Chicken anaemia virus evades host immune responses in transformed lymphocytes

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Abstract

Chicken anaemia virus (CAV) is a lymphotropic virus that causes anaemia and immunosuppression in chickens. Previously, we proposed that CAV evades host antiviral responses in vivo by disrupting T-cell signalling, but the precise cellular targets and modes of action remain elusive. In this study, we examined gene expression in Marek’s disease virus-transformed chicken T-cell line MSB-1 after infection with CAV using both a custom 5K immune-focused microarray and quantitative real-time PCR at 24, 48 and 72 h post-infection. The data demonstrate an intricate equilibrium between CAV and the host gene expression, displaying subtle but significant modulation of transcripts involved in the T-cell, inflammation and NF-κB signalling cascades. CAV efficiently blocked the induction of type-I interferons and interferon-stimulated genes at 72 h. The cell expression pattern implies that CAV subverts host antiviral responses and that the transformed environment of MSB-1 cells offers an opportunistic advantage for virus growth.

Chicken anaemia virus (CAV) is a small, non-enveloped DNA virus that contains a single-stranded circular genome of 2.3 kb and causes significant economic losses in the production of young birds worldwide. In older birds CAV causes a subclinical disease that compromises vaccination and aggravates secondary coexisting infections, especially with Marek’s disease virus [1, 2]. The CAV genome encodes three partially overlapping open reading frames that are translated to produce the structural VP1 protein (52 kDa), the putative immunomodulatory VP2 protein (24 kDa) and VP3 (13 kDa), which induces apoptosis in infected cells. VP3 or apoptin also induces tumour-selective apoptosis in a range of human cancer cells and is a potential anti-cancer therapeutic agent [3].

CAV targets erythroid and lymphoid progenitor cells in the bone marrow and thymus, respectively, while B-cells appear unaffected by the virus [4, 5]. The mechanisms that the virus employs to trigger apoptosis, evade host anti-viral processes and exploit the host cellular resources have not been elucidated. We previously reported that in 2-week-old chickens at 14 days post-infection (p.i.) with CAV, pro-apoptotic genes such as the Bcl-2 family member Bcl2-antagonist/killer 1 (BAK1) are upregulated in thymus cells, which is consistent with the extensive apoptosis of thymus cells that is observed macroscopically following viral infection [6]. We also observed a significant virus-induced modulation of the expression of genes involved in TCR signalling. A virus-induced dysregulation of TCR signalling may provide a strategic mechanism for virus escape from the host immune system.

Received 5 October 2017; Accepted 8 January 2018

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Keywords: Chicken anaemia virus; T-lymphocytes; immune evasion; microarray.

Abbreviations: ANOVA, analysis of variance; Bcl, B-cell lymphoma; BIRC5, baculoviral (inhibitor of apoptosis domain) repeat containing 5; BLAST, Basic Local Alignment Search Tool; CARD, caspase recruitment domain family; CCT4, chaperonin-containing T-complex polypeptide subunit 4; CD, cluster of differentiation; CXCL, chemokine (C-X-C motif) ligand; DEDD, death effector domain; DIDD1, death inducer obliterator 1; HSP, heat-shock protein; ICP, infected-cell polypeptide; IFI6, interferon alpha-inducible protein 6; IFIT5, interferon-induced protein with tetratricopeptide repeats 5; IFN, interferon; IκB, inhibitor of nuclear factor kappa-B; IL, interleukin; JAK, Janus kinase; K6, kDa, kilodaltons; MAPK, mitogen-activated protein kinase; meq, Marek’s virus EcorR-S-encoded protein; MIF, macrophage migration inhibitory factor; MxA, Myxovirus (influenza virus) resistance 1; NF-κB, nuclear factor kappa-light chain enhancer of activated B cells; OASL, 2′-5′-oligoadenylate synthetase Like; pp38, phosphorylated 38 kDa phosphoprotein; SATB1, special AT-rich sequence-binding protein 1; SLA, Src-like adaptor; STAT, signal transduction and activator of transcription; TGF, transforming growth factor; Th, helper T-cell; Treg, regulatory T-cell; VP, viral protein.

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The original microarray data produced in this study have been deposited in the public database ArrayExpress, with the accession number: E-MEXP-1229.

†Deceased

Supplementary material is available with the online version of this article.
However, given the complexity of genetic and epigenetic alterations in *in vivo* conditions, which can mask important cell signalling events, an *in vitro* system may be more appropriate to systematically dissect the mechanisms involved in viral pathogenesis. CAV can grow in chick embryos and a limited number of transformed B- and T-lymphoblastoid cell lines [7–10]. The most commonly used cell line is the immortalized Marek’s disease virus (MDV)-transformed chicken lymphoblastoid cell line MDCC-MSB-1 (MSB-1), which consists of MDV virus-transformed chicken lymphocytes [including mature helper T-lymphocytes (CD3+, CD4+, CD8+, TCR2+)] derived from an MDV T-cell splenic lymphoma. MDV is another economically important poultry disease and is caused by an avian α-herpesvirus that frequently coexists with CAV in infected chicken flocks [11, 12]. Crowley et al. used a 20K-oligo microarray chip (ARK Genomics, Roslin Institute, UK, now Edinburgh Genomics) and showed that CAV infection of MSB-1 cells for 24 and 48 h induced transcripts involved in inflammation, apoptosis and antiviral activity [13]. Due to the rudimentary annotation of early chicken genome assemblies (2.1 genome assembly), this earlier study identified many transcripts that lacked annotation [13]. Our study aimed to confirm and extend this study by assessing CAV-induced host gene expression using a more extended time frame of infection, a lower multiplicity of infection (m.o.i.) and an in-house immune-focused microarray chip. This chip was developed by Edinburgh Genomics (Roslin Institute) and had a better coverage of immune-related genes than the other existing chicken microarray chips [14]. The data from the study were reannotated with the latest chicken genome assembly (Gallus_gallus-5.0; GCA_000002315.3). Employing an immune-focused array can circumvent the problems typically associated with universal arrays, such as the extensive amounts of biological information produced or the elimination of relevant data through multiple-testing correction to significance levels.

MSB-1 cells grow in suspension culture and require subculturing at intervals of around 3 days and are resistant to infection by most other avian viruses except retroviruses [15, 16]. The level of cell-associated infectivity reaches a maximum between 36 and 48 h after infection and coincides with the period when the maximal levels of viral antigen can be detected by immunofluorescence [17]. We have found that cell-free infectivity peaks between 72 and 84 h p.i. (data not shown), in agreement with previous studies [17]. We conducted a time-course investigation (at 24, 48 and 72 h p.i.) of the host gene expression profile in CAV-infected MSB-1 cells using both a 5K immune-focused microarray cDNA array [14] and quantitative RT-PCR (qRT-PCR). MSB-1 cells were grown and infected with the Cux-1 CAV strain at an m.o.i. of 1 as previously described [15]. Six replicate samples from Cux-1-infected and mock-infected MSB-1 cells were collected at each time point and total RNA was extracted. Total RNA was isolated, evaluated and divided for microarray analyses and qRT-PCR, using previously described procedures and qRT-PCR primers [6]. Samples were processed and hybridized on the immune-focused cDNA microarray representing 5026 chicken genes (the study’s data have been deposited in ArrayExpress; accession: E-MEXP-1229). Genes with a false discovery rate (FDR) of more than 5% in either set of data were excluded from further analyses. The cut-off fold gene expression change was set at ≥±1.5. Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA) was used to associate differentially expressed genes with biological pathways and functional gene networks, matrix2png v1.2.1 was used for data clustering and R v3.3.3 was used for the multidimensional scaling (MDS) of microarray samples. Analyses of the differences between transcripts from mock and infected cells found with qRT-PCR were carried out using one-way ANOVA and a Bonferroni multiple comparisons test, with *P* < 0.05 considered to be significant. For both microarrays and qRT-PCR assays a reference design was used where each experimental sample was compared against a common reference sample, which was obtained by pooling all samples at each time point. This enabled samples to be directly compared with each other.

Prior to differential gene expression analysis, the data from the microarray probes were used for MDS, which displays sample variability in two-dimensional space (Fig. 1a). MDS analysis demonstrated that samples were differentiated by time point along coordinate 1, while coordinate 2 separated samples by treatment status (i.e. infected versus mock samples). Samples from infected (24 h) and mock-infected cells (24 and 48 h), and infected cells at 48 and 72 h were clustered together in two groups, while the mock-infected cells at 72 h were distinct from the rest of the samples, possibly due to the extended culture duration. Using a stringent set of criteria, we found a total of 385 transcripts with FDR < 0.05 that were differentially expressed (DE) in CAV-infected cells compared with mock-infected cells at all time points (Fig. 1b and Supplementary Material). The most noticeable changes in host gene expression occurred between 48 and 72 h p.i. (Fig. 1b). More transcripts (156) were downregulated than were upregulated (119) at 72 h p.i. and only 18 transcripts overlapped between 48 and 72 h (Fig. 1c and Supplementary Material). Overall there were different expression trends at different time points, implying distinct cascades of host gene response.

DE transcripts were updated in annotation terms and gene IDs using probe sequence information and BLAST analysis. The list of updated gene IDs and the expression data were then processed into Ingenuity pathway software and analysed for known ‘canonical’ pathways and biological functions (full analysis is presented in Supplementary Material). Analysis identified diverse biological process categories at 48 h, with the more abundant categories including cell-to-cell signalling and interaction, tissue development and morphology, immune response and molecular transport, possibly relating to the virus’s need for the host machinery to replicate itself. The most enriched pathways were involved in T-cell signalling. At 72 h the most abundant biological function categories were cell death, cell growth and proliferation, and immunological disease, while the most affected pathways were purine
and pyrimidine metabolism, integrin and glucocorticoid receptor signalling, and T-cell signalling.

In the heatmap rendering of T-cell receptor signalling, inflammation and cytokine signalling pathways in mock- and CAV-infected cells (Fig. 2a) a complex gene expression pattern is shown, suggestive of deregulated T-cell, proinflammatory and innate immune responses. The activation of mammalian T-cells involves a complex cascade of multiple signal transduction pathways that is initiated at the membrane T-cell receptors (TCR) and results in the transcription of multiple genes within the nucleus. Infection studies in chicken T-cells are compromised by a lack of understanding of the chicken T-cell activation/signalling circuits and further impaired by the partial understanding of the overall chicken immune system, including the type I IFN response, which is the first line of host defence upon virus infection. Downregulation of genes involved in TCR signalling, such as those for TCRα, TCRβ and CD3ε, and upregulation of the genes for CD80 and CD83 and the negative TCR signalling regulator, SLA (Fig. 2a), corroborate the CAV-induced T-cell dysfunction that we described in vivo [6] and were not reported by Crowley et al. [13].

At 24 h p.i. a fatty acid desaturase (FADS6) was upregulated and protein kinase delta 1 (PRKCD) was downregulated. PRKCD plays a critical role in the control of growth, differentiation and apoptosis [18]. At 48 and particularly 72 h p.i., the pro-inflammatory cytokine MIF, the MAPK signalling components MAP3K3 and CXXC5, the pro-inflammatory ligand receptor, IFNγ-R2, STAT3 and NF-κB1 were upregulated, showing late activation of the type II IFN response, the inflammation response and NF-κB signalling cascades (Fig. 2a, b). A wide range of viruses have been reported to activate the NF-κB pathway to promote virus replication, as NF-κB regulates numerous target genes involved in the host cell cycle and immune responses [19]. Interpretation of CAV infection data is complicated by the presence of integrated and circular copies of the MDV-1 genome [20]. MDV’s ICP4, pp38 and meq genes are involved in the maintenance of the transformation of MSB-1 cells [21]. MDV miRNAs in MSB-1 cells target the mRNA of IL-18, a proinflammatory cytokine that stimulates IFN-γ production in T-cells [22]. Furthermore, NF-κB is central in MDV neoplastic transformation and the MDV Meq oncoprotein may augment NF-κB transcription by targeting the IKK complex [23]. The observed induction of the type II IFN response and the NF-κB complex in CAV-infected MSB-1 cells at 72 h (Fig. 2b) cannot be attributed to MDV, as normalized comparison between infected and mock samples should have negated any effect of MDV. However,
whether aberrant signalling by MDV proteins (undetectable by microarrays) can exacerbate the induction of NF-κB in the samples infected with CAV could not be determined in this study.

To identify inherent gene expression trends in the cells that may conceal or intensify CAV-induced changes, such as an effect of the number of copies of MDV, we also compared the basal gene expression of mock MSB-1 cells across the time course (Fig. 2c and full dataset in the Supplementary Material). We did not observe DE genes involved in the NF-κB complex, but instead we identified a time-dependent upregulation (especially at 72 h) of proliferation- and apoptosis-related TGF-β1 (consistent with the transformed phenotype of MSB-1 cells) and downregulation of transcripts encoding chaperonin proteins (CCT4, HSPA8 and HSP90B1), which ensure correct protein folding and prevent apoptosis [24]. CAV VP3 requires a transformed environment to induce apoptosis [25], and the transformed phenotype of MSB-1 cells may offer an opportunistic advantage to VP3 and to CAV infection. Induction of pro-apoptotic genes (DEDD, DIDO1, CARD11) at 48 h and anti-apoptotic genes (BIRC5 or inhibitor of apoptosis 5) at 72 h in CAV-infected cells compared to mock-infected (Fig. 2a) confirm that apoptosis is an integral part of the pathogenesis of the virus. Viruses have been reported to either induce or block apoptosis or to do both at different stages during infection [26]. There is evidence that apoptosis is a prerequisite for CAV replication and facilitates virus escape from the cell [27, 28]. Nevertheless, it cannot be ruled out that the apoptosis seen is also part of a host defence mechanism to prevent CAV spread by early cell death [26].

Combining IPA functional network analysis with differential gene expression analysis (see the Supplementary Material) helped uncover high-confidence genes, such as SATB1, a pleiotropic genome organizer, which was downregulated
at 72 h, and SLA, which inhibits TCR signalling [29] and was upregulated at 72 h p.i. Both genes were found in our in vivo studies to be downregulated by CAV infection and were not found to be differentially regulated by Crowley et al. [13]. MSB-1 cells are distinct both functionally and phenotypically from lymphocytes in vivo and the virus-specific modulation of these genes both in vivo and in vitro implicates their involvement in a viral mechanism for the suppression of host antiviral responses, possibly by modulating host TCR signalling, although this requires experimental confirmation.

Validation of gene expression results by qRT-PCR demonstrated a high level of agreement in the measurement of the expression of five randomly selected genes (SLA, TGF-β1, SATB1, IFN-β and STAT3) with both technologies (Pearson’s correlation coefficient r=0.987 and P=0.0018, Fig. 2d). For further validation of the microarray results and for consistency with our previous in vivo study [6] we used qRT-PCR to examine the expression levels of transcripts for the signature pro-inflammatory [IL-1β, IL-2, IL-6, IL18 and CXCLi2 (CAF/interleukin-8)], type I IFN (IFN-α and IFN-β), Th1 (IL-3, IL-12α, IL-12β and IFN-γ), Th2 (IL-4 and IL-13) and Treg (IL-10 and TGF-β4) cytokines. Only transcripts that showed significant differential expression are presented in Fig. 3. The results demonstrated subtle overall changes in the transcription of cytokines. At 24 h p.i. the expression of IFN-α, TGF-β4 and IL-13 was moderately induced, while at 48 h we noticed a small but significant induction of IFN-α, IFN-β, TGF-β4 and IL-3, followed by downregulation of these transcripts at 72 h. At 72 h only the expression of IL-1β was increased, while that of other cytokines was either unchanged (IL-6) or downregulated (IL-4, IL-10, IL-13 and IL-18). The induction of IFN-α and IFN-β at 24 and 48 h is consistent with the observations of Crowley et al. [13]. However, there is a discrepancy between their study, which reported that IL-2, IFN-γ, IL-12α and IL-12β were DE, and our study, which did not detect any regulation in the expression of these genes. In chickens, as in mammals, interferons are key modulators of immune response; they are induced following viral infection and initiate a signalling cascade through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway, leading to the transcriptional regulation of hundreds of IFN-regulated genes (IRGs), which induce an antiviral state in the cells [30–32]. However, analysis of our microarray results showed that host induction of IFN-α at 24 h and IFN-α and IFN-β at 48 h by CAV was not accompanied by differential expression of canonical chicken IRGs, such as Mx1, IFIT5, IFI6 and OASL at either 48 or 72 h. This, together with the downregulation of IFN-α and IFN-β at 72 h, suggest that both IFN induction and signalling cascades are efficiently blocked by the virus between 48–72 h. Future studies should aim to identify the mediators that CAV uses to disarm the host type-I IFN response.

![Fig. 3.](image) qRT-PCR analysis of RNA samples from CAV-infected cells at 24, 48 and 72 h p.i. with a cytokine panel reveals modest transcription changes of immune-related genes. Extracted total RNA of CAV-infected and mock-infected MSB-1 cells at each time point was subjected to reverse transcription followed by quantitative PCR using specific primer sets for 10 cytokines normalized against GAPDH (using the ΔΔCt method). The data are representative from three independent experiments. One-way ANOVA with Bonferroni’s post hoc test was used to analyse the data. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001. Broken lines represent fold change=1.
In this study, we used both qRT-PCR and an immune-focused microarray to screen host responses over 72 h of CAV infection in MSB-1 cells. While overall there was agreement with the gene expression trends described by Crowley et al. [13], there were also many differences. These may have been due to the lower m.o.i. used in our study, and the more up-to-date chicken genome annotation (Gallus gallus 5.0 assembly) that helped us identify new transcripts that may be involved in the pathogenicity of CAV. Crowley et al. reported that CAV infection of MSB-1 cells resulted in an initial wave of inflammatory, anti-apoptotic and anti-viral gene expression changes at 24 h, followed by alterations in genes associated with immunosuppression at 48 h. Instead, our study showed minimal immune response at 24 h, a moderate antiviral response at 48 h and an increased regulation of NF-κB- and apoptosis-related transcripts at 72 h. Our results should prove helpful in elucidating the pathogenic mechanisms of CAV and for future work on CAV in MSB-1 cells i.e. RNA interference studies targeting CAV viral genes.

Funding information
This research was undertaken with financial support from the Biotechnology and Biological Sciences Research Council (http://www.bbsrc.ac.uk) (grant numbers BBS/B/00182, BBS/B/00360 and BBS/B/0028X. L.R., D.W.B., E.J.G. and P.K. were also supported by BBSRC Institute Strategic Programme grants (ISPR3 In innate Immunity and Endemic Disease) (BB/J004227/1) and (ISPR1 Analysis and Prediction in Complex Animal Systems) (BB/J004235/1). E.S.G. is currently supported by the BBSRC via grant BB/K002465/1 [Developing Rapid Responses to Emerging Infections of Poultry (DODREVPI)]. E.J.G. is currently supported by BBSRC Strategic Programmes (ISP1 Blueprints for Healthy Animals (BB/P013732/1) and ISP2 Control of Infectious Diseases (BB/P013740/1)].

Acknowledgements
Our thanks go to Kirsty Jensen for her technical assistance and advice, and to Dave Waddington, who helped extensively with the data analysis.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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