seen and became dominant in infected cells, shown with an arrow in Fig. 2(b), but was not seen in mock-infected cells. Flow cytometry revealed that in Tula virus-infected cells the G1/G2-ratio was altered (Fig. 2b), which could imply that the cell cycle was affected by infection. We also observed membrane blebbing that often occurs in apoptotic cells (data not shown). Taken together, TULV induced typical apoptosis in Vero E6 cells. Therefore, TULV infection may serve as a good model to investigate how the replication of hantaviruses interacts with host-cell apoptotic programmes.

Caspase 3 activation and PARP cleavage in Vero E6 cells dependent on the replication of Tula virus

The direct involvement of caspases in hantavirus-induced apoptosis has not yet been described. Caspase activation plays a central role in apoptotic cell death (Budihardjo *et al.*, 1999; Earnshaw *et al.*, 1999). The cleavage of the nuclear 116 kDa PARP protein to a smaller 85 kDa inactive form is regarded as a hallmark of caspase-mediated apoptosis. Inactivation of PARP through proteolytic cleavage facilitates chromosomal DNA fragmentation that is generally considered a part of the downstream apoptotic programme. As shown in Fig. 3(a), immunoblot analysis of TULV-infected Vero E6 cells (m.o.i. 0.1) revealed that a substantial amount of PARP cleavage (85 kDa form of PARP) was visible 5 days p.i.; the activation of caspase 3 took place 1 day prior to the enhanced PARP cleavage at 4 days p.i. Next, we asked whether virus replication is required for the activation of caspase 3 and PARP cleavage, and found that UV-inactivated Tula virus did not give these effects (Fig. 3b). To investigate further the involvement of caspases, a broad-spectrum caspase inhibitor, z-VAD-fmk, was used to study its ability to block the Tula virus induced apoptosis. At 100 µM it efficiently prevented the cleavage of PARP as shown in an immunoblot (Fig. 4a). z-VAD-fmk also prevented apoptosis as shown by TUNEL staining (Fig. 4b). TUNEL and anti-TULV N protein double staining demonstrates that cell death occurs in the Tula virus-infected cells, and not bystander cells (Fig. 4b).

Regulation of Bcl-2 family members during TULV infection

Previously, Bcl-2 downregulation has been suggested to contribute to both Hantaan and Prospect Hill virus-induced apoptosis in Vero E6 cells (Kang *et al.*, 1999). Due to the fact that balance between the functions of anti- versus



pro-apoptotic Bcl-2 family members is critical for the commitment of cells to apoptosis, we followed the expression levels of three key members of the Bcl-2 protein family during TULV infection (m.o.i. 0.1). Shown in Fig. 5, we found that TULV regulated the isoforms of Bcl-2, Bcl-X_L and Bax (Tsujiimoto & Croce, 1986; Oltvai *et al.*, 1993) differently. Infection suppressed only non-membrane forms of Bcl-2 and Bax, Bcl-2 β and Bax α , while other isoforms, Bcl-2 α , Bax β and Bcl-X_L, remained unaltered. Bcl-2 family functions involve homo- and heterodimerizations and further studies are needed to explain the functional significance of the present findings, which seemingly would lead to an impairment of anti-apoptotic potential.

IFN- α does not contribute significantly to TULV-induced apoptosis of Vero E6 cells

Hantavirus infections regulate IFN-inducible genes including MxA and MxB (Temonen *et al.*, 1995; Geimonen *et al.*, 2002; Nam *et al.*, 2003), and to some extent their replication can be inhibited by type I IFNs. The role of type I IFNs as apoptosis modulators has recently emerged (Barber, 2001; Chawla-Sarkar *et al.*, 2003). In many virus infections, e.g. in the case of influenza A, IFNs are required for the initiation of apoptosis in cell culture. In view of the prominent role of IFNs in virus–host interactions, we sought to evaluate the role of IFNs in hantavirus-induced apoptosis. Firstly, we tested, using external IFN- α , whether the MxB protein could serve as an indicator of endogenous IFN activity (Fig. 6a), and found that it did. Once we had set up this assay, we proceeded to follow the endogenous level of MxB at various time points during hantavirus infection and could not detect any apparent induction of endogenous MxB (Fig. 6b). With the MxB induction in Fig. 6(a) as a reference, IFN activity induced during TULV infection was lower than the externally induced effect by 20 IU ml⁻¹. Secondly, we treated Tula virus-infected Vero E6 cells with two concentrations of IFN- α (20 or 100 IU ml⁻¹) to see if this would accelerate the course of apoptosis. Fig. 6(c) shows that IFN- α treatment caused no significant increase

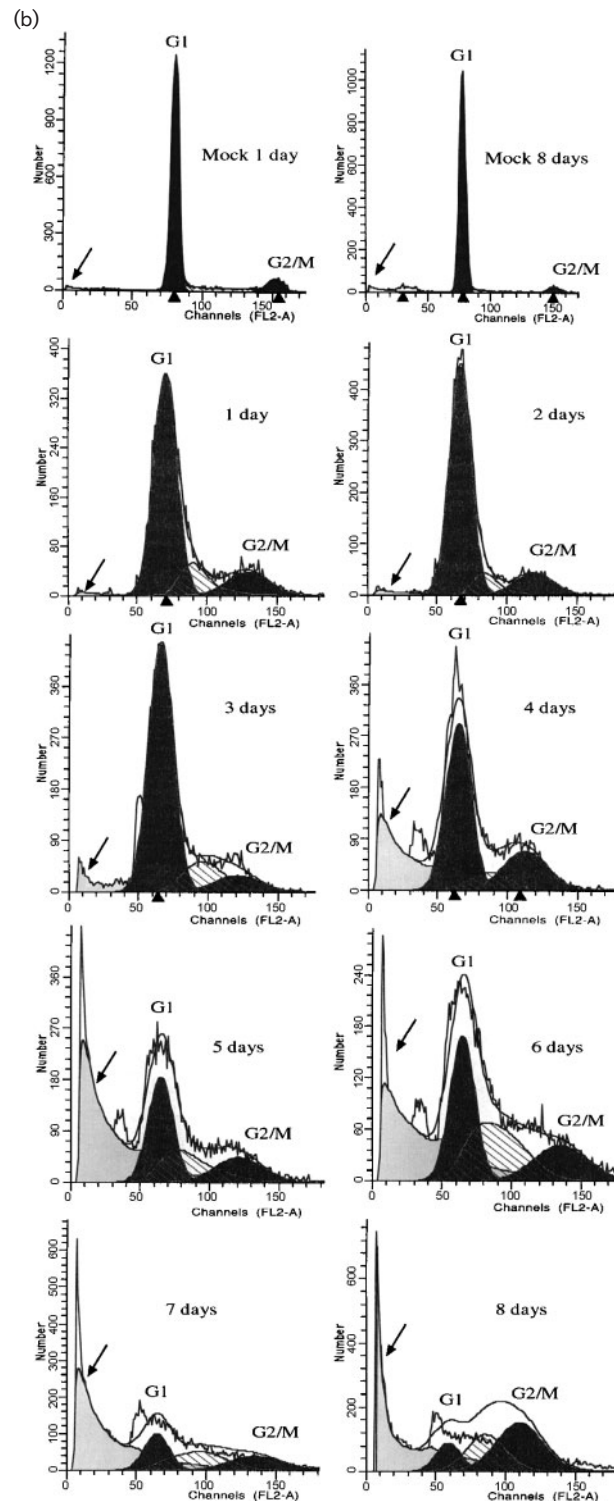


Fig. 2. TULV (Moravia strain 5302) (m.o.i. 0.1) induced apoptosis of Vero E6 cells. (a) DNA fragmentation induced by TULV infection. Lanes from left to right represent the uninfected cells (Mock), infected cells 1–7 days p.i. and DNA marker. (b) FACS analysis of Tula virus mock-infected and infected Vero E6 cells at 1–8 days p.i. with propidium iodide staining. Each day post-infection, Vero E6 cells were collected for flow cytometry analysis. Sub G1 peak marked with an arrow indicates the apoptotic cell population.

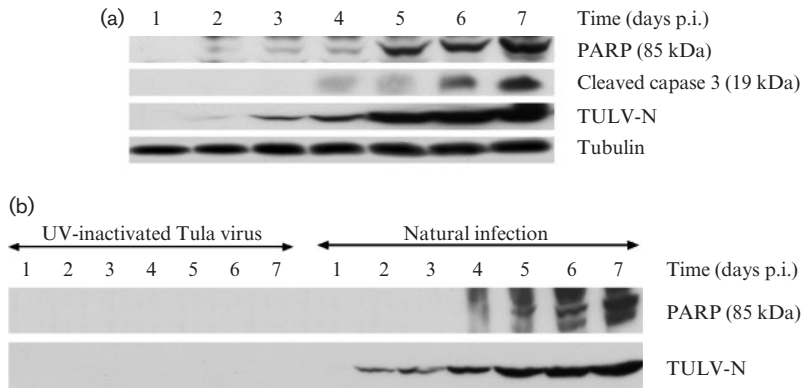


Fig. 3. TULV infection triggered PARP cleavage and activation of caspase 3. (a) Vero E6 cells in 10 cm diameter plates were infected (m.o.i. 0.1) and samples were prepared each day post-infection. The cleaved form of PARP (85 kDa), activated cleaved caspase 3 (19 kDa) and expression of TULV-N are shown. Tubulin expression is provided as an internal sample quantity control. (b) Absence of PARP activation in cells treated with UV-inactivated Tula virus.

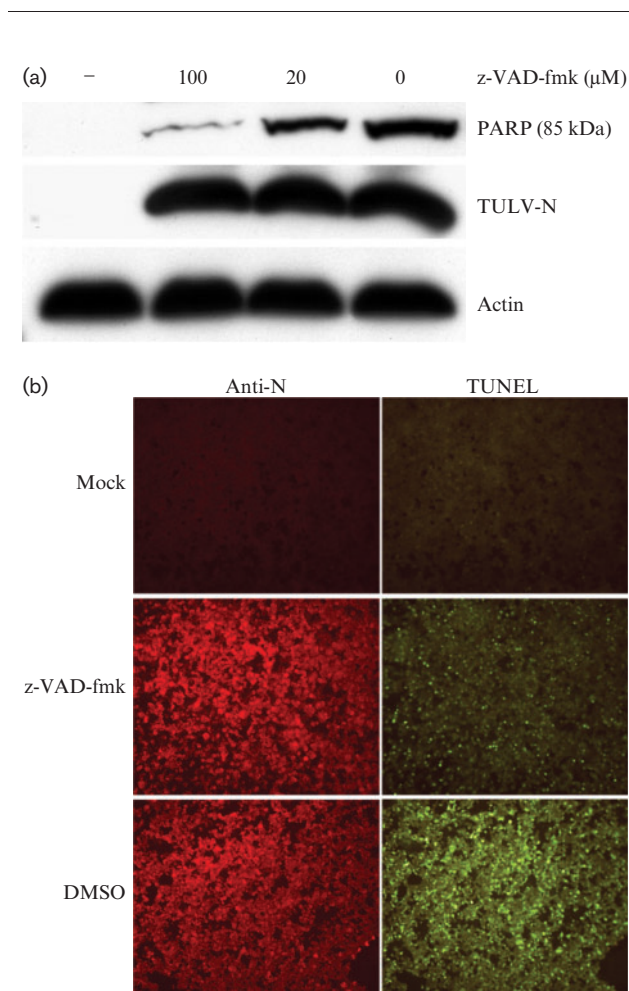


Fig. 4. Inhibition of apoptosis by z-VAD-fmk. (a) Immunoblot analysis shows the inhibition of PARP cleavage by z-VAD-fmk. z-VAD-fmk caspase inhibitor concentrations used in the experiment are indicated and immunoblots of PARP along with TULV-N protein are shown. Actin is provided as an internal marker of total cell sample quantity of SDS-PAGE. (b) Inhibition of apoptosis by TUNEL staining of TULV-infected Vero E6 cells with (middle panel) and without (bottom panel) treatment at 100 μ M z-VAD-fmk. Mock controls of anti-TULV-N protein and TUNEL stainings are provided (top panel).

in PARP cleavage in infected cells. Taken together, these data suggest that IFN- α does not play a critical role in TULV induced apoptosis, but we cannot rule out its regulatory role in prevention of apoptosis.

Involvement of TNF-R1 mediated signal pathway in Tula virus-infected Vero E6 cells

Hantavirus infections of humans typically lead to elevated cytokine levels of which most attention has been given to TNF- α (Linderholm *et al.*, 1996; Temonen *et al.*, 1996; Mori *et al.*, 1999; Sundstrom *et al.*, 2001). We have shown here that after a hantavirus infection, TNF-R1 is upregulated and that an enforced treatment of infected cells with recombinant human TNF- α enhanced the TULV-induced apoptosis of Vero E6 cells (Fig. 7a and b). The TNF superfamily of ligands and receptors is critical for host-cell defence against viral invasion since they regulate both cell death and survival. Several viruses have been found to modulate TNF superfamily mediated signal pathways (Benedict *et al.*, 2003). A slight increase of TNF-R1 mRNA has been observed in hantavirus-infected HEK293 cell line (Markotic *et al.*, 2003). To evaluate the role of TNF signalling of TULV-induced apoptosis, we conducted two experiments: one

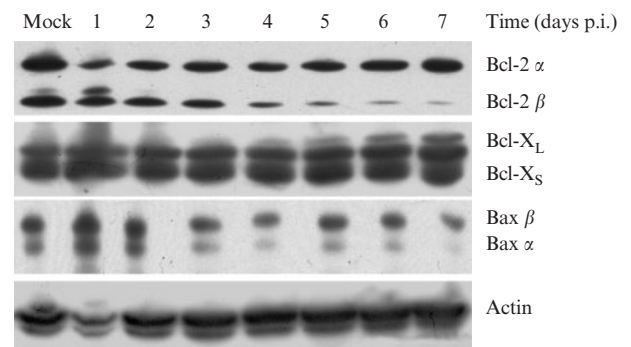


Fig. 5. Time course of expression of Bcl-2 and related molecules Bax and Bcl-X_L during TULV infection (m.o.i. 0.1) of Vero E6 cells. Immunoblots of infected cell lysates were made as indicated using actin expression as an internal marker of sample quantity of SDS-PAGE.

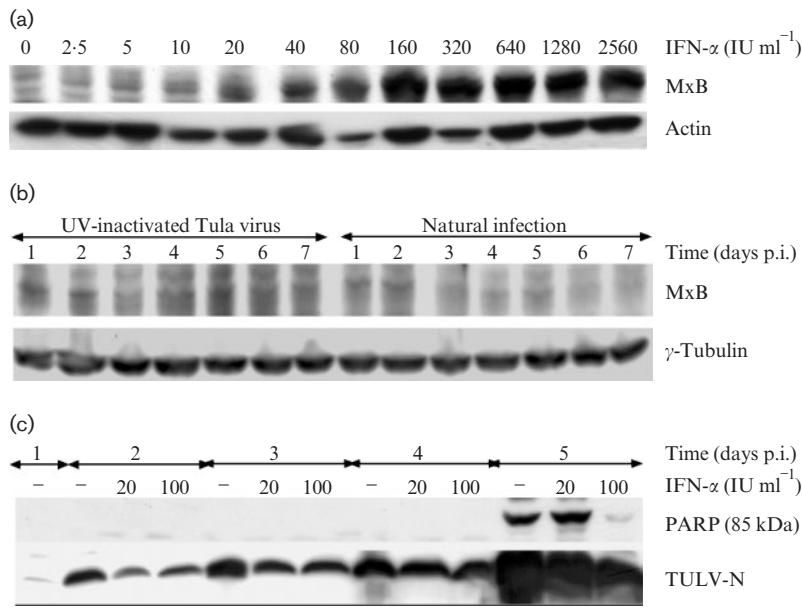


Fig. 6. Role of IFN- α in TULV-induced apoptosis of Vero E6 cells. (a) Titration of IFN- α effects on Vero E6 cells using MxB protein level as an indicator. Increased concentrations of IFN- α from 2.5 to 2560 IU ml⁻¹ were added to culture medium and cell cultures were incubated for 24 h. Cells were collected for immunoblot analysis with MxB rabbit polyclonal antibody. Actin expression is provided as an internal marker of sample quantity of SDS-PAGE. (b) Endogenous MxB level in TULV-treated (UV-inactivated virus) and TULV-infected Vero E6 cells between 1–7 days p.i. (c) External IFN- α induction of PARP cleavage. IFN- α (0, 20 or 100 IU ml⁻¹) was added to the culture medium of TULV-infected Vero E6 cells (m.o.i. 0.1) on day 2 p.i. Samples were prepared and analysed by immunoblotting with MxB antibody.

was to follow the expression level of TNF-R1 by immunoblot analysis, the other was to see the effects of enforced treatment of TNF- α to Vero E6 cells during the course of infection. Fig. 7(a) shows that TNF-R1 was induced 5 days

p.i. by live TULV and not by UV-inactivated virus. Fig. 7(b) shows that treatment of TULV-infected Vero E6 cells with recombinant TNF- α advances the course of apoptosis, as judged by using PARP cleavage as a marker. Furthermore,

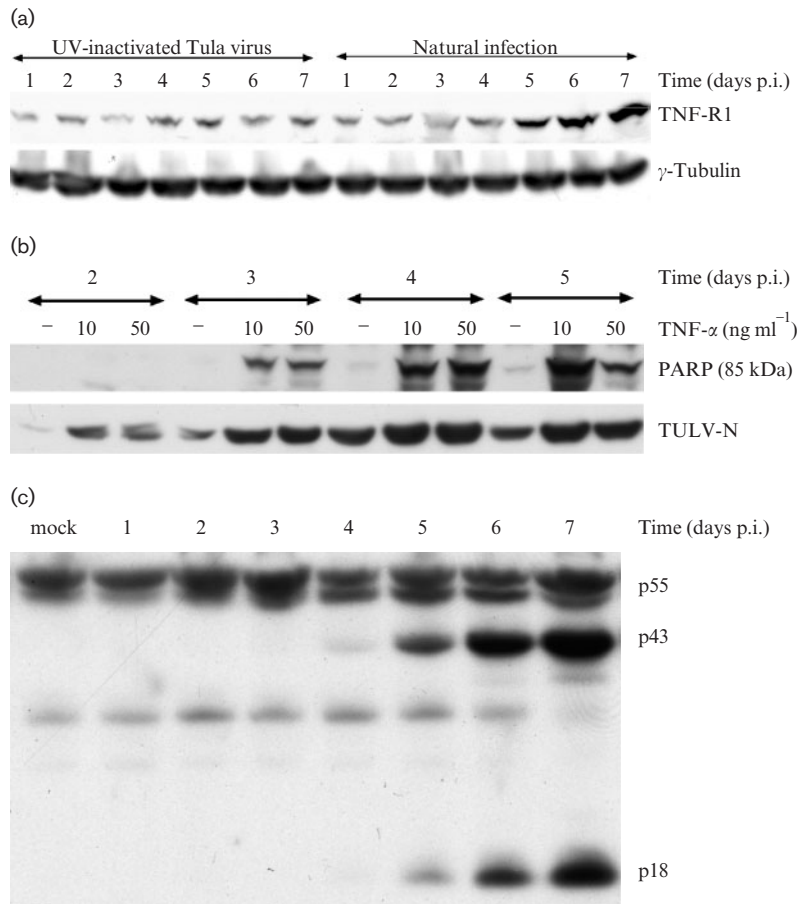


Fig. 7. Involvement of TNF-R1-mediated signal pathways in TULV induced apoptosis. (a) TNF-R1 (p55) in Vero E6 cells following treatment with UV-inactivated virus or TULV infection 1–7 days p.i. The immunoblots show expression of TNF-R1 in cell lysates and γ -tubulin is provided as an internal control of sample quantity of SDS-PAGE. (b) Treatment with TNF- α (0, 10 or 50 ng ml⁻¹) in TULV-infected (m.o.i. 0.1) Vero E6 cells. Cell lysates were collected each day until 7 days p.i. and analysed by immunoblotting with antibodies against PARP and TULV-N protein. (c) The activation of caspase 8. Vero E6 cells were infected with Tula virus (m.o.i. 0.1) and samples were prepared each day post-infection. The procaspase 8 (55 kDa) and the cleaved caspase 8 (43 and 18 kDa) were detected by monoclonal antibody (1C12) against caspase 8.

the involvement of the TNF-R1 signal pathway is supported by the observed activation of caspase 8 (Fig. 7c), which is often induced by members of the TNF receptor superfamily. In summary, TNF-R1 mediated signal pathway has the potential to participate in the TULV triggered apoptosis in Vero E6 cells.

DISCUSSION

The study of virus-induced apoptosis has proved to be very important for better understanding of the mechanisms of pathogenesis of many virus infections. So far, two reports (Kang *et al.*, 1999; Markotic *et al.*, 2003) have documented that hantavirus infection is able to cause programmed cell death directly or indirectly in cultured Vero E6 or HEK293 cells. In addition, apoptosis has been recorded in lymphocytes (Akhmatova *et al.*, 2003). However, the molecular mechanisms that hantaviruses employ in apoptotic pathways are unknown, and the presence of apoptotic cells in infected tissues *in vivo* has not yet been proven. In the present report, we show that TULV (Moravia strain 5302) replication in Vero E6 cells initiates a characteristic apoptotic programme involving caspase 8 activation.

The apoptotic programme is mainly executed by caspases. Caspases have been classified into two groups: upstream initiator caspases such as caspase 8 or 9 that cleave and activate other caspases, and downstream effector caspases including caspase 3, 6 and 7 that are responsible for disassembling the structural components of the cell (Budihardjo *et al.*, 1999; Earnshaw *et al.*, 1999). We found that caspase 8 and 3 are activated (Fig. 7c and Fig. 3a, respectively) during hantavirus infection cycle as early as 4 days after infection with the m.o.i. as low as 0.1. Caspase 3 is a major enzyme in the cleavage of PARP that became evident 5 days p.i. It seems that caspase activation is critical for the following events as we demonstrated that z-VAD-fmk efficiently blocked PARP cleavage. Furthermore, we identified that caspase 8 was activated on day 4 p.i. (Fig. 7c). Caspase 8 is an essential component of the death receptor induced signal complex (known as DISC) including TNF-R1 (Budihardjo *et al.*, 1999). It is worth noting that induction of TNF-R1 was seen on day 5 after Tula virus infection, 1 day later than the activation of caspase 3. This could suggest that TNF-R1 mediated signalling pathway might accelerate apoptosis at a late stage of infection.

TNF- α has multifunctional effects on cells including pro-inflammatory responses and host resistance to viral pathogens. It is produced by many cell types, such as activated macrophages, natural killer cells, endothelial cells, T and B lymphocytes, and epithelial cells (Aggarwal & Vilcek, 1992). Pro-inflammatory cytokines such as TNF- α and IL6 have been implicated in the pathogenesis of both HFRS and HPS (Temonen *et al.*, 1996; Mori *et al.*, 1999). However, the molecular mechanism of their functions in these two diseases is unclear. Here, we sought to determine how

IFN- α or TNF- α mediated signal pathways contribute to the TULV-induced apoptosis of Vero E6 cells. We found that TNF- α , but, surprisingly not IFN- α , induced apoptosis during Tula virus replication in Vero E6 cells. TNF-R1 was upregulated 5 days p.i. when massive apoptosis took place. In addition, enforced treatment of TNF- α significantly advanced the time course of apoptosis. These results suggest that TNF- α , through its interaction with TNF-R1, accelerates the apoptotic process of Vero E6 cells. This is consistent with a recent report; a slight increase of TNF-R1 mRNA in hantavirus induced cytopathic effects and apoptosis in cultured HEK293 cells (Markotic *et al.*, 2003).

The present study provides the first insight into understanding the relationship at the molecular level between a hantavirus and the apoptotic process of host cells. From the study of e.g. influenza virus-induced apoptosis, we have learnt, first of all, to appreciate the delicate relationship between the virus and host (Lowy, 2003). There are many worthwhile questions to investigate further regarding hantavirus-induced apoptosis. For example why do some hantaviruses appear to cause programmed cell death of Vero E6 cells more readily than others? Which, the hantavirus or the host, will benefit from apoptosis? Is apoptosis a mechanism in the pathogenesis of HFRS and/or HPS or is it a mechanism to avoid disease? To answer these questions, TULV as a strong apoptosis inducer may serve as a good model. The ability to induce apoptosis could also represent a more general difference between a pathogenic and pathogenic hantavirus strains leading to variations in the gene regulation of cell-mediated immunity.

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REFERENCES

- Aggarwal, B. B. & Vilcek, J. (editors) (1992). *Tumor necrosis factor: structure, function and mechanism of action*. New York, NY: Marcel Dekker.
- Akhmatova, N. K., Yusupova, R. S., Khaiboullina, S. F. & Sibiryak, S. V. (2003). Lymphocyte apoptosis during hemorrhagic fever with renal syndrome. *Russ J Immunol* **8**, 37–46.
- Antic, D., Wright, K. E. & Kang, C. Y. (1992). Maturation of Hantaan virus glycoproteins G1 and G2. *Virology* **189**, 324–328.
- Balachandran, S., Roberts, P. C., Kipperman, T., Bhalla, K. N., Compans, R. W., Archer, D. R. & Barber, G. N. (2000). Alpha/beta interferons potentiate virus-induced apoptosis through activation of the FADD/caspase-8 death signaling pathway. *J Virol* **74**, 1513–1523.
- Barber, G. N. (2001). Host defense, viruses and apoptosis. *Cell Death Differ* **8**, 113–126.
- Benedict, C. A., Banks, T. A. & Ware, C. F. (2003). Death and survival: viral regulation of TNF signaling pathways. *Curr Opin Immunol* **15**, 59–65.

- Budihardjo, I., Oliver, H., Lutter, M., Luo, X. & Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 15, 269–290.
- Chao, D. T. & Korsmeyer, S. J. (1998). Bcl-2 family: regulators of cell death. *Annu Rev Immunol* 16, 395–419.
- Chawla-Sarkar, M., Lindner, D. J., Liu, Y. F., Williams, B. R., Sen, G. C., Silverman, R. H. & Borden, E. C. (2003). Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 8, 237–249.
- Cory, S. & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2, 647–656.
- Earnshaw, W. C., Martins, L. M. & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 68, 383–424.
- Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. & Mackow, E. R. (1998). β_3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure. *Proc Natl Acad Sci U S A* 95, 7074–7079.
- Gavrilovskaya, I. N., Brown, E. J., Ginsberg, M. H. & Mackow, E. R. (1999). Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by β_3 integrins. *J Virol* 73, 3951–3959.
- Geimonen, E., Neff, S., Raymond, T., Kocer, S. S., Gavrilovskaya, I. N. & Mackow, E. R. (2002). Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses. *Proc Natl Acad Sci U S A* 99, 13837–13842.
- Kanerva, M., Mustonen, J. & Vaheri, A. (1998). Pathogenesis of puumala and other hantavirus infections. *Rev Med Virol* 8, 67–86.
- Kang, J. I., Park, S. H., Lee, P. W. & Ahn, B. Y. (1999). Apoptosis is induced by hantaviruses in cultured cells. *Virology* 264, 99–105.
- Khaiboullina, S. F. & St Jeor, S. C. (2002). Hantavirus immunology. *Viral Immunol* 15, 609–625.
- Kolakofsky, D. & Hacker, D. (1991). Bunyavirus RNA synthesis: genome transcription and replication. In *Bunyaviruses*, pp. 143–159. Edited by D. Kolakofsky & D. Hacker. Heidelberg: Springer.
- Li, X. D., Mäkelä, T. P., Guo, D., Soliymani, R., Koistinen, V., Vapalahti, O., Vaheri, A. & Lankinen, H. (2002). Hantavirus nucleocapsid protein interacts with the Fas-mediated apoptosis enhancer Daxx. *J Gen Virol* 83, 759–766.
- Linderholm, M., Ahlm, C., Settergren, B., Waage, A. & Tarnvik, A. (1996). Elevated plasma levels of tumor necrosis factor (TNF)-alpha, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients with hemorrhagic fever with renal syndrome. *J Infect Dis* 173, 38–43.
- Lowy, R. J. (2003). Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms. *Int Rev Immunol* 22, 425–449.
- Markotic, A., Hensley, L., Geisbert, T., Spik, K. & Schmaljohn, C. (2003). Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells. *J Gen Virol* 84, 2197–2202.
- Melen, K., Keskinen, P., Ronni, T., Sareneva, T., Lounatmaa, K. & Julkunen, I. (1996). Human MxB protein, an interferon-alpha-inducible GTPase, contains a nuclear targeting signal and is localized in the heterochromatin region beneath the nuclear envelope. *J Biol Chem* 271, 23478–23486.
- Meyer, B. J. & Schmaljohn, C. S. (2000). Persistent hantavirus infections: characteristics and mechanisms. *Trends Microbiol* 8, 61–67.
- Mori, M., Rothman, A. L., Kurane, I., Montoya, J. M., Nolte, K. B., Norman, J. E., Waite, D. C., Koster, F. T. & Ennis, F. A. (1999). High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. *J Infect Dis* 179, 295–302.
- Nam, J. H., Hwang, K. A., Yu, C. H. & 7 other authors (2003). Expression of interferon inducible genes following Hantaan virus infection as a mechanism of resistance in A549 cells. *Virus Genes* 26, 31–38.
- Nijhawan, D., Honarpour, N. & Wang, X. (2000). Apoptosis in neural development and disease. *Annu Rev Neurosci* 23, 73–87.
- Oltvai, Z. N., Milliman, C. L. & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74, 609–619.
- Plyusnin, A. & Morzunov, S. P. (2001). Virus evolution and genetic diversity of hantaviruses and their rodent hosts. *Curr Top Microbiol Immunol* 256, 47–75.
- Plyusnin, A., Vapalahti, O. & Vaheri, A. (1996). Hantaviruses: genome structure, expression and evolution. *J Gen Virol* 77, 2677–2687.
- Schmaljohn, C. S., Hasty, S. E., Dalrymple, J. M. & 8 other authors (1985). Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science* 227, 1041–1044.
- Schmaljohn, C. S., Schmaljohn, A. L. & Dalrymple, J. M. (1987). Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order. *Virology* 157, 31–39.
- Strasser, A., O'Connor, L. & Dixit, V. M. (2000). Apoptosis signaling. *Annu Rev Biochem* 69, 217–245.
- Sundstrom, J. B., McMullan, L. K., Spiropoulou, C. F., Hooper, W. C., Ansari, A. A., Peters, C. J. & Rollin, P. E. (2001). Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. *J Virol* 75, 6070–6085.
- Tanaka, N., Sato, M., Lamphier, M. S., Nozawa, H., Oda, E., Noguchi, S., Schreiber, R. D., Tsujimoto, Y. & Taniguchi, T. (1998). Type I interferons are essential mediators of apoptotic death in virally infected cells. *Genes Cells* 3, 29–37.
- Temonen, M., Lankinen, H., Vapalahti, O., Ronni, T., Julkunen, I. & Vaheri, A. (1995). Effect of interferon-alpha and cell differentiation on Puumala virus infection in human monocyte/macrophages. *Virology* 206, 8–15.
- Temonen, M., Mustonen, J., Helin, H., Pasternack, A., Vaheri, A. & Holthöfer, H. (1996). Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. *Clin Immunol Immunopathol* 78, 47–55.
- Tsujimoto, Y. & Croce, C. M. (1986). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci U S A* 83, 5214–5218.
- Vapalahti, O., Kallio-Kokko, H., Närvänen, A. & 7 other authors (1995). Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. *J Med Virol* 46, 293–303.
- Vapalahti, O., Lundkvist, Å., Kukkonen, S. K. & 10 other authors (1996). Isolation and characterization of Tula virus, a distinct serotype in the genus *Hantavirus*, family *Bunyaviridae*. *J Gen Virol* 77, 3063–3067.
- Vapalahti, O., Mustonen, J., Lundkvist, Å., Henttonen, H., Plyusnin, A. & Vaheri, A. (2003). Hantavirus infections in Europe. *Lancet Infect Dis* 3, 653–661.