Apoptosis versus survival of African horse sickness virus serotype 4-infected horse peripheral blood mononuclear cells

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Abstract

Expanding on our previous work, this study used transcriptome analysis of RNA sequences to investigate the various factors that contributed to either inducing apoptosis that resulted in cell death or promoting the survival of African horse sickness virus serotype 4 (AHSV4)-infected horse peripheral blood mononuclear cells (PBMC) after 24 h. Apoptosis is a host defence mechanism that prevents virus replication, accumulation and spread of progeny viruses. AHSV4-infected PBMC were killed via the intrinsic and the perforin/granzyme pathways of apoptosis during the attenuated AHSV4 (attAHSV4) *in vivo* primary and secondary immune responses. Trained innate immunity played an important role in circumventing viral interference that resulted in the elimination of AHSV4-infected PBMC through the intrinsic and the extrinsic pathways of apoptosis during the virulent AHSV4 (virAHSV4) *in vitro* secondary immune response. Oxidative stress in conjunction with IRE1α pro-apoptotic signaling played a major role in the induction of the intrinsic pathway of apoptosis and cytotoxic lymphocytes induced the perforin/granzyme or extrinsic pathways of apoptosis. In contrast, AHSV4-infected PBMC survived during the virAHSV4 *in vitro* primary immune response, which allows unrestrained viral replication. The virAHSV4 interference with the innate immune response resulted in impaired NK cell responses and delayed immune responses, which together with the antioxidant defence system promoted AHSV4-infected PBMC survival.

Keywords:
African horse sickness virus, Apoptosis, Secondary necrosis, Oxidative stress, Cytotoxicity, Antioxidant defence system

Abbreviations [1]

[1]**Abbreviations:** AHS, African horse sickness; AHSV4, African horse sickness virus serotype 4; dsRNA, double stranded RNA; PBMC, peripheral blood mononuclear cells; DAMPs, damage-associated molecular patterns; PRRs, pattern recognition receptors; CTLs, cytotoxic T lymphocytes; DISC, death inducing signaling complex; MOMP, mitochondrial outer membrane permeabilization; NK, natural killer; ROS, reactive oxygen species; ER, endoplasmic reticulum; UPR, unfolded protein response; RIDD, regulated IRE1-dependent decay; ISR, integrated stress response; MAMs, mitochondria-associated membranes

1. Introduction

Programmed cell death can be categorized into inflammatory (e.g. necroptosis and pyroptosis) and non-inflammatory (e.g. apoptosis) cell death (Green and Llambi, 2015). Inflammatory cell death is characterized by plasma membrane rupture, also known as cell lysis that results in the release of intracellular damage-associated molecular patterns (DAMPs) into the extracellular environment. DAMPs are recognized by pattern recognition receptors (PRRs), which activates the inflammatory response. Apoptosis is induced during various housekeeping processes that include embryogenesis, normal tissue development and regular cell turnover. Apoptosis and the rapid clearance of apoptotic cells by phagocytes are essential during homeostasis and also play a major role in the immune response (Elmore, 2007; Green and Llambi, 2015).

Apoptosis is a non-inflammatory cell death because apoptotic cells retain their membrane integrity (Elmore, 2007; Green and Llambi, 2015). However, when apoptotic cells are not cleared via phagocytosis in a timely manner, they can progress to a secondary necrotic state, known as secondary necrosis or late apoptosis. This results in the rupture of the plasma membrane (cell lysis) and the release of intracellular DAMPs that induce the inflammatory response (Green and Llambi, 2015). Importantly, cells undergoing apoptosis *in vitro* will progress to secondary necrosis (cell lysis) (Elmore, 2007). For example, *in vitro* peripheral blood mononuclear cells (PBMC) studies have shown that in addition to the large populations of apoptotic cells, 7.9% and 30% late apoptotic cells (cell lysis) were also detected 24 h after apoptosis was induced with cycloheximide treatments (Baskić et al., 2006) and ionizing radiation (Beer et al., 2014), respectively. Similarly, between 20% to 70% cell lysis were detected in human immunodeficiency virus (HIV) Gag and Nef expressing PBMC target cells that were killed by antigen-specific effector CD8+ T cells, also known as cytotoxic T cells or cytotoxic T lymphocytes (CTLs), *ex vivo* within 24 h (van Baalen et al., 2008). Crucially, CTLs do not kill their target cells by directly causing lysis to the cell membranes, CTLs induce apoptosis in target cells (Jerome et al., 2003; Golstein and Griffiths, 2018) and the apoptotic cells progress to secondary necrosis (cell lysis) *in vitro* (Jerome et al., 2003).

There are two main apoptosis signaling pathways, the intrinsic pathway (or mitochondrial pathway) and the extrinsic pathway (or death receptor pathway). Both pathways converge on the activation of the apoptotic effectors or executioners that are responsible for dismantling the target cell through the proteolytic cleavage of intracellular proteins (Elmore, 2007; Green and Llambi, 2015). Proteolytic cleavage by the apoptotic effectors, caspase 3, 6, and 7 result in changes in the morphology of the cell that includes DNA fragmentation, membrane blebbing, the formation of apoptotic bodies and the cell surface exposure of phosphatidylserine (Elmore, 2007; Julien and Wells, 2017; Nainu et al., 2017). In addition, the perforin/granzyme pathway (or granule exocytosis pathway) induces caspase-dependent apoptosis and/or caspase-independent apoptosis (Elmore, 2007; Martínez-Lostao et al., 2015; Nutt and

Huntington, 2019). The intrinsic pathway of apoptosis is induced in response to numerous cellular stresses (Redza-Dutordoir and Averill-Bates, 2016; Jeng et al., 2018). In contrast, cytotoxic lymphocytes selectively kill target cells (e.g. virus-infected cells) through the extrinsic or the perforin/granzyme pathways of apoptosis (Martínez-Lostao et al., 2015; Nutt and Huntington, 2019). The cytotoxic lymphocytes physically interact with target cells via the formation of an immunological synapse to induce apoptosis (Kumar, 2018; Nutt and Huntington, 2019).

Viruses are obligate intracellular pathogens that require the basic machinery of their host cells to replicate (Croft et al., 2017; Nainu et al., 2017). Apoptosis and the subsequent phagocytosis of virus-infected apoptotic cells are important host defence mechanisms and crucial to prevent virus replication and spread (Barber, 2001; Croft et al., 2017; Nainu et al., 2017). The induction of apoptosis can result in the elimination of the virus-infected cells before the virus has completed its replication to generate sufficient progeny viruses. Therefore, many viruses have developed various strategies to interfere with this pathway. Multiple viruses inhibit the premature or early apoptosis of virus-infected cells to prolong cell survival, thus allowing the accumulation of maximum progeny viruses. Additionally, upon completion of viral replication, some viruses induce apoptosis to facilitate the release and spread of the progeny viruses without activating an inflammatory response (Iannello et al., 2006; Croft et al., 2017; Zhou et al., 2017).

The current study focused on African horse sickness virus (AHSV), a double stranded RNA (dsRNA) virus and member of the orbivirus genus of the family *Reoviridae* that causes African horse sickness (AHS). AHS is a disease that primarily affects equids and is the most economically significant disease of equines throughout the world (Dennis et al., 2019). We have previously indicated that virulent AHSV serotype 4 (virAHSV4) interference with the innate immune response during the primary immune response also included unrestrained viral replication because of the survival of virAHSV4-infected horse PBMC. The survival of virus-infected PBMC together with impaired innate immune responses and an unregulated inflammatory response likely give rise to an excessive inflammatory response that causes immunopathology that might contribute to the pathogenesis of AHS in a naïve horse. In contrast, AHSV4-infected PBMC were eliminated via apoptosis during the attenuated AHSV4 (attAHSV4) primary and secondary immune responses and the virAHSV4 secondary immune response (Faber et al., 2021).

Expanding on our previous work, this study identified the effector cells and various cell stresses that were responsible for inducing apoptosis and subsequent cell death of AHSV4-infected PBMC during the attAHSV4 primary and secondary immune responses and the virAHSV4 secondary immune response. The different factors that played a role in promoting the survival of AHSV4-infected PBMC during the virAHSV4 primary immune response were identified. Transcriptome analysis of the global primary and secondary immune responses induced in horse PBMC by the attAHSV4 (*in vivo*) and the

virAHSV4 (*in vitro*) after 24 h were used to investigate cell death versus survival of AHSV4-infected PBMC.

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 Department of Agriculture, Forestry and Fisheries (DAFF) under Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984).

2.2. Transcriptome analyses of horse peripheral blood mononuclear cells (PBMC)

As illustrated in Supplementary Fig. S1, five horses were each immunized twice subcutaneously (days 0 and 21) with the attAHSV4 vaccine as described previously (Faber et al., 2016; Pretorius et al., 2016). The 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). Immune transcriptome data was generated after PBMC and RNA isolation followed by RNA sequencing and analyses. The *in vitro* transcriptome data sets comprised of naïve (day 0) and immune (day 38) PBMC stimulated for 24 h with the virAHSV4 that were normalised to their respective 24 h unstimulated naïve and immune PBMC control transcriptome data sets. The *in vivo* transcriptome data sets comprised of day 1 and day 22 PBMC that were normalised to the naïve PBMC (day 0) transcriptome data set. Infection and replication of AHSV4 in PBMC was confirmed and the comparison of *in vivo* with *in vitro* transcriptome data sets was validated as described previously (Faber et al., 2021). Monocytes in circulation are major target cells for AHSV replication (Clift and Penrith, 2010) and PBMC consist of monocytes, dendritic cells, NK cells, T cells and B cells (Sen et al., 2018). As such, it is plausible that AHSV4-infected PBMC specifically is AHSV4-infected monocytes. Since there is a correlation shown between the *in vitro* and *in vivo* gene expression profiles in PBMC (Cheng et al., 2015; Tapia-Calle et al., 2017), the attAHSV4 *in vivo* and the virAHSV4 *in vitro* transcriptome data sets were compared with each other in this study. Venn diagrams were drawn using the Draw Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Heat maps were generated using shinyheatmap (Khomtchouk et al., 2017). The first exposure/encounter to AHSV4 immune responses induced in naïve PBMC (day 0, collected before the first immunization) that was stimulated *in vitro* with virAHSV4 for 24 h and the *in vivo* immune responses activated in PBMC (day 1, collected 24 h after the first immunization) will be referred to as primary immune responses. The re-exposure/re-encounter to AHSV4 immune responses induced in immune PBMC (collected on day 38, 17 days after the second immunization) 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) will be referred to as secondary immune responses.

2.3. Statistical analysis

The significance of results for the comparative transcriptome data was determined by the Student's *t*-test, Baggerley's test and empirical analysis of digital gene editing (EDGE). Normalized fold change values $\geq \pm 1{,}2$ and *P*-values $\leq 0{,}05$ were regarded as significant.

3. Results and Discussion

3.1. Enrichment analysis of differentially expressed genes associated with apoptosis

STRING v11 analysis (KEGG Pathways, Biological Process and Reactome Pathways) demonstrated that many differentially expressed genes were significantly enriched in various pathways associated with apoptosis that included positive and/or negative regulation pathways during the virAHSV4 (Table 1) and attAHSV4 (Table 2) primary and secondary immune responses. At least double the number of genes in the apoptosis pathways (KEGG Pathways) were up-regulated during the virAHSV4 secondary immune response (Table 1), the attAHSV4 primary and secondary immune responses (Table 2) compared to the virAHSV4 primary immune response (Table 1). Interestingly, similar to the virAHSV4 primary immune response, which included the impaired immune responses (Faber et al., 2021), it was also shown that less than twice the number of genes were up-regulated in the apoptosis pathways during the highly pathogenic influenza A virus H5N1 primary immune response compared to less pathogenic strains (Muramoto et al., 2014). The limited gene expression involved with apoptosis, the innate immune response and activation of the adaptive immune response during the highly pathogenic influenza A virus H5N1 primary immune response after 24 h resulted in the inability to control early viral replication that gave rise to an excessive inflammatory response and a more severe disease (Muramoto et al., 2014). Furthermore, no genes associated with the apoptotic execution phase, apoptotic cleavage of cellular proteins (Reactome Pathways) and apoptotic cell clearance (Biological Process) were significantly up-regulated during the virAHSV4 primary immune response (Table 1 and Fig. 1A), indicating that AHSV4-infected PBMC were not killed. Similarly, it was demonstrated that the survival of ovine bluetongue virus (BTV)-infected conventional dendritic cells was promoted during the infection (Hemati et al., 2009) and the down-regulation of FOS in red deer BTV-infected epithelial cells prevented apoptosis that permitted effective viral replication during the early stages of the infection (Galindo et al., 2012). The survival of AHSV4-infected PBMC during the virAHSV4 primary immune response showed that uncontrolled viral replication potentially contributes to the pathogenesis of AHS in a naïve horse. In contrast, the up-regulation of genes involved with the apoptotic execution phase, apoptotic cleavage of cellular proteins (Reactome Pathways) and apoptotic cell clearance (Biological Process) during the virAHSV4 secondary immune response (Table 1 and Fig. 1B) and the attAHSV4 primary (Table 2 and Fig. 2A) and secondary (Table 2 and Fig. 2B) immune responses, demonstrated

that AHSV4-infected PBMC were eliminated via apoptosis and that apoptotic cells were phagocytized. This showed that apoptosis and the clearance of virus-infected apoptotic cells are conceivably crucial host defence mechanisms to limit virus replication and spread.

Table 1. The total significant up-regulated (black, positive) and down-regulated (red, negative) differentially expressed genes associated with apoptosis during the virAHSV4 (vAHSV4) primary and secondary immune responses (KEGG Pathways, Biological Process and Reactome Pathways). Genes not differentially expressed nor significantly up- regulated or down-regulated in the pathways are indicated with (-). The false discovery rates (FDR) are shown in the table.

STRING v11 analysis	***vAHSV4***							
	Primary immune response				**Secondary immune response**			
	Up	**FDR**	**Down**	**FDR**	**Up**	**FDR**	**Down**	**FDR**
KEGG Pathways (total genes in pathway)								
Apoptosis (135)	25	1,43E-14	-23	2,34E-13	59	1,14E-32	-35	1,82E-19
Natural killer cell mediated cytotoxicity (124)	18	1,34E-08	-22	3,71E-13	45	5,24E-21	-26	4,51E-13
Biological Process (total genes in pathway)								
Extrinsic apoptotic signaling pathway (93)	14	4,18E-07	-12	7,64E-06	34	1,06E-16	-16	3,75E-07
Intrinsic apoptotic signaling pathway (158)	21	1,81E-09	-10	6,40E-03	31	1,89E-09	-18	1,10E-05
Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (31)	7	7,32E-05	-		9	2,30E-04	-4	4,61E-02
Intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator (30)	4	1,68E-02	-5	2,55E-02	6	1,36E-02	-	
Positive regulation of oxidative stress-induced cell death (16)	-		-5	3,40E-04	7	2,00E-04	-	
Negative regulation of oxidative stress-induced cell death (44)	8	7,10E-05	-		8	6,20E-03	-8	4,50E-04
Negative regulation of intrinsic apoptotic signaling pathway by p53 class mediator (22)	4	7,30E-03	-		-		-	
Nucleotide-excision repair, DNA damage recognition (23)	6	1,60E-03	-		-		-	
Regulation of sequestering of calcium ion (113)	15	5,71E-07	-9	3,00E-03	28	1,61E-10	-11	3,50E-03
Positive regulation of mitochondrial calcium ion concentration (10)	-		-3	8,80E-03	-		-	
Positive regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway (34)	-		-4	2,22E-02	8	1,70E-03	-5	1,58E-02
Positive regulation of release of cytochrome c from mitochondria (29)	-		-4	1,49E-02	6	1,20E-02	-4	4,32E-02
Apoptotic cell clearance (29)	-		-		7	3,20E-03	-5	1,05E-02
Reactome Pathways (total genes in pathway)								
TP53 Regulates Transcription of Cell Death Genes (42)	-		-4	3,28E-02	12	1,57E-05	-	
Detoxification of Reactive Oxygen Species (35)	6	1,00E-03	-		-		-8	6,52E-05
Apoptotic execution phase (51)	-		-		15	4,15E-06	-	
Apoptotic cleavage of cellular proteins (37)	-		-		12	2,74E-05	-	

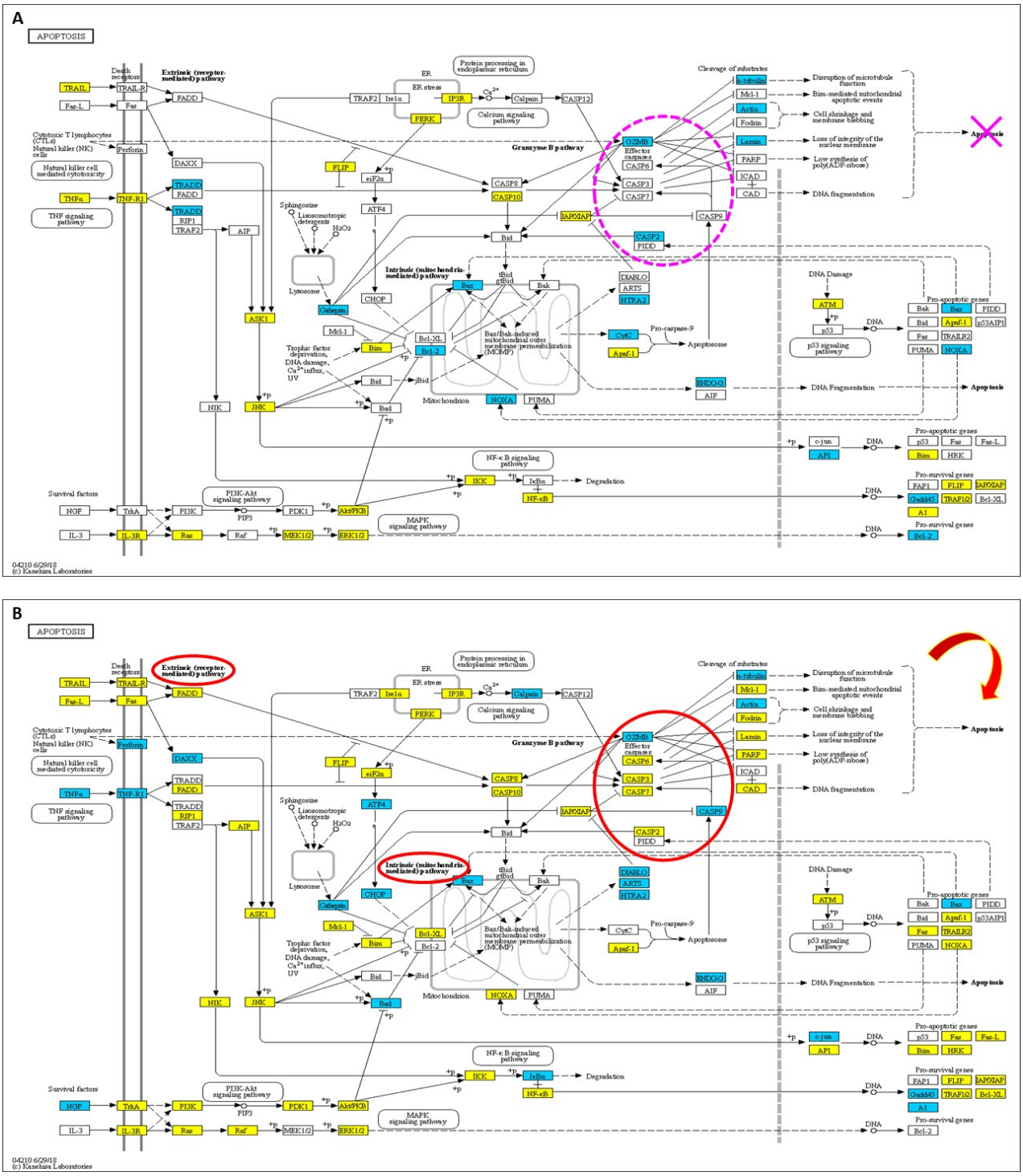

Fig. 1. Apoptosis pathways (http://www.genome.jp/kegg/pathway.html) during (A) the *in vitro* virAHSV4 primary immune response and (B) the *in vitro* virAHSV4 secondary immune response. The yellow genes are up-regulated and the blue genes are down-regulated. Only the significantly up-regulated and down-regulated genes are shown in colours in the figure. The pink cross in (A) indicates that apoptosis was not induced and the pink circle shows the down-regulated or not significantly expressed effectors of apoptosis during the virAHSV4 primary immune response. The red arrow in (B) indicates that apoptosis was induced and the red circles show the specific pathways and the up-regulated effectors of apoptosis during the virAHSV4 secondary immune response.

Table 2. The total significant up-regulated (black, positive) and down-regulated (red, negative) differentially expressed genes associated with apoptosis during the attAHSV4 (aAHSV4) primary and secondary immune responses (KEGG Pathways, Biological Process and Reactome Pathways). Genes not differentially expressed nor significantly up- regulated or down-regulated in the pathways are indicated with (-). The false discovery rates (FDR) are shown in table.

STRING v11 analysis	***aAHSV4***							
	Primary immune response				**Secondary immune response**			
	Up	**FDR**	**Down**	**FDR**	**Up**	**FDR**	**Down**	**FDR**
KEGG Pathways (total genes in pathway)								
Apoptosis (135)	53	8,65E-25	-15	1,08E-07	62	3,53E-32	-25	1,97E-14
Natural killer cell mediated cytotoxicity (124)	44	1,49E-19	-10	2,00E-04	51	1,71E-23	-15	1,07E-07
Biological Process (total genes in pathway)								
Extrinsic apoptotic signaling pathway (93)	35	1,50E-15	-6	2,41E-02	35	9,49E-16	-11	6,93E-05
Intrinsic apoptotic signaling pathway (158)	33	5,08E-09	-9	8,90E-03	34	1,06E-09	-14	8,92E-05
Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (31)	12	6,45E-06	-		13	1,08E-06	-4	1,94E-02
Intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator (30)	7	8,00E-03	-		6	2,33E-02	-	
Positive regulation of oxidative stress-induced cell death (16)	5	1,15E-02	-		6	2,40E-03	-	
Negative regulation of oxidative stress-induced cell death (44)	8	1,40E-02	-6	1,20E-03	-		-6	2,60E-03
Negative regulation of intrinsic apoptotic signaling pathway by p53 class mediator (22)	-		-		-		-	
Nucleotide-excision repair, DNA damage recognition (23)	-		-		-		-	
Regulation of sequestering of calcium ion (113)	22	7,09E-06	-6	4,50E-02	26	3,72E-08	-7	3,51E-02
Positive regulation of mitochondrial calcium ion concentration (10)	3	2,10E-04	-		3	2,10E-04	-	
Positive regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway (34)	6	3,91E-02	-		9	1,00E-03	-	
Positive regulation of release of cytochrome c from mitochondria (29)	6	2,22E-02	-		7	7,60E-03	-	
Apoptotic cell clearance (29)	8	1,80E-03	-		7	3,30E-03	-4	1,60E-02
Reactome Pathways (total genes in pathway)								
TP53 Regulates Transcription of Cell Death Genes (42)	12	6,79E-05	-		14	2,72E-06	-	
Detoxification of Reactive Oxygen Species (35)	-		-6	2,20E-04	-		-8	9,73E-05
Apoptotic execution phase (51)	12	3,30E-04	-		12	2,30E-04	-	
Apoptotic cleavage of cellular proteins (37)	9	4,50E-03	-		9	1,30E-03	-	

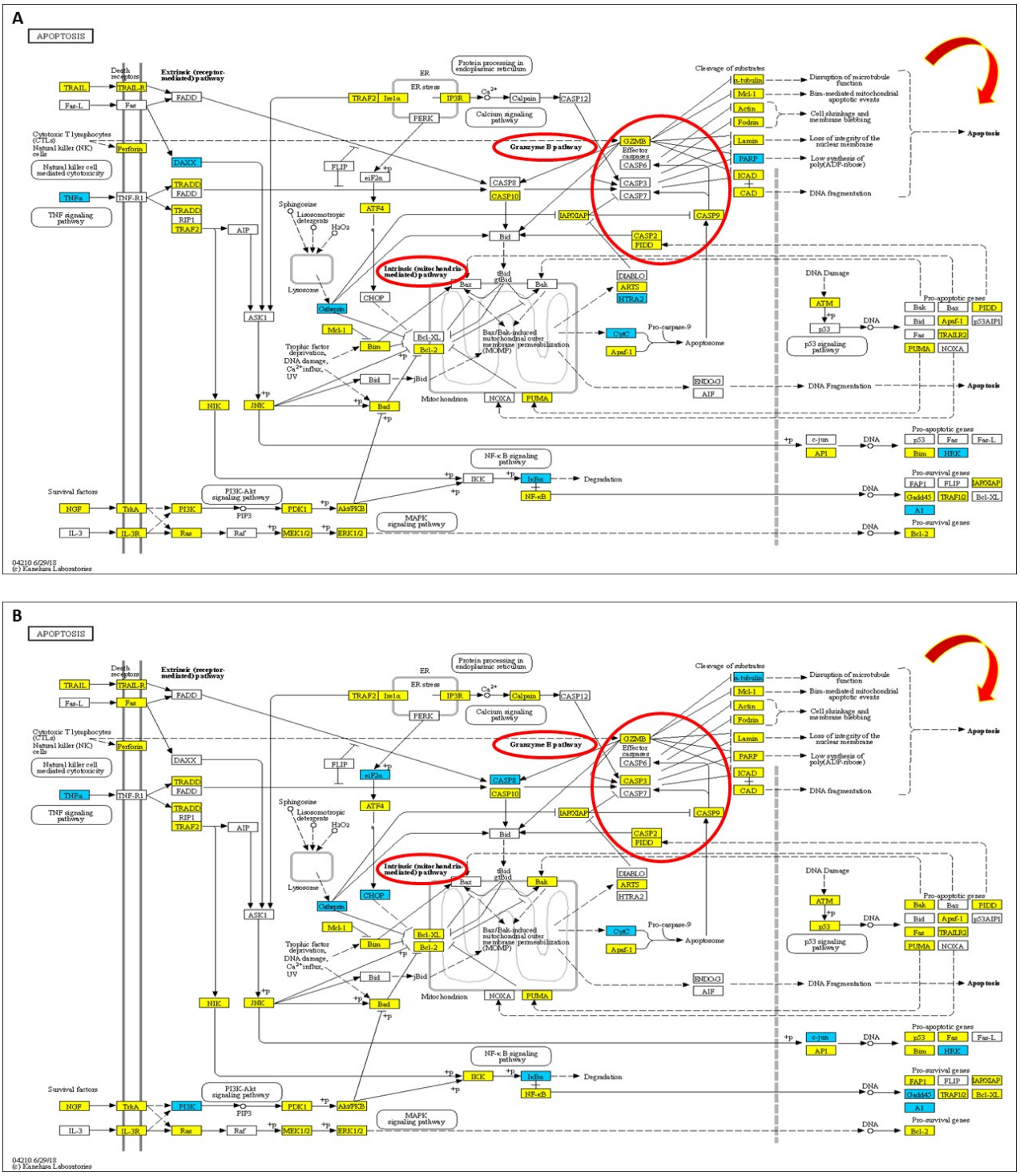

Fig. 2. Apoptosis pathways (http://www.genome.jp/kegg/pathway.html) during (A) the *in vivo* attAHSV4 primary immune response and (B) the *in vivo* attAHSV4 secondary immune response. The yellow genes are up-regulated and the blue genes are down-regulated. Only the significantly up-regulated and down-regulated genes are shown in colours in the figure. The red arrow indicates that apoptosis was induced and the red circles show the specific pathways and the up-regulated effectors of apoptosis during the attAHSV4 primary (A) and secondary (B) immune responses.

However, the survival of virAHSV4-infected PBMC during the primary immune response in this study as well as the survival of BTV-infected conventional dendritic (Hemati et al., 2009) and epithelial cells (Galindo et al., 2012), are contradictory to BTV and AHSV studies where the induction of early apoptosis in mammalian cell lines were observed. The BTV-induced (Hemati et al., 2009; Saminathan et al., 2020) as well as virulent and UV-inactivated AHSV4-induced (Vermaak and Theron, 2015) extrinsic and intrinsic pathways of apoptosis in mammalian cells are suggested to be triggered by outer capsid proteins, VP2 and VP5, during viral uncoating and is independent of viral replication. Briefly, after AHSV (Dennis et al., 2019) and BTV cell entry, the uncoating of the outer capsid proteins, VP2 and VP5, occurs in endosomes that is followed by the release of the viral core particle into the cytoplasm where it becomes transcriptionally active (Forzan et al., 2007). It is not well understood why this virus-induced apoptosis in mammalian cell lines prior to viral replication may be beneficial for BTV and AHSV since viruses are dependent on viable cells for replication and propagation (Croft et al., 2017) and many viruses typically inhibit premature apoptosis or induce apoptosis at a later stage (Zhou et al., 2017). The AHSV study proposed that the short replication time of orbiviruses in mammalian BHK-21 cells, where progeny viruses are observed at 12 h post infection might potentially allow the adequate generation of progeny viruses before the infected cells are severely damaged during the execution phase of apoptosis (Vermaak and Theron, 2015). On the other hand, apoptosis occurs very rapidly, taking between 2-3 h from induction to completion (Elmore, 2007). As such, it seems that the pro-apoptotic effects of the AHSV outer capsid proteins, VP2 and VP5, in mammalian BHK-21 cells are highly cell type specific, similar as proposed for BTV in mammalian cell lines (Hemati et al., 2009). Importantly, unlike the AHSV outer capsid proteins-induced apoptosis in mammalian BHK-21 cells (Vermaak and Theron, 2015), the induction of apoptosis in AHSV4-infected PBMC was a host defence mechanism during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses in this study, as will be discussed in this paper.

3.2. Apoptosis via the extrinsic or perforin/granzyme pathways

Cytotoxicity includes inducing apoptosis in target cells via either the extrinsic pathway or the perforin/granzyme pathway (Nutt and Huntington, 2019). Much more genes in the extrinsic apoptotic signaling pathway (Biological Process) were up-regulated during the virAHSV4 secondary immune response (Table 1), the attAHSV4 primary and secondary immune responses (Table 2) than the virAHSV4 primary immune response (Table 1). The extrinsic pathway of apoptosis is activated by the binding of death receptors, FAS and TRAILR, expressed on the plasma membrane of the target cells (e.g. virus-infected cells) to their respective ligands, FASL and TRAIL, expressed on the effector cells. This results in the recruitment of FADD, pro-caspase 8 and pro-caspase 10, leading to the formation of the death inducing signaling complex (DISC) that activates pro-caspase 8 (Green and Llambi, 2015; Martínez-Lostao et al., 2015; Zhou et al., 2017). Caspase 8 activates the executioner caspases (e.g. caspase 3 and caspase 7), which in turn cleave of the intracellular components (Green and Llambi, 2015;

Julien and Wells, 2017; Zhou et al., 2017). In addition, caspase 3 and caspase 7 activate caspase 6 (Green and Llambi, 2015). The up-regulation of TRAIL, TRAILR, FASL, FAS, FADD, CASP8, CASP10, CASP3, CASP6 and CASP7 showed that apoptosis was induced via the extrinsic pathway during the virAHSV4 secondary immune response (Fig. 1B and Fig. 3). TRAIL, TRAILR, FAS (secondary only) and CASP10 were up-regulated during the attAHSV4 primary and secondary immune responses (Fig. 2 and Fig. 3). However, FADD, CASP3, CASP6, CASP7 and CASP8 were not significantly expressed during the attAHSV4 primary immune response (Fig. 2A and Fig. 3) and FADD, CASP6 and CASP7 were not significantly expressed and CASP8 was down-regulated during the attAHSV4 secondary immune response (Fig. 2B and Fig. 3). TRAIL and CASP10 were up-regulated during the virAHSV4 primary immune response, while TRAILR, FADD, CASP3, CASP6, CASP7 and CASP8 were not significantly expressed (Fig. 1A and Fig. 3). The results indicated that apoptosis was not induced through the extrinsic pathway during the attAHSV4 primary and secondary immune responses or the virAHSV4 primary immune response.

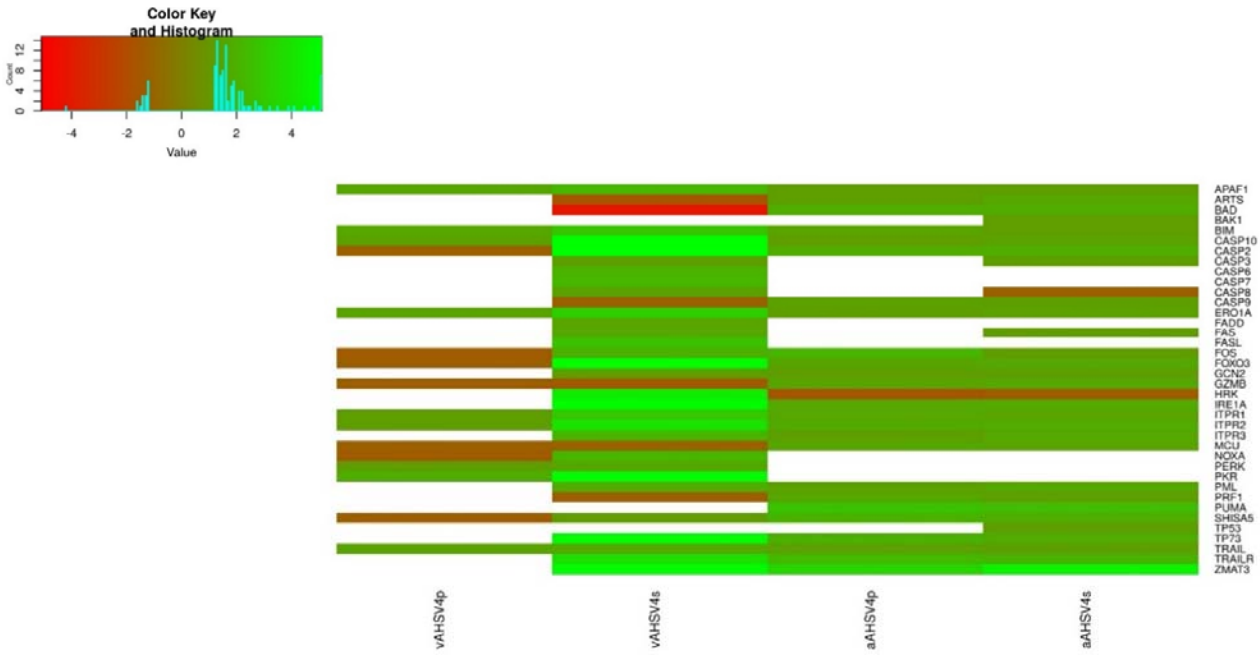

Fig. 3. Heat map of the normalized fold change values of some of the major pro-apoptotic genes that were significantly up-regulated (green) and down-regulated (red) during the virAHSV4 (vAHSV4) and the attAHSV4 (aAHSV4) primary (p) and secondary (s) immune responses. Using a red and green colour scale, the lower values are illustrated in darker shades of red and the higher values in darker shades of green. Genes not differentially expressed nor significantly up-regulated or down-regulated are shown in white. The normalized fold change values and pro-apoptotic gene functions are shown in Supplementary Table S1.

During the perforin/granzyme pathway of apoptosis, the lytic granules that contain perforin and granzyme B are secreted from the cytotoxic lymphocytes via exocytosis (degranulation) and released into the immunological synapse. Perforin mediates cell entry by forming pores in the plasma membranes of target cells. This allows the entry of granzyme B into the cytoplasm of the target cell

where it can induce both caspase-dependent and -independent apoptosis. Granzyme B activates the executioner caspases and/or directly cleaves intracellular proteins (Elmore, 2007; Martínez-Lostao et al., 2015; Nutt and Huntington, 2019). The up-regulation of PRF1, GZMB and CASP3 (secondary only) demonstrated that apoptosis was induced through the perforin/granzyme pathway in AHSV4-infected PBMC during the attAHSV4 primary and secondary immune responses (Fig. 2 and Fig. 3). During the attAHSV4 primary immune response, it seemed that GZMB was responsible for the direct cleavage of substrates. GZMB activated CASP3 and the substrates were cleaved by CASP3 and/or GZMB during the attAHSV4 secondary immune response. In contrast, PRF1 and GZMB were down-regulated during the virAHSV4 secondary immune response (Fig. 1B and Fig. 3). PRF1 was not significantly expressed and GZMB was down-regulated during the virAHSV4 primary immune response (Fig. 1A and Fig. 3). This showed that apoptosis was not induced via the perforin/granzyme pathway during the virAHSV4 primary or secondary immune responses.

3.3. Effector cells inducing the perforin/granzyme or extrinsic pathways of apoptosis

The effector cell types that may be responsible for the induction of the perforin/granzyme or extrinsic pathways of apoptosis were considered. Multiple genes associated with Natural killer cell mediated cytotoxicity (KEGG Pathways) were up-regulated during the virAHSV4 secondary immune response (Table 1) and attAHSV4 primary and secondary immune responses (Table 2). Whereas some genes were up-regulated during the virAHSV4 primary immune response (Table 1). Importantly, as illustrated in Fig. 4A, the various genes in the Natural killer cell mediated cytotoxicity pathway are also representative of cytotoxicity mediated by effector CD8+ T cells (CTLs) (Martínez-Lostao et al., 2015; Nutt and Huntington, 2019) and the unconventional T cells that include γδ T cells (Lawand et al., 2017) and NKT cells (Balato et al., 2009). Cell markers associated with this pathway including CD226, CD244, NCR2 (primary), NCR3 (secondary), LFA1 (composed of ITGAL and ITGB2), CD1A, CD1B, CD8A, CD8B and MHC class I genes were up-regulated (Fig. 4B) while NKG2D was down-regulated during the attAHSV4 primary and secondary immune responses. During the virAHSV4 primary immune response, NCR3 and CD226 were up-regulated (Fig. 4B) and CD8B, CD8A and ITGB2 were down-regulated. CD8A, CD226, NCR1, NCR2, CD1A, CD1B and CD1C were up-regulated (Fig. 4B), while CD8B, ITGB2 and NKG2D were down-regulated during the virAHSV4 secondary immune response.

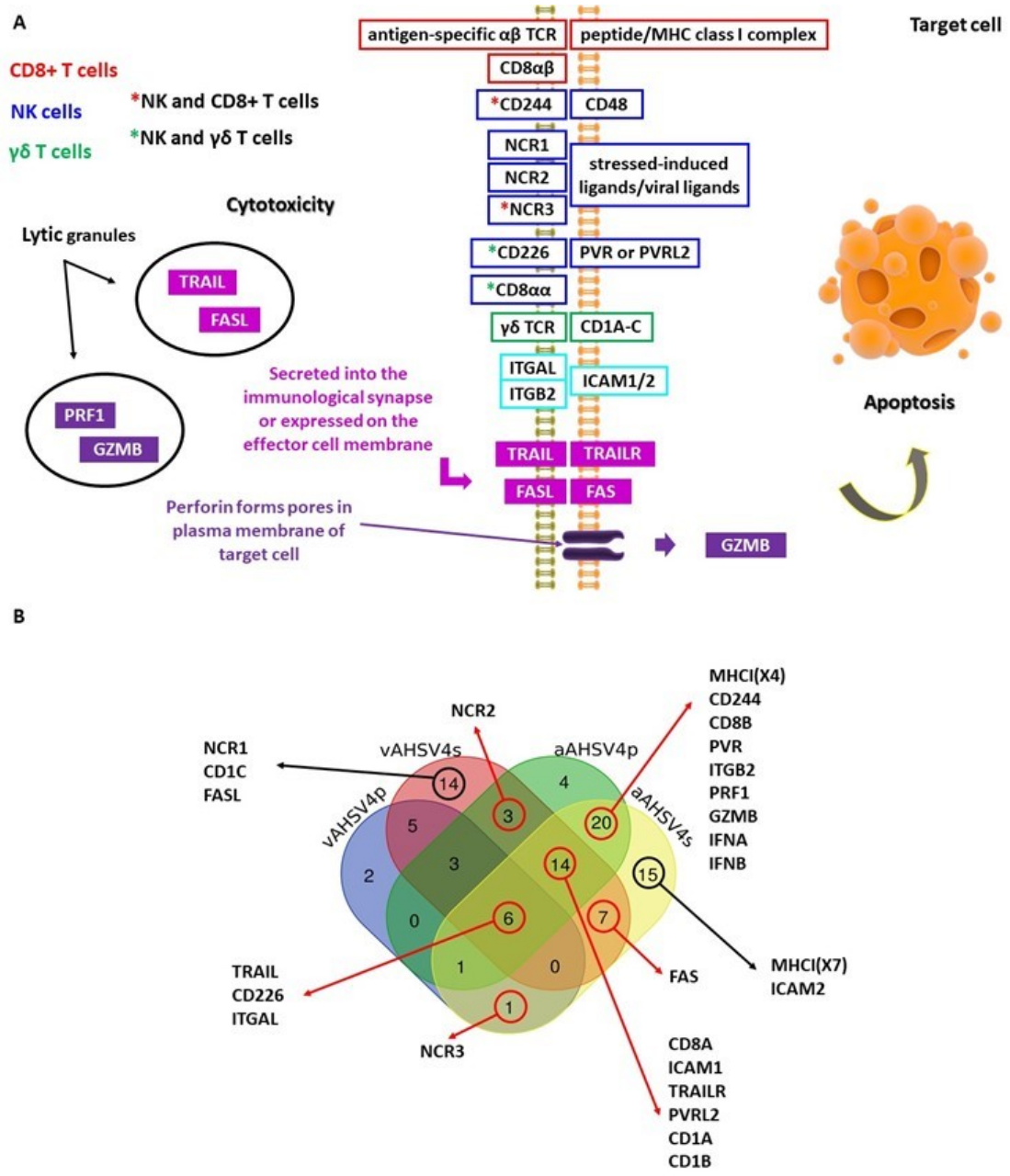

Fig. 4. (A) Some of the genes involved in cytotoxicity that include activating receptors and markers expressed on NK cells, CD8+ T cells and/or γδ T cells as well as their respective ligands expressed on target cells that were up-regulated during the virAHSV4 and/or the attAHSV4 primary and/or secondary immune responses. (B) The Venn diagram of the unique (black circle) and overlapping (red circle) genes involved in cytotoxicity that were significantly up-regulated during the virAHSV4 (vAHSV4) and/or the attAHSV4 (aAHSV4) primary (p) and secondary (s) immune responses. Genes illustrated in (A) are shown in the Venn diagram (black and red arrows). Cellular components used in this picture were obtained from www.somersault1824.com. NKT cells are not included since they were not involved in the elimination of AHSV4-infected PBMC.

The TCRs of NKT cells and γδ T cells recognize lipid antigens presented by the CD1 proteins, a family of non-polymorphic MHC class I-related molecules (Opasawatchai and Matangkasombut, 2015; Vermijlen et al., 2018). CD1D was not significantly expressed during the attAHSV4 or virAHSV4

primary and secondary immune responses. This indicated that NKT cells were not activated through their TCRs and thus not involved in inducing apoptosis (Balato et al., 2009) in AHSV4-infected PBMC during any of the immune responses. Similarly, the release of perforin and granzymes from the γδ T cells towards the target cells is only activated by the combined effects of γδ TCR and NKG2D signaling as well as LFA1 (Paul and Lal, 2016; Lawand et al., 2017). The down-regulation of NKG2D showed that γδ T cells probably did not play a role in the elimination of AHSV4-infected PBMC via the perforin/granzyme pathway of apoptosis during the attAHSV4 primary and secondary immune responses.

During the attAHSV4 primary immune response, antigen-specific naïve CD8+ T cells were activated, as indicated by the up-regulation of the CD8αβ heterodimer (Fig. 4B), MHC class I genes (Fig. 4B and Supplementary Table S2) and genes associated with the CD8+ T cell response (Supplementary Table S3). During the primary immune response, naïve CD8+ T cells require several days to undergo clonal expansion and differentiate into effector CD8+ T cells before they can exert their effector functions (Janeway et al., 2001). As such, it was too early for effector CD8+ T cells to have played a role in the elimination of AHSV4-infected PBMC. The combined up-regulation of CD226, NCR2, CD244 and LFA1 indicated that NK cells (Kumar, 2018) were most likely responsible for the elimination of AHSV4-infected PBMC via the perforin/granzyme pathway of apoptosis during the attAHSV4 primary immune response. The cytotoxicity of NK cells, specifically the release of lytic granules that contain perforin and granzymes (Janeway et al., 2001; Nutt and Huntington, 2019) into the immunological synapse is controlled by several receptors and is only activated when LFA1 is engaged together with a synergistic receptor pair (Kumar, 2018). In addition, we have previously shown that multiple type I and type III IFNs were up-regulated during the attAHSV4 primary immune response (Faber et al., 2021) that conceivably played a crucial role in activating the effector functions of NK cells (Janeway et al., 2001; Nutt and Huntington, 2019). In fact, type I IFNs can increase the activity of NK cells up to 100-fold (Barber, 2001). Type I IFNs and NK cells play a crucial role in keeping the early virus infection under control (Barber, 2001; Janeway et al., 2001; Nutt and Huntington, 2019) while the antigen-specific CD8+ T cells undergo clonal expansion (Janeway et al., 2001).

Antigen-specific memory CD8+ T cells were activated during the attAHSV4 secondary immune response; as shown by the up-regulation CD8αβ heterodimer (Fig. 4B), MHC class I genes (Fig. 4B and Supplementary Table S2) and genes associated with the CD8+ T cell response (Supplementary Table S3). The much greater numbers of antigen-specific memory CD8+ T cells populate and recirculate through both secondary lymphoid organs and peripheral tissues throughout the host. This allows them to provide immune surveillance and immediately respond to the pathogen re-encounter by eliminating virus-infected cells through their cytotoxic functions upon re-activation (Valbon et al., 2016; Martin and Badovinac, 2018; Nutt and Huntington, 2019). Therefore, antigen-specific memory CD8+ T cells most likely played the

dominant role in the killing of AHSV4-infected PBMC via the perforin/granzyme pathway of apoptosis during the attAHSV4 secondary immune response. However, NCR3 (Barrow et al., 2019) and CD244 (Janeway et al., 2001) could have been expressed on NK cells and/or CD8+ T cells, demonstrating that NK cells potentially contributed to the elimination of AHSV4-infected PBMC.

Neither antigen-specific naïve CD8+ T cells nor memory CD8+ T cells were activated during the virAHSV4 primary and secondary immune responses, as observed by the down-regulated MHC class I genes (Supplementary Table S2) and the predominantly down-regulated or not significantly expressed genes associated with CD8+ T cell activation (Supplementary Table S4). Additionally, CD8B and CD8A, the CD8αβ heterodimer that is expressed on CD8+ T cells (Li et al., 2013) were down-regulated during the virAHSV4 primary immune response. CD8B was down-regulated and CD8A (Fig. 4B) was up-regulated during the virAHSV4 secondary immune response, indicating that the CD8αα homodimer was probably expressed on γδ T cells and NK cells (Li et al., 2013). Collectively, viruses interfere with every step of the MHC class I antigen processing and presentation pathway as well as MHC class I surface expression to prevent or delay antigen-specific recognition and killing of virus-infected cells by effector CD8+ T cells (CTLs) (Iannello et al., 2006). Similarly, it appears that the virAHSV4 interferes with the MHC class I antigen processing and presentation pathway by facilitating the down-regulation of MHC class I expression to evade and delay the CD8+ T cell response. This correlates with our previous observation where antigen-specific memory CTL-induced cell lysis (apoptotic cells that progressed to secondary necrosis) were absent in PBMC isolated from three horses immunized with the OBP polyvalent AHSV live attenuated vaccine after 24 h stimulation with the virAHSV4 *in vitro* during the secondary immune response (Faber et al., 2016). This was surprising since it is known that antigen-specific effector memory CD8+ T cells can immediately execute their cytotoxic functions after re-exposure to peptide/MHC class I complexes displayed on the cell surface of virus-infected cells (Valbon et al., 2016; Martin and Badovinac, 2018). Furthermore, it was shown that after antigen-specific effector CD8+ T cells induced apoptosis in HIV Gag and Nef expressing PBMC target cells, secondary necrotic cells (cell lysis) were already detected within 24 h, *ex vivo* (van Baalen et al., 2008). It therefore seems that virAHSV4 reduces the expression of both intracellular and cell surface MHC class I molecules, similar to the influenza A viruses (Koutsakos et al., 2019) and the rotavirus (Holloway et al., 2018). In the Faber et al., (2016) study, cell lysis (secondary necrotic cells) were observed at 96 h stimulation, a direct result from the activation of antigen-specific memory CTLs and subsequent killing of virAHSV4-infected PBMC during the secondary immune response. Thus, it is plausible that virAHSV4 uses mechanisms similar to the influenza B virus (Koutsakos et al., 2019) and coxsackievirus (Cornell et al., 2007) to delay the cell surface expression of peptide/MHC class I complexes by either retaining them in the ER or redirecting them away from the cell surface at 24 h. However, peptide/MHC class I complexes are able to traffic back and remain displayed on the cell surface long enough to effectively activate antigen-specific memory CTLs at 96 h.

During the virAHSV4 primary immune response, NK cell activating receptor NCR3 could have been expressed on either activated or resting NK cells (Barrow et al., 2019; Nutt and Huntington, 2019). However, apoptosis was not induced via the extrinsic or the perforin/granzyme pathways during the virAHSV4 primary immune response. It is likely that virAHSV4 interference with the type I and type III IFN responses (Faber et al., 2021) played a major role in the impaired NK cell responses and their inability to eliminate AHSV4-infected PBMC during the virAHSV4 primary immune response. In fact, without NK cells, uncontrolled viral replication allows the viral load to reach high quantities early during the infection that can be fatal for the host (Janeway et al., 2001).

In the absence of LFA1, a synergistic pair of activating receptors of NK cells can induce the degranulation of lytic granules but this occurs without polarization (Kumar, 2018). It is therefore plausible that the perforin/granzyme pathway of apoptosis was not activated during the virAHSV4 secondary immune response because the complete form of LFA1 was not expressed (ITGB2 was down-regulated). In addition to being stored in lytic granules and secreted into the immunological synapse, FASL and TRAIL are also up-regulated on the cell membranes (Fig. 4A) of activated cytotoxic lymphocytes (Martínez-Lostao et al., 2015). Thus, FASL and TRAIL were probably expressed on the plasma membranes of activated NK cells (Martínez-Lostao et al., 2015) and/or γδ T cells (Lawand et al., 2017). Where NK cells activated via CD226, NCR1 and/or NCR2 as well as γδ T cells activated through their TCRs and CD226 were possibly responsible for the elimination of AHSV4-infected PBMC via the extrinsic pathway of apoptosis during the virAHSV4 secondary immune response. Despite virAHSV4 interference with the type I and type III IFN responses, the virAHSV4 secondary immune response was as robust and well developed as the attAHSV4 primary and secondary immune responses (Faber et al., 2021). The much faster and stronger innate immune response that included the killing of AHSV4-infected PBMC was conceivably due to trained innate immunity. Innate immune cells, specifically monocytes, macrophages and NK cells undergo long-term translational and epigenetic changes/modifications after the primary immune response, which allows these cells to mount a much more rapid and robust non-specific secondary immune response (Nainu et al., 2017; Cronkite and Strutt, 2018; Rusek et al., 2018). Additionally, it was also demonstrated that γδ T cells respond more rapidly and robustly during the secondary infection (Paul and Lal, 2016; Vermijlen et al., 2018). The enhanced secondary responses of γδ T cells also appear to be due to trained innate immunity (Dantzler and Jagannathan, 2018).

3.4. Apoptosis via the intrinsic pathway

Several genes in the intrinsic apoptotic signaling pathway (Biological Process) were up-regulated during the virAHSV4 (Table 1) and the attAHSV4 (Table 2) primary and secondary immune responses. Pro-apoptotic genes, APAF1 and BIM (BCL2L11) were up-regulated during the virAHSV4 primary immune response (Fig. 1A and Fig. 3). APAF1, BIM, HRK, NOXA (PMAIP1) and CASP2 were up-

regulated during the virAHSV4 secondary immune response (Fig. 1B and Fig. 3). APAF1, BIM, PUMA (BBC3), BAD, ARTS (SEPT4), CASP2, CASP9 and BAK1 (secondary only) were up-regulated during the attAHSV4 primary and secondary immune responses (Fig. 2 and Fig. 3). Exposure to different cellular stresses induce the transcriptional or post-translational activation of various pro-apoptotic BH3-only proteins that include BIM, PUMA, NOXA, BAD and BID. The BH3-only proteins are responsible for either the direct or indirect activation of BAX and BAK, the pro-apoptotic effectors that initiate mitochondrial outer membrane permeabilization (MOMP) through the formation of pores in the mitochondrial outer membrane that results in the release of cytochrome c (CYCS), DIABLO, HTRA2, ARTS and other mitochondrial pro-apoptotic proteins into the cytosol. In the cytoplasm, CYCS, APAF1 and pro-caspase 9 form the apoptosome, where pro-caspase 9 is activated (Elmore, 2007; Green and Llambi, 2015; Jeng et al., 2018). Caspase 9 activates the executioner caspases (e.g. caspase 3 and caspase 7) resulting in the cleavage of the intracellular components (Julien and Wells, 2017).

The up-regulation of CASP9 and CASP3 demonstrated that apoptosis was likely induced through the intrinsic pathway during the attAHSV4 secondary immune response (Fig. 2B and Fig. 3). However, CASP3 could have been activated by either CASP9 or GZMB in the perforin/granzyme pathway (Martínez-Lostao et al., 2015; Nutt and Huntington, 2019). While CASP9 was up-regulated, the executioner caspases were not significantly expressed, indicating that apoptosis was probably not induced through the intrinsic pathway via CASP9 during the attAHSV4 primary immune response. Likewise, apoptosis was also not induced through the intrinsic pathway via CASP9 during the virAHSV4 primary and secondary immune responses.

Caspase 2 is activated in response to several different types of cell stress that include DNA damage, reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress and function as an initiator and/or as an executioner caspase (Redza-Dutordoir and Averill-Bates, 2016). The up-regulation of CASP2 showed that apoptosis was induced through the intrinsic pathway via CASP2 during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. Since BID was not significantly expressed during any of the immune responses (Fig. 1 and Fig. 2), it seemed that CASP2 functioned as an executioner caspase and contributed to the cleavage of the apoptotic substrates (Redza-Dutordoir and Averill-Bates, 2016). In contrast, apoptosis was not induced through the intrinsic pathway via CASP2 during the virAHSV4 primary immune response.

Unlike the virAHSV4 primary immune response (Table 1), genes associated with the positive regulation of MOMP involved in apoptotic signaling pathway and the release of cytochrome c from mitochondria (Biological Process) were up-regulated during the virAHSV4 secondary immune response (Table 1) and the attAHSV4 primary and secondary immune responses (Table 2). This showed the intrinsic pathway of apoptosis only contributed to the killing of AHSV4-infected PBMC during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses.

3.5. Cellular stresses and the intrinsic pathway of apoptosis

There is immense crosstalk and feedback cycles between the various cell stress pathways that ultimately either induce the intrinsic pathway of apoptosis or promote cell survival. The intrinsic pathway of apoptosis is induced in response to cellular stresses that include oxidative stress, DNA damage and ER stress (Redza-Dutordoir and Averill-Bates, 2016; Zhou et al., 2017; Jeng et al., 2018). Oxidative stress occurs when the production of ROS overwhelms the antioxidant defence system (Redza-Dutordoir and Averill-Bates, 2016; Rehman et al., 2018). Genes in the intrinsic apoptotic signaling pathway in response to ER stress and DNA damage by p53 class mediator as well as the regulation of sequestering of calcium ion (Biological Process) were up-regulated during the virAHSV4 (Table 1) and the attAHSV4 (Table 2) primary and secondary immune responses.

Several insults (e.g. oxidative stress, calcium flux and viral infections) can disrupt ER homeostasis. This results in the accumulation of unfolded or misfolded proteins inside the ER lumen and ER stress, which activates the signaling pathways of the unfolded protein response (UPR). Three resident ER transmembrane proteins, ATF6, IRE1α (ERN1) and PERK (EIF2AK3), initiate the UPR. The initial phase of UPR is a pro-survival response that aims to restore homeostasis. However, the UPR will switch to pro-apoptotic signaling during unresolvable ER stress conditions (Dufey et al., 2014; Grootjans et al., 2016; Gong et al., 2017). IRE1α (Fig. 3) and JNK downstream in the IRE1α pro-apoptotic signaling pathway were up-regulated during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. JNK promotes apoptosis via various mechanisms. Additionally, the regulated IRE1-dependent decay (RIDD) pathway degrades mRNAs encoding pro-survival proteins. IRE1α also cleaves microRNAs that inhibit the expression of pro-apoptotic proteins (e.g. caspase 2) (Cao and Kaufman, 2014; Gong et al., 2017). The IRE1α pro-apoptotic signaling pathway played a role in promoting the intrinsic pathway of apoptosis in response to ER stress during the virAHSV4 secondary immune response and attAHSV4 primary and secondary immune responses. Specifically, via JNK activation, facilitating the expression of CASP2 (Fig. 3) and RIDD activity that possibly degraded pro-survival genes.

In addition to phosphorylating eIF2α as will be discussed below, PERK also activates the NRF2/KEAP1 pathway, a crucial antioxidant defence system that plays a central role in protecting cells against oxidative stress (Cao and Kaufman, 2014; Gong et al., 2017; Lee, 2018). The NRF2/KEAP1 antioxidant defence system was activated during the virAHSV4 primary and secondary immune responses as indicated by the up-regulation of PERK (Fig. 3), NRF2 (Fig. 5) and the down-regulation of KEAP1. In contrast, NRF2 was down-regulated (Fig. 5) and KEAP1 was up-regulated during the attAHSV4 primary and secondary immune responses. During oxidative stress and ER stress, NRF2 dissociates from KEAP1 and translocate to the nucleus where it induces the expression of many cytoprotective, antioxidant and detoxification target genes (e.g. SODs) that promote cell survival and redox

homeostasis (Cao and Kaufman, 2014; Gong et al., 2017; Lee, 2018). It is not possible to determine if virAHSV4 interference was responsible for the selective activation of the PERK-mediated NRF2/KEAP1 pathway without future studies since the UPR is a common adaptive pathway activated by numerous stresses in response to a virus infection (Dufey et al., 2014; Grootjans et al., 2016; Gong et al., 2017). Additionally, the pathways of UPR are initially activated in response to a virus infection and then targeted and manipulated by the virus, as observed during dengue virus (DENV) infections (Perera et al., 2017). Certain viruses can also directly activate specific pathways of the UPR (Grootjans et al., 2016) as well as positively modulate the NRF2/KEAP1 pathway to reduce ROS levels during oxidative stress to promote the survival of virus-infected cells (Lee, 2018). The fact that the PERK-mediated NRF2/KEAP1 pathway was only activated during the virAHSV4 primary and secondary immune responses at the 24 h time points indicated the possibility that virAHSV4 specifically targets this pathway.

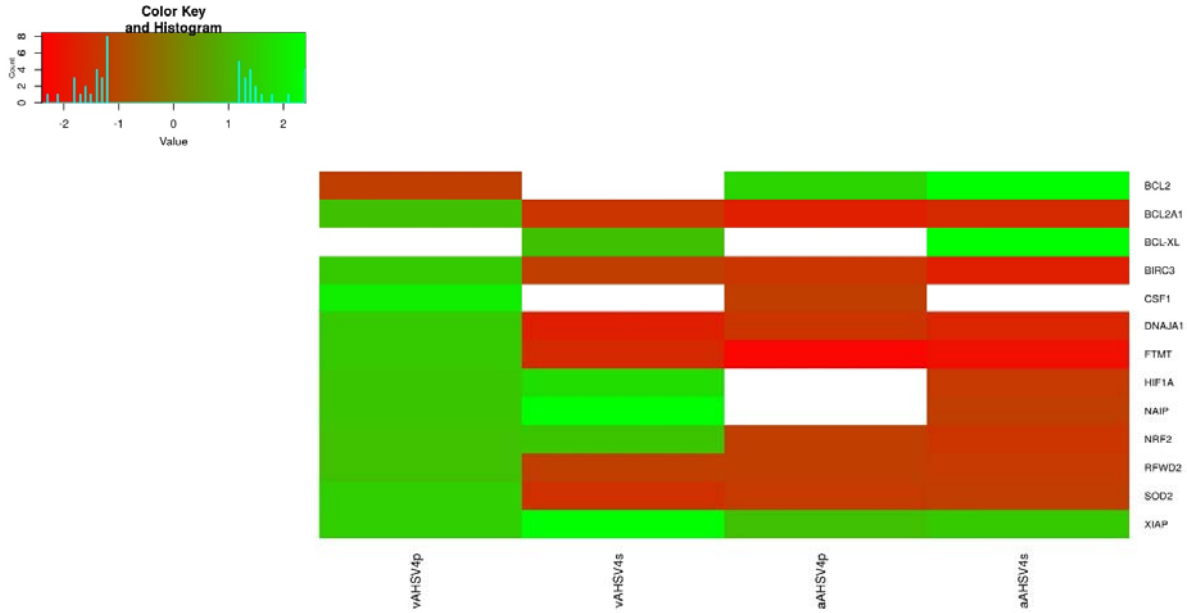

Fig. 5. Heat map of the normalized fold change values of a few of the main anti-apoptotic and pro-survival genes that were significantly up-regulated (green) and down-regulated (red) during the virAHSV4 (vAHSV4) and the attAHSV4 (aAHSV4) primary (p) and secondary (s) immune responses. Using a red and green colour scale, the lower values are illustrated in darker shades of red and the higher values in darker shades of green. Genes not differentially expressed nor significantly up-regulated or down-regulated are shown in white. The normalized fold change values, anti-apoptotic and pro-survival gene functions are shown in Supplementary Table S5.

In the virAHSV4 primary immune response, genes involved with the negative regulation of oxidative stress-induced cell death (Biological Process) and the detoxification of reactive oxygen species (Reactome Pathways) (Table 1) that included SOD2 and FTMT (Fig. 5) were up-regulated. This indicated that ROS levels were kept under control by the antioxidant defence system and cell survival was promoted at this time point allowing the virus to replicate. This specifically affects mitochondrial

ROS production since SOD2 (target gene of NRF2) resides in the mitochondria (Azadmanesh and Borgstahl, 2018; Rehman et al., 2018). The pro-survival effects of SOD2 is similar to that detected in rotavirus-infected cells, where it was shown that SOD2 reduced ROS levels that resulted in prolonged cell survival, which allowed the accumulation of rotavirus progeny viruses (Gac et al., 2010). FTMT is localized in the mitochondria where it is involved in storing cellular iron as well as in protecting the mitochondria from oxidative stress by reducing ROS levels (Campanella et al., 2009).

In contrast, mitochondrial ROS production was not controlled by the antioxidant defence system during the virAHSV4 secondary immune response (Table 1) and the attAHSV4 primary and secondary immune responses (Table 2). This was indicated by the up-regulation of genes associated with the positive regulation of oxidative stress-induced cell death (Biological Process), whereas genes involved with the negative regulation of oxidative stress-induced cell death (Biological Process) and the detoxification of reactive oxygen species (Reactome Pathways) were down-regulated. This included the down-regulation FTMT as well as SOD2 (Fig. 5) downstream of NRF2 during the virAHSV4 secondary immune response and the down-regulation of SOD2 possibly downstream of ATF4 during the attAHSV4 primary and secondary immune responses since the SODs are also target genes of ATF4 (Cao and Kaufman, 2014). As such, oxidative stress due to excessive mitochondrial ROS production likely played a role in inducing the intrinsic pathway of apoptosis in AHSV4-infected PBMC during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. The down-regulation of SOD2 could have been mediated by CASP2 (Fig. 3) or degraded by RIDD. Caspase 2 can inhibit antioxidants (e.g. SODs) (Redza-Dutordoir and Averill-Bates, 2016) and RIDD can degrade pro-survival genes (Cao and Kaufman, 2014; Gong et al., 2017). IRE1α pro-apoptotic signaling conceivably played a role in the down-regulation of SOD2, either via the up-regulation of CASP2 and/or RIDD activity, during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses.

Additionally, the results of TP53 (p53) signaling supported that ROS levels were reduced during the virAHSV4 primary immune response as opposed to excessive ROS production during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. The target genes of TP53 are in pathways that include cell cycle arrest, DNA repair and apoptosis. At low levels, ROS activates TP53 that in turn induces downstream target genes that promote DNA repair and cell survival (Redza-Dutordoir and Averill-Bates, 2016). Similarly, genes were up-regulated in the negative regulation of intrinsic apoptotic signaling pathway by p53 class mediator and nucleotide-excision repair-DNA damage recognition (Biological Process). Genes were down-regulated associated with TP53 regulates transcription of cell death genes (Reactome Pathways) (Table 1) during the virAHSV4 primary immune response. There were likely only low levels of ROS present in virAHSV4-infected PBMC, since the great majority of cellular ROS production occur in the mitochondria (Azadmanesh

and Borgstahl, 2018; Rehman et al., 2018) which was detoxified by SOD2 and reduced by FTMT. Therefore, low levels of ROS possibly promoted TP53-mediated DNA repair and virAHSV4-infected PBMC survival during the primary immune response.

In contrast, high levels of ROS promote apoptosis, where activated TP53 induces the transcription of pro-apoptotic genes that are important for activating the intrinsic pathway of apoptosis (e.g. BID, BAX, PUMA and NOXA) (Redza-Dutordoir and Averill-Bates, 2016). Similarly, genes involved with TP53 regulates transcription of cell death genes (Reactome Pathways) were up-regulated during the virAHSV4 secondary immune response (Table 1) and the attAHSV4 primary and secondary immune responses (Table 2). This included TP53 pro-apoptotic target genes PUMA (attAHSV4), NOXA (virAHSV4), ZMAT3 and SHISA5 during the attAHSV4 primary and secondary immune responses and the virAHSV4 secondary immune response (Fig. 3). This showed that there were probably high levels of ROS present due to excessive mitochondrial ROS production. In turn, the high levels of ROS and/or ROS-induced damage to DNA promoted TP53-mediated apoptosis of AHSV4-infected PBMC during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses.

PERK as well as the other members of the eIF2α kinase family that include HRI (EIF2AK1), PKR (EIF2AK2) and GCN2 (EIF2AK4) phosphorylate eIF2α, which leads to the inhibition of global mRNA translation and the preferential translation of selected genes (e.g. ATF4). This is known as the integrated stress response (ISR) (Grootjans et al., 2016; Pakos-Zebrucka et al., 2016). Interestingly, it is likely during the activation of the ISR that overlapping AHSV genes (e.g. NS3A and NS4) are translated (Supplementary Fig. S2). The ISR, like the UPR, is a common adaptive pathway activated to restore cellular homeostasis. During severe ER stress, the ISR (Pakos-Zebrucka et al., 2016) and PERK switch to pro-apoptotic signaling, where CHOP activates the transcription of BIM (Dufey et al., 2014) and ERO1A (Pakos-Zebrucka et al., 2016) that promote the intrinsic pathway of apoptosis. CHOP is an important target gene of ATF4 and the main mediator of ER stress induced apoptosis (Cao and Kaufman, 2014; Pakos-Zebrucka et al., 2016; Gong et al., 2017). The ISR was activated via PKR and/or PERK during the virAHSV4 primary and secondary immune responses (Fig. 3), as well as via GCN2 during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses (Fig. 3). The up-regulation of CHOP target genes, BIM and ERO1A (Fig. 3), demonstrated that the ISR signaling pathway played a role in promoting the intrinsic pathway of apoptosis in response to ER stress during the virAHSV4 and attAHSV4 primary and secondary immune responses.

There is a functional connection and crosstalk between ER stress and calcium-mediated mitochondrial ROS production that induces the intrinsic pathway of apoptosis (Cao and Kaufman, 2014; Redza-Dutordoir and Averill-Bates, 2016; Feno et al., 2019). This was also observed by the up-regulation of genes involved with ER stress and the uptake of calcium by the mitochondria. Where GCN2, BIM,

ERO1A, PML, MCU and the IP3Rs (ITPR1-3) were up-regulated during the attAHSV4 primary and secondary immune responses (Fig. 3). PKR, PERK, BIM, ERO1A, ITPR1 and ITPR2 were up-regulated during the virAHSV4 primary and secondary immune responses (Fig. 3). Additionally, GCN2, PML and ITPR3 were up-regulated during the virAHSV4 secondary immune response (Fig. 3). During ER stress, ERO1A increases the production of ROS in the ER that results in oxidative stress (Cao and Kaufman, 2014; Pakos-Zebrucka et al., 2016). In addition, ERO1A via ROS production induces IP3R-mediated calcium leakage from the ER into the cytoplasm that is taken up by mitochondria through the mitochondria-associated membranes (MAMs) that leads to calcium-mediated mitochondrial ROS production (Cao and Kaufman, 2014; Redza-Dutordoir and Averill-Bates, 2016). The localization of PML at the MAMs promotes apoptosis, where PML regulates calcium release from the IP3Rs from the ER during ER stress that result in the influx of calcium into the mitochondria (Hsu and Kao, 2018). MCU, located in the inner mitochondrial membrane, is the major mediator of cytoplasmic calcium influx into the mitochondria (Cui et al., 2019; Feno et al., 2019). This creates a vicious cycle where calcium release from the ER and mitochondrial ROS production disrupts homeostasis that results in apoptosis (Cao and Kaufman, 2014; Pakos-Zebrucka et al., 2016; Redza-Dutordoir and Averill-Bates, 2016; Feno et al., 2019).

Similarly, ER stress and calcium-mediated mitochondrial ROS production contributed to the induction of the intrinsic pathway of apoptosis in AHSV4-infected PBMC during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. During the attAHSV4 primary and secondary immune responses, genes were up-regulated that are involved in the positive regulation of mitochondrial calcium ion concentration (Table 2). MCU (Fig. 3) likely mediated the influx and overload of calcium in the mitochondria, whereas the down-regulation of the antioxidant defence system (Fig. 5) gave rise to excessive calcium-mediated mitochondrial ROS generation. During the virAHSV4 secondary immune response, the down-regulation of MCU (Fig. 3) possibly reduced calcium uptake by the mitochondria. However, the plausible earlier accumulation of calcium in the mitochondria together with the down-regulation of both SOD2 and FTMT (Fig. 5) potentially allowed calcium-mediated mitochondrial ROS production.

In contrast, in spite of ER stress and pro-apoptotic ISR signaling that induced the up-regulation of BIM and ERO1A, AHSV4-infected PBMC did not undergo apoptosis during the virAHSV4 primary immune response. The down-regulation of MCU (Fig. 3) probably limited calcium influx into the mitochondria. This was further demonstrated by the down-regulation of genes involved with the positive regulation of mitochondrial calcium ion concentration (Table 1). Whereas FTMT and SOD2 (Fig. 5) played a crucial role in reducing and detoxifying mitochondrial ROS. This likely protected the AHSV4-infected PBMC from oxidative stress and promoted their survival. Additionally, much less pro-apoptotic genes (Fig. 3) as well as more anti-apoptotic and pro-survival genes (Fig. 5) were up-regulated during the

virAHSV4 primary immune response compared to the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses. In addition to FTMT (Campanella et al., 2009) and SOD2 that also protected rotavirus-infected cells (Gac et al., 2010), other important pro-survival genes that possibly promoted the survival of AHSV4-infected PBMC during the virAHSV4 primary immune response include CSF1 (Faber et al., 2021) and HIF1A (Fig. 5). It was shown that some viruses stabilize HIF-1α to promote virus-infected cell survival (Palazon et al., 2014). In addition to down-regulated MCU, the down-regulation of FOS (Fig. 3) conceivably promoted AHSV4-infected PBMC survival similar as observed with BTV-infected cells (Galindo et al., 2012).

Based on its location in the inner mitochondrial membrane, microRNAs were potentially responsible for the down-regulation of MCU during the virAHSV4 primary and secondary immune responses, similar as in certain cancers. Upon translocating into the mitochondria, miR-25 selectively degrades MCU in prostate and colon cancers, which reduces the uptake of calcium by the mitochondria. This prevents cancer cells from undergoing apoptosis and promotes their survival (Cui et al., 2019; Feno et al., 2019). Since miR-25 is present in horse PBMC (Parkinson et al., 2017), it is possible that miR-25 or another microRNA that down-regulates MCU could have been up-regulated in horse PBMC only in response to the virAHSV4 and not the attAHSV4. Similar as shown that certain microRNAs were only expressed in response to the virulent peste des petits ruminants virus (PPRV) and not the PPRV vaccine (Yang et al., 2019). This needs to be investigated in a future study.

4. Conclusion

This study identified the effector cells and various cell stresses that were responsible for inducing apoptosis and subsequent cell death of AHSV4-infected PBMC. Apoptosis specific transcripts were detected during the attAHSV4 primary and secondary immune responses (*in vivo*) and the virAHSV4 secondary immune response (*in vitro*), but these were absent in the virAHSV4 primary response. Accordingly, the different factors that played a role in promoting the survival of AHSV4-infected PBMC during the virAHSV4 primary immune response were identified.

In contrast to mammalian BHK-21 cells (Vermaak and Theron, 2015), the results of this study indicated that the elimination of AHSV4-infected PBMC via apoptosis during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses were exclusively induced as a host defence mechanism and not activated by viral proteins. This was further supported by the lack of cell death during the virAHSV4 primary immune response. Unlike the proposed direct induction of apoptosis by outer capsid proteins during viral uncoating in both virulent and UV-inactivated AHSV4-infected mammalian BHK-21 cells (Vermaak and Theron, 2015), viral protein(s) promote the survival of virAHSV4-infected horse PBMC. The anti-apoptotic and/or pro-survival strategies of virAHSV4 proteins observed included interference with type I and type III IFN responses, the down-regulation of MHC class I genes and the potential selective activation of the PERK-mediated NRF2/KEAP1 pathway

during the virAHSV4 immune responses. This promoted the survival of AHSV4-infected PBMC during the virAHSV4 primary immune response. In contrast, these anti-apoptotic and/or pro-survival effects were overcome and countered by trained innate cells during the virAHSV4 secondary immune response that resulted in the elimination of AHSV4-infected PBMC via apoptosis (host defence mechanism). The lack of immune evasion by proteins of the attAHSV4 likely due to alterations of certain genes of the genome during the attenuation process resulted in well-developed immune responses that included the elimination of AHSV4-infected PBMC via apoptosis (Faber et al., 2021), induced by effector cells and cellular stresses (host defence mechanism) during the attAHSV4 immune responses.

AHSV4-infected PBMC were killed through the perforin/granzyme pathway of apoptosis induced by NK cells during the attAHSV4 primary immune response and mainly by antigen-specific memory CD8+ T cells and potentially NK cells during the secondary immune response. AHSV4-infected PBMC were killed through NK cell and/or γδ T cell-induced extrinsic pathway of apoptosis during the virAHSV4 secondary immune response. Despite virAHSV4 interference with the type I and type III IFN responses, the fast kinetics and increased effector responses of NK cells (Nainu et al., 2017; Cronkite and Strutt, 2018; Rusek et al., 2018) and γδ T cells (Dantzler and Jagannathan, 2018) due to trained innate immunity resulted in the elimination of AHSV4-infected PBMC during the virAHSV4 secondary immune response. It appears that NK cells and γδ T cells play a crucial role in controlling the early virAHSV4 secondary infection until the host can overcome virAHSV4 interference with the MHC class I antigen processing and presentation pathway and antigen-specific memory CTLs can be activated.

It seemed that high levels of ROS due to the lack of detoxification played a main role in driving the various cell stress pathways towards cell death. It appeared that oxidative stress in conjunction with IRE1α pro-apoptotic signaling that included CASP2 expression and RIDD activity were the major factors responsible for the induction of the intrinsic pathway of apoptosis during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses at the 24 h time points. The results of this study are in agreement that ROS plays a dominant role in the different cell stress pathways that induces the intrinsic pathway of apoptosis (Redza-Dutordoir and Averill-Bates, 2016). It is possible that trained innate immunity in AHSV4-infected PBMC, specifically AHSV4-infected monocytes, resulted in the rapid activation of IRE1α pro-apoptotic signaling during the virAHSV4 secondary immune response; where RIDD activity were likely crucial in countering the pro-survival effects of the NRF2/KEAP1 pathway by mediating the down-regulation of SOD2 and other pro-survival genes.

Apoptosis and the phagocytosis of virus-infected apoptotic cells are major host defence mechanisms and crucial to prevent virus replication and spread (Barber, 2001; Croft et al., 2017; Nainu et al., 2017). Similarly, in this study, the elimination of AHSV4-infected PBMC via apoptosis and the clearance of

apoptotic cells possibly restrict virus replication and prevents the accumulation of progeny viruses as well as limit their spread during the virAHSV4 secondary immune response and the attAHSV4 primary and secondary immune responses.

AHSV4-infected PBMC survived during the virAHSV4 primary immune response. Specifically, virAHSV4 interference with the type I and type III IFN responses likely contributed to the impaired NK cell responses and their inability to eliminate AHSV4-infected PBMC via the extrinsic or perforin/granzyme pathways of apoptosis. While it needs to be determined if virAHSV4 is able to positively modulate the NRF2/KEAP1 antioxidant defence system in horse PBMC, it is clear that the PERK-mediated NRF2/KEAP1 pathway via SOD2 played an essential role in protecting AHSV4-infected PBMC against oxidative stress during the virAHSV4 primary immune response. It seemed that the detoxification of mitochondrial ROS played the major role in promoting the survival of AHSV4-infected PBMC in the majority of cell stress pathways during the virAHSV4 primary immune response. Furthermore, virAHSV4 interference with the innate immune response resulted in delayed immune responses (Faber et al., 2021). It is therefore plausible that IRE1α was not activated due to the delayed immune responses during the virAHSV4 primary immune response. As such, the lack of IRE1α pro-apoptotic signaling potentially promoted the survival of AHSV4-infected PBMC, specifically to up-regulate CASP2 and RIDD activity to degrade pro-survival genes (Cao and Kaufman, 2014; Gong et al., 2017). The survival of virAHSV4-infected PBMC indicated that uncontrolled viral replication and spread to neighbouring cells might allow AHSV4 to reach high quantities early during the infection, which likely contributes to inducing excessive inflammatory responses and the pathogenesis of AHS in a naïve horse.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgements

The Economic competitiveness support programme, ARC-OVR, South Africa, provided funding for this study.

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