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# Hantavirus infection in type I interferon receptor-deficient (A129) mice

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**Abstract**

Type I interferon receptor knockout mice (strain A129) were assessed as a disease model of hantavirus infection. A range of infection routes (intramuscular, intraperitoneal and intranasal) were assessed using minimally passaged Seoul virus (strain Humber). Dissemination of virus to the spleen, kidney and lung was observed at 5 days after intramuscular and intraperitoneal challenge, which was resolved by day 14. In contrast, intranasal challenge of A129 mice demonstrated virus tropism to the lung, which was maintained to day 14 post-challenge. These data support the use of the A129 mouse model for future infection studies and the *in vivo* evaluation of interventions.

## INTRODUCTION

Hantaviruses are negative-sense RNA viruses (order *Bunyavirales*, family *Hantaviridae*) reservoired in a wide range of small animal hosts, including rodents, bats, moles, shrews, fish and reptiles [1–3]. At present, only rodent-borne hantaviruses are associated with severe illness in humans, typified by two disease syndromes; hantavirus pulmonary syndrome (HPS) associated with the Americas and haemorrhagic fever with renal syndrome (HFRS) associated with Europe and Asia [4]. However, as more knowledge develops on hantavirus disease it is becoming clearer that many features of HFRS and HPS overlap.

The predominant aetiological agents of HPS are Sin Nombre orthohantavirus (SNV) and Andes orthohantavirus (ANDV), which are endemic in North and South America, respectively. Experimental infection of deer mice, the natural host of SNV, has been used to study tissue tropism, viral load kinetics and therapeutic evaluation [5, 6]; however, no pathological changes were observed in deer mice, limiting their use as an HPS disease model. Experimental infections of ANDV in Syrian hamsters resulted in the development of HPS disease symptoms [7] and this model has become a useful tool to study ANDV pathogenesis [8]. Interestingly, however, Syrian hamsters do not exhibit significant clinical signs following infection with SNV and attempts to adapt SNV by serial passage through hamsters have not led to increased pathogenicity [9]. Other studies have used hamsters that were immunosuppressed with a combination of dexamethasone and cyclophosphamide prior to challenge with SNV and showed evidence of a vascular leak syndrome, mimicking HPS disease in humans [10]. The observation of increased severity in immunosuppressed hamsters has been seen for other similar hantaviruses (Bayou orthohantavirus, Black Creek Canal orthohantavirus, Cano Delgadito orthohantavirus, Choclo orthohantavirus, Laguna Negra orthohantavirus and Maporal orthohantavirus), with lethality rates of up to 100% [11]. More recently, non-human primate (NHP) models of HPS have been sought to advance the preclinical development of therapeutic strategies. To this end, rhesus macaques have been shown to be susceptible to SNV, resulting in thrombocytopenia, leucocytosis and interstitial pneumonia [12].

With respect to HFRS, 150000 cases are reported annually, with case fatality rates ranging from <1–15% [13]. China accounts for 90% of total HFRS cases worldwide, mainly caused by Hantaan orthohantavirus (HTNV) and Seoul

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**Abbreviations:** ANDV, Andes orthohantavirus; DMEM, Dulbecco's Modified Eagle Medium; DOBV, Dobrava-Belgrade orthohantavirus; H and E, haematoxylin and eosin; HFRS, haemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome; HTNV, Hantaan orthohantavirus; i.c., intracranial; i.m., intramuscular; i.p., intraperitoneal; NSG, NOD scid gamma; PBS, phosphate-buffered saline; p.f.u., Plaque forming units; PMN, polymorphonuclear; PUUV, Puumala orthohantavirus; s.c., subcutaneous; SCID, severe combined immunodeficiency; SEOV, Seoul orthohantavirus; SNV, Sin Nombre orthohantavirus.

orthohantavirus (SEOV) [14]. Other hantaviruses, such as Puumala orthohantavirus (PUUV), Dobrava–Belgrade orthohantavirus (DOBV) and related viruses, contribute to the remaining 10% of HFRS, which occurs predominantly in Europe. SEOV has a unique global distribution due to the worldwide dispersal of its reservoir host (*Rattus* sp). Confirmed human SEOV infections have been reported in Asia [Japan [15], Republic of Korea [16], PR China [17], Indonesia [18]), Europe [19] and the Americas (USA [20] and Brazil [21]]. This is a result of its association with the Norwegian/brown rat (*Rattus norvegicus*), a cosmopolitan species, which, through movements accompanying human activities (e.g. trade, travel and migration via railways and through seaports), represents an emerging and widely distributed natural reservoir host of SEOV over much of the globe [22].

Currently, small animal models that faithfully recapitulate human HFRS disease are unavailable. Early pathogenicity studies in suckling mice [23] and rats [24] with HTNV challenge through multiple routes, including intracranial (i.c.), intraperitoneal (i.p.), intramuscular (i.m.) and subcutaneous (s.c.), resulted in lethal disease with widespread viral dissemination characterized by histological lesions in the brain, liver, lung and spleen. The age of the mice was critical to the disease outcome; 100% lethality only occurred in 3-day-old mice, but lethality decreased rapidly with age and it was only 50% lethal in 1-week-old mice and not lethal in 2-week-old animals.

Based on the success of the ANDV/HPS disease model established in Syrian hamsters (*Mesocricetus auratus*), similar studies with HTNV, SEOV, DOBV, or PUUV have been carried out [7, 25]. Unfortunately, each of these experimental infections resulted in asymptomatic disease, limiting their use.

Humanized mice infected with HTNV show progressive weight loss and inflammatory disease reminiscent of an immunopathology and, interestingly, the non-humanized parental NOD *scid gamma* (NSG) strain demonstrated similar viral loads to the hNSG/HLA-A12 mice, but no disease manifestations [26]. Severe combined immunodeficiency (SCID) mice inoculated with SEOV and HTNV died within 35 days after infection [27]. Similarly, 2-week-old mice have been shown to be susceptible to HTNV [28]. Whilst some commonly available laboratory mouse strains (C57/BL6, BALB/c, AKR/J and SJL/J) have been shown to be susceptible to HTNV infection, this has relied on intraperitoneal challenge with a high dose [$10^5$ plaque-forming units (p.f.u.)], unfortunately not mimicking the natural route of infection. Moreover, the cause of severe disease and mortality was manifestly a neurological disorder [29] and unrelated to the clinical sequelae recognized in human HFRS. However, a study focusing on PUUV experimental infection in cynomolgus macaques has provided evidence of HFRS-type disease [30], which despite being mild, represents important progress and suggests future possibilities for the development of new HFRS disease models in NHPs. Nevertheless, the availability of a small animal model that recapitulates HFRS is an important gap that is urgently required to understand pathogenesis and accelerate the design and evaluation of interventions prior to their assessment in NHP disease models and human clinical trials. This is important due to the wide geographical range and ability to cause severe human disease [19, 31, 32], including direct transmission evidence from wild rodent populations [33, 34] and laboratory rat colonies [35]. For SEOV in particular, where recent findings show evidence of efficient transmission to humans from pet rats [36, 37], including from rats bred for exotic pet food in 'feeder units' [38], the need for a small animal disease model is especially acute. A serosurveillance study of UK pet rat owners demonstrated hantavirus-specific antibodies in 34% (27/79) of those owners, the majority of which (26/27) were seropositive for SEOV [39]. Similar zoonotic outbreaks of SEOV associated with pet rats and commercial breeder units have since been reported in humans living and working in close contact with rats from the USA and Canada [40], Belgium [41], Sweden [42], France [43] and the Netherlands [44].

The objective of this work was to investigate the suitability of type I interferon receptor-deficient mice (strain A129) as an informative HFRS or viral kinetics model of SEOV infection. In previous studies these have been shown to be sensitive to a range of different viral pathogens [45–48], enabling evaluation of vaccinology and therapeutics.

## METHODS

### Virus

SEOV (strain Humber) was isolated from a rat associated with human disease by three passages of lung homogenate in VeroE6 cells (European Collection of Cell Cultures, UK) [34]. The virus is widely available through the European Virus Archive goes Global (EVAg) collection [49].

*In vitro* culture of SEOV in many different cell lines does not show cytopathic effects and thus titration by standard $TCID_{50}$ or plaque assay is not possible. Therefore, SEOV was titrated on VeroE6 cells in 96-well plates, alongside heat-inactivated SEOV, using a quantitative RT-PCR readout. This was performed by eight 10-fold serial dilutions in a 96-well dilution plate containing Dulbecco's Modified Eagle Medium (DMEM). A 50 µl aliquot of each dilution was transferred to a 96-well cell culture plate containing VeroE6 cells and incubated at 37 °C for 60 min; the plate was then removed, 50 µl of 20% FBS+DMEM was added to each well, and then the plate was incubated at 37 °C for 11 days. After day 11, the plate was snap-frozen by transferring it to −80 °C. Upon removal and once thawed, each well was resuspended via gentle pipetting. The suspended dilution (100 µl) was transferred to an S-block (Qiagen) containing 300 µl RLT Buffer (Qiagen) and $\beta$-mercaptoethanol (Sigma) and resuspended via pipetting until homogenous. After 10 min of inactivation, 200 µl of absolute ethanol was added per well. A plate cover was then applied to the S-block, it was inverted to ensure homogeneity and fumigated in formaldehyde vapour overnight before being removed from containment level 3 the following morning.

The samples in the S-block were extracted using the BioSprint 96 One-For-All kit (Qiagen) on the Kingfisher Flex purification system, and a SEOV-specific RT-PCR [50] was performed on extracted RNA. In order to increase sensitivity to the SEOV Humber sequence, the degenerative probe SEOV TMGB2 was modified such that nucleotide 9, A, was replaced with R (bold text) to give SEOV TMGBX : F-TCAATGGG**R**ATACAACT-NFQ-MGB.

The reaction composition was as follows: 1 µl of 18 µM SEOV F, 1 µl of 9 µM SEOV R, 0.3 µl of 25 µM SEOV TMGBX probe, 1 µl $MgSO_4$, 10 µl 2× reaction mix from the Superscript III Platinum One-step qRT-PCR kit (Invitrogen), 0.9 µl water, 0.8 µl Superscript III *Taq* and 5 µl of template. Reverse transcription was performed at 50 °C for 10 min, followed by denaturation at 95 °C for 2 min and amplification cycling for 45 cycles at 95 °C for 10 s per cycle; a final extension was performed at 60 °C for 40 s. $C_t$ values for each dilution were acquired.

Spearman–Kärber analysis was performed in order to determine an equivalent $TCID_{50}$ value. A positive result was determined as any $C_t$ value below the lowest $C_t$ value obtained from the heat-inactivated samples.

### Animal experiments

Male mice (aged 6–8 weeks) with deficiencies in their type I IFN receptor (strain A129, Marshalls BioResources, UK) were inoculated with $3\times10^6$ $TCID_{50}$ equivalents via the intramuscular, intranasal or intraperitoneal routes. We based these challenge doses on data from previous studies with SEOV ranging from $10^4$ p.f.u. [51] to $10^6$ $TCID_{50}$ [52], and since our intention was to assess the susceptibility to infection we used concentrations at the higher end of this range.

Animals were monitored for up to 14 days post-challenge with their temperature, weights and clinical signs (i.e. ruffled fur, hunched posture, lethargy) monitored on a daily basis. Temperatures were recorded by indwelling temperature chips. A randomly selected group of animals were culled 5 days post-challenge to assess virus dissemination at this time point. Group sizes are stated in the relevant figure legends and the data are representative of a single biological replicate.

### Measurement of viral burden

At necropsy, samples of blood, saliva, urine, spleen, lung, liver and kidney were collected and immediately frozen at −80 °C for virological analysis. Blood was collected into RNAprotect tubes (Qiagen) and saliva into dry tubes. Tissue samples were placed into tubes containing RNAlater (Qiagen), weighed and homogenized using ceramic beads with an automated homogenizer (PreCellys). Tissue samples and biological fluids (blood, saliva) were extracted using the BioSprint 96 One-For-All Vet extraction kit (Qiagen). The SEOV-specific real-time RT-PCR assay was utilized for the detection of viral RNA using the QuantStudio RT-PCR system. Synthetic RNA was used to construct an eight-point standard curve of $10^7$ to $10^{10}$ copies to enable quantification of genome copies in each sample. Fluorescence was detected in the FAM channel during the extension step and the threshold was set at 1 000 000 ΔRn. Viral RNA was expressed as genome copies $g^{-1}$ or $ml^{-1}$.

### Histological processing

Samples of spleen, lung, liver and kidney were fixed in 10% neutral buffered saline and processed routinely to paraffin wax. Sections were cut at 3–5 µm, stained with haematoxylin and eosin (H and E) and examined microscopically. Pathological changes relating to infection were assessed by a pathologist who was blind to the groups in order to prevent bias.

## RESULTS

### Adult A129 mice do not show clinical signs of disease after challenge with SEOV by any of multiple routes

To assess disease outcomes after exposure to SEOV, A129 mice were challenged with $3\times10^6$ $TCID_{50}$ by either the intraperitoneal, intranasal or intramuscular route. A common sign of illness in rodents is loss of weight, but no substantial or sustained weight loss was observed, and animals increased in weight over the course of the study, indicating that they were in good health (Fig. 1). In addition, temperatures remained stable, with no differences compared to the natural fluctuations seen in the animals given phosphate-buffered saline (PBS) (Fig. 1). Animals were also monitored for clinical signs of disease, with animals scoring normal at all time points.

### Detection of viral RNA in blood and tissues varied with challenge route in the A129 mice

After challenge with SEOV, three mice from each group were culled at day 5 post-challenge and then at the end of the study (day 14 post-challenge) to assess viral burden by RT-PCR in blood and tissues (Fig. 2). A129 mice challenged by the intraperitoneal or intramuscular routes showed widespread distribution of SEOV, with viral RNA detected in the blood, lung, kidney, spleen and liver when culled at 5 days post-challenge. However, by 14 days post-challenge the virus had cleared, and RT-PCR results were all negative. In contrast, A129 mice challenged by the intranasal route had localized RNA signals in the blood and lung, which were maintained at day 14 post-challenge, indicating an established SEOV infection.

### Only minor histological changes were observed in SEOV-challenged A129 mice

No major changes attributable to SEOV infection were observed in any of the groups of the A129 mice challenged. However, hepatocyte degeneration accompanied by polymorphonuclear (PMN) cell infiltration and focal mononuclear cell infiltration were noted in the liver. These were observed with increasing frequency in animals challenged via the intraperitoneal route (Fig. 3a). Occasionally, small necrotic foci were observed in the liver of inoculated animals (Fig. 3b). In addition, animals challenged via the intraperitoneal route showed a very mild increase in the number of PMNs within

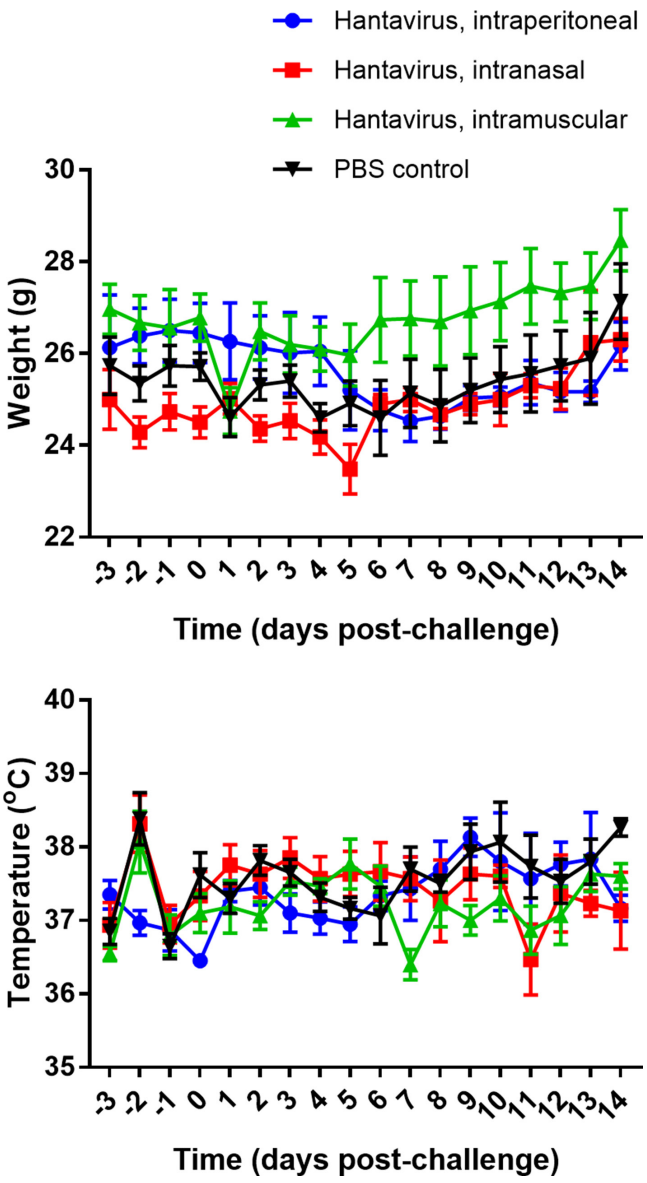


**Fig. 1.** Clinical data from A129 mice challenged with SEOV by different routes. A129 mice were challenged by the intraperitoneal, intranasal or intramuscular routes with $3\times10^6$ $TCID_{50}$ of SEOV. (a) Weight of A129 mice. (b) Temperature of A129 mice. Lines show the mean values ($n$=6/group to day 5 post-challenge and $n$=3/group to day 14 post-challenge), with error bars denoting standard error.

the splenic red pulp in two animals (Fig. 3c) and in the alveolar walls of three animals (Fig. 3d).

## DISCUSSION

SEOV contributes to a significant burden of global zoonosis. Importantly, its efficient aerosol transmission from rodents, which are increasingly kept as pets, makes it a serious public health risk. The development of vaccines and therapies to prevent and treat SEOV-induced HFRS have been hampered by the absence of an informative animal model. Here, we have attempted to address this gap by characterizing experimental infections of SEOV in type I interferon receptor knockout mice (strain A129) to establish a suitable infection or HFRS disease model that could be used to develop and assess new medical countermeasures. While many different studies have been undertaken by many different institutes and groups, the development of an animal model that faithfully recapitulates HFRS type disease caused by SEOV has been fraught with difficulties [52, 53].

Given that the *in vitro* passage history of viruses, including hantaviruses, has been reported to have a dramatic impact on the disease course when used experimentally to infect animals [2], and similarly, that a virus isolated from the wildlife reservoir is often more pathogenic in an experimental animal infection than a virus that has been passaged multiple times in cell culture [7, 12], we sought to use a challenge SEOV stock with minimal passage history that was as close to an isolation from the wildlife reservoir as possible. In addition, three different inoculation routes were assessed: intranasal, intraperitoneal and intramuscular. Intranasal and intraperitoneal routes were used in an attempt to increase the efficiency of infection, especially in the lungs [9]. These routes also mimic the natural route of human exposure through inhalation of aerosolized SEOV in dried excreta or secreta from infected rodents [54, 55]. The intramuscular route was assessed because direct transmission via bites is an important route for the circulation of the virus in rodent populations in nature [1]. Additionally, the transmission of hantavirus infection to humans through biting has also been reported [56].

Whilst Syrian hamster models of hantavirus pulmonary syndrome have been widely reported with ANDV infection [7, 57, 58], these have been difficult to fully characterize because of the deficiency of hamster-specific reagents. Susceptibility to SEOV has been previously assessed by others in Norway (brown) rats [51, 53], suckling mice [59] and Mongolian gerbils [60], with subclinical, disseminated infection seen in most models apart from the suckling mice study in which 100% lethality was observed. Therefore, in order to understand the pathogenesis of disease and ultimately to assess intervention strategies there is still a requirement to assess other adult small animals and HFRS disease models.

SEOV-challenged A129 mice remained healthy. In contrast, HTNV infection induces a rapid course of disease progression after challenge of type I interferon receptor knockout mice with a similar viral inoculum, with animals meeting clinical endpoints by 7 days post-infection [29]. Whilst it is likely that this is due to the differences between SEOV and HTNV, the genetic background of the parental mouse strain may also play a significant role in contributing to the observed pathological differences [61], clearly demonstrated in work on two identical strains of Zika virus [46]. In the current study, animals were procured from an established colony based on a 129S7/SvEvBrd background and were genetically typed. Unfortunately, the parental strain of the mice used in the HTNV experiments was not reported [29], and thus it is not possible to build on this hypothesis.

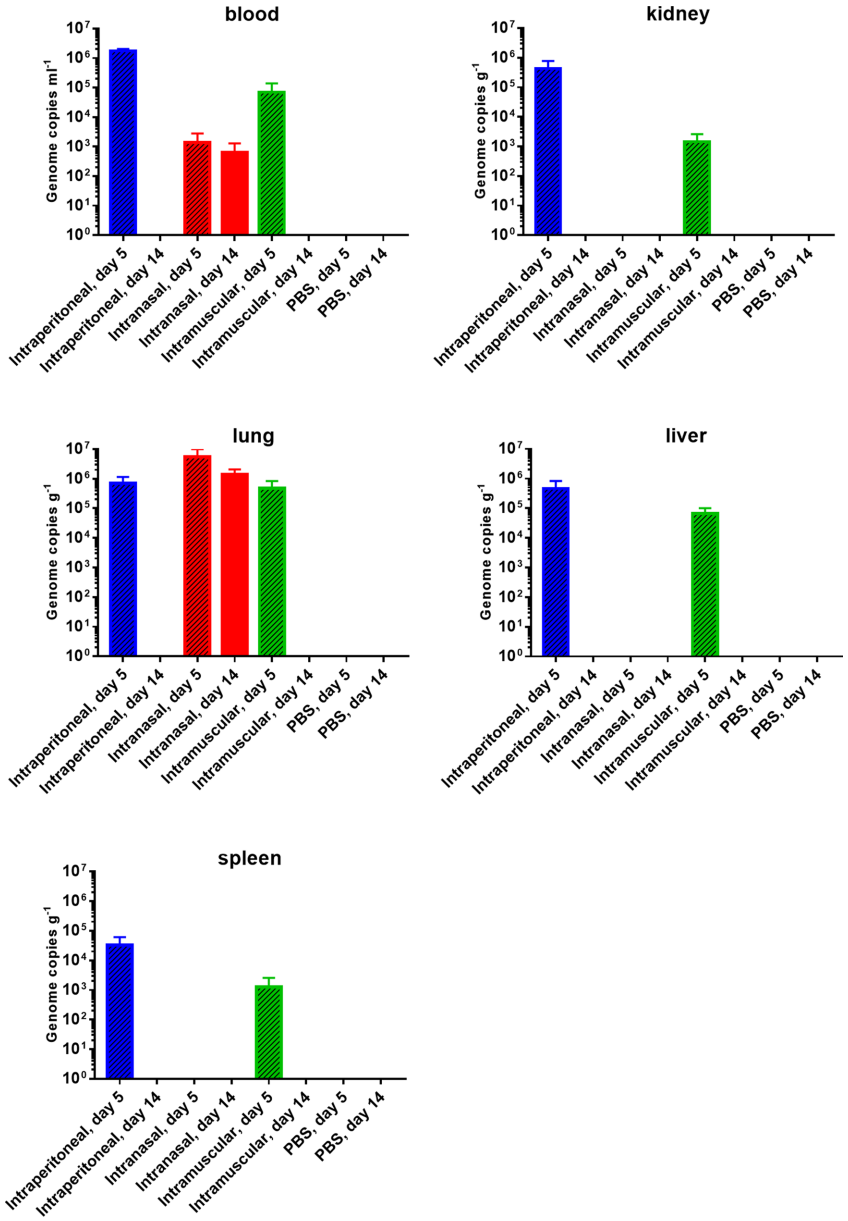


**Fig. 2.** Viral RNA levels in A129 mice challenged with SEOV by different routes. Animals were challenged by the intraperitoneal, intranasal and intramuscular routes with $3\times10^6$ (A129 mice) $TCID_{50}$ of SEOV. At 5 days post-challenge, three A129 mice per group were culled to assess SEOV genome copies in each indicated tissue. Bars show the mean values with error bars denoting the standard error.

In A129 mice, SEOV RNA was widely disseminated to the blood, lung, kidney, spleen and liver, when delivered via the intraperitoneal and intramuscular routes, with a transient infection such that viral RNA had cleared by day 14 post-challenge. However, when delivered via the intranasal route, the primary organ affected was the lung and viral RNA was still at high levels at day 14 post-challenge, demonstrating persistence. While many early reports of SEOV disease have described classic HFRS type features, there is growing evidence from human clinical studies of disease for involvement of the pulmonary system [62–65], confirming overlap between HPS and HFRS disease outcomes. Notably, this evidence underscores that the natural tropism of SEOV observed in this study is recapitulated in A129 mice. Similarly, data from experimental infections of PUUV in Syrian hamsters showed that the highest titre of viral RNA was detected in lung samples, suggesting its suitability for viral replication [25].

Whilst viral RNA was detected in local sites, histopathological changes were minimal in this study. Other groups have reported that infection with HPS-causing hantaviruses in wild rodents is associated with histopathological changes, including pulmonary oedema and periportal hepatitis [66, 67].

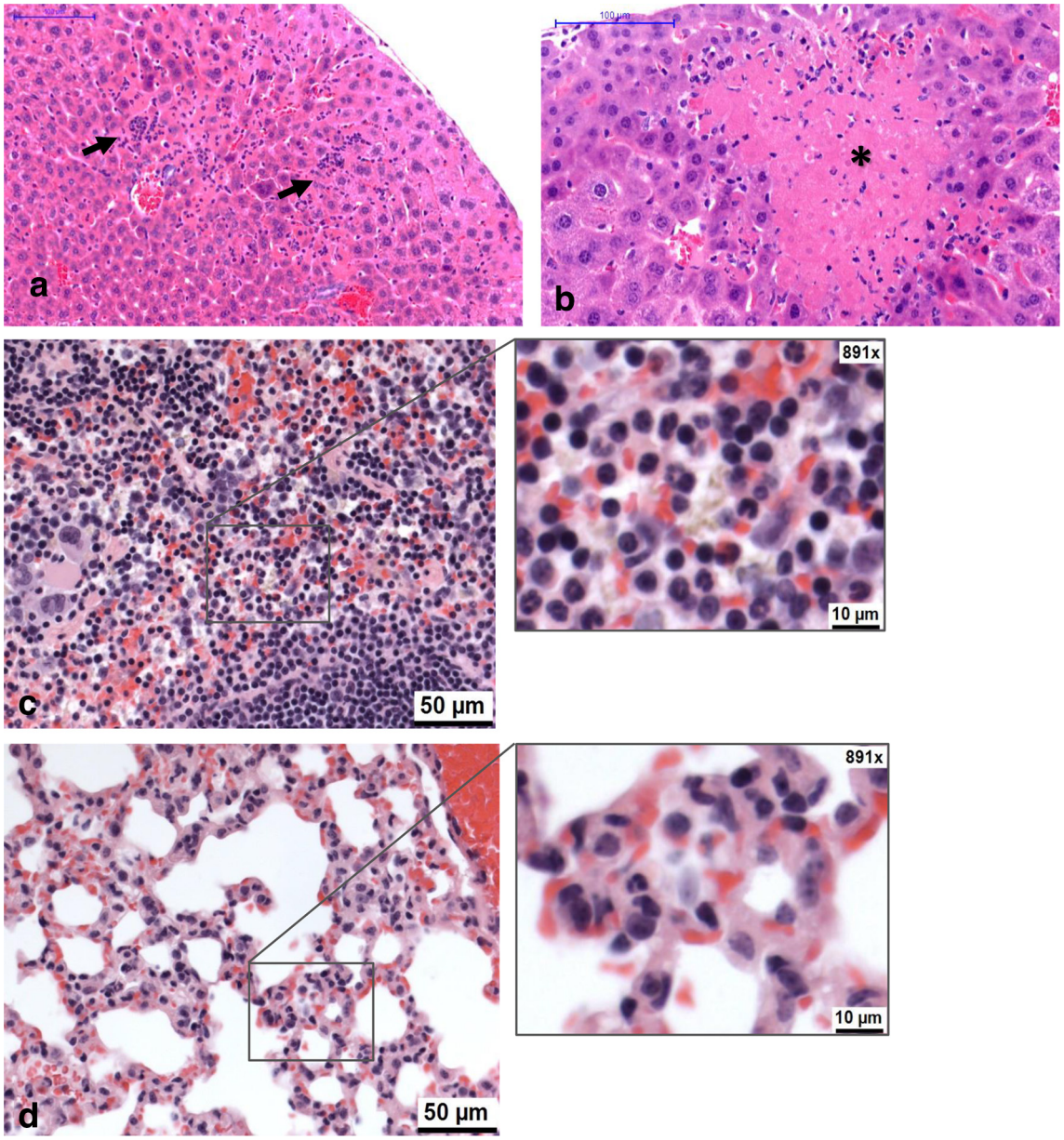


**Fig. 3.** Histological findings in the tissues of A129 mice challenged with SEOV via the intraperitoneal route. (a) Liver. Small foci of hepatocyte degeneration (arrows) and inflammatory cell infiltration (intraperitoneal challenge, day 5). (b) Liver. Small necrotic foci (*) within the liver parenchyma). (c) Spleen. A small increased in mature PMNs scattered diffusely in the red pulp. (d) Lung. A small increase in the prominence of PMNs within the alveolar walls.

However, when using deliberate experimental inoculations of SNV in deer mice, the natural host, consistent pathological changes were not observed [5].

The detection of virus replication in our work, as measured by the accumulation of viral RNA, without pathological changes suggests that virus replication is not the only factor that contributes to disease [8]. Hantavirus infections are associated with vascular leakages that are believed to be primarily immune mediated. Indeed, it is widely believed that there is an immunopathological basis for HFRS disease, as evidenced by the immune complex in kidney [68] and complement activation [69]. In addition, mice models with restricted immune functions, including SCID [27] and newborn models [70], show an early death phenomenon. It has been suggested that SEOV infection of macrophages and lymphocytes could reduce the immune response and thus contribute to persistent infection [71]. Although the A129 mice used in our study had abrogated type I IFN receptors, it is surprising that the uncoupling of the IFN response pathway was not enough to lead to disease, as hantaviruses have evolved mechanisms to

inhibit production of type I IFN upon infection [72, 73]. Thus our data suggest that other pathways must be disengaged in order to exert virulence. This aligns with hantavirus studies utilizing endothelial cells where the presence of at least two cell signalling pathways was demonstrated; one that regulates antiviral IFN signalling and another that enhances the normal hypoxia–VEGF–mTOR signalling pathway, affecting endothelial cell permeability [74].

While our challenge virus in this study was minimally passaged in cell culture, it was nevertheless amplified in Vero-derived cells. It is possible that propagation on this cell line alters the biological properties, as has been observed for PUUV, which was no longer able to reproducibly infect bank voles, its natural reservoir [75]. As such, challenge materials for NHP studies have been sourced by exclusively passaging the hantavirus in the natural rodent reservoirs [12, 30]. However, regulatory requirements to carefully limit the number of animals used in scientific research, as part of the NC3Rs approach (National Centre for Reduction, Replacement and Refinement) [76], mean that the use of live animals for viral propagation is difficult to justify. Furthermore, the inherent differences in batches of virus stocks incorporate additional variations in challenge preparations. Whilst consideration for a batch of virus to be produced by a single institute and deposited in an accessible biobank collection, e.g. the European Virus Archive (EVA) [49], may help circumvent some of the issues, alternatives should be considered. Instead of using generic cell lines for virus propagation, the use of cell lines from the reservoir host should be considered for future work, for example those available from bank voles [77].

Whilst A129 mice are deficient in their type I interferon receptor, the type II interferon responses associated with adaptive immune responses are unaffected [78]. These mice therefore retain a utility for vaccine studies and are capable of demonstrating protective responses from lethal viral infections such as Crimean–Congo haemorrhagic fever virus [79]. Transgenic mice are widely available and are more amenable to inducing immunosuppression by other means. Chemical immunosuppression of hamsters has demonstrated the extension of disease parameters after challenge with SNV which results in a vascular leak syndrome [10]. Whilst similar approaches have been used for other viruses, including West Nile virus [80] and severe acute respiratory syndrome (SARS) [81], regular treatment is required to maintain immunosuppression. For the SNV study [10], drugs were delivered intraperitoneally with three loading doses before challenge, followed by subsequent daily dosing up to 13 days post-challenge. Therefore, adverse effects from the multiple injections may interfere with natural disease progression, as it is likely that an inflammatory immune response at the injection sites will occur. In addition, the contribution of the immune system is compromised due to the immunosuppressive agents affecting a range of immune cell types and thus limiting the potential use of the model in vaccine efficacy studies.

Medical countermeasures for hantaviruses are urgently required. The growing public health burden of these zoonotic agents is increasing due to (i) rising rates of urbanization, for example in China, where such conditions favour prolonged hantavirus epidemics due in part to the higher population volumes and poor living conditions [82] where rats thrive and (ii) increased interest in pet rats and other exotic companion animals that require large-scale production of rats for food. Animal models will increasingly be required as the pipeline of vaccines and other interventions against hantaviruses require efficacy testing. These new vaccines include pan-hantavirus DNA vaccines [83].

Whilst the development of a disease model brings the useful benefit to characterizing and understanding pathogenesis, including better definition of experimental end points, faithful reproduction of human disease in a small animal is not always possible. In the hantavirus field, even the most commonly used hamster models challenged with doses as high as 20000 p.f.u. do not always develop disease [7]. Our results demonstrate that adult A129 mice show evidence of SEOV infection after challenge, with animals challenged intranasally showing a tropism to the lung that is maintained for at least 14 days. This model offers a useful approach to assess antivirals and vaccine candidates against SEOV.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All procedures with animals were undertaken according to the UK Animals (Scientific Procedures) Act 1986. These studies were approved by the ethical review process of Public Health England, Porton Down, UK, and by the Home Office, UK, via an establishment licence (PEL PCD 70/1707) and project licence (30/3147).

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