African horse sickness virus NS4 protein is an important virulence factor and interferes with JAK-STAT signaling during viral infection

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Abstract

African horse sickness virus (AHSV) non-structural protein NS4 is a nucleocytoplasmic protein that is expressed in heart, lung, and spleen of infected horses, binds dsDNA, and colocalizes with promyelocytic leukemia nuclear bodies (PML-NBs). The aim of this study was to investigate the role of AHSV NS4 in viral replication, virulence and the host immune response. Using a reverse genetics-derived virulent strain of AHSV-5 and NS4 deletion mutants, we showed that knockdown of NS4 expression has no impact in cell culture, but results in virus attenuation in infected horses. RNA sequencing (RNA-seq) was used to investigate the transcriptional response in these horses, to see how the lack of NS4 mediates the transition of the virus from virulent to attenuated. The presence of NS4 was shown to result in a 24 hour delay in the transcriptional activation of several immune system processes compared to when the protein was absent. Included in these processes were the RIG-I-like, Toll-like receptor, and JAK-STAT signaling pathways, which are key pathways involved in innate immunity and the antiviral response. Thus, it was shown that AHSV NS4 suppresses the host innate immune transcriptional response in the early stages of the infection cycle. We investigated whether AHSV NS4 affects the innate immune response by impacting the JAK-STAT signaling pathway specifically. Using confocal laser scanning microscopy (CLSM) we showed that AHSV NS4 disrupts JAK-STAT signaling by interfering with the phosphorylation and/or translocation of STAT1 and pSTAT1 into the nucleus. Overall, these results showed that AHSV NS4 is a key virulence factor in horses and allows AHSV to overcome host antiviral responses in order to promote viral replication and spread.

Keywords

African horse sickness virus, AHSV, NS4, JAK-STAT signaling, innate immunity

1. Introduction

African horse sickness (AHS) is a noncontagious, highly infectious vector-borne disease that can have an up to 95% mortality rate in horses (Mellor & Hamblin 2004). AHS is endemic to sub-Saharan Africa and the Arabian Peninsula, but has also caused epidemics in areas such as Spain, Portugal and India (Howell 1962; Mellor & Hamblin 2004; Carpenter et al. 2017). In the first half of 2020, an outbreak of AHS occurred in Thailand, the first of its kind in that country (OIE 2020). AHS is caused by African horse sickness virus (AHSV), a member of the *Orbivirus* genus (family *Reoviridae*) of which bluetongue virus (BTV) is the prototype virus. The disease manifests in different forms ranging in severity, and the main target organs of AHSV are the heart, lungs, and spleen (Clift et al. 2009; Clift & Penrith 2010). The incidence of AHS and its geographical distribution are linked to the availability of

the virus and susceptible hosts, but also to the presence of competent vector species, in this case certain species of *Culicoides* biting midges (Du Toit 1944; Venter et al. 2000). Due to its severity, AHS is a World Organization for Animal Health (OIE)-listed disease. As a result, strict control measures are in place to restrict the circulation of AHSV. This causes a substantial economic burden on affected countries, in addition to the huge impact the disease has on animal health (Zientara et al. 2015).

The AHSV virion is a non-enveloped triple-layered icosahedral particle that contains 10 double-stranded RNA (dsRNA) segments (Seg-1 to Seg-10) that encode 7 structural proteins (VP1-7) and 4 non-structural proteins (NS1-4) (Oellermann et al. 1970; Verwoerd et al. 1972; Bremer 1976; Roy 1996; Manole et al. 2012; Zwart et al. 2015). The viral core is composed of two concentric layers of VP3 and VP7 (Grimes et al. 1998), and houses the ten dsRNA segments as well as VP1, VP4 and VP6 which form the enzymatic machinery of the core (Urakawa et al. 1989; Stauber et al. 1997; Ramadevi et al. 1998). The core is surrounded by a diffuse outer capsid layer composed of VP2 and VP5 (Hewat et al. 1992; Manole et al. 2012), with VP2 being the most variable AHSV protein and main determinant of serotype (Potgieter et al. 2003).

In southern Africa, AHS is controlled by the use of a live attenuated vaccine (LAV). While this LAV provides protection against all AHSV serotypes, it has safety limitations, does not allow for the differentiation of infected from vaccinated animals (DIVA), and has not been licensed outside of Africa (Mellor & Hamblin 2004; Weyer et al. 2016). Other vaccine strategies that have been explored include inactivated, recombinant and sub-unit vaccines (reviewed in Calvo-Pinilla et al. 2020), however none of these have reached the market. The recent development of reverse genetics systems now allows for the rescue of live AHSV vaccine candidates engineered by design. This system has been used to develop Entry Competent Replication Abortive (ECRA) (formerly known as disabled infection single cycle, DISC) and AHS disabled infectious single animal (DISA) vaccine candidates (van de Water et al. 2015; Lulla et al. 2016; Lulla et al. 2017; van Rijn et al. 2018) that are DIVA compliant and have the potential to be used as a platform for all serotypes (Calvo-Pinilla et al. 2020).

During AHSV infection, four non-structural proteins are expressed that play key roles in the viral replication cycle. The NS1 protein of orbiviruses assembles into hollow tubular structures (Huismans & Els 1979), and BTV NS1 has been shown to be a positive regulator of viral protein synthesis (Boyce et al. 2012; Kerviel et al. 2019). NS2 forms cytoplasmic viral inclusion bodies (Thomas et al. 1990; Uitenweerde et al. 1995), which are the sites of viral assembly (Kar et al. 2007). NS3/A are involved

in viral trafficking and release (Beaton et al. 2002; Celma & Roy 2009; Celma & Roy 2011; Ferreira-Venter et al. 2019), and BTV NS3 acts as an interferon (IFN) antagonist (Chauveau et al. 2013). The recently discovered NS4 protein is the smallest of the AHSV proteins (144 to 154 amino acids (aa), and its function in the viral replication cycle remains unclear. NS4 is encoded from a second open reading frame on Seg-9, overlapping but out-of-phase with that of VP6, and is present in all AHSV serotypes (Firth 2008; Belhouchet et al. 2010; Belhouchet et al. 2011; Ratinier et al. 2011; Zwart et al. 2015). BTV NS4 is highly conserved (Ratinier et al. 2011), however two clades of AHSV NS4 exist, i.e. NS4-I and NS4-II, with only 52% aa identity between the clades (Zwart et al. 2015).

Whilst AHSV replicates exclusively in the cytoplasm, NS4 is a nucleocytoplasmic protein, with each type of NS4 displaying a unique intracellular distribution in mammalian cell culture (Boughan et al. 2020). The NS4 proteins of both AHSV and BTV bind double-stranded DNA (dsDNA), but not dsRNA, suggesting a role for NS4 in nuclear virus-host interactions (Belhouchet et al. 2011; Zwart et al. 2015). Whilst BTV NS4 is also present in the cytoplasm and nucleus, it shows an intranuclear nucleolar localization (Belhouchet et al. 2011; Ratinier et al. 2011) not observed for AHSV NS4 (Zwart et al. 2015). It was recently shown that all forms of AHSV NS4 colocalize with promyelocytic leukemia nuclear bodies (PML-NBs) within the nucleus (Boughan et al. 2020). One of the roles of PML-NBs in the cell is involvement in antiviral defense via activation of innate immunity, and many viruses have evolved antagonistic effector proteins that modify PML-NBs, thereby disabling host cell intrinsic defences (Scherer & Stamminger 2016).

Cytoplasmic viruses often encode nuclear proteins that act to suppress the host immune response (Zakaryan & Stamminger 2011; Chen et al. 2016). Innate immunity is the first line of defense against an invading pathogen. Upon infection with an RNA virus innate immunity is initiated when dsRNA or ssRNA is recognized by host PRRs (Kell & Gale 2015), ultimately leading to the production of type I IFN α and β (Randall & Goodbourn 2008). The IFN response, which is central to innate immunity and the fight against viral infections, is mediated by the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway. Whilst some viruses encode proteins that act as direct IFN antagonists, others counteract the action of type I and type II IFNs. BTV was shown to modulate the IFN-I response by interfering with the JAK-STAT signaling pathway, even though it was unclear which proteins were involved (Doceul et al. 2014). BTV NS4 has subsequently been shown to modulate the IFN-response by downregulating mRNA levels of IFN-I and interferon stimulated genes (ISGs) and downregulating the activities of certain promoters (Ratinier et al. 2016). NS4 is not required for BTV replication in insect cells, mammalian cells, or type I IFN receptor $^{-/-}$ (IFNAR$^{(-/-)}$) mice

(Ratinier et al. 2011). It does, however, confer a replication advantage to BTV in mammalian cells in an IFN-induced state as well as *in vivo* in the host (Ratinier et al. 2011; Ratinier et al. 2016). BTV NS4 was therefore concluded to be an IFN antagonist and a key virulence factor in the host (Ratinier et al. 2016).

The localization of AHSV NS4 to the nucleus, its ability to bind dsDNA, and its interaction with PML-NBs suggest that NS4 may play a role in counteracting the host innate immune response. In this study we investigated the role of AHSV NS4 in viral replication, virulence and the host immune response. We used a virulent strain of AHSV-5 and describe the use of reverse genetics to generate NS4 deletion mutants that were assayed both *in vitro* and *in vivo*. We show that NS4 does not affect replication in cell culture, but is an important virulence factor in horses. Furthermore, our results showed that AHSV NS4 suppresses the host innate immune response at early stages of infection, potentially by interfering with the JAK-STAT signaling pathway at the point at which STAT1 translocates into the nucleus.

2. Methods

2.1. Cells

BSR cells (a clone of BHK-21 cells and a gift from Piet van Rijn, Wageningen Bioveterinary Research, The Netherlands) and BSR-T7/5 cells (BSR cells that constitutively express T7 polymerase, used with permission from Ulla Buchholz, Department of Clinical Virology, Federal Research Center for Virus Diseases of Animals, Tubingen, Germany) (Buchholz et al. 1999) were maintained as monolayers in Minimal Essential Medium with Earle's Balanced Salts and L-Glutamine (MEM, HyClone) supplemented with 5% fetal bovine serum (FBS, Gibco®), 1.2% fungizone (Sigma-Aldrich®), 1% non-essential amino acids (NEAA, Lonza), and 1% penicillin and streptomycin (pen/strep, Lonza). Additionally, 1 mg/ml Geneticin (Invitrogen™) was added to every second passage of BSR-T7/5 cells. Vero cells (ATCC CCL-81) were maintained as monolayers in Dulbecco's Modified Eagle Medium with high glucose, L-Glutamine and sodium pyruvate (DMEM, HyClone) supplemented with 5% FBS, 1.2% fungizone and 1% pen/strep. All cells were grown at 37°C, with 5% carbon dioxide (CO_2) and 90% humidity.

2.2. Generation of recombinant strains rAHSV-5, rAHSV-5minNS4 and rAHSV-5ΔNS4

The reverse genetics rescue of "synthetic" virulent AHSV-5 from the AHSV-5 reference strain HS 30/62 was performed as described previously using expression vectors and synthetic single stranded RNA (ssRNA) (van de Water et al. 2015; Potgieter et al. 2017), or using a plasmid-based reverse

genetics protocol as described by Boughan et al. (2020). Here, this strain is referred to as rAHSV-5. The AHSV-5 NS4 deletion mutant, rAHSV-5minNS4, was rescued using a plasmid-based reverse genetics protocol as described by Boughan et al. (2020). The virus stock was stored at 4°C. The Seg-9minNS4 plasmid contained a Seg-9 with the nucleotide substitutions T194C, T197C, T305C, T377C, T401C, T449C, T452C, T455C, T467C and A551G (synthesized by GenScript Corporation). This resulted in the M1T, M2T, M38T, M62T, M70T, M86T, M87T, M88T, M92T, N120S substitutions in the NS4 ORF, which would abolish NS4 expression. The synthetic virus rAHSV-5ΔNS4 was kindly provided by S. Boughan (University of Pretoria) and included the mutant Seg-9 described above plus two additional modifications, G209A and T332A, which introduced two stop codons at amino acids positions 6 and 47 respectively in the NS4 ORF. None of the mutations to Seg-9 affected the coding region of VP6, which is expressed from an out-of-phase overlapping reading frame on Seg-9. Virus titers were determined by endpoint dilution in BSR-T7/5 cells and expressed as $TCID_{50}$/ml (50% tissue infective dose per milliliter) (Reed & Muench 1938) and converted to pfu/ml (plaque forming units per milliliter) by multiplying by 0.7.

2.3. Isolation of dsRNA and sequencing of Seg-9

BSR cells seeded in 6-well plates were infected with rAHSV-5, rAHSV-5minNS4 or rAHSV-5ΔNS4 at a multiplicity of infection (MOI) of 0.1 and harvested when full cytopathic effect (CPE) was observed. Total RNA was isolated using TRI Reagent™ solution (Invitrogen™) according to the manufacturer's instructions. Thereafter, 2 M lithium chloride was used to precipitate and remove single-stranded RNA (ssRNA) by centrifugation prior to ethanol precipitation of dsRNA. The dsRNA pellet was washed with 96% ethanol, resuspended in nuclease-free water and quantified using NanoDrop™ ND-1000 spectrophotometry (Thermo Scientific™). The dsRNA was then denatured with DMSO (1.6µl/500ng dsRNA, 95°C for 2mins) used as a template for complementary DNA (cDNA) synthesis using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific™) with random hexamer primers. Polymerase chain reaction (PCR) was used to amplify Seg-9 using Seg-9-specific primers (Fwd CTCA TGTCTTCGGCATTACTC and Rev GCAAGCCCCTATCTACAGTAAATAAG) and DreamTaq PCR Master Mix (Thermo Scientific™). Amplicons were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing kit (v3.1, Applied Biosystems) and the Applied Biosystems 3500xl genetic analyser (Life Technologies).

2.4. Virus growth curves

Virus growth curves were carried out in BSR cells. Cell monolayers in 24-well plates were infected with the appropriate virus at a MOI of 0.1 pfu/ml. At 12, 24, 36, 48 and 72 hpi cells were harvested

by scraping and total virus collected. Virus titre was determined by endpoint dilution in BSR cells followed by immunoperoxidase monolayer assay (IPMA) after 72 h, according to standard procedures (Wensvoort et al. 1986). Briefly, cells were fixed with 50% methanol:50% acetone and blocked with 1% tryptone (Merck) and 0.05% Tween-20 (Sigma-Aldrich®) in 1 x phosphate buffered saline (PBS). Immunostaining was done with rabbit anti-NS2 serum (Uitenweerde et al. 1995) diluted in blocking solution. Peroxidase-conjugated Protein A and AEC reagent (Sigma-Aldrich®) were used for protein detection and chromogen development. Titres were expressed as $\log_{10}TCID_{50}$/ml and each experiment was performed independently in triplicate from two different virus stocks. Statistical analyses were done using one-way ANOVA (P<0.05) in IBM® SPSS® Statistics Software (v26).

2.5. Horse trials

Horse trials were conducted at the insect-free facilities situated at Deltamune, Roodeplaat, South Africa. Permission to perform the vaccine trials were obtained from the South African Department of Agriculture Fisheries and Forestry (DAFF) and from the Deltamune Ethical Committee (DEC, PD14-16, PD15-11, PD15-18, PD16-27, PD17-23). Table 2 provides a summary of the trials that were undertaken. Susceptibility of the horses to AHS was determined using a commercial blocking ELISA (Eurofins Technologies Ingenasa). After inoculation, rectal temperatures were taken daily and viremia was tested from daily bleeds in EDTA using real-time RT-PCR targeting Seg-4 (VP4) or Seg-5 (NS1) (Potgieter et al. 2017; van Rijn et al. 2018). Clinical signs were monitored daily by qualified veterinarians.

2.6. Collection of EDTA blood and PBMC isolation

EDTA blood was collected aseptically from the jugular vein of three horses inoculated with rAHSV-5minNS4 and one horse inoculated with rAHSV-5 (Table 2) using sodium heparin containing vacutainers on days 0 (before viral inoculation), 1 and 2 for all animals, and on day 4 for the horse inoculated with rAHSV-5. This resulted in 3 biological replicates per day in the case of horses inoculated with rAHSV-5minNS4 and one sample per day for rAHSV-5. Blood was transferred to the laboratory immediately (<2 h) and peripheral blood mononuclear cells (PBMCs) were isolated via density gradient centrifugation. Blood samples were layered over Histopaque-1077® (Sigma-Aldrich®) and centrifuged at 900 x g for 30 minutes (min) at room temperature (RT). The opaque interface containing PMBCs was collected, rinsed, resuspended in sterile 1 x PBS and centrifuged at 250 x g for 10 min at RT. The PBMC pellet was resuspended in a 1:3 mixture of sterile 1 x PBS and TRI Reagent® (Sigma-Aldrich). Cells were stored at -70°C until RNA isolation.

2.7. Total RNA isolation and transcriptome sequencing

Total RNA was isolated according to the TRI Reagent® protocol and resuspended in LiChlorosolv® Water (Merck). Additional RNA purification was done using the RNeasy® Mini kit (Qiagen), with the inclusion of the DNase digestion step, and the RNA eluted in LiChlorosolv® Water. RNA integrity was assessed using the Experion™ Automated Electrophoresis System (BioRad Laboratories) at the ACGT Microarray Facility and Ion Torrent Sequencing Facility of the University of Pretoria and was found to be high quality RNA. Between 2.3 and 4.6 µg total RNA of each sample was sent to Novogene (Hong Kong) for Illumina transcriptome sequencing with the Illumina HiSeq (Illumina, USA). Raw reads were processed, and clean reads obtained using in-house perl scripts (Novogene). On average, 38.2 million clean reads per library were generated.

2.8. Bioinformatic analyses of transcriptome data

The clean paired-end reads were mapped to Ensembl release 84 of the *Equus caballus* reference genome EquCab2.0 (Wade et al. 2009) using TopHat v2.0.12. Levels of gene expression were determined using HTSeq software (v0.6.1, -m union). The read count values were used as input data for differential gene expression analysis. At this point, the data for the three biological replicates of rAHSV-5minNS4 was combined for each day (Table 1). Differential gene expression of the samples with biological replicates (rAHSV-5minNS4) was analysed using DESeq (v1.10.1, padj<0.05) and its inherent normalization method. Samples without biological replicates (rAHSV-5) were normalised using TMM and then analysed using DEGSeq (v1.12.0, $\log_2$FoldChange>1 & qval<0.005). Differentially expressed genes were compared to the list of innate immunity-related genes in InnateDB v5.4 (Breuer et al. 2013). KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis of DEGs in KEGG pathways was done with KOBAS (v2.0). KEGG pathways were filtered to include membrane transport (3), signal transduction (33), signaling molecules and interaction (5), as well as immune system pathways (21), resulting in a total of 62 pathways that were investigated in more detail. KEGG pathways with corrected pval<0.05 were considered significantly enriched.

2.9. Immunofluorescence and confocal microscopy

For the detection of NS4, BSR-T7/5 cells were grown on sterile coverslips in 24-well plates and infected at a MOI of 0.1-1 pfu/ml. At 24 hours post infection (hpi) cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich®) for 30 min at RT followed by permeabilisation with 0.2% Triton X-100 (TX-100) for 10 min at RT. Primary labeling was done with rabbit anti-NS4 serum produced at Deltamune (Pty) Ltd (Boughan et al. 2020) followed by secondary labeling with Alexa

Fluor™ (AF) 488 goat anti-rabbit (Invitrogen™, A-11034). Nuclei were stained with 10 µg/µl 4',6-diamidino-2-phenylindole (DAPI, Roche Applied Science). For the detection of STAT1 and pSTAT1, Vero cells were grown on sterile coverslips in 24-well plates and infected at a MOI of 0.1-1 pfu/ml. At 24 hpi cells were left untreated, or were treated with 200 ng/ml of IFN-γ, (GenScript USA, Human Z02915-100 or CHO expressed Z02986-50) for 30 min to 5 h at 37°C, with CO_2 and 90% humidity. Cells were fixed and permeabilised as described above. Simultaneous primary labeling was done using anti-NS1 (Eurofins Technologies Ingenasa) in conjunction with anti-STAT1 (Abcam, ab109320) or anti-pSTAT1 (LifeSpan Biosciences, STAT1 pSer727, LS-C352894/67027). Simultaneous secondary labeling was done using AF 594 goat anti-mouse (Invitrogen™, A11005) or AF 488 goat anti-rabbit (Invitrogen™, A110344); nuclei were stained with DAPI. All coverslips were mounted onto glass slides with VECTASHIELD Mounting Medium (Vector Laboratories). Immunofluorescence was visualized using a Zeiss LSM 880 Confocal Laser Scanning Microscope (CLSM) coupled to an Airyscan detector.

2.10. Western blotting

Monolayers of BSR-T7/5 cells were infected with rAHSV-5, rAHSV-5minNS4 or rAHSV-5ΔNS4 at a MOI of 1.5 and harvested at 24 hpi. All samples were resuspended in 2/3 volume 3 x protein solvent buffer (PSB, 0.188 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, 0.005% bromophenol blue) and heated to 95°C for 3 min. NS4 proteins were resolved by 15% SDS-PAGE and were transferred to Protran® nitrocellulose membranes (Amersham™) via electroblotting (100V for 1 hour) in the presence of Towbin's transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol pH 8.3). Blocking was done with 1% skim milk powder in 1 x PBS and membranes were incubated in the appropriate primary antibodies diluted in blocking solution. Rabbit anti-NS4 serum (GenScript) (Zwart et al. 2015) was used for Western blotting. Rabbit anti-NS1 serum was raised at the animal facilities of Deltamune (Pty) Ltd against baculovirus expressed NS1. Mouse anti-β-actin (Sigma-Aldrich®, A2228) was used as a loading control. Detection was done using peroxidase-conjugated Protein A (Calbiochem®) via standard procedures.

3. Results

3.1. AHSV NS4 knockout is attenuated in experimentally infected horses

AHSV NS4 is expressed in the spleen of infected horses, suggesting that AHSV NS4 may function in immunity (Zwart et al. 2015). In addition to this, BTV NS4 is an IFN-antagonist and virulence factor in the host (Ratinier et al. 2016). Based on this information, we set out to investigate the effect of abolishing NS4 expression on the virulent AHSV-5 reference strain. We rescued rAHSV-5, a

"synthetic" virulent virus, from expression vectors and synthetic ssRNA (van de Water et al. 2015; Potgieter et al. 2017), or from plasmids only (Boughan et al. 2020). The AHSV NS4 deletion mutant, rAHSV-5minNS4, was made by introducing mutations to the plasmid containing Seg-9, such that the start codon and the downstream methionine codons were changed to threonine. The rescued viruses were sequenced, viral replication confirmed by RT-PCR and the expression of NS2 confirmed by immunoperoxidase monolayer assay (IPMA, not shown). Horses were then inoculated with rAHSV-5 and rAHSV-5 minNS4 as part of vaccine trials. A compilation of the results from five vaccine trials is shown in Table 2. For the purpose of this study, only the safety data is presented. All four control horses exposed to rAHSV-5 showed clinical symptoms and died, or had to be euthanized, six to eight days after inoculation. Post-mortem examination showed that all four horses had classical lesions of the disease such as interstitial lung edema, alveolar edema (froth in the trachea), and that some, but not all, had hydropericardium (Supplementary Figure 1). In contrast, despite evidence of virus replication, none of the thirteen horses inoculated with rAHSV-5minNS4 showed any clinical signs of AHS, independent of the route of inoculation. These results showed that rAHSV-5 is virulent in experimentally infected horses, whereas rAHSV-5minNS4 is fully attenuated.

3.2. AHSV NS4 knockout has no impact in cell culture

Next, we wanted to determine how NS4 mediates the transition from virulence to attenuation. We began by characterizing the viruses in more detail in cell culture. Preliminary data indicated that there was a low level of NS4 expression in some rAHSV-5minNS4-infected cells (not shown). Therefore, we rescued an additional NS4 deletion mutant, rAHSV-5ΔNS4 (Boughan et al. 2020), which contained two additional mutations to Seg-9 resulting in the introduction of two stop codons in the NS4 ORF (Fig. 1a). The migration pattern of the dsRNA segments isolated from rAHSV-5, rAHSV-5minNS4 and rAHSV-5ΔNS4 were comparable to AHSV-5Ref (Fig. 1b) and Seg-9 of each virus was sequenced to confirm the presence of the mutations. Western blot analysis of infected BSR-T7/5 cell lysates was used to test for NS4 expression. NS4 was expressed by rAHSV-5, while rAHSV-5minNS4 and rAHSV-5ΔNS4 did not express NS4, and all viruses expressed NS1 to comparable levels, confirming viral infection and replication (Fig. 1c).

CLSM was used to investigate the intracellular localization of NS4. In the case of rAHSV-5, NS4 showed a homogeneous, predominantly cytoplasmic distribution with weaker labeling also observed throughout the nucleus of infected BSR-T7/5 cells (Fig. 1d). This corresponded to the profile described previously for all AHSV strains expressing NS4-II (Boughan et al. 2020). The same pattern was observed at a low intensity in some cells infected with rAHSV-5minNS4 (Fig. 1d), confirming the

preliminary data which suggested some low-level expression of NS4 from rAHSV-5minNS4. NS4 was not detected via Western blot for this virus, likely due to immunoblotting not being as sensitive as CLSM. None of the horses inoculated with rAHSV-5minNS4 died or showed any clinical signs, irrespective of this potential leaky NS4 expression. No NS4 was observed in rAHSV-5ΔNS4-infected cells (Fig. 1d), confirming that the expression of NS4 was fully abolished by the additional mutations to Seg-9.

Next, we assessed the replication kinetics of AHSV-5Ref, rAHSV-5 and rAHSV-5ΔNS4 in BSR cells. Cells were infected at a MOI of 0.1 and total virus titres were determined using a combination of $TCID_{50}$ and IPMA at 12, 24, 36, 48 and 72 hpi (Fig. 2). The replication of rAHSV-5 was comparable to AHSV-5Ref, and the lack of NS4 expression did not change the overall replication kinetics of rAHSV-5 in BSR cells. Furthermore, no significant differences in the replication of the viruses were observed. Taken together, these results show that we had rescued AHSV NS4 knockout strains comparable to rAHSV-5, except for their ability to express NS4.

3.3. AHSV NS4 suppresses transcription of genes involved in host innate immunity

RNA sequencing (RNA-seq) was subsequently used to compare the transcriptional responses in horses following inoculation with rAHSV-5 or rAHSV-5minNS4. Three of the horses inoculated with rAHSV-5minNS4 (Table 2, horses 2, 5 and 7, trial 2) and one horse inoculated with rAHSV-5 (Table 2, horse 16, trial 2) as part of the vaccine trials (Section 3.1) were used for this part of the study and the comparisons done are listed in Table 1.

Figure 3 provides an overview of the number of the number of differentially expressed (DE) genes in the transcriptome datasets. The full lists of DE genes are provided in Supplementary Table 1. A total of 28, 191, and 750 genes were DE on day 1, 2 or 4 respectively in the control horse inoculated with rAHSV-5 (Fig. 3a). Overall, fewer genes were DE in the horses inoculated with rAHSV-5minNS4, 44 on day 1 and 12 on day 2. The number of genes common to both datasets, or unique to a dataset, on days 1 and 2 are shown in Fig. 3b. Three genes were DE in both datasets on day 1, while the remaining genes were unique. On day 2, 9 genes were common to both datasets. Table 3 shows the top ranked upregulated DE genes in each comparison. RNA levels of three known ISGs namely OAS2, OAS3 and MX1 were upregulated on day 1 in the control horse inoculated with rAHSV-5 (Table 3, NS4_D1). Of the 172 genes upregulated on day 2, at least 48 were innate immunity-related genes and included additional ISGs and important regulators of immunity such as IRF7 (Table 3) and STAT1 and STAT2 (Supplementary Table 1). Also upregulated on day 2 was PML, which is involved in IFN

signaling and forms part of PML-NBs with which NS4 colocalises (Choi et al. 2006; El Bougrini et al. 2011; Chen et al. 2015; Kim & Ahn 2015; Boughan et al. 2020). This trend continued to day 4, with at least 82 genes predicted to be involved in innate immunity (Table 3 and Supplementary Table 1). More genes were transcriptionally upregulated on day 1 than on day 2 in the horses inoculated with rAHSV-5minNS4 (Fig. 3a), many of which were innate immunity-related and included several ISGs (Table 3, minNS4_D1). Furthermore, the pathogen recognition receptor (PRR) DDX58, as well as STAT1 and IRF7, were upregulated on day 1 in these horses, a day earlier than in the control horse.

At least three of the downregulated genes on day 1 in the control horse were predicted to be involved in innate immunity, decreasing to at least two on day 2 (Table 4, NS4_D1 and NS4_D2). The number of downregulated genes increased considerably from day 2 to day 4 (347 genes, Supplementary Table 1). Very few genes were downregulated on days 1 and 2 in the horses inoculated with rAHSV-5minNS4 (Table 4, minNS4_D1 and minNS4_D2).

To further define the function of the DE genes, we mapped them to canonical pathways in KEGG (Supplementary Table 2) the results of which are summarized in Fig. 4. Five immune system pathways (platelet activation, leukocyte transendothelial migration, intestinal immune network for IgA production, hematopoietic cell lineage and the chemokine signaling pathway) were enriched on day 1 in the control horse (blue bars, NS4_D1), all of which by downregulated genes (Supplementary Table 2). In contrast, 6 immune system pathways (the Toll-like and RIG-I-like receptor signaling pathways, Fc gamma R-mediated phagocytosis, cytosolic DNA-sensing pathway, complement and coagulation cascades and the chemokine signaling pathway) were enriched by upregulated genes on day 1 in the horses inoculated with rAHSV-5minNS4 (blue bars, minNS4_D1), and included the Toll-like and RIG-I-like receptor signaling pathways, which are hallmarks of innate immunity. The JAK-STAT signaling pathway was also enriched on day 1 in these horses (blue bars, minNS4_D1). These pathways were not enriched on day 1 in the control horse.

The Toll-like and RIG-I-like receptor signaling pathways were activated on day 2 in the control horse, 24 h later than in the horses inoculated with rAHSV-5minNS4. Also activated on day 2 in the control horse were the JAK-STAT, MAPK and NF-kappa B signaling pathways. These signal transduction pathways were enriched by upregulated genes except for NF-kappa B which was enriched by both up-and down-regulated genes. Evidence of adaptive immunity (e.g. natural killer cell mediated cytotoxicity and the B cell receptor signaling pathway) was also observed on day 2. Overall, more immune system processes were transcriptionally enriched on day 2 for rAHSV-5, indicating a 24-hour

delay in the onset of a full immune response in this horse. An increase in the overall number of enriched pathways for rAHSV-5 was observed from day 2 to day 4 in the control horse (red bars, NS4_D4) and all processes that were enriched on day 2 were still enriched on day 4, to a greater extent. Immune system pathways enriched on day 4 included both innate and adaptive immune system processes. All pathways enriched on day 4 were enriched by upregulated genes.

Taken together, the results of this section indicated that the presence of NS4 (rAHSV-5) results in many immune response pathways to be transcriptionally activated 24 hours later than when the protein is absent (rAHSV-5minNS4). Furthermore, this appears to be due to a delay in the innate immune response specifically.

3.4. AHSV NS4 affects the translocation of STAT1 into the nucleus

The previous set of results showed that the JAK-STAT signaling pathway was activated on day 1 in the horses inoculated with rAHSV-5minNS4, but only on days 2 and 4 (not day 1) in the horse inoculated with rAHSV-5. To investigate whether AHSV NS4 affects the innate immune response by interfering with the JAK-STAT signaling pathway specifically, Vero cells were infected with rAHSV-5 or rAHSV-5ΔNS4, and treated with IFN-γ at 24 hpi. These cells cannot produce interferon alpha or beta but do produce interferon lambda. The cells also have an intact IFN-signaling pathway and can respond to interferon treatment (Desmyter et al. 1968; Osada et al. 2014). Under normal circumstances, treatment with IFN-γ (type-II IFN) should result in phosphorylation, homodimerization and the subsequent nuclear translocation of STAT1 (Fleming 2016). We focus here on STAT1, as it is the first protein to be involved in innate immunity following the synthesis of interferon. Following IFN treatment, the cells were processed for CLSM and labeled either for STAT1 or for AHSV NS1 (Fig. 5). The cellular localization of STAT1 was predominantly cytoplasmic in both uninfected and virus-infected cells. Upon treatment with IFN-γ, STAT1 translocated into the nucleus of uninfected cells as expected. In contrast, STAT1 remained cytoplasmic in the majority of rAHSV-5-infected cells. Labeling of non-structural protein NS1, which is highly expressed following AHSV infection and forms tubular aggregates in the cytoplasm, allowed us to distinguish infected from uninfected cells in the same field. This clearly illustrated the difference in the STAT1 localization following IFN-γ treatment in the presence versus absence of rAHSV-5 replication. However, in rAHSV-5ΔNS4-infected cells, which would sustain normal viral replication just lacking NS4 expression, the interferon treatment resulted in nuclear translocation of STAT1 (Fig. 5). This indicated that the presence of NS4 results in the cytoplasmic retention of STAT1.

To verify these results the experiments were repeated, but labeling was done with an antibody specific to the phosphorylated form of STAT1 (pSTAT1) (Fig. 6). Prior to the IFN treatment, little to no phosphorylated STAT1 (pSTAT1) could be detected in the nuclei of uninfected or virus-infected cells (Fig. 6). This was to be expected, as the presence of IFN is required to activate the phosphorylation of STAT1. After treatment with IFN-γ a strong nuclear signal was observed for pSTAT1 in uninfected cells, as well as in rAHSV-5ΔNS4-infected cells. No nuclear pSTAT1 was however observed in rAHSV-5-infected cells (Fig. 6). Taken together, these results suggest that AHSV NS4 interferes with the phosphorylation and/or translocation of STAT1 and pSTAT1 into the nucleus.

4. Discussion

The role of AHSV non-structural protein NS4 in the viral replication cycle and pathogenesis remains largely unknown. A recent study showed that NS4 is expressed by all nine AHSV serotypes and colocalizes with PML-NBs, suggesting a possible role in counteracting the innate immune response (Boughan et al. 2020). The present study aimed to investigate the role of AHSV NS4 in viral replication, pathogenicity and the host immune response, through the analysis of AHSV NS4 deletion mutants. We showed that AHSV NS4 is an important virulence factor in horses, and that the absence or very low expression thereof results in virus attenuation. Furthermore, NS4 suppresses the innate immune response early in the replication cycle and suggest that one way in which it does this is by interfering with the translocation of STAT1 into the nucleus.

The vaccine trials described in this study showed that rAHVS-5 was highly pathogenic in horses, irrespective of the way in which the viruses were rescued. In contrast, despite signs of viral replication, all horses inoculated with rAHSV-5minNS4 survived and showed no clinical signs of disease, no matter the route of inoculation. These results confirmed that like BTV NS4 (Ratinier et al. 2016), AHSV NS4 is a virulence factor in the host and the absence thereof results in a safe, highly attenuated virus.

Based on the colocalization of AHSV NS4 with PML-NBs in the nucleus (Boughan et al. 2020) as well as the similarities observed to date for BTV NS4 and AHSV NS4, we investigated if NS4 mediates the transition from virulence to attenuation by interfering with host innate immunity. RNA-seq data showed activation of the innate IFN response within 24 hours (day 1) of inoculation with rAHSV-5minNS4 as evidenced by the rapid upregulation of the important regulators of immunity, IRF7 and STAT1, as well as many ISGs. Further confirmation of this was the activation of the Toll-like and RIG-I-like receptor signaling pathways, which play an important role in innate immunity, observed on this

day. Similar to what was observed on day 1 in horses inoculated with attenuated AHSV-4 (Pretorius et al. 2016), several other immune system and signal transduction pathways, including JAK-STAT signaling, were also activated on day 1 in the horses inoculated with rAHSV-5minNS4.

Although several ISGs were also upregulated on day 1 in the presence of NS4 (rAHSV-5), they were upregulated to a lower level. Furthermore, the immune system pathways observed on day 1 were enriched by downregulated genes and importantly did not include the immune system pathways that were activated on day 1 in the horses inoculated with rAHSV-5minNS4. Therefore, although innate immunity had been activated, it was not as strong an activation as in the absence of NS4. Thus, NS4 alone is unable to entirely inhibit mRNA expression of the host IFN response. These findings are similar to what has been observed in RNA-seq analyses done on nascent RNA isolated from BTV8ΔNS4 and BTV8wt infected and uninfected A549 cells at 12 hpi (Ratinier et al. 2016).

An immune transcriptional response was observed on day 2 in the horse exposed to rAHSV-5, with the activation of immune system and signal transduction pathways such as the RIG-I-like receptor, Toll-like receptor, and JAK-STAT signaling. These pathways were already activated on day 1 in the absence of NS4 (rAHSV-5minNS4). Overall, the RNA-seq results indicated that the presence of NS4 (rAHSV-5) results in several immune response pathways only being transcriptionally activated 24 hours later than when exposed to rAHSV-5minNS4. Furthermore, this appears to be due to a delay in upregulation of innate immunity mRNAs specifically.

After the innate immune response has been initiated following the recognition of an RNA virus, the newly secreted type I IFNs bind to the IFN-α/β receptor IFNAR (Randall & Goodbourn 2008; Fros et al. 2010), leading to the phosphorylation of the tyrosine kinases, TYK2 and JAK1. This leads to the phosphorylation of the cytoplasmic transcription factors STAT1 and STAT2, which dimerize, bind to importin-α and together with IFN regulatory factor 9 (IRF9), form the IFN-stimulated gene factor 3 (ISGF3) complex (Randall & Goodbourn 2008). This complex translocates into the nucleus where it binds to IFN-stimulated response elements (ISREs) present in ISGs leading to their expression. In contrast to type I IFNs which are induced by viral infection, type II IFN (IFN-γ) is induced by mitogenic or antigenic stimuli and only by certain types of cells (Samuel 2001; Randall & Goodbourn 2008). IFN-γ is also mediated by the JAK-STAT pathway and binds to the IFNGR receptor, activating JAK1 and JAK2 which leads to the phosphorylation of two STAT1 molecules which homodimerize into the γ activation factor. This factor translocates into the nucleus and binds to the gamma-activation sequence on ISGs, stimulating their transcription (Fleming 2016). Several hundred ISGs can be

expressed as a result of the IFN response and together they lead to a potent antiviral state (Randall & Goodbourn 2008).

To promote their replication and spread, many viruses have evolved countermeasures against innate immunity, particularly the IFN response. One such measure involves encoding IFN antagonizing proteins (Randall & Goodbourn 2008). These proteins are often multifunctional, especially in the case of RNA viruses, with roles that vary in importance depending on the stage of the virus replication cycle (Randall & Goodbourn 2008). Examples of innate immune system antagonists include rotavirus VP3 and NSP1, Ebola virus protein VP35, Newcastle disease virus (NDV) V protein, Rabies virus phosphoprotein, and several nonstructural proteins within the arboviruses (Basler et al. 2000; Huang et al. 2003; Hollidge et al. 2011; Oksayan et al. 2012; Morelli et al. 2015). Within the orbiviruses BTV NS3 and NS4 are known IFN antagonists, and like NDV V protein, BTV NS4 is an important virulence factor (Huang et al. 2003; Chauveau et al. 2013; Ratinier et al. 2016).

Another way of counteracting IFN-mediated innate immunity is by disrupting the normal functioning of the JAK-STAT signaling pathway (Fleming 2016). For example, many viral proteins can 1) prevent the nuclear translocation of STAT1/2, 2) prevent the phosphorylation of STAT1/2 or 3) promote the nuclear export of STAT1/2 (Melén et al. 2004; Ashour et al. 2009; Mazzon et al. 2009; Fros et al. 2010; Röthlisberger et al. 2010; Fros et al. 2013; Goertz et al. 2018; Feng et al. 2019; Mitra et al. 2019). It is also possible for viruses to inhibit nuclear translocation after STAT has already been activated and bound to importin-α, as is the case in rotavirus infection (Holloway et al. 2009; Holloway et al. 2014). While it is known that BTV inhibits the JAK-STAT signaling pathway by blocking the phosphorylation and nuclear translocation of STAT1 early in infection, or by downregulating the expression of JAK1 and TYK2 later in the infection cycle (Doceul et al. 2014), The exact BTV protein(s) involved in this remains unknown. Our results suggest that NS4 may be a candidate.

Here, the JAK-STAT signaling pathway was transcriptionally activated on day 1 in the absence of NS4 (rAHSV-5minNS4) but only on days 2 and 4 in the presence of NS4 (rAHSV-5). Thus, we investigated whether AHSV NS4 affects the innate immune response by interfering with the JAK-STAT signaling pathway specifically. Upon treatment with IFN-γ STAT1 was generally retained in the cytoplasm in rAHSV-5-infected cells, but translocated into the nucleus in cells infected with rAHSV-5ΔNS4. rAHSV-5ΔNS4 sustains normal replication, just lacks NS4, thus the presence of NS4 results in the cytoplasmic retardation of STAT1, effectively blocking type II IFN-induced JAK-STAT signaling. The mechanism by which AHSV NS4 interferes with STAT1 nuclear translocation requires further

investigation, but the absence of pSTAT1 in rAHSV-5 infected cells suggest that NS4 could inhibit phosphorylation of STAT1. AHSV NS4 is found in both the nucleus and the cytoplasm therefore it is possible that the protein may act at the stage of STAT1 import into, or export out of, the nucleus.

Within the nucleus AHSV NS4 colocalizes with PML-NBs (Boughan et al. 2020) which are found as distinct foci and are involved in a diverse range of cellular processes including antiviral defense against DNA and RNA viruses (Bernardi & Pandolfi 2007; Scherer & Stamminger 2016). By associating with NF-κB, STAT1, STAT2 and ISG promoters, PML regulates type I IFN signaling by regulating the ISGF3 complex and promoting ISG expression (Chen et al. 2015; Kim & Ahn 2015). PML is also involved in type II IFN signaling by affecting STAT1 DNA binding and upregulating STAT1 phosphorylation (Choi et al. 2006; El Bougrini et al. 2011). As a result of its antiviral function, viruses have developed several ways to antagonize PML-NBs (Scherer & Stamminger 2016). A well-studied PML-NB antagonist is the human cytomegalovirus (HCMV) immediate early protein 1 (IE1), a structural analogue of NS4-II (Boughan et al. 2020). IE1 downregulates ISG transcription by interacting with STAT1 and STAT2 and by binding to PML, effectively sequestering ISGF3 (Paulus et al. 2006; Huh et al. 2008; Krauss et al. 2009; Scherer et al. 2014; Kim & Ahn 2015; Scherer et al. 2016). The present study showed that although AHSV NS4 was able to interfere with the translocation of STAT1 into the nucleus it was not able to completely abolish it. Due to its ability to bind dsDNA it is possible that AHSV NS4 prevents STAT1 binding to ISG promoters in cells in which STAT1 translocates into the nucleus, and in that way inhibits ISG expression (Zwart et al. 2015). Furthermore, due to the structural similarities of AHSV NS4 and HCMV IE1 and the colocalization of NS4 with PML-NBs we suggest that NS4 may act in a similar manner to IE1 and bind to PML and/or STAT1 in the nucleus.

Many RNA viruses that replicate in the cytoplasm make use of nucleocytoplasmic trafficking of viral proteins to carry out various roles in viral replication and pathogenesis as well as in the modulation of the immune response. In this study we show that abolishing AHSV NS4 expression from rAHSV-5 results in virus attenuation. Thus, AHSV NS4 was shown to be a determinant of virus virulence. It appears that NS4 mediates the transition from virulent to attenuated by the differential triggering of innate immunity. We show that one way in which AHSV NS4 interferes with the IFN response is by interfering with the nuclear accumulation of STAT1 during JAK-STAT signaling. As NS4 is found in both the nucleus and the cytoplasm it is possible that the protein may interact directly with STAT1 in either compartment, essentially inhibiting nuclear import or promoting nuclear export of STAT1.

Further studies need to be carried out to determine the mechanism by which AHSV NS4 acts and whether other viral proteins are involved.

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Ethical approval

Deltamune Ethical Committee approval numbers PD14-16, PD15-11, PD15-18, PD16-27 and PD17-23.

CRediT authorship contribution statement

Gayle V. Wall: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing - original draft **Isabella M. Wright:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing - review & editing **Carin Barnardo:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization **Baltus J. Erasmus:** Conceptualization; Methodology; Validation; Writing - review & editing **Vida van Staden:** Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing **A. Christiaan Potgieter:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing

Abbreviations

AF, Alexa Fluor™; AHS, African horse sickness; AHSV, African horse sickness virus; aa, amino acid(s); BTV, Bluetongue virus; cDNA, complementary DNA; CLSM, confocal laser scanning microscopy; CPE, cytopathic effect; DAPI, 4',6-diamidino-2-phenylindole; DE, differentially expressed; DEG, differentially expressed gene(s); DMEM, Dulbecco's Modified Eagle Medium; DISA, disabled

infectious single animal; DISC, disabled infection single cycle; DIVA, differentiation of infected from vaccinated animals; ds, double-stranded; ECRA, Entry Competent Replication Abortive; FBS, fetal bovine serum; HCMV, human cytomegalovirus; hpi, hours post infection; IE1, HCMV immediate early protein 1; IFN, interferon; IFNAR$^{(-/-)}$, type I IFN receptor $^{-/-}$; IPMA, immunoperoxidase monolayer assay; IRF9, IFN regulatory factor 9; ISG(s), interferon stimulated genes; ISGF3, IFN-stimulated gene factor 3 complex; ISRE, IFN-stimulated response element(s); JAK-STAT, Janus kinase-signal transducers and activators of transcription; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAV, live attenuated vaccine; MEM, Minimal Essential Medium; MOI, multiplicity of infection; min, minutes; NDV, Newcastle disease virus; NEAA, non-essential amino acids; OIE, World Organization for Animal Health; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PCR, polymerase chain reaction; pen/strep, penicillin and streptomycin; PFA, paraformaldehyde; pfu/ml, plaque forming units per milliliter; PML-NB(s), promyelocytic leukemia nuclear bodies; PSB, protein solvent buffer; pSTAT1, phosphorylated STAT1; RNA-seq, RNA sequencing; PRR, pathogen recognition receptor(s); RT, room temperature; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; seg, segment; ss, single-stranded; $TCID_{50}$/ml, 50% tissue infective dose per milliliter; TX-100, Triton X-100.

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Figure captions

Fig. 1. Characterization of AHSV NS4 knockouts. (a) Schematic representation of AHSV-5 Seg-9 and the positions of the wild type (WT) VP6 and NS4 ORFs. Specific amino acid changes are shown in minNS4 and ΔNS4 and the introduction of stop codons illustrated by an * (b) Agarose gel showing the dsRNA profiles of AHSV-5Ref, rAHSV-5, rAHSV-5minNS4 and rAHSV-5ΔNS4. (c) Western blot analysis of rAHSV-5, rAHSV-5minNS4 and rAHSV-5ΔNS4 infected BSR-T7/5 cells. Cells were infected, harvested and assayed for the presence of NS4, NS1 or ß-actin in whole cell lysates. (d) Localization of NS4 in infected BSR-T7/5 cells. Cells infected with rAHSV-5, rAHSV-5minNS4 or rAHSV-5ΔNS4 were fixed at 24 hpi and prepared for immunofluorescence and CLSM using anti-NS4 primary and AF 488 secondary antibodies. Nuclei were stained with DAPI. Scale bars, 10 µm.

Fig. 2. *In vitro* replication kinetics of AHSV-5Ref, rAHSV-5 and rAHSV-5ΔNS4 in BSR cells. Cells were infected with AHSV-5Ref (black), rAHSV-5 (light grey) or rAHSV-5ΔNS4 (dark grey) at an MOI of 0.1, and viral titres determined from 12 to 72 hpi by endpoint dilution analysis and expressed as $\log_{10}$TCID50/ml. Experiments were performed twice independently, each time in triplicate. The error bars represent 95% confidence interval.

Fig. 3. Overview of the differentially expressed genes in the transcriptome datasets of total RNA isolated from the PBMCs of inoculated horses) Bar graph showing the total number of up- and downregulated genes on days 1, 2 and 4 for the horse inoculated with rAHSV-5 (designated NS4) and days 1 and 2 for the horses infected with rAHSV-5minNS4 (designated minNS4). In all cases the data was normalised to day 0. b) Venn diagrams showing the number of genes shared between the datasets on days 1 and 2 when normalised to day 0.

Fig. 4. KEGG pathways enriched by the differentially expressed genes on days 1, 2 and 4 in horses that were inoculated with rAHSV-5 or rAHSV-5minNS4. Pathways involved in membrane transport are listed in orange, signaling molecules and interaction in purple, signal transduction in black and the immune system in gold.

Fig. 5. NS4 interferes with nuclear translocation of STAT1. Vero cells were infected with rAHSV-5 or rAHSV-5ΔNS4 and then treated with IFN-γ, fixed and labeled for AHSV NS1 and STAT1. Nuclei were stained with DAPI. Scale bars, 10 µm. Open arrow heads indicate AHSV-infected cells in which STAT1 was retained in the cytoplasm.

Fig. 6. NS4 interferes with STAT1 phosphorylation or pSTAT1 nuclear translocation. Vero cells were infected with rAHSV-5 or rAHSV-5ΔNS4 and then treated with IFN-γ, fixed and labeled for AHSV NS1 and pSTAT1. Nuclei were stained with DAPI. Scale bars, 10 µm. Open arrow heads indicate AHSV-infected cells lacking nuclear pSTAT1.

Figures

Fig 1

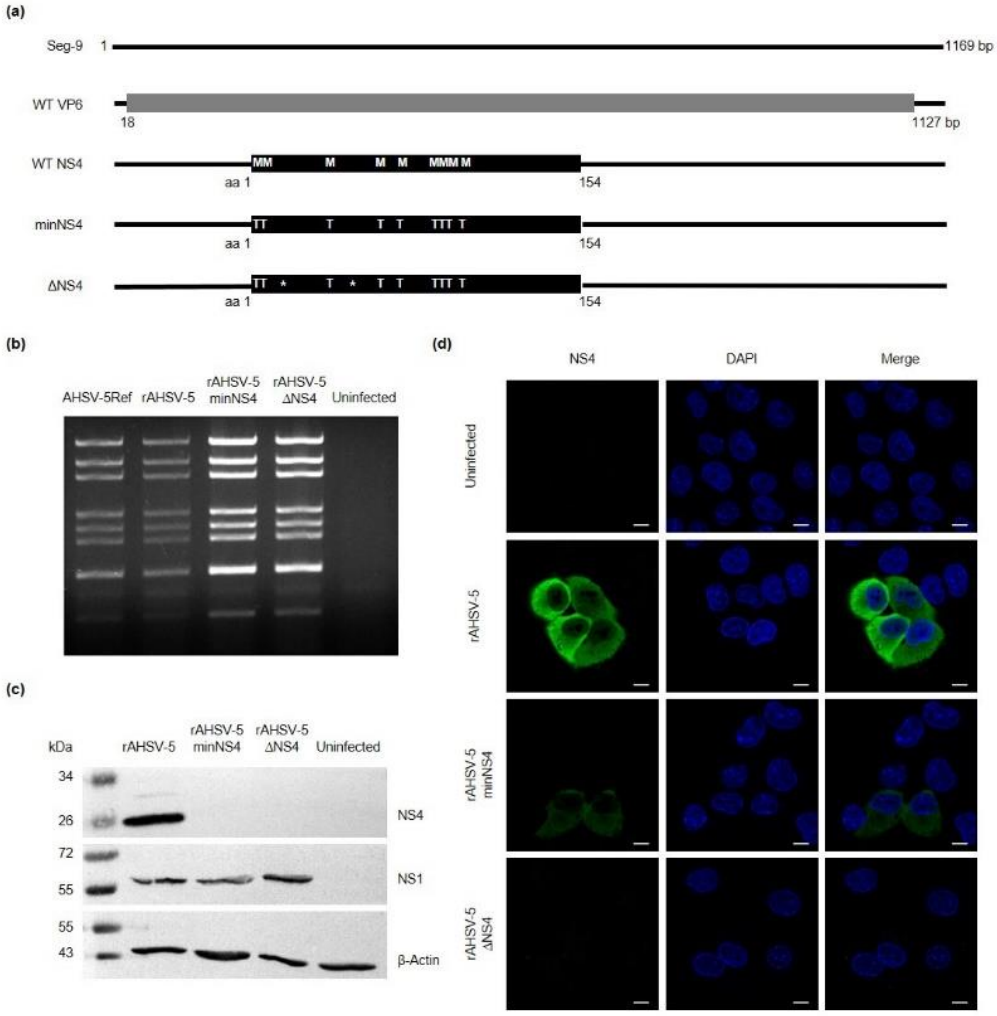


Fig 2

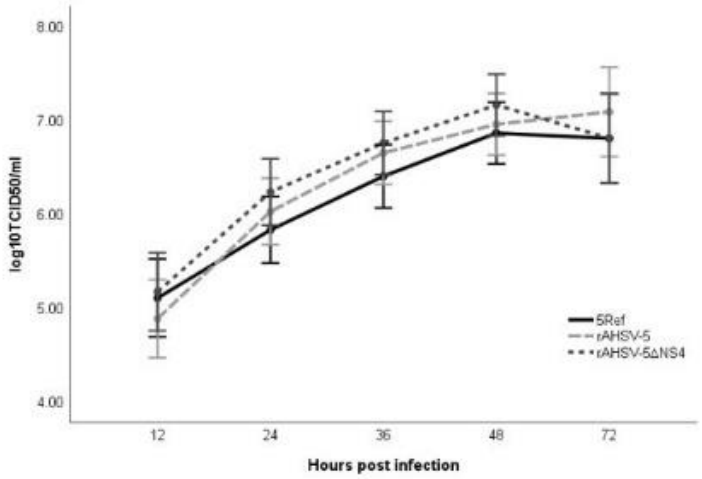
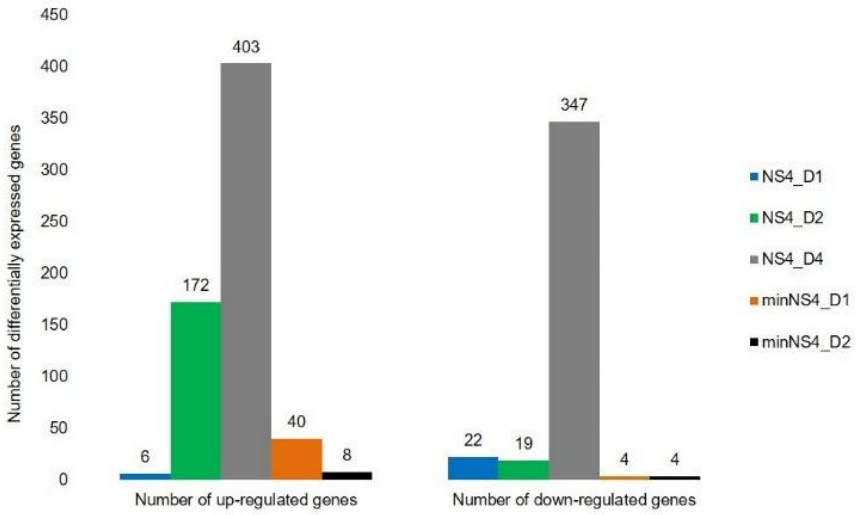

8.00
7.00
6.00
5.00
4.00
log10TCID50/ml
12
24
36
48
72
Hours post infection
5Ref
rAHSV-5
rAHSV-5ΔNS4

Fig 3

a)


Number of differentially expressed genes
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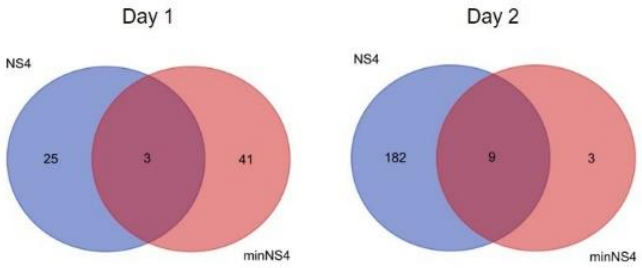
400
350
300
250
200
150
100
50
0
403
172
40
6
8
347
22
19
4
4
Number of up-regulated genes
Number of down-regulated genes
NS4_D1
NS4_D2
NS4_D4
minNS4_D1
minNS4_D2

b)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NS4
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3
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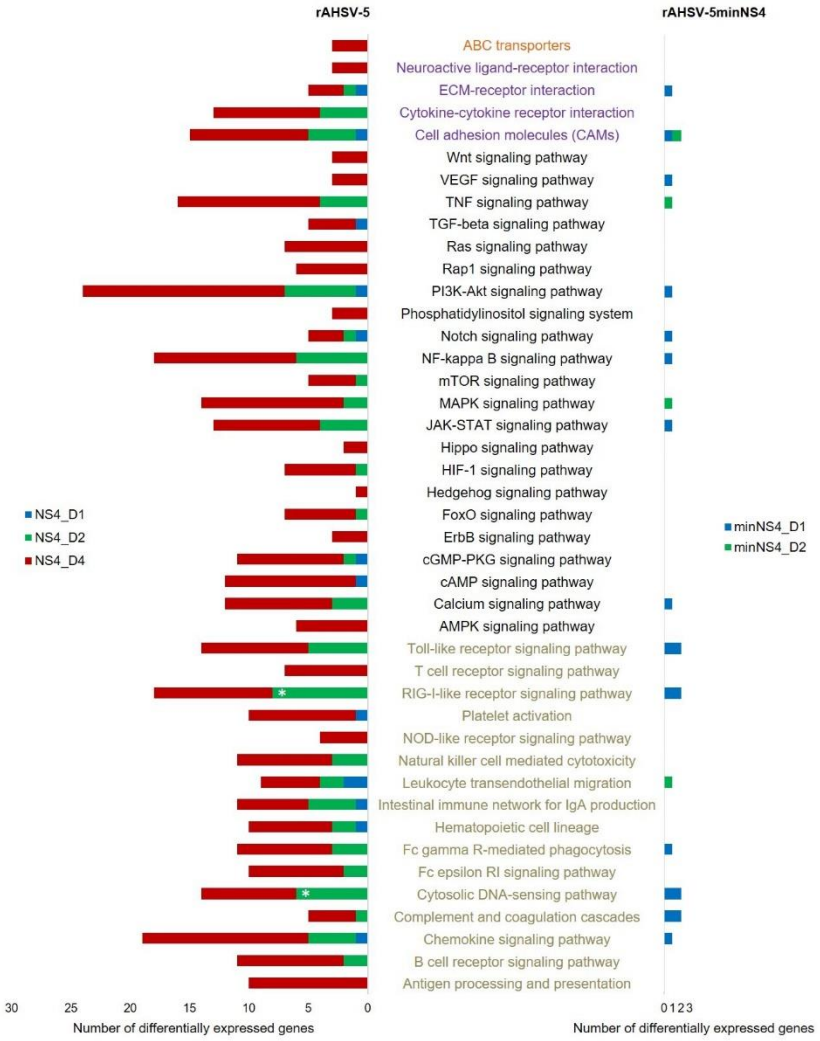
minNS4
Day 2
NS4
182
9
3
minNS4

Fig 4


rAHSV-5
rAHSV-5minNS4
ABC transporters
Neuroactive ligand-receptor interaction
ECM-receptor interaction
Cytokine-cytokine receptor interaction
Cell adhesion molecules (CAMs)
Wnt signaling pathway
VEGF signaling pathway
TNF signaling pathway
TGF-beta signaling pathway
Ras signaling pathway
Rap1 signaling pathway
PI3K-Akt signaling pathway
Phosphatidylinositol signaling system
Notch signaling pathway
NF-kappa B signaling pathway
mTOR signaling pathway
MAPK signaling pathway
JAK-STAT signaling pathway
Hippo signaling pathway
HIF-1 signaling pathway
Hedgehog signaling pathway
FoxO signaling pathway
ErbB signaling pathway
cGMP-PKG signaling pathway
cAMP signaling pathway
Calcium signaling pathway
AMPK signaling pathway
Toll-like receptor signaling pathway
T cell receptor signaling pathway
RIG-I-like receptor signaling pathway
Platelet activation
NOD-like receptor signaling pathway
Natural killer cell mediated cytotoxicity
Leukocyte transendothelial migration
Intestinal immune network for IgA production
Hematopoietic cell lineage
Fc gamma R-mediated phagocytosis
Fc epsilon RI signaling pathway
Cytosolic DNA-sensing pathway
Complement and coagulation cascades
Chemokine signaling pathway
B cell receptor signaling pathway
Antigen processing and presentation
NS4_D1
NS4_D2
NS4_D4
minNS4_D1
minNS4_D2
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0 1 2 3
Number of differentially expressed genes
Number of differentially expressed genes

Fig 5

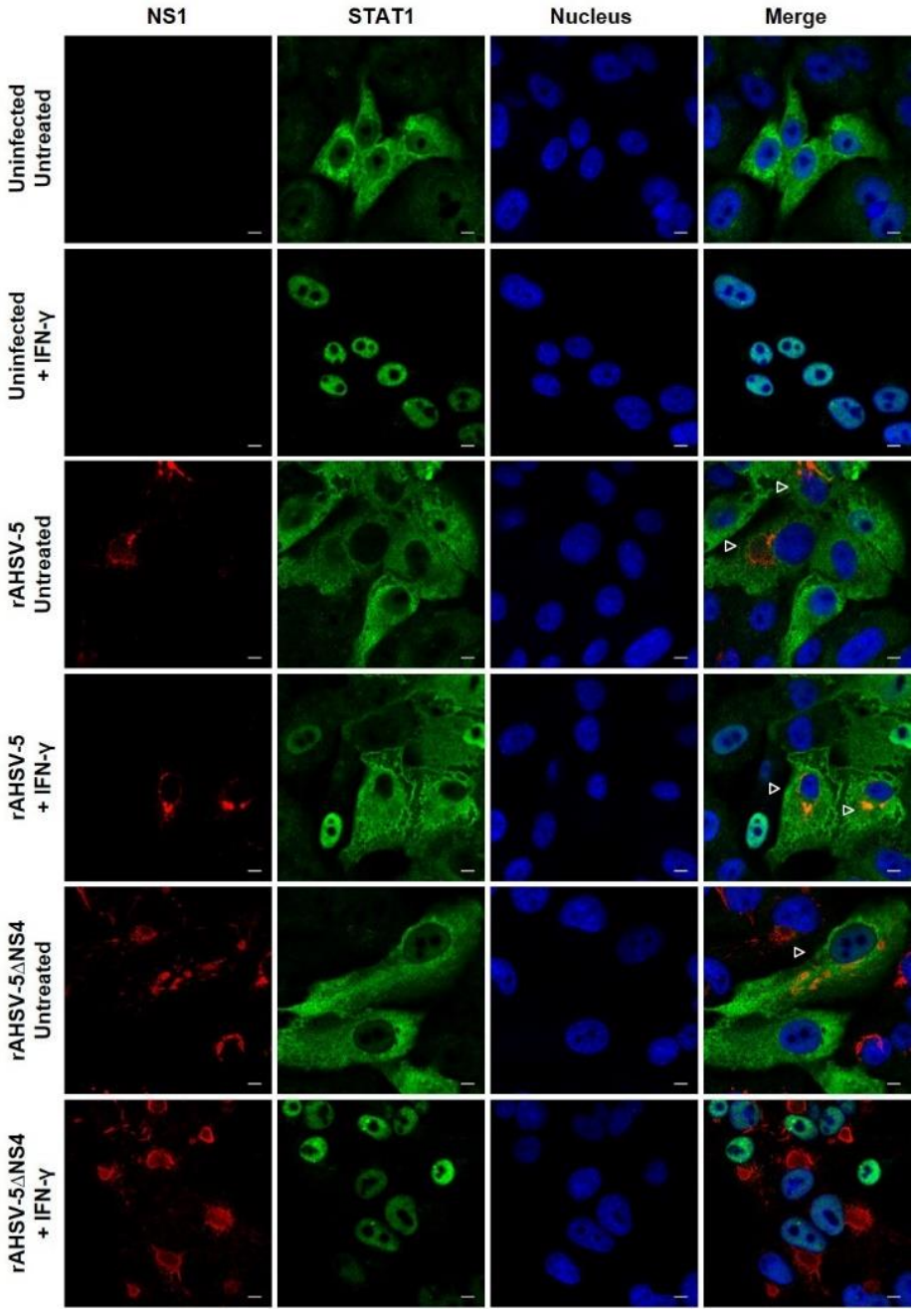
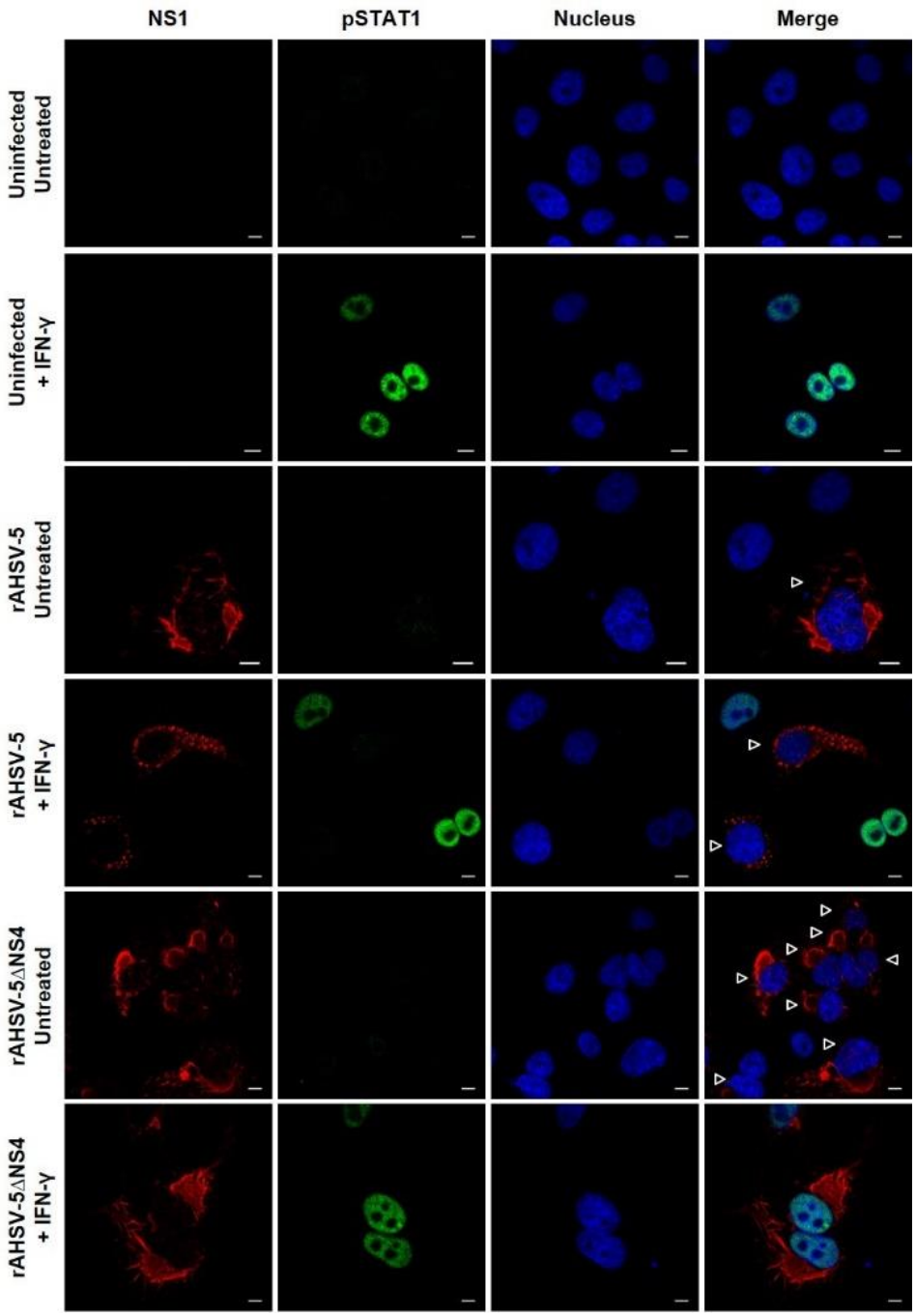

NS1
STAT1
Nucleus
Merge
Uninfected Untreated
Uninfected + IFN-γ
rAHSV-5 Untreated
rAHSV-5 + IFN-γ
rAHSV-5ΔNS4 Untreated
rAHSV-5ΔNS4 + IFN-γ

Fig 6


NS1
pSTAT1
Nucleus
Merge
Uninfected Untreated
Uninfected + IFN-γ
rAHSV-5 Untreated
rAHSV-5 + IFN-γ
rAHSV-5ΔNS4 Untreated
rAHSV-5ΔNS4 + IFN-γ

Tables

Table 1. Summary of data sets and comparisons in the transcriptome analysis

Day (D)	Number of horses	Virus injected	Sample name		Comparisons done (referred to as)
			Individual	Combined	
0	1	rAHSV-5	NS4_D0	-	-
1	1	rAHSV-5	NS4_D1	-	NS4_D1 vs NS4_D0 (NS4_D1)
2	1	rAHSV-5	NS4_D2	-	NS4_D2 vs NS4_D0 (NS4_D2)
4	1	rAHSV-5	NS4_D4	-	NS4_D4 vs NS4_D0 (NS4_D4)
0	3	rAHSV-5minNS4	minNS4_D0_1	minNS4_D0	-
		rAHSV-5minNS4	minNS4_D0_2		
		rAHSV-5minNS4	minNS4_D0_3		
1	3	rAHSV-5minNS4	minNS4_D1_1	minNS4_D1	minNS4_D1 vs minNS4_D0 (minNS4_D1)
		rAHSV-5minNS4	minNS4_D1_2		
		rAHSV-5minNS4	minNS4_D1_3		
2	3	rAHSV-5minNS4	minNS4_D2_1	minNS4_D2	minNS4_D2 vs minNS4_D0 (minNS4_D2)
		rAHSV-5minNS4	minNS4_D2_2		
		rAHSV-5minNS4	minNS4_D2_3		

Table 2. Safety data obtained from five vaccine trials in horses.

Horse number	Trial number	Virus	Virus titer determined on BSR (log10 $TCID_{50}$/ml)	Infection route	Lowest Cq value	AHS clinical signs	Died / Euthanized	Neutralization titer (day 28)	Alveolar edema
794	1	rAHSV-5	5.80	IV	24.8	Yes	Yes	-	Yes
16[#]	2	rAHSV-5	5.80	IV	19.5	Yes	Yes	-	Yes
12	4	rAHSV-5 (pl)	6.66	IV	26.8	Yes	Yes	-	Yes
15	5	rAHSV-5 (pl)	6.66	IV	21.8	Yes	Yes	-	Yes
B14	1	rAHSV-5minNS4	6.80	IV	35.2*	No	-	1024	-
453	1	rAHSV-5minNS4	6.80	IV	30.3	MR	-	≥4096	-
9	3	rAHSV-5minNS4	5.67	IV	35.2*	No	-	128	-
14	3	rAHSV-5minNS4	5.67	IV	36.8*	No	-	128	-
2[#]	2	rAHSV-5minNS4	5.67	IM	34.5*	No	-	32	-
4	2	rAHSV-5minNS4	5.67	IM	34.1*	No	-	32	-
5[#]	2	rAHSV-5minNS4	5.67	IM	34.3*	No	-	64	-
6	2	rAHSV-5minNS4	5.67	IM	36.3*	No	-	16	-
7[#]	2	rAHSV-5minNS4	5.67	IM	34.7*	No	-	32	-
2	3	rAHSV-5minNS4	5.67	SC	ND	No	-	64	-
4	3	rAHSV-5minNS4	5.67	SC	ND	No	-	128	-
5	3	rAHSV-5minNS4	5.67	SC	ND	No	-	128	-
6	3	rAHSV-5minNS4	5.67	SC	ND	No	-	16	-

rAHSV-5 (pl) = Recombinant AHSV-5 rescued from plasmids only.

IV = Intravenous. IM = Intramuscular. SC = Subcutaneous. MR = Mild reaction.

*Horse tested positive only two or three times during a 14-day period.

[#] Horses from which total RNA was isolated from PBMCs for RNA-seq.

Table 3. Top upregulated differentially expressed genes on days 1, 2 and/or 4 as compared to day 0 for the horse infected with rAHSV-5 (designated NS4) and for the horses infected with rAHSV-5minNS4 (designated minNS4). For genes differentially expressed on the same day for both NS4 and minNS4, the log_2FC is indicated in bold.

	rAHSV-5							rAHSV-5minNS4			
Gene	**NS4_D1**		**NS4_D2**		**NS4_D4**		**Gene**	**minNS4_D1**		**minNS4_D2**	
	Rank[a]	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**		**Rank[a]**	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**
OAS3*	U1	**1.62**	U16	**4.4304**	U11	6.4897	Novel02004	U1	5.6909	-	-
#00000021220	U2	1.5197	U23	4.1436	U64	3.5219	MX2*	U2	5.5855	U2	**3.4715**
MX1*	U3	**1.3585**	U8	5.9236	U10	6.6216	Novel00397	U3	5.3207	U1	**4.0086**
STAB1	U4	1.2245	-	-	-	-	Novel02003	U4	5.189	-	-
TGM3	U5	1.1218	-	-	-	-	IFIT5*	U5	4.3476	-	-
OAS2*	U6	**1.0924**	U25	**4.0317**	U24	5.0045	IFI44	U6	4.2385	U3	**1.9847**
APOBEC3Z1B	-	-	U1	8.0631	U2	9.007	MX1*	U7	**3.7577**	-	-
Novel00397	-	-	U2	**7.7817**	U1	11.001	HERC5*	U8	3.4031	-	-
MX2*	-	-	U3	**7.5321**	U3	8.5137	#00000000284	U9	3.2036	U4	**1.6982**
Novel02003	-	-	U4	7.2867	U5	7.4986	HERC6	U10	3.1709	-	-
Novel00818	-	-	U5	7.2274	U4	7.5143	Novel01303	U11	3.0508	-	-
DDX60	-	-	U6	6.9685	U7	7.3932	DDX58*	U12	2.8417	-	-
Novel01228	-	-	U7	6.3226	U22	5.0633	SPHK1*	U13	2.8402	-	-
CCL2*	-	-	U9	5.7699	U12	6.2304	OASL	U14	2.8347	-	-
Novel02081	-	-	U10	5.4359	U19	5.241	Novel01876	U15	2.825	-	-
IFIT5*	-	-	U11	5.3356	U21	5.1826	OAS3*	U16	**2.8087**	U6	**1.5056**
ISG15*	-	-	U12	5.2103	U8	7.0132	C1R*	U17	2.7992	-	-
#00000000284	-	-	U13	**4.788**	U17	5.5516	RTP4	U18	2.7486	-	-
HERC5*	-	-	U14	4.4969	U44	4.0961	FBXO39	U19	2.7377	-	-
IFIT1*	-	-	U15	4.459	U45	4.0605	IRF7*	U20	2.6988	-	-
HSD11B1	-	-	U17	4.4046	U13	5.9152	GBP2*	U21	2.6869	-	-
IFI44	-	-	U18	**4.344**	U48	3.9876	C2*	U22	2.65	-	-
CXCL10*	-	-	U19	4.2425	U26	4.8572	ITSN1	U23	2.6337	-	-
ETV7	-	-	U20	4.2387	U20	5.2229	CMPK2	U24	2.6184	U5	**1.6838**
Novel02002	-	-	U21	4.2387	U55	3.7706	#00000005126	U25	2.6017	-	-
IFI6*	-	-	U22	4.162	U15	5.7488	#00000012132	U26	2.5958	-	-
IFIH1*	-	-	U24	4.103	U37	4.3226	IFI6*	U27	2.558	-	-
HERC6	-	-	U26	4.0114	U56	3.7248	OAS2*	U28	**2.5476**	U7	**1.3382**
Novel01303	-	-	U27	3.9936	U38	4.313	XAF1	U29	2.5432	-	-
IRG1	-	-	U28	3.9694	U47	4.0036	HSD11B1	U30	2.4699	-	-
OAS1*	-	-	U29	3.9188	U29	4.7622	OAS1*	U31	2.4688	-	-
IFIT3*	-	-	U30	3.8494	U67	3.4453	Novel02081	U32	2.381	-	-
#00000010185	-	-	U31	3.8455	U73	3.2855	IRG1	U33	2.3481	-	-
IFI44L	-	-	U32	3.794	U79	3.1244	Novel00654	U34	2.317	-	-
IRF7*	-	-	U33	3.7091	U16	5.6167	SPATS2L	U35	2.2573	-	-
XAF1	-	-	U34	3.693	U35	4.4591	STAT1*	U36	2.2151	-	-
FBXO39	-	-	U35	3.6748	U53	3.803	ZCCHC2	U37	2.1628	-	-
RTP4	-	-	U36	3.67	U36	4.4495	#00000024875	U38	2.1312	-	-
EIF2AK2*	-	-	U37	3.5122	U102	2.6891	SIGLEC1*	U39	2.1219	U8	**1.3016**
CMPK2	-	-	U38	**3.495**	U31	4.6301	DTX3L	U40	1.9868	-	-
SAMD9L	-	-	U39	3.4872	U113	2.4844					
EPSTI1	-	-	U40	3.4791	U80	3.0907					
RSAD2*	-	-	U41	3.3533	U23	5.0315					
RNF213	-	-	U42	3.1751	U39	4.2855					
OASL	-	-	U43	3.1098	U34	4.5074					
GBP5	-	-	U44	3.0003	U204	1.7655					
DDX58*	-	-	U45	2.9652	U76	3.2151					
CD274*	-	-	U46	2.8545	-	-					
ISG20*	-	-	U47	2.8413	U52	3.8241					

[a]Ranks of upregulated genes in each comparison, [b]Log_2Fold change, *Involved in innate immunity according to InnateDB, #ENSECAG

Table 4. Top downregulated differentially expressed genes on days 1, 2 and/or 4 as compared to day 0 for the horse infected with rAHSV-5 (designated NS4) and for the horses infected with rAHSV-5minNS4 (designated minNS4). For genes differentially expressed on the same day for both NS4 and minNS4, the log_2FC is indicated in bold.

	rAHSV-5							rAHSV-5minNS4			
Gene	**NS4_D1**		**NS4_D2**		**NS4_D4**		**Gene**	**minNS4_D1**		**minNS4_D2**	
	Rank[a]	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**		**Rank[a]**	**log_2FC[b]**	**Rank[a]**	**log_2FC[b]**
ANKRD12	D1	-2.95	-	-	D287	-1.0903	U3	D1	-2.993	NA	-
KIF20B	D2	-2.793	-	-	-	-	SCARNA2	D2	-2.5864	NA	-
SMC4	D3	-2.549	-	-	D30	-1.6915	5_8S_rRNA	D3	-2.4694	NA	-
CCDC88A*	D4	-2.2886	-	-	-	-	FN1	D4	-2.407	NA	-
JCHAIN	D5	-2.2698	-	-	D302	-1.0696	RETN	NA	-	D1	-3.5695
FGL2	D6	-1.9536	-	-	D175	-1.2734	MMP1	NA	-	D2	-1.8825
SSB	D7	-1.9388	-	-	D188	-1.2502	DUSP1*	NA	-	D3	-1.5657
PPIG	D8	-1.8348	-	-	D190	-1.2466	MMP9*	NA	-	D4	**-1.3939**
AKAP9	D9	-1.5708	-	-	D35	-1.6584					
RAD50	D10	-1.4676	-	-	D71	-1.5008					
CIR1	D11	-1.4044	-	-	D97	-1.4272					
DEK	D12	-1.3508	-	-	D67	-1.5134					
EIF5B	D13	-1.2612	-	-	D285	-1.0931					
#00000007621	D14	-1.1675	-	-	D47	-1.5644					
TRAT1*	D15	-1.1375	-	-	D163	-1.2897					
CCAR1	D16	-1.1033	-	-	D187	-1.2502					
ROCK1	D17	-1.0914	-	-	D240	-1.1531					
#00000020878	D18	-1.0788	-	-	-	-					
KTN1	D19	-1.0587	-	-	D41	-1.595					
SEC62	D20	-1.0497	-	-	D26	-1.7396					
TAX1BP1*	D21	-1.0428	-	-	D279	-1.1015					
ITGA4	D22	-1.0419	-	-	-	-					
FN1	-	-	D1	-4.6842	D1	-4.2612					
SEPP1	-	-	D2	-2.4215	D7	-2.136					
#00000007258	-	-	D3	-1.9357	D33	-1.6645					
ALDH3A1	-	-	D4	-1.8954	-	-					
GAS2L1	-	-	D5	-1.8869	-	-					
DDIT4	-	-	D6	-1.6516	-	-					
MMP9*	-	-	D7	**-1.4784**	D75	-1.4914					
#00000008721	-	-	D8	-1.3927	-	-					
#00000009556	-	-	D9	-1.38	D299	-1.0721					
VAT1	-	-	D10	-1.3546	D212	-1.2103					
IRF2BP1*	-	-	D11	-1.2827	-	-					
FCGRT	-	-	D12	-1.1567	-	-					
ZNF683	-	-	D13	-1.137	-	-					
CHST2	-	-	D14	-1.127	D50	-1.5565					
LRRC4B	-	-	D15	-1.0873	-	-					
GSN	-	-	D16	-1.0166	-	-					
ZFP36L1	-	-	D17	-1.0132	D116	-1.3927					
MED25	-	-	D18	-1.0061	-	-					
Novel02103	-	-	D19	-1.0036	-	-					
MS4A2	-	-	-	-	D2	-2.6698					
LRRCC1	-	-	-	-	D3	-2.3101					
ALDH1A1	-	-	-	-	D4	-2.2793					
MMP1	-	-	-	-	D5	-2.2367					
KIAA1551	-	-	-	-	D6	-2.1409					
CCNG1	-	-	-	-	D8	-2.0402					
Novel01276	-	-	-	-	D9	-1.9883					
SIRT1*	-	-	-	-	D10	-1.9774					

[a]Ranks of downregulated genes in each comparison, [b]Log_2Fold change, *Involved in innate immunity according to InnateDB, #ENSECAG

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