Global Transcriptomic Profiling of Bovine Endometrial Immune Response *in vitro*. II. Effect of Bovine Viral Diarrhea Virus on the Endometrial Response to Lipopolysaccharide[1]

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Running title: Effect of BVDV on the Endometrial Response to LPS

Summary sentence: Infection of bovine endometrial cells with non-cytopathic bovine viral diarrhea virus *in vitro* suppressed many innate immune genes that typically respond to LPS, suggesting mechanisms that may predispose the uterus to postpartum bacterial endometritis.

Keywords:Endometrium; gene expression; bovine viral diarrhea virus, innate immunity, uterine disease.

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ABSTRACT

Infection with non-cytopathic bovine viral diarrhea virus (ncpBVDV) is associated with uterine disease and infertility. This study investigated the influence of ncpBVDV on immune functions of the bovine endometrium by testing the response to bacterial lipopolysaccharide (LPS). Primary cultures of mixed epithelial and stromal cells were divided into 4 treatment groups (CONT, BVDV, CONT+LPS, BVDV+LPS) and infected with ncpBVDV for 4 days followed by treatment with LPS for 6 h. Whole-transcriptomic gene expression was measured followed by Ingenuity Pathway Analysis. There was differential expression of 184 genes between CONT and BVDV treatments, showing interplay between induction and inhibition of responses. Up-regulation of *TLR3,* complement, chemotactic and TRIM factors by ncpBVDV all suggested an on-going immune response to viral infection. Down-regulation of inflammatory cytokines, chemokines, *CXCR4* and serine proteinase inhibitors suggested mechanisms by which ncpBVDV may simultaneously counter the host response. Comparison between BVDV+LPS vs. CONT+LPS treatments showed 218 differentially expressed genes. Canonical pathway analysis identified the key importance of interferon signaling. Top down-regulated genes were *RSAD2*, *ISG15*, *BST2*, *MX2*, *OAS1*, *USP18*, *IFIT3*, *IFI27*, *SAMD9*, *IFIT1* and *DDX58* whereas *TRIM56*, *C3*, *OLFML1* were most up-regulated. Many of these genes are also regulated by IFNT during maternal recognition of pregnancy. Many innate immune genes that typically respond to LPS were inhibited by ncpBVDV including those involved in pathogen recognition, inflammation, interferon response, chemokines, tissue remodeling, cell migration and cell death/survival. Infection with ncpBVDV can thus compromise immune function and pregnancy recognition thereby potentially predisposing infected cows to postpartum bacterial endometritis and reduced fertility.

INTRODUCTION

Uterine bacterial infection and endometritis affect a significant number of dairy cows after calving and are associated with impaired reproductive performance and increased risk of reproductive culling [1]. In addition to the bacterial causes of postpartum uterine disease, certain viruses are considered as important etiological factors. Infection with bovine viral diarrhea virus (BVDV) [2, 3] and bovine herpes virus (BoHV)-4 [4] are both associated with reproductive disease. BVDV is an important single-stranded RNA (ssRNA) virus that causes widespread infection of cattle worldwide [5, 6]. BVDV can use the reproductive system to maintain and spread itself in cattle populations [2] and has been demonstrated in the uterine endometrium and other reproductive tissues of infected cows [7, 8].

Bacterial causes of bovine uterine disease such as *Escherichia coli* and *Trueperella pyogenes* have been considerably studied. Much less is known about the actions of viruses in reproductive tissues although BoHV-4 shows marked tropism for bovine endometrial epithelial and stromal cells, replicating and inducing intense cytopathic effects in cells infected in vitro [9]. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFA) are secreted by both immune and non-immune cells in response to invading microorganisms. An increase of BoHV-4 replication and cytopathic effect was observed in BoHV-4-infected and TNFA-treated bovine endometrial stromal cells, highlighting possible mechanisms through which bacterial infection of the uterus in postpartum cows may activate a persistent viral infection to cause chronic uterine disease or endometritis [10].

BVDV strains in cattle may exist as one of two biotypes, cytopathic (cp) and noncytopathic (ncp). These have marked differences in their viral mechanisms including cytopathogenicity, induction of interferon response and the ability to cause persistent infection [11]. Cytopathic strains are rare and are usually found in association with outbreaks of mucosal

disease. Noncytopathic strains predominate in nature and are associated with the most clinically severe form of acute BVDV infection [5]. While infection with ncpBVDV is associated with poor fertility [2, 12, 13] the underlying mechanisms are not clear. Cattle infected with ncpBVDV appeared increasingly susceptible to infection with bacteria and other viral pathogens [14, 15]. There is evidence that BVDV can lead to immunosuppression of the innate immune response in infected cells by a variety of mechanisms. These include inhibition of the interferon response [16], inhibition of lipopolysaccharide (LPS)-induced antimicrobial peptide expression [17] and interference with signaling, chemotactic, phagocytic, microbicidal and other functions of innate immune cells [18, 19]. These mechanisms have been studied in a variety of immune cell types [19, 20] but there is a paucity of information on the influence of BVDV on the innate immune functions of the bovine endometrium.

Our hypothesis is that infection with ncpBVDV leads to immunosuppression in bovine endometrium making cows less able to clear to bacterial infections after calving. In this study we utilized an *in vitro* model to investigate the influence of ncpBVDV infection on the bovine endometrial immune response to bacterial LPS using whole-transcriptomic profiling.

MATERIALS AND METHODS

Source and Propagation of Non-cytopathic BVDV

The ncpBVDV (Pe515nc strain) was acquired from the BVDV Research Group, Royal Veterinary College, UK. The Pe515nc strain is a virologically cloned non-cytopathogenic virus isolated from a cow diagnosed with mucosal disease. Virus isolation in calf testis was subsequently followed by expansion of the virus stock [21]. To provide adequate infectious BVDV for the

present experiments, the virus stock was first propagated in BVDV-free Madin-Darby bovine kidney (MDBK) epithelial cells (ATCC). After incubation, the cells were lysed, centrifuged at 800 x g and 4ºC for 10 min to allow for sedimentation of cell debris and aliquots of the supernatant containing the propagated BVDV were preserved at -80ºC. The concentration of the propagated BVDV was determined using a virus serial dilution microtitre assay in MDBK cells with a ten-fold serial dilution of the BVDV suspension ranging from neat to 10^{-8}. The plate was incubated for 5 days then the cells were washed in phosphate buffered saline (PBS), fixed in 80% acetone at -20ºC for 20 min prior to immunostaining using a primary BVDV-specific mouse monoclonal antibody (VLA, Weybridge, Surrey, UK) as described below. The 50% tissue culture infective dose ($TCID_{50}$) per mL was calculated and the Pe515nc virus used in the subsequent study had a $TCID_{50}$ of 5 x 10^5 per ml.

Screening of Experimental Samples for BVDV

The uterine tissues and fetal bovine serum (FBS) used in the cell culture experiments were first confirmed to be free of BVDV infection or contamination respectively using Trizol RNA extraction (Invitrogen) followed by conventional Reverse Transcription-PCR as described previously [22, 23]. RNA integrity was confirmed using agarose gel electrophoresis. Reverse transcription of 1 µg RNA was performed using the GoScript RT System (Promega). Conventional PCR was performed using the G-Storm thermal cycler (Gene Technologies) and the Qiagen Multiplex PCR kit (Qiagen). The primer pair for the PCR was selected from the highly conserved 5′ non-coding/non-structural coding regions of the pestivirus BVDV genome strain NADL [24]. The 5′→3′ nucleotide sequences of the primers in the NADL strain were: forward (ATGCCCWTAGTAGGACTAGCA; position 108-128) and reverse

(TCAACTCCATGTGCCATGTAC; position 395-375) with an expected product size of 288 bp. A BVDV-positive control was prepared using the pT7Blue-2 blunt vector, linearized (Novagen). Reverse transcription-negative controls (sample cDNA replaced by respective RNA) were included to verify that PCR reagents and reaction volumes were free of genomic DNA contamination. Beta-actin (*ACTB*) was measured as an endogenous reference gene to validate PCR efficiency (data not shown). Further experiments were continued only with endometrium that initially tested negative for BVDV.

Infection of Bovine Endometrial Cells with ncpBVDV

Fresh and apparently healthy bovine reproductive tracts in the early luteal phase of the estrous cycle were collected at slaughter from the local abattoir and preserved temporarily in ice. Primary mixed endometrial epithelial and stromal cell cultures were prepared as outlined in Oguejiofor et al. [25]. These contained both surface and glandular epithelium. Isolated cells were allocated at 5 x 10^5 cells/well to sterile 24-well plates (Nunc). They were cultured in growth medium (GM) which comprised MEM (PAA) containing 10% FBS and 1% antibiotic solution (100 IU/ml penicillin + 100 µg/ml streptomycin; Sigma) for 4 days and then divided into 4 treatment groups (CONT, BVDV, CONT+LPS and BVDV+LPS). Wells treated with virus were maintained in separate plates from the untreated controls to prevent cross-contamination. For the cell cultures specified as the non-infected control, the GM was replaced with 0.25 ml of maintenance medium (MM) containing 5% FBS only. Endometrial cell cultures were infected with Pe515nc in 0.25 ml of MM at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell and then incubated for 3 h to allow for virus adsorption. The volume in each well was then made up to 1 ml with MM and the plates incubated for 4 days with the MM changed after 2 days. After incubation,

culture media were discarded and the specified cultures treated with 100 ng/ml lipopolysaccharide (ultra-pure LPS from *E. coli* 0111:B4 strain; Invivogen) in warm MM for 6 h. For immunostaining, endometrial cell cultures were washed in PBS and then fixed in 80% acetone at -20ºC for 20 min.

Six separate batches of cells each obtained from an individual cow were used in the main experiment and analyzed as biological replicates. The cells isolated from each cow were divided into the 4 treatment groups (CONT, BVDV, CONT+LPS and BVDV+LPS) with 6 wells per treatment. These cultures were established on four different occasions but the same batches of reagents were used for all cultures to minimize variations in culture conditions. Endometrial cell cultures were validated using specific immunocytochemical staining as previously described [26]. The relative proportions of each cell type after 8 days of culture were evaluated using image analysis software (ImageJ version 1.44; Research Services Branch, NIMH/NIH, Bethesda, USA). The stromal cells comprised 9.5% of the population present before LPS challenge (range 7-12% for cultures derived from n = 6 cows). There was negligible contamination with immune cells (< 0.001%). No endothelial cells were present based on morphological appearance although these were not specifically stained for.

Demonstration of ncpBVDV Infected Cells

The infection of bovine endometrial cells with ncpBVDV was validated by both conventional PCR and gel electrophoresis using isolated total RNA as described above and by indirect enzyme (alkaline phosphatase) immunostaining. Briefly, the fixed endometrial cell cultures were washed in PBS. A 1:100 dilution of the primary BVDV-specific mouse monoclonal antibody (VLA) was made in PBS-NRS-T diluent made of PBS containing 5% normal rabbit serum (NRS; Invitrogen-Gibco) and 0.05% Tween 20 (BDH Prolabo). The cells were incubated

with the primary antibody for 45 min at 37ºC and then washed in PBS. A 1:1000 dilution of the secondary antibody (Anti-Mouse IgG-Alkaline Phosphatase; Sigma) was made in PBS-NRS-T diluent and then incubated on the cells for 45 min at 37ºC. The cells were washed in PBS and each well was covered in 50 µL of SIGMAFAST BCIP/NBT substrate solution (Sigma) for 10-20 min at room temperature until suitable staining developed.

Assessment of Endometrial Cell Viability

Bovine endometrial cell viability was assessed following infection with ncpBVDV and/or exposure to LPS using the MTS reduction assay method as described in [25].

Isolation, Quantitative and Qualitative Analysis of RNA

Isolation of total RNA from the experimental bovine endometrial cell cultures was performed using Qiagen RNeasy Mini spin columns following the manufacturer's protocol. All RNA samples showed concentrations of ≥230 ng/µl. Total RNA samples for microarray analysis were assessed for optimum quality using the Agilent 2200 TapeStation. The RNA integrity numbers (RINs) for all the experimental RNA samples were ≥ 9.

Microarray Hybridization and Statistical Analysis

Twenty-four arrays were used to analyze the respective 24 samples representing total RNA from the four endometrial cell culture treatment groups (CONT, BVDV, CONT+LPS, and BVDV+LPS) with cell cultures from six different cows for each treatment group. Whole-transcriptome gene expression was measured by Affymetrix Bovine Gene 1.1 ST 24-Array containing the probes for 23,000 transcripts (Affymetrix). Processing, normalization and further

analysis of the microarray data were performed using RMA16 built in Genespring GX software version 12.5 (AgilentTechnologies) using the annotation files provided by the company which had annotation of over 95% of the probes.

Differences in gene expression between the treatment groups of bovine endometrial cells that met the cut-off -1.2≤ Fold Change ≥1.2 were next compared using a one way ANOVA with randomized block design (in which treatment was taken as the fixed effect and cow as the random effect) and paired *t*-test with *P* values adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) method. The differentially expressed genes (DEGs) were considered significant based on the adjusted *P* value of < 0.05. A hierarchical cluster analysis of the DEG was performed showing a heat map of expression levels for the overall gene expression pattern by treatment group (Suppl. Fig. S1).

Ingenuity Pathway Analysis (IPA)

Array data were next analyzed through the use of Ingenuity Pathway Analysis (IPA; QIAGEN, www.qiagen.com/ingenuity). IPA was used to query the Ingenuity Knowledge Base in order: (1) to determine the biological functions and pathways associated with the resultant DEGs between the different experimental groups; (2) to undertake network analysis to show the interaction between the genes in a list of DEGs and how these may represent significant biological functions; (3) to perform a downstream effects analysis to identify the biological processes, diseases or toxicological functions affected in the bovine endometrial cells and (4) to perform an upstream regulator analysis to evaluate the number of known targets of a given transcription regulators that were present in the list of DEGs.

Real-time RT-PCR Validation of Microarray Data

Following the microarray analysis, a total of 15 DEGs and 2 endogenous reference genes were selected for validation of array data by qRT-PCR as described previously [25]. Analysis using linear mixed-effects model showed that there were no significant differences in the mRNA expression of the two reference genes *RN18S1* and *ACTB* in bovine endometrial cells after infection with ncpBVDV or exposure to LPS or the combination of both treatments when compared to the control. The mRNA expression was calculated using a known standard curve. The mean mRNA concentration ± SEM of 6 samples measured for *RN18S1* were 8.4 ± 1.46, 8.8 ± 0.95, 8.9 ± 1.01 and 8.5 ± 0.75 µg/µgRNA in the CONT, BVDV, CONT+LPS and BVDV+LPS groups respectively. The equivalent values for *ACTB* were 11 ± 1.99. 10.7 ± 1.54, 11.1 ± 1.7 and 9.9 ± 1.39 fg/µgRNA respectively. The mRNA expression values of the 15 selected genes were therefore normalized to *RN18S1* and *ACTB* by dividing the sample value for each gene with the corresponding sample normalization factor derived from geNorm version 3.4 [27].

Statistical Analysis of qRT-PCR Data

For each gene, the mean qRT-PCR expression values were derived from duplicate samples then the normalized qRT-PCR expression data were compared between the four treatment groups of endometrial cell cultures using IBM SPSS Statistics for Windows, Version 20.0). A linear mixed-effects model was used with the exposure to LPS or infection with ncpBVDV or combination of both treatments held as the fixed effect while the cow was considered as the random effect. Significant differences between means were confirmed using Fisher's LSD post hoc test. Data are presented as mean ± SEM. Results were considered significant when $P < 0.05$.

RESULTS

This paper describes the influence of infection with ncpBVD on the endometrium and so includes the comparisons between (i) BVDV vs. CONT and (ii) BVDV+LPS vs. CONT+LPS. The effect of LPS alone (CONT vs LPS) has been described in a separate paper [25].

Validation of Experimental Methods

Infection of the cultured endometrial cells with ncpBVDV for 4 days and/or exposure to 100 ng/ml LPS for 6 h did not significantly alter the number of viable cells. The absorbance values at 490 mm in the cell viability assay were 2.0 ± 0.05, 2.0 ± 0.08, 2.1 ± 0.05 and 2.1 ± 0.03 in the CONT, BVDV, CONT+LPS and BVDV+LPS treatment groups respectively (mean ± SD of 4 samples per group). At Day 8 of culture in the main experiment, prior to LPS treatment, the average number of endometrial cells per culture for the CONT /non-infected wells did not differ significantly from the BVDV-infected wells for the six different batches of cells cultured from the different cow samples. The cell numbers at this stage were 181,667 ± 2,189 and 183,542 ± 2,671 respectively (mean ± SD). Both epithelial and stromal cells in infected cultures showed positive staining for BVDV (Fig. 1) and BVDV RNA was, detected in the endometrial cultures of the *in vitro* infected groups but not in the non-infected groups by conventional PCR (Fig. 2). This confirmed that the control cultures were not contaminated with virus but that there was successful infection of the treated wells. The number of detectable probes (22,024) and their mean expression values (6.72, range 4.2-13.4) were identical between treatment groups confirming that the LPS and BVD treatments did not bias the results by altering the number of expressed genes.

Effects of BVDV on the Innate Immune Response in Bovine Endometrial Cells (BVDV vs.CONT)

After four days of infection, ncpBVDV significantly altered the expression of 184 genes with fold changes ≥1.2 in bovine endometrial cells compared with the control. Of these, 121 genes (66%) were up-regulated whereas 63 genes (34%) were down-regulated (Suppl. Table S1 and Suppl. Fig. S1). Half of the most up-regulated genes (8/15 with ≥1.4-fold increase) had known activities related to immunity, inflammation, the complement pathway and tissue remodeling (*TRIM56, C3, CHI3L1, ANK2*, *C1S, CIR, VCAM1, XPNPEP2)* (Table 1). HBA is a sub-unit of bovine hemoglobin. For another four highly up-regulated genes, however, their function is largely unknown *(OLFML1*, *ZBTB2*, *DTWD2*, *SCUBE2*). Among the seven most down-regulated genes (≥1.4-fold decrease), four had established immune function (*CDH6*, *SERPINB2, CALCA* and *IL33)* while little is known regarding *MAMDC2, PHOSPHO1* and *SSLP1*.

Diseases and Functions and Canonical Pathways Analysis

From the total of 184 genes altered by ncpBVDV infection, 164 genes were mapped and analyzed with IPA. The five most significant disease processes and biological functions identified (all $P<0.001$) were cellular movement, hematological system development and function, immune cell trafficking, inflammatory response and cell-to-cell signaling and interaction (Table 2). The three most significant canonical pathways whose expression was significantly altered by ncpBVDV infection ($P<0.001$) are also shown in Table 2 and Suppl. Table S2. There was significant down-regulation of many of the genes involved in the pathways for agranulocyte or granulocyte adhesion and diapedesis (*CCL4*, *CCL26*, *CCL27*, *CXCR4*, *IL18*, *IL33*, *MMP12*, *MMP13* and *MYL3*). Of these *CCL4*, *IL18* and *IL33* are also involved in communication between innate and adaptive immune cells. On the other hand, ncpBVDV infection significantly up-regulated genes involved in liver X receptor/retinoid X receptor (LXR/RXR) activation (*APOD*,

C3, *C4A/C4B* and *TLR3*), NF-κB signaling (*GHR*, *TGFBR3*, *TLR3* and *TNFAIP3*), cAMP-mediated signaling (*ADORA2A*, *AGTR1*, *ENPP6*, *PDE1A* and *PRKAR2B*) and the complement system (*C3*, *C1R*, *C1S* and *C4A/C4B*).

Interaction Network Analysis

IPA identified 14 network functions altered by ncpBVDV infection. The top three networks containing 16 or more focus molecules are listed in Table 2. Network 1 (score 34) contained 19 DEGs involved in cellular movement, connective tissue disorders and metabolic disease (Fig. 3). There was decreased expression of the chemokine receptor *CXCR4* which interacted directly with the up-regulation of chemotactic cytokine *CXCL3*, and indirectly with the down-regulation of the cell-to-cell adhesion factor *CDH2*.

Network 2 (score 33) contained 19 DEGs involved in developmental disorder, hereditary disorder and immunological disease (Fig. 4). There was down-regulation of the pro-inflammatory cytokine *IL33* which indirectly, through the extracellular signal-regulated kinase (ERK) complex, induced the down-regulation of the chemotactic cytokines *CCL26* and *CCL27*. In addition, the increased expression of complement component 3 (*C3*) interacted directly with the up-regulation of components of the classical complement activation pathway (*C4A*/*C4B*, *C1R* and *C1S*).

Network 3 (score 27) contained 16 DEGs involved in cellular movement, hematological system development and function and immune cell trafficking (Suppl. Fig. S2). NcpBVDV infection increased the expression of the dsRNA-recognition receptor *TLR3*. The decreased expression of the IL1 pro-inflammatory cytokine complex also had a direct interaction with the down-regulation of the pro-inflammatory cytokine *IL18* as well as an indirect connection with the up-regulation of *TNFAIP3*, a known inhibitor of TNF-induced NF-kappa B responses. In addition, there was down-

regulation of the matrix metalloproteinases (*MMP12* and *MMP13*) and the proteinase inhibitors *SERPINB10* and *SERPINB2*.

Upstream Regulator Analysis

There was predicted upstream inhibition ($P < 0.01$) of *ERK* (a *MAPK*) and *JNK* intracellular signal transducers (group factors), *EDN1* (a cytokine), *TGFB1* (a growth factor), *FN1* (an enzyme), and *PPARA* (a ligand-dependent nuclear receptor). On the contrary, there was predicted upstream activation ($P < 0.001$) of tumor necrosis factor receptor type 1-associated DEATH domain (*TRADD*).

Effects of ncpBVDV on the Innate Immune Response of Bovine Endometrial Cells to Bacterial LPS (BVDV+LPS vs. CONT+LPS)

The effect of ncpBVDV infection for 4 days on the immune response to LPS was examined after 6 h LPS exposure: expression of 218 genes was significantly altered. Of these 123 (56%) were up-regulated whereas 95 (44%) were down-regulated (Suppl. Table S3 and Suppl. Fig. S1). Six of the most up-regulated genes (≥ 1.5-fold increase) also appeared in the list affected by BVDV treatment alone (*TRIM56*, *C3*, *OLFML1*, *ZBTB2*, *DTWD2, HBA*) so validating these results (Table 3). In addition *ALPL,* a phosphatase which is known to dephosphorylate LPS, was also up-regulated 1.5 fold. The most down-regulated genes (≥ 2-fold decrease) were mainly interferon response genes (*RSAD2*, *ISG15*, *BST2*, *MX2*, *OAS1Y*, *USP18*, *IFIT3*, *OAS1Z*, *IFI27*, *IFIT1* and *MX1*).

Diseases and Functions and Canonical Pathways Analysis

From the total of 218 DEGs, 193 were mapped and analyzed with IPA. The most significant disease processes and biological functions associated with them were infectious disease, post-translational modification, protein folding, antimicrobial response, inflammatory response, cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking and inflammatory disease. The most significant canonical pathways associated with the DEGs are shown in Table 4 and Suppl. Table S4. There was a significant down-regulation of the genes involved in the pathways for the activation of IRF by cytosolic PRRs (*ADAR*, *DDX58*, *DHX58*, *IFIH1*, *ISG15* and *STAT1*), interferon signaling (*IFIT1*, *IFIT3*, *MX1* and *STAT1*), oncostatin M signaling (*MMP1*, *MMP13* and *STAT1*), role of PRRs in recognition of bacteria and viruses (*CCL5*, *DDX58*, *EIF2AK2* and *IFIH1*) and role of RIG1-like receptors in antiviral innate immunity (*DHX58*, *IFIH1* and *DDX58*). Several of these genes (*CCL5*, *MMP1* and *MMP12)* plus *MMP13* and *MYL3* were also identified as being involved in agranulocyte adhesion and diapedesis. On the contrary, there was significant up-regulation of genes involved in the pathways for complement system (*C3*, *C1R*, *C1S* and *SERPING1*) and eicosanoid signaling (*PTGES*, *PTGDR* and *PLA2G4F*).

Network Analysis

The top three identified networks are shown in Table 4. Network 1 (score 33) showed inhibition of the components of a type I IFN response and contained 19 DEGs involved in infectious disease, post-translational modification and protein folding (Fig. 5). The down-regulation of the type I IFN transcription regulatory factor complex *ISGF3* and several IFNA inducible genes (*IFI27*, *MX1*, *IFIT1*, *IFIT3*, *IFI6*) had both direct and indirect interactions with the decreased expression of genes

involved in intracellular pathogen recognition and antiviral signaling (*DHX58*, *DDX58*, *IFIH1* and *EIF2AK2*) and a host of other IFN-stimulated genes (*IFIT5*, *BST2*, *HERC5*, *RSAD2* and *ISG15*). Network 2 (score 30) contained 19 DEGs involved in dermatological diseases and conditions, hereditary disorder and immunological disease (Fig. 6). There was increased expression of the complement regulatory factor *SERPING1* which had a direct interaction with the up-regulation of components of the classical complement 1 pathway (*C1R* and *C1S*). On the other hand, there was down-regulation of the matrix metalloproteinases (*MMP1* and *MMP12*) which interacted indirectly with the up-regulation of *PTGES,* an enzyme involved in the synthesis of PGE_2.

Upstream Regulator Analysis

The top upstream regulators with a predicted inhibition state ($P < 0.001$) were transcription regulators (IRF3 and IRF7), cytokines (IFNL1, IFNA2, and IFNB1), a transmembrane receptor (TLR3), lipopolysaccharide (Suppl. Fig. S3) and IFN type 1 (Suppl. Fig. S4).

Validation of Microarray Gene Expression Data by qRT-PCR

A comparison of the array and qRT-PCR data for the 15 selected DEGs showed similar patterns of changes in gene expression for the respective treatment groups (Table 5). The qPCR data are also illustrated in Figs. 7 and 8. Infection of bovine endometrium with ncpBVDV suppressed the response to LPS with respect to several interferon responsive genes (*BST2, GBP5, ISG15, MX2, RSAD2)* and the chemokine *CCL5* but did not influence the LPS induced up-regulation of *CX3CL1* and *IL1A*. ncpBVDV infection increased the expression of *TRIM56* and *C3* and caused an increase in *RND1* and *PTGES* compared with LPS alone. It did not alter the up-regulation caused by LPS with respect to *VCAM1*. Expression levels of *STAT1* and *AMIGO2* were numerically lower

following treatments with ncpBVD and/or LPS in comparison with CONT but this did not achieve statistical significance.

DISCUSSION

Effects of ncpBVDV on Bovine Endometrial Innate Immune Response (BVDV vs. CONT)

BVDV is an important viral pathogen of cattle that can invade the reproductive tissues and reduce conception rates [2, 6]. Here, we investigated the effects of an *in vitro* established infection with ncpBVDV on the ability of bovine endometrial cells to respond to LPS four days later. Immunostaining confirmed that both the epithelial and stromal uterine cells became infected, consistent with previous studies showing BVDV distribution in the uterine endometrium of infected cows [7, 8]. Infection with a relatively low dose (0.1 MOI) of ncpBVDV did not appear to compromise endometrial cellular integrity and function. This is consistent with previous studies in which ncpBVDV did not produce the cytopathic effects typically observed in cpBVDV-infected cells [28]**.** The effects of BVDV alone on changes in gene expression after 4 days of infection were more subtle than the changes induced by the addition of LPS [29].

Viral recognition by toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) triggers an immune response in the host cells characterized by the activation of NF-κB, MAP kinases, and IRFs and the expression of type I interferon and inflammatory cytokines which are important for eliminating the viruses [30]. ssRNA viruses such as BVDV are recognized by TLR7 and TLR8 [31]. There is no available information on the expression of TLRs in the endometrium of ncpBVDV-infected cows. The expression of *TLR2*, *TLR3* and *TLR4* was not altered after 24 h of

ncpBVDV infection in bovine fetal muscle fibroblast cells (BFM) or MDBK epithelial cells [29]. In contrast, infection of bovine monocytes with ncpBVDV significantly increased the expression of *TLR3* in early infection (1 h) whereas *TLR7* was up-regulated at 24 h post-infection [32]. In this study, an increased expression of *TLR3* was observed after four days. TLR3 is known to recognize dsRNA viruses and can also detect dsRNA intermediates generated during the replication of ssRNA viruses in infected cells [33].

The expression of the pro-inflammatory cytokines *TNF*, *IL1B* and *IL6* were down-regulated in bovine monocytes after 24 h of ncpBVDV infection [27] but *IL6* was not altered in BFM or MDBK cells at this time [29]. Here, ncpBVDV down-regulated expression of *IL18* and *IL33* after 4 days. These inflammatory cytokines belonging to the IL1 family are constitutively expressed in mucosal or barrier cell types, where they act as regulators of innate and acquired immune responses by amplifying both Th1 and Th2 responses [34]. The down-regulation of both *IL18* and *IL33* suggests that the ncpBVDV infection can disrupt an important bridge between early and late immune response. Chemokines have important functions in leucocyte trafficking during inflammation. Infection with ncpBVDV increased expression of *CXCL3*, *CCL28* and *CX3CL1* whereas *CCL4*, *CCL26*, *CCL27* and the chemokine receptor *CXCR4* were down-regulated. Similarly, ncpBVDV down-regulated the expression of several chemokines in infected bovine macrophages [35] while the presence of a fetus persistently infected with ncpBVDV caused a down-regulation of *CXCR4* mRNA in the blood of the dam [36]**.**

Interferon gamma (IFNG), a type II IFN, has an important role in the restriction of BoHV-4 replication in bovine endometrial stromal cells, possibly limiting viral reactivation from a latent state to a chronic uterine disease [37]. Moreover, Type I IFNs protect uninfected cells against viral pathogens, prevent viral replication and serve as a key link to the activation of an adaptive immune

response [38]. There is evidence that ncpBVDV can evade the innate immune system by the activity of viral proteins which inhibit the induction of a type I IFN response [16]. In support of this, there was no evidence of an IFN response at 4 days post infection with ncpBVDV. Although this may have occurred at an earlier time point, other *in vivo* and *in vitro* studies have likewise described the absence of a type I IFN response following infection with ncpBVDV, unlike cpBVDV which triggered marked IFN induction [39, 40]. Similarly rabies, another RNA virus, is able to counteract transcriptional activation of interferon genes and interferon signaling pathways [41]. In contrast, experimental infection with ncpBVDV induced type I IFN in calves, pregnant dams and the fetus [42,43].

Here, infection with ncpBVDV up-regulated the expression of the anti-apoptotic factors, tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) and arrestin, beta 1 (*ARRB1*) but down-regulated the pro-apoptotic genes death associated protein-like 1 (*DAPL1*), B-cell CLL/lymphoma 2-Like 15 (*BCL2L15*) and the phagocytosis-related genes, c-mer proto-oncogene tyrosine kinase (*MERTK*) and transglutaminase 2 (*TGM2*). This may play an important role in the absence of cytopathic effects. *In vitro* studies demonstrate infection with cpBVDV, but not ncpBVDV, induces apoptosis in bovine cells [44].

Infection with ncpBVDV altered the expression of intercellular adhesion factors in endometrium. L-Selectin (*SELL*) and the vascular cell adhesion molecule *VCAM1* were up-regulated whereas the cadherins *CDH2* and *CDH6* and endomucin (*EMCN*) were down-regulated. The functional roles of adhesion molecules in cell proliferation [45] and leucocyte migration [46] are facilitated by the cleavage and proteinase activity of matrix metalloproteinases. In this study, infection with ncpBVDV decreased the expression of *MMP12* and *MMP13*. Furthermore, the biological functions of cell adhesion molecules and immune cell migration are intimately linked

to activation of the actin cytoskeleton [47]. Here, expression of myosin, light chain 3 (*MYL3*) was decreased while Rho family GTPase 1 (*RND1*) was increased. RND1 is an important modulator of actomyosin contractility [48]. Viruses seem to induce rearrangements of cytoskeletal filaments in infected cells so that they can utilize them as tracks during attachment, endocytosis, replication and exocytosis [49].

Infection with ncpBVDV also triggered an increase in the expression of the complement components *C3*, *C1R*, *C1S*, *C4A* and *C4B*. The complement pathway is important in immune clearance of viral pathogens by direct inactivation of virions, by triggering inflammation and through regulation of antibody and immune cells effector functions [50]. The expression of several serine peptidase inhibitors including *SERPINB2*, *SERPINB10* and *SERPINA14* was decreased. The Kazal type serine peptidase inhibitors *SPINK5*, *SPINK7* and *SPINK9* were also decreased. These inhibitors limit damage caused to virally infected cells by serine proteases.

Finally, infection with ncpBVDV up-regulated the expression of the tripartite motif (TRIM) factors *TRIM56* and *TRIM38*. Previously, the expression of TRIM56 was up-regulated by BVDV infection, and TRIM56 acted as an antiviral host factor against BVDV by suppressing intracellular viral RNA replication [51]. TRIM38 is thought to limit the excessive production of type I IFNs in response to viral infection by mediating degradation of TRIF, a critical adaptor of the TLR3-mediated immune response [52]. In addition, TRIM38 negatively regulates inflammatory processes by inhibiting TNFα- and IL1B–triggered activation of NF-κB and induction of downstream cytokines [53].

In summary, the response of bovine endometrial epithelial and stromal cells after four days of ncpBVDV infection showed interplay between induction and inhibition of immune and inflammatory response genes. The mRNA up-regulation of *TLR3* in addition to increased

expression of complement, chemotactic and TRIM factors all suggest an on-going immune response to viral infection. On the other hand, ncpBVDV appeared to counter the host immune response by down-regulating the expression of many immune-related genes, including inflammatory cytokines, chemokines, *CXCR4*, serine proteinase inhibitors and *S100A12* antimicrobial factor. In addition ncpBVDV appeared to inhibit IFN response and apoptosis signaling in infected cells. The altered expression of cell adhesion and extracellular matrix factors may inhibit leucocyte migration and promote ncpBVDV replication and proliferation in infected endometrial tissue.

Effects of ncpBVDV on the Innate Immune Response of Infected Bovine Endometrial Cells to Bacterial LPS (BVDV+LPS vs. CONT+LPS)

Infection with ncpBVDV has been associated with reduced conception rates in dairy cows although the mechanisms are uncertain [12,13]. Uterine disease is a major factor in reducing fertility [1]. Bacterial infection of endometrial stromal cells was previously linked to BoHV-4 replication in virus-infected endometrial cells [54]. Moreover, an increase in BoHV-4 replication and cytopathic effect was associated with the presence of the pro-inflammatory molecule TNFA [10]. This suggests a possible mechanism whereby the interaction between an existing viral infection and a consequent inflammatory response to bacterial infection may compromise uterine function leading to endometritis. In addition, there is evidence that animals infected with BVDV are more vulnerable to infection with other pathogens [5]. Therefore, we hypothesized that ncpBVDV infection may interfere with the immune functions of the endometrium thereby predisposing the uterus to bacterial infection and endometritis.

In support of our hypothesis, prior infection with ncpBVDV inhibited the expression of several LPS-inducible genes that play important roles in both intra-cellular recognition of bacteria and the consequent activation of an innate immune response. There was significant inhibition of the RLRs *DDX58*, *IFIH1* and *DHX58*, the protein kinase *EIF2AK2*, and the adenosine deaminase *ADAR*. DDX58 is important in intracellular recognition of bacterial mRNA, leading to the activation of NF-kB and interferon pathways of the innate immune system [55]. EIF2AK2 is required for MAPK and NF-kB signaling and the induction of pro-inflammatory cytokines in response to bacterial LPS [56]. These intracellular receptors are important in antiviral immune response, and many viruses have developed counter-measures against them [57]. For instance, BVDV is thought to evade recognition by PRRs in the host cells via the RNase activity of the viral protein E^{rns} which degrades viral RNA [16]. Also, the dsRNA-editing enzyme ADAR can inhibit BVDV replication and BVDV non-structural protein 4A (NSA4) may bind ADAR to favor viral replication [58].

There is some previous evidence that BVDV can interfere with the production and function of inflammatory cytokines and chemokines, although the precise mechanisms are not clear. For example, ncpBVDV-infected monocytes suppressed LPS-stimulated IL1 activity in uninfected cells, although *IL1* mRNA expression remained unaltered [59]. Similarly, infected macrophages showed decreased production of TNF following stimulation with heat-inactivated *Salmonella dublin* or LPS [60]. In this study ncpBVDV did not have a pronounced effect on the mRNA expression of inflammatory cytokines including *TNF* and *IL1A* at the time of assessment of cellular response to LPS. There was, however, a slight increase in the expression of *CCL28,* a chemokine which may play dual roles as a chemoattractant and broad-spectrum antimicrobial protein [61]. More remarkably, ncpBVDV significantly suppressed the induction of *CCL5* by LPS.

CCL5 is an important chemokine which provides linkage between mucosal innate and adaptive immunity [62].

Type I IFNs regulate multiple immune cell types and are also crucial in linking innate to adaptive immunity [63]. Bacterial molecular patterns are now recognized to activate distinctive pathways that merge with those activated by viruses and lead to high-level type I IFN production, which is crucial for host resistance against different species of pathogenic bacteria [64,65]. However, ncpBVDV can inhibit this IFN response via the activity of two viral proteins: N^{pro} is a proteinase which degrades the transcription factor IRF3 thereby preventing the activation of INFB genes and E^{rns} degrades viral RNA, with a strong preference for ssRNA [66]. Prior infection with ncpBVDV inhibited the induction of IFN in bovine calf testis cells infected with a secondary virus [67] and in bovine macrophages and MDBK cells exposed to Poly(I:C), a dsRNA ligand [39, 67]. We provide new evidence of this suppressive activity in the present study in which prior infection of endometrial cells with ncpBVDV significantly inhibited the expression of many genes involved in IFN response to LPS including *IFI6*, *IFI27*, *MX1*, *MX2*, *IFIT1*, *IFIT3*, *IFIT5*, *RSAD2*, *BST2*, *ISG15*, *HERC5*, *HERC6*, *OAS1* and *USP18*. Indeed several ISGs were among the genes whose expression was suppressed to the greatest extent. The antiviral effects of several ISGs have been described [68]. The induction of ISGs by different bacteria or bacterial ligands suggests a broader role in innate antimicrobial defenses, but their functions against bacterial pathogens are yet to be clearly defined. LPS induces the expression of RSAD2 (viperin) [69], an important broad-spectrum antiviral factor which also modulates TLR signaling including TLR7 and TLR9 [70]. BST2 induces NF-kB activity [71] and may mediate monocyte migration through the endothelium to the inflammation site [72].

The type I IFN cytokine IFNT is released by the bovine conceptus during the maternal recognition of early pregnancy [73] and this induces the endometrial up-regulation of many ISGs including *MX2*, *BST2*, *RSAD2*, *ISG15 OAS1* and USP18 [74] which are thought to have important functions in implantation and embryonic survival [75]. Many of these IFNT-induced genes in the early pregnant bovine endometrium were among the ISGs suppressed by ncpBVDV in the present study. The possible interaction between a ncpBVD infection and the presence of an embryo in modulating the immune responses of the endometrium are therefore of considerable potential interest but require further study.

Other potentially important down-regulated genes included signal transducer and activator of transcription 1 (*STAT1*), guanylate-binding proteins *GBP4* and *GBP5* and sterile alpha motif domain containing 9 (*SAMD9*). Activation of STAT1 by an LPS-induced type I IFN may promote innate immunity against gram-negative bacteria [76]. GBP4 and GBP5 belong to the GBP gene family that enable host defense against bacteria and viruses [77]. Infection with ncpBVDV also suppressed the LPS-induced up-regulation of *MMP1*, *MMP12* and *MMP13*. The MMPs regulate inflammation and innate immunity by modulating tissue remodeling and leucocyte recruitment [78].

Infection with ncpBVDV suppressed LPS-induced up-regulation of several genes involved in the balance between cell death and survival. There was down-regulation of poly (ADP-ribose) polymerase family, member 14 (*PARP14*) and deltex 3 like, E3 ubiquitin ligase (*DTX3L*) which both regulate DNA damage repair and post-translational protein modification [79,80]. In addition, ncpBVDV suppressed the up-regulation of XIAP associated factor 1(*XAF1*) and promyelocytic leukemia (*PML*) both of which have important roles in the activation [81] and

regulation [82] of apoptosis. The suppression of LPS-induced apoptotic factors may interfere with pro-inflammatory apoptosis and the elimination of pathogens.

BVDV was previously observed to stimulate the production of PGE_2 in infected cell culture and monocytes [83]. In this study, ncpBVDV increased the expression of *PLA2G4F* and *PTGES* (*mPGES-1*) in infected endometrial cells following exposure to LPS. PLA2G4F belongs to the family of PLA2 enzymes that are essential for the initial release of AA in prostaglandin biosynthesis [84] while PTGES subsequently catalyzes the isomerization of PGH_2 to PGE_2 [85]. PGE_2 suppresses acute inflammatory mediators, resulting in its predominance at late/chronic stages of immunity [86]. High levels of PGE_2 were secreted by endometrial cells from cows with clinical endometritis [87]. Thus, ncpBVDV infection may increase PGE_2 production by the endometrium upon bacterial infection, potentially compromising the endometrial innate immune response.

In summary, prior ncpBVDV infection inhibited the expression of many genes typically up-regulated in bovine endometrium following exposure to LPS (Fig. 9). Many of these genes are important in innate immune responses to both viruses and bacteria involving pathogen recognition, IFN response, inflammatory response, chemokine activity, tissue remodeling and cell migration, and cell death/survival. This study thus provides new evidence to corroborate previous reports of immune suppression observed in BVDV-infected animals and extends this to the uterine environment. NcpBVDV, like many other viruses, may have evolved this interference with the cellular immune signaling mechanisms as a means of evading the host's immune response. We infer from the present findings that ncpBVDV infection can compromise the uterine immune system during the critical postpartum period when the cow's reproductive tract becomes contaminated with pathogens. Furthermore, ncpBVDV infection may have important

consequences for the establishment of pregnancy by altering the signal given to the endometrium
550 by IFNT. These actions could contribute to the significant economic losses due to uterine disease, infertility and culling of affected animals.

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FIGURE LEGENDS

FIG. 1. Demonstration of ncpBVDV-infected cells using indirect alkaline phosphatase immunostaining. A purple cytoplasmic staining was positive for BVDV antigen. Photomicrographs represent: (A) MDBK cells infected with ncpBVDV; (B) control non-infected MDBK cells; (C) epithelial cells (e) and stromal cells (s) from cultured bovine endometrium infected with ncpBVDV and (D) control non-infected endometrial cells.

FIG. 2. Validation of ncpBVDV infection of bovine endometrial cell culture groups using PCR. Target BVDV gene product was expressed in the infected groups (BVDV; BVDV+LPS) but not in the non-infected groups (Control; Control+LPS). Expected product sizes: BVDV (288 bp); *ACTB* (91 bp).

FIG. 3. IPA network 1. The genes altered by ncpBVDV infection in bovine endometrial cells were identified in networks involved in cellular movement, connective tissue disorders, and metabolic disease (Score = 34 with 19 focus molecules). The relationship is described as either a direct interaction (solid line) or an indirect interaction (dashed line) while the intensity of the color indicates the level of up-regulation (red) or down-regulation (green) of the respective molecules

FIG. 4. IPA network 2. The genes altered by ncpBVDV infection in bovine endometrial cells were identified in networks involved in developmental disorder, hereditary disorder, and immunological disease (Score = 33 with 19 focus molecules). See Fig. 1 for explanation of symbols.

FIG. 5. IPA network 1. The altered genes in ncpBVDV-infected bovine endometrial cells after exposure to LPS vs. LPS alone were identified in networks involved in infectious disease, post-translational modification, and protein folding (Score = 33 with 19 focus molecules). See Fig. 1 for explanation of symbols.

FIG. 6. IPA network 2. The altered genes in ncpBVDV-infected bovine endometrial cells after exposure to LPS vs. LPS alone were identified in networks involved in dermatological diseases and conditions, hereditary disorder, and immunological disease (Score = 30 with 19 focus molecules). See Fig. 1 for explanation of symbols.

FIG. 7. Quantitative RT-PCR analysis of selected genes for the validation of microarray data. Bovine endometrial cells were infected with ncpBVDV for 4 days followed by treatment with 100 ng/ml LPS for 6 h. Expression values represent mean ± SEM (n = 6) in arbitrary units (AU) after normalization to *RN18S1* and *ACTB*. Treatment groups were compared using linear mixed-effects model. The significant differences between CONT vs. BVDV and CONT+LPS vs. BVDV+LPS are shown: *P < 0.05; **P < 0.01.

FIG. 8. Quantitative RT-PCR analysis of selected genes for the validation of microarray data. Bovine endometrial cells were infected with ncpBVDV for 4 days followed by treatment with 100 ng/ml LPS for 6 h. Expression values represent mean ± SEM (n = 6) in arbitrary units (AU) after normalization to *RN18S1* and *ACTB*. Treatment groups were compared using linear mixed-effects model. The significant differences between CONT vs. BVDV and CONT+LPS vs. BVDV+LPS are shown: *P < 0.05; **P < 0.01.

FIG. 9. Summary of ncpBVDV-induced suppression of the innate immune response to bacterial LPS in bovine endometrial cells. (1) NcpBVDV infection and replication in bovine endometrial cells. (2) NcpBVDV production of viral proteins. (3 & 4) Viral protein (Erns) degrades viral RNA to prevent recognition by TLRs [16] and RLRs (5) Viral protein (Npro) inhibits type-I interferon expression by proteasomal degradation of IRF3 [16]. (6) Unknown viral effect. (7) TLR4 recognizes bacterial LPS on the cell surface in association with the co-receptor MD2 and additional proteins such as CD14 and LPS-binding protein, which activates inflammatory and innate immune response [88]. However, the infection with ncpBVDV inhibited the LPS-induced up-regulation of several genes involved in pathogen recognition, interferon response, inflammatory response, chemokine activity, tissue remodeling and cell migration, and cell death/survival. ECM (extracellular matrix). *LPS-inducible genes suppressed by ncpBVDV

TABLE 1. Genes whose expression was most altered by ncpBVDV infection in bovine endometrial cells.[#]

Gene Symbol	FC	Function
Top up-regulated (≥ 1.4-fold increase)		
TRIM56	1.9**	Protein modification, immune response
OLFML1	1.7**	Olfactomedin-like protein
C3	1.6**	Complement activation, immune response
ZBTB2	1.5**	May be involved in transcriptional regulation
CHI3L1	1.5*	Inflammation and tissue remodeling
DTWD2	1.4**	-
ANK2	1.4**	Cell motility, activation, and proliferation
SCUBE2	1.4*	Tumor suppressor
C1S	1.4**	Activation of complement pathway
HBA	1.4*	Hemoglobin subunit
C1R	1.4**	Activation of complement pathway
APOD	1.4*	Apolipoprotein
VCAM1	1.4*	Cell adhesion and cell-cell recognition
XPNPEP2	1.4*	Metalloproteinase and inflammatory activity
ENPP6	1.4**	Fatty acid hydrolysis
Top down-regulated (≥ 1.4-fold decrease)		
CDH6	-1.5*	Cell-cell adhesion and cell differentiation
MAMDC2	-1.5*	-
CALCA	-1.4*	Calcium metabolism
SERPINB2	-1.4*	Plasminogen Activator Inhibitor
PHOSPHO1	-1.4**	Pyrophosphatase activity
SSLP1	-1.4*	Member of retrotransposon-derived Ly-6 superfamily
IL33	-1.4*	Cytokine activity, immune response

[#]Microarray analysis showing the fold change (FC) in gene expression based on BH-adjusted *P* value: *($P < 0.05$); **($P < 0.01$); paired *t*-test

TABLE 2. Ingenuity Pathway network analysis associated with the genes significantly altered by ncpBVDV infection in bovine endometrial cells showing: (1) the top five disease processes and biological functions; (2) the top three canonical pathways and (3) the top three network functions.

P Value	No. genes	Disease processes and biological functions
< 0.001	43	Cellular movement
< 0.001	37	Hematological system development and function
< 0.001	28	Immune cell trafficking
< 0.001	40	Inflammatory response
< 0.001	39	Cell-to-cell signaling and interaction
	Ratio*	Canonical pathways
< 0.001	14/192	Agranulocyte adhesion and diapedesis
< 0.001	13/182	Granulocyte adhesion and diapedesis
< 0.001	5/112	Communication between innate and adaptive immune cells
Score	Focus Molecules	Network#
34	19	Cellular movement, connective tissue disorders, metabolic disease
33	19	Developmental disorder, hereditary disorder, immunological disease
27	16	Cellular movement, hematological system development and function, immune cell trafficking

*The number of genes in the list of DEGs that participate in the canonical pathway divided by the total number of genes that are known to be associated with the pathway in the Ingenuity knowledge base.

#A limit of 35 genes was set for each generated network. The networks were scored based on the number of the network-eligible molecules that are present in the list of DEGs. A higher network score corresponds to a lower probability of finding the observed number of the DEGs in a given network by chance.

TABLE 3. The genes altered to the greatest extent in ncpBVDV-infected bovine endometrial cells after exposure to bacterial LPS. [#]

Gene symbol	Fold Change	Function
Top up-regulated (≥ 1.5-fold increase)		
TRIM56	1.8***	Protein modification, immune response
C3	1.6***	Complement activation, immune response
OLFML1	1.6**	Olfactomedin-like protein
ZBTB2	1.6***	May be involved in transcriptional regulation
HBA	1.5***	Hemoglobin subunit
ALPL	1.5**	Alkaline phosphatase, dephosphorylates LPS
DTWD2	1.5***	-
Top down-regulated (≥ 2-fold decrease)		
RSAD2	5.1***	Interferon inducible, immune response
ISG15	4.6*	Interferon inducible, protein modification
BST2	4.1**	Interferon inducible, signal transduction
MX2	4.1*	Interferon inducible, GTPase activity
OAS1Y	3.9**	Interferon inducible, immune response
USP18	3.3*	Protein modification, ISG15-specific protease
IFIT3	3.2*	Interferon inducible, immune response
OAS1Z	2.7**	Interferon inducible, immune response
IFI27	2.5**	Interferon inducible, immune response
SAMD9	2.5*	May regulate cell proliferation and apoptosis
IFIT1	2.3**	Interferon inducible, immune response
DDX58	2.2*	Cell migration, immune response.
HERC6	2.1*	Protein modification
MX1	2.0**	Interferon inducible, induction of apoptosis
HERC5	2.0*	Protein modification, immune response

[#]Microarray analysis showing the fold change (FC) in gene expression based on BH-adjusted *P* value: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$); paired *t*-test.

TABLE 4. Ingenuity Pathway network analysis associated with the genes significantly altered by ncpBVDV infection in bovine endometrial cells treated with LPS for 6 h showing: (1) the top six disease processes and biological functions; (2) the top three canonical pathways and (3) the top three network functions.

P Value	No. genes	Disease processes and biological functions
< 0.05	26	Infectious disease
< 0.01	17	Post-translational modification
< 0.001	4	Protein folding
< 0.05	12	Antimicrobial response
< 0.05	32	Inflammatory response
< 0.05	28	Cell-to-cell signaling and interaction
< 0.05	36	Hematological system development and function
< 0.05	25	Immune cell trafficking
	Ratio*	Canonical pathways
< 0.001	6/73	Activation of IRF by cytosolic PRRs
< 0.001	4/36	Interferon signaling
< 0.001	4/35	Oncostatin M signaling
< 0.01	5/109	Role of PRRs in recognition of bacteria and viruses
< 0.01	3/49	Role of RIG1-like receptors in antiviral innate immunity
< 0.01	4/35	Complement system
Score	Focus Molecules	Network#
33	19	Infectious disease, post-translational modification, protein folding
30	19	Dermatological diseases and conditions, hereditary disorder, immunological disease
29	18	RNA post-transcriptional modification, endocrine system development and function, molecular transport

*The number of genes in the list of DEGs that participate in the canonical pathway divided by the total number of genes that are known to be associated with the pathway in the Ingenuity knowledge base.

#A limit of 35 genes was set for each generated network. The networks were scored based on the number of the network-eligible molecules that are present in the list of DEGs. A higher network score corresponds to a lower probability of finding the observed number of the DEGs in a given network by chance.

TABLE 5. Expression of selected genes in bovine endometrial cells compared by both qRT-PCR analysis and Affymetrix microarray

	BVDV (compared to CONT)				BVDV+LPS (compared to CONT+LPS)			
	qRT-PCR*		Microarray[#]		qRT-PCR*		Microarray[#]	
	FC	*P*	FC	*P*^	FC	*P*	FC	*P*^
TRIM56	2.5 up	**	1.9 up	**	1.9 up	NS	1.8 up	**
C3	2.2 up	**	1.6 up	**	1.9 up	*	1.6 up	**
CX3CL1	2.0 up	NS	1.3 up	*	1.0	-	1.0	-
PTGES	1.3 up	NS	1.0	-	1.4 up	*	1.3 up	*
RND1	1.3 up	NS	1.2 up	*	1.3 up	**	1.0	-
BST2	2.5 down	NS	1.0	-	14.3 down	*	4.1 down	**
CCL5	2.3 down	NS	1.0	-	2.8 down	*	1.6 down	**
ISG15	2.2 down	NS	1.0	-	20 down	*	4.6 down	*
MX2	1.4 down	NS	1.0	-	2.6 down	*	4.1 down	*
RSAD2	1.3 down	NS	1.0	-	11.1 down	**	5.1 down	**
STAT1	1.7 down	NS	1.0	-	1.5 down	NS	1.2 down	*
GBP5	1.3 up	NS	1.0	-	2.9 down	*	1.9 down	*
VCAM1	1.1 up	NS	1.4 up	*	1.1 down	NS	1.0	-
AMIGO2	1.6 down	NS	1.0	-	1.3 down	NS	1.0	-
IL1A	1.0	-	1.0	-	1.0	-	1.0	-

FC: fold change in expression; NS no significant difference.

*Comparison by linear mixed-effects model; [#]comparison by paired *t*-test; ^BH-adjusted $P < 0.05$.

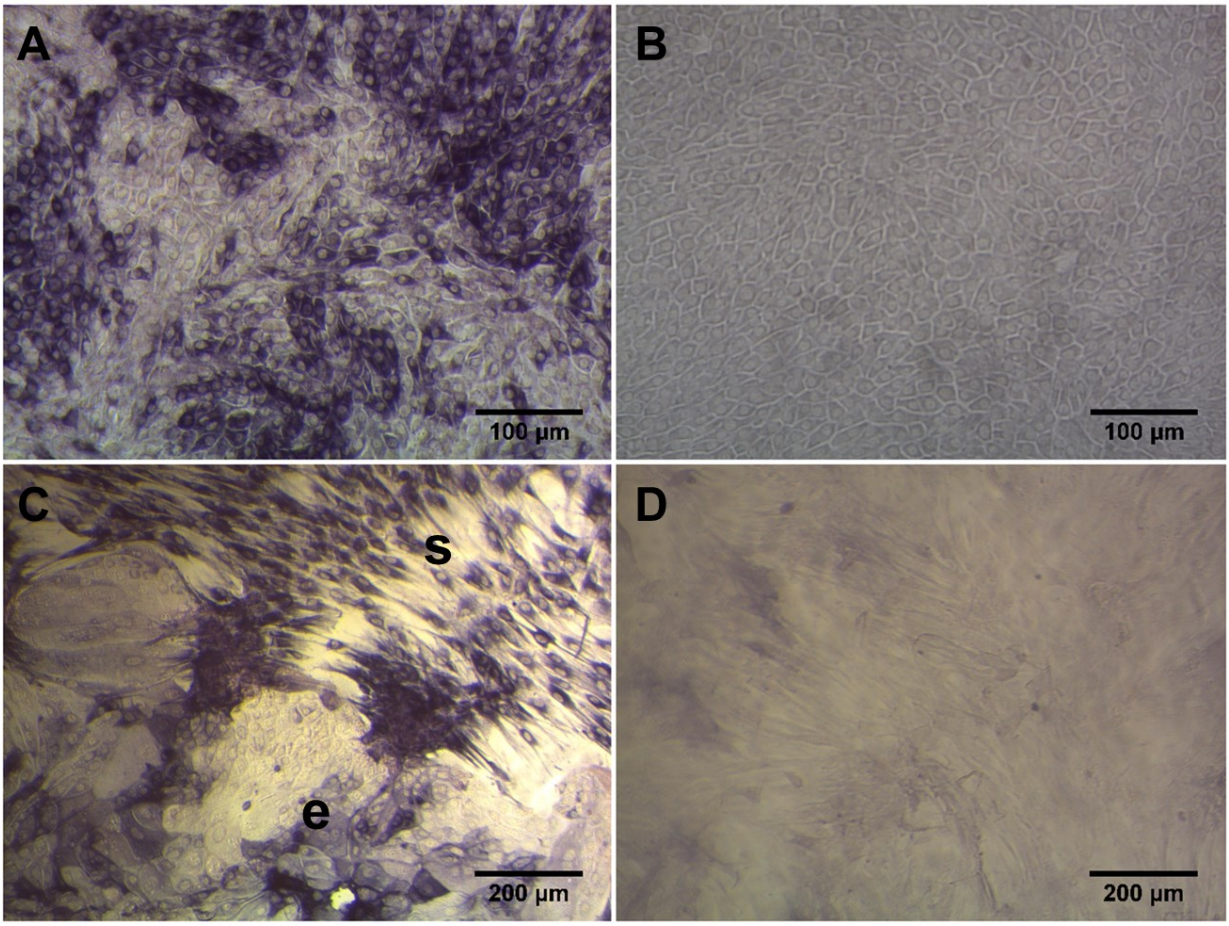


FIG. 1.

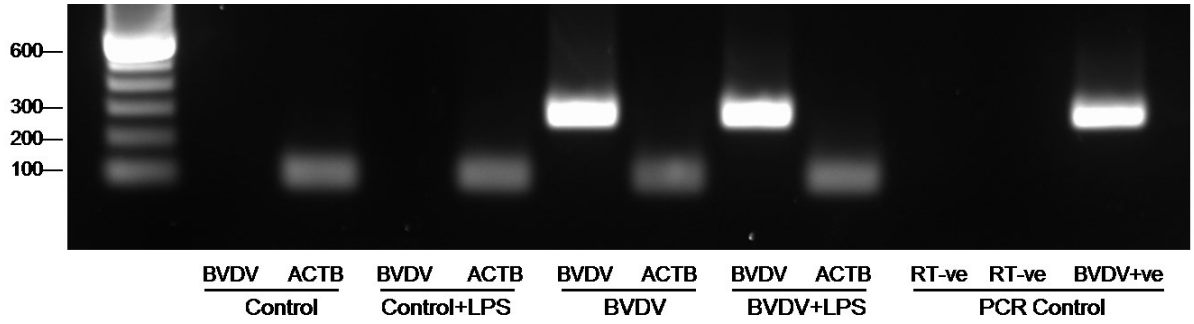

600
300
200
100
BVDV ACTB
Control
BVDV ACTB
Control+LPS
BVDV ACTB
BVDV
BVDV ACTB
BVDV+LPS
RT-ve RT-ve BVDV+ve
PCR Control

FIG. 2.

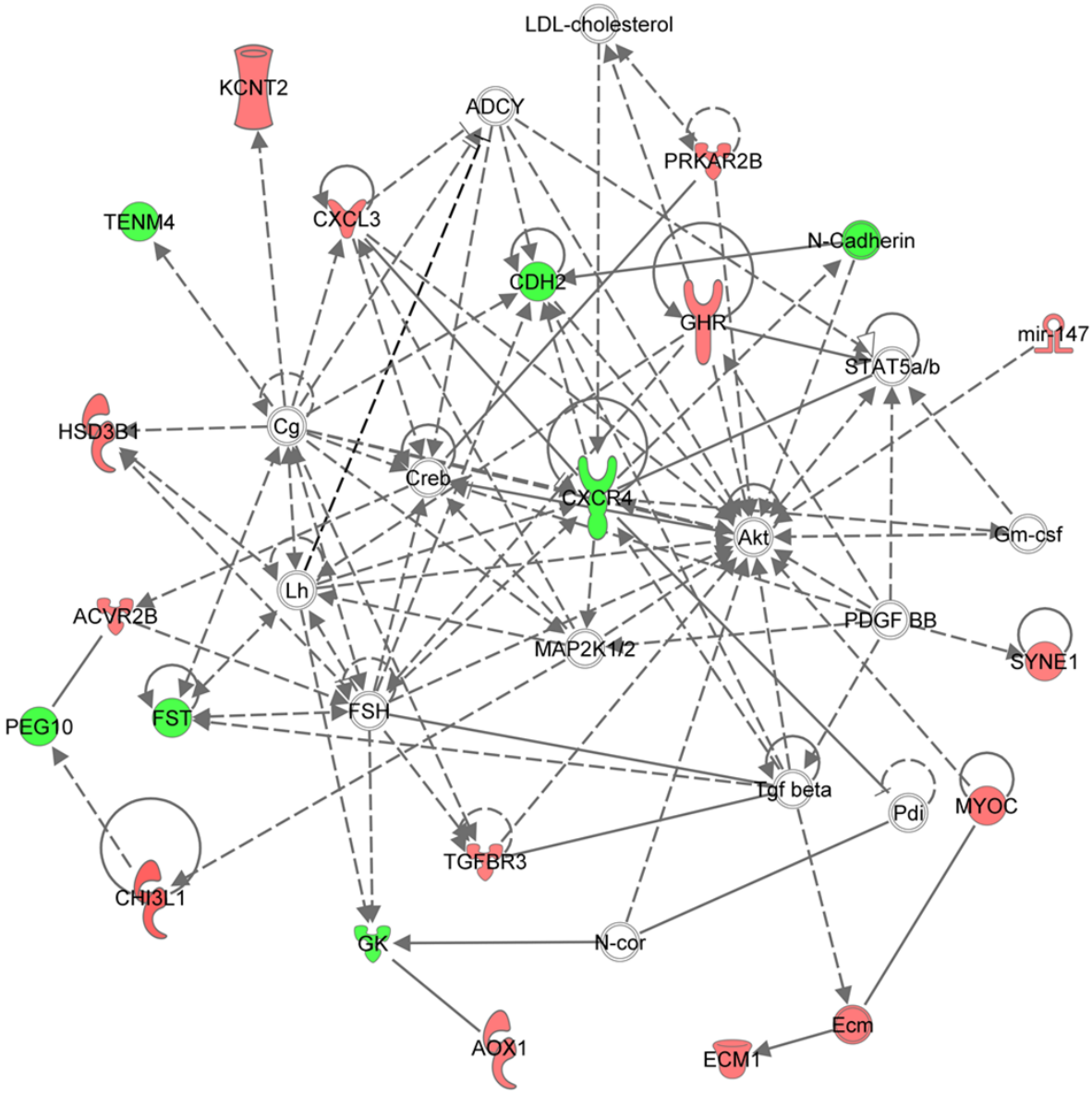

LDL-cholesterol
KCNT2
ADCY
PRKAR2B
TENM4
CXCL3
N-Cadherin
CDH2
GHR
mir-147
STAT5a/b
HSD3B1
Cg
Creb
CXCR4
Akt
Gm-csf
Lh
ACVR2B
PDGF BB
MAP2K1/2
SYNE1
PEG10
FST
FSH
Tgf beta
Pdi
MYOC
TGFBR3
CHI3L1
GK
N-cor
Ecm
AOX1
ECM1

FIG. 3.

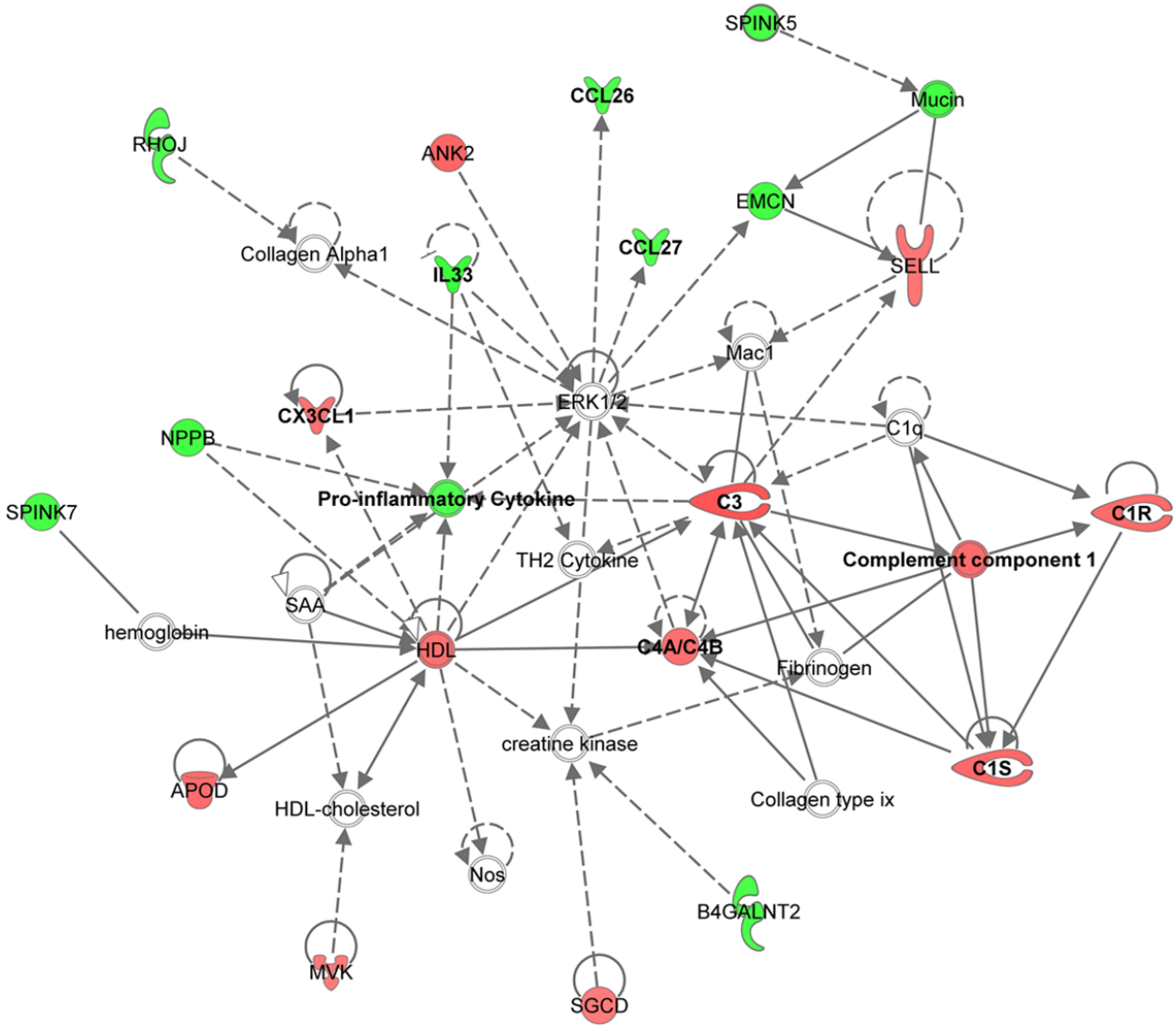

SPINK5
Mucin
CCL26
RHOJ
ANK2
EMCN
Collagen Alpha1
IL33
CCL27
SELL
Mac1
ERK1/2
CX3CL1
C1q
NPPB
Pro-inflammatory Cytokine
C3
C1R
SPINK7
TH2 Cytokine
Complement component 1
SAA
hemoglobin
HDL
C4A/C4B
Fibrinogen
creatine kinase
C1S
APOD
HDL-cholesterol
Collagen type ix
Nos
B4GALNT2
MVK
SGCD

FIG. 4.

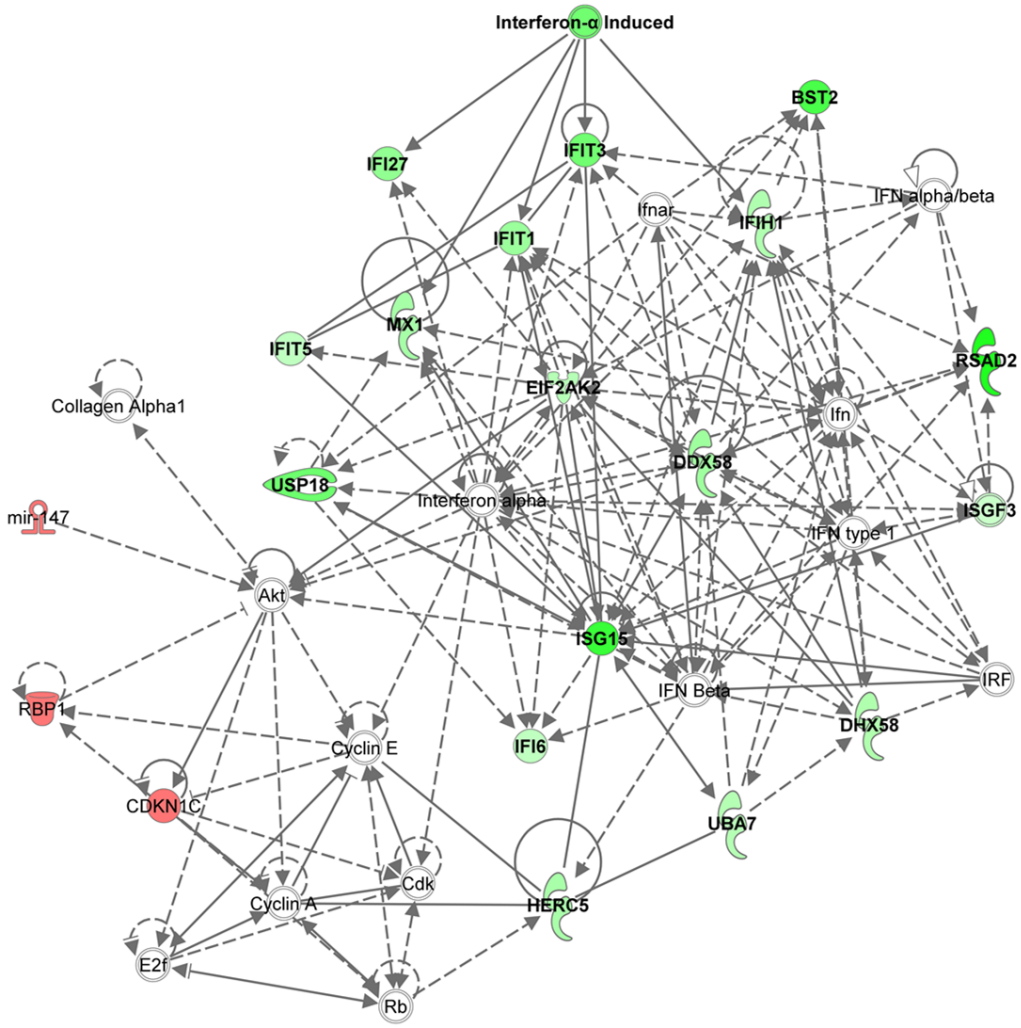

Interferon-α Induced
BST2
IFI27
IFIT3
IFN alpha/beta
Ifnar
IFIH1
IFIT1
MX1
IFIT5
RSAD2
Collagen Alpha1
EIF2AK2
Ifn
DDX58
USP18
Interferon alpha
ISGF3
mir-147
IFN type 1
Akt
ISG15
IRF
IFN Beta
RBP1
DHX58
Cyclin E
IFI6
CDKN1C
UBA7
Cdk
Cyclin A
HERC5
E2f
Rb

FIG. 5.

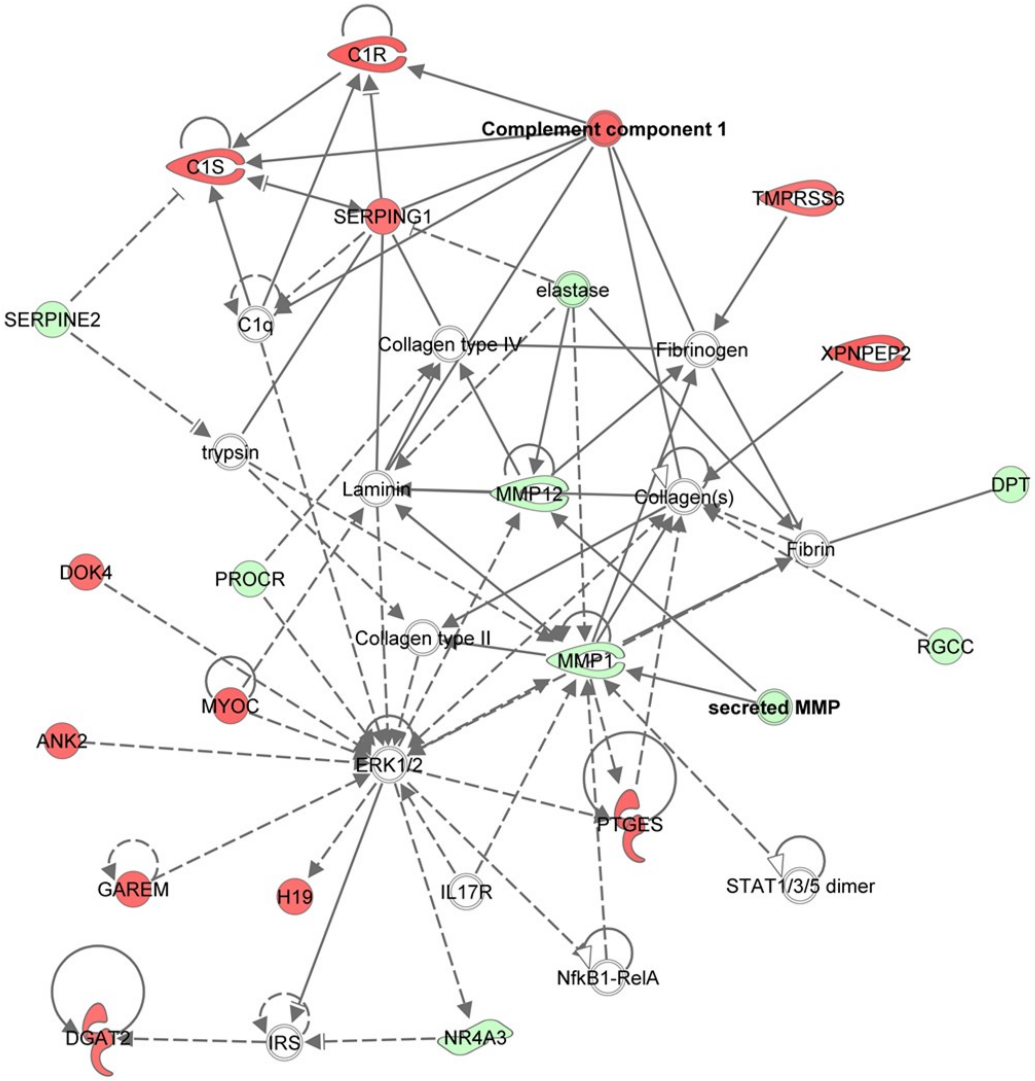

C1R
Complement component 1
C1S
SERPING1
TMPRSS6
elastase
SERPINE2
C1q
Collagen type IV
Fibrinogen
XPNPEP2
trypsin
Laminin
MMP12
Collagen(s)
DPT
Fibrin
DOK4
PROCR
Collagen type II
MMP1
RGCC
MYOC
secreted MMP
ANK2
ERK1/2
PTGES
GAREM
H19
IL17R
STAT1/3/5 dimer
NfkB1-RelA
DGAT2
IRS
NR4A3

FIG. 6.

FIG. 7

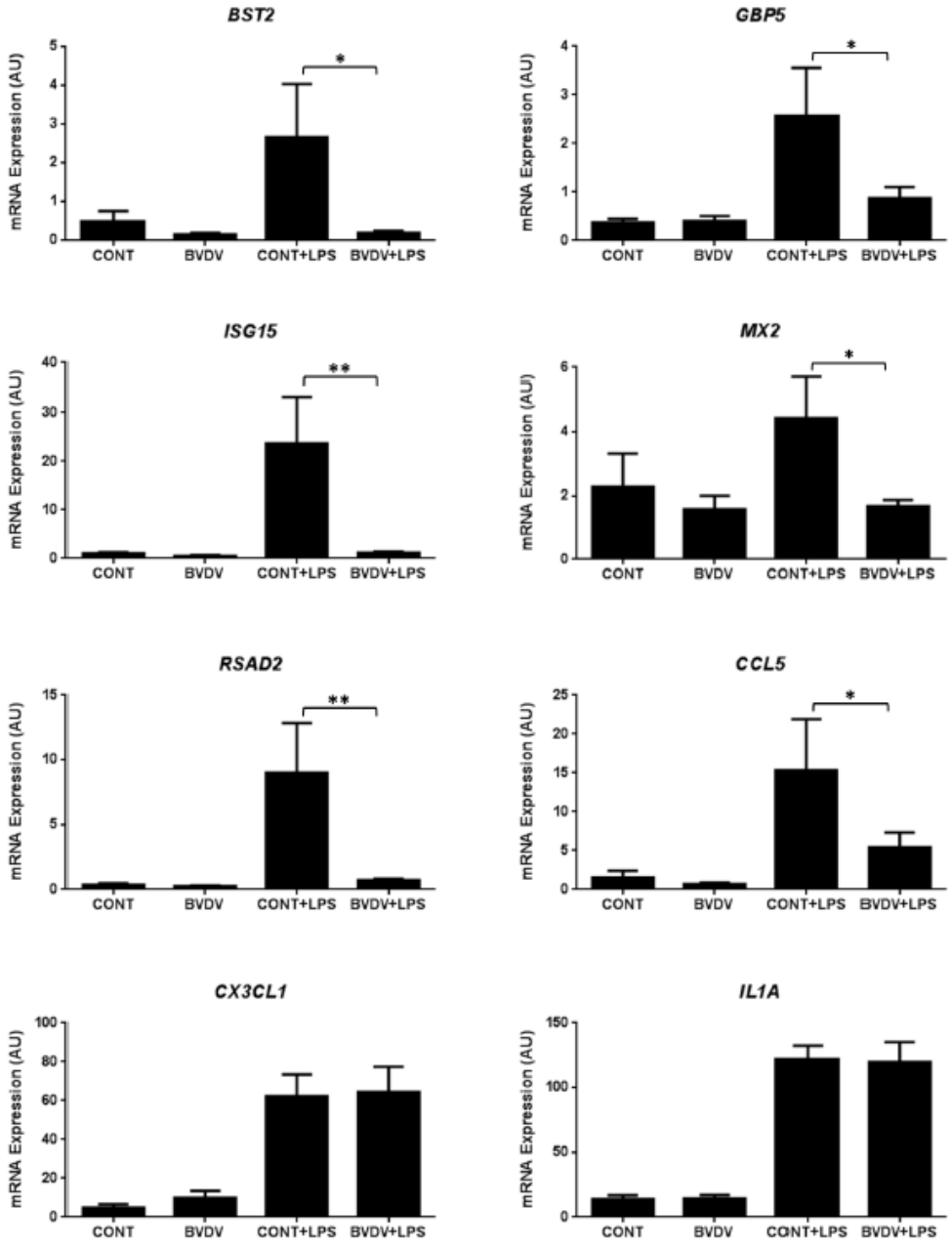
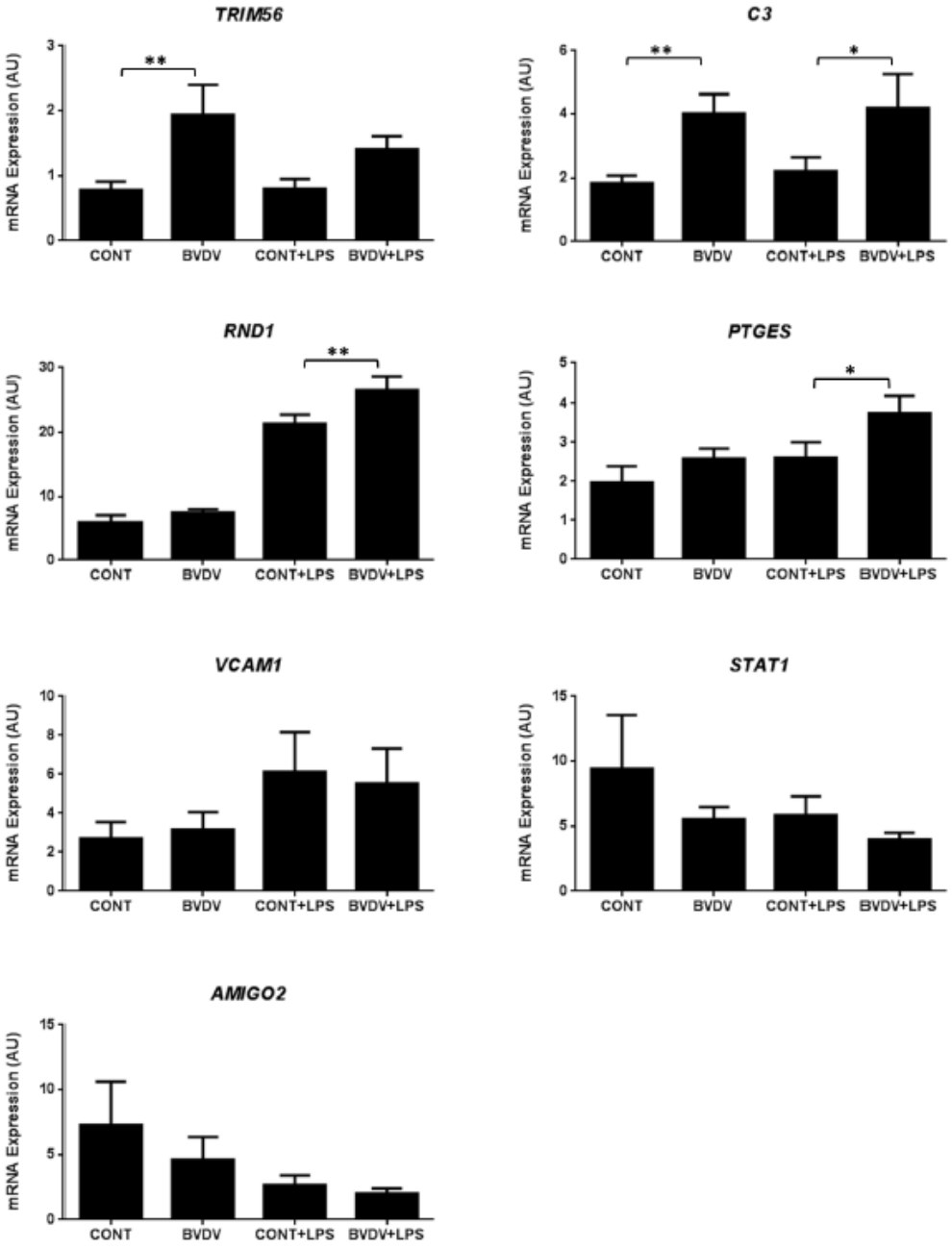

BST2
GBP5
ISG15
MX2
RSAD2
CCL5
CX3CL1
IL1A
mRNA Expression (AU)
CONT
BVDV
CONT+LPS
BVDV+LPS
*
**

FIG. 8


TRIM56
C3
RND1
PTGES
VCAM1
STAT1
AMIGO2
mRNA Expression (AU)
CONT
BVDV
CONT+LPS
BVDV+LPS
**
*

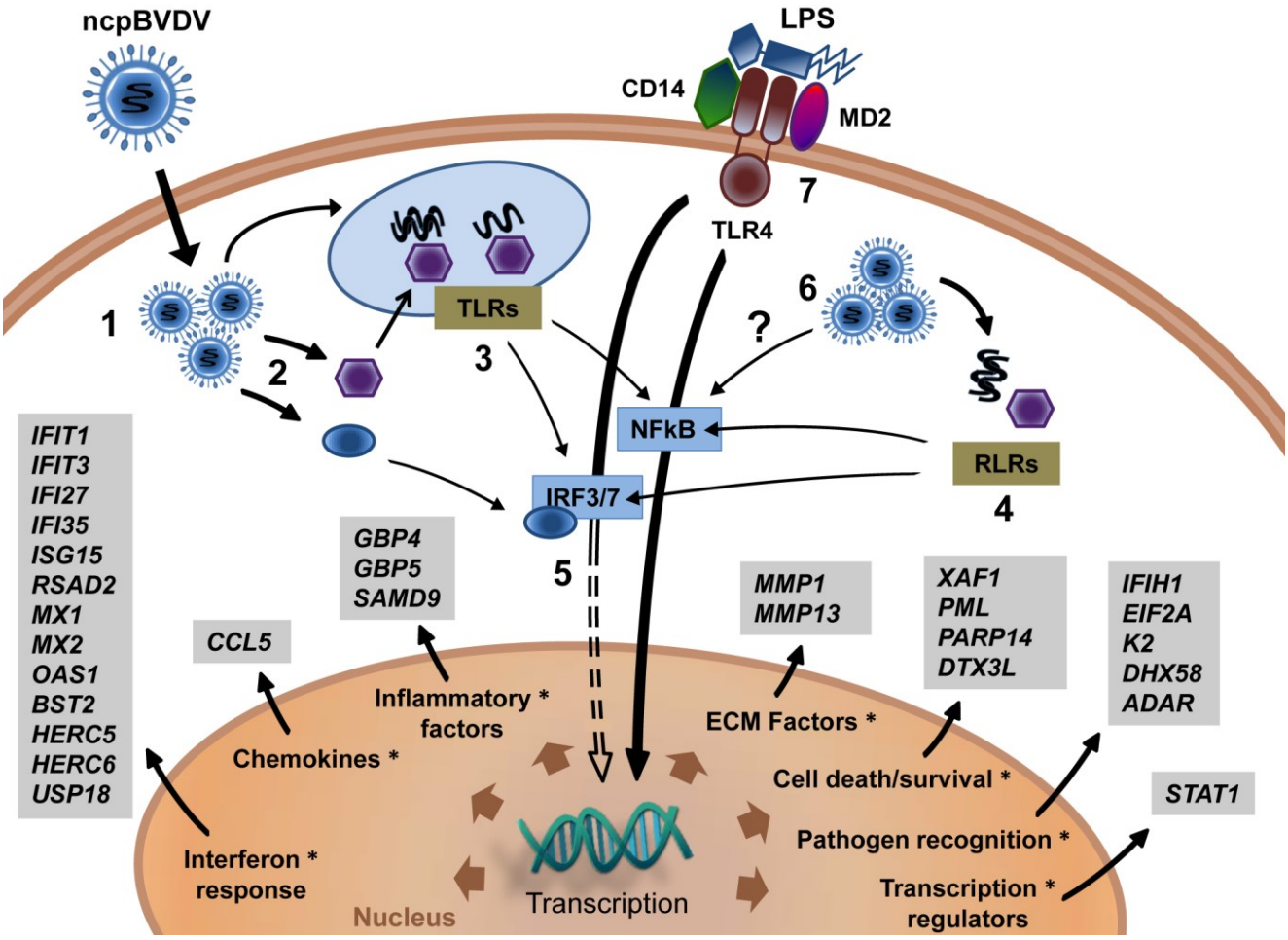

ncpBVDV
LPS
CD14
MD2
TLR4
7
1
2
TLRs
3
?
6
NFkB
RLRs
4
IRF3/7
5
IFIT1
IFIT3
IFI27
IFI35
ISG15
RSAD2
MX1
MX2
OAS1
BST2
HERC5
HERC6
USP18
GBP4
GBP5
SAMD9
CCL5
Inflammatory * factors
Chemokines *
MMP1
MMP13
ECM Factors *
XAF1
PML
PARP14
DTX3L
Cell death/survival *
IFIH1
EIF2A
K2
DHX58
ADAR
STAT1
Pathogen recognition *
Transcription * regulators
Interferon * response
Nucleus
Transcription

FIG. 9

SUPPLEMENTAL TABLES (see attached files)

SUPPL. TABLE S1. A list of the differentially expressed genes in bovine endometrial cells following infection with ncpBVDV in vitro (BVDV vs. CONT).

SUPPL. TABLE S2. Genes identified in the most significant IPA canonical pathways (BVDV vs. CONT).

SUPPL. TABLE S3. A list of the differentially expressed genes in ncpBVDV-infected bovine endometrial cells following treatment with LPS *in vitro* (BVDV+LPS vs. CONT+LPS).

SUPPL. TABLE S4. Genes identified in the most significant IPA canonical pathways (BVDV+LPS vs. CONT+LPS).

SUPPLEMENTAL FIGURE LEGENDS

SUPPL. FIG. S1. A hierarchical cluster analysis showing the expression patterns of significantly differentially expressed genes between the cells treated with control (CONT), lipopolysaccharides (LPS), bovine virus diarrhoea virus (NCP) and NCP+LPS. Gene expression of each sample was analysed with Affymetrix Bovine Gene 1.1 ST Array and normalised with RNA16. The bars are genes and their colours indicate the normalized expression intensities from 5.4 (blue) to 10.8 (red).

SUPPL. FIG. S2. IPA network 3. The genes altered by ncpBVDV infection in bovine endometrial cells were identified in networks involved in cellular movement, hematological system development and function, and immune cell trafficking (Score = 27 with 16 focus molecules). The relationship is described as either a direct interaction (solid line) or an indirect interaction (dashed line) while the intensity of the color indicates the level of up-regulation (red) or down-regulation (green) of the respective molecules.

SUPPL. FIG. S3. IPA analysis of the altered genes in ncpBVDV-infected bovine endometrial cells after exposure to LPS showing the predicted inhibition of LPS upstream regulator; $P < 0.001$.

SUPPL. FIG. S4. IPA analysis of the altered genes in ncpBVDV-infected bovine endometrial cells after exposure to LPS showing the predicted inhibition of IFN type 1 upstream regulator; $P < 0.001$.