Virulent African horse sickness virus serotype 4 interferes with the innate immune response in horse peripheral blood mononuclear cells *in vitro*

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Highlights

- Continued type I and type III IFN antiviral responses were induced during the attenuated AHSV4 immune response.
- Virulent AHSV4 interference with the innate immune response in naïve PBMC may cause immunopathology.
- Fast kinetics of immune cells during the secondary immune response was essential to overcome virulent AHSV4 interference.

Abstract

African horse sickness (AHS) is caused by African horse sickness virus (AHSV), a double stranded RNA (dsRNA) virus of the genus *Orbivirus*, family *Reoviridae*. For the development of new generation AHS vaccines or antiviral treatments, it is crucial to understand the host immune response against the virus and the immune evasion strategies the virus employs. To achieve this, the current study used transcriptome analysis of RNA sequences to characterize and compare the innate immune responses activated during the attenuated AHSV serotype 4 (attAHSV4) (*in vivo*) and the virulent AHSV4 (virAHSV4) (*in vitro*) primary and secondary immune responses in horse peripheral blood mononuclear cells (PBMC) after 24 h. The pro-inflammatory cytokine and chemokine responses were negatively regulated by anti-inflammatory cytokines, whereas the parallel type I and type III IFN responses were maintained downstream of nucleic acid sensing pattern recognition receptor (PRR) signalling pathways during the attAHSV4 primary and secondary immune responses. It appeared that after translation, virAHSV4 proteins were able to interfere with the C-terminal IRF association domain (IAD)-type 1 (IAD1) containing IRFs, which inhibited the expression of type I and type III IFNs downstream of PRR signalling during the virAHSV4 primary and secondary immune responses. Viral interference resulted in an impaired innate immune response that was not able to eliminate virAHSV4-infected PBMC and gave rise to prolonged expression of pro-inflammatory cytokines and chemokines during the virAHSV4 induced primary immune response. Indicating that virAHSV4 interference with the innate immune response may give rise to an excessive inflammatory response that causes immunopathology, which could be a major contributing factor to the pathogenesis of AHS in a naïve horse. Viral interference was overcome by the fast kinetics and increased effector responses of innate immune cells due to trained innate immunity and memory T cells and B cells during the virAHSV4 secondary immune response.

Keywords:
AHSV, immune evasion, innate immune response, immunopathology, antiviral treatment, transcriptome sequencing

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[1] **Abbreviations:** AHS, African horse sickness; AHSV4, African horse sickness virus serotype 4; PBMC, peripheral blood mononuclear cells; PRR, pattern recognition receptor; IAD1, C-terminal IRF association domain (IAD)-type 1; dsRNA, double stranded RNA

1. Introduction

African horse sickness (AHS) is an insect-transmitted, infectious but noncontagious disease that is regarded as one of the most lethal diseases of equids with a 90% mortality rate (Carpenter et al., 2017). AHS is caused by African horse sickness virus (AHSV), a double stranded RNA (dsRNA) virus and a member of the genus *Orbivirus* of the family *Reoviridae*. AHSV is transmitted by *Culicoides* biting midges (Zientara et al., 2015; Dennis et al., 2019). The underlying pathology of AHSV in the target organs is direct virus-induced endothelial cell damage (Zientara et al., 2015; Dennis et al., 2019) that results in hemostatic abnormalities and increased vascular permeability that leads to effusions and oedemas (Zientara et al., 2015). It was also shown that an excessive inflammatory response contributes to the pathogenesis of AHS (Carrasco et al., 1999; Clift and Penrith, 2010; Zientara et al., 2015). This is also characteristic of Bluetongue virus (BTV), where several studies have indicated that there is a strong correlation between excessive pro-inflammatory cytokine and chemokine production (e.g. TNF-α, IL-1β, IL-6 and CXCL8) that contributes to immunopathology during BTV infections and the pathogenesis of the disease (Maclachlan et al., 2014; Vitour et al., 2014; Marín-López et al., 2016). Importantly, an excessive inflammatory response that cause immunopathology is more likely to occur with viruses that can interfere with the innate immune response (Rouse and Sehrawat, 2010).

The innate immune response is the first line of host defence, it is non-specific, very rapid and activates the adaptive immune response. Activation of the innate immune response leads to the production of pro-inflammatory cytokines, chemokines and various inflammatory mediators that induce the inflammatory response (Janeway et al., 2001; Moser and Leo, 2010). The innate immune response is activated when pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). The PRRs that recognize RNA viruses include the Toll-like receptors (TLRs) (e.g. TLR3 and TLR7/8) (De Nardo, 2015; Jiménez-Dalmaroni et al., 2016), retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs) (Liu et al., 2017; Chow et al., 2018) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Zhong et al., 2013; Kim et al., 2016). The RLR, TLR and NLR signalling pathways converse at the activation of the NF-κB and MAPKs transcription factors that induce the expression of pro-inflammatory cytokines and chemokines. This includes the parallel activation of IRF3 and IRF7, the main transcription factors responsible for type I and type III IFN expression.

Type I and type III IFNs are regarded as the major antiviral cytokines. They are part of the earliest host defence against infections and play a critical role in limiting viral replication and spread (Schneider et al., 2014; García-Sastre, 2017; Lee and Ashkar, 2018; Nelemans and Kikkert, 2019). Type I IFNs facilitate the recruitment and activate the effector functions (e.g. cytotoxicity) of NK cells and CD8+ T cells, known as cytotoxic T cells (CTLs) and increase the surface expression of co-stimulatory molecules and MHC molecules on dendritic cells (Lee and Ashkar, 2018). Type I and type III IFN signalling result in the expression of hundreds of interferon-stimulated genes (ISGs) that induce an antiviral state in both infected and neighbouring cells (García-Sastre, 2017; Nelemans and Kikkert, 2019). In addition, type I IFN signalling simultaneously dampens damaging

inflammatory responses that can result in immunopathology (Lee and Ashkar, 2018). Type I IFN signalling induces the expression of IL-10 (González-Navajas et al., 2012; Rojas et al., 2017; Lee and Ashkar, 2018) and inhibits the functions of the NLRP1 and NLRP3 inflammasomes (González-Navajas et al., 2012). The type I IFN responses also apply to the type III IFNs since they signal through the same JAK/STAT pathways (Schneider et al., 2014; Nelemans and Kikkert, 2019) and have similar functions (García-Sastre, 2017).

To successfully replicate and survive in infected host cells, pathogenic viruses have developed numerous strategies to escape, interfere, dampen, delay and/or counter the innate and adaptive immune responses. These various strategies are collectively known as immune evasion (Iannello et al., 2006). In particular, viruses must be able to overcome the innate immune type I and type III IFN-mediated antiviral response to replicate and spread. As such, viruses have many strategies to evade and interfere with several steps in the pathways responsible for type I and type III IFN production and/or signalling (García-Sastre, 2017; Nelemans and Kikkert, 2019). This includes members of the *Reoviridae* family, BTV (Chauveau et al., 2013; Doceul et al., 2014; Ratinier et al., 2016; Avia et al., 2019), Rotavirus (Morelli et al., 2015) and Mammalian reoviruses (MRV) (Stanifer et al., 2017).

In order to develop new generation vaccines, it is essential to understand the global immune responses induced and the mechanisms involved in protective immunity of the live attenuated vaccine (Pulendran and Ahmed, 2011). Similarly, for the development of antiviral treatments, it is important to understand the host immune response against a pathogenic virus and the immune evasion strategies that virus employ to combat these immune responses (Condotta and Richer, 2017; Lee, 2018). Importantly, several studies showed that there is a correlation between *in vitro* and *in vivo* gene expression profiles. This was demonstrated with bovine peripheral blood mononuclear cells (PBMCs) stimulated *in vitro* with *Mycobacterium bovis (M. bovis*) that reflected the gene expression observed in PBMC (*in vivo*) of *M. bovis*-infected cattle (Cheng et al., 2015). Similarly, the immune responses induced by different Influenza vaccine formulations in human PBMC monocyte-derived dendritic cells *in vitro* matched the vaccine-specific *in vivo* immune responses (Tapia-Calle et al., 2017). Additionally, based on the correlation of biomarker gene expression of PBMC stimulated with Influenza vaccines *in vitro* and the corresponding *in vivo* vaccination responses, it was indicated that the *in vitro* PBMC assay potentially reflects the actual *in vivo* immune responses induced in human PBMC (Sasaki et al., 2018). As such, there was a high probability that the attenuated AHSV serotype 4 (attAHSV4) *in vivo* and *in vitro* immune responses in horse PBMC will reflect each other. Thus, the attAHSV4 *in vivo* and the virulent AHSV4 (virAHSV4) *in vitro* transcriptome data sets were compared with each other in this study. Therefore, this current study used transcriptome analysis of the global primary and secondary immune responses induced in horse PBMC by the attAHSV4 (*in vivo*) and the virAHSV4 (*in vitro*) to characterize and compare the similarities and/or differences in the innate immune responses. The knowledge generated by this study may contribute to the development of new generation AHS vaccines, broaden our understanding of the pathogenesis of AHS and potentially be used in the development of antiviral treatments.

2. Materials and methods

2.1. Immunization of horses

Five AHSV naïve 30 month-old horses were previously immunized (Faber et al., 2016; Pretorius et al., 2016). Briefly, the five horses were each immunized twice subcutaneously with the attAHSV4 vaccine strain using 5 x 10^4 viable virus in 2 ml DMEM medium (Lonza) prepared at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR). They received their first immunization on day 0 and received the second immunization on day 21. All animal vaccination protocols were approved by the animal ethics committees at the ARC-OVR, the University of Pretoria and Onderstepoort Biological Products (OBP), Ltd. The study was also approved by the Department of Agriculture, Forestry and Fisheries (DAFF) Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984).

2.2. Collection of blood and isolation of horse peripheral blood mononuclear cells (PBMC)

This study used PBMC prepared when horses were bled before immunization on day 0 (naïve) and at several time points that included day 1 (24 h after the first immunization with attAHSV4), day 22 (24 h after the second immunization) and day 38 (17 days after the second immunization) (Faber et al., 2016; Pretorius et al., 2016). Blood (50 ml per horse) was collected in BD Vacutainer®- EDTA tubes (Becton, Dickinson) and PBMC was isolated on a density gradient medium (Histopaque®-1077; Sigma-Aldrich®) as described previously (Pretorius et al., 2012). The PBMC was washed three times and the live cells were counted using GIBCO® trypan blue stain (Invitrogen) and resuspended at 4 x 10^6 cells/ml in cRPMI (GIBCO® RPMI+GlutaMAX™-I (Invitrogen) supplemented with 55 mM 2-mercaptoethanol and 1% (v/v) of 100x GIBCO® Pen Strep (Invitrogen) and 10% heat inactivated horse serum (Invitrogen).

2.3. Total RNA isolation and transcriptome sequencing

Naïve PBMC (collected on day 0 before the first immunization) and immune PBMC (collected on day 38, 17 days after the second immunization) were stimulated *in vitro* with a live viable virAHSV4 field isolate (5 x10^3 virus/ml) and incubated for 24 h. In addition, unstimulated naïve and immune PBMC controls were incubated for 24 h. Total RNA was isolated from the 24 h virAHSV4 stimulated and unstimulated naïve (day 0) and immune (day 38) PBMC of the five horses according to the TRI® Reagent (Sigma Aldrich®) protocol. Contaminating genomic DNA was removed by using a DNA-free kit (Ambion) according to the instructions of the manufacturer. The total RNA quality was quantified using a spectrophotometer ND-1000 Nanodrop® (Thermo Scientific) where samples with an absorbance ratio value (A260/A280) of 1.8–2.0 were considered for further analysis and assessed by agarose gel electrophoresis. The isolated total RNA for the five horses were pooled at each time point (100 ng of each sample) and at least 250 µg total RNA mix was sent to the Agricultural Research Council-Biotech Platform (ARC-BTP) (South-Africa) for Ilumina transcriptome sequencing with the Illumina HiSeq (Illumina, San Diego, CA, USA). These total RNA samples were tested for quality and purity using the qubit fluorometric quantification instrument (Thermofisher Scientific, USA).

The RNA libraries were prepared by using the TruSeq Stranded mRNA Sample Prep Kit (Illumina) as per manufacturer's instructions.

2.4. Bioinformatics analysis of the transcriptome data

The RNA-seq data generated from the PBMC were analysed using CLC Genomics Workbench version 9.5.2 (http://www.clcbio.com/products/clc-genomics-workbench/). To specifically focus on immune modulation by these cells, the reads were mapped, using the CLC RNA-seq analysis tool, to preselected *Equus caballus* (*E. caballus*) immune reference gene sequences (n=2333) obtained from the NCBI server (http://www.ncbi.nlm.nih.gov/). In addition, the RNA-seq data from the previous *in vivo* study (Pretorius et al., 2016), were re-analysed. This was done and included in this study because the reads were previously mapped to a combination of pre-selected equine or bovine immune reference gene sequences. Since then, the *E. caballus* genome sequence had been annotated and published. The RNA-seq reads from all the samples were imported and adapters as well as low quality base reads were trimmed. The AHSV specific reads were also mapped against AHS viral gene transcripts obtained from the NCBI server (http://www.ncbi.nlm.nih.gov/).

Using the CLC transcriptomics function, transcriptome data sets from naïve (day 0) and immune (day 38) PBMC stimulated *in vitro* for 24 h with the virAHSV4 were normalised to their respective 24 h unstimulated naïve and immune PBMC control transcriptome data sets. The *in vivo* transcriptome data sets from PBMC (day 1), collected 24 h after the first immunization with attAHSV4 and PBMC (day 22), collected 24 h after the second immunization were normalised to the naïve PBMC (collected on day 0 before the first immunization) transcriptome data set. The immune responses induced upon the first exposure/encounter to AHSV4 will be referred to as primary immune responses in this paper. They include the immune responses activated in naïve PBMC (collected on day 0 before the first immunization with attAHSV4) that was stimulated *in vitro* with the virAHSV4 for 24 h and the *in vivo* immune responses induced in PBMC (day 1), collected 24 h after the first immunization with attAHSV4. The recall or memory immune responses activated upon re-exposure/re-encounter to AHSV4 will be referred to as secondary immune responses in this paper. They include the immune responses induced in immune PBMC (collected on day 38, 17 days after the second immunization with attAHSV4) that was stimulated *in vitro* with the virAHSV4 for 24 h and the *in vivo* immune responses activated in PBMC (day 22), collected 24 h after the second immunization with attAHSV4.

Comparative transcriptomic experiments allowed for appropriate statistical analysis including Baggerley's test on proportions and the empirical analysis of digital gene editing (EDGE) tool. The expressed transcripts were filtered based on false discovery rate (FDR) corrected P-values <0.05 and fold change $\geq \pm 1.2$ visualised in volcano plots. Experimental fold change calculations resulted in some samples with infinite (∞) fold increase calculation results. This occurs when one of the mean RPKM data sets had no reads present and has a mean value of zero. These were recalculated in excel using the RPKM+1 formula by adding one to both the control and stimulated RPKM values before division of samples to determine fold increase. Scatter plots were

compiled using normalised data sets. All significant fold change values were visually correlated with the sequence map generated in CLC and those that did not have any reads but generated a normalised RPKM value were corrected by changing the RPKM value to "0" and applying the RPKM +1 formula as described above. The RPKM values of selected housekeeping genes (https://www.sigmaaldrich.com/) of the attAHSV4 *in vivo*, virAHSV4 *in vitro* and their respective controls transcriptome data sets were compared to validate the normalisation between data sets.

KEGG Pathways, Reactome Pathways, Biological Process and Molecular Functions were identified using STRING v11 (http://string-db.org/) analysis. KEGG immune pathways were visualised using the KEGG Pathway database (http://www.genome.jp/kegg/pathway.html). Immune gene function analysis was done using STRING v11 (http://string-db.org/), Uniprot (http://www.uniprot.org/uniprot) and GeneCards (www.genecards.org/). The Interferome database v2.01 (www.interferome.org) was used to identify the interferon-stimulated genes (ISGs).

2.5. Statistical analysis

The significance of results for the comparative transcriptome data was determined by the Student's *t*-test, Baggerley's test and EDGE. Fold change values $\geq \pm 1.2$ and *P*-values ≤ 0.05 were regarded as significant.

3. Results and Discussion

3.1. Expression of African horse sickness virus serotype 4 (AHSV4) structural and non-structural genes in horse peripheral blood mononuclear cells (PBMC)

The presence of viral mRNA was determined in transcriptome data sets to confirm that the PBMC were infected with the attAHSV4 *in vivo* and with the virAHSV4 field isolate after *in vitro* stimulation for 24 h. There were no viral transcripts detected in the *in vivo* naïve PBMC (collected on day 0 before the first immunization) nor after 24 h in the *in vitro* unstimulated control PBMC collected on days 0 (before the first immunization) and 38 (17 days after the second immunization) (Fig. 1). The absence of viral transcripts in the unstimulated immune PBMC (day 38) indicated that no residual virus was present in the PBMC and that the attAHSV4 was effectively cleared by the immune response *in vivo*. Transcripts for the attAHSV4 structural and non-structural genes could be detected in the *in vivo* PBMC collected on days 1 and 22 (Fig. 1A). Viral transcripts for the virAHSV4 structural and non-structural genes were detected in the *in vitro* 24 h stimulated naïve PBMC (day 0) and immune PBMC (day 38) (Fig. 1B).

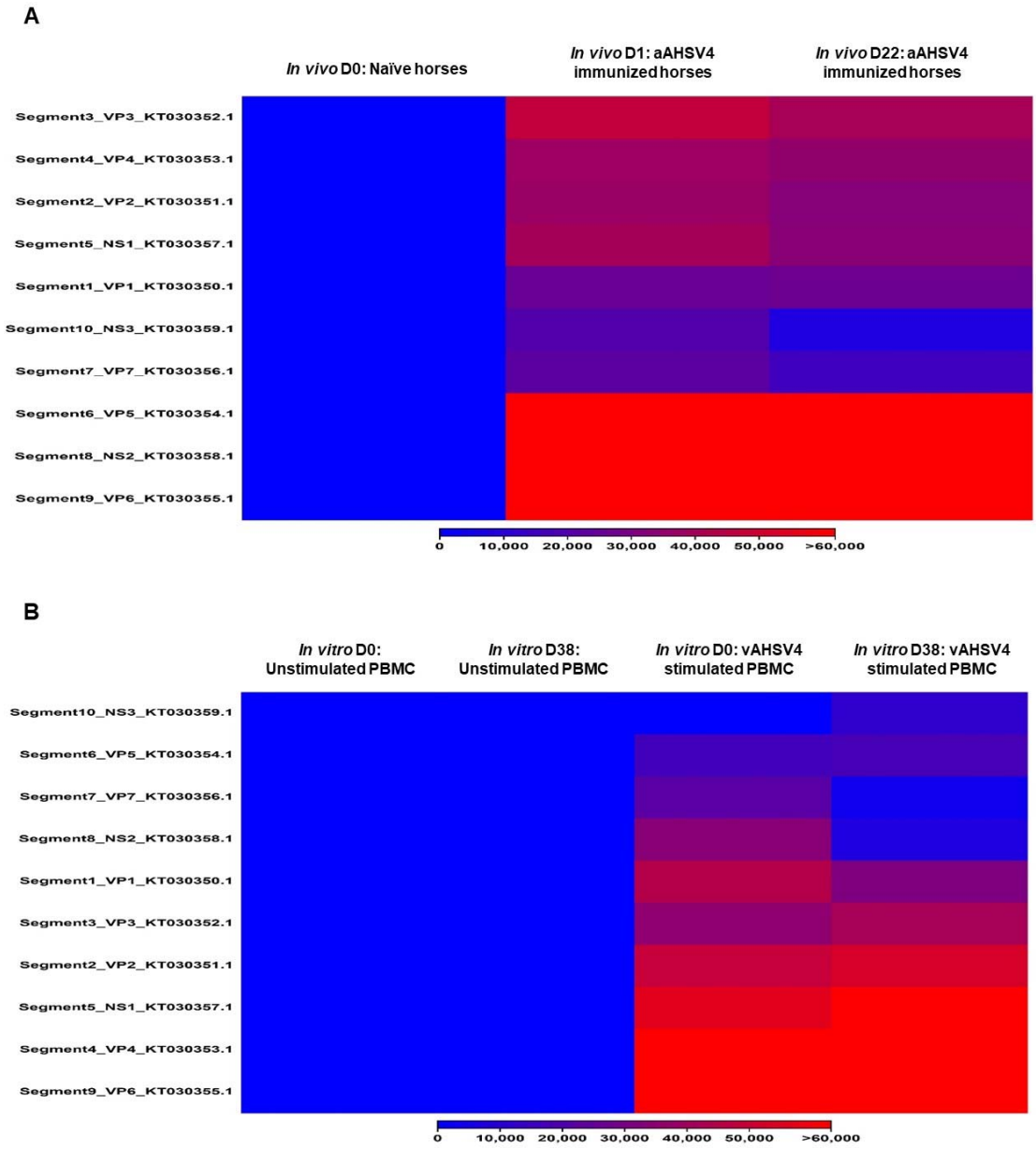

Fig. 1. Heat map of (A) the attAHSV4 (aAHSV4) gene transcripts and (B) the virAHSV4 (vAHSV4) gene transcripts. Using a blue and red colour scale, the levels of expression of the different viral transcripts ranges from no expression (blue) to highest copies (red).

The presence of viral transcripts in PBMC on day 1 and day 22 collected from immunized horses, demonstrated that attAHSV4 could infect and replicate in PBMC. This was also observed in naïve PBMC (day 0) and immune PBMC (day 38) stimulated for 24 h *in vitro* with virAHSV4, confirming infection and replication of the virAHSV4 in PBMC. PBMC consist of monocytes, dendritic cells, NK cells, T cells and B cells (Sen et al., 2018). Monocytes and macrophages in circulation are some of the main target cells for AHSV replication (Clift and Penrith, 2010). It is therefore highly probable that AHSV4 infection and replication in PBMC specifically occurred in monocytes. Interestingly, there was evidence of varying levels of expression for the different viral transcripts. The typical two-stage replication cycle may account for the differences observed in the viral mRNA expression (Matsuo et al., 2010; Kaname et al., 2013). It also appears, as observed with the

varying levels of expression, that not all of the viral mRNAs of a specific replication stage are immediately expressed after cell entry or at the same time, but rather in different stages. However, an in depth study with several different time points is required to evaluate this.

3.2. Identification of differentially expressed up-regulated and down-regulated immune genes in horse peripheral blood mononuclear cells (PBMC)

The total number of differentially expressed up- and down-regulated immune genes were identified resulting from a total of seven data sets listed in Supplementary Table S1. These transcriptome data sets contained between 3.77-28.82 million reads, after trimming and between 8.09%-11.42% of the total transcripts mapped to the pre-selected immune reference mRNA track. The attAHSV4 *in vivo* and virAHSV4 *in vitro* transcriptome data sets showed an even distribution of genes up-regulated and down-regulated at each time point on the scatter plots (Supplementary Fig. S1) and in volcano plots (Supplementary Fig. S2). Similar RPKM values of the selected housekeeping genes were observed for the various controls, attAHSV4 *in vivo* and virAHSV4 *in vitro* transcriptome data sets (Supplementary Fig. S3). This showed a correlation between the *in vivo* and *in vitro* gene expression profiles, which indicated that the attAHSV4 *in vivo* and virAHSV4 *in vitro* transcriptome data sets could be compared with each other. Numerous differentially expressed immune genes for the attAHSV4 and virAHSV4 transcriptome data sets were shown to be up- and down-regulated with significant RPKM fold change of >±1.2 and a FDR corrected P-value of <0.05, based on Baggerley's test (Fig. 2).

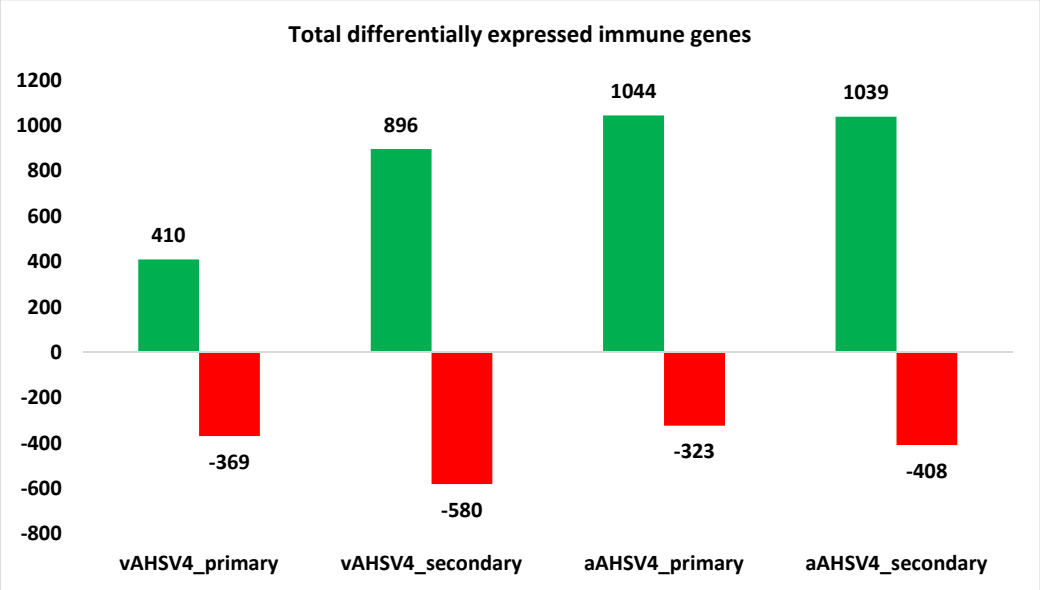

Fig. 2. The total up-regulated (green, positive) and down-regulated (red, negative) immune genes with a fold change (normalized values) of ≥ 1.2 and *P*-values ≤ 0.05 (significant) during the virAHSV4 (vAHSV4) and the attAHSV4 (aAHSV4) primary and secondary immune responses.

However, at least double the number of differentially expressed immune genes were up-regulated during the attAHSV4 primary (1044) and secondary (1039) immune responses as well as the virAHSV4 secondary

immune responses response (896) compared to the virAHSV4 primary immune response (410) (Fig. 2). Similarly, STRING v11 analysis demonstrated that less genes associated with the innate and adaptive immune responses (Reactome Pathways) as well as activation of various immune cells (Biological Process) were up-regulated during the virAHSV4 primary immune response (Fig. 3 and Supplementary Table S2) than during the attAHSV4 primary immune response (Fig. 3 and Supplementary Table S3). The differences in their gene expression indicated that the virAHSV4, similar as other members of the *Reoviridae* family, likely have strategies to interfere with the innate immune response (discussed later). Multiple genes associated with the innate immune response and the adaptive immune response were up-regulated during both the virAHSV4 (Fig. 3 and Supplementary Table S2) and the attAHSV4 (Fig. 3 and Supplementary Table S3) secondary immune responses. The well-developed immune responses showed that virAHSV4 interference was circumvented during the memory immune response (discussed later).

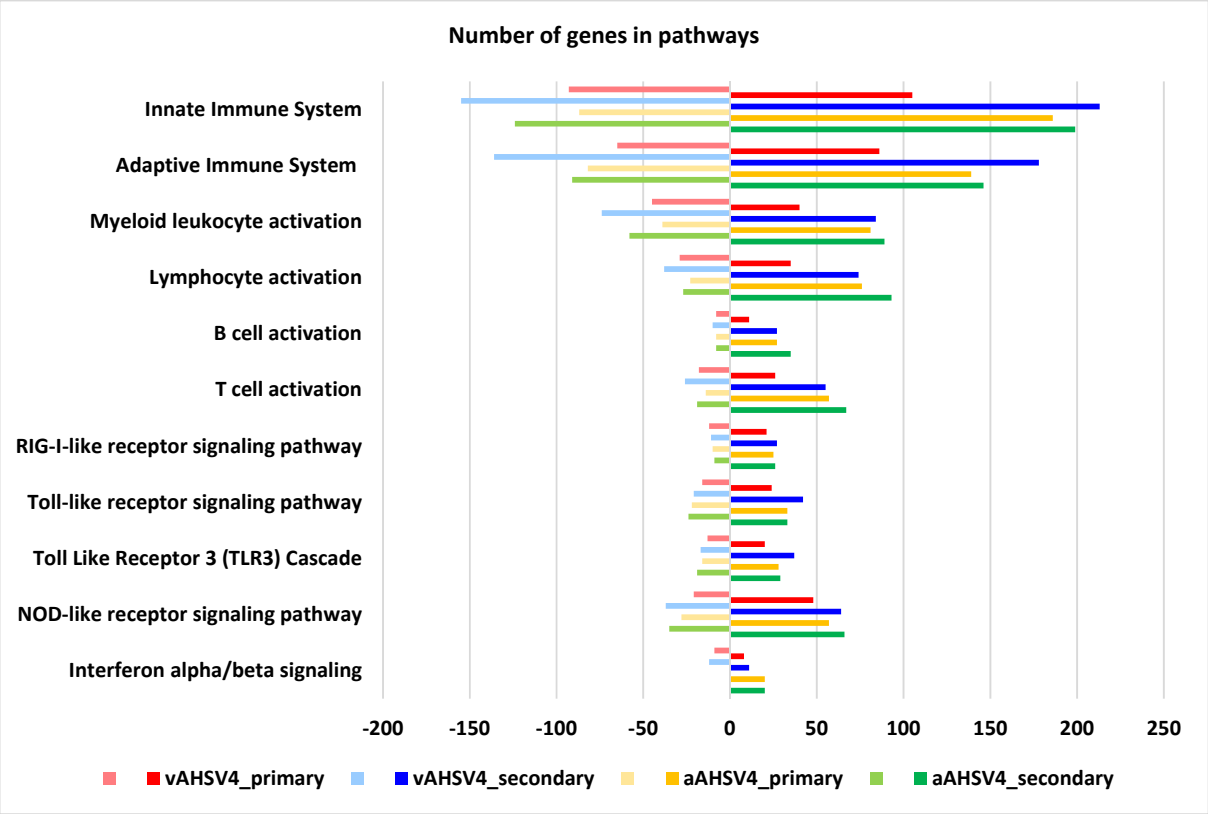

Fig. 3. The total number of differentially expressed up-regulated (positive, dark shade colours) and down-regulated (negative, light shade colours) genes in various immune pathways during the virAHSV4 (vAHSV4) and the attAHSV4 (aAHSV4) primary and secondary immune responses. The values, false discovery rates and STRING v11 analysis (e.g. KEGG Pathways, Reactome Pathways and Biological Process) are shown in Supplementary Tables S2 and S3.

Significant differences in the gene expression profiles were also observed in transcriptome comparisons between a virulent Dengue virus (DENV1) strain and its attenuated derivative (Sessions et al., 2013), a highly pathogenic Influenza A virus H5N1 strain and less pathogenic strains (Muramoto et al., 2014) and a virulent Vaccinia virus (VACV) strain and an attenuated VACV (Israely et al., 2019). More than twice the number of genes were up-regulated in pathways that included the innate immune response and activation of the adaptive

immune response during the attenuated primary immune responses compared to their respective virulent virus immune responses. In the attenuated viral infections, the lack of immune evasion resulted in rapid and robust innate and adaptive immune responses. In contrast, the reduced levels of gene expression during the virulent viral primary immune responses were due to innate immune evasion (Sessions et al., 2013; Israely et al., 2019; Liu et al., 2017). It was shown that the limited gene expression early during the highly pathogenic H5N1 strain (Muramoto et al., 2014) and the virulent VACV (Israely et al., 2019) infections resulted in weak innate and delayed adaptive immune responses that contributed to a severe disease (Muramoto et al., 2014; Israely et al., 2019). Specifically, the impaired immune responses early during the highly pathogenic H5N1 infection resulted in the failure to restrain viral replication and the development of excessive inflammatory response at later stages, which gave rise to a more severe disease (Muramoto et al., 2014).

3.3. Activation of the innate immune response

STRING v11 analysis (KEGG Pathways) showed that numerous differentially expressed genes were significantly enriched in the RLR, TLR and NLR signalling pathways during both the virAHSV4 (Fig. 3 and Supplementary Table S2) and attAHSV4 (Fig. 3 and Supplementary Table S3) primary and secondary immune responses. It is important to take into consideration that the transcriptome data sets were generated 24 h after attAHSV4 immunizations and virAHSV4 stimulations. Since the innate immune response can be activated within several minutes to a few hours (Janeway et al., 2001; Moser and Leo, 2010), certain genes in some of the PRR signalling pathways that were up-regulated earlier may be down-regulated after 24 h and vice versa. Hence, several downstream events likely already occurred and others were in the process of taking place. Therefore, some regulatory mechanisms (e.g. negative regulation of the inflammatory response) may be operational at the current time points.

3.3.1. The retinoic acid-inducible gene-I (RIG-I) like receptor (RLR) signalling pathway

Two key proteins mediate this pathway, RIG-I and MDA, which recognize viral dsRNA in the cytoplasm specifically during cytoplasmic viral replication in host cells. RIG-I preferably binds to short 5′ triphosphate (5’ppp) dsRNA with blunt 5′ ends while MDA5 binds to long dsRNA (>1,000 bp-2000bp) with no end specificity. Both RIG-I and MDA5 signal through the adaptor protein, MAVS (Dixit and Kagan, 2013; Roers et al., 2016; Liu et al., 2017; Chow et al., 2018). MDA was up-regulated during the virAHSV4 primary immune response. RIG-I and MAVS were up-regulated during the virAHSV4 and attAHSV4 primary and secondary immune responses (Table 1 and Supplementary Fig. S4A-D).

It is evident from literature that both RIG-I and MDA5 are also activated by the other members of the *Reoviridae* family that include BTV (Maclachlan et al., 2014; Vitour et al., 2014), MRV (Stanifer et al., 2017; Lanoie et al., 2019) and Rotavirus (Lanoie et al., 2019). This implies that the viral dsRNA is detected in the host cell cytoplasm during viral replication. However, due to transcription of their dsRNA genomes that occur within viral cores, the dsRNA genomes of the *Reoviridae* family members are not freely exposed in the cytoplasm (Mohl and Roy, 2014; Lanoie et al., 2019). Furthermore, like host mRNA (Roers et al., 2016; Chow

et al., 2018), the 5′ppp of the newly synthesized *Reoviridae* ssRNA transcripts from each of the dsRNA segments are capped and methylated before they are extruded into the cytoplasm as full-length mRNA copies (Mohl and Roy, 2014). The 5′ ends of the secondary structures of the viral ssRNA will therefore mimic host mRNA duplexes and not activate RIG-I (Roers et al., 2016; Chow et al., 2018). Thus, it remains largely unknown how the RLRs are activated by these viruses during infections.

Table 1. Some of the key up-regulated (positive) and down-regulated (negative) genes associated with PRR signalling as well as pro-inflammatory cytokines, chemokines and anti-inflammatory cytokines with a fold change (normalized values) of ≥ 1.2 and *P*-values ≤ 0.05 (significant) during the virAHSV4 (vAHSV4) and the attAHSV4 (aAHSV4) primary and secondary immune responses. Using a green, yellow and red colour scale, the lower values are illustrated in darker shades of green and the higher values in darker shades of red. The non-significant genes that include the not significantly up-regulated, down-regulated nor expressed genes (no values, white) are included in the table.

Genes	vAHSV4		aAHSV4	
	Primary	Secondary	Primary	Secondary
PRRs				
RIG-I	1.2	1.3	1.3	1.3
MDA5	1.5			
MAVS	1.2	1.6	1.2	1.4
TLR3	1.5			1.3
TLR7	1.5	2.7	1.2	-1.2
TLR8	1.3	1.7		
MYD88		1.3	1.4	1.3
TRAM		2.1		
TIRAP		2.9	1.2	
TRIF	-1.4	-2.3	2.2	2.3
NOD2	1.2	1.3	2.2	2.1
RIPK2		-1.3	-1.2	-1.2
Transcription factors				
IRF3	-1.2	-1.5	1.2	1.3
IRF7	1.5	-1.9	1.3	1.2
MAPK1	1.2	3.1	1.2	1.3
MAPK3	-1.2	-1.3		
MAPK4			1.8	1.9
MAPK6		1.7		
MAPK7	-1.3		1.4	1.2
MAPK8	1.2	1.3		
MAPK9			-1.2	-1.2
MAPK10		3.7	1.5	1.5
MAPK11	-1.2	-1.2		1.5
MAPK12	-1.3	-1.9		1.4
MAPK14	1.4	1.9	1.2	1.2
MAPK15			1.6	2.5
RELA			1.4	1.4
NFKB1	1.2	1.2	1.2	1.3
RELB	1.5	-1.6	1.2	1.5
NFKB2		-1.4		1.2

Genes	vAHSV4		aAHSV4	
	Primary	Secondary	Primary	Secondary
Type I and type III IFNs				
IFNL1			11.7	7.8
IFNL3			3.5	3.6
IFNA1			10.2	5.1
IFNA2			4.3	3.1
IFNA4			3.3	6.8
IFNB			3.7	2.1
IFND1			3.1	4.4
IFNE			1.4	3.1
IFNW			3.2	5.7
Pro-inflammatory cytokines and chemokines				
IL12A	-5.8	-8.1	1.2	
IL12B		1.7	-1.2	1.4
IL1B	4.5	-1.5	-5.7	-21.1
IL18	1.4	-1.7	-1.3	-1.6
IL6	1.4	1.3	-1.5	-2.3
TNF	1.3	-1.2	-2.5	-2.7
CCL3		-1.5	-2.3	-7.5
CCL4		-2.1	-1.8	-3.9
CCL5		-1.4	1.3	1.2
CXCL1	1.2	-1.6	-3.3	-8.8
CXCL2	2.4	-1.4	-2.4	-2.6
CXCL3		-1.5	-2.4	-6.4
CXCL8	2.1		-1.4	-3.8
CSF1	2.1		-1.2	
IFNG		-2.2		-1.3
Anti-inflammatory cytokines				
IL4		4.9	1.6	1.2
IL10	-2.1	-1.9	1.3	1.2
IL13		8.8		1.3
TGFB2			2.4	2.1
TGFB3		1.2	1.9	1.6

However, RIG-I also binds to 3′-untranslated regions (3'-UTRs) of viral secondary RNA structures. This was demonstrated with several ssRNA viruses that included Chikungunya virus (CHIKV) (Sanchez David et al., 2016), Hepatitis C virus (HCV) (Uzri and Gehrke, 2009), Influenza virus (Davis et al., 2012) and Measles virus (MeV) (Runge et al., 2014). Since the 5′ppp of newly synthesized *Reoviridae* ssRNA transcripts is masked from recognition, we propose that RIG-I binds to the 3'-UTRs of the secondary RNA structures. The viruses with segmented dsRNA genomes must package the correct number of ssRNA segments into the viral cores. The *Reoviridae* family achieve this with specific RNA-RNA interactions between the different ssRNA

segments mediated by sequences located in the 3'-UTRs of the secondary RNA structures. This results in the formation of supramolecular RNA complexes before packaging into the assembling capsid (Fajardo et al., 2015; Fajardo et al., 2016). MDA5 has been reported to bind to RNA organized in large secondary structures that contain both ssRNA and dsRNA regions (Dixit and Kagan, 2013; Chow et al., 2018) that likely adopts web-like conformations (Dixit and Kagan, 2013). As such, we speculate that the larger supramolecular RNA complexes are recognized by MDA5.

3.3.2. The Toll-like receptor (TLR) signalling pathway

TLR signalling during the virAHSV4 and attAHSV4 primary and secondary immune responses mainly occurred through TLR3, TLR7 and TLR8. TLR3 signals via the adaptor protein TRIF, whereas TLR7 and TLR8 signal through MYD88 (Table 1 and Supplementary Fig. S5A-D). TLR3 was up-regulated during the primary virAHSV4 and the secondary attAHSV4 immune responses (Table 1). TLR3 recognizes dsRNA inside intracellular compartments thus most likely after viral phagocytosis and degradation in endosomes by antigen presenting cells (De Nardo, 2015; Jiménez-Dalmaroni et al., 2016; Chow et al., 2018). Alternatively, viral RNA will also be detected by TLR3 during AHSV4 entry into cells at early and later stages in the infection. TLR3 is also activated by other *Reoviridae* family members that include MRV (Stanifer et al., 2017; Lanoie et al., 2019) and BTV (Dai et al., 2015). Although TLR3 was not significantly expressed in all the data sets, STRING v11 analysis (Reactome Pathways) showed that several genes associated with the TLR3 signalling cascade were up-regulated during the virAHSV4 (Fig. 3 and Supplementary Table S2) and attAHSV4 (Fig. 3 and Supplementary Table S3) primary and secondary immune responses. This indicated that dsRNA was likely recognized by TLR3 at an earlier stage during both the virAHSV4 secondary and the attAHSV4 primary immune responses.

Both TLR7 and TLR8 were up-regulated during the virAHSV4 primary and secondary immune responses (Table 1). TLR7 was up-regulated during attAHSV4 primary immune response (Table 1). TLR7 and TLR8 recognize ssRNA inside intracellular compartments (De Nardo, 2015; Jiménez-Dalmaroni et al., 2016). During viral degradation in endosomes, the degradation products of the dsRNA genome that include single strands of the dsRNA or ssRNA fragments can be recognized by TLR7 and TLR8 (Chow et al., 2018). It was also demonstrated that TLR7 is activated by other *Reoviridae* family members. TLR7 was activated in plasmacytoid dendritic cells during a Rotavirus infection (Deal et al., 2010) and TLR7 was up-regulated in both sheep and goat PBMC infected with BTV16 (Singh et al., 2016).

3.3.3. The nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) signalling pathway

In addition to bacterial peptidoglycan components, NOD2 can also sense viral ssRNA in the cytoplasm (Zhong et al., 2013; Kim et al., 2016). NOD2 and MAVS were up-regulated by both the virAHSV4 and attAHSV4 (Table 1 and Supplementary Fig. S6A-D). Adaptor protein RIPK2 was down-regulated during the attAHSV4 primary and secondary immune responses and the virAHSV4 secondary immune response (Table 1). This

finding correlates with the fact that recognition of viral ssRNA by NOD2 induces the recruitment of MAVS but not RIPK2 that leads to the activation of IRF3 and/or IRF7 and the production of type I IFNs. In contrast, NOD2 primarily recruits RIPK2 during bacterial infections that results in the activation of NF-κB and the MAPKs and the subsequent expression of pro-inflammatory cytokines, chemokines and anti-microbial peptides (Zhong et al., 2013; Kim et al., 2016). Similarly, the BTV transcriptome study also showed that the NLR signalling pathway was activated in both sheep and goat PBMC infected with BTV16 at 72 h post infection (Singh et al., 2016).

3.3.4. The inflammatory response downstream of pattern recognition receptor (PRR) signalling

Several NF-κB and the MAPKs transcription factors were up-regulated in the RLR, TLR and NLR signalling pathways during the virAHSV4 and the attAHSV4 primary and secondary immune responses (Table 1). Multiple pro-inflammatory cytokines and chemokines that included CSF1, TNF-α, IL-1β, IL-18, IL-6, CXCL1, CXCL2 and CXCL8 were up-regulated during the virAHSV4 primary immune response (Table 1 and Supplementary Fig. S7A). In contrast, many pro-inflammatory cytokines and chemokines (e.g. TNF-α, IL-1β, IL-18, CXCL1-CXCL3) were selectively down-regulated downstream of NF-κB during the virAHSV4 secondary immune response (Supplementary Fig. S7B) and the attAHSV4 primary (Supplementary Fig. S7C) and secondary (Supplementary Fig. S7D) immune responses (Table 1). Therefore, the expression of pro-inflammatory cytokines and chemokines was prolonged during the virAHSV4 primary immune response as compared to their down-regulation during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses at the 24 h time points (Table 1).

TNF-α, IL-1β and IL-6 are regarded as prototypic pro-inflammatory cytokines. They are endogenous pyrogens that promote fever and induce the production of acute phase proteins from hepatocytes (Arango Duque and Descoteaux, 2014). TNF-α and IL-1β in particular, are powerful activators of the inflammatory response (Mai et al., 2013; Arango Duque and Descoteaux, 2014). The inflammatory response is crucial for the recruitment of blood circulating leukocytes to the site of infection, eliminating and limiting the spread of pathogens (Janeway et al., 2001; Moser and Leo, 2010; Netea et al., 2017). However, an excessive inflammatory response can lead to the overproduction of pro-inflammatory cytokines (known as a cytokine storm), increased leukocyte recruitment and vascular permeability that cause damage to cells and tissues (Janeway et al., 2001; Rouse and Sehrawat, 2010; Arango Duque and Descoteaux, 2014; Netea et al., 2017). Furthermore, overproduced TNF-α and IL-1β are potent inducers of damaging inflammatory responses (Mai et al., 2013; Arango Duque and Descoteaux, 2014; Akdis et al., 2016) and hemostatic abnormalities (Mai et al., 2013). Thus, the continued induction of these pro-inflammatory cytokines may contribute to immunopathology and a severe disease outcome during virAHSV infections in a naïve horse.

The persistent up-regulation of CXCL1, CXCL2 (Arango Duque and Descoteaux, 2014) and CXCL8 (Arango Duque and Descoteaux, 2014; Akdis et al., 2016) during the virAHSV4 primary immune response may play a role in the unrestrained recruitment and activation of neutrophils that contributes to tissue damage. It was

demonstrated during AHSV infections that pro-inflammatory cytokines and chemokines produced by pulmonary intravascular macrophages recruit neutrophils that contributed to the tissue damage in lungs (Carrasco et al., 1999).

Additionally, M-CSF (CSF1) was only up-regulated during the virAHSV4 primary immune response (Table 1). CSF1 is another cytokine that most likely contributes to immunopathology during AHSV infections. CSF1 signalling is responsible for the generation of the monocyte and macrophage lineage from hematopoietic progenitor cells and plays a crucial role in promoting their survival (Becher et al., 2016; Meyer et al., 2018). Monocytes and macrophages (Clift and Penrith, 2010) as well as pulmonary intravascular macrophages (Carrasco et al., 1999) were shown to play major roles in contributing to AHS pathogenesis during AHSV infections via production of pro-inflammatory cytokines and chemokines (Carrasco et al., 1999; Clift and Penrith, 2010). It is therefore very possible that CSF1 will play a prominent role in the facilitating immunopathology during the AHSV infections. Similarly, CSF1 was identified as the major pro-inflammatory cytokine that drives monocyte and macrophage mediated immunopathology in Coxsackievirus B3-induced myocarditis in mice (Meyer et al., 2018). Furthermore, in addition to microvascular endothelial cells, monocytes, macrophages (Clift and Penrith, 2010) and pulmonary intravascular macrophages (Carrasco et al., 1999) are the main target cells for AHSV replication. As a major pro-survival factor for monocytes and macrophages (Becher et al., 2016; Meyer et al., 2018), CSF1 may play a critical role in promoting the survival of these AHSV-infected target cells which may contribute to a severe disease outcome.

Unfortunately, it is not possible without investigating additional time points for transcriptome data analyses to determine if the up-regulated pro-inflammatory cytokines and chemokines will develop into an excessive inflammatory response during the virAHSV4 primary immune response. However, it was demonstrated that monocyte/macrophage produced pro-inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) were up-regulated from day 1 post BTV infection in IFN-α receptor gene knock-out mice (IFNAR−/−) mice. The expression of the pro-inflammatory cytokines significantly increased on day 2 that gave rise to an excessive inflammatory response, which contributed to immunopathology during the BTV infection (Marín-López et al., 2016). As such, the prolonged up-regulation of pro-inflammatory cytokines and chemokines during the virAHSV4 primary immune response as compared to their down-regulation during the virAHSV4 secondary and the attAHSV4 primary and secondary immune responses at the 24 h time points, may be an indication that an excessive inflammatory response was in the process of developing.

Several anti-inflammatory cytokines were up-regulated during the attAHSV4 primary (IL-4, IL-10, TGF-β2 and TGF-β3) and secondary (IL-4, IL-10, IL-13, TGF-β2 and TGF-β3) immune responses and the virAHSV4 secondary immune response (IL-4, IL-13 and TGF-β3) (Table 1). In contrast, these anti-inflammatory cytokines were down-regulated or not significantly expressed during the virAHSV4 primary immune response (Table 1). Thus, the selective down-regulation of the pro-inflammatory cytokines and chemokines in the RLR, TLR and NLR signalling pathways downstream of NF-κB during the attAHSV4 primary and secondary

immune responses and the virAHSV4 secondary immune response was mediated by anti-inflammatory cytokines (Supplementary Fig. S7B-D). Anti-inflammatory cytokines that include IL-4, IL-13, IL-10 (Opal and DePalo, 2000) and TGF-β (Arango Duque and Descoteaux, 2014; Sanjabi et al., 2017) negatively regulate the inflammatory response to prevent immunopathology (Arango Duque and Descoteaux, 2014; Akdis et al., 2016). Anti-inflammatory cytokines can directly inhibit the production of pro-inflammatory cytokines, suppress pro-inflammatory cytokine signalling and prevent the inflammatory cascade from progressing (Mai et al., 2013).

Anti-inflammatory cytokines are crucial to prevent damaging inflammatory responses during both innate and adaptive immune responses during acute viral infections (Rouse and Sehrawat, 2010; Rojas et al., 2017). In fact, studies have demonstrated that the early (e.g. typically before 24 h) expression of anti-inflammatory cytokines play protective roles during acute viral infections by inhibiting pro-inflammatory responses, which prevented immunopathology. This was shown with virulent Influenza A virus infections (Furuya et al., 2015) and Simian immunodeficiency virus (SIV) infections (Katsikis et al., 2011). Interestingly, the strict early negative regulation of pro-inflammatory cytokines by anti-inflammatory cytokines demonstrated how bats are able to be asymptomatic reservoirs for highly pathogenic viruses (Kacprzyk et al., 2017). Thus, the up-regulation of several anti-inflammatory cytokines during the attAHSV4 primary and secondary immune responses and the virAHSV4 secondary immune response at the 24 h time points indicated that the early down-regulation of pro-inflammatory cytokines and chemokines might possibly be important to prevent immunopathology during AHSV4 infections.

3.3.5. The interferon (IFN) response downstream of pattern recognition receptor (PRR) signalling

Both IRF3 and IRF7, the main type I and type III IFN transcription factors downstream of the RLR, TLR and NLR signalling pathways, were up-regulated during the attAHSV4 primary and secondary immune responses (Table 1). In contrast, both IRF3 and IRF7 were down-regulated during the virAHSV4 secondary immune response (Table 1). IRF3 was down-regulated and IRF7 was up-regulated during the virAHSV4 primary immune response (Table 1). As expected, IRF signalling correlated with IFN expression with several type I IFNs (IFN-α1, IFN-α2, IFN-α4, IFN-β, IFN-δ1, IFN-ε and IFN-ω) and type III IFNs (IFN-λ1 and IFN-λ3) being up-regulated during the attAHSV4 primary and secondary immune responses (Table 1). Whereas, neither type I nor type III IFNs were significantly expressed during the virAHSV4 primary and secondary immune responses at the 24 h time points (Table 1). However, STRING v11 analysis (Reactome Pathways) showed that genes involved in type I IFN signalling were up-regulated during both the virAHSV4 (Fig. 3 and Supplementary Table S2) and attAHSV4 (Fig. 3 and Supplementary Table S3) primary and secondary immune responses. This appeared to be indicative of earlier type I IFN production and subsequent signalling events.

Further evidence of earlier type I and type III IFN signalling were from the expression of interferon-stimulated genes (ISGs). The Interferome database v2.01 (www.interferome.org) identified numerous ISGs downstream

of type I IFN, type III IFN and IFN-γ signalling during both the virAHSV4 and attAHSV4 the primary and secondary immune responses. The ISGs identified by the Interferome database v2.01 (www.interferome.org) as well as their functions are shown in Supplementary Table S4.

The lack of type I and type III IFN expression during the virAHSV4 primary and secondary immune responses seemed to be a consequence of virAHSV4 interference at the 24 h time points. This was also observed with *Reoviridae* family members BTV (Chauveau et al., 2013; Doceul et al., 2014; Ratinier et al., 2016; Avia et al., 2019), Rotavirus (Morelli et al., 2015) and MRV (Stanifer et al., 2017) that initially induce type I IFN responses, but interfered with type I IFN expression and/or signalling at later stages during the infection.

The earlier type I and type III IFN expression and signalling during the virAHSV4 primary and secondary responses indicated that the virAHSV4 is not able to interfere with the innate immune response during the initial stages of infection. It appeared that viral interference is dependent on virus replication. This was also demonstrated with MRV, where the inhibition of type I and type III IFN production was dependent on virus replication and was mediated via non-structural protein μNS (Stanifer et al., 2017). Similarly, the non-structural proteins, BTV NS3 (Chauveau et al., 2013; Doceul et al., 2014; Avia et al., 2019), BTV NS4 (Ratinier et al., 2016; Avia et al., 2019) and NSP1 of Rotavirus (Morelli et al., 2015) are responsible for interference with the immune response.

In contrast, proteins of the attAHSV4 do not interfere with the type I and type III IFN responses (Table 1). It appears that certain genes of the attAHSV4 genome are altered during the attenuation process that prevents immune evasion. Similar to the attenuated DENV1 (Sessions et al., 2013), the attenuated VACV (Israely et al., 2019) and the attenuated Modified Vaccinia virus Ankara (MVA) (Volz and Sutter, 2017). More specifically, the attenuated MVA lost the ability to interfere with the immune response because many genes involved in immune evasion are truncated or inactivated in the MVA genome (Volz and Sutter, 2017).

3.4. Viral interference

It is not possible to definitely conclude that proteins of the virAHSV4 interfere with type I and type III IFN expression or signalling without additional transcriptome data sets and experiments. Nonetheless, by comparing the attAHSV4 and virAHSV4 immune responses, our group have identified a possible mechanism through which virAHSV4 seems to interfere with type I and type III IFN expression as well as signalling. It appears that viral interference could have been responsible for the down-regulation of IRF3 and IRF7 during the virAHSV4 secondary immune response and IRF3 during the primary immune response (Table 1). Earlier IFN signalling likely induced the expression of IRF7 during virAHSV4 primary immune response. Although IRF7 is an ISG, IRF7 requires phosphorylation induced downstream of PAMP or DAMP recognition by PRRs, for its nuclear translocation and subsequent transcription of type I and type III IFNs (Schneider et al., 2014). As such, it is possible that virAHSV4 was still in the process of interfering with IRF7 during the primary immune response. Additionally, IRF9 was also down-regulated during the virAHSV4 secondary immune

response. The JAK/STAT signalling pathway is also a major target for virus interference (García-Sastre, 2017). By targeting IRF9, virAHSV4 prevents it from being recruited to the STAT1/STAT2 heterodimer to inhibit the formation of the ISGF3 complex. Since there is no ISGF3 complex to translocate to the nucleus to induce transcription of ISGs (Schneider et al., 2014; Lee and Ashkar, 2018), virAHSV4 blocks the expression of ISGs downstream of type I and type III IFN signalling. Furthermore, IRF4 was down-regulated during the virAHSV4 primary immune response. IRF5 downstream of TLR signalling was down-regulated during the virAHSV4 primary and secondary immune responses (Supplementary Fig. S5A-B). In contrast, IRF4 and IRF5 (Supplementary Fig. S5C-D) were up-regulated during the attAHSV4 primary and secondary immune responses. Similar to Rotavirus (Morelli et al., 2015), it seems that the virAHSV4 is able to interfere with the C-terminal IRF association domain (IAD)-type 1 (IAD1) containing IRFs to inhibit type I and type III IFN production and signalling (Fig. 4). The IAD1 containing IRFs include IRF3-IRF9 (Morelli et al., 2015).

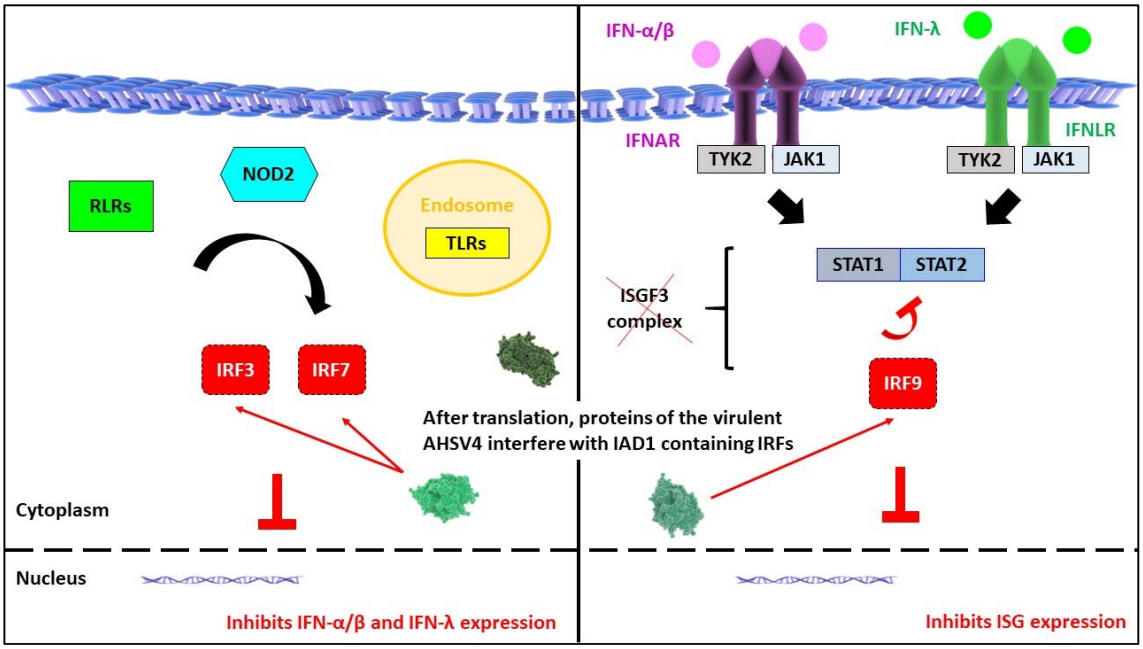

Fig. 4. After translation, proteins of virAHSV4 interfere with IAD1 containing IRFs. Interference with IRF3 and IRF7 inhibits the expression of type I and type III IFNs downstream of PRR signalling (See Supplementary Fig. S4-S6 to view all the genes downstream of the various PRRs). Interference with IRF9 inhibits the expression of ISGs downstream of type I and type III IFNs signalling. Where the recruitment of IRF9 to the STAT1/STAT2 heterodimer is prevented, which in turn inhibits the formation of the ISGF3 complex. Cellular components used in this picture were obtained from www.somersault1824.com.

In addition to dsRNA viruses that include *Reoviridae* family members, BTV (Chauveau et al., 2013; Doceul et al., 2014; Ratinier et al., 2016; Avia et al., 2019), Rotavirus (Morelli et al., 2015) and MRV (Stanifer et al., 2017), viral interfere with type I IFN production and/or signalling has been demonstrated with multiple ssRNA viruses in humans. Including the emerging bat-borne viruses, severe acute respiratory syndrome-coronavirus (SARS-CoV), Middle East respiratory syndrome (MERS)-CoV (Nelemans and Kikkert, 2019; Banerjee et al., 2020), Ebola and Marburg viruses (Basler, 2015) as well as Influenza A viruses (Liu et al., 2017), DENV (Nelemans and Kikkert, 2019) and Coxsackievirus B3 (Liu et al., 2017; Zhang et al., 2018). Viral interference

with type I IFN production and/or signalling enables ssRNA viruses to cause pathology in humans (Nelemans and Kikkert, 2019; Banerjee et al., 2020). Where excessive pro-inflammatory responses are induced because of unrestrained viral replication that results in immunopathology and a severe disease outcome (Basler, 2015; Mandl et al., 2018; Nelemans and Kikkert, 2019; Banerjee et al., 2020).

The virAHSV4 primary immune response (Fig. 3 and Supplementary Table S2) was not as well-developed as the attAHSV4 primary immune response (Fig. 3 and Supplementary Table S3) at the 24 h time points. As indicated by the fewer differentially expressed genes involved in the innate and adaptive immune responses as well as the activation of both innate and adaptive immune cells (Fig. 3 and Supplementary Table S2). virAHSV4 interference resulted in an impaired innate immune response and the delayed activation of the adaptive immune response during the virAHSV4 primary immune response, *in vitro*. Furthermore, virAHSV4 interference with the type I and type III IFN responses likely contributed to the ineffective NK cell responses that did not induce apoptosis in virAHSV4-infected PBMC (Unpublished). NK cells are crucial to keep the primary viral infection under control while the antigen-specific CTL responses develop to effectively eliminate virus infected-cells (Janeway et al., 2001). Therefore, the lack of apoptosis as well as the up-regulation of various pro-survival genes played a role in promoting the survival of virAHSV4-infected PBMC during the virAHSV4 primary immune response (Unpublished). It therefore seemed that unrestrained viral replication together with the inhibition of the type I IFN immunoregulatory functions and the down-regulated anti-inflammatory cytokines failed to control the inflammatory response that resulted in the prolonged expression of pro-inflammatory cytokines and chemokines.

Despite virAHSV4 interference, the *in vitro* virAHSV4 secondary immune response (Fig. 3 and Supplementary Table S2) was as robust and well developed as the attAHSV4 primary and secondary immune responses (Fig. 3 and Supplementary Table S3). The accelerated and increased effector responses of innate immune cells due to trained innate immunity (Cronkite and Strutt, 2018; Rusek et al., 2018) and the fast kinetics of memory Th cell and B cell responses (Janeway et al., 2001; Moser and Leo, 2010) were able to overcome virAHSV4 interference and control the infection. The enhanced responses of trained NK cells and γδ T cells were responsible for the early elimination of virAHSV4-infected PBMC through the extrinsic pathway of apoptosis (FASL/FAS and TRAIL/TRAILR) during the virAHSV4 secondary immune response. The memory CD8+ T cell responses (Unpublished) were delayed due to virAHSV4 interference with the MHC class I antigen processing and presentation pathway.

The inability to interfere with the immune response resulted in uninterrupted type I and type III IFN (Table 1) antiviral responses and the down-regulation of pro-inflammatory cytokines and chemokines by anti-inflammatory cytokines during the attAHSV4 primary and secondary immune responses. This is similar to that observed by bat-borne ssRNA viruses that do not interfere with type I IFN production and/or signalling in bats, in contrast to infections in humans (Banerjee et al., 2020). As such, robust type I IFN responses are induced and maintained to limit virus replication, while the parallel activation of pro-inflammatory cytokines is

dampened during RNA virus infections in bats (Banerjee et al., 2020). The early tight regulation of pro-inflammatory cytokines plays a critical role in preventing immunopathology induced by excessive inflammatory responses (Mandl et al., 2018). The dampening of pro-inflammatory cytokines is mediated by the early, within 24 h, production of anti-inflammatory cytokines (e.g. IL-10) (Kacprzyk et al., 2017).

The lack of immune evasion gave rise to a well-developed innate immune response and the early and effective activation of the adaptive immune response during the attAHSV4 primary immune response, *in vivo* (Fig. 3 and Supplementary Table S3). The virus infection was controlled by NK cells through the elimination of attAHSV4-infected PBMC via the perforin/granzyme pathway of apoptosis (Unpublished) during the primary immune response. In addition to no viral interference, the rapid and enhanced effector responses of both trained innate immune cells (Cronkite and Strutt, 2018; Rusek et al., 2018) and memory CD8+ T cells, Th cells and B cells (Janeway et al., 2001; Moser and Leo, 2010) played a role in controlling the virus infection during the attAHSV4 secondary immune response, *in vivo*. Primarily antigen-specific memory CD8+ T cells (CTLs) were responsible for the early elimination of attAHSV4-infected PBMC via the perforin/granzyme pathway of apoptosis (Unpublished) during the secondary immune response.

3.5. Conclusion

Similar PRR signalling pathways were activated during all of the immune responses. The major difference between the virAHSV4 and attAHSV4 innate immune responses was virAHSV4 interference with type I and type III IFN responses at the 24 h time points. The virAHSV4 interference during the primary immune response resulted in an impaired innate immune response, survival of virus-infected PBMC and an unregulated inflammatory response that contributed to the prolonged expression of pro-inflammatory cytokines and chemokines. Therefore, virAHSV4 interference with the innate immune response may give rise to an excessive inflammatory response that causes immunopathology, which could be a major contributing factor to the pathogenesis of AHS in a naïve horse. We speculate that virAHSV immune evasion is likely responsible for the monocyte, macrophage (Clift and Penrith, 2010) and pulmonary intravascular macrophage (Carrasco et al., 1999) excessive inflammatory responses that contributes to the pathogenesis of AHS.

In contrast, the lack of viral interference during the attAHSV4 primary immune response culminated in controlling the early AHSV4 infection while the adaptive immune response was developing. Therefore, the inability of attAHSV4 to interfere with the immune response may be a crucial factor associated with the loss of virulence in horses. The lack of innate immune evasion likely play an important role in protective immunity upon the first exposure to attAHSV4 in a naïve horse.

The accelerated and enhanced responses of both innate immune cells due to trained innate immunity and memory T cells and B cells played a major role in the effective regulation of the inflammatory response and controlling the virus infection during the attAHSV4 and the virAHSV4 secondary immune responses. In fact, this was essential to overcome virAHSV4 interference during the virAHSV4 secondary immune response.

Demonstrating that the fast kinetics and increased effector functions of the secondary immune response likely play a crucial role in contributing to protective immunity in attAHSV4 vaccinated horses during a virAHSV4 infection.

Subunit vaccines often contain highly purified recombinant proteins or synthetic antigens that are poorly immunogenic because they do not contain PAMPs that can be recognized by PRRs to activate the innate immune response. It is therefore crucial to include adjuvants in new generation subunit vaccines, specifically adjuvants that mimics the immune responses induced by the whole organism attenuated vaccine (Karch and Burkhard, 2016; Vasou et al., 2017). AHS subunit vaccine studies should consider using synthetic RNA adjuvants that are recognized by the RLRs, NLR (NOD2) and TLRs (TLR3 and TLR7/8) to activate the innate immune response and to mimic the same immune responses induced by the attAHSV4.

Immunotherapy may prove to be a viable option in the attempt to develop antiviral treatments for horses suffering from AHS. Based on the different cytokine expression profiles during the attAHSV4 and the virAHSV4 primary immune responses at the 24 h time points. This study proposes that type I and/or type III IFN treatments as well as anti-inflammatory cytokine treatments (e.g. IL-10 and/or TGF-β) in conjunction with blocking certain pro-inflammatory cytokines, specifically TNF-α, IL-1β and CSF1, should be included in future studies developing antiviral treatments for horses suffering from AHS. Immunotherapy via cytokine treatments or blocking the functions of specific cytokines has been shown to be effective in various diseases. Type I IFN treatments were shown to improve host survival during various viral infections that include SARS-CoV, MERS-CoV (Nelemans and Kikkert, 2019), Influenza A virus (Li et al., 2018) and Ebola virus (Konde et al., 2017). The therapeutic potential of anti-inflammatory cytokines in limiting immunopathology and promoting host survival was demonstrated with TGF-β during Influenza virus infections (Furuya et al., 2015; Sanjabi et al., 2017) as well as with recombinant IL-10 treatments during Murine cytomegalovirus (MCMV) (Tang-Feldman et al., 2010) and Encephalomyocarditis virus (EMCV) (Nishio et al., 1999) infections in mice. Additionally, recombinant IL-10 treatments did not enhance viral replication or delay viral clearance (Nishio et al., 1999; Tang-Feldman et al., 2010). Blocking TNF-α (Shi et al., 2013) and IL-1β (Cavalli and Dinarello, 2018) resulted in a significant down-regulation of excessive inflammatory immune responses and promoted host survival. Since pro-inflammatory cytokine and chemokine production by monocytes and macrophages play a role in the pathogenesis of AHS, an attractive therapeutic option is to block the CSF1/CSF1R axis. The effectiveness of blocking the CSF1/CSF1R axis is well established in cancer studies and is in clinical development (Anfray et al., 2019). Additionally, blocking the CSF1 axis did not compromise the defence mechanisms of the innate immune response (Meyer et al., 2018). Therefore, anti-CSF1 and/or anti-CSF1R therapies could limit the infiltration of monocytes into target organs as well as prevent CSF1-mediated survival of AHSV-infected monocytes and macrophages.

Studies showed that there is a correlation between *in vitro* and *in vivo* gene expression profiles in PBMC (Cheng et al., 2015; Tapia-Calle et al., 2017; Sasaki et al., 2018), which implied that this might also be the case for attAHSV4 *in vivo* and *in vitro* gene expression in horse PBMC. Furthermore, the RPKM values of the

selected housekeeping genes indicated that the attAHSV4 *in vivo* and virAHSV4 *in vitro* transcriptome data sets could be compared with each other. However, an *in vitro* study where PBMC is stimulated with attAHSV4 is required to verify a correlation between *in vitro* and *in vivo* gene expression. Similarly, while this study served as a proof of concept that virAHSV4 interferes with the innate immune response in horse PBMC *in vitro*, these results need to be confirmed with additional studies. This includes a future *in vitro* study using attAHSV4 and virAHSV4, where multiple early and later time point transcriptome data sets are needed for a more comprehensive characterization of the innate immune responses and virAHSV4 interference.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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