Transcriptomic analysis of bovine monocytes in response to non-cytopathic bovine viral diarrhea virus infection

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Research article

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Abstract

Background: Monocytes are significant players in the detection of invading pathogens, particularly in pathogen defense. Bovine Viral Diarrhea Virus (BVDV) can cause a persistent infection and immune suppression if animals are infected with an non-cytopathic (ncp) biotype. However, its exact role in ncp BVDV1-infected bovine monocytes remains poorly understood.

Results: RNA sequencing (RNA-seq) was used to investigate the effect of ncp BVDV1 infection on the transcriptional profile of bovine monocytes. Compared with the non-infected cells, 9959 and 7977 differentially expressed gene (DEGs) were identified at 2 and 24 h hpi, respectively. These DEGs were associated with signal transduction, immune response, apoptotic process, cellular process , binding and cellular component. The differential expression profiles of select the type I interferon signaling pathway , interferon (IFN)-stimulated genes (ISGs), and genes involved in the innate immune response, including IRF7, DDX3X, TLR13, DDX58(RIG-I), MVAS, TLR9, TRAF6, IRF1, IFIT1, STAT1, ISG20, TRIM25, MX1,NLRX1, CYLD, SIKE1 and ZAP70 were confirmed by real-time quantitative PCR and consistent with the RNA-seq data.

Conclusion: Our transciptome anslysis provides useful initial data towards better understanding of the infection mechanisms used by ncp BVDV1, while highlighting the potential molecular relationships occurring between the virus and the host's immune response.

Background

Bovine viral diarrhea virus (BVDV) is a small positive-sense single-stranded enveloped RNA virus that is part of the *Pestivirus* genus of the *Flaviviridae* family and a viral pathogen of cattle. BVDV infection is found in cattle of all ages and breeds worldwide, with a huge economic impact due to production and reproductive losses[1, 2]. BVDV can also be categorized under two biotypes based on their growth characteristics in cell cultures. The rare cytopathic (CP) biotype will damage tissue cultures and the much more common noncytopathic (ncp) will not[3, 4].The pathogenesis of the disease caused by BVDV is complex and involves persistent infection (PI) and immune suppression with the ncp biotype during early gestation, followed by an acute infection by a cp biotype[5].

In recent years, the understanding that the vertebrate innate immune system represents the first defense against pathogens has emerged. The synthesis and secretion of type I interferons (IFN-I) is the first step in the induction of cellular innate immune responses [6] to viral infections or double-stranded RNA (dsRNA). dsRNA is a common pathogen-associated molecular pattern (PAMP) that is recognized by a variety of pattern recognition receptors (PRRs). Currently identified PRRs can be divided into the Toll-like receptors (TLRs), the retinoic acid-inducible gene I (RIG-I) receptor-like receptors (RLRs), and the NOD-like receptors (NLR). All three PRRSs recognize the viral PAMPs [7].Each of the three receptors cooperates with their adaptor proteins to deliver information in the presence of viral PAMPs and interferons. The Toll-like receptors affect the Total Recordable Injury Frequency (TRIF) adaptor protein, the Cyclic GMP-AMP

synthase (cGAS) receptor effects the Stimulator of interferon genes (STING) adaptor protein, and the RIG-I receptor interacts with the Mitochondrial antiviral-signaling (MAVS) adaptor protein. These connecting molecules recruit and activate downstream signaling pathways to produce corresponding inflammatory cytokines, chemokines, and Type I (IFN-α/β) and III (IFN- g) interferons [8, 9]. Many studies have confirmed that innate immune evasion affects the survival and adaptive immune response of virus. [10].

Previous studies have revealed many crucial discrepancies in the results of ncp and cp biotypes in variety of cell types. ncp BVDV does not induce IFN synthesis in cultured cells in vitro, suggesting that this could be a defense mechanism to evade innate host immunity that might be critical for establishing persistent infections [11, 12, 13]. However, the molecular mechanisms by which BVDV evades host cell immunity remains unclear. RNA-seq has recently been developed for transcriptomics analysis of BVDV infection in MDBK cells. Research was performed with microarrays that showed gene expression changes in host cells infected with ncp BVDV compared with cp BVDV [14, 15]. However, it was reported that a large number of genes related to BVDV invasion and replication mechanisms still need to be characterized to better understand the interaction between BVDV pathogenetic mechanisms and host immune defenses. Although ncp BVDV is thought to play an important role in BVDV-infected bovine monocytes [16-19], little is known about how BVDV disturbs host cell gene expression profiles. During this research, we showed that BVDV infected bovine monocyte gene expression profiles, which provided meaningful information from genetic analyses that could not be discerned using biochemical methods.

Materials And Methods

Animals

Nine conventionally reared, healthy BVDV-free cows from the Shihezi University Animal Experimental Station were used. All animal experiments were approved by the Shihezi State University Institutional Animal Care and Use Committee. The blood samples of all animals were confirmed to be free of BVDV, infectious bovine rhinotracheitis virus, bovine parainfluenza virus, and Mycoplasma bovis infection using enzyme linked immunosorbent assay (ELISA) kits (Idexx Labs Inc., USA) and by reverse transcription PCR (RT-PCR) or PCR for viral nucleic acid detection [18].All animal serums were negative identified by Bovine viral diarrhoea virus(BVDV) antibody test kit(Idexx Labs Inc., USA)

Cell preparation

Blood samples (150 ml) were obtained by jugular puncture into blood collection tubes (16 x 100 mm, Tyco Healthcare). Bovine PBMCs were isolated as previously reported elsewhere [16, 17]. Briefly, PBMCs were separated from the blood on a Histopaque gradient (1.077 g/ml, Amersham Biosciences) and resuspended in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Invitrogen), 5 x 10^{-5} M 2-Mercaptoethanol, and 100 IU/ ml gentamicin (Invitrogen). For monocyte isolation,, 40 ml of the PBMC suspensions (5 x 10^{8} cells) were added to Petri dishes (150 x 25 mm, BD Biosciences) for 2 hours at 37°C. Non-adherent cells were removed, and adherent cells were washed two times with PBS. The yield of

recovered adherent cells was 20-30% of the initial PBMC. Adherent cells were then incubated with a CD14 monoclonal antibody (MM61A, VMRD) followed by incubation with mouse anti-IgG1 microbeads (Miltenyi Biotech, Auburn, CA). Using magnetic bead separation technology, $CD14^+$ monocytes were selected using the manufacturer's instructions (Miltenyi Biotech), yielding a final monocyte concentration that was 2-3% of the original PBMC population[18,19].

BVDV stocks and infection

The ncp BVDV1 strain was named BVDV-LC,which isolated from the feces of a cattle with diarrhea, (GenBank accession NO. MK102095, previously identified in our lab), and was classified as genotype 1q and non-cytopathic (ncp). For titration of virus stock, four replicates of 10-fold serially diluted virus (starting from 1/10) were inoculated on MDBK cell monolayer in 96-well culture plates. After 48 h incubation, the culture plates were fixed at 4 °C for 30 min with ice cold absolute ethyl alcohol and subjected to immunofluorescence staining with fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-BVDV (VMRD, USA) antibody (diluted 1:1000). The fluorescence signals were observed undera fluorescence microscopy (ZEISS) and viral titers was expressed as the 50% tissue culture infective dose (TCID50)/mL by Reed-Muench method.

For infection of bovine monocytes, virus dilutions were made in DMEM 4Mm L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 10% horse serum. 5×10^6 monocytes were added to each well of a 6 well tissue culture plate and adsorbed with ncp BVDV1 biotypes at the same MOI of 0.1 for 2 h.

RNA extraction

After infection 2 h and 24 h, at least 10^7 cells were pooled in one tube. Cells were harvested and prepared for RNA extraction. The monocytes were rinsed three times with ice-cold PBS, and whole RNA was extracted by DNase digestion using the RNeasy mini kit following the manufacturer's instructions (Qiagen). RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher) and stored at -80℃. For RNA-seq analysis, the RNA quality was assessed with an Agilent Bioanalyzer (Agilent Technologies).

RNA sequencing and analysis

The RNA elimination was performed using Ribo-Zero rRNA removal kit (Epicenter, USA) following the manufacturer’s instructions. Illumina sequencing libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocol. RNA sequencing was performed on the Illumina HiSeq 4000 platform at the Novogene Bioinformatics Institute (Beijing, China).

The sequence data of the reference genome was checked against the NCBI database. The filtered mass reads were aligned with the reference genomic sequence using Bowtie 2. Relative transcript abundance was metered as fragments per kilobase per million mapped reads (FPKM). Genes were clustered into functionally relevant groups using the eggNOG (Genetic Evolution Genealogy: Unsupervised Orthologous

Group) database and the metabolic pathways were analyzed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Visualization of mapping outcomes and differentially expressed gene (DEG) analysis was performed using the CLRNASeqTM program (ChunLab, Korea). ncp BVDV1 expression and infected monocyte gene expression were contrasted with gene expression from the inoculum and uninfected monocytes. The fold change in gene expression was calculated from three different sample types.

Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

DEGs were examined by RT-qPCR to confirm the accuracy of the sequencing data. Primers were made using Primer 5.0 software (Table 1). cDNA was synthesized from 2 μg of total RNA using a reverse transcription kit (Fisher Scientific, USA) and by following the manufacturer's instructions. Gene expression was quantified using a LightCycler 480 PCR platform (Roche Applied Science, Indianapolis, IN, USA). The RT-qPCR procedure began with an initial step of 10 minutes at 95 °C followed by 45 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. Gene transcription levels were quantified relative to GAPDH gene expression using the relative cycle threshold (ΔCT) method.

Statistical analysis

All data are shown as means ± SE. Differences were evaluated using ANOVA followed by the Student's t-test. Statistical significance was defined as $P < 0.05$.

Results

RNA sequence analysis of ncp BVDV1-infected monocytes

The results show that more differential expression, either up- or down-regulated was seen at 2 h post-infection (hpi), the expression of 9959 genes was significantly changed compared with those of the controls, of which 4968 genes were upregulated, and 4991 genes were down-regulated (Fig.1A, Additional file 1:Table S1). At 24 hpi, the expression of 7977 genes was significantly different from that of the controls, with 4184 genes up-regulated and 3793 genes down-regulated (Fig.1A, Additional file 2:Table S2). These significantly altered genes were involved in signal transduction, immune response, apoptotic process, cellular process , binding and cellular component, with most of the differential genes being involved in signal transduction.

The DEGs were filtered to determine which genes were present at both of the time points (2 hpi and 24 hpi). At 2 hpi time point, 9959 genes responded to ncp BVDV1 infection, and at 24 hpi time point, 7977 genes responded; however, only 5709 genes were common at both time points (Figure. 1A). Two libraries were packaged and analyzed against the non-redundant NCBI database using the BLAST program. Next, we mapped and annotated the genetic libraries using the Gene ontology (GO) database. To identify pathways involved in immune activation after ncp BVDV1 infection of bovine monocytes, transcript records were further analyzed. The GO terms classify the function of BVDV-infected cell transcripts,

offering 4014 transcripts involved in molecular functions, 4476 involved in biological processes, both and 3341 involved in discretely cellular processes (Figure. 1B). Analysis revealed that differentially expressed genes are involved in a variety of biological processes, including immune responses and immune system procedures, cell death, macromolecular localization, apoptosis, cell death regulators, antigen processing and presentation, protein localization, and others (Figure. 1C, Additional file 3:Table S3, Additional file 4:Table S4).

To further define DEGs function, KEGG pathway/enrichment analysis was performed. DEGs were significantly enriched in 20 pathways（Fig.1D and E）. A total of 126 and 113 DEGs show Ribosome pathway enrichment in 2 hpi and 24 hpi, respectively. This pathway was mainly responsible for gene expression regulation and protein translation. Many significantly DEGs were involved in immune responses such as with the TNF signaling, B cell receptor signaling pathway, Fc epsilon RI signaling pathway, T cell receptor signaling pathway and NF-κB signaling pathway (Fig.1D and E, Additional file 5:Table S5, Additional file 6:Table S6). These DEGs, which include TRIM25, TRAF6, TRAF3, IL-1β, CD14, BCL2, TICAM1/2, TNIP3, TNFSF13B, ZAP70, TRAF2, TRAF2, TNFSF11, PIDD1, CARD11, LYN, BLNK, BCAP, BCL-10, CD81, CD19, MEK1/2, AP1, BCL10, Ikkα, Ikkγ, Ikkβ, PD-1, CD45, LCK, CBL, ZAP70, LAT, etc, play an important role in activating or inhibiting innate and adaptive immunity (Table 2, Additional file 1:Table S1, Additional file 2:Table S2). These DGEs were further analyzed (GO: 0006955) (Figure.2A), A list of genes that were differentially expressed between 2 hpi and 24 hpi is shown in Table 2. Most of the upregulated genes were related to the immune system. Upregulated genes included IL6 (3.1 fold), TNFSF13B (2.5 fold), CD14 (2.8 fold), IFI27 (5.7 fold), RNF125 (1.8 fold) and MAPK12 (2.4 fold), and downregulated genes included IRF7 (1.08 fold), TLR13 (2.2 fold), ZAP70 (2.9 fold), TLR8 (1.5 fold) , TLR9 (1.5 fold), IFITM1 (1.8 fold), and IGHG2 (2.9 fold). Some genes (eg, IFI30, JUN, CD40, LTBP2, IKBKB, CCL5, and MAPK11) were up-regulated at 2 hpi and down-regulated at 24 hpi. Other genes, such as EIF2AK2, DHX58, DDX3X, IFI6, TIRAP, STING, and IFIT1 were downregulated at 2 hpi and upregulated at 24 hpi (Table 2).

Through careful examination of the information set (Figure. 2B and Table 2), several ISGs [20] mainly produced by type I interferons, were differentially expressed in ncp BVDV1-infected cells. Figure.2A and B show stratified heat maps of all of the up or down regulated genes in ncp BVDV1-infected cells, and they were all associated with immune responses and type 1 IFN signaling. Overall, the RNA-seq analysis indicated that ncp BVDV1 infection resulted in different expression of genes related to inherited immunity, type I IFNs, stimulatory cytokines, and cell signaling pathways, leading to the creation of antiviral responses in monocytes.

Partial validation of the DEGs involved in the type 1 IFN pathway by RT-qPCR

Sequences found using RNA-seq analysis were validated with RT-qPCR to assess if mRNA expression was upregulated or downregulated in relation to the genes and to the immune responses. The genes were identified if they were recognized as being involved in type I IFN responses, were reported to be related to innate antiviral immune responses [21, 22], and if they were common to both infected groups. Using GAPDH as an internal control, the transcription levels of 31 DEGs were measured (between the 2 and 24

hour time points) with RT-qPCR with the primers listed in Table 1. In conclusion, the RNA-seq and RT-qPCR results from bovine monocytes at 2 and 24 hpi were fairly consistent (Figure. 3). IRF7, TLR13, ZAP70, IFITM1 were all down-regulated 2 hours and 24 hpi with ncp BVDV1 virus. DDX3X, IFIT1, STING were down-regulated 2 hpi and up-regulated 24 hpi. The expression of IKBKB, TRAF1, IRF5 was up-regulated 2 hpi and down-regulated 24 hpi. EF2AK2, IFI27, IFI30, MX1, MX2 and other genes were up-regulated sharply 2 hpi compared with 24 hpi.

Discussion

The complex and unique nature of BVDV continues to challenge infectious disease researchers,veterinarians,and the cattle industry. The different genotypes and biotypes of BVDV ability to induce persistently infection, as well as its ability to interfere both innate and adaptive immunity of the host, make it difficult for prevention and control[5]. Although previous research [18] evaluated the effect of cp and ncp BVDV infection of bovine monocytes to determine their role in viral immune suppression and uncontrolled inflammation by using proteomics, this study was the first to analyze transcriptionally diversified gene expression after in vitro ncp BVDV infection of bovine monocytes. In this study, using RNA-seq, we demonstrated that most of the DEGs between ncp BVDV1 infected monocytes for 2 and 24 hours, involved immune responses, suggesting that ncp BVDV1 infection plays an important role in bovine monocytes. We observed increased levels in a variety of antiviral genes such as IFI27, IFI30, MX1, TRIM25, DDX41, ISG20, STAT1, TRAF6, CD14, CD40, IL1B, IL6, MAPK12, MAPK13, TNFSF13B, and TBKBP1 (Table 2), It seems to play a fundamental role in ncp BVDV1 infection in monocytes. The expression levels of the IFI27, IFI30, ISG20, STAT1, TRAF6 and TRIM25 genes obtained from the RNA-seq data were confirmed by the real-time PCR mRNA expression data (Table 1 and Figure. 3). These proteins might be upregulated in bovine monocytes because of the ncp BVDV1 infection. However, we observed that type I IFN genes were reduced such as IRF7, DDX3X, DDX58, TLR13, TLR9, STING, and IFIT1, which was possibly caused by the ncp BVDV1 viral particles[12, 13]. On the other hand, upregulated DEGs, such as NLRX1, CYLD, SIKE1, and RNF125, were shown to interfere with host immune responses in previous documents .NLRX1 acts as a negative regulator of MAVS-mediated antiviral response by inhibiting virus-induced RLH (RIG-like helicase)-MAVS interaction[23-25].CYLD (Cylindromatosis) is a deubiquitin enzyme. CYLD removes the K63-connected ubiquitin chain of a specific substrate through its own deubiquitin activity and negatively regulates multiple signal pathways including NF- kappa B[26].SIKE1 (Suppressor of IKBKE 1) was classified as an endogenous inhibitor . Inhibits TLR3-mediated activation of interferon-stimulated response elements (ISRE) and the IFN-beta promoter. May act by disrupting the interactions of IKBKE or TBK1 with TICAM1/TRIF, IRF3 and DDX58/RIG-I[27,28].

RNF125 is an E3 ubiquitin protein ligase that mediates ubiquitin and subsequent proteasome degradation of target proteins. As a negative regulator of type I interferon production, it can mediate DDX 58 / RIG-I ubiquitination in "Lys-181", resulting in DDX 58 / RIG-I degradation[29].

Our research showed that DHX16, DHX34, DHX37, DHX58 (Table 2) were not significantly DEGs after ncp BVDV1 infection, but DHX58 expression was increased (Figure. 3). In other studies, viral dsRNA

stimulation did not induce high expression of DEAD-box proteins, and viral dsRNA was shown to be degraded by some proteases, such as BVDV Erns protein [30, 31]. We found that DHX16, DHX34, DHX37, DHX58 DDX3X, DDX10, DDX51, DDX54, and DDX55 were differentially expressed after the ncp BVDV1 infection of monocytes. Post-downregulation was not significant (Table 2), but DHX41 expression was significantly increased, suggesting that DDX41 plays an important role in RNA binding and secondary structure changes [32, 33].

Toll-like receptors (TLRs) are one of the most essential innate immune receptors that identify many types of PAMPs from different pathogens and activate downstream immune responses. TLRs are grouped into two groups: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11, which are concerned with membrane proteins that detect microbial membrane components, while TLR3, TLR7, TLR8, and TLR9 are concerned with nucleic acid recognition of proteins, cells, intracytoplasmic molecules, and microorganisms [34, 35]. Nevertheless, studies showed that infections and administrative mechanisms the TLR13 signaling pathway in mollusks. In this study, TLR8, TLR9, and TLR13 expression in BVDV infected bovine monocytes were downregulated (Table 2 and Figure. 3), which may be the reason for the decreased type 1 IFN expression that was seen [36- 40].

This study found common groups of genes related to important cellular processes at 2 hpi. For example, inhibition of NF-κB activation was induced by upregulated IKKE and SIKE1, negative regulators of the NF-κB signaling pathway, and by downregulated p65/RelA-NF-κB [27]. SIKE1 directly interacts with TBK1 to inhibit the TBK1-AKT signalling pathway, Which could further lead to blocked or delayed antiviral responses. These results are interesting because they show that infected living cells exhibit strong immunosuppressive phenotypes. In fact, the results also show downregulation of vital immune response genes, for example, the PIDD1, IKBKG, IKBKB, INFGR2, PRKCQ and EIF2AK2 genes (Table 2). Moreover, the expression of IKKA and SIKE1 transcripts indicates the blocking of NF-κB in infected cells, which were support by the RT-qPCR results (Figure. 3 and Table 2).

Our research demonstrates that there is antiviral regulation into bovine monocytes upon activation, as shown by upregulation of the NLRX1, CYLD, SIKE1, ATG12, and RNF125 genes (Table 2 and Figure. 3). These findings are vital for the understanding of the molecular mechanisms by which ncp BVDV1 evades innate immunity. NLRX1 is involved in antiviral signaling. By inhibiting the virus-induced RLH (RIG-like helicase)-MAVS interaction, NLRX is a negative regulator of the MAVS-mediated antiviral response [23]. RNF125 mediates DDX58/RIG-I ubiquitination inducing DDX58/RIG-I degradation of Lys-181 [29]. SIKE1, IKK-epsilon, and TBK1 genes are physiological inhibitors that act on viral and TLR3-triggered IRF3 to inhibit TLR3-mediated interferon-stimulated response elements (ISRE) and IFN-β promoters. Activation of the ISRE and IFN-β promoters can be achieved by disrupting the interaction between IKBKE or TBK1 and TICAM1/TRIF, IRF3, and DDX58/RIG-I, which does not inhibit the NF-κB activation pathway [27,28].

Type I IFNs are key mediators of host immune responses to viral infections. These IFNs induce the expression of many ISGs of which some could be antiviral. Besides, they regulate innate and adaptive immunity by activating immature DCs, strengthening NK cell responses, and boosting the survival and

effector functions of T and B lymphocytes [41]. In this research, a significant decrease in ZAP70, TNFSF11, V-TCR, and CARD11 was regulated in ncp BVDV1-infected bovine monocytes. ZAP70 regulates T cell activation by modulating TCR expression on T cell surfaces [42, 43]. CARD11 is involved in the costimulatory signals necessary for T cell receptor (TCR)-mediated T cell activation. CARD11 connects with the DPP4 and guides T cell multiplication and NF-κB activation in a T cell receptor/CD3-dependent manner [44]. TNFSF11 is a necessary regulator of T cell and dendritic cell interactions and might regulate T cell-dependent immune responses [45]. Most serine/threonine kinase-expressing genes are increased upon ncp BVDV1 infection of monocytes, which is quite interesting because only MAP2K6 is significantly reduced. MAP2K6 plays a vital role in regulating cytokine to cytokine responses, particularly as it relates to the induction of IL-6 production. IL-6 is increased 2 hpi and is decreased by 24 hpi (Table 2 and Figure. 2) [46, 47].

Conclusions

In conclusion, the lack of knowledge on how BVDV evades host immune responses causes improper control of this disease. Our studies have identified IRF7, DDX3X,, TLR13, TLR9, STING, and IFIT1 as key downregulatory molecules in ncp BVDV1 infected monocytes. Conversely, NLRX1, CYLD, SIKE1, ATG12, and RNF125 act as upregulatory molecules. There also appears to be negative regulation of IFN production. On the basis of previously reported studies and current information, we feel that the underlying role of these genes are to defend against ncp BVDV1 infection. More research is needed to clarify the mechanism by which ncp BVDV1 upregulates and downregulates these various molecules and to determine the role of these genes in the pathogenesis of BVDV, which will thus enhance our understanding of BVDV replication and pathogenesis.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors’ contributions

YH and CC contributed to the study conception and design. YH, JH and JY wrote the manuscript. CC, CX and FZ checked and revised it. YH, JY, XM, XH, XZ and TG performed the experiments and analysed the data. All authors read and approved the final version of the manuscript.

Ethics approval

This study was carried out in accordance with the recommendations of National Standards for Laboratory Animals of the People's Republic of China(GB149258–2010). The protocol was approved by the Shihezi State University Institutional Animal Care and Use Committee. Cattles used for the study were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable

Conflicts of interest

The authors have no conflicts of interest to declare.

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Abbreviations

BVDV: Bovine Viral Diarrhea Virus; ncp: non-cytopathic; ISGs: interferon stimulated genes; IRF7: interferon regulatory factor 7; DDX3X: DEAD-Box Helicase 3 X-Linked; TLR13: Toll-like receptor TLR 13; TRAF6: TNF receptor-associated factor 6; IFIT1: Interferon-induced protein with tetratricopeptide repeats 1; STAT1:

Signal transducer and Activator of transcription 1; ISG20: interferon-stimulated gene 20 kDa; TRIM25: Tripartite Motif Containing 25; MX1: MX Dynamin Like GTPase 1; NLRX1: the nucleotide-binding oligomerization domain, leucine rich repeat containing X1; CYLD: Cylindromatosis, turban tumor syndrome; SIKE1: Suppressor Of IKBKE 1; ZAP70: Zeta chain of T cell receptor-associated protein kinase 70; PAMP: pathogen-associated molecular pattern; PRRs: pattern recognition receptors; TLRs: Toll-like receptors; RIG-I: retinoic acid-inducible gene I; NLR: NOD-like receptors; TRIF: Total Recordable Injury Frequency; cGAS: Cyclic GMP-AMP synthase; STING: Stimulator of interferon genes ; MAVS: Mitochondrial antiviral-signaling; PBMC: peripheral blood mononuclear cells; TCID50: The 50% tissue culture infectious dose; mock: Uninfected control; FPKM: fragments per kilobase per million mapped reads; DEG: differentially expressed gene; GO: Gene ontology; NF-κB: nuclear factor kappa-B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR: real-time quantitative reverse transcription-polymerase chain reaction;

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Tables

Table 1. Primers used for real-time PCR.

Name	5′Sequence	3′Sequence
IRF7	CGGCGCTTCTCGGAGGCAGAGA	CCCCTGGGGCTCACGAGCAAACA
DHX58	TGTGCTCCTACTGGTTGTGGA	AACGACAACTTTTCCCTTT
DDX3X	GCTGGCCTAGACCTGAACTC	CTTTTGCACTGGAGTTGGGC
MAVS	ATGGGCCAGTTCAGAACCCAG	CTGGGGTAGGCGCCGCCGGTA
IKBKB	GCCATGATGAATCTGCTGCG	TGATCCCAAACTCCGTCTGC
TRAF6	TTGCCATGAAAAGATGCAGAGG	AGCCTGGGCCATCATTCTC
TRAF1	CTTCATGCTGCTGGACCAGA	GAGTCCACCTCCACGTTCAG
IRF5	TGCCAGTGCAAGGTGTTCTGG	AGGTTTGCAGTCAGGCCACT
IRF9	CTCTGCCCCATTCCCATCTC	TGGCCAAGTCTCTGCAGAAG
IRF1	GGGCCTCAGCACCTTCCATC	GCCAGCGCCGCCTCATCATTTC
STAT1	CAGCAGAATTCCGACACCT	CAGTGACGATGAGAGGACCCT
EIF2AK2	GATGGTTTCAGTGCTGCGTG	CTGGAGACACGGAAGAGCTG
TRIM25	GGGGACGAGTTTGAGTTTCTGG	AGCGTCCCCTGGGATGGTGAGCT
TLR13	GCTGCATGAATGTTGCCCAA	GCTGCTCTCTGCTTCTTCCA
TLR9	CTGGAGAAGCTGGACATGCA	TGAAGGGCTGGCTGTTGTAG
IFI27	TTTGCCAGTAGGAGCCAAGG	AGGAGGAGGCAGTGATTCCT
IFI30	CTACACCTTGTCTGCCGGTT	GGCTTTGCTGAGTCAGGGAT
ISG20	GGCCCGAGGGTGACATCAC	CAGCGCCTTGAAGTCGTGCT
ZAP70	ACGCCAAGATCAGCGACTT	GGGCGCATACCACTTGAGC
SIKE1	ACCAGGTCAGGCAGAGG	CTTTCAGGACCGGTTCAGCATC
RNF125	GACACTCTGGTTTGCCTCAGT	ACTAAACGGCAAAGTGGACA
CYLD	AAGGCTACAGGATCTACCTCAG	GTGGTTGTGAGTCAACAGAAGA
NLRX1	GACCCAGATGCCTGTGGGC	TTGCGCACCAGCGTGCTCTTG
STING	GCGTTGGGACTGAGTCTTGA	TGGTGGTGTTTCTGGCTGTT
IFITM1	CTGACGACCACGGTGATCAA	TGATGTCGCCGACCATCTTC
IFITM3	GCTTAAGGAGGAGCACGAGG	TGAACAGGGACCACACGATG
IFIH1	TCTTATTGGCGCTGGACACA	CCTTCTTCTGCCACTGTGGT
IFI6	AAAAGATACTCAGAGGAAAACAGC	CATCAGTGAGGAGGCCAACGAGT
MX1	CCTGCGGCCGTAGTCTCTGCTGTCT	CATGGGGTGTGAAAGGCTATTAC
MX2	CTGCCCGCCATCGCTGTTATCG	AGCTGACTTTGCCTTTCCACTCG
IFIT1	TCTAAACAGGGCCTTGCAGG	GCACTTCAGCCATGCAAACA
GAPDH	GATTGTCAGCAATGCCTCCT	GGTCATAAGTCCCTCCACGA

Table 2. Differentially expressed genes associated with immune response (GO: 0006955 and GO: 0060337) at 2 hpi and 24 hpi

Gene ID	Genename	Protein	log2Fold Change		regulation
			2 h	24 h	
10012559	IRF7	Interferon regulatory factor 7	-1.0805	-0.3302	Down
100137737	PIDD1	p53-induced death domain-containing protein 1	-1.4506	-0.6936	Down
100138357	TLR13	Toll-like receptor 13	-2.2337	-1.4470	Down
100300510	V-TCR	Viral T-cell receptor beta chain-like T17T-22	-2.4117	-0.8672	Down
101902461	IGHM	Immunoglobulin heavy constant mu	-2.0308	-0.6729	Down
100139670	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1, IFIT-1	-0.6036	0.5688	Down/UP
101904342	Unkwon		-2.0912	-0.5966	Down
101905399	MP2K1	Dual specificity mitogen-activated protein kinase kinase 1, MAP kinase kinase 1	1.6315	0.8817	Up
107131142	TN13B	Tumor necrosis factor ligand superfamily member 13B	2.5355	0.6563	Up
280826	IL6	Interleukin-6	3.1302	1.7687	Up
280831	JUN	Transcription factor AP-1 (Activator protein 1, AP1)	0.2522	-0.4913	Up/ Down
280872	MX1	Interferon-induced GTP-binding protein Mx1	0.2973	1.3411	Up
280991	AKT3	RAC-gamma serine/threonine-protein kinase	-0.3475	-0.1926	Down
281048	CD14	Monocyte differentiation antigen CD14	2.7904	1.6051	Up
281073	IKKA	Inhibitor of nuclear factor kappa-B kinase subunit alpha	0.3704	0.4059	Up
281251	IL1B	Multifunctional fusion protein (Includes: Interleukin-1; Interleukin-1 beta)	2.1884	0.9302	Up
281408	PLAU	Urokinase-type plasminogen activator	1.2409	0.9751	Up
281440	RAC1	Ras-related C3 botulinum toxin substrate 1	1.1334	0.4546	Up
281499	SPP1	Osteopontin,OPN	-2.8820	-1.3743	Down
281534	TLR2	Toll-like receptor 2	0.3926	0.4348	Up
281850	IGHG1	Immunoglobulin heavy constant gamma 1	-2.9087	-0.6335	Down
281854	IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta	0.4542	-0.5201	Up/ Down
533223	IKKE	Inhibitor of nuclear factor kappa-B kinase epsilon subunit homolog 1, IKBKE	1.6054	0.4558	UP
281855	IKBKG	NF-kappa-B essential modulator, NEMO	-0.6517	-0.3850	Down
281857	IL12B	Interleukin-12 subunit beta, IL-12B	-0.7333	-1.8455	Down
281987	PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	-1.9601	-0.4078	Down
282023	PTGS2	Prostaglandin G/H synthase 2,cox-2	-1.5531	-0.4333	Down
282152	BCL2L1	Bcl-2-like protein 1, Bcl2-L-1 (Apoptosis regulator Bcl-X)	2.0242	1.7502	Up
282170	CCL3	C-C motif chemokine 3, SCYA3	-0.8464	-0.4205	Down
282258	IFNAR2	Interferon alpha/beta receptor 2	-0.4874	-0.9526	Down
282307	PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha, PI3-kinase regulatory subunit alpha, PI3K regulatory subunit alpha	-0.8549	-0.5501	Down
282527	TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A, TNFR1	-0.2498	-0.4574	Down
282602	TLR9	Toll-like receptor 9	-1.4592	-1.0121	Down
286849	CD40	CD40 ligand, CD40-L	1.5229	-0.4701	Up/ Down
286883	MAP2K6	Dual specificity mitogen-activated protein kinase kinase 6, MAP kinase kinase 6	-1.2655	-0.7933	Down
327712	CCL5	C-C motif chemokine 5, SCYA5	1.2276	-0.8678	Up/ Down
347700	EIF2AK2	Interferon-induced, double-stranded RNA-activated protein kinase, PKR	-0.4040	1.2915	Down/ Up
404109	IGHG2	Immunoglobulin heavy constant gamma 2	-2.9326	-0.4829	Down
407131	CD80	T-lymphocyte activation antigen CD80	-0.3815	0.2950	Down/ Up
407237	TLR6	Toll-like receptor 6	1.0333	0.9866	Up
414347	CCL4	C-C motif chemokine 4, SCY4A	-0.5452	-1.5640	Down
444881	MYD88	Myeloid differentiation primary response protein MyD88	0.3157	0.7378	Up
493720	FADD	FAS-associated death domain protein	0.8561	0.4406	Up
497199	CFLAR	CASP8 and FADD-like apoptosis regulator	0.6936	0.6236	Up
504507	TNFSF13B	Tumor necrosis factor ligand superfamily member 13B	2.8432	0.8164	Up

504509	ZAP70	Tyrosine-protein kinase ZAP-70	-2.9472	-0.8753	Down
504653	LTBR	Tumor necrosis factor receptor superfamily member 3	1.4498	0.6032	Up
504727	RIPK1	Receptor-interacting serine/threonine-protein kinase 1	0.5676	0.6115	Up
505901	PRKCQ	Protein kinase C theta type	-1.6829	-0.5520	Down
506604	ISG20	Interferon-stimulated gene 20 kDa protein	1.0151	1.3866	Up
506848	SIKE1	Suppressor of IKBKE 1	0.2907	0.6086	Up
507484	CASP8	Caspase-8	0.7014	0.4480	Up
508233	RELA	Bifunctional (p)ppGpp synthase/hydrolase RelA	1.0480	0.4738	Up
505276	DDX41	Probable ATP-dependent RNA helicase DDX41	1.2221	1.864	Up
528483	DDX10	Probable ATP-dependent RNA helicase DDX10	-0.6339	-0.4593	Down
507418	DDX51	Probable ATP-dependent RNA helicase DDX51	-0.8559	-0.9751	Down
510826	DDX52	Probable ATP-dependent RNA helicase DDX52		0.3368	
535126	DDX54	Probable ATP-dependent RNA helicase DDX54	-0.3170	-0.4606	Down
508581	DDX55	Probable ATP-dependent RNA helicase DDX55	0.7844	0.9073	Up
508728	DDX56	Probable ATP-dependent RNA helicase DDX56		-0.4749	Down
508378	DHX58	Probable ATP-dependent RNA helicase DHX58	-0.4027	0.7525	Down/ Up
506405	DHX16	Probable ATP-dependent RNA helicase DHX16	0.3052	0.2765	Up
506965	DHX34	Probable ATP-dependent RNA helicase DHX34	0.5960	0.4518	Up
508890	LCK	Tyrosine-protein kinase Lck	-2.1462	-0.9103	Down
509855	IRF9	HUMAN	-0.43701	0.3477	Down
510093	DDX3X	ATP-dependent RNA helicase DDX3X	-0.3125	0.3726	Down/ Up
510393	BLNK	B-cell linker protein	-0.8093	-0.6531	Down
510427	TICAM1	TIR domain-containing adapter molecule 1	1.8071	0.7284	Up
615930	IFI30	Gamma-interferon-inducible lysosomal thiol reductase, GILT	1.2038	-0.3533	Up/ Down
510814	STAT1	Signal transducer and activator of transcription 1-alpha/beta	0.6806	1.0941	Up
510923	TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	0.7529	1.0850	Up
512373	TKFC	Triokinase/FMN cyclase,DAK	-0.8394	-0.9184	Down
512913	IFI6	Interferon alpha-inducible protein 6	-0.6431	0.4839	Down/ Up
512943	MAPK12	Mitogen-activated protein kinase 12	2.3863	1.0708	Up
513038	CTSK	Cathepsin K	1.3518	0.6320	Up
513836	TNFSF11	Tumor necrosis factor ligand superfamily member 11	-6.6088	-2.5936	Down
514735	LAT	Linker for activation of T-cells family member 1	-1.6198	-0.6212	Down
514889	IFNGR2	Interferon gamma receptor 2, IFN-gamma receptor 2, IFN-gamma-R2	0.3675	-0.2189	Up/ Down
515377	CARD11	Caspase recruitment domain-containing protein 11	-1.5137	-0.7376	Down
515515	SYK	Tyrosine-protein kinase SYK	0.6282	0.5178	Up
515640	IL1R1	Interleukin-1 receptor type 1	0.8671	0.5319	Up
517948	PIK3CB	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform	1.2348	0.7218	Up
519541	RNF125	E3 ubiquitin-protein ligase RNF125	1.8224	1.2301	UP
523962	MAP3K1	Mitogen-activated protein kinase kinase kinase 1	0.4868	0.4092	Up
526469	MAP2K4	Dual specificity mitogen-activated protein kinase kinase 4, MAP kinase kinase 4	0.8372	0.6697	Up
529757	LTBP2	Latent-transforming growth factor beta-binding protein 2	2.1305	-0.7872	Up/ Down
530884	TNFRSF11A	Tumor necrosis factor receptor superfamily member 11A	0.9452	0.7040	Up
531079	TIRAP	Toll/interleukin-1 receptor domain-containing adapter protein	-1.0706	0.4089	Down/ Up
531391	MAPK3	Mitogen-activated protein kinase 3, MAP kinase 3	0.6463	0.7910	Up
532262	TLR8	Toll-like receptor 8	-1.4958	-0.5007	Down
533141	TBK1	Serine/threonine-protein kinase TBK1	0.9298	0.5684	Up
533199	MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1, MAP kinase kinase 1, MEK1	1.7738	1.1024	Up
533216	IKBKE	Inhibitor of nuclear factor kappa-B kinase subunit epsilon	1.5211	0.4558	Up
533661	TMEM173	Stimulator of interferon genes protein, ERIS, MITA, STING	-0.7046	0.2969	Down/ Up
533692	IRAK4	Interleukin-1 receptor-associated kinase 4	0.8041	0.4558	Up
534125	MAPK9	Mitogen-activated protein kinase 9	0.4329	0.5851	Up
534492	MAPK14	Mitogen-activated protein kinase 14, MAP	0.9916	1.0225	Up

		kinase 14			
534996	LYN	Tyrosine-protein kinase Lyn	1.1763	0.4651	Up
535327	MAPK13	Mitogen-activated protein kinase 13, MAP kinase 13	2.3265	2.0249	Up
535470	PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	0.4819	0.2186	Up
535622	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	0.8805	0.2926	Up
536421	CYLD	Ubiquitin carboxyl-terminal hydrolase CYLD	1.5235	0.9096	UP
539124	TRAF6	TNF receptor-associated factor 6	1.0078	0.5226	Up
539974	NLRX1	NLR family member X1	2.3460	0.6370	Up
540203	TAB2	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2	0.5059	0.5130	Up
540824	BCL10	B-cell lymphoma/leukemia 10	0.5599	0.2978	Up
540944	TRAF1	TNF receptor-associated factor 1	0.4740	-0.4845	Up/ Down
613464	IFITM1	Interferon-induced transmembrane protein 1	-1.8299	-1.4268	Down
613753	LY96	Lymphocyte antigen 96	-0.8608	-0.2878	Down
613845	IRF2BPL	Interferon regulatory factor 2-binding protein-like	1.2784	1.3427	Up
614838	PIK3R5	Phosphoinositide 3-kinase regulatory subunit 5, PI3-kinase regulatory subunit 5	0.6818	-0.1916	Up/ Down
615340	IRF5	Interferon regulatory factor 5	0.3004	-0.8504	Up/ Down
615930	IFITM3	Interferon-induced transmembrane protein 3	1.2038	-0.3533	Up/ Down
617420	IFI27	Interferon alpha-inducible protein 27	2.5072	5.7561	Up
618508	MAVS	Mitochondrial antiviral-signaling protein, Ips1, Visa	0.6098	0.6317	Up
618906	MAPK11	Mitogen-activated protein kinase 11, MAP kinase 11	1.3666	-0.4751	Up/ Down
767903	ATG12	Ubiquitin-like protein ATG12	0.6840	0.5125	Up
767932	AZI2	5-azacytidine-induced protein 2, NAP1, TBKBP2	0.7515	0.4546	Up
767939	IFRD2	Interferon-related developmental regulator 2	-0.4901	-0.9573	Down
785603	TBKBP1	TANK-binding kinase 1-binding protein 1, TBK1-binding protein 1	2.1172	1.6489	Up
787278	MAP2K7	Dual specificity mitogen-activated protein kinase kinase 7, MAP kinase kinase 7, MAPKK 7	-0.5272	-0.3009	Down
789216	IRF1	Interferon regulatory factor 1	0.2692	0.5236	Up
Novel01841	Novel01841	Unkown	-3.4592	-1.2632	Down
Novel01841	Novel02373	Unkown	-2.3094	-0.8320	Down

Additional Files Legends

Additional file 1:Table S1. Differentially expressed genes between mock and ncp BVDV1 infected samples at 2 hpi.(XLS 1601 kb)

Additional file 2:Table S2. Differentially expressed genes between mock and ncp BVDV1 infected samples at 24 hpi.(XLS 1281 kb)

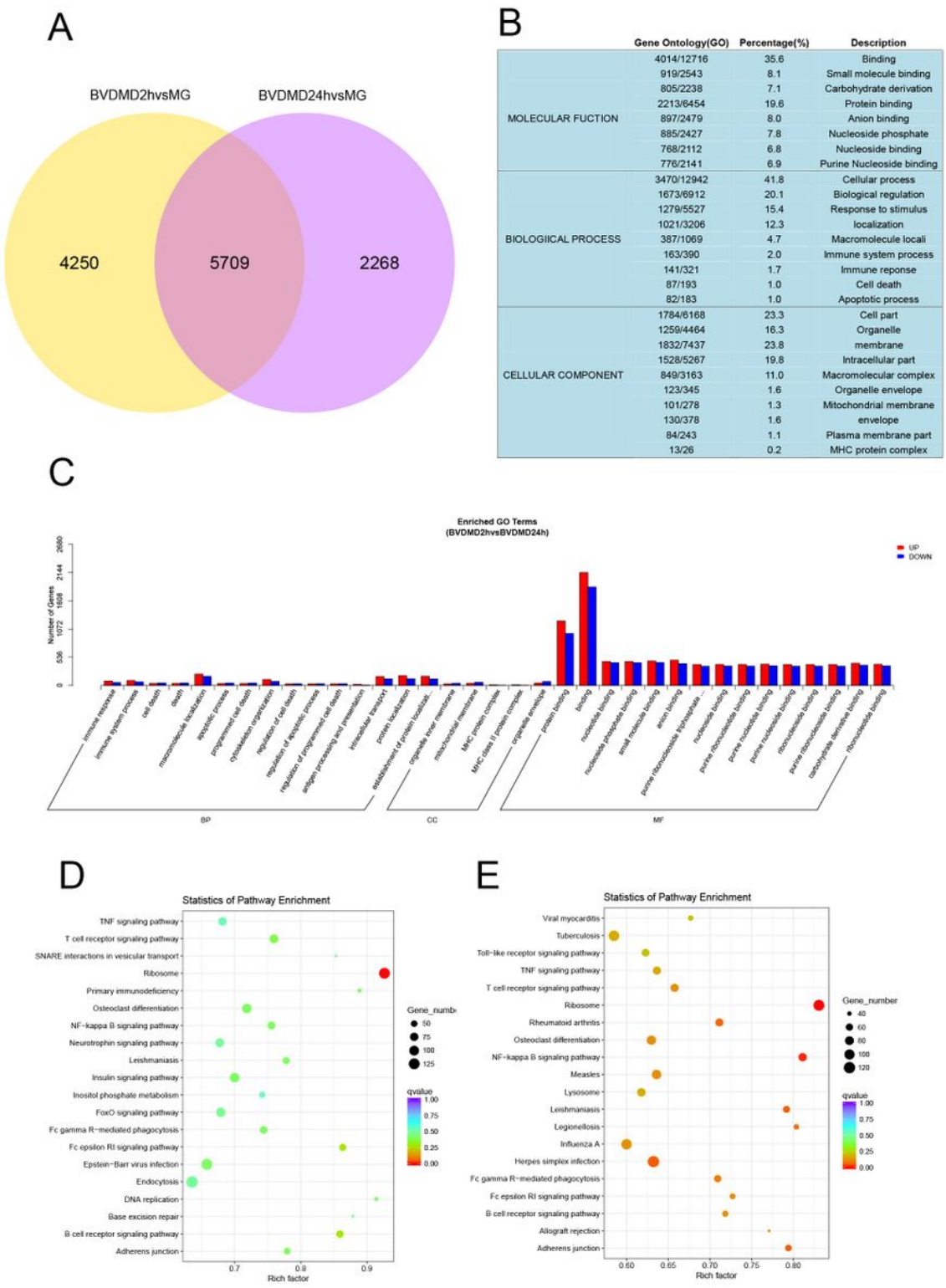
Additional file 3: Table S3. GO enrichment results of the DEGs between mock and ncp BVDV1 infected samples at 2 hpi. (XLS 2632 kb)

Additional file 4: Table S4. GO enrichment results of the DEGs between mock and ncp BVDV1 infected samples at 24 hpi. (XLS 2147 kb)

Additional file 5: Table S5. KEGG pathway enrichment result of the DEGs between mock and ncp BVDV1 infected samples at 2 hpi. (XLS 549 kb)

Additional file 6: Table S6. KEGG pathway enrichment result of the DEGs between mock and ncp BVDV1 infected samples at 24 hpi. (XLS 469 kb)

Figures



	Gene Ontology(GO)	Percentage(%)	Description
MOLECULAR FUCTION	4014/12716	35.6	Binding
	919/2543	8.1	Small molecule binding
	805/2238	7.1	Carbohydrate derivation
	2213/6454	19.6	Protein binding
	897/2479	8.0	Anion binding
	885/2427	7.8	Nucleoside phosphate
	768/2112	6.8	Nucleoside binding
	776/2141	6.9	Purine Nucleoside binding
BIOLOGIICAL PROCESS	3470/12942	41.8	Cellular process
	1673/6912	20.1	Biological regulation
	1279/5527	15.4	Response to stimulus
	1021/3206	12.3	localization
	387/1069	4.7	Macromolecule locali
	163/390	2.0	Immune system process
	141/321	1.7	Immune reponse
	87/193	1.0	Cell death
	82/183	1.0	Apoptotic process
CELLULAR COMPONENT	1784/6168	23.3	Cell part
	1259/4464	16.3	Organelle
	1832/7437	23.8	membrane
	1528/5267	19.8	Intracellular part
	849/3163	11.0	Macromolecular complex
	123/345	1.6	Organelle envelope
	101/278	1.3	Mitochondrial membrane
	130/378	1.6	envelope
	84/243	1.1	Plasma membrane part
	13/26	0.2	MHC protein complex

Figure 1

Gene ontology (GO) and KEGG analysis of DEGs at 2 hpi and 24 hpi. (A) A Venn diagram depicting the distribution of differentially expressed transcripts from ncp BVDV1 infected bovine monocytes (BIC) at 2h and 24h.(B) GO assignments are shown for the differentially expressed genes (DEGs) in BIC. Molecular

functions, Biological processes, and Cellular components (C) GO analysis of DEGs between 2 and 24 h after infection. (D) KEGG database analysis of signal pathways involved in DEGs 2 h after infection (E) KEGG database analysis of signal pathways involved in DEGs 24 h after infection.

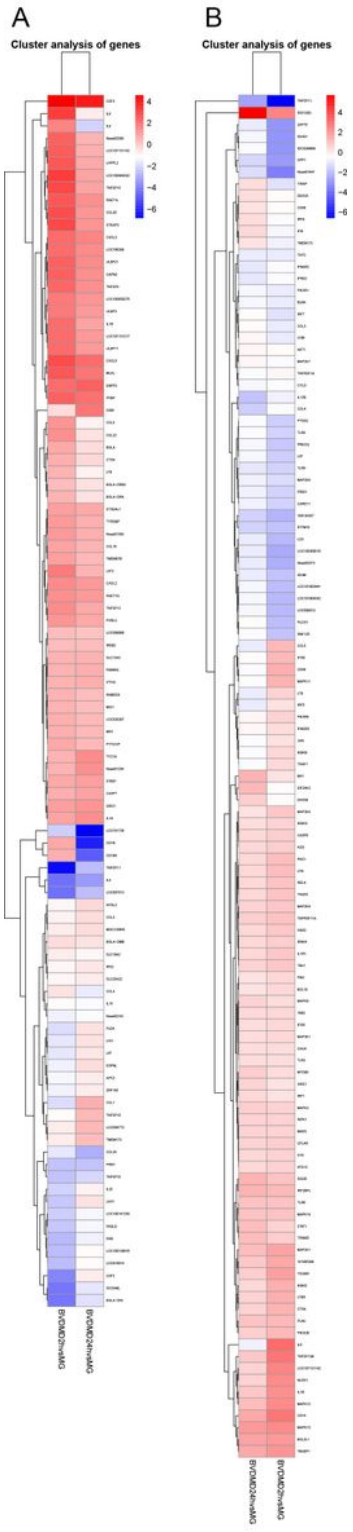


Figure 2

DEGs profiling of cellular genes involved in the immune responses and the type I interferon pathway in bovine monocytes after infection with ncp BVDV1. Heat maps show genes related to the immune

responses (left: GO: 0006955) and the type I interferon signaling pathway (right: GO: 0060337) that were either upregulated or downregulated upon ncp BVDV1 infection of bovine monocytes. The color coding represents a normalized expression of genes in monocytes infected with ncp BVDV1 (see color key). Gene upregulation is denoted in red and gene downregulation is denoted in blue.

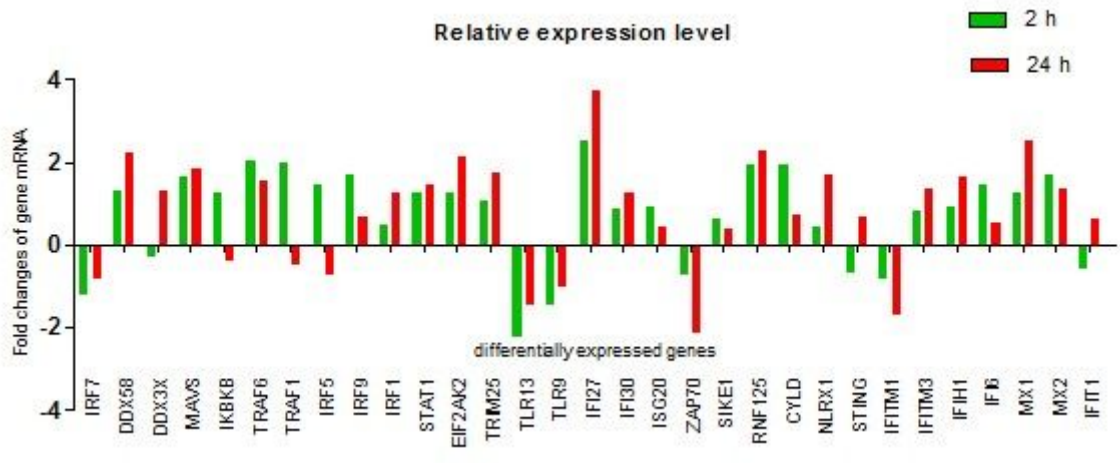


Figure 3

Partial validation of the DEGs involved in the type 1 IFN pathway by RT-qPCR. Bovine monocytes were isolated as described in the materials and methods section. The monocytes were infected with ncp BVDV1 and harvested 2h and 24h post-infection. RT-qPCR was performed on extracted RNA to amplify the selected genes using specific primers. Relative mRNA expression of the indicated genes is shown. Values are expressed as the fold change in with ncp BVDV1 of the 2h and 24h time points post-infection compared with mock-infected cells. Expression was normalized with GAPDH expression (the housekeeping gene). The results represent the mean and standard deviation (SD) of three samples from one experiment ($P < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile6TableS6.xls.xls
- Additionalfile3TableS3.xls.xls
- Additionalfile1TableS1.xls
- Additionalfile5TableS5.xls.xls
- Additionalfile4TableS4.xls.xls
- Additionalfile2TableS2.xls.xls