Screening of differentially expressed transcripts in infectious bursal disease virus-induced apoptotic chicken embryonic fibroblasts by using cDNA microarrays

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Infectious bursal disease virus (IBDV) induces apoptosis and immunosuppression. To understand the molecular mechanisms involved in the pathogenesis of infectious bursal disease (IBD) and the host-directed antiviral responses, cDNA microarrays were used to identify the differentially expressed transcripts in IBDV-infected chicken embryonic fibroblasts. The results suggest a general suppression of surface receptors, including CD40 ligand and SEMA4D. These are related to T- and B-cell activation and differentiation, which may contribute to the immunosuppression of IBD. In addition, activation of genes involved in Toll-like receptor- and interferon (IFN)-mediated antiviral responses was detected. In particular, upregulation of Toll-like receptor 3, a double-stranded (ds) RNA receptor, and MX1, an IFN-inducible antiviral GTPase, may represent the possible host-directed defence responses against the virus and its dsRNA genome. Interestingly, several lines of evidence suggest the modulation of G protein-coupled receptors and receptor tyrosine kinase signalling pathways, especially the possible transactivation of epidermal growth factor receptor by lysophosphatidic acid. Alteration of these may contribute to the previously reported activation of mitogen-activated protein kinases upon IBDV infection, resulting in macrophage activation and inflammatory responses. Additionally, numerous target genes and inducers of nuclear factor kappa B (NF-κB) were upregulated profoundly, implying that IBDV may modulate host-cell survival and apoptosis to support its replication and facilitate viral spread through NF-κB activation. In summary, this investigation of host-gene expression unravelled the candidate physiological pathways involved in host–virus interaction on a molecular level, providing a foundation for researchers to design experiments based on testable hypotheses targeting individual genes.

INTRODUCTION

Infectious bursal disease (IBD) is an immunosuppressive disease in young chickens, caused by a bisegmented, double-stranded (ds) RNA virus of the family *Birnaviridae*, *Infectious bursal disease virus* (IBDV) (Dobos *et al.*, 1979). Its pathogenesis is characterized by severe necrosis and lymphoid depletion in the bursa (Burkhardt & Muller, 1987), although marked atrophy without severe inflammation was also reported (Rosenberger *et al.*, 1985). Previous studies demonstrated that infection of IBDV induces apoptosis in peripheral blood lymphocytes (Vasconcelos & Lam, 1994), bursa (Vasconcelos & Lam, 1995) and thymus (Tanimura & Sharma, 1998). Moreover, *in vitro*

apoptosis was also reported in infected tissue cultures (Tham & Moon, 1996) and cell lines overexpressing its major capsid protein VP2 or non-structural protein VP5 (Fernandez-Arias *et al.*, 1997; Yao *et al.*, 1998). These results suggest a possible role for apoptosis in the pathogenesis of IBD. Recent studies showed that reduction of IgM$^+$ cells in the bursa may be due to dramatic infiltration of T cells (Tanimura & Sharma, 1998). T cells may also promote the production of inflammatory factors that accentuate tissue destruction. Nitric oxide, produced by macrophages activated by T cell-derived cytokines such as gamma interferon (IFN-γ), can promote cellular destruction in both virus-infected and virus-free cells (Yeh *et al.*, 2002).

Interactions between viruses and host cells include a complex interplay of molecular pathways directed by the host to prevent virus replication, and countermeasures by the

A table showing transcripts with at least threefold up- or downregulation and a figure showing a summary of the possible pathways involved in NF-κB activation and apoptosis in IBDV-infected cells are available as supplementary material with the online version of this paper.

virus to favour its propagation. Characterization of the gene-expression profiles of virus-infected host cells provides an efficient means to understand the molecular basis of viral disease pathogenesis, e.g. nuclear factor kappa B (NF-κB)-induced apoptosis in reovirus infection (O'Donnell et al., 2006). To understand the molecular mechanisms involved in IBDV-induced pathogenesis, the gene-expression profile of IBDV-infected chicken embryonic fibroblasts (CEFs) was investigated by using cDNA microarrays.

METHODS

Virus culture and purification. IBDV vaccine strain D78 was purchased and propagated in secondary CEFs according to the manufacturer's instructions (VR-2041; ATCC). The virus was purified by using CsCl gradient purification and desalted by using dialysis tubing as described by Xue & Lim (2001). The collected virus was further purified by centrifugation through a 20 % sucrose cushion at 35 000 r.p.m. in an SW55 rotor (Beckman) at 4 °C for 4 h. The purified virus was resuspended in TNE [10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA] and stored at −20 °C for future use. The titre of the purified virus was determined by using a plaque assay as described by Muller et al. (1986).

Cell-culture maintenance, infection and sampling. Secondary CEF cultures were used in all of the following experiments and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen) in 5 % CO_2 at 37 °C. All secondary CEF cultures were seeded at 1×10^5 cells in 100 mm plates, and seven plates were prepared for each sampling time point of both the mock- and IBDV-infected groups. At 24 h after seeding, the culture medium in each plate was removed and the cells were infected with purified virus at an m.o.i. of 10 in 1.5 ml DMEM, whereas the mock-infected group was treated with an equal volume of TNE. At 2 h post-infection (p.i.), each plate was replenished with 8 ml DMEM supplemented with 1 % FBS. The progression of apoptosis, cell viability and viral growth was monitored from 0 to 96 h p.i. at intervals of 12 h. For the Annexin V–phycoerythrin (PE) apoptosis detection assay and Hoechst 33342 staining assay (described below), one 100 mm plate was sampled for each of the two assays at each of the time points indicated and the assays were carried out immediately after sampling. For RNA extraction, CEFs from five 100 mm plates of the mock- or IBDV-infected group at each of the time points were collected, pooled and stored at −20 °C for batch RNA extraction at the end of the experiment.

Assessment of apoptosis and cell viability. Typical indicators of apoptosis, including chromatin condensation and plasma-membrane asymmetry, were monitored during the course of infection. To detect chromatin condensation, mock- and IBDV-infected CEFs were stained with Hoechst 33342 (Molecular Probes) in PBS at 37 °C for 5 min. At least 500 cells from each of three random fields of view were assessed by fluorescence microscopy. Asymmetry of plasma membranes was monitored by dual staining with Annexin V–PE and 7-aminoactinomycin D according to the manufacturer's instructions (Annexin V : PE Apoptosis Detection kit I; BD Biosciences). Data were acquired by using a Coulter Epics Elite flow cytometer. At least 1×10^5 cells were counted for each data point. In addition, the viability of CEFs was assessed by a trypan blue exclusion assay as described elsewhere (Lombardo et al., 2000). All three assays were performed in triplicate and each data point was presented as a mean with the standard deviation (SD) indicated.

RNA extraction. Approximately 5×10^6 cells were pooled from five plates at each time point and total RNA was extracted by using an RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was stored at −20 °C and used for cDNA microarray analysis and quantitative real-time PCR.

Assessment of viral growth by real-time PCR. The copy number of both viral genomes was assessed by using real-time PCR. The extracted RNA (20 ng) was reverse-transcribed by using SuperScript II (Invitrogen) according to the manufacturer's instructions. For real-time PCR, SYBR green PCR mix (Applied Biosystems) and an iCycler Real-time PCR system (Bio-Rad) were used according to the manufacturers' instructions. Information on individual primer sequences is available on http://web.hku.hk/~h0003414/. For absolute quantification of both viral genome copies, standard curves based on serially diluted plasmids containing the corresponding amplicons were constructed according to the procedures described by Patel et al. (2003). Amplification of both amplicons was performed in triplicate at each time point. The final data were expressed as genome copies (ng total RNA)$^{-1}$.

Reverse transcription, cDNA labelling, hybridization and image analysis. Total RNA samples collected from four time points, 24, 48, 72 and 96 h p.i., were chosen for cDNA microarray analysis. The microarray contains 13 007 usable features, representing approximately 12 000 unique mRNAs with coverage of 24 different chicken tissues or cell types (Burnside et al., 2005). In this study, pairing comparison and a duplicated repeat dye-swap experimental design were used (Churchill, 2002), i.e. two sets of dye-swapped slides were used at each time point. The processes of reverse transcription, cDNA labelling, hybridization and data analysis were carried out according to procedures described by Burnside et al. (2005). Briefly, 5 µg total RNA was amplified by using a linear T7 promoter-based mRNA amplification method incorporating amino-allyl dUTP, followed by random-primer labelling with Cy3 or Cy5 (Amersham Biosciences). After hybridization, array images were captured by using a GenePix 4000A scanner (Axon Instruments) and the image intensity was analysed by GenePix 3.0 software (Molecular Devices Corporation). Normalization of the Cy3 : Cy5 fluorescent signal was carried out by assuming equivalent global hybridization of test and reference probes to genomic chicken DNA. Expression of transcripts in IBDV-infected cells was compared with that in mock-infected cells from the same time point and expressed as fold change. Transcripts with P values ≤ 0.05 were considered to be significantly regulated.

Analysis of gene-expression data. Hierarchical clustering analysis was performed by using CLUSTER 3.0 (University of Tokyo). The clustering result was visualized by TreeView 1.0.13 (http://jtreeview.sourceforge.net). Physiological functions of the transcripts were annotated according to the original annotation (Fred Hutchinson Cancer Research Center, Seattle, WA, USA), as well as the BLAST results from Ensembl (http://www.ensembl.org) and GenBank (http://www.ncbi.nih.gov).

Confirmation of microarray data by real-time PCR. To confirm the transcript-expression pattern in IBDV-infected CEFs detected by microarray analysis, specific primers were designed on transcripts selected from each of the clusters for real-time PCR analysis. Information on individual primer sequences is available at http://web.hku.hk/~h0003414/. SYBR green PCR master mix (Applied Biosystems) and an iCycler Real-time PCR system (Bio-Rad) were used according to the manufacturers' instructions. All amplifications were repeated in triplicate at each time point. The relative expression (RE) of each gene was calculated based on the normalization with the Ct value of β-actin, i.e. expression levels of the target gene relative to those of β-actin (RE$_{\beta\text{-actin}}$). RE$_{\beta\text{-actin}} = 2^{\Delta Ct}$, when $\Delta Ct = (Ct_{\beta\text{-actin}} - Ct_{\text{target gene}})$. The final data were expressed as fold change compared with the mock-infected control, calculated as RE$_{\beta\text{-actin}}$(IBDV-infected)/ RE$_{\beta\text{-actin}}$(mock-infected).
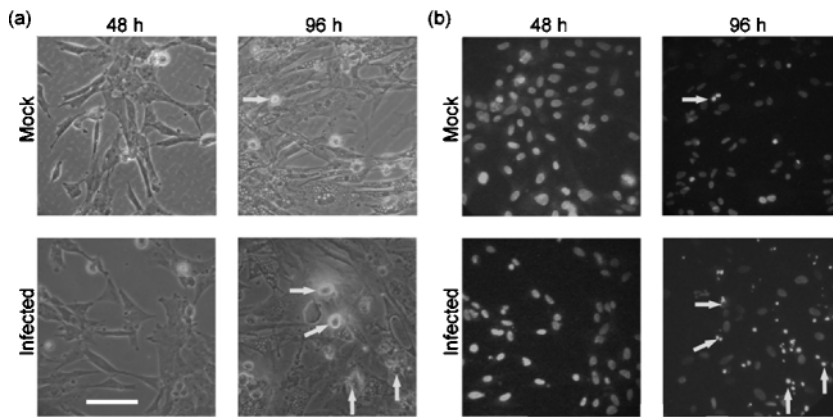
Fig. 1. (a) Cytopathic effects (CPE) in IBDV-infected CEFs at 48 and 96 h p.i. viewed under light microscopy (×100). Arrows indicate cells with CPE. (b) Chromatin morphology stained with Hoechst 33342 and viewed under a fluorescence microscope (×100) at 48 and 96 h p.i. Arrows show the fragmented nuclei characteristic of apoptosis. Bar, 15 μm.

RESULTS AND DISCUSSION

Progression of apoptosis and viral growth during the course of IBDV infection

At 72 h p.i., cytopathic effects, i.e. shrinkage and detachment (Fig. 1a) and chromatin condensation (Figs 1b, 2b), were observed in a substantial proportion of IBDV-infected cells, but not in mock-infected cells. After 48 h p.i., the viability of IBDV-infected cells was significantly lower ($P < 0.05$) than that of mock-infected cells (Fig. 2a). Moreover, in IBDV-infected cells, the proportion of late apoptotic cells increased rapidly throughout the course of infection, whilst the proportion of early apoptotic cells reached its peak (approx. 20 %) after 72 h p.i. (Fig. 2c, d). These results show that IBDV infection induces apoptosis in CEFs as early as 48 h p.i. and that approximately 50 % of the infected cells were apoptotic by 96 h p.i., as indicated by chromosome condensation and nuclear fragmentation. In addition, the copy number of both genome segments showed synchronized dynamics (Fig. 2e) and coincided with the onset of apoptosis in IBDV-infected cells at around 48 h p.i.

Global gene expression of IBDV-infected CEFs

In total, 214 transcripts were either induced or repressed by more than threefold for at least one of the time points assayed. Of these genes, 190 can be assigned well-defined physiological functions. Based on their distinct expression patterns, they were categorized into 10 clusters (Fig. 3). Clusters 1, 2, 3, 4, 5 and 8 contained approximately 60 % of the significantly regulated transcripts with an elevated expression pattern during the course of infection. The remainder (about 40 %) showed a generally suppressed expression pattern and were categorized into clusters 6, 7, 9 and 10. The change in expression and corresponding gene names of the 190 annotated transcripts are shown in Supplementary Table S1, available in JGV Online. It is noted that these differentially regulated transcripts are likely to be from productively infected cells, but may also be from antigen-negative cells in their vicinity that can be
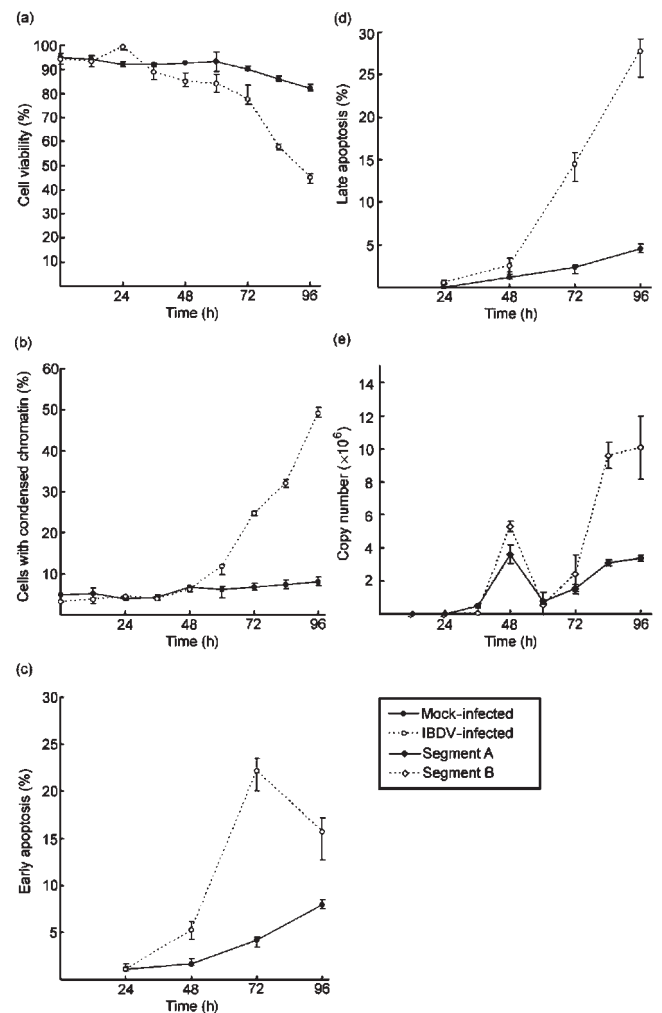

Fig. 2. Time course of: (a) cell viability, detected by trypan blue exclusion assay; (b) percentage of cells with condensed chromatin, detected by Hoechst 33342 staining; (c) early apoptosis; (d) late apoptosis, detected by flow cytometry; (e) virus titre, detected by real-time PCR of segment A in IBDV-infected CEFs. Error bars represent SD of the mean data.
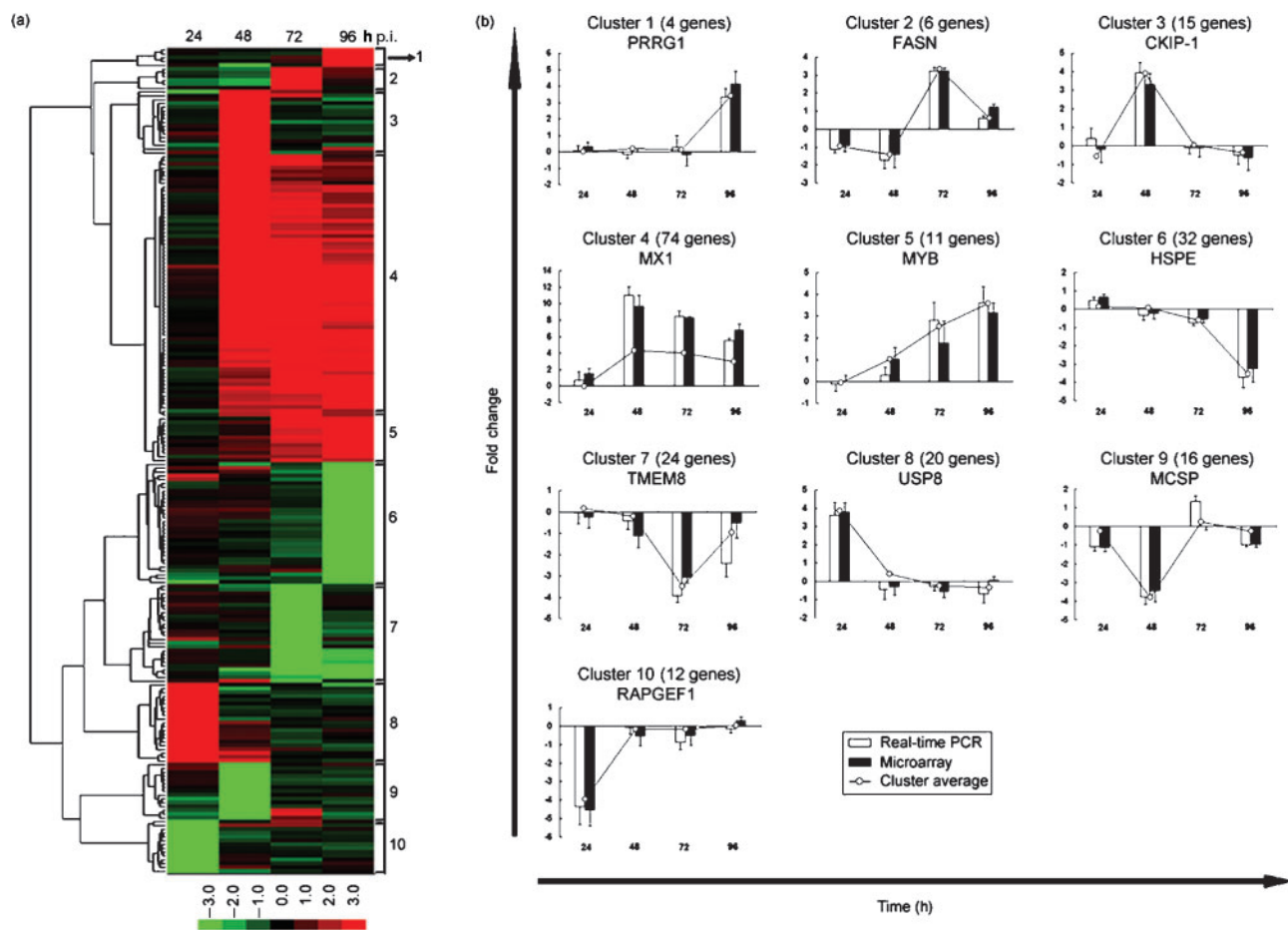
Fig. 3. Expression pattern of significantly regulated transcripts detected by microarray and real-time PCR. (a) Expression pattern of the 214 transcripts. Expression changes (fold) are represented as indicated in the colour scale. The transcripts were classified into 10 clusters according to their expression patterns. (b) Averaged expression profiles of the 10 expression clusters and the quantitative real-time PCR result of one selected transcript from each of the clusters. The number of genes is stated above the graph of each cluster. Error bars represent SD.

modulated by products of the infected cells (Jungmann *et al.*, 2001).

Confirmation of microarray data by using real-time PCR

The RE levels of transcripts representing distinct expression patterns were assessed by real-time PCR (Fig. 3b). The real-time PCR results generally correlated well with the microarray results, irrespective of their signal strength and expression patterns.

Transcriptional regulation of immunity-related surface receptors and their ligand

Expression of several cell-surface receptors and their ligands involved in immune responses, including T- and B-cell receptor (TCR and BCR) signalling-related molecules (PD-L1, CD72 and CD40L, LY6E) and members of

the semaphorins–plexins family (Plexin-C1 and Sema4D), was altered significantly (Table 1). Despite the fact that some of these genes may not trigger downstream effectors or interaction partners in CEFs, their crucial roles in T- and B-cell signalling and their potential roles in the immunosuppressive effects of IBDV should not be overlooked. PD-L1 is a ligand for programmed death-1 (PD-1) on lymphocytes, and ligation of PD-1 delivers an inhibitory signal to T-cell activity. PD-L1, which is upregulated in this study, is reported to be upregulated upon dsRNA stimulation and proposed to be associated with the viral escape mechanisms from the host immune system by inhibition of T-cell activation (Tsuda *et al.*, 2005). Lymphocyte antigen 6 complex (LY6E), which is upregulated in IBDV-infected CEFs, participates in TCR complex signalling and plays a regulatory role in T-cell development (Saitoh *et al.*, 1995). Liu *et al.* (2003) suggested LY6E as a candidate gene for Marek's disease resistance in chickens. A previous study indicated that LY6E has a negative-feedback role to limit
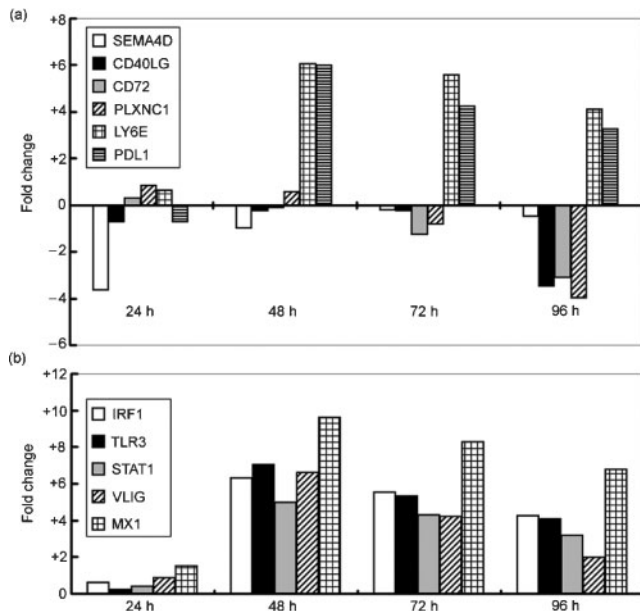
Fig. 4. Expression patterns of functionally grouped genes. (a) Positive and negative regulators of T and B cells were up- and downregulated from fourfold to sixfold, showing a generally reciprocal expression pattern. (b) Synchronous upregulation of genes related to the IFN pathway in IBDV-infected CEFs.

interleukin-2 (IL-2) production (Kosugi *et al.*, 1994) and, therefore, simple assays of the concentration of extracellular IL-2 in infected cells may serve as an indicator of the role of LY6E. CD40/CD40 ligand (CD40L) signalling, which plays a critical role in immune responses (van Kooten & Banchereau, 2000) and with which a number of viruses interfere (Klein *et al.*, 1999; Kornbluth, 2000), was downregulated in this study. The upregulation of these two negative regulators of T-cell activation, i.e. PD-L1 and LY6E, and the downregulation of CD40L may play a role in the immunosuppression that is characteristic of IBDV infection.

Semaphorins represent a novel class of immunoregulatory molecules. Accumulating data suggest the influences of a semaphorin, Sema4D (CD100), that can affect the behaviour of B cells, dendritic cells (DCs) and monocytes, including the enhancement of CD40-mediated B-cell proliferation and immunoglobulin production (Takegahara *et al.*, 2005). Sema4D was shown to be upregulated in immature DCs by the Tat protein of human immunodeficiency virus (HIV) and is likely to facilitate the expression of chemoattractants for activated T cells and macrophages (Izmailova *et al.*, 2003). CD72 is a main receptor of Sema4D in B cells to determine the undergoing of apoptosis or proliferation in B cells. Binding of Sema4D to CD72 appears to inhibit negative signalling by CD72, leading to increased B-cell activation (Nitschke & Tsubata, 2004). In addition, plexin-C1 was recently demonstrated to be involved in soluble Sema4D-mediated signalling, although the mechanism is unknown. Interestingly, expression of Sema4D, CD72

and plexin-C1 was downregulated in this study and, therefore, we speculate that they may have a role in the suppression of Sema4D-mediated activation of immune cells in IBDV-induced immunosuppression. Additionally, the expression of B-cell adaptor (BCAP) for phosphoinositide 3-kinase (PI3K), which connects BCR to the PI3K pathway and triggers B-cell proliferation and differentiation (Okada *et al.*, 2000), was significantly modulated. The expression patterns of these positive and negative regulators of T- and B-cell activation and differentiation showed a general reciprocal correlation (Fig. 4a), suggesting the possible functional correlation of these genes in modulation of immune responses. Given the fact that these molecules play crucial roles in B-cell activation and differentiation, a possible link between their altered expression and the immunosuppressive effects of IBDV can be inferred. The physiological roles of these genes, especially in B- and T-cell activation and differentiation, are summarized in Table 1.

Transcriptional regulation of genes related to antiviral responses

Toll-like receptor 3 (TLR3) is a dsRNA receptor that plays key roles in antiviral responses through cross-priming of multiple physiological pathways, including the activation of IFN responses, NF-κB and mitogen-activated protein kinase (MAPK) pathways, as well as the caspase cascade (Schroder & Bowie, 2005). In this study, the expression of TLR3 was upregulated significantly and correlated with the peak of dsRNA genome copy number at 48 h p.i., implying possible roles of TLR3 in dsRNA-initiated antiviral responses in IBDV-infected CEFs. The altered expression of TLR3 has been linked to enhanced responsiveness to viral infection, which was reported in a number of viruses, such as respiratory syncytial virus (Groskreutz *et al.*, 2006), vaccinia virus (Harte *et al.*, 2003) and hepatitis C virus (HCV) (Li *et al.*, 2005).

IFN-induced expression of antiviral genes is mediated by the JAK-STAT signalling cascade and regulated by IFN-regulatory factors (IRFs) (Chelbi-Alix *et al.*, 2006). In our study, STAT1 and IRF1 were upregulated. STAT1 plays a key role in signal transduction of IFN responses and hence is a target for modulation of the JAK-STAT signalling cascade (Chelbi-Alix *et al.*, 2006). Consequently, the upregulation of STAT1 in IBDV-infected CEFs may be interpreted as a host-initiated elevation of the IFN-mediated antiviral response or as an autoregulatory feedback mechanism in the case of post-translational viral inhibition of its activities. IRF1 was initially identified as a transcription factor that activates the IFN-α/β genes. Viral infection or dsRNA was demonstrated to induce the expression of IRF1, and its expression is crucial for dsRNA-induced IFN responses (Espert *et al.*, 2004). Overexpression of IRF1 has been demonstrated to suppress the replication of HCV (Kanazawa *et al.*, 2004). Recent studies have demonstrated that both TLR3 and STAT1 contribute to dsRNA- plus IFN-γ-induced apoptosis (Rasschaert *et al.*, 2005) and that IFN-β-induced

Table 1. Genes modulated by IBDV infection

GenBank accession no.	Symbol	Name	Related function(s)	Fold change*				Cluster
				24	48	72	96	
Immunity-related surface receptors and their ligands								
BU361094	PD-L1	CD274 antigen	Inhibitor of T-cell activation	−0.73	6.00	4.22	3.26	4
BU324702	LY6E	Lymphocyte antigen Ly6E precursor	Positive regulator of T-cell proliferation	0.64	6.03	5.57	4.10	4
AI982044	CD40L	CD40 ligand	Positive regulator of B-cell activation and antibody production	−0.71	−0.23	−0.23	−3.48	6
BU326894	Sema4D	CD100	Positive regulator of B-cell aggregation and differentiation	−3.66	−0.98	−0.21	−0.47	10
BU481163	CD72	CD72 antigen	Important regulator of B-cell signalling and activation	0.29	−0.13	−1.24	−3.10	6
BU259201	PLXNC1	Plexin C1	Required for CD100/SEMA4D to modulate cytokine production from monocytes and their movement	0.83	0.56	−0.82	−3.98	6
BF845749	BCAP	B-cell adaptor	Adaptor protein that connects PI3K to BCR	−1.35	−2.07	3.27	0.34	2
Antiviral responses								
BU242827	TLR3	Toll-like receptor 3	dsRNA receptor that activates type I IFN	0.20	7.03	5.33	4.06	4
BG625531	STAT1	Signal transducer and activator of transcription 1	Key signal transducer of IFN signalling	0.40	4.95	4.30	3.19	4
AI980049	IRF1	IFN-regulatory factor 1	Transcription factor that activates IFN-α/β genes	0.62	6.29	5.52	4.24	4
BU449782	STAT4	Signal transducer and activator of transcription 4	Key signal transducer of IFN signalling	0.37	4.30	1.91	0.39	4
BU457953	Mx1	Myxovirus resistance 1	IFN-induced antiviral protein	1.50	9.61	8.26	6.77	4
BU425330	VLIG-1	Very large inducible GTPase-1	IFN-induced antiviral protein	0.84	6.59	4.20	1.95	4
GPCRs and RTKs								
BU415940	CCR11	Chemokine (C-C motif) receptor 11	Chemokine receptor that plays roles in orchestrating leukocyte movements	−0.32	2.12	4.42	4.50	4
BI175781	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Phospholipase to catalyse LPA production	−0.41	3.09	3.64	2.23	4
BU450109	RGS5	Regulator of G-protein signalling 5	GAP that inactivates G-protein signalling pathways	0.51	−0.20	−3.01	−0.03	7
BG625427	RGS18	Regulator of G-protein signalling 18	GAP that inactivates G-protein signalling pathways	0.22	0.97	−1.59	−3.17	6
BU316243	PLC	Phospholipase C	PLC responsible for PKC activation	0.44	−0.11	−0.60	−3.01	6
BU447975	ADAMTS1	Disintegrin-like and metalloprotease with thrombospondin type 1 motif, 4	Possible HB-EGF sheddase	−0.09	5.14	4.57	3.35	4
BU456745	MMP15	Matrix metalloproteinase-like 1	Possible HB-EGF sheddase	3.35	3.90	−0.22	−0.64	8
BU346048	DNM2	Dynamin 2	Positive regulator of EGFR and LPA receptor endocytosis	−1.63	−5.74	3.06	−1.45	9
BU377943	Rab5	RAB5A, member of RAS oncogene family	Positive regulator of EGFR and LPA receptor endocytosis	0.00	−0.29	−1.00	−3.16	6
BU320637	EPS8	Epidermal growth factor receptor pathway substrate 8	Participates in EGFR signalling through Rac and endocytosis through Rab5	0.01	1.84	3.50	3.47	4
BU131791	USP8	Ubiquitin-specific protease 8	Negative regulator of EGFR ubiquitination level	3.78	−0.26	−0.54	0.06	8
AJ445837	PIK4CA	Phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	Required for endosomal trafficking and degradation of the EGFR	−0.66	−3.55	0.06	0.34	9
Intrinsic/extrinsic apoptotic pathway and NF-κB pathway								
BU399268	TRAIL	TNF (ligand) superfamily, member 10	Soluble factor inducing apoptosis through binding with its death receptors	0.15	3.16	3.53	3.38	4

Table 1. cont.

GenBank accession no.	Symbol	Name	Related function(s)	Fold change*				Cluster
				24	48	72	96	
BU421348	ARTS-1	Type 1 TNFR shedding aminopeptidase regulator	Sheddase promoting the shedding of TNFR1	0.21	5.37	4.49	3.61	4
BU393044	HIPK3	Homeodomain-interacting protein kinase 3	Inhibitor of apoptosis induced by extrinsic pathway and Fas-mediated JNK activation	−0.40	0.40	2.47	3.08	5
AJ394568	BAR	Bifunctional apoptosis regulator	Inhibitor of apoptosis induced by both extrinsic and intrinsic pathways	−0.25	3.17	2.23	1.69	4
AI980572	IAP1	Baculoviral IAP repeat-containing 4	Inhibitor of apoptosis by inactivating caspases	0.00	3.62	3.40	2.78	4

*Indicated fold changes are microarray results of IBDV-infected CEFs at 24, 48, 72 and 96 h p.i.

TLR3 upregulation required STAT1 and, in part, IRF1 (Heinz et al., 2003). Therefore, we speculate that the upregulation of TLR3, STAT1 and IRF1 may play a role inIFN-mediated antiviral responses to IBDV in infected CEFs.

In addition, two IFN-induced antiviral GTPases, Mx1 and the very large inducible GTPase-1 (VLIG-1), were also upregulated. Mx proteins are important IFN-induced antiviral effectors in mammals, birds and teleost fish (Mac-Micking, 2004). The human MxA protein has a broad antiviral effect against all kinds of viruses, although the mechanism is largely unknown. A previous report demonstrated the antiviral activity of Atlantic salmon Mx1 against another birnavirus, infectious pancreatic necrosis virus (Larsen et al., 2004), implying a potential role for the Mx1 protein in combating dsRNA viruses and birnaviruses in particular. VLIG-1 is a recently identified antiviral GTPase induced upon IFN-γ activation, but its functional significance in antiviral responses awaits clarification (MacMicking, 2004). Additionally, an IFN-inducible transmembrane protein, IFITM3, is also upregulated in this study, although its function is unknown. The expression patterns of these IFN-related genes are similar, indicating a possible functional correlation of these genes in induction of IFN (Fig. 4b). The physiological roles of these antiviral response-related genes are summarized in Table 1.

Transcriptional regulation of G protein-coupled receptor (GPCR)- and receptor tyrosine kinase (RTK)-related genes

The families of GPCRs and RTKs represent two prominent pathways for controlling a variety of physiological responses, including cell survival and apoptosis through the MAPK signalling cascade (Liebmann, 2001). According to our microarray data, several lines of evidence suggest the modulation of GPCR and RTK signalling pathways in IBDV-infected CEFs, including the altered expression of a GPCR, as well as genes involved in ligand production, signal transduction and receptor internalization of these two pathways.

A chemokine receptor, CCR11, belonging to the rhodopsin family of GPCRs, is upregulated in this study. CCR11 is a receptor for several lymphocyte-attracting chemokines that play roles in orchestrating temporal and spatial leukocyte movements during immune surveillance and inflammation (Schweickart et al., 2000). Lysophosphatidic acid (LPA) is an important lipid mediator that binds to GPCR of the Edg family, which affects cell proliferation, survival and migration. Production of LPA involves sequential actions of multiple phospholipases, including ENPP2, which catalyses LPA production (Xie & Meier, 2004) and is upregulated in this study. Overexpression of ENPP2 may lead to excessive LPA production and enhanced stimulation of LPA-targeted GPCR (Kishi et al., 2006). Antagonist-bound GPCRs activate various downstream effectors, whereas such activation is inhibited by GTPase-activating proteins (GAPs) called

regulators of G protein signalling (RGSs). Two RGSs, RGS5 and RGS18, were downregulated, which may enhance the responsiveness of specific GPCRs in IBDV-infected CEFs. In particular, RGS18 was shown previously to be down-regulated upon TLR3 activation (Shi *et al.*, 2004). Interestingly, RGS18 is reported to inhibit LPA-mediated MAPK activation (Shi *et al.*, 2004) and, therefore, the suppression of RGS18 and upregulation of ENPP2 in IBDV may enhance LPA-mediated GPCR signalling. Phospholipase C delta (PLC) is an immediate downstream effector of GPCR and is responsible for initiation of protein kinase C (PKC)-mediated activation of the MAPK pathway (Wu & Cunnick, 2002). PLC is downregulated in IBDV-infected CEFs at 48 h p.i., representing a possible intervention in GPCR-mediated signal transduction. A wide range of viruses were reported to manipulate the GPCR signalling pathway through expression of a virally encoded GPCR. This presumably aids the virus to elude the host immune response, renders infected cells sensitive to chemokines and promotes virus dissemination or modulates the cellular properties of infected cells (Smit *et al.*, 2003).

RTKs include the majority of growth-factor receptors, such as platelet-derived growth-factor receptor (PDGFR), epidermal growth-factor receptor (EGFR) and nerve growth-factor receptor (NGFR), and they play important roles in control of cell survival and growth. Activation of RTKs relies primarily on binding with their ligands, whilst trans-activation of RTKs by GPCR is a newly identified, alternative pathway of RTK activation (Wu & Cunnick, 2002). Binding of LPA to its specific GPCRs is reported to trans-activate a number of RTKs, including EGFR (Hong *et al.*, 2002), NGFR (Moughal *et al.*, 2004) and PDGFR (Inoue, 2002). Whether the upregulation of ENPP2 in IBDV-infected CEFs will result in an elevated level of LPA and transactivation of these RTKs warrants further investigation. One possible mechanism of EGFR transactivation by LPA requires the GPCR-induced cleavage of an EGFR pro-ligand, heparin-binding EGF (HB-EGF), which is possibly mediated by proteases of the ADAM and matrix metallo-proteinase (MMP) families (Wu & Cunnick, 2002). Members of each of these two protease families, ADAMTS1 and MMP15, are upregulated in IBDV-infected CEFs. Although ADAMTS1 and MMP15 were not shown previously to cleave HB-EGF, based on the growing list of HB-EGF sheddases (Higashiyama & Nanba, 2005), the possible roles of these two metalloproteinases in LPA-induced EGFR transactivation should not be overlooked.

Antagonist binding and activation of most GPCRs and RTKs result in endocytosis and degradation of the receptor–ligand complexes, ultimately attenuating the receptor signals and diminishing the level of cell activation (Ferguson, 2001). Interestingly, the expression of a number of genes related to EFGR endocytosis was found to be altered significantly, including DNM2, Rab5, EPS8, USP8 and PIK4CA. Among them, DNM2 and Rab5 are reported to enhance both EGFR and LPA receptor endocytosis, whilst ESP8 was shown to inhibit Rab5-mediated EGFR

endocytosis (Rami & Rabie, 1990; Murph *et al.*, 2003; Stang *et al.*, 2004). A recent report demonstrated that PIK4CA is required for endosomal trafficking and degradation of the EGFR (Minogue *et al.*, 2006). On the other hand, over-expression of USP8 reduces the ubiquitination level of EGFR and delays its degradation in EGF-stimulated cells (Mizuno *et al.*, 2005). Based on the altered expression levels and their reported roles in EGFR signalling, a general suppression of EGFR endocytosis and degradation in IBDV-infected cells seems likely. Although defective endocytic trafficking of EGFR was reported to enhance EGF signalling (Vieira *et al.*, 1996), further experiments are needed to investigate the possible effects of these genes on EFGR signalling in IBDV-infected CEFs. Other than cell proliferation and oncogenesis, activation of EGFR also plays a critical role in tumour necrosis factor alpha (TNF-α)-induced IL-8 secretion, which is important in inflammatory responses (Chokki *et al.*, 2006). Induction of IL-8 gene transcription by IBDV has been reported previously (Kim *et al.*, 1998), and Khatri & Sharma (2006) demonstrated that IBDV uses cellular signal-transduction machinery, in particular the MAPK and NF-κB pathways, to elicit macrophage activation. They speculated that the increased production of IL-8 by macrophages may contribute to bursal inflammatory responses. In summary, the expression of a number of genes related to the GPCR and RTK families, in particular LPA receptors and EGFR, was altered, showing a generally enhanced responsiveness of both pathways (summarized in Fig. 5). Artificial manipulations of these two signalling pathways, e.g. by addition of exogenous LPA or LPA inhibitors and EGFR inhibitors, may be employed as alternative ways to investigate the possible roles of LPA receptors and EGFR in IBDV-induced apoptosis. The roles of these genes in the GPR and RTK signalling pathways are summarized in Table 1.

Transcriptional regulation of genes involved in the intrinsic/extrinsic apoptotic pathway and NF-κB pathway

Apoptosis is initiated through two interrelated pathways, the extrinsic and intrinsic pathways. In the extrinsic pathway, engagement of the cell-death receptors has been shown to trigger the activation of MAPK/JNK and caspases 3 and 8 (Baetu & Hiscott, 2002). The expression of TRAIL, which is a ligand of death receptor 4/5, was upregulated significantly in IBDV-infected CEFs. Upregulation of TRAIL has been related to the pathogenesis of a number of viruses, e.g. influenza A virus (Zhou *et al.*, 2006) and measles virus (Vidalain *et al.*, 2000). The release of soluble TRAIL has also been related to the induction of apoptosis by bystander cells in HIV-1 (Zhang *et al.*, 2001) and reovirus (Clarke & Tyler, 2003) infection. Jungmann *et al.* (2001) reported the induction of apoptosis by IBDV replication in productively infected cells as well as in antigen-negative cells in their vicinity, implying an indirect apoptotic mechanism, which may be mediated by an enhanced release of TRAIL suggested by the upregulation
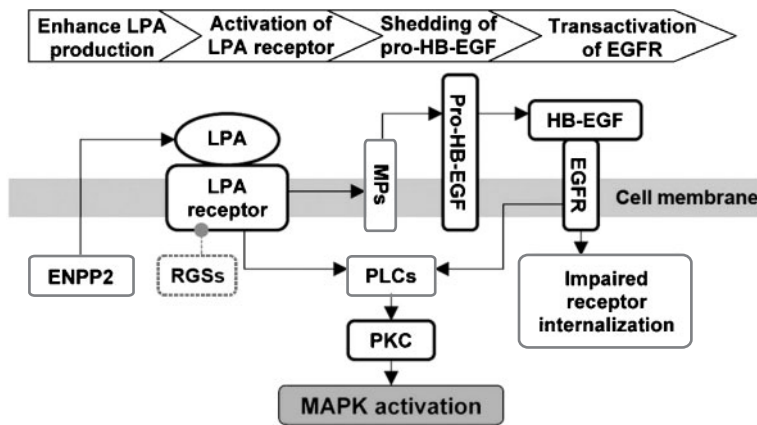
Fig. 5. Possible transactivation of EGFR induced by enhancement of LPA production. Up- and downregulated transcripts are highlighted in grey and grey dotted boxes, respectively. Positive- and negative-regulatory pathways are represented by black solid arrows and grey dotted lines, respectively. The upper panel summarizes this proposed model in four indicated steps from left to right. For details of this proposed model, please refer to the text.

of TRAIL observed in this study. A previous report demonstrated that IFN-γ enhances TRAIL-induced apoptosis through IRF1 (Park *et al.*, 2004). It is worthy to note that the upregulation of STAT1, which is a key regulator of the IFN-γ pathway, synchronized with that of TRAIL and IRF1 in IBDV-infected CEFs, implies a functional coordination of these genes in the induction of apoptosis by IBDV. TNF is a cytokine that plays a key role in immune function and its activity can be regulated by shedding of membrane-bound TNF receptor (TNFR) to soluble TNFR (sTNFR), which competes with cell-surface TNF receptors and thus decreases TNF bioactivity (Cui *et al.*, 2002). Shedding of TNFR1 requires an aminopeptidase named ARTS-1, which is upregulated significantly in IBDV-infected CEFs, suggesting the possible elevation of extracellular concentrations of sTNFR1. Elevated levels of extracellular sTNFR1 have been associated with the modulation of host immune defences and hepatic inflammation in hepatitis viruses (Tai *et al.*, 2001). HIPK3, a Fas-associated death domain (FADD)-interacting kinase, which prevents FADD-mediated caspase 8 and JNK activation and thereby inhibits death receptor-mediated apoptosis, was also upregulated significantly. The potent upregulation of ARTS-1 and HIPK3 may contribute to the control of death receptor-mediated apoptosis in IBDV-infected cells.

In the intrinsic apoptotic pathway, Bcl-2 and Bcl-xL play key roles in controlling the release of cytochrome *c* from mitochondria. BAR, a Bcl-2/Bcl-XL-interacting protein that inhibits FADD–caspase 8 interaction and suppresses BAX-mediated apoptosis (Roth *et al.*, 2003), was upregulated significantly in the early- to mid-phase of infection. In addition to BAR, a member of the inhibitor of apoptosis (IAP) family, IAP1, which inhibits apoptosis by blocking the activities of caspases, was also upregulated significantly. Blocking of apoptosis is a common strategy employed by viruses to evade host defence mechanisms that limit virus replication by killing infected cells (Hiscott *et al.*, 2001). The anti-apoptotic effects of BAR and IAP-1, which act on both the intrinsic and extrinsic apoptotic pathways, may have a profound influence on the enhancement of virus replication in the early stage of infection, as the expression

of both genes peaked at 24 h p.i. and then decreased gradually.

NF-κB is a transcription factor that has a crucial role in the regulation of cell survival, protecting cells from apoptosis under most circumstances and accelerating apoptosis in others. NF-κB is activated via degradation of the inhibitor of NF-κB (IκB). NFKBIA, a component of IκBα, is upregulated significantly in IBDV-infected CEFs. As IκBα itself is an NF-κB target gene, its upregulation is considered to be a negative-feedback mechanism that limits NF-κB activation, and thereby evidence of NF-κB activation. Numerous NF-κB target genes of diverse physiological functions showed a similarly upregulated expression pattern (Fig. 6), suggesting the possible activation of NF-κB in IBDV-infected CEFs. Among the upregulated NF-κB target genes is a receptor-interacting protein (RIP), RIPK2, which was demonstrated to interact with IKK and to be a potent activator of NF-κB (McCarthy *et al.*, 1998). Also, PDPK1, a critical regulator of cell survival by activating the NF-κB pathway (Tanaka *et al.*, 2005), is upregulated in the early stage of IBDV infection. These two genes may play a role in mediating the anticipated NF-κB activation in IBDV-infected CEFs. In addition, several NF-κB-activating receptors or ligands, including TRAIL, TLR3 and LY6E, were found to be upregulated (Fig. 6) and may be responsible for initiation of signal transduction of NF-κB activation. Although these genes were previously reported to be NF-κB target genes (http://people.bu.edu/gilmore/nf-kb/target/index.html), the direct correlation between NF-κB activation and the upregulation of these genes has to be further confirmed experimentally. Due to the fact that NF-κB regulates numerous target genes that have profound influences on the host cell cycle and immune responses, a wide range of viruses were reported to activate the NF-κB pathway to promote virus replication, block virus-induced apoptosis and modulate the immune responses against the virus through the viral proteins, as well as viral and synthetic dsRNA (Hiscott *et al.*, 2001). VP5, a non-structural protein of IBDV, which was initially identified as an apoptotic inducer (Yao & Vakharia, 2001) but later found to be anti-apoptotic at the early stage of virus infection,
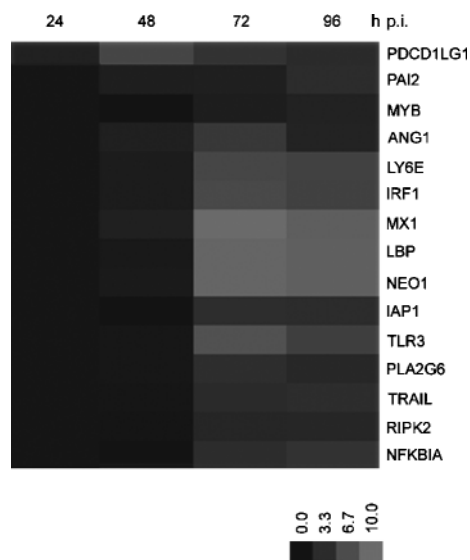
Fig. 6. Expression patterns of NF-κB target genes. A number of NF-κB target genes showed three- to tenfold upregulation in the late stage of IBDV infection in CEFs, implying the possible activation of NF-κB. The change (fold) in transcript levels is represented as indicated in the scale bar.

was reported to activate the NF-κB pathway, which is required for IBDV-induced apoptosis (Liu & Vakharia, 2006). Khatri *et al.* (2005) demonstrated that the IBDV-induced activation of macrophages requires NF-κB activation. Therefore, the upregulation of numerous NF-κB target genes detected in this study agrees with previous reports. The roles of altered genes involved in the intrinsic/extrinsic apoptotic pathways are summarized in Table 1 and Supplementary Fig. S1, available in JGV Online.

Conclusions and implications

Among the 214 significantly altered transcripts, genes that have possible roles in pathogenesis or host defence responses against the virus were classified into four categories listed below. Firstly, genes for immunity-related surface receptors and ligands, which may alter lymphocyte activation and differentiation. Secondly, genes involved in antiviral responses, which include both the TLR- and IFN-signalling pathways. Thirdly, genes related to the GPCR- and RTK-signalling pathways, which may participate in inflammation, macrophage activation and controlling the mitogenic responsiveness of host cells. Lastly, genes involved in apoptosis and cell survival, which may be responsible for virus-induced apoptosis leading to destruction of host immune cells or, alternatively, for virus-induced cell survival to support virus replication. This report pinpoints the candidate genes involved in host defensive responses against the virus, as well as virus-directed mechanisms of pathogenesis. These proposed pathways and candidate genes provide a foundation for researchers to formulate testable hypotheses and develop specific experimental approaches to test their hypotheses targeting individual genes.

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