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The A2 gene of alcelaphine herpesvirus-1 is a transcriptional regulator affecting cytotoxicity in virus-infected T cells but is not required for malignant catarrhal fever induction in rabbits

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Abstract

Alcelaphine herpesvirus-1 (AIHV-1) causes malignant catarrhal fever (MCF). The A2 gene of AIHV-1 is a member of the bZIP transcription factor family. We wished to determine whether A2 is a virulence gene or not and whether it is involved in pathogenesis by interference with host transcription pathways. An A2 gene knockout (A2⁻ AIHV-1) virus, revertant (A2revAIHV-1) virus, and wild-type virus (wtAIHV-1) were used to infect three groups of rabbits. A2⁻ AIHV-1-infected rabbits succumbed to MCF, albeit with a delayed onset compared to the control groups, so A2 is not a critical virulence factor. Differential gene transcription analysis by RNAseq and qRT-PCR validation of a selection of these was performed in infected large granular lymphocyte (LGL) T cells obtained in culture from the MCF-affected animals. A2 was involved in the transcriptional regulation of immunological, cell cycle and apoptosis pathways. In particular, there was a bias towards $\gamma\delta$ T cell receptor (TCR) expression and downregulation of $\alpha\beta$ TCR. TCR signalling, apoptosis, cell cycle, IFN- γ and NFAT pathways were affected. Of particular interest was partial inhibition of the cytotoxicity-associated pathways involving perforin and the granzymes A and B in the A2⁻ AIHV-1-infected LGLs compared to controls. In functional assays, A2⁻ AIHV-1-infected LGLs were significantly less cytotoxic than wtAIHV-1- and A2revAIHV-1-infected LGLs using rabbit corneal epithelial cells (SIRC) as targets. This implies that A2 is involved in a pathway enhancing the expression of LGL cytotoxicity. This is important as virus-

infected T cell cytotoxicity *in vivo* has been suggested as a potential mechanism of disease induction in MCF.

Keywords

Malignant catarrhal fever; Herpesvirus; bZIP protein; Transcription regulation; Cytotoxicity

1. Introduction

Malignant catarrhal fever (MCF) is a usually-fatal lymphoproliferative disease of a range of ungulates, caused by gammaherpesviruses of the genus macavirus (Plowright, 1990; Russell et al., 2009). The two best characterised MCF viruses are alcelaphine herpesvirus-1 (AIHV-1) and ovine herpesvirus-2 (OvHV-2). A feature of MCF is that these viruses induce subclinical infections in their natural, reservoir hosts but can induce often dramatic and fatal disease in the MCF-susceptible animals that include cattle, deer, bison and pigs. Clinical signs in MCF-susceptible animals include fever, inappetence, ocular and nasal discharge, corneal opacity and epithelial erosions in the mouth, oesophagus and alimentary tract (Liggitt and DeMartini, 1980a,b; Russell et al., 2009). Histologically, the disease is characterised by the accumulation of lymphocytes (CD8+ T cells predominantly) in multiple tissues (Dewals et al., 2008; Dewals and Vanderplasschen, 2011; Nelson et al., 2010). Vasculitis and epithelial damage in particular and possibly tissue necrosis (at least for OvHV-2 MCF) are thought to be responsible for the severity of disease or death in MCF (Russell et al., 2009). The detection of virus in the tissues has been difficult, probably because of the low copy number of virus DNA in infected cells. However recent results (Palmeira et al., 2013) indicate that a much higher proportion of the accumulating T cells in the tissues is infected than was previously thought. Enrichment for infected T cells can be achieved by the culture of MCF-affected animal lymphoid tissue that develops into lymphoblastoid cells (Reid et al., 1983, 1989). These lymphoblastoid cells are usually termed large granular T lymphocyte cells (LGL) and are an important resource for studying virus–host interactions in MCF (Burrells and Reid, 1991; Schock et al., 1998; Swa et al., 2001).

AIHV-1 is maintained in wildebeest (*Connochaetes taurinus*), its natural reservoir host, and is therefore a problem to MCF-susceptible cattle and antelope in eastern and southern Africa and in zoological collections where wildebeest are present. OvHV-2 is maintained in sheep as the natural reservoir host and affects MCF-susceptible animals worldwide wherever sheep are present (Russell et al., 2009). Transmission from wildebeest calves or lambs to MCF-susceptible animals is *via* nasal and ocular secretions and saliva. This is inefficient as sporadic disease involving small numbers of animals is normally seen, and susceptible animals can co-exist with reservoir species animals without apparent disease. However, outbreaks involving many animals in a herd are occasionally recorded, particularly in species thought to be more susceptible to MCF such as bison, some species of deer and Bali cattle (Russell et al., 2009). An important consideration in MCF research is that the virus has adapted to give highly efficient infection in the reservoir species where there is no apparent disease. However, infection of the disease-susceptible hosts is usually sporadic and often

fatal. Thus, the mechanism of pathogenesis of these viruses is of great interest. The virus genes will not have undergone evolutionary adaptation in the MCF-susceptible animals as these are unable to transmit the virus horizontally to other animals in the herd. This is probably because the viruses are cell-associated for the most part in the susceptible species animals, unable to undergo the full productive life cycle. Whereas AIHV-1 can be isolated from the tissues of MCF-affected animals for propagation in tissue culture, OvHV-2 cannot and is only produced as virions in the upper respiratory tract of sheep (Taus et al., 2006, 2010). For this reason, vaccine control of MCF is currently being developed for AIHV-1 MCF where virulent (wild-type) and attenuated virus can be obtained (Haig et al., 2008; Russell et al., 2012a).

MCF can be reproduced in experimental infections of rabbits and hamsters with AIHV-1 and OvHV-2 (Reid et al., 1986; Jacoby et al., 1988; Anderson et al., 2007). The disease is similar to that seen in cattle and these experimental animals are very useful for exploring disease pathogenesis.

In order to better understand MCF, a BAC clone of the AIHV-1 genome has been generated (Dewals et al., 2006). This stabilises the viral genome and allows the deletion and insertion of genes that may be involved in virus pathogenesis. A2 is a positional homologue of genes that in some other gammaherpesviruses play an important role in pathogenesis. These include: LMP-1 of EBV (Young and Murray, 2003; Raab-Traub, 2012; Damania et al., 2000); K1 of HHV-8 (Wang et al., 2004); and STP/tip of HVS (Tsygankov, 2005), all of which are involved in virus-induced transformation of cells, and M1 of MHV-68 (Krug et al., 2013) that acts as a superantigen for particular CTL cells. Furthermore, A2 encodes a basic leucine zipper family protein-homolog that may be involved in host and/or virus gene transcriptional control. We hypothesise that the A2 gene product might be involved in MCF pathogenesis by way of dysregulation of host transcriptional pathways. To address this we have constructed an A2 gene knockout AIHV-1 (A2⁻ AIHV-1) and an A2 gene reinsertion (revertant) control (A2revAIHV-1) and compared these to wild-type AIHV-1 (wtAIHV-1) in a rabbit infection model of MCF to determine whether the A2 gene product is involved in the development of MCF *in vivo*. Also, we have determined the putative function of A2 product by comparing global transcription levels of LGLs developed from animals infected with each of A2⁻ AIHV-1, A2revAIHV-1 and wtAIHV-1, and performing cytotoxicity assays based on the outcome of this analysis.

2. Materials and methods

2.1. Animals, cells and virus

Specific-pathogen-free female New-Zealand white rabbits aged between 2 and 3 months were obtained from Harlan Laboratories, UK. AIHV-1 C500 strain, originally isolated from an ox in Kenya (Plowright et al., 1975) has been maintained by passage in rabbits and culture in bovine turbinate cells (BT cells). For cytotoxicity assays, rabbit SIRC cells (corneal epithelial cell line, ATCC CCL60) were used as target cells for the LGLs. These represent a cell type that is damaged in MCF animals. Also, in preliminary experiments, these gave a lower background control lysis than BT cells (data not shown), and were therefore used in all the assays presented in this study.

2.2. Virus bZIP phylogeny

Predicted amino acid sequences for A2 and Ov2 were aligned using MUSCLE (Edgar, 2004). bZIP domains for all Human bZIP proteins as well as viral A2 and Ov2 were aligned using clustalW and a phylogenetic tree was derived using <http://www.phylogeny.fr/>. Viral bZIP sequences were gathered using a search of the NCBI protein database. The sequences were trimmed to their bZIP domain as previously described (Deppmann et al., 2006) and a phylogeny was generated using the phylogeny.fr platform.

2.3. Generation of ORF A2 deleted AIHV-1 (A2 AIHV-1) and A2 revertant AIHV-1 (A2revAIHV-1)

A2 AIHV-1 was constructed using recombineering of the AIHV-1 C500 BAC plasmid and galactokinase (galK) selection (Warming et al., 2005). For production of the A2 AIHV-1 BAC plasmid, the recombination fragment consisted of a galK flanked by 56-bp sequences corresponding to the regions directly flanking the AIHV-1 A2 coding sequence. This fragment was produced by PCR using the pgalK vector as a template, the forward chimeric primer A2H1galKF, and the reverse chimeric primer A2H2galKR (Table 1). Production of the A2revAIHV-1 plasmid was performed from a selected A2 AIHV-1 BAC clone with a recombination fragment produced by PCR using the wtAIHV-1 BAC plasmid as template and primers A2H1TTAFwd and A2H2ATGRev (Table 1). A2 AIHV-1 and A2rev AIHV-1 were transfected and propagated in EBL-Cre cells to excise the BAC cassette. Further propagation of A2 AIHV-1 and A2revAIHV-1 virus was done in Bovine Turbinate (BT) cells to a maximum of 5 passages. The absence of the A2 gene and integrity of the reinserted A2 gene was confirmed by southern blotting.

2.4. Virus plaque assay

Growth kinetics of wtAIHV-1, A2 AIHV-1 and A2revAIHV-1 virus in culture were analysed using a multistep growth curve as described in Dewals et al. (2006). Briefly, 12 well plates were seeded with BT cells overnight and infected with an MOI of 10^{-3} AIHV-1. After 1 h incubation, virus suspension was removed and fresh IMDM (containing Glutamax, 10% FCS, 1% pen/strep) added. Samples of AIHV-1-infected BT cells were collected on days 2, 4, 6 and 8 of culture and their PFU measured by plaque assay as described in Vanderplasschen et al. (1993) with the following minor modifications: freshly infected BT were overlaid with 2% carboxymethylcellulose (Sigma cat#21902) and incubated for 5 days, followed by fixation with neutral buffered formalin, acetone and methanol and immunohistochemistry with mAb 15-A (anti-AIHV-1) primary and HRP anti-mouse secondary antibodies (Dako EnVision, cat# K4006) to detect plaques. Plaque numbers and plaque size were measured optically with plaque diameters recorded using an eyepiece graticule.

2.5. Infection of rabbits with A2 AIHV-1, wtAIHV-1 and A2revAIHV-1

Two experiments involving three groups of three animals per experiment were performed. Rabbits were inoculated intravenously with either wtAIHV-1, A2 AIHV-1 or A2revAIHV-1 infected BT cells. Each AIHV-1 inoculated animal received 7.5×10^9 viral genomic copies as calculated by quantitative PCR (Traul et al., 2005) within $(1-3) \times 10^6$ viable BT cells.

Some of the virus is cell-associated and determining virus infectious dose is difficult and inaccurate. We infected with infected cells and not with viral particles. We normalised the infection dose on the quantity of viral DNA copies. A negative control group of 4 specific-pathogen-free New-Zealand white rabbits was included in the study and inoculated with mock-infected BT cells. Rabbits were examined daily for MCF disease endpoint indicators and twice daily once their rectal temperature was above 40 °C and were euthanased on the second day of this febrile response.

2.6. Tissue sampling, processing and culture

Weekly intravenous blood samples were collected into K₃EDTA just prior to and during infection with AIHV-1. Samples of kidney, liver, lung, mesenteric lymph node (MLN), popliteal lymph node (PLN) and spleen were collected at necropsy and thin slices (~5 mm) were placed immediately into plastic cassettes and immersed in formalin fixative for 24 h at room temperature. Cassettes were transferred to 70% ethanol before processing to paraffin wax. Standard 5 µm-thick serial sections were cut and mounted on Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany) and dried overnight at 37 °C. One section from each tissue was stained with haematoxylin and eosin (H&E).

2.7. LGL cells

MLN cell suspensions from the infected groups of animals were also prepared under aseptic conditions in order to attempt development of large granular lymphocyte (LGL) cells in culture. The MLN cell suspension was cultured in IMDM (containing Glutamax, 10% FCS, 100 µg/ml penicillin/50 µg/ml streptomycin) and IL-2 (Invitrogen, UK; 150 ng/ml) at 1×10^7 cells per ml in 10 ml aliquots in upright 25 cm² flasks at 37 °C in an atmosphere of 5% CO₂ in air in an incubator. After 2–3 weeks, with weekly refeeding and expansion in medium with IL-2, LGLs had developed in most of the cultures. Further phenotypic characterisation of the cells was by flow cytometry analysis using rabbit alloantigen-specific monoclonal antibodies (see below).

2.8. AIHV-1 DNA detection by quantitative PCR

DNA was extracted from the tissues (including the LGLs) using the QIAGEN DNAeasy blood and tissue kit (cat# 69504). Quantitative PCR was performed on ORF A3 as described in Traul et al. (2005). Briefly, a 546 bp AIHV-1 ORF A3 PCR product was used as a standard (using PCR primers AHVTeg-F and AHVTeg-R). ORF A3 qPCR primers within the standard sequence were used along with probe FAMAIHV-1 and analysed in a Roche Lightcycler 480 system. Similarly, for host gene quantitative amplification a 375 bp genomic DNA sequence of the rabbit β-globin gene was amplified using traditional PCR, purified and used as a standard. Rabbit β-globin qPCR primers within the standard sequence were used along with a β-globin FAM probe as described in Boudry et al. (2007).

2.9. Antibodies and flow cytometry analysis of cells

Monoclonal antibodies directed against rabbit CD4 (KEN-4, AbD Serotec), CD8 (ISC29E, VMRD, WSU, Pullman, USA), Pan T (RTH21A, VMRD), CD25 (KEI-ALPHA1, AbD Serotec), and pan B (MRB29A, Moredun Research Institute) were used according to the

manufacturer's/users instructions for flow cytometry analysis of LGLs. mAb 15-A (VMRD) raised against AIHV-1 glycoprotein complex gp115 (Li et al., 1994) was also used for IHC of AIHV-1 infected BT cells for plaque assays.

2.10. LGL RNA for RNAseq analysis and quantitative RT-PCR of host genes

LGLs that had established a relatively stable phenotype, good growth characteristics and a good viral DNA load (Table 2) were cultured free from the presence of exogenous IL-2 for 1.5–2 h (to remove the effect of IL-2 signalling) before extracting RNA using the QIAGEN RNAeasy kit. A DNase step was included for samples that were to undergo RNAseq. RNA from LGL lines isolated from rabbits 244.2, 675.3 (A2 AIHV-1+ LGLs), 281.4, 635.5 (wtAIHV-1+LGLs), 318.7 and 320.9 (revAIHV-1+ LGLs) all had RIN > 7.5. Each of the duplicate LGL samples from each experimental group was compared to LGL samples in the other experimental groups to determine statistically significant ($P < 0.05$, Baggerly test with FDR correction) changes in gene transcription associated with the presence and absence of the A2 gene of AIHV-1. Duplicate merged sample comparisons revealed similar results. RNA from these and additional LGLs cultured from wtAIHV-1-, A2 AIHV-1- and A2revAIHV-1-infected rabbits (Table 2) were tested using two step qRT-PCR. Key genes identified from RNAseq or Metacore pathway analysis (see below) that were up or down-regulated in A2 AIHV-1 LGLs compared to wtAIHV-1 LGLs or A2revAIHV-1 LGLs were chosen for further analysis using gene specific primers and probes (Table 1) and qRT-PCR relative quantification to 3 rabbit housekeeping genes (SDHA, β -actin and HPRT). Of these SDHA gave the most consistent results. cDNA was synthesised from RNA for qPCR using the Roche Transcriptor First Strand cDNA Synthesis Kit and random hexamer primers. qPCR was performed using Roche LightCycler 480 and LightCycler 480 Probe Master Kit. Standard curves were created using serial dilutions of PCR amplicons alongside cDNA samples. A range of 1.93–2.05 for the standard curve efficiency was used to define an optimised assay. Results were expressed as the relative quantification of gene transcript (as cDNA) compared to SDHA using the standard curve method (Bolha et al., 2012).

2.11. The LGL cytotoxicity assay and the detection of perforin and granzymes A and B

LGL cytotoxicity was measured in the LGLs using a commercial LDH release assay (CytoTox96- Non-Radioactive Cytotoxicity Assay, Promega cat#G1780) with SIRC target cells according to the manufacturer's directions. Effector to target ratios of 10:1, 3:1 and 1:1 were used and cells were co-cultured for 4 h before being centrifuged at $250 \times g$ for 4 min and supernatant collected. The following controls were used; effector cell spontaneous release, target cell spontaneous release, target cell maximum release (lysed cells), culture medium background and volume correction control. The culture medium consisted of phenol red-free IMDM containing 5% (v/v) of 70 °C-inactivated FCS, 100 μ g/ml penicillin/50 μ g/ml streptomycin and glutamax. Percentage cytotoxicity for each target cell to effector cell ratio was calculated as follows:

$$\% \text{Cytotoxicity} = \frac{(\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous})}{(\text{Target Maximum} - \text{Target Spontaneous})} \times 100$$

Granzyme A release of LGL lines was quantified using a BLT esterase assay (which detects the tryptase activity of granzyme A) as described previously (Haig et al., 1996). Culture supernatant from co-cultured LGL and SIRC cells were added to a 96 well flat bottomed plates and 200 µl of a mixture of 0.3 mM-BLT and 0.33 mM-dithio-bis-(2-nitrobenzoic acid) in PBS pH 7.2 added. Incubation was for 1 h at 37 °C. The reaction was terminated by placing the plates on ice and adding 5ul of 5 mM-Pefabloc (Sigma). Plates were read on an ELISA plate reader at A410. Granzyme B was measured using the colorimetric substrate N-Acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (Ac-IEPD-pNA) in 25 mM HEPES buffer (pH 7.5) containing 0.1% CHAPS, 5% (w/v) sucrose, 5 mM DTT, 2 mM EDTA. Supernatant was incubated for 48 h at 37 °C and read at A410 nm.

2.12. Transcription and differential gene expression

50 bp cDNA reads were generated using the ABI SOLiD platform. These were trimmed to remove SOLiD adapters and filtered to remove low quality reads. Reads below quality score 0.05, with greater than two ambiguous nucleotides or shorter than 15 bp after trimming were removed. Remaining reads were aligned to the *Oryctolagus cuniculus* reference genome (oryCun2, Ensembl version 66) using CLC Genomics workbench 5.5. Read counts per gene and normalised RPKM expression values were generated and compared to calculate fold change values (Baggerly test with FDR corrected *P* value). Reads which did not map to the *Oryctolagus cuniculus* genome were re-mapped against the AIHV-1 sequence (GenBank: AF005370.1).

2.13. Pathway analysis

Putative human orthologues of the *Oryctolagus cuniculus* genes that were differentially expressed in the A2 AIHV-1 LGLs when compared to wtAIHV-1 and/or A2revAIHV-1 (*P* < 0.05) were identified using Ensembl (Flicek et al., 2012). Enriched pathways, gene ontologies and networks within these orthologue gene lists were identified using MetaCore (Thompson Reuters).

The raw data from this study has been deposited in the European nucleotide archive at <http://www.ebi.ac.uk/ena/home> accession code PRJEB4799.

3. Results

3.1. The A2 gene and predicted protein

The predicted amino acid sequence of A2 of AIHV-1 is shown in Fig. 1. This is derived from the genome sequence of AIHV-1 (ref, GenBank: AF005370.1). Also shown is a comparison with the equivalent Ov2 gene of OvHV-2 (Hart et al., 2007). A bioinformatic analysis of the A2 gene was performed, indicating that A2 is a member of the basic leucine zipper family of transcription regulator proteins (Fig. 2A). A similar comparison with other viruses indicated that several herpesviruses contained bZIP domain proteins phylogenetically-related to A2 and O2 (Fig. 2B). These included: MEQ of gallid herpesvirus-2 (Marek's disease virus type 1), the bZLF1 proteins of HHV-4 (Epstein Barr virus) and macacine herpesvirus 4, and bZIP of macacine herpesvirus 5.

3.2. Construction of the recombinant viruses

Fig. 3A shows the construction of the recombinant viruses. Fig. 3B shows that the A2 gene was successfully deleted in the A2 AIHV-1, but was present in the wild-type and revertant viruses (wtAIHV-1 and A2revAIHV-1). Fig. 3C shows that the A2 AIHV-1 grew in culture and therefore the A2 gene product is not essential for virus replication. The *in vitro* growth of A2 AIHV-1 was significantly slower than that of wtAIHV-1 and A2revAIHV-1 viruses ($P < 0.002$ for wtAIHV-1 or A2revAIHV-1 *versus* A2 AIHV-1 on day 8). Fig. 3D shows that plaque sizes (diameters) were not significantly different between the three groups of cultured infected cells. This indicates that the A2 gene product is involved in a pathway affecting either the rate of growth of the virus in culture or virus yields per cell.

3.3. Infection of rabbits with A2 AIHV-1 delayed the onset but did not prevent the development of MCF

To determine the effect of A2 in AIHV-1 infection, A2 AIHV-1 and A2revAIHV-1 were used along with wtAIHV-1 in two *in vivo* experiments.

Fig. 4A shows the pooled survival plot for the two experiments. Five of the six A2 AIHV-1-infected animals showed a delay in onset on MCF compared to the other two groups, with the exception of animal YF8 that succumbed on day 17 after infection (Fig. 4 legend gives the animal identities in each group), which was around the same time as the wtAIHV-1 and A2revAIHV-1-infected animals. In spite of this single animal outlier, the delay was significant for the A2 AIHV-1-infected group as a whole compared to wtAIHV-1 and A2revAIHV-1-infected animals ($P = 0.03$). Fig. 4B shows that the viral DNA load (viral DNA copies/ 10^5 β -globin copies) in the MLNs of the rabbits post-mortem were not significantly-different between the three infection groups.

Infection of rabbits with each of the virus variants induced MCF that was generally typical of that seen in rabbits as described previously (Anderson et al., 2007; Dewals et al., 2008). Clinically, the animals exhibited inappetence, depression and pyrexia. In one of the A2 AIHV-1 virus-infected animals (YM9), there was a more marked weight loss than in the other animals. Histological examination of the tissues showed that in all animals infected with wtAIHV-1, A2 AIHV-1 and A2revAIHV-1, there were lesions diagnostic of MCF. These included lymphoid architecture disruption in the lymphoid tissue (MLN, PLN and spleen) with evidence of secondary lymphoid follicles. In the non-lymphoid tissue (kidney, liver, lung) large interstitial and perivascular accumulations of lymphocytes were seen and there was widespread vasculitis. Unlike OvHV-2 MCF (Buxton et al., 1984; Anderson et al., 2007), there were very few areas of necrosis in the tissues. In A2 AIHV-1-infected animals 227.1 and 675.3 an 'atypical' form of MCF was seen with more severe haemorrhaging in the lungs. The pathology and histopathology of the chronic weight loss animal YM9 presented as typical MCF. Thus, we conclude that A2 is involved in virulence as it affects the onset of MCF, but is not a major virulence gene essential for the development of MCF in rabbits.

3.4. Transcriptomic and qRT-PCR analysis of LGLs revealed that the A2 gene product is involved in transcriptional regulation of multiple host genes and pathways

LGLs were cultured from rabbits in each of the experimental groups (Table 2). Their phenotype and virus DNA load was recorded at a time at which the LGLs had developed a relatively stable phenotype, in respect of large granular lymphocyte morphology and expression of T cell alloantigens, prior to the various analyses of them. In addition, virus DNA load at around the time a selection of them was used in the qRT-PCR validation assays and the cytotoxicity assays is shown (Table 2, final column). Two LGL lines from each of the three experimental groups from the first of the two infection experiments were submitted for RNAseq analysis. These were: LGL lines isolated from rabbits 244.2, 675.3 (A2 AIHV-1+ LGLs), 281.4, 635.5 (wtAIHV-1+ LGLs), 318.7 and 320.9 (revAIHV-1+ LGLs). These all exhibited good growth in culture, a similar LGL gross phenotype with a mixture of lymphoblasts and granulated cells and yielded high quality RNA.

The raw data were filtered and trimmed (Section 2). On average, 11% of the reads per sample were removed. The filtered lists were mapped against the rabbit genome achieving an average mapping efficiency of 52%. Whilst the expression of AIHV-1 genes varied between the samples, mapping depth was too low in some to confidently call statistically robust changes in gene expression. For the sample with highest RNAseq coverage (A2revAIHV-1, 318.7 LGL, >40-fold coverage of the known genes) the sequenced strain showed 99.974% identity to the published wtAIHV-1 sequence.

Expression analysis was performed to identify gene transcripts differentially expressed in A2 AIHV-1 LGLs when compared to wtAIHV-1 and A2revAIHV-1 (FDR corrected $P < 0.05$). These lists indicated that there were more host genes down-regulated than up-regulated in the A2 AIHV-1 LGLs compared to the wtAIHV-1 or A2revAIHV-1 LGLs. Those transcripts showing statistically-significant differential abundances in the comparison are shown in Tables 3A and 3B. Table 4 shows pathways differentially affected by the absence of A2 (analysed by Metacore). A range of transcripts were subjected to qRT-PCR analysis to validate or not a selection of the RNAseq results (Fig. 5). This was applied to an extended number of LGLs beyond those used for the RNAseq analysis (Table 2, final column). The principal affected pathways are those of the immune response, cell cycle, apoptosis, cytotoxicity, transcription control and signal transduction (Tables 3A, 3B and 4). Within the immune response, T cell receptor, IL-2 and IFN- γ signalling pathways were affected by the absence of the A2 gene, indicating A2 is involved in their regulation.

There was a clear down-regulation of T cell receptor (TCR) $\gamma\delta$ chain transcripts and upregulation of $\alpha\beta$ TCR transcripts in the A2 AIHV-1 LGLs compared to wtAIHV-1 or A2revAIHV-1 LGLs indicating that the A2 gene product is involved in a pathway supporting $\gamma\delta$ T cell receptor expression. We were unable to determine if the LGLs contained $\gamma\delta$ chains as there are no antibodies available to detect these in the rabbit. The RNAseq data was clear and we did not validate this by qRT-PCR. The phenotype of the LGLs is shown in Table 2. Most of the LGLs contained >40% CD8⁺ cells, except A2 AIHV-1 LGL 675.3. Proportions between 10 and 60% of the LGLs were CD4⁻, CD8⁻. All lacked the B cell antigen B220.

Of the qRT-PCR-validated results, cytotoxicity-associated pathways were affected, notably those involving granzyme A and B and the FAS pathway. The IL-2 transcript was increased, whereas the IFN- γ transcript was decreased in the A2 AIHV-1 LGLs compared to the others (Fig. 5).

There were many transcription and cell-signalling molecules differentially expressed in A2 AIHV-1 LGLs *versus* the others (Tables 3A, 3B and 4). Amongst these, FOSB and CAPN5 (a FOSB homologue), downregulated in A2 AIHV-1 LGLs compared to the others could be potential binding partners for A2. The NFAT transcription factor was downregulated in A2 AIHV-1 LGLs compared to the others when the LGLs were analysed by qRT-PCR (Fig. 5). We conclude that the A2 gene product is involved in gene transcriptional regulation in multiple pathways as described in Table 4.

3.5. The A2 gene product is involved in a pathway that enhances the granzyme/perforin pathway leading to constitutively-activated cytotoxicity in LGLs

During the course of these studies, it was observed that the A2 AIHV-1 LGLs were less cytotoxic for target fibroblast or epithelial cells than the other LGLs infected with virus containing A2. In addition, the Metacore pathway analysis showed that the perforin/granzyme pathway was significantly downregulated in the A2 AIHV-1 LGLs compared to the other LGLs. This prompted us to analyse cytotoxicity transcripts in more detail. From the RNAseq analysis ($n = 2$ for each group of LGLs), the transcripts for granzyme A and granzyme B were on the borderline of significance (4.2-fold reduction for granzyme A in A2 AIHV-1 LGLs compared to either wtAIHV-1 LGLs or A2revAIHV-1 LGLs, $P = 0.052$; 3.5-fold reduction for granzyme-B in A2 AIHV-1 LGLs compared to either wtAIHV-1 LGLs or A2revAIHV-1 LGLs, $P = 0.050$). Also the NKG2D transcript was downregulated in A2 AIHV-1 LGLs compared to the others, which is a potential cytotoxicity regulator, although levels of this were low. These cytotoxicity transcripts in all the LGL lines (Table 2) were analysed by qRT-PCR showing that those for FAS (but not FAS ligand), granzyme A, granzyme B, perforin and NKG2D were all significantly downregulated in A2 AIHV-1 LGLs compared to either wtAIHV-1 LGLs or A2revAIHV-1 LGLs (Fig. 5).

In order to determine whether the A2 AIHV-1 LGLs were less cytotoxic to target epithelial (SIRC) cells, a cytotoxicity assay was performed using the LGLs listed in Table 2 (final column). Fig. 6A shows that the A2 AIHV-1 LGLs were significantly less cytotoxic than either the wtAIHV-1 LGLs or the A2revAIHV-1 LGLs in this assay. Granzymes A (Fig. 6B) and B (Fig. 6C) were measured in the culture supernates. Although less of both was produced by the A2 AIHV-1 LGLs compared to the others, this was significant only for granzyme A in the A2 AIHV-1 LGL supernates at a 10:1 E:T ratio compared to the A2wtAIHV-1 or A2revAIHV-1 samples ($P = 0.04$). We conclude that the A2 gene product is involved in transcriptional regulation of the perforin/granzyme pathway and functions to enhance the cytotoxicity in AIHV-1-infected LGLs.

4. Discussion

In this study we wished to determine whether A2 is a virulence gene and whether it interferes with host transcription to affect pathogenicity.

Phylogenetic analyses of the A2 gene and its product show that it is a member of the basic leucine zipper proteins involved in many aspects of cell and tissue function. The bZIP domain of A2 and O2 was related to proteins found in other herpesviruses, most of which are in the gammaherpesvirinae subfamily. These included BZLF-1 (Zta) of EBV (HHV-4) and K-bZIP (K8) of KSHV (HHV-8, Sinclair, 2003). BZLF-1 and K-bZIP interact with many viral and cellular proteins (Sinclair, 2003). Interestingly, the BZLF-1 of EBV is involved in the latent to lytic virus life cycle progression, whereas K-bZIP does not do this, but is involved in virus replication (Sinclair, 2003). MEQ of Marek's disease virus (MDV, gallid herpesvirus-2) is responsible for the oncogenic properties of MDV that binds to JUN for this effect (Lupiani et al., 2004). Other viruses also have basic leucine zipper molecules, notably the HBZ of HTLV-1 (a retrovirus) (Zhao and Matsuoka, 2012). HBZ represses both viral and cellular gene expression (Zhao and Matsuoka, 2012). So, prior to performing detailed studies of the A2 protein and its interactions with binding partners, we reasoned that it would be useful to determine which genes and pathways were affected by the presence and absence of A2.

Lack of A2 (A2⁻ AIHV-1) did not affect the ability of the virus to grow in culture, although growth rate was slower or the virus per cell was less in the A2⁻ AIHV-1-infected cell cultures compared to those of the control virus-infected cells. In rabbits infected with the different viruses, all developed MCF. In all animals infected with A2⁻ AIHV-1 except one, the onset of clinical disease was delayed compared to the animals infected with A2 intact virus. A2 is therefore not essential for the production of virus or for the infection of rabbits and induction of disease, but does affect the time to disease onset.

In order to determine the role of A2 in infected cells, LGLs from the MCF-affected rabbits were grown in culture and global gene transcription measured by RNAseq. Both individual gene transcription and pathway data highlighted genes and pathways apparently affected by A2. Immune response transcripts were particularly affected, which is not surprising given the LGLs are virus-altered T/NK cells. There was a bias towards $\gamma\delta$ TCR usage that may explain previous observations that LGLs from various species infected with AIHV-1 or OvHV-2 can exhibit a $\gamma\delta$ T cell phenotype (where antibodies are available to detect this) or have a T cell phenotype predominantly CD4-CD8- (Burrells and Reid, 1991; Schock et al., 1998; Swa et al., 2001). Most LGLs in the rabbit and in cattle are predominantly CD8+, but this could still be present on $\gamma\delta$ TCR+ cells. Recently, CD8+ $\gamma\delta$ T cells were described in MCF lesions in bison (Nelson et al., 2010). It is not known what the target cell is in the T cell lineage for initial virus infection in MCF. A study of TCR usage by LGLs from MCF affected animals has not been done, but would be worthwhile. The LGLs subjected to RNAseq analysis exhibited features typically seen in LGLs, namely a mixture of lymphoblastoid and granular lymphocytes. However, there were some variations in cell-surface phenotype (alloantigens), which is also a feature of these cells. Whereas many LGL lines express the CD8 molecule, not all do and there are some that express neither CD8 nor CD4 (Burrells and Reid, 1991; Schock et al., 1998; Swa et al., 2001). There is a lack of antibody reagents to probe phenotype further (e.g. $\gamma\delta$ cells). The two lines, 675.3 and 244.2 in the A2 deleted AIHV-1 group differed markedly in the expression of CD8. However, importantly, their RNAseq profiles were similar, as were those of the individual lines when

compared to each other within the other LGL groups. In this study we show that A2 is involved in the regulation of T cell receptor phenotype and it is possible that the virus may regulate other aspects of T cell phenotype. In recent studies involving single cell transcriptomics within highly phenotypically-defined T cells, it is apparent that the transcriptomic phenotype is not necessarily predicted by the presence of the same alloantigens on the cells (Shalek et al., 2013) in the same activation state. We therefore believe that any differences in LGL – surface phenotype are not important in this study.

$\alpha\beta$ TCR signalling and CD28 co-receptor signalling were upregulated in LGLs lacking A2, indicating A2 is involved in a pathway down-regulating their expression. We have previously shown that MCF virus-infected LGLs show a constitutive activation of the TCR-associated *src* kinases *Lck* and *Fyn* and downstream MAPK (Swa et al., 2001). Transcripts for these were not differentially expressed in A2 AIHV-1 LGLs *versus* the others, so are not regulated at the transcriptional level by A2.

Other elements of immune responsiveness regulated by A2 include the IFN- γ , IL-1 and IL-2 pathways. IFN- γ production by LGLs has been detected previously (Schock et al., 1998). The function of many cytokines in MCF pathogenesis is unknown. We have previously shown that IL-15 is expressed in the tissues of MCF-affected animals, and that the receptor is expressed on the LGLs, whereas IL-15 is not produced by them (Anderson et al., 2008). IL-15 is involved in the recruitment, proliferation and activation of cytotoxic cells including T cells and NK cells (Waldmann, 2006) and is therefore a candidate effector of MCF pathogenesis.

The role of IL-2 in MCF pathogenesis has been the subject of speculation (Meier-Trummer et al., 2009). The original observation was that the IL-2 transcript was low or lacking in LGLs from OvHV-2-infected cattle (Schock et al., 1998). Expression could not be achieved in these cells even after stimulation with mitogens. Most LGLs (AIHV-1 or OvHV-2 infected from cattle or rabbits) studied so far show variable levels of IL-2 mRNA, with the majority exhibiting low or absent levels of the transcript (D. Haig, unpublished). In this study, most of the wtAIHV-1 LGLs and A2revAIHV-1 LGLs expressed low levels of IL-2mRNA, that were significantly increased in the A2 AIHV-1 LGLs (Fig. 5). Other pathways were affected by the absence of A2 in the virus-infected LGLs. Apoptosis pathways were downregulated in the wtAIHV-1 LGLs and A2revAIHV-1 LGLs compared to A2 AIHV-1 LGLs. It is clearly an advantage to an infecting virus to prevent apoptosis in order to survive and replicate within the host cells.

Cell cycle effects of A2 were recorded, particularly affecting the G1/S phase. T lymphocyte accumulation in the tissues, which contain virus-infected cells, are a feature of MCF lesions and these are recruited to non-lymphoid tissue and show evidence of cell division/proliferation. Transcription/signalling molecules influenced by A2 included the nuclear factor NFAT, involved in regulating immune responses. As A2 is a basic leucine zipper protein, an effect on transcription and other transcriptional regulators is expected.

The main focus of the study was the role of A2 in LGL cytotoxicity, prompted by the observation that the A2 AIHV-1 LGLs did not kill fibroblasts or epithelial cells whereas the

wtAIHV-1 LGLs and A2revAIHV-1 LGLs did. LGLs are indiscriminately (non-MHC-restricted) cytotoxic for a range of target cells (Russell et al., 2009). Cytotoxicity-associated pathways and genes were down-regulated in A2 AIHV-1 LGLs compared to the other control LGLs. These include NKG2D, perforin and the granzymes A and B. FAS was also affected, which is part of the FAS–FAS ligand interaction that can control T cell cytotoxicity (Ehrenschrwender and Wajant, 2009). However, FAS-ligand transcript abundance was not affected by the absence of A2 in AIHV-1-infected LGLs (Fig. 5). The A2 AIHV-1 LGLs were significantly less cytotoxic for target SIRC epithelial cells than the wtAIHV-1 LGLs or the A2revAIHV-1 LGLs. Cytotoxic activity of virus-infected T cells in MCF has been implicated in causing the endothelial cell (vasculitis) and epithelial cell damage in multiple tissues that causes the demise of the animals in MCF (Russell et al., 2009). A reduced cytotoxicity of A2 AIHV-1-infected LGLs as well as a possible reduced growth of this virus (indicated *in vitro*, Fig. 3C and D) compared to the others may have contributed to the delay seen in most A2 AIHV-1-infected rabbits in developing MCF, provided the hypothesis that it is the cytotoxic activity of virus-infected T cells that is responsible for the tissue damage in MCF is correct (Russell et al., 2009).

Our results are broadly in line with other analyses looking at transcription profiles in the tissues of cattle with OvHV-2 MCF or AIHV-1 MCF (Meier-Trummer et al., 2009; Palmeira et al., 2013; Russell et al., 2012a,b), in spite of focussing on differential transcript profiles in LGLs affected by the presence and absence of the A2 gene. In these studies, one looked at lymphoid tissue from OvHV-2 MCF-affected cattle (Meier-Trummer et al., 2009) and the others at lymphoid tissue and non-lymphoid kidney tissue in AIHV-1-affected cattle (Russell et al., 2012a,b; Palmeira et al., 2013). In all cases transcript detection was achieved using microarray. Transcripts for TCR usage, inflammatory responses, cytotoxicity-associated genes, cell cycle and apoptosis genes were broadly similar to our analysis of those affected by A2 presence or absence in LGLs. IFN- γ was upregulated. However, whereas in the OvHV-2 MCF study in cattle, significant depression of IL-2 transcript was seen, this was not apparent in the AIHV-1 MCF cattle study (Meier-Trummer et al., 2009; Russell et al., 2012a,b). The authors of the OvHV-2 study speculated that MCF may be a disease of IL-2 deficiency (Meier-Trummer et al., 2009). These studies compared to our own herein indicate that A2 may have a function, at least in part, in the regulation of similar pathway genes both in LGLs and by extrapolation in the tissues of MCF-affected animals. The use of LGLs has advantages over analyses of gene transcription in the tissues of animals with MCF. Firstly, the LGLs are an enriched population of virus-infected cells that allows virus–host interaction to be studied efficiently and effectively in infected cells. Virus-infected T cells in the tissues of MCF-affected animals are only a proportion of the total T cells and other cells, but are probably the source of the LGLs developed in culture. The similarities in the tissue *versus* LGL transcription analyses would support this view. The LGLs are viable, whereas the tissues of MCF-affected animals are undergoing disease-induced damage and dead or dying cells could affect the transcription profiles detected. As the LGLs are developed in culture, usually with exogenously-added IL-2 there is a possibility that they do not represent accurately the virus-infected cells *in vivo*. Interestingly, LGLs can be developed in the absence of exogenous IL-2, although this is a less efficient way of generating the cells (Swa et al., 2001). A pilot comparison of RNAseq from an infected lymph node compared to LGL

analysis indicated that there was better resolution and detail in the LGL analysis (unpublished result).

Thus, the absence of A2 was associated with transcriptional changes in many genes, none of which affected the ability of the A2 AIHV-1 to induce MCF. Other work with recombinant viruses lacking the A5 gene (a chemokine receptor orthologue), failed to have any measurable effect on the pathogenesis of MCF (Boudry et al., 2007). Recently however, it has been demonstrated that AIHV-1 lacking the virus ORF73 latency gene was able to establish a transient infection, but T cell accumulations and disease were not seen (Palmeira et al., 2013). This suggests that virus latency programming drives infected T cell accumulation and is involved in a pathway inducing disease. The actual effector mechanism responsible for disease is not known.

5. Conclusions

The A2 gene product of AIHV-1 has sequence and predicted structural properties consistent with basic leucine zipper transcriptional activation proteins. A2 is involved in the transcriptional regulation of many host genes and pathways, but in spite of this, it is not necessary for the induction of MCF. The relative lack of cytotoxicity of the LGLs from MCF-affected rabbits infected with A2 AIHV-1 compared to virus containing A2 did not affect the ability of the A2 deleted virus to induce MCF, although the onset of disease was delayed compared to controls. This opens up new avenues for research on the mechanism of disease induction in MCF.

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Abbreviations

MCF	malignant catarrhal fever
AIHV-1	Alcelaphine herpesvirus-1
A2 AIHV-1	A2 gene knockout AIHV-1
A2revAIHV-1	A2 revertant AIHV-1
wtAIHV-1	wild-type AIHV-1
LGL	large granular lymphocytes
SIRC	Statens Seruminstitut rabbit cornea cells

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A2   1  MSQNSNSSENPSERKKRYVMCDLTEEQERRRSINRRASNFKKRRRIFEEQOEKGLINL
OV   1  -----MSQNKKEPKKTYPRMCDLTEEQERRRSINRRASNFKKRLQEHEQOEKRELRDL

A2   61  KYENSRLRCQMEKKKDEIRILREWLNYHKCTTLQNYNTGPEPRVKVENSLEMQCATAFL
OV   56  IYTNMCLRGEMEKKKEIRRLQYWLSTHNCLKLEG-----ATPSNSSSGHGGEVTNDFY

A2   121  NLDQQYTNNINIPETVSGNNTTNGFAAATATLHTNCYEKTLANNNNFEAKLNCEVLPS
OV   111  NSEQHLWSPQV-----GGSGCYFDPPAGSTWQMEDRGEGTASSSPFTTEGSEQVLEE

A2   181  FTS----ALDDLSDWNNLYNL
OV   164  LFPETWLSVDISFDTENMLHSL

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Fig. 1.

Predicted amino acid sequence of A2 and Ov2 gene products. A2: UniProtKB/Swiss-Prot: O42037.1. Ov2: NCBI Reference Sequence: YP 438127.1. MUSCLE sequence alignment.

Fig. 2.

(A) Phylogenetic tree of bZIP domains showing the position of A2 and Ov2. (B) Phylogenetic tree showing the relationship between the bZIP domains of A2 and O2 with those of other herpesvirus proteins. The viruses are mainly of the gammaherpesvirinae subfamily (lymphocryptovirus or rhadinovirus genera), apart from gallid herpesvirus-2 (Marek's disease virus) of the alphaherpesvirinae subfamily and cyprinid herpesvirus-1 (currently unassigned). The numbers on the plots refer to the branch support values ranging

from 0 to 1 which were obtained using a bootstrapping statistical approach. Higher values reflect the strength of evidence for the phylogenetic relationship represented.

Fig. 3.

(A), BAC recombinant virus generation strategy. (B) Presence of GALK (Middle panel) and absence of A2 gene (right hand panel) in A2 AIHV-1 and presence of the A2 gene in the A2revAIHV-1 (right hand panel) using southern blot analysis. HindIII was used to digest the BAC clones. (C) Growth of wtALHV-1, A2revALHV-1 and A2 AIHV-1 in BT cells in culture. Groups of triplicate wells of 12 well plates were seeded with BT cells overnight and infected with an MOI of 10^{-3} of each AIHV-1. SD for replicate wells was within 2% of the mean value at each time point and for each group. The experiment was repeated with the same overall result. (D) Plaque diameters for each group (from the virus growth assay). * $P < 0.02$ for the comparison between A2 AIHV-1 and either A2revAIHV-1 or wtAIHV-1. ** $P < 0.01$ for the comparison between A2 AIHV-1 and either A2revAIHV-1 or wtAIHV-1. *** $P < 0.002$ for the comparison between A2 AIHV-1 and either A2revAIHV-1 or wtAIHV-1.

Fig. 4.

(A) Kaplan Meier survival plot for rabbits infected with each of wtALHV-1, A2revALHV-1 and A2 AIHV-1. $P = 0.03$ for the longer survival of A2 AIHV-1 compared to either wtAIHV-1 or revAIHV-1. (B) Viral DNA copy number per 10^5 β -globin copies in the MLN of the rabbits in each group at necropsy. Rabbit identities (survival in days) were as follows: wtALHV-1 group: YU3(17); PR2(18); XX1(21); 281.4(26) 635.5(17); 717.6(34). A2revAIHV-1 group: RF6(19); RA5(22); YO4(23); 318.7(34); 320.9(31). A2 AIHV-1 group: YF8(17); YM9(51); 227.1(42); 244.2(38); 675.3(43).

Fig. 5.

(A) qRT-PCR for host (rabbit) transcripts in the LGLs identified as part of the RNAseq analysis ($n = 5$ for A2 AIHV-1 group; $n = 5$ for wtAIHV-1 LGL and $n = 3$ for A2revAIHV-1 LGL). * indicates $P < 0.05$ for the comparison of A2 AIHV-1 LGL (A2 LGL) with either wtAIHV-1 LGL (wt LGL) or A2revAIHV-1 LGL (A2rev LGL).

Fig. 6.

LGL cytotoxicity against SIRC cells at various effector to target cell ratios (E:T). (A) Cytotoxicity assay. (B) Granzyme A release from LGLs into the culture supernates. (C) Granzyme B release from LGLs into the culture supernates. Mean with standard deviation error bars for LGLs in each of the three groups: wtAIHV-1-infected LGLs (wt LGL), A2revAIHV-1-infected LGLs (A2rev LGL) and A2 AIHV-1-infected LGLs (A2 LGL). * $P < 0.02$ for A2 AIHV-1 comparison with either A2revAIHV-1 LGLs or wtAIHV-1 LGLs. ** $P < 0.008$ for A2 AIHV-1 comparison with either A2revAIHV-1 LGLs or wtAIHV-1 LGLs. *** $P = 0.004$ for A2 AIHV-1 comparison with either A2revAIHV-1 LGLs or wtAIHV-1 LGLs.

Table 1

Primer and probe sequences for rabbit and virus gene transcript detection by qRT-PCR.

Gene	Fwd sequence	Rev sequence	Probe sequence
β -actin	ccagatcatgttcgagacgtt	cagaggcgtacaggacag	
HPRT	gctttccttggtcaagcagt	cacttcgaggggtccttttc	
SDHA	accgtgaagggtctgact	tttctagctcgaccacagagg	
A2H1galKF	cccttaatacatattcctggcactattcacattcaagtacttt cttttttcagttcctgttgacaattaatcatcggca	gtctctcaaaatattagctgtgtagctgctatagt cactcttacaatcctcaaaatcagcactgtcct gtcctt	
A2H1-TTA	cccttaatacatattcctggcactattcacattcaagta ctttcttttttcagttttataaattgtacaagttgttcc		
A2H2-ATG		gtctctcaaaatattagctgtgtagctgctatagt cactcttacaatcctcaaaatgtcccaaaa ctcaaatcttg	
A2	tgctttggatgatcttttatcaat	ccccacaaaacacacacctta	
NFkB1	atcccaatggggaaggctct	acggcaggctattgctca	
Fas	acagctatgaccgggactcg	cagtgagccagcaatacagag	
FasL	gcctctgcctcctctgatg	gaaggactctctgagctcgg	
FoxP3	ctctgcaccttccaagc	cacttgacacgccattt	
Gran-A	gtgtgtaagaatcattggagg	tctgctcatgtcacagt	actcctcattcaagacctacatgg
Caspase	gatgtcctcaaggcgaagat	cccagcttccttaagtctca	
Cathepsin	catcacactgaatgacgagga	ctcgaaggcgaagctcac	
Perforin	tgctgctgttacagttttc	cgtggagggtgtgaaatc	ccgctgcacctgacttcag
Gran-B	gaagctcaataaatgtcacctg	gggatggctcttctcacg	
IL-2	agtgtctagaagaagaactcaaac	agatcccttttagtttcagaactgt	
NKG2D	tgctcatctctcaatgccag	cacacaaatccatagccagt	cggcatctaccgtaaggcagctg
NFATC2	cagcccccaggaagagtt	ggttcttctcgatgggattc	
IFN- γ	tggaaagaggagagtgaaca	tactcttcttgatgacctcatggt	
NCR1	caccagaagcaggcact	cagaaagcagctggtactctc	
LAIR1	cagtaccgctgcatctccta	ggacgtcctcagggatca	

Housekeeping gene controls: β -actin, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and succinate dehydrogenase, subunit A (SDHA). The remainder are host genes and are described in Tables 3A and 3B. Universal ProbeLibrary (UPL) single probes were utilised from Roche Applied Science.

Table 2

LGL cells developed in culture.

LGL ID	Phenotype ^a	Log Viral DNA load ^b	Log viral DNA load ^c
WT: YU3	CD8 (69%); CD4 (5%)	3.86	4.62
WT: PR2	CD8 (60%); CD4 (7%)	4.73	3.83
WT: XX1	CD8 (59%); CD4 (4%)	3.41	3.01
WT: 281.4*	CD8 (60%); CD4 (<2%)	5.3	3.10
WT: 635.5*	CD8 (73%); CD4 (2%)	5.80	5.88
REV: RF6	CD8 (47%); CD4 (10.8%)	5.57	5.78
REV: 318.7*	CD8 (46%); CD4 (<2%)	5.95	6.06
REV: 320.9*	CD8 (96%); CD4 (<2%)	4.00	<i>d</i>
DEL: YF8	CD8 (68%); CD4 (7%)	3.47	3.82
DEL: YJ7	CD8 (64%); CD4 (8%)	5.41	4.82
DEL: YM9	CD8 (85%); CD4 (2.5%)	5.08	4.98
DEL: 244.2*	CD8 (94%); CD4 (<2%)	5.50	5.67
DEL: 675.3*	CD4 (44%); CD8 (4%)	4.94	4.65

^aPhenotype of LGLs by FACs analysis using rabbit alloantigen-specific antibodies. B cells were <1% in all LGLs analysed.

^bViral DNA load (copies of AIHV-1 DNA/10⁵ β-globulin DNA copies) at establishment of the LGL line with a stable phenotype.

^cViral DNA load within 1–2 weeks of use in cytotoxicity and qRT-PCR assays.

^dnot included for qRT-PCR or cytotoxicity assays as the log viral load of the LGLs had declined to <2.5.

* These LGLs used in the RNAseq analysis.

Table 3A

Transcripts up-regulated in A2 AIHV-1LGLs compared to A2revAIHV-1LGLs and wtAIHV-1 LGLs.

Gene ID/description	Fold change
T cell/lymphoid receptors	
T cell receptor alpha variable 5.	294.7
CD5. Receptor possibly regulating T cell proliferation.	65.5
CD28. Co-stimulatory signal for T cells. Receptors are CD80 and CD86.	26.8
CD200. Ig superfamily. Inhibitory signal for macrophage lineage.	22.9
CD6. The gene product is important for continuation of T cell activation.	20.5
T cell receptor alpha constant.	5.0
CD2. On T cells and NK cells.	3.3
Cytokines/chemokines	
CXCL10. Chemokine (C-X-C motif) ligand 10. Interferon-gamma-inducible.	24.4
Chemokine (C-C motif) ligand 3-like 1.	13.9
TIFA. TRAF-interacting protein with forkhead-associated domain.	5.2
SOCS3. Suppressor of cytokine signalling.	4.1
Transcription and signalling	
TIAM2. T-cell lymphoma invasion and metastasis 2.	33.8
ZEB2. Zinc finger E-box binding homeobox 2. Transcriptional inhibitor.	6.6
INPP1. Inositol polyphosphate-1-phosphatase.	5.6
TFDP2. Transcription factor Dp-2 (E2F dimerization partner 2).	4.4
NFKB1. Transcription factor.	2.9
Cell cycle and apoptosis	
PDCD1. Programmed cell death 1.	46.7
DENND2D. DENN/MADD domain containing 2D.	12.9
UBASH3A. Ubiquitin associated and SH3 domain containing A.	11.0
LYRM7. Lyr motif containing protein.	6.3
BCL11B. B-cell CLL/lymphoma 11B (zinc finger protein).	5.2
S10AA RABIT. S100 calcium binding protein A10.	4.9
Caspase 8. Apoptosis.	2.0
Cyclin-dependent kinase 2. Cell cycle G1/S phase transition.	1.9
Miscellaneous	
WDR27. WD repeat domain 27.	55.6
CRYBG3. Beta-gamma crystallin domain containing 3.	14.2
TATA box binding protein (TBP)-associated factor, RNA polymerase I.	11.8
C18orf1. Chromosome 18 open reading frame 1.	11.3
BAMBI. BMP and activin membrane-bound inhibitor homolog.	10.2
SLC46A3. Solute carrier family 46, member 3.	7.6
CYP4V2. Cytochrome P450, family 4, subfamily V, polypeptide 2.	7.2
ST6GAL1. ST6 beta-galactosamide alpha-2,6-sialyltransferase 1.	6.7
Growth hormone inducible transmembrane protein.	6.3
FAM115C. Family with sequence similarity 115, member C.	6.0

Gene ID/description	Fold change
GGH. Gamma-glutamyl hydrolase.	5.9
RNF144A. Ring finger protein 144A.	4.6
Dihydrofolate reductase-like 1. Thymidylate biosynthesis pathway.	3.5

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Table 3BTranscripts downregulated in A2 AIHV-1 LGLs *versus* A2revAIHV-1LGLs and wtAIHV-1 LGLs.

Gene ID/description	Fold change
T Cell/NK cell/other cell receptors	
CD163. Acute phase-regulated receptor.	-328
GPR97. G protein-coupled receptor 97. Possibly an orphan receptor	-301.4
T cell receptor delta variable 3.	-267.5
NKG2D. Killer cell lectin-like receptor. T and NK cell cytotoxicity	-149.0
LAIR-1. Leucocyte-associated immunoglobulin-like receptor 1.	-140.6
T cell receptor delta constant.	-127.6
LGR4. Leucine-rich repeat containing G protein-coupled receptor 4.	-92.0
vKIT/cKIT. Stem cell factor (kit-ligand) receptor. Haematopoiesis.	-82.5
NTRK2. Neurotrophic tyrosine kinase, receptor, type 2	-77.1
T cell receptor gamma variable 10.	-58.2
UNC5C. Unc-5 homolog C. Netrin receptor.	-57.2
Natural cytotoxicity triggering receptor 1.	-53.2
CD63. Mediates signal transduction events, complexes with integrins.	-52.4
GPR125. Orphan receptor.	-49.5
CD200 receptor-1. Myeloid down-regulatory signal.	-38.2
CD22. Mediates B-cell-B-cell interactions.	-30.0
GPR56. Tumour inhibition, neuron development, cytotoxic T cells.	-26.4
T cell receptor gamma variable 3 (three transcript IDs).	-13/-21/-22.3
GPR65. Receptor for glycosphingolipid psychosine (PSY).	-11.7
KLRG1. Killer cell lectin-like receptor subfamily G member 1.	-7.5
GPR4. Proton-sensing receptor coupled to several G-proteins.	-3.9
Cytokines and their receptors	
IL-1 alpha. Pro-inflammatory cytokine.	-115.0
FAM20A. Family with sequence similarity 20, member A.	-98.1
IGFBP2. Insulin-like growth factor binding protein 2.	-31.2
IL1R1. IL-1 receptor type 1.	-29.9
CCR8. Receptor for the chemokine CCL1/SCYA1/I-309.	-28.8
EBF1. Early B cell factor 1.	-23.9
IRAK3. Interleukin-1 receptor-associated kinase 3.	-20.2
CLCF1. Cardiotrophin-like cytokine factor 1. Gp130 cytokine family.	-12.1
Interleukin 18 (interferon-gamma-inducing factor).	-11.2
Transcription and signalling	
LRRN1. Leucine rich repeat neuronal 1.	-126.7
LDB2. LIM domain binding 2. Adaptor protein.	-63.7
DNM1. Dynamin 1 GTPase involved in endocytosis, cytokinesis.	-53.3
RRAGD. Ras-related GTP binding D.	-50.6
FOXP3. Transcription factor in regulatory T cells.	-43.7
ZNF608. Zinc finger protein 608.	-42.1

Gene ID/description	Fold change
ARAP3. ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3.	-38.1
PPEF1. Serine/threonine protein phosphatase.	-38.0
SEC14-like 2. May have transcriptional activation activity.	-32.8
SPRY2. Sprouty homolog 2 (Drosophila).	-27.4
JAG1. Jagged 1. Signalling through notch 1.	-26.9
SHISA2. Shisa homolog 2. ER membrane.	-24.3
CAPN5. Homologue of FosB that interacts with Jun proteins.	-23.8
ANXA3. Annexin A3. Regulation of cellular growth and signal transduction.	-18.7
BCAR3. Breast cancer anti-oestrogen resistance 3.	-11.4
FOSB. Interacts with Jun proteins enhancing their DNA binding activity.	-9.1
Cytoskeletal, metabolism, miscellaneous	
Beta casein.	-494.0
Alcohol dehydrogenase 4 (class II), pi polypeptide.	-253.8
CHST4. Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4.	-241.9
CDH17. Cadherin 17, LI cadherin.	-222.8
Casein alpha s1.	-184.0
Clusterin. Secreted chaperone also in the cell cytosol.	-175.6
MFG8. Milk fat globule-EGF factor 8 protein.	-123.0
CNTN1. Cytoskeleton, cell motility, activation, proliferation, contact.	-102.5
B7N2P8 RABIT. Transketolase-like 1. Mediates glycolysis and biosynthesis.	-91.2
Acyloxyacyl hydrolase. Removes secondary acyl chains.	-88.5
ITGAX. Integrin, alpha X (complement component 3 receptor 4 subunit).	-83.1
ATP8B1. ATPase, aminophospholipid transporter, class I, type 8B, member 1	-80.3
ANK1. Ankyrin 1.	-73.4
LEPR. Leptin receptor. Regulates fatty tissue mass.	-72.8
POPDC3. Popeye domain containing 3.	-66.3
DSC2. Desmocollin 2. Subfamily of the cadherin superfamily.	-61.1
A5HC47 RABIT. Cathepsin H.	-60.2
CADPS. Ca ⁺⁺ -dependent secretion activator.	-59.5
CHST15. CHO(N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase.	-51.4
KRT84. Keratin 84.	-47.7
WDFY3. WD repeat and FYVE domain containing 3.	-28
PMP22. Peripheral myelin protein 22. Integral membrane protein.	-25.9
KLK11. Kallikrein-related peptidase 11. Serine protease.	-20.3
WBP5. WW domain binding protein 5.	-17.7
GFOD1. Glucose-fructose oxidoreductase domain containing 1.	-16.3
STC2. Stanniocalcin 2.	-15.1
Q2N0M8 RABIT. Kv channel interacting protein 2.	-14.8

Table 4

Pathways affected by A2 gene deletion in the LGLs.

Immune response (principal genes affected):*Downregulated in A2 LGLs versus wt or revA2 LGLs:*IFN- γ signalling pathway (IFN- γ ↓; SOCS3 ↑ CLCF-1 ↓ IL-18 ↓)T cell receptor signalling pathway (γ δTCR↓)

NFAT in the immune response (NFAT ↓)

IL-2 activation and signalling (IL-2 ↓; IL-2R α ↓, IL-2R β and γ ↓)IL-15 signalling (IL-2R β and γ ↓)IL-1 signalling pathway (IL-1 α ↓; IL-1R1 ↓; IRAK3 ↓)

NKG2D signalling pathway (NKG2D ↓)

Th1 Th2 differentiation (IFN- γ ↓)*Up-regulated in A2 LGLs versus wt or revA2 LGLs:*TCR signalling (α βTCR↑)

CD28 signalling (CD28↑)

Transcription/signal transduction/cell cycle (principal genes affected):*Downregulated in A2 LGLs versus wt or revA2 LGLs:*

GPCR signalling (GPR65 ↓; GPR4 ↓; GPR56 ↓)

Up-regulated in A2 LGLs versus wt or revA2 LGLs:

Regulation of G1/S transition (CDK-2- ↑; S10AA_RABIT ↑)

JNK pathway signalling (FOSB ↑; CAPN5 ↑)

Apoptosis/cytotoxicity*Downregulated in A2 LGLs versus wt or revA2 LGLs:*

Granzyme A and granzyme B signalling (Granzyme A and B ↓; NKG2D ↓; NCTR1 ↓; GPR56 ↓)

Up-regulated in A2 LGLs versus wt or revA2 LGLs:

Apoptosis regulation by mitochondrial proteins and p53 pathway (PCD-1 ↑; DENND2D ↑; UBASH3A ↑; BCL11B ↑)

OthersCytoskeleton remodelling (ANK-1 ↑; CNTN-1 ↑ DSC-2 ↑; PMP-22 ↑; ARAP-3 ↑) (*upregulated*)

Metacore analysis. Genes that were statistically up or down regulated in A2 LGLs versus wt or revA2 LGLs are shown in parentheses. For definitions of these refer to Tables 3A and 3B. Arrows indicate whether a gene transcript is up or down regulated in A2 LGLs versus wtAIHV-1 LGLs or revA2AIHV-1 LGLs.