The impact of *Escherichia coli* contamination products present in recombinant African horse sickness virus serotype 4 proteins on the innate and humoral immune responses

Erika Faber[1, 2*], Selaelo Ivy Tshilwane[2], Mirinda Van Kleef [1, 2] and Alri Pretorius[1, 2]

[1]Agricultural Research Council-Onderstepoort Veterinary Research, Private Bag X5, Onderstepoort 0110, South Africa.

[2]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa.

Highlights

- *E. coli* contamination products in recombinant proteins effect the immune response.
- Synergistic PRR and cytokine signalling may result in hyperinflammation.
- LPS induced robust innate-like B cell and polyreactive antibody-mediated responses.
- Antibody responses of innate-like B cells may contribute to endotoxin tolerance.
- Experimental data could be misinterpreted due to *E. coli* contamination.

* Corresponding author:

Mailing address: Agricultural Research Council-Onderstepoort Veterinary Research, Private Bag X05, Onderstepoort, 0110, South Africa

Tel: +27 12 529 9425

Fax: +27 12 529 9417

Email address: FaberE@arc.agric.za

Abstract

Transcriptome analysis was used to characterize the *in vitro* primary and secondary immune responses induced in horse peripheral blood mononuclear cells (PBMC) stimulated for 24 h with the individual recombinant proteins of a virulent AHSV serotype 4 (AHSV4) field isolate (rAHSV4 proteins) that were previously expressed in *Escherichia coli* (*E. coli*). The results showed that the *E. coli* contamination products greatly affected the innate and humoral immune response transcripts. Hence, the impact of *E. coli* contamination products present in the individual rAHSV4 proteins on the translational immune response was determined. The combined amplification effects of synergistic pattern recognition receptors (PRRs), TNF-α and IL-1β signalling induced potent pro-inflammatory responses that were too overwhelming for the anti-inflammatory cytokines and regulators to control. In addition to inducing robust B cell and antibody-mediated responses, lipopolysaccharide (LPS) activation of the innate-like B cells and subsequent polyreactive (natural) antibody responses could potentially contribute to endotoxin tolerance.

Keywords:

Recombinant proteins, *Escherichia coli*, lipopolysaccharide, endotoxin tolerance, innate-like B cells, polyreactive (natural) antibodies, hyperinflammation

Abbreviations:

ADCC, antibody-dependent cellular cytotoxicity; AHS, African horse sickness; AHSV4, African horse sickness virus serotype 4; APCs, antigen-presenting cells; BCRs, B cell receptors; DAMPs, damage-associated molecular patterns; DAP, diaminopimelic acid; *E. coli*, *Escherichia coli*; FcR, Fc receptor; FcεRI, Fc epsilon RI; FcγR, Fc gamma R; FO B cells, follicular B cells; GCs, germinal centers; IgG, immunoglobulin G; LAL, Limulus Amebocyte Lysate; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MPLA, monophosphoryl lipid A; MZ B cells, marginal zone B cells; NLRs, nucleotide-binding and oligomerization domain (NOD)-like receptors; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; PC, phosphorylcholine; PRRs, pattern recognition receptors; PtC, phosphatidylcholine; rAHSV4 proteins, recombinant proteins of a virulent AHSV4 field isolate; RLRs, retinoic acid-inducible gene-I (RIG-I)-like receptors; TAP, transporter associated with antigen processing; TCRs, T cell receptors; Tfh cells, follicular helper T cells; TLRs, Toll-like receptors

1. Introduction

Although live attenuated African horse sickness virus (AHSV) vaccines are successful in endemic areas, they have many shortcomings and there is growing concern over the safety of their use. Many studies are focussing on developing alternative new generation African horse sickness (AHS) vaccines (Calvo-Pinilla et al., 2018; Dennis et al., 2019). It is therefore important to identify the individual AHSV proteins that contain CD4+ T cell, CD8+ T cell and/or B cell epitopes and investigate their ability to induce cellular and humoral immune responses for future selection as potential new generation AHS vaccine candidates.

Transcriptome analysis was used in this study to characterize the global *in vitro* primary and secondary immune responses induced in horse peripheral blood mononuclear cells (PBMC) stimulated for 24 h with the individual structural and non-structural recombinant proteins of a virulent AHSV serotype 4 (AHSV4) field isolate (rAHSV4 proteins) that were previously expressed in *Escherichia coli* (*E. coli*). *E. coli* expression systems have several advantages that include low cost, high yields and rapid expression (Schwarz et al., 2014). However, recombinant proteins expressed in *E. coli* inherently contain low levels of bacterial contamination products that are recognized by pattern recognition receptors (PRRs), which activate the innate immune response. The *E. coli* contamination products may include bacterial DNA, lipoproteins, outer wall proteins and lipopolysaccharide (LPS), also referred to as endotoxin (Wakelin et al., 2006; Schwarz et al., 2014). LPS is regarded as the major product responsible for the contamination of recombinant proteins because of its heat stability and binding affinity to many different surfaces (Schwarz et al., 2014). LPS from *E. coli* is one of the most powerful activators of the innate immune response and induces potent inflammatory responses (Karch and Burkhard, 2016; Steimle et al., 2016; Vasou et al., 2017).

The recently discovered low endotoxin recovery phenomenon that is the failure of the Limulus Amebocyte Lysate (LAL) based assays to detect the presence of LPS due to a masking effect caused by chelators or detergents present in buffer formulations commonly used for recombinant proteins, is of great concern (Schwarz et al., 2017; Reich et al., 2019). The LAL assays are the standard methods used for detecting and quantifying endotoxin contamination in recombinant proteins (Wakelin et al., 2006; Schwarz et al., 2017; Reich et al., 2019). It has been demonstrated that masked LPS induced the activation of the innate immune response and that the LAL based assays failed to detect LPS even though the recombinant proteins expressed in *E. coli* still contained LPS. The false negatives obtained by the LAL assays may compromise the studies that use these assays (Schwarz et al., 2017). Immune responses induced in response to *E. coli* contamination products are frequently incorrectly attributed to recombinant proteins, which can lead to the misinterpretation of the experimental results (Wakelin et al., 2006; Schwarz et al., 2014, 2017). As an example, tolerance induced by LPS contamination was initially wrongly attributed to ovalbumin (Wakelin et al., 2006).

This study identified and explored the impact of the *E. coli* contamination products present in the rAHSV4 proteins on both the innate and adaptive humoral immune responses during the primary and secondary immune responses. However, unlike B cells that react to a wide range of different types of antigens that include protein and non-protein antigens, CD4+ T cells and CD8+ T cells can only respond to protein antigens (Moser and Leo, 2010; Murphy et al., 2017). Specifically, linear peptide fragments that were degraded from proteins, where CD4+ T cells and CD8+ T cells recognize specific peptide/MHC complexes displayed on the surfaces

of antigen-presenting cells (APCs) (Moser and Leo, 2010; Rock et al., 2016; Murphy et al., 2017). In contrast to the innate and humoral immune responses and apart from activating APCs, the *E. coli* contamination products did not have a direct effect on the T cell responses, which were rAHSV4 protein-specific. Therefore, the individual rAHSV4 protein-specific CD4+ T cell and CD8+ T cell responses will only be discussed in a future paper.

2. Materials and methods

2.1. Ethical statement

All animal vaccination protocols were approved by the animal ethics committees at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR), the University of Pretoria and Onderstepoort Biological Products (OBP), Ltd. The study was also approved by the Department of Agriculture, Land Reform and Rural Development (DALRRD), previously known as Department of Agriculture, Forestry and Fisheries (DAFF) Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984).

2.2. Immunization of horses and isolation of peripheral blood mononuclear cells (PBMC)

Five AHSV naïve 30 month-old horses were vaccinated with the attenuated AHSV4 vaccine (days 0 and 21). Horses were bled and PBMC isolated on day 0 (naïve/before immunization) and at several time points that included day 1, day 22 and day 38 as previously described (Faber et al., 2016; Pretorius et al., 2016) and illustrated in Supplementary Fig. S1.

2.3. Total RNA isolation and transcriptome sequencing

Naïve PBMC was collected on day 0 before the first immunization with attenuated AHSV4 and day 38 vaccine-exposed PBMC, referred to as immune PBMC in this paper, was collected 17 days after the second immunization with attenuated AHSV4. Naïve and immune PBMC were stimulated *in vitro* with the individual structural and non-structural rAHSV4 proteins (10 μg/ml) and incubated at 37 ◦C in a humidified 5% CO2 incubator (United Scientific) for 24 h. The soluble fractions of VP1-1, VP1-2, VP2-2, VP3-1, VP6, NS1, NS2 and NS3 as well as the denatured/unfolded fractions of VP2-1, VP3-2, VP4, VP5 and VP7 (referred to as insoluble rAHSV4 proteins in this paper) that were previously expressed in *E. coli* (Faber et al., 2016) were used in this study. Unstimulated naïve PBMC (day 0) and unstimulated immune PBMC (day 38) were included as the negative controls. Total RNA was isolated from each sample (Tri-reagent protocol) and contaminating genomic DNA removed (DNA-free kit [Ambion]). 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, according to the requirements of the ARC-Biotechnology platform (South Africa). The isolated total RNA for the five horses (five biological repeats) were pooled at each time point (100 ng of each sample) and at least 250 μg total RNA mix was sent for sequencing with the Illumina HiSeq (Illumina, San Diego, CA, USA). Importantly, the five biological repeats were individually stimulated and RNA isolated before pooling, resulting in a total of 26 different biological samples. At the ARC-Biotechnology platform additional RNA quality controls that included determining RNA concentration

(Qubit) and evaluating the quality and integrity of the RNA by using LabChip GX Touch HT Nucleic Acid Analyzer and LabChip GX Software Version 5.3.2115.0 (Perkin Elmer) were performed. Only the total RNA samples that complied with these requirements were further processed. These RNA libraries were prepared with the TruSeq Stranded mRNA Sample Prep Kit (Illumina) and subsequently sequenced (1-4 lanes for each sample tested). For comparison in CLC, the mean of samples with more than one lane were used. Of note, there were no transcriptome data sets generated for NS2. Although the NS2 immune RNA sample complied with the requirements of the ARC-Biotechnology platform and could be sequenced, the NS2 naïve RNA sample did not, and therefore both NS2 naïve and immune were excluded from this study.

2.4. Bioinformatics analysis of the transcriptome data

The RNA sequencing (RNA-seq) data generated from the individual rAHSV4 proteins stimulated naïve PBMC and immune PBMC as well as the unstimulated controls were imported, trimmed and analysed using CLC Genomics Workbench version 9.5.2 (http://www.clcbio.com/products/clc-genomics-workbench/). CLC quality trimming was based on the Phred score and sequences with low quality scores (< 0.05) were removed. CLC quality trimming parameter settings (the limit of low quality sequence allowed: 0.05, only two ambiguous nucleotides were allowed and one terminal nucleotide was removed at the 3' and 5' end, respectively). CLC RNA-seq analysis mapping options (Mismatch cost: 3, Insertion cost: 3, Deletion cost: 3, Length fraction: 0.8, Similarity fraction: 0.8, Auto-detect paired distances, Strand specific: both, Maximum number of hits for a read: 10). The average length of the reads after quality and adaptor trimming was 92 bp in all the data sets. The transcript sequences were mapped, using the CLC RNA-seq analysis tool, to preselected *Equus caballus* (*E. caballus*) immune reference orthologous gene transcript sequences (n=2333) obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (http://www.genome.jp/kegg/pathway.html). (NCBI *Equus caballus* Annotation Release ID: 102, date: 2015-11-20. KEGG Pathway database update: 2021-12-03). After mapping, between 8-12 % of the reads mapped as pairs with an average distance of 0-400 bp. The transcript sequences were also mapped against AHSV4 gene transcripts as well as the viruses database (taxid:10239) obtained from the NCBI server (http://www.ncbi.nlm.nih.gov/).

Using CLC transcriptomics analysis, the data sets from naïve or immune PBMC stimulated *in vitro* with the individual rAHSV4 proteins were normalised to their respective unstimulated PBMC control transcriptome data sets. Normalise CLC (Method: Quantile using original expression values). The immune responses induced in naïve PBMC stimulated with the rAHSV4 proteins will be referred to as primary immune responses and the memory immune responses induced in immune PBMC stimulated with the rAHSV4 proteins will be referred to as secondary immune responses in this paper. Importantly, the secondary immune responses refer to the memory immune responses against the individual rAHSV4 proteins and not responses arising from re-exposure to the *E. coli* contamination products.

All the transcript sequences that mapped to the *E. caballus* immune reference orthologous gene transcript sequences were converted to mean reads per kilo base per million mapped reads (RPKM) values by the CLC software, and the RPKM values were used to determine the fold change. Comparative transcriptomic experiments allowed for appropriate CLC 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 > ±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.

KEGG pathways, Reactome pathways and Biological processes were identified using search tool for retrieval of interacting genes/proteins (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, Uniprot (https://www.uniprot.org/) and GeneCards (https://www.genecards.org/). The heat maps were generated using shinyheatmap (Khomtchouk et al., 2017).

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 in CLC. Normalized fold change values ≥ ±1.2 and *P*-values ≤ 0.05 were regarded as significant differential expression and these genes were included in the analyses.

3. Results and Discussion

The total number of differentially expressed up-regulated and down-regulated immune genes were identified from a total of twenty-six transcriptome data sets (See Supplementary Table S1 and Fig. S2-S5 for statistical analyses of the data and sequencing quality). Numerous differentially expressed immune genes for the rAHSV4 proteins transcriptome data sets were shown to be up-regulated 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 (Supplementary Fig. S6). Of note, all of the rAHSV4 proteins induced predominantly similar innate and humoral immune response gene expression profiles in horse PBMC. This as well as the specific types of innate and humoral immune responses showed that there were contaminating *E. coli* products present in all of the rAHSV4 protein preparations, and the effects of these products will be discussed throughout this paper.

3.1. The innate immune response

STRING v11 analysis indicated that the Toll-like receptors (TLRs), the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) were the main PRR signalling pathways that contributed to the activation of the innate immune response. Numerous differentially expressed genes were significantly enriched in the innate immune system (Reactome Pathways), TLR, NLR and RLR signalling pathways (KEGG Pathways) during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1). Recombinant proteins do not contain pathogen-associated molecular patterns (PAMPs) and are therefore unable to activate the innate immune response (Karch and Burkhard, 2016; Vasou et al., 2017). Thus, we hypothesised that *E. coli*

contamination products (e.g. LPS, lipoproteins and peptidoglycan) present in the rAHSV4 protein samples used for stimulation were responsible for the activation of the innate immune response.

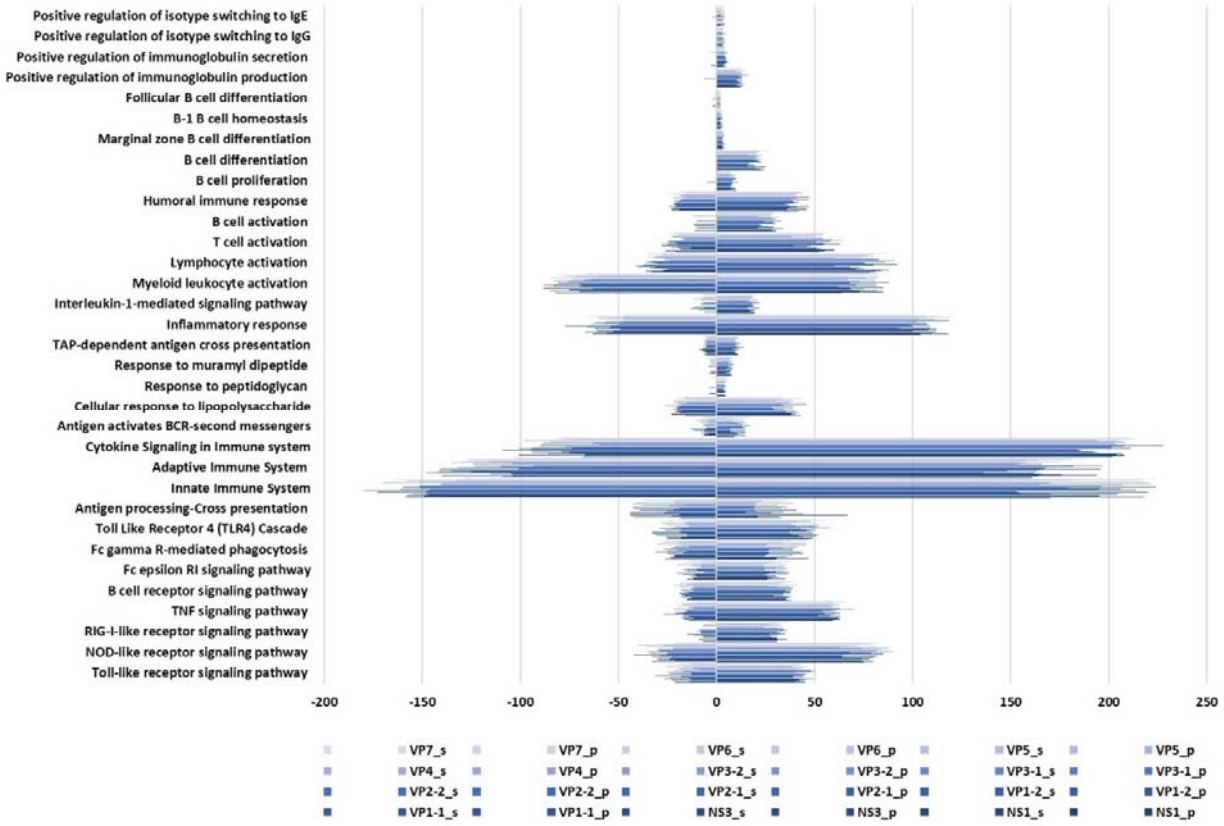

Fig. 1. The total significant up-regulated (positive) and down-regulated (negative) differentially expressed genes during the rAHSV4 proteins primary (p) and secondary (s) immune responses associated with the innate immune response, adaptive immune response and the humoral immune response. The values, false discovery rates (FDR) and STRING v11 analysis (e.g. KEGG Pathways, Reactome Pathways and Biological Process) for the individual rAHSV4 proteins are shown in Supplementary Table S2A-L.

Genes associated with cellular response to lipopolysaccharide (Biological process), Toll Like Receptor 4 (TLR4) Cascade (Reactome Pathways) (Fig. 1) and TLR4 (Fig. 2) during the primary and secondary immune responses of all of the rAHSV4 proteins tested were up-regulated. This showed that the rAHSV4 proteins were likely contaminated with LPS since TLR4 recognizes LPS (De Nardo, 2015; Vidya et al., 2018). The up-regulation of TLR1 and/or TLR2 during a few of the rAHSV4 proteins primary and secondary immune responses (Fig. 2) demonstrated that some of the rAHSV4 proteins probably contained *E. coli* contamination products that included lipoproteins, as observed by the formation of the TLR1/TLR2 heterodimer during VP5 primary immune response (Fig. 2). TLR2 heterodimerizes with TLR1 or TLR6 and recognizes bacterial lipoprotein moieties (De Nardo, 2015; Vidya et al., 2018).

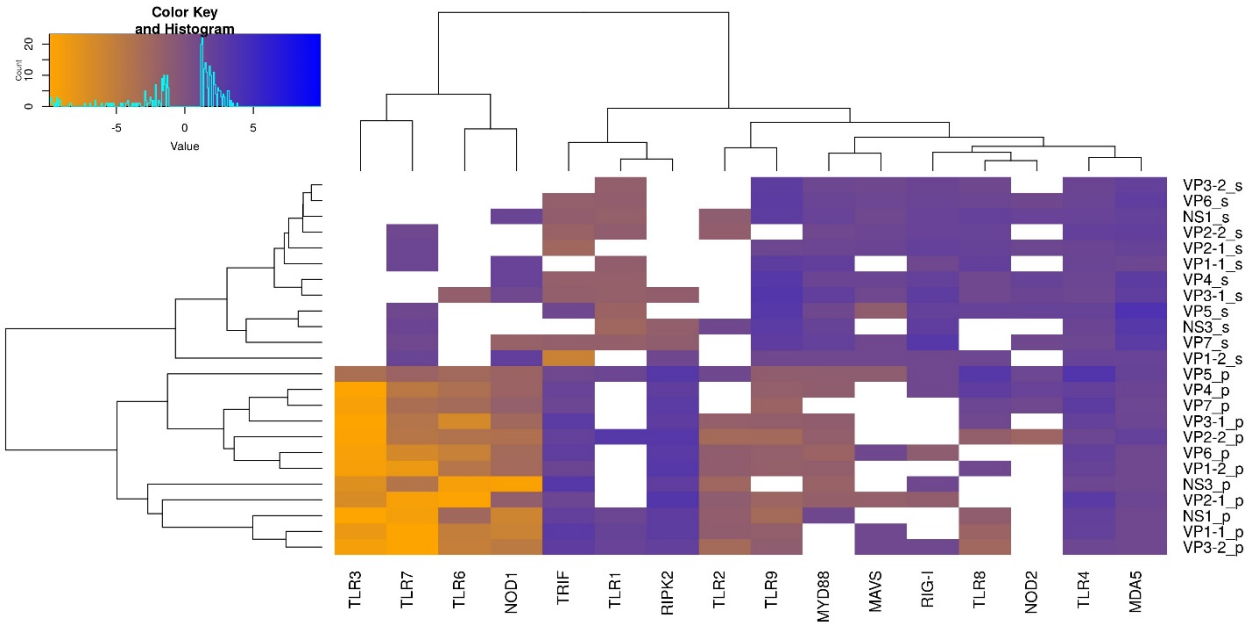

Fig. 2. Heat map of the normalized fold change values of the significant up-regulated (blue) and down-regulated (orange) TLRs, NLRs, RLRs and a few of the major adaptor proteins during the rAHSV4 proteins primary (p) and secondary (s) immune responses. Using an orange and blue colour scale, the lower values are illustrated in darker shades of orange and the higher values in darker shades of blue. Genes not differentially expressed nor significantly up-regulated or down-regulated are shown in white.

The cytoplasmic sensors, NOD1 and/or NOD2 as well as adaptor protein RIPK2 were up-regulated during many of the rAHSV4 proteins primary and/or secondary immune responses (Fig. 2). Bacterial peptidoglycan derivatives, diaminopimelic acid (DAP) activates NOD1 and muramyl dipeptide (MDP) activates NOD2. Both NOD1 and NOD2 signal through adaptor protein RIPK2 that results in the activation of NF-κB as well as the MAPKs and the subsequent expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Kim et al., 2016; Platnich and Muruve, 2019). Additionally, genes involved in the response to peptidoglycan and/or response to muramyl dipeptide (Biological process) were up-regulated during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1). This indicated that the rAHSV4 proteins were likely also contaminated with peptidoglycan components from *E. coli* that activated NOD1 and/or NOD2. The *E. coli* contamination products conceivably gained entry into the cytoplasm to activate the NLRs during the cytosolic pathway of antigen cross-presentation of the rAHSV4 proteins. Genes involved in antigen processing-cross presentation (Reactome Pathways) and antigen processing and presentation of exogenous peptide antigen via MHC class I, transporter associated with antigen processing (TAP)-dependent (Biological process) were up-regulated during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1). In the cytosolic pathway of antigen cross-presentation, exogenous protein antigens are internalized through endocytosis or phagocytosis. The internalized proteins are exported out of endosomal or phagosomal compartments into the cytoplasm and degraded by the proteasome into peptide fragments after which the peptide fragments can be transported by TAP into the ER lumen to enter the classical MHC class I pathway (Rock et al., 2016; Cruz et al., 2017).

Interestingly, the nucleic acid sensing PRRs, TLR7, TLR8, TLR9, MDA5 and/or RIG-I were up-regulated during most of the rAHSV4 proteins primary and/or secondary immune responses (Fig. 2). There was no

AHSV4 mRNA present in the horse PBMC (Supplementary Fig. S7), similarly, the transcript sequences did not map to any of the viruses in the viruses database (taxid:10239). Thus, viral RNA from AHSV4 or other viruses (taxid:10239) were not responsible for the activation of TLR7, TLR8 or the RLRs. Bacterial DNA could not have activated TLR9 because the contaminating DNA was removed with DNase added during protein purification. Moreover, TLR9 was down-regulated during the rAHSV4 proteins primary immune responses (Fig. 2). In addition to apoptosis, pyroptosis was induced during the rAHSV4 proteins primary immune responses and necroptosis was induced during the rAHSV4 proteins secondary immune responses. It therefore seemed that damage-associated molecular patterns (DAMPs), plausibly extracellular mitochondrial RNA and DNA (Grazioli and Pugin, 2018) that were released after the inflammatory cell deaths and phagocytosed, were responsible for the activation of the nucleic acid sensing TLRs inside intracellular compartments. Similarly, cleaved self-RNA products functioned as DAMPs (unpublished) that activated the RLRs.

3.1.1. The imbalance between the pro-inflammatory and anti-inflammatory responses

Activation of the innate immune response by PRR signalling results in the production of pro-inflammatory cytokines and chemokines that induce the inflammatory response as well as the activation of the adaptive immune response via antigen presentation (Moser and Leo, 2010; Murphy et al., 2017). Similarly, many pro-inflammatory cytokines and chemokines (Table 1) as well as multiple genes in the inflammatory response, myeloid leukocyte activation, lymphocyte activation, T cell activation, B cell activation (Biological process), adaptive immune system and cytokine signalling in immune system (Reactome Pathways) (Fig. 1) were up-regulated during all of the rAHSV4 proteins primary and secondary immune responses. This demonstrated that *E. coli* contamination products functioned as adjuvants by activating robust innate, inflammatory and adaptive immune responses.

The prototypic pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 (Arango Duque and Descoteaux, 2014) and chemokines CXCL1, CXCL2, CXCL3, CXCL6 and/or CXCL8 (IL-8), which recruit neutrophils (Palomino and Marti, 2015) were up-regulated (Table 1). The pro-inflammatory cytokines and chemokines were up-regulated in the TLR, NLR and RLR signalling pathways downstream of NF-κB and the MAPKs. All of the TLR signalling pathways (De Nardo, 2015; Vidya et al., 2018), the RLR signalling pathway (Chow et al., 2018) as well as NLR/RIPK2 signalling (Kim et al., 2016; Platnich and Muruve, 2019) activate NF-κB and the MAPKs that in turn, induce the expression of pro-inflammatory cytokines and chemokines. Additionally, many genes were up-regulated in the TNF signalling pathway (KEGG Pathways) and interleukin-1-mediated signalling pathway (Biological process) during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1). Similar to PRR signalling, both TNF-α and IL-1β signalling also activate the MAPKs and NF-κB pathways that result in the expression of pro-inflammatory cytokines and chemokines. In fact, like the TLRs, IL-1β/IL-1R1 also signals via the MYD88-dependent pathway (Turner et al., 2014).

Table 1. The normalized fold change values of the significant up-regulated (positive) pro-inflammatory cytokines, chemokines and type I IFNs during the rAHSV4 proteins primary (p) and secondary (s) immune responses. None of these genes was significantly down-regulated. Genes not differentially expressed nor significantly up-regulated or down-regulated are indicated with (-).

	IL1B	TNF	IL6	IFNA1	IFNB1	CXCL1	CXCL2	CXCL3	CXCL6	CXCL8
NS1_p	893,7	10,9	28,4	6,6	10,1	30,4	619,4	88,1	964,9	304,7
NS1_s	1,8	2,4	3,5	-	-	1,6	1,3	1,5	-	2,1
NS3_p	634,5	5,8	21,1	6,5	-	13,4	272,9	32,5	589,2	134,9
NS3_s	1,7	1,5	2,5	-	-	2,5	1,4	1,3	-	1,9
VP1-1_p	773,3	8,1	22,3	-	-	25,4	480,3	71,6	866,4	225,3
VP1-1_s	1,7	1,5	1,9	-	-	1,6	1,4	1,2	-	1,6
VP1-2_p	1415,6	10,1	17,2	-	4,6	37,1	1019,2	137,9	1402,6	282,4
VP1-2_s	1,8	1,2	5,2	-	-	1,2	-	-	-	1,4
VP2-1_p	1030,3	8,7	42,1	12,5	-	29,1	568,2	90,5	1185,9	417,1
VP2-1_s	1,6	1,2	3,5	-	-	1,3	-	-	-	1,2
VP2-2_p	686,4	6,7	20,5	-	-	17,6	311,5	47,8	576,9	178,9
VP2-2_s	1,8	1,5	6,4	-	-	1,4	-	-	-	1,5
VP3-1_p	901,2	8,2	31,6	-	6,6	28,5	534,2	92,7	959,1	180,6
VP3-1_s	1,3	2,2	1,4	-	-	1,4	-	-	-	1,7
VP3-2_p	518,6	8,5	16,2	-	-	13,9	343,7	62,7	595,2	112,2
VP3-2_s	1,9	1,7	4,5	-	-	1,8	1,3	1,2	-	1,6
VP4_p	859,1	9,5	22,4	-	-	21,4	464,1	91,4	830,6	218,5
VP4_s	1,8	2,1	6,1	-	-	1,6	1,2	1,3	-	1,9
VP5_p	949,6	8,8	22,9	-	-	23,1	582,7	98,8	980,2	245,3
VP5_s	1,9	3,2	5,9	-	6,2	1,6	-	-	-	1,6
VP6_p	868,5	12,5	25,9	-	-	29,2	641,8	85,7	1070,9	286,2
VP6_s	2,1	2,3	5,6	-	-	1,8	1,4	1,5	-	2,1
VP7_p	882,3	5,5	19,3	-	-	27,5	594,6	89,3	953,8	225,7
VP7_s	1,4	1,2	3,5	-	-	1,3	-	-	-	1,4

The inflammatory response must be strictly regulated to prevent the development of excessive pro-inflammatory responses that can cause immunopathology (Mai et al., 2013; Arango Duque and Descoteaux, 2014). Several anti-inflammatory cytokines that include IL-4, IL-13, IL-10, TGF-β3, CSF2 and/or CSF3 as well as negative regulators of TLR signalling (e.g. TOLLIP, SOCS1 and/or TRIM32) were up-regulated during the rAHSV4 proteins primary and/or secondary immune responses (Table 2). Although CSF2 (Sadeghi et al., 2016) and CSF3 (Boneberg and Hartung, 2002) are not generally regarded as typical anti-inflammatory cytokines, they have anti-inflammatory effects on monocytes. Anti-inflammatory cytokines play an essential role in the regulation of the inflammatory response (Mai et al., 2013; Arango Duque and Descoteaux, 2014). Likewise, TLR signalling must be tightly regulated, while it is crucial for the activation of the innate immune response, prolonged stimulation of TLRs can induce excessive inflammatory responses that can be damaging for the host (Vidya et al., 2018; Sheats, 2019).

Table 2. The normalized fold change values of the significant up-regulated (positive) and down-regulated (negative) anti-inflammatory cytokines and negative regulators of TLR and/or IL-1R1 signalling during the rAHSV4 proteins primary (p) and secondary (s) immune responses. Genes not differentially expressed nor significantly up-regulated or down-regulated are indicated with (-).

	IL4	IL10	IL13	TGFB3	CSF2	CSF3	TRIM32	SOCS1	TOLLIP
NS1_p	3,4	8,9	-	49,2	310,2	578,9	-1,2	1,2	-
NS1_s	8,8	-1,6	18,3	1,5	-1,5	1,8	2,1	5,8	2,7
NS3_p	-	21,6	-	22,3	52,2	229,7	-	-3,2	-1,8
NS3_s	56,4	-1,4	116,2	6,3	-1,2	2,6	1,3	6,9	3,3
VP1-1_p	-	12,7	2,8	26,9	309,9	376,8	-2,5	-1,7	-1,6
VP1-1_s	-	-2,9	-	2,1	-2,3	1,6	-	2,2	3,2
VP1-2_p	1,7	6,1	-	55,4	335,3	567,8	-1,7	1,5	-1,2
VP1-2_s	4,9	-	15,3	1,2	-1,2	-	1,8	3,6	1,7
VP2-1_p	-	7,4	-	37,7	251,9	483,1	-1,7	-1,6	-4,1
VP2-1_s	13,3	-	-	1,2	-	-	1,2	2,7	1,7
VP2-2_p	-	2,5	-	20,1	317,4	306,2	-1,3	1,2	-2,4
VP2-2_s	29,9	-1,4	30,7	-	-1,3	1,2	1,2	2,7	2,1
VP3-1_p	-	6,9	-	47,9	208,1	513,2	-1,2	-1,7	-
VP3-1_s	-	-	24,2	-	-1,5	1,8	1,3	2,1	1,7
VP3-2_p	-	2,9	-	11,9	284,6	318,9	-2,4	-	-1,2
VP3-2_s	8,4	-1,7	26,1	1,4	-1,4	1,7	-	3,8	3,6
VP4_p	1,2	6,1	-	40,9	324,8	397,99	-1,4	-	-
VP4_s	6,9	-1,7	10,2	1,2	-	1,3	1,5	6,8	2,5
VP5_p	3,1	5,2	2,4	50,2	342,7	298,8	-	-	-1,8
VP5_s	-	-	25,9	-	-	1,5	-4,7	2,9	3,1
VP6_p	-	11,7	-	28,4	444,5	688,4	-2,4	-1,7	-3,1
VP6_s	18,2	-1,6	34,5	1,2	-	1,6	1,5	5,3	2,5
VP7_p	3,3	5,6	-	57,5	213,4	416,8	-1,6	-	-
VP7_s	31,2	-4,4	28,1	1,2	-	1,2	1,2	4,5	1,7

TLR and IL-1β/IL-1R1 signalling through the MYD88-dependent pathway was being negatively regulated during most of the rAHSV4 proteins primary immune responses, where IL-10 was responsible for the down-regulation of MYD88 (Knödler et al., 2009) and SOCS1 probably inhibited the phosphorylation of IRAK1 (Vidya et al., 2018). TRIM32 (Yang et al., 2017) negatively regulated TLR4 signalling via TRIF, whereas TOLLIP (Kowalski and Li, 2017) and SOCS1 (Vidya et al., 2018) limited IL-1β/IL-1R1 and TLR signalling via the MYD88-dependent pathway through their inhibitory effects on IRAK1 during most of the rAHSV4 proteins secondary immune responses. Additionally, TOLLIP can aggregate with TLR4 at the cellular and lysosome membranes that inhibits all TLR4-mediated signalling (Kowalski and Li, 2017). In contrast, TLR7 and/or TLR9 signalling via MYD88/IRAK4/TRAF6/IRF5 that leads to the expression of pro-inflammatory cytokines in plasmacytoid dendritic cells (Vidya et al., 2018) was not negatively regulated during the rAHSV4 proteins secondary immune responses.

It is well documented that LPS/TLR4 signalling induces the secretion of large amounts of pro-inflammatory cytokines (Schwarz et al., 2017; Vasou et al., 2017) and exceptionally strong inflammatory responses (Steimle et al., 2016; Schwarz et al., 2017). Thus, the negative regulation of TLR4 signalling via both TRIF and MYD88 during the rAHSV4 proteins secondary immune responses may explain why the pro-inflammatory cytokines and chemokines were not as highly up-regulated as during the primary immune

responses (Table 1). It is conceivable that the faster kinetics in the negative regulation of TLR4-mediated signalling was due to trained innate immunity during the rAHSV4 proteins secondary immune responses, where monocytes, macrophages and NK cells induce much more rapid immune responses after the re-exposure to the same or different PAMPs (Rusek et al., 2018). Nonetheless, the fact that multiple pro-inflammatory cytokines and chemokines were still up-regulated (Table 1) despite the up-regulation of several anti-inflammatory cytokines (Table 2) and the negative regulation of TLR and IL-1β signalling, indicated that the inflammatory response was not effectively controlled at the 24 h time points during the rAHSV4 proteins secondary immune responses.

Additional experiments are required to determine if the up-regulated pro-inflammatory cytokines and chemokines will give rise to excessive inflammatory responses that may cause immunopathology during the rAHSV4 proteins primary and secondary immune responses. However, excessive inflammatory responses have been observed in horse PBMC (Pacholewska et al., 2017) and rabbit PBMC (Jacquier et al., 2015) stimulated with LPS for 24 h, *in vitro*. This was demonstrated with the up-regulation of IL-1β, IL-6 and several chemokines that recruit neutrophils, CXCL2, CXCL3, CXCL6 and CXCL8 in horse PBMC (Pacholewska et al., 2017). The up-regulation of TNF-α, IL-6 and IL-1β in rabbit PBMC indicated that an excessive inflammatory response was in the process of developing (Jacquier et al., 2015).

The overproduction of TNF-α and IL-1β induces an acute inflammatory response that is characteristic of septic shock (Arango Duque and Descoteaux, 2014; Sheats, 2019). High levels of circulating TNF-α, IL-1β and IL-6 are present in horses during LPS-induced sepsis (Sheats, 2019). Additionally, LPS-induced systemic inflammation and/or sepsis in horses are associated with an increased accumulation of neutrophils in target organs and/or multiple organ dysfunction. The effector functions of neutrophils can be damaging to host tissues in sepsis (Sheats, 2019). High levels of CXCL8 are often present in the blood of septic patients (Palomino and Marti, 2015; Sheats, 2019). As such, the up-regulation of TNF-α, IL-1β, IL-6 and CXCL8 together with the other chemokines that recruit neutrophils (Table 1) during all of the rAHSV4 proteins primary and secondary immune responses after 24 h may be an indication that excessive inflammatory responses were likely to develop.

Furthermore, unlike the horse PBMC (Pacholewska et al., 2017) and rabbit PBMC (Jacquier et al., 2015) studies, the rAHSV4 proteins contained unknown quantities of LPS as well as other *E. coli* contamination products (e.g. lipoproteins and peptidoglycan). LPS/TLR4 signalling together with other *E. coli* contamination products that include peptidoglycans that activate the NLRs (Schwarz et al., 2014) and lipoproteins that activate TLR2 (Wakelin et al., 2006) as well as cytokines (e.g. IL-1β and TNF-α) have synergistic effects that induce potent inflammatory responses (Wakelin et al., 2006; Schwarz et al., 2014). The combined amplification effects of various PRR signalling pathways activated by both DAMPs and PAMPs give rise to a vicious cycle of hyperinflammation (Netea et al., 2017; Sheats, 2019). This is further intensified by TNF-α and IL-1β signalling that induce their own and each other's expression (Turner et al., 2014), which creates feedback loops. While IL-1β signalling was inhibited during the rAHSV4 proteins primary and secondary immune responses, both TNF-α and IL-1β signalling have autocrine and paracrine effects (Netea et al., 2017). As such, while autocrine IL-1β signalling was inhibited, paracrine IL-1β signalling potentially acted on neighbouring PBMCs, thus creating more feedback cycles. It appeared that

the robust expression of pro-inflammatory cytokines and chemokines were too overwhelming for the anti-inflammatory cytokines and other anti-inflammatory mediators to control during the rAHSV4 proteins primary and secondary immune responses. In fact, the balance between pro-inflammatory cytokines and anti-inflammatory cytokines is assumed to be the decisive factor in inflammatory disease progression; where the expression of predominantly pro-inflammatory cytokines or the insufficient expression of anti-inflammatory cytokines result in the development of inflammatory diseases (Mai et al., 2013). It seemed that the combined amplification effects of various PRR signalling pathways activated by both DAMPs and PAMPs as well as TNF-α and IL-1β signalling may plausibly give rise to hyperinflammation during the rAHSV4 proteins primary and secondary immune responses.

3.1.2. The expression of type I interferons (IFNs) downstream of Toll-like receptor (TLR) signalling

IFN-α was up-regulated during NS1, NS3 and VP2-1 primary immune responses. IFN-β was up-regulated during NS1, VP1-2 and VP3-1 primary immune responses and during VP5 secondary immune response (Table 1). In addition to pro-inflammatory cytokine and chemokine production, TLR4, the nucleic acid sensing TLRs (De Nardo, 2015; Vidya et al., 2018) and RLR/MAVS signalling (Chow et al., 2018) also activate IRF3 and/or IRF7, which induce the expression of type I IFNs. In contrast, type I IFNs are not expressed downstream of NOD1 or NOD2 signalling via RIPK2 (Kim et al., 2016; Platnich and Muruve, 2019). Interestingly, it has been demonstrated that the RLRs function as regulators of inflammation in response to LPS, where RLR signalling results in the exclusive expression of pro-inflammatory cytokines and chemokines (Imaizumi et al., 2013; Moser et al., 2016). Similarly, it appeared that RLR signalling, like NLR signalling, only induced the expression of pro-inflammatory cytokines and chemokines in this study, whereas type I IFNs were exclusively induced downstream of TLR signalling. Specifically TLR4 signalling via TRIF, as observed by the down-regulation of TLR7, TLR8 and TLR9 during NS1 primary immune response and the down-regulation of MYD88 during NS3, VP1-2, VP2-1 and VP3-1 primary immune responses. MAVS was not significantly expressed or down-regulated and both TLR4 and TRIF were up-regulated during NS1, NS3, VP1-2, VP2-1 and VP3-1 primary immune responses (Fig. 2). Although RIG-I, MDA5, MAVS, TLR7, TLR8 and/or TLR9 and MYD88 were up-regulated during several of the rAHSV4 proteins secondary immune responses (Fig. 2), the fact that MAVS was down-regulated and that both TRIF (Fig. 2) and IFN-β (Table 1) were significantly up-regulated only during VP5 secondary immune response indicated that TLR4 signalling via TRIF was responsible for the expression of type I IFNs at the 24 h time points.

It is plausible that the expression of fewer type I IFNs downstream of TLR signalling only (possibly via TLR4/TRIF) compared to the robust expression of pro-inflammatory cytokines and chemokines downstream of TNF-α, IL-1β and all the PRR signalling pathways may contribute to the overproduction of pro-inflammatory cytokines and chemokines. Since type I IFN signalling also dampens excessive inflammatory responses (Faber et al., 2021), it is likely that the type I IFN responses were too weak to effectively exert immunoregulatory functions in the overwhelmingly pro-inflammatory microenvironment during the immune responses induced by rAHSV4 proteins.

3.2. The humoral immune response

3.2.1. Recognition of antigens by B cell receptors (BCRs) and antibodies

STRING v11 analysis (KEGG Pathways, Reactome Pathways and Biological process) demonstrated that robust humoral immune responses were induced as observed by the multiple up-regulated genes associated with the humoral immune response during the rAHSV4 proteins primary and secondary immune responses (Fig. 1). B cell receptors (BCRs), as secreted antibodies, also known as immunoglobulins, predominantly bind to conformational/discontinuous epitopes and to a much lesser extent, linear/continuous epitopes located on the outer surface of an intact antigen. It is essential for proteins to be folded into their native conformations (three-dimensional structures) for recognition of conformational epitopes by BCRs and antibodies. Some investigators presume that the specific BCRs and antibodies that recognize linear epitopes on the intact protein also recognizes the denatured/unfolded protein (Najar et al., 2017; Sanchez-Trincado et al., 2017). Others view that most of the recognized linear epitopes of the denatured/unfolded protein are different from those of the native protein. Antibodies induced against the linear epitopes of the denatured protein will most likely not recognize the linear epitopes of the native protein and vice versa (Greenfield et al., 2018). This was further supported with the demonstration that antibody-specific responses against linear epitopes are depended on their display on the three-dimensional structures of the proteins (Forsström et al., 2014).

As illustrated in Supplementary Fig. S8, the soluble rAHSV4 proteins, VP6, NS1 and NS3 were probably in their native conformations, whereas it is unknown if VP1-1, VP1-2, VP2-2 and VP3-1 folded into some sort of three-dimensional structures. The insoluble rAHSV4 proteins, VP2-1, VP3-2, VP4, VP5 and VP7 were solubilized under denaturing conditions and thus considered denatured/unfolded.

Similar as T cell receptors (TCRs), a nearly infinite number of unique BCRs are generated during VDJ recombination. Nonetheless, as part of the collective repertoire of the host, there will only be a few naïve CD4+ T cells, CD8+ T cells and B cells that express the unique receptors to specifically recognize antigens from any given pathogen during any particular infection (Moser and Leo, 2010; Murphy et al., 2017). Thus, there is a small possibility that anyone of the soluble or insoluble rAHSV4 proteins could have been recognized by naïve B cells during the *in vitro* primary immune responses. However, this is strictly dependent on whether naïve B cells with the BCRs specific for epitopes on that individual soluble or insoluble rAHSV4 protein were present in the naïve PBMC population. Additionally, the specific B cell epitopes recognized in response to the individual soluble or insoluble rAHSV4 proteins during the primary immune responses is most likely not reflective of the specific B cell epitopes recognized by naïve B cells in response to intact AHSV4.

Although antigen-specific B cell responses and antibodies potentially could have been induced against some of the individual soluble or insoluble rAHSV4 proteins during the *in vitro* primary immune responses, this is not the case during the *in vitro* secondary immune responses. The immune PBMC were isolated from horses that were immunized twice with the attenuated AHSV4 vaccine. As such, the antigen-specific BCRs of memory B cells and antibodies will probably recognize epitopes exposed on the surface of the intact AHSV4. During AHSV infections, serotype-specific neutralizing antibodies are predominantly detected against VP2 (Calvo-Pinilla et al., 2018; Dennis et al., 2019). Neutralizing antibodies bind to conformational

epitopes on VP2 and it is therefore a requirement to express recombinant VP2 in a soluble form (Calvo-Pinilla et al., 2018). In addition to VP2, the only other AHSV proteins with surface exposed regions and thus accessible to BCRs and antibodies are VP5 (Russell et al., 2018; Dennis et al., 2019) and VP7 (Russell et al., 2018).

Additionally, after binding to the AHSV-infected cell membrane during virus replication, the extracellular domain of NS3 (Avia et al., 2019) is exposed to the extracellular environment where it can be recognized by BCRs and antibodies (Mathebula et al., 2017). While this remains to be investigated, there is a possibility that several if not all of the individual AHSV proteins could be released into the extracellular environment where they can be recognized by BCRs and antibodies at various stages during AHSV infections. However, this will be highly dependent on both the stage of AHSV replication as well as the loss of plasma membrane integrity (Supplementary Fig. S9).

Since the insoluble rAHSV4 proteins were denatured/unfolded and it has been shown that the recognition of linear epitopes is dependent on a strict conformational component (Forsström et al., 2014), it appears unlikely that VP2-1, VP3-2, VP4, VP5 and VP7 were recognized by memory B cells. In contrast, it is more plausible that the soluble rAHSV4 proteins, VP6, NS1 and NS3 (native conformations) and potentially VP1-1, VP1-2, VP2-2 and VP3-1, if their structures contained the same discontinuous epitopes recognized on intact VP1, VP2 and VP3, could have been recognized by memory B cells. It is also conceivable that only NS3 and VP2-2 were recognized by memory B cells since they are exposed to the extracellular environment. NS1, VP6 and possibly VP1-1, VP1-2 and VP3-1 could have been recognized by memory B cells only if the conditions described in Supplementary Fig. S9 occurred, but this is currently unknown.

Crucially, all of the rAHSV4 proteins were contaminated with *E. coli* products that included LPS, which is a potent activator of B cells. The T cell-independent type 1 antigens, like LPS, have an intrinsic B cell activating activity and are regarded as B cell mitogens (Murphy et al., 2017). LPS induces the activation, proliferation and differentiation of B cells in a BCR-independent and TLR4-dependent manner (Hoffman et al., 2016; Murphy et al., 2017). Additionally, LPS activates B cells through the dual engagement of the BCRs and TLR4 (Xu et al., 2012; Cyster and Allen, 2019), where the lipid A moiety of LPS engages TLR4 and its repetitive polysaccharidic moiety crosslinks the BCR (Xu et al., 2012). The fact that potent B cell and antibody-mediated responses were induced during all of the soluble and insoluble rAHSV4 proteins primary and secondary immune responses (Fig. 1) indicated that LPS likely played the dominant role in the activation of humoral immune responses.

3.2.2. B cell responses

There are three major subsets of B cells, B1 B cells, marginal zone (MZ) B cells and follicular (FO) B cells (Hoffman et al., 2016; Sebina and Pepper, 2018; Cyster and Allen, 2019), which are also present in horse PBMC (Prieto et al., 2017). Similarly, in this study, genes associated with B1 B cell homeostasis and MZ B cell differentiation (Biological process) were up-regulated during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1 and 3). Genes involved with FO B cell differentiation (Biological process) were up-regulated during all of the rAHSV4 proteins secondary immune responses (Fig. 3). This indicated that B1 B cells and MZ B cells were present in the naïve and immune PBMC populations.

Additionally, memory FO B cells were also present in the immune PBMC populations, which was to be expected since the immune PBMC were isolated from horses that were immunized twice with the attenuated AHSV4 vaccine.

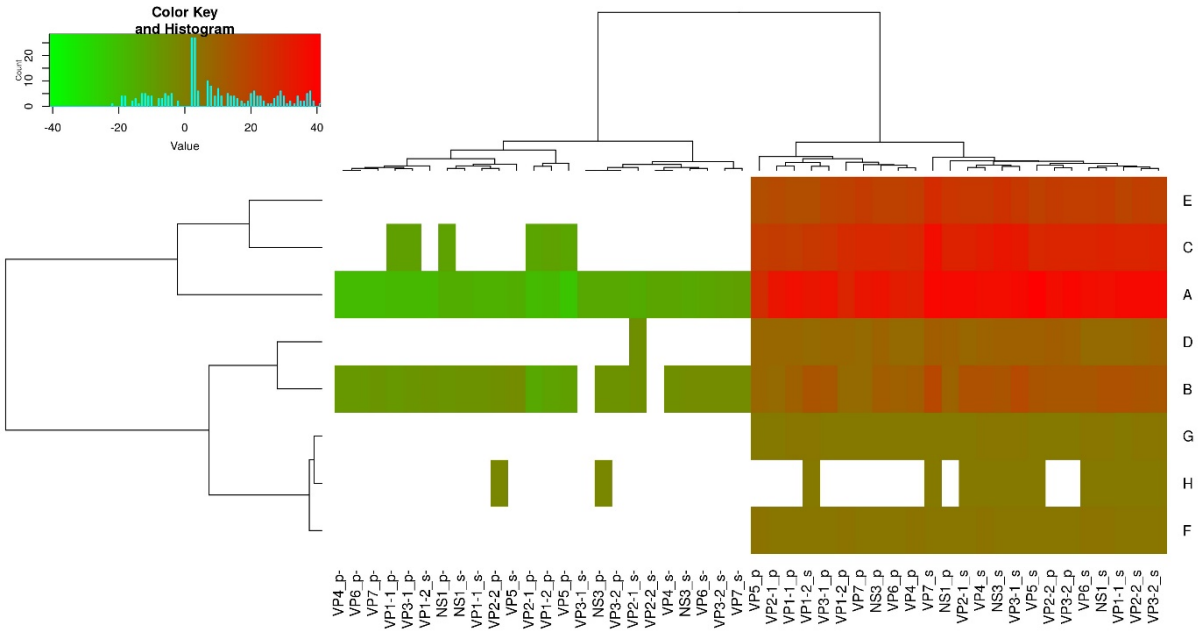

Fig. 3. Heat map of the total number of significant genes involved with B cell responses (A-H) that were up-regulated (red) during the rAHSV4 proteins primary (p) and secondary (s) immune responses and down-regulated (green) during the rAHSV4 proteins primary (p-) and secondary (s-) immune responses. Using a green and red colour scale, the lower values are illustrated in darker shades of green and the higher values in darker shades of red. A: B cell receptor signalling pathway, B: Antigen activates BCR leading to generation of second messengers, C: B cell activation, D: B cell proliferation, E: B cell differentiation, F: Marginal zone B cell differentiation, G: B1 B cell homeostasis and H: Follicular B cell differentiation. Genes not differentially expressed nor significantly up-regulated or down-regulated in the pathways are shown in white. The values, FDRs and STRING v11 analysis (e.g. KEGG Pathways, Reactome Pathways and Biological Process) for the individual rAHSV4 proteins are shown in Supplementary Table S2A-L.

The innate-like B cells, B1 B cells and MZ B cells mainly express polyreactive BCRs, constitutively express TLRs and respond to T cell-independent antigens (e.g. LPS) (Hoffman et al., 2016; Cyster and Allen, 2019). In response to T cell-independent antigens, B1 B cells and MZ B cells rapidly differentiate into short-lived extrafollicular plasmablasts (Sebina and Pepper, 2018) that secrete low-affinity polyreactive antibodies also known as natural antibodies (Palma et al., 2018; Maddur et al., 2020). The T cell-independent innate-like B cell responses do not result in the formation of immunological memory (Moser and Leo, 2010; Murphy et al., 2017). FO B cells predominantly express monoreactive BCRs, respond to T cell-dependent antigens (e.g. viral proteins) and are activated with conventional T cell help, specifically follicular helper T (Tfh) cells (Hoffman et al., 2016; Cyster and Allen, 2019). The help provided by Tfh cells drives FO B cells toward both extrafollicular and germinal center (GC) pathways (Cyster and Allen, 2019). Some of the activated FO B cells differentiate into extrafollicular short-lived plasmablasts that secrete antigen-specific low-affinity antibodies and early memory B without entering the B cell zones or GCs (Hoffman et al., 2016; Sebina and Pepper, 2018). Activated FO B cells that express BCL6 migrate back to the B cell zones and participate in the formation of GCs (Hoffman et al., 2016). The GC response mediates the diversification of BCRs and antibodies (e.g. clonal expansion, class-switch recombination, also known as isotype switching and affinity maturation that

includes somatic hypermutation and clonal selection). The continued interactions of FO B cells with Tfh cells in the GC result in the generation of long-lived plasma cells that produce high-affinity, isotype-switched antibodies and GC-memory B cells that express high-affinity, isotype-switched BCRs. The T cell-dependent FO B cell responses are responsible for the generation of long-term immunological memory (Hoffman et al., 2016; Sebina and Pepper, 2018; Cyster and Allen, 2019).

The activation of B cells is initiated after binding to their cognate antigens, which subsequently induces BCR signalling that triggers both the proliferative and differentiation programs (Sebina and Pepper, 2018). Likewise, B cells recognized antigens that activated BCR signalling, proliferation and differentiation during the rAHSV4 proteins primary and secondary immune responses (Fig. 1). As observed by the up-regulation of genes in the B cell receptor signalling pathway (KEGG Pathways), antigen activates BCR leading to generation of second messengers (Reactome Pathways), B cell activation, B cell proliferation and B cell differentiation (Biological process) (Fig. 3).

The robust B cell responses (Fig. 3) demonstrated that B1 B cells and MZ B cells recognized LPS from *E. coli* and that the innate-like B cells were the major B cell subsets that were activated during all of the soluble and insoluble rAHSV4 proteins primary and secondary immune responses (discussed further in section 3.2.3). Additionally, antigen-specific naïve FO B cells potentially could have recognized a few of the soluble and/or insoluble rAHSV4 proteins and antigen-specific memory FO B cells possibly recognized some of the soluble rAHSV4 proteins.

3.2.3. Antibody-mediated responses

Several genes associated with antibody-mediated responses were up-regulated during all of the rAHSV4 proteins primary and secondary immune responses. That includes genes involved with Fc epsilon RI (FcεRI) signalling pathway, Fc gamma R (FcγR)-mediated phagocytosis (KEGG Pathways), the positive regulation of immunoglobulin production, positive regulation of immunoglobulin secretion, positive regulation of isotype switching to immunoglobulin G (IgG) isotypes and/or positive regulation of isotype switching to IgE isotypes (Biological process) (Fig. 1). In addition to neutralizing antibodies that bind to pathogens and prevent them from entering cells, the non-neutralizing antibodies have a wide range of Fc receptor (FcR)-mediated effector functions. These include antibody-mediated complement activation, antibody-dependent cellular cytotoxicity (ADCC) and the activation of various immune cells that may lead to cytokine production, degranulation and/or antibody-dependent phagocytosis (Hoffman et al., 2016; Lu et al., 2018).

Based on the kinetics, only the innate-like B cells, B1 B cells and/or MZ B cells, low-affinity polyreactive (natural) antibody responses could have played a role in any effector functions during the rAHSV4 proteins primary immune responses at the 24 h time points. That is because the extrafollicular FO B cell low-affinity monoreactive antibody responses require between 3-7 days (Carter et al., 2017) and the GC-FO B cell high-affinity monoreactive antibody responses take about 7 days (Hoffman et al., 2016) to develop during a primary immune response. The B1 B cell and MZ B cell low-affinity polyreactive (natural) antibody responses can be detected within 1-3 days (Hoffman et al., 2016; Cyster and Allen, 2019). B1 B cells and MZ B cells produce polyreactive (natural) antibodies in health and disease (Palma et al., 2018; Maddur et al., 2020). Natural

antibodies do not lack specificity; they are polyreactive because the specific epitopes recognized by them are present on both self-antigens and a variety of pathogens. Polyreactive (natural) antibodies play crucial roles in the maintenance of immune tolerance (Maddur et al., 2020) and homeostasis. For example, many polyreactive (natural) antibodies are involved in the clearance of apoptotic cells. Because of their polyreactivity, natural antibodies also serve in the innate immune response (Palma et al., 2018; Maddur et al., 2020).

During recall immune responses, memory B cells recirculate through secondary lymphoid organs and are rapidly activated upon antigen re-exposure. The extrafollicular memory B cells enter GCs to generate high-affinity isotype-switched secondary memory B cells and long-lived plasma cells (Hoffman et al., 2016). The GC-memory B cells re-enter GC to produce further diversified secondary memory B cells and long-lived plasma cells, whereas some populations of GC-memory B cells directly differentiate into plasmablasts and rapidly secrete high-affinity antibodies (Hoffman et al., 2016; Cyster and Allen, 2019). Since some antigen-specific GC-memory B cells can immediately play a role during the secondary immune response, it cannot be ruled out that memory B cell high-affinity rAHSV4 protein-specific antibody responses potentially contributed to the effector functions during some of the rAHSV4 proteins secondary immune responses. However, the robust antibody responses observed during all of the rAHSV4 proteins secondary immune responses indicated that it was more likely that the innate-like B cells low-affinity polyreactive (natural) antibody responses played the dominant role in the effector functions during the secondary immune responses.

All of the soluble and insoluble rAHSV4 proteins primary and secondary immune responses had predominantly the same gene expression profiles associated with antibody-mediated effector functions. Therefore, the KEGG pathway pictures of the FcγR-mediated phagocytosis pathway (Fig. 4) and the FcεRI signalling pathway (Fig. 5) of insoluble VP4 secondary immune response was shown as a representative. Insoluble VP4 (denatured/unfolded and no surface exposure) was selected because it was unlikely to be recognized by high-affinity VP4-specific antibodies during the secondary immune response, which demonstrated that the innate-like B cells low-affinity polyreactive (natural) antibodies were plausibly responsible for the observed effector functions during the secondary immune responses, like during the primary immune responses.

Independent of the cytokine microenvironment, through the dual engagement of the BCR and TLR4, LPS directly induces class-switch recombination to IgG3 (Xu et al., 2012). Similarly, the up-regulated genes associated with BCR signalling, positive regulation of isotype switching to IgG isotypes (Fig. 1) and TLR4 (Fig. 2) during the rAHSV4 proteins primary and secondary immune responses, indicated that LPS conceivably activated the innate-like B cells via the dual engagement of BCRs and TLR4. In turn, the innate-like B cells secreted isotype-switched polyreactive (natural) IgG, possibly IgG3, that likely played a role in antibody-dependent phagocytosis. This was demonstrated by the up-regulated genes involved with the FcγR-mediated phagocytosis during all of the rAHSV4 proteins primary and secondary immune responses (Fig. 1), similar as during insoluble VP4 secondary immune response (Fig. 4).

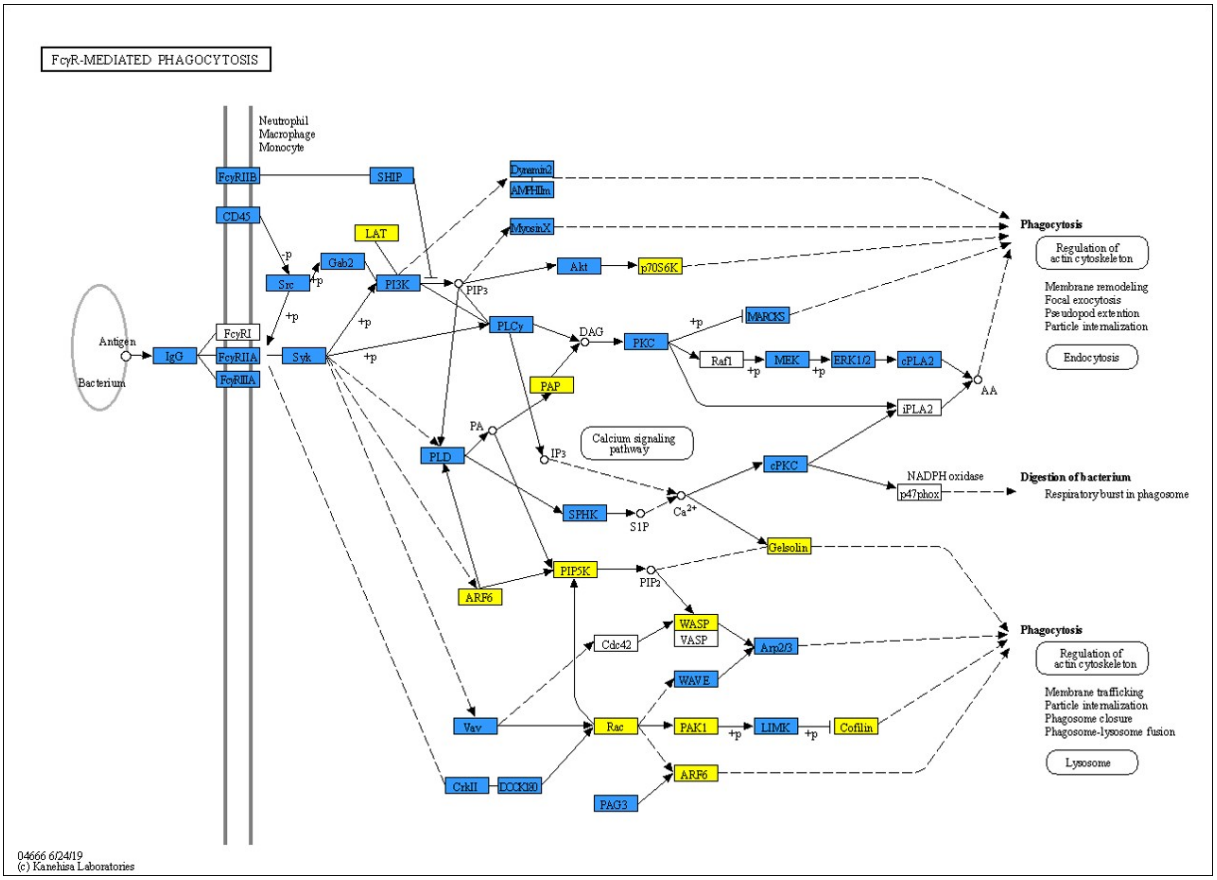

Fig. 4. The FcγR-mediated phagocytosis pathway (http://www.genome.jp/kegg/pathway.html) during VP4 secondary immune response (the blue genes are up-regulated and the yellow genes are down-regulated). Only the significantly up-regulated and down-regulated genes are shown in colours in the figure.

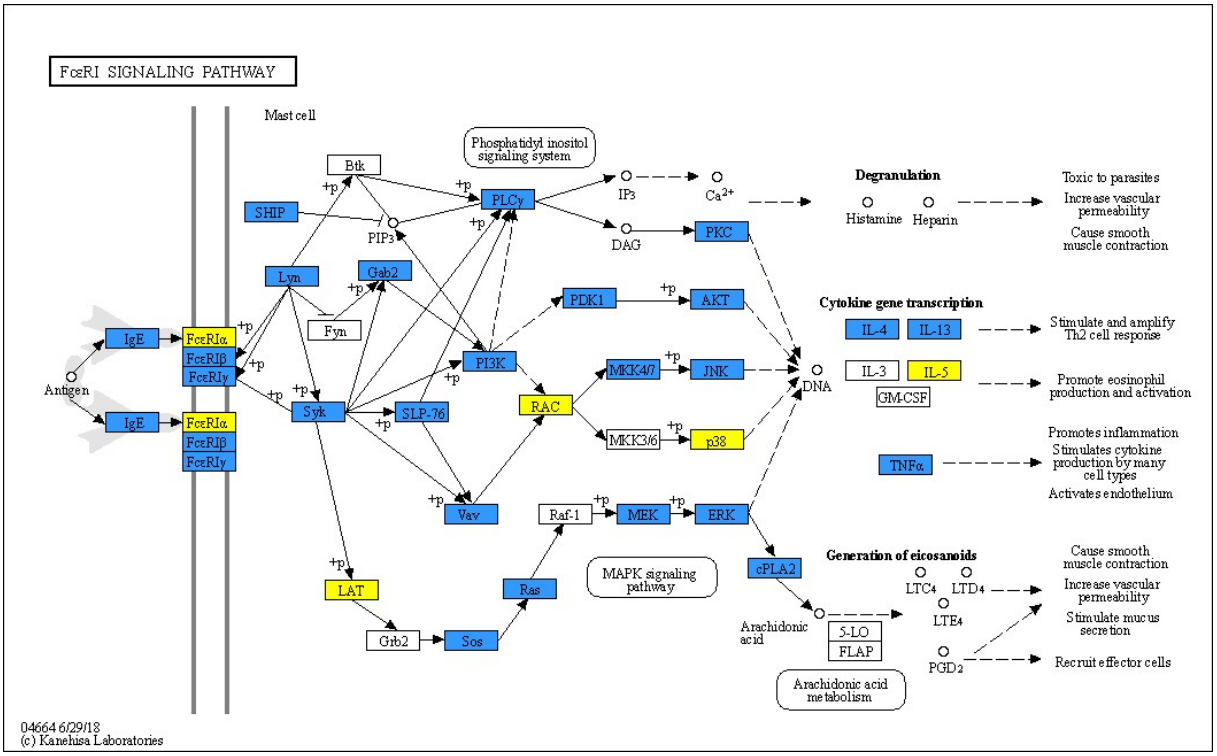

Fig. 5. The FcεRI signalling pathway (http://www.genome.jp/kegg/pathway.html) during VP4 secondary immune response (the blue genes are up-regulated and the yellow genes are down-regulated). Only the significantly up-regulated and down-regulated genes are shown in colours in the figure.

In response to LPS, B1 B cells rapidly differentiate into plasma cells that secrete polyreactive (natural) antibodies that recognize phosphatidylcholine (PtC) (Yang et al., 2007). The majority of B1 B cell-derived polyreactive (natural) antibodies recognize self and microbial phosphorylcholine (PC)-containing antigens (Grönwall et al., 2012). PC is a major immunodominant epitope present in the cell membranes of Gram-positive bacteria and LPS of Gram-negative bacteria (Weismann and Binder, 2012; Palma et al., 2018). Self-PC is a cryptic epitope of oxidized PtC that can only be recognized by antibodies when exposed on apoptotic cell membranes (Weismann and Binder, 2012). It is plausible that LPS induced the production of polyreactive (natural) antibodies that recognize PC-containing antigens, which in turn, bound to self-PC exposed on apoptotic cell membranes during the rAHSV4 proteins primary and secondary immune responses. As such, like demonstrated during insoluble VP4 secondary immune response (Fig. 4), FcγR cross-linking induced antibody-dependent phagocytosis (Lu et al., 2018) conceivably contributed to the clearance of the apoptotic cells in this study.

Genes associated with the positive regulation of isotype switching to IgE isotypes and the FcεRI signalling pathway were up-regulated during the rAHSV4 proteins primary and secondary immune responses (Fig. 1), similar as during insoluble VP4 secondary immune response (Fig. 5).

LPS mimics the immune responses induced in clinical sepsis, which include hyperinflammation and immunosuppression (or sepsis-induced immunoparalysis) (Peters van Ton et al., 2018). Immunosuppression is known as endotoxin tolerance, which is characterized by an impaired inflammatory response upon a secondary challenge with LPS (Peters van Ton et al., 2018; Liu et al., 2019). The major characteristics of endotoxin tolerance include the down-regulation of pro-inflammatory mediators and the overproduction of anti-inflammatory cytokines after LPS re-exposure (Liu et al., 2019). However, the various factors that contribute to endotoxin tolerance remain largely unknown. Studies have shown that FcεRI signalling in monocytes and dendritic cells result in the production of the anti-inflammatory cytokines (Shin and Greer, 2015). This also appeared to be the case in this study, similar as observed during insoluble VP4 secondary immune response (Fig. 5). In addition to apoptotic cell membranes, the self-PC head group of PtC can also be exposed (Weismann and Binder, 2012) or released (Shin and Greer, 2015) when the host cell membranes are damaged. High levels of polyreactive (natural) IgE antibodies that recognize PC are produced in response to tissue damage caused by helminth infections, surgery and burns. Polyreactive (natural) IgE promotes dendritic cell recognition of self-antigens (e.g. self-PC) that are released during tissue damage via FcεRI, which contributes to immune tolerance to self (Shin and Greer, 2015). Importantly, polyreactive (natural) antibodies maintain immune tolerance as well as bind to both self-antigens and foreign-antigens (Maddur et al., 2020), which include LPS of Gram-negative bacteria (Yang et al., 2007; Weismann and Binder, 2012; Palma et al., 2018). It is therefore possible that innate-like B cell activation by LPS and subsequent polyreactive (natural) antibody production contribute to endotoxin tolerance.

As such, the polyreactive (natural) IgE antibodies potentially bound to LPS-PC and/or the self-PC head group released when the host cell membranes were damaged during the inflammatory cell deaths in this study. In turn, either LPS-PC and/or self-PC, IgE/FcεRI mediated signalling in dendritic cells and/or monocytes conceivably played a role in maintaining immune tolerance to self-PC, which will most likely also induce tolerance to LPS. Since the exact mechanisms that play a role in endotoxin tolerance are mostly

unknown (Peters van Ton et al., 2018; Liu et al., 2019), sepsis studies should consider investigating the potential role that polyreactive (natural) antibodies of innate-like B cells play in contributing to endotoxin tolerance.

The results demonstrated that LPS induced potent B cell and antibody-mediated responses. While antigen-specific naïve FO B cells and memory FO B cells/high-affinity antibodies potentially recognized some of the rAHSV4 proteins, it was just not possible to distinguish between the LPS and the rAHSV4 protein-specific humoral immune responses.

It is clear that *E. coli* contamination products present in recombinant proteins is a much bigger problem than generally thought. Horses are very sensitive to LPS (Pacholewska et al., 2017; Sheats, 2019) and LPS/TLR4 signalling in horse monocytes are even more pro-inflammatory compared to other species (Sheats, 2019). As such, great care should be taken when using recombinant proteins that are expressed in *E. coli*, either for evaluating their potential to be used as vaccine candidates or to be tested as AHS subunit vaccines in horses. Their use may compromise the results of the study or potentially induce deleterious inflammatory responses and/or endotoxin tolerance in horses.

4. Conclusion

New generation subunit vaccines frequently contain highly purified recombinant proteins or synthetic antigens that are poorly immunogenic because they do not contain PAMPs that can be recognized by PRRs. They will therefore not be able to activate the innate immune response without the inclusion of adjuvants (Karch and Burkhard, 2016; Vasou et al., 2017). Because of their immunostimulatory effects, many studies are investigating the use of PRR agonists as adjuvants to be included in the vaccine formulations. As an example, the TLR4 agonist monophosphoryl lipid A (MPLA), a detoxified derivative of LPS, is used as an adjuvant in human licensed vaccines (Karch and Burkhard, 2016; Vasou et al., 2017). Likewise, the *E. coli* contamination products present in the rAHSV4 proteins functioned as adjuvants by activating the innate and adaptive immune responses, where LPS activated the TLR4 signalling pathway, lipoproteins activated the TLR1/TLR2 signalling pathway and peptidoglycan components induced NOD1 and/or NOD2 signalling via RIPK2. Additionally, after the inflammatory cell deaths it appeared that mitochondrial RNA were responsible for the activation of TLR7 and/or TLR8 and mitochondrial DNA activated TLR9. Cleaved self-RNA products functioned as DAMPs (unpublished) that activated the RLR signalling pathway, RIG-I and/or MDA5, during the rAHSV4 proteins primary and secondary immune responses.

However, unlike MPLA, that induces significantly fewer pro-inflammatory cytokines than LPS (Vasou et al., 2017); LPS/TLR4 signalling induces very strong pro-inflammatory responses (Steimle et al., 2016; Schwarz et al., 2017). Additionally, LPS it is not a good adjuvant because high levels of LPS cause septic shock (Karch and Burkhard, 2016). Furthermore, synergy occurs between LPS, other *E. coli* contamination products and cytokine signalling (Wakelin et al., 2006; Schwarz et al., 2014). Similarly, although LPS/TLR4 signalling could have been the initial main driver responsible for potent pro-inflammatory responses, synergistic PAMP/PRR, DAMP/PRR, TNF-α and IL-1β signalling culminated in the overproduction of pro-inflammatory cytokines and chemokines. Despite the up-regulation of several anti-inflammatory cytokines and the negative regulation of TLR and IL-1β signalling, the feedback cycles and

combined amplification effects of PRR, TNF-α and IL-1β signalling induced powerful pro-inflammatory responses that were too overwhelming for the anti-inflammatory cytokines and other anti-inflammatory mediators to control. This indicated that excessive inflammatory responses were likely to develop during the rAHSV4 proteins primary and secondary immune responses at the 24 h time points.

In addition to the plausible induction of hyperinflammation in the innate immune response, this study also demonstrated that the *E. coli* contamination products present in the rAHSV4 proteins have a considerable effect on the humoral immune response. LPS activated potent B cell and antibody-mediated responses during the rAHSV4 proteins primary and secondary immune responses. This highlights the possible danger of incorrectly attributing LPS-induced humoral immune responses to rAHSV4 protein-specific B cell and/or antibody-mediated responses. Additionally, this study indicated that the activation of the innate-like B cells by LPS and subsequent polyreactive (natural) antibody responses, likely via IgE/FcεRI signalling in dendritic cells and/or monocytes could potentially contribute to endotoxin tolerance.

E. coli contamination products, especially LPS, present in recombinant proteins may lead to the misinterpretation of the experimental data (Wakelin et al., 2006; Schwarz et al., 2014, 2017). This should be of particular concern for studies that rely on the LAL based assays to detect the presence of LPS due to the false negatives obtained because of the low endotoxin recovery phenomenon (Schwarz et al., 2017; Reich et al., 2019). It is advisable to use mammalian and insect expression systems (Wakelin et al., 2006) or genetically modified *E. coli* strains to avoid endotoxin contamination altogether. Alternatively, researches should consider using the cell-based TLR4-NF-κB-luciferase reporter gene assay to detect the presence of LPS since it is able to detect masked endotoxin (Schwarz et al., 2014, 2017).

This study used pooled RNA-seq analysis from five biological repeats to avoid false result interpretation that may arise due to individual genetic divergence when there is a large heterogeneity within a population (within-group biological variability). However, this was also a limitation of this study because a pooled RNA sample does not give an indication of the individual immune response of each animal in the group. Analysis of immune responses induced in individual horses should be included in future studies. Additionally, while a batch effect was not detected in this study, the various processes to conclusively rule this out will be implemented in our future experiments.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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