Acute bovine viral diarrhoea virus infection inhibits expression of interferon tau-stimulated genes in bovine endometrium

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) can evade host detection by down-regulation of interferon signalling pathways. Infection of cows with non-cytopathic (ncp) BVDV can cause early embryonic mortality. Upregulation of type I interferon stimulated genes (ISGs) by blastocyst-secreted interferon tau (IFNT) is a crucial component of the maternal recognition of pregnancy (MRP) in ruminants. This study investigated the potential of acute BVDV infection to disrupt MRP by modulating endometrial ISG expression. Endometrial cells from 10 BVDV-free cows were cultured and treated with 0 or 100 ng/ml IFNT for 24 h in the absence or presence of ncpBVDV infection to yield 4 treatment groups: CONT, ncpBVDV, IFNT or ncpBVDV+IFNT. ncpBVDV infection alone only up-regulated *TRIM56*, but reduced mRNA expression of *ISG15, MX2, BST2* and the pro-inflammatory cytokine *IL1B.* As anticipated, IFNT treatment alone significantly increased expression of all 17 ISGs tested. In contrast to the limited effect of ncpBVDV alone, the virus markedly inhibited IFNT-stimulated expression of 15 ISGs tested (*ISG15, HERC5, USP18, DDX58, IFIH1, IFIT1, IFIT3, BST2, MX1, MX2, RSAD2, OAS1Y, SAMD9, GBP4* and *PLAC8*), together with ISG15 secreted protein. Only *TRIM56* and *IFI27* expression was unaltered. *IL1B* expression was reduced by the combined treatment. These results indicate that acute ncpBVDV infection may decrease uterine immunity and lead to MRP failure through inhibition of IFNT-stimulated endometrial ISG production. This in turn could reduce fertility and predispose cows to uterine disease, while evasion of the normal uterine immune response by ncpBVDV may contribute to maintenance and spreading of this economically important disease.

INTRODUCTION

Early embryonic death is a major cause of poor reproductive performance in cattle, with most losses occurring before day 16 of pregnancy [1]. There is strong evidence to suggest that infection with bovine viral diarrhoea virus (BVDV) is one of many potential causes of pregnancy failure. BVDV is a single-stranded (ss)RNA virus in the genus *Pestivirus*. It causes a significant disease of cattle which is endemic in the majority of countries worldwide, leading to major economic losses [2].

BVDV is able to replicate in many types of tissues including the reproductive tract [3, 4]. Reproductive losses are one of the consequences of this disease: if cows develop an acute infection with non-cytopathic (ncp) BVDV shortly before or during the first six months of gestation then the pregnancy can be adversely affected [2, 3]. This includes early embryonic development as conception rates fell by up to 44% following experimental infections either nine days before or four days after insemination [5]. The review by Fray *et al*. [2] cited many similar results which have been reported following ncpBVDV infection in the field, in spite of the occasional report to the contrary. Acutely infected animals are usually able to eliminate the virus within 10-14 days post infection [6]. There is, however, evidence that transmissible virus can persist for much longer periods in some animals which have apparently recovered [7]. Bulls can continue to shed virus into semen for some time after initial BVDV infection due to continued viral replication in the seminal vesicles and prostate gland [8], but there is little information available regarding the survival time of BVDV in the female reproductive tract. Bielanski *et al*. [9, 10] infected heifers with BVDV by i.v. inoculation or by breeding to a persistently infected (PI) bull and detected virus in the uterus between 7-16 days later, while ncpBVDV was isolated from the uterocervical mucus of a heifer 24 days after initial infection [5]. Kirkland *et al.* isolated BVDV from degenerate fetuses of cows slaughtered approximately 38 days after exposure to BVDV [11]. Firat *et al*. [12] found BVDV antigen in macrophage-like cells of the endometrium in 23% of 65 cows examined in a slaughterhouse survey, but the previous history of these animals was unknown.

BVDV is known to use a variety of strategies to inhibit host defence mechanisms [13-15]. The virus is detected by TLR3 or TLR7/8 located in intracellular compartments such as endosomes and the endoplasmic reticulum or by the pattern recognition receptor DDX58 in the cytoplasm which detects ssRNA. The downstream signalling pathway from the TLR involves transcription interferon regulatory factor 3 or 7 (IRF3, IRF7) which usually act to up-regulate transcription of type 1 IFN. The BVDV protein N^{pro} acts to target IRF3 towards proteasomal degradation, so inhibiting downstream signalling and preventing the rise in IFN. GBP4, an IFN-inducible GTPase, can also inhibit this

pathway by disrupting interactions between TRAF6 and IRF7 while leaving NFKB signalling intact [15-18].

We showed previously that infection of cultured bovine endometrial cells with a non-cytopathic strain of BVDV inhibited many of the immune pathways normally activated in response to a challenge with bacterial lipopolysaccharide (LPS), including downregulation of many interferon stimulated genes (ISGs) [19]. This observation is pertinent as pregnancy recognition in cows is initiated by interferon tau (IFNT), which is a Type I interferon. The conceptus trophectoderm starts secreting IFNT into the uterine lumen on around day 8 of gestation, with production increasing dramatically during elongation [20, 21]. When IFNT reaches a sufficient threshold level (normally by day 16 of pregnancy in cows), the development of oxytocin receptors is inhibited, so preventing luteolysis and ensuring maintenance of the pregnancy [22-24]. Additional actions of IFNT help the uterine endometrium to develop a receptive environment for implantation. These include changes in the production and/or localization of steroid hormone receptors, cytokines, prostaglandins (PGs), growth hormones and their receptors and nutrient transporters (reviewed by Bazer [25]). Among over 500 uterine endometrial genes which were significantly differentially expressed during IFNT-initiated maternal recognition of pregnancy (MRP) in cows, the greatest upregulation was in a group of ISGs, including *MX2, BST2, RSAD2, ISG15, OAS1, USP18, IFI44, ISG20, SAMD9, EIF4E* and *IFIT2, etc*. [22, 23, 26, 27]. The mechanisms by which these ISGs act on the endometrium to ensure MRP are not yet fully understood but they are likely to have crucial roles in modulation of uterine immunity, stromal remodelling, stimulating hyperplasia of the endometrial glands and development of the uterine vasculature [25, 28]. Their interruption may therefore potentially lead to pregnancy failure.

Investigations to date have failed to determine precisely how ncpBVDV can prevent the establishment of a successful pregnancy. As both IFNT and BVDV alter the expression of multiple ISGs, we hypothesised that infection with BVDV might interfere with the normal pregnancy recognition signalling pathways. In support of this we demonstrated previously that acute ncpBVDV infection inhibited the stimulatory effect of IFNT on uterine PGE_2 production [29]. It has been shown

previously that an increase in PGE_2 production is important for MRP, both by enhancing IFNT production and by having direct luteotrophic actions on the corpus luteum [27, 30].

In the present study we therefore investigated the effect of an acute ncpBVDV infection on IFNT-induced signalling mechanisms in bovine endometrial cells. We utilized mixed cultures of epithelial and stromal cells as both cell types are important in the innate immune response. The epithelium constitutes the first line of defence to pathogens that invade the uterus while the stroma is exposed to virus reaching the uterus via the circulation. Interaction between epithelium and stroma is essential for the MRP in response to IFNT [19, 25, 31, 32]. Both cell types express pattern recognition receptors such as TLRs to enable detection of both bacteria and viruses [33]. The viral infection in our experimental model (Supplementary Fig. 1) was established before the IFNT treatment, in order to mimic the most likely *in vivo* situation in which a cow becomes infected before or during mating. While previous studies by us and others have investigated the effects of IFNT and BVDV alone this is, to the best of our knowledge, the first to determine how these two treatments may interact in regulating ISG expression. Seventeen candidate ISGs were selected. ISG15, HERC5, TRIM56 and USP18 are all involved in ubiquitin-like modification of target proteins through the process such as ISGylation [34-36]. DDX58 and IFIH1 (also known as RIG1 and MDA5 respectively) are both cytosolic sensors of viral RNA [16]. IFIT1, IFI27, IFIT3, MX1, MX2, RSAD2, SAMD2, GBP4, OASY1 and BST2 are all regulators of immunity with known antiviral activity [37]. PLAC8 is up-regulated by IFNT in the endometrium with various potential roles described relating to immunity, differentiation and/or proliferation [38]. IL1B was also included. This is not an ISG but represented a major pro-inflammatory cytokine which is thought to participate in signalling between the conceptus and endometrium during MRP in cows [39].

MATERIALS AND METHODS

All reagents and consumables were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or VWR International (Lutterworth, Leicestershire, UK) unless otherwise stated. All culture media

contained 50,000 units/l penicillin and 50 mg/l streptomycin and were certified BVDV free. BVDV free fetal bovine serum (FBS; PAA, Somerset, UK) was used for the cell isolation and culture. This was carried out under sterile conditions. The cells were cultured at 37°C with 5% CO_2.

Animals, cell isolation and culture

The overall experimental design is summarised in Supplementary Fig. 1. The experiment was carried out using BVDV free bovine endometrial samples and FBS as confirmed with a PCR method used in our laboratory with the primer pair: forward (ATGCCCWTAGTAGGACTAGCA; position 108-128) and reverse (TCAACTCCATGTGCCATGTAC; position 395-375) [29]. The expected product size is 288 bp, covering the highly conserved 5′ non-coding/non-structural coding regions of the pestivirus BVDV genome strain NADL [40]. The BVDV-positive control was prepared using the pT7Blue-2 blunt vector, linearized (Novagen, Cambridge, MA02139, USA). The testing system also included a reverse-transcription-negative control and a reference gene *ACTB* (see Table 1 for its primers). Isolation and culture of bovine uterine endometrial cells (a mixture of primary epithelial and stromal cells) were carried out following the protocol established in our group [29, 31]. Briefly, fresh uteri from 10 mature cows in the early luteal phase of the oestrous cycle were collected at a local abattoir and placed on an ice during transport to the laboratory. The cycle stage was estimated by the presence of a newly-formed corpus haemorrhagicum in one of the ovaries. The uteri appeared healthy based on visual inspection at both collection and dissection. Strips of intercaruncular endometrium were dissected and put into Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12 medium) (Sigma). After the strips were chopped into 1 mm^3 cubes using a mechanical tissue chopper (McIIwain Laboratory Engineering, Guilford, Surrey, UK), about 40 g of the chopped tissue were placed into two 50 ml sterile vials and mixed with 30 ml digestive solution containing 100 mg bovine serum albumin (BSA, Sigma), 50 mg trypsin III (Worthington, Lakewood, NJ 08701, USA) and 50 mg collagenase A (Roche, Welwyn Garden City, UK) per 100 ml of Hanks' balanced salt solution (HBSS; Sigma). The vials were briefly centrifuged at 100 × g and 10°C for up to 1 min to allow the cells to settle. The supernatant was then removed and replaced with 30 ml of the above

digestive solution. This significantly increased the effective concentration of the digestion and thus the yield of endometrial cells. After incubation for 90 min at 37°C with 5% CO_2 and manual mixing every 30 min, the cell suspension was filtered through a 100 µm mesh into 50 ml falcon vials containing 10% FBS and 3 µg/ml trypsin inhibitor (Sigma) with HBSS added to 50 ml and centrifuged at 100 × g at 10°C for 10 min. Following two repetitions of the above washing procedures, the cells were suspended with the culture medium (DMEM/F12 medium with 10% FBS) and plated in 24 well IWAKI micro plates (Scitech DIV, Asahi Techno Glass, Japan) at 2 ml per well containing 0.5×10^5 cells (day 1). Culture medium was changed every 48 h to allow the cells to grow. The composition of the cell population was confirmed using immunocytochemical staining validated in our laboratory and showed that the stromal and epithelial cells constituted about 10% and 90% respectively of the population on day 8 of culture [31], when IFNT challenge was carried out, with negligible presence of immune cells (data not shown).

Infection of bovine endometrial cells with ncpBVDV

The ncpBVDV (Pe515nc strain) was provided by the BVDV Research Group at the Royal Veterinary College, UK. This type 1 strain was isolated from a cow diagnosed with mucosal disease and virologically cloned as non-cytopathogenic virus and the consensus sequence of the E2 region was established [41, 42]. The virus stock was propagated in BVDV-free Madin-Darby bovine kidney epithelial cells to achieve a 50% tissue culture infective dose ($TCID_{50}$) of 5×10^5 per ml following the method described in detail previously [19, 31]. This propagated ncpBVDV was kept at -80°C until use.

The endometrial cells from 10 cows used in the present study were confirmed to be initially BVDV negative. Cells from each cow were taken as a batch and grown in two 24-well plates as described previously (day 1, see Supplementary Fig. 1). After the cells had grown for 4 days, reaching about 70% confluence, FBS in the culture medium was reduced to 5% (maintenance medium, MM) to prevent overgrowth of the cells. One plate was designated as the non-infected control and the other

plate for the ncpBVDV infection. These plates were subsequently maintained separately to avoid possible cross-contamination. To infect the cells with ncpBVDV, the wells were inoculated with 0.25 ml of MM containing Pe515nc BVDV at a multiplicity of infection (MOI) of 0.1 for 3 h. For the cells designated as the non-infected controls, 0.25 ml MM was added to each well following the above procedures. The volume in all wells was made up to 1 ml with MM and the medium was changed after two days. Details of how the treatment times and doses for ncpBVDV infection were optimized have been described previously [19, 31]. The infection of the endometrial cells with ncpBVDV was confirmed using both the PCR method described above with the extracted RNA and an indirect enzyme (alkaline phosphatase) immunostaining procedure as described previously [19, 29].

Treatment of bovine endometrial cells with IFNT

IFNT (recombinant ovine IFNT, Cell Sciences, Canton, USA) was prepared in MM at 100 ng/ml. Four days after the ncpBVDV infection (day 8 of culture) 1 ml of this IFNT enriched medium was added to the designated wells and the incubation continued for a further 24 h. The same procedure, but with 1 ml MM only, was performed for the other cells. The cells from each of the 10 replicate cows therefore included 4 treatment groups: Control (CONT), ncpBVDV, IFNT and IFNT+ncpBVDV. The treated cells were used for total RNA extraction (on day 9). The cell viability after exposure to the infection and treatment was also examined in a separate experiment with a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following the supplied protocol as described previously [31].

ISG15 protein measurement

The culture medium was collected 24 h following IFNT challenge and stored at -20 ^{o}C. Concentrations of ISG15 protein in the medium were quantified using an enzyme-linked immunosorbent assay (ELISA) (Insight Biotechnology, Middlesex, UK) following the supplier's protocol.

RNA extraction

Total RNA in the treated cells was extracted using RNeasy Mini Kits (Qiagen, Manchester, UK) following the supplier's protocol. Each treatment comprised 6 duplicated wells in each plate and these were pooled for total RNA extraction (one pooled RNA sample for each of four treatments in each cow). The concentrations and purity of RNA were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilminton, USA). The RNA was aliquoted as 1 ug/tube in 0.2 ml PCR tubes and stored at -80°C until reverse transcription (RT).

Primer design and PCR

The primers were designed using a "Primer 3" web based programme (http://frodo.wi.mit.edu/primer3) with the DNA sequences obtained from GenBank at NCBI (http://www.ncbi.nlm.nih.gov/Database/index.html). According to the recommendation by PCRBiosystems (London, UK) who supplied the reagents for cDNA synthesis (RT) and real time PCR (qPCR), the amplicon length for most of the genes was set to 100-200 bp with the predicted melting temperature of around 60 °C using the default Primer 3 settings. Their alignment specificity was checked using the Blast tool at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and an Amplify tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx) was used to check the primer quality. The detailed information of the primers is given in Table 1. The primers were made by Eurofins MWG Operon (Ebersberg, Germany).

One µg of total RNA was treated with DNase to eliminate potential genomic DNA contamination using a RQ1 RNase-Free DNase kit (Promega) and then reverse transcribed into cDNA in a 20 µl reaction volume using a cDNA synthesis kit supplied by PCRBiosystems following the supplier's protocol. Each 20 µl reaction contained 4 µl 5 × cDNA synthesis mix, 1 µl 20 × RTase and 1 µg of DNase-Treated RNA sample in 15 µl PCR grade water. RT for all samples was performed in one assay to minimize variation. The resulting cDNA was diluted in nuclease free water up to 100 µl to

achieve a concentration of 10 ng/µl and used for qPCR and conventional PCR. The conventional PCR for the tested genes was carried out using the G-Storm thermal cycler (G-Storm Ltd, Somerset, UK), Qiagen Multiple PCR kit (Qiagen) and the primers listed in Table 1 following the methods described previously [29]. The specificity of primers was verified using electrophoresis on a 2% (w/v) agarose gel. The cDNA amplicon for each gene was purified using a QIAquick PCR purification kit (Qiagen) and their quality and concentrations were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies Inc., Wilminton, USA). It was stored at -80°C for use in the qPCR standard curve and annealing temperature optimization.

qPCR analysis for gene expression

An absolute qPCR method was used to quantify the mRNA expression of all selected genes, including 18 target genes (*BST2, MX1, MX2, RSAD2, USP18, OAS1Y, ISG15, GBP4, IFI27, IFIT1, IFIT3, DDX58, PLAC8, IFIH1, HERC5, TRIM56, SAMD9* and *IL1B*) and four potential reference genes (*GAPDH, RPL19, ACTB* and *18SrRNA*) using methods reported previously [43]. A temperature gradient (55-65°C) qPCR with 8 identical reactions was carried out to determine the annealing temperature which produced maximal amplicon and the amplicon-specific melting temperatures of the primers using a gradient function of the qPCR machine (CFX96 Real-Time System DNA, Bio-Rad Laboratories, CA, USA). Each reaction contained 2 ng of the DNA standard, 10 µl Sygreen Mix (PCRBiosystems), 0.8 µl of each 10 µM forward and reverse primer and nuclease free water added up to 20 µl. The standard curve contained 8 concentrations from 1 to 1×10^{-7} ng/ml prepared using the purified DNA for each gene. Each qPCR assay contained the standard curve, no template control and sample cDNA for a testing gene from all cows in duplicate. Each qPCR vial contained 5 µl of cDNA standard or samples, 10 µl Sygreen Mix (PCRBiosystems), 0.8 µl of 10µM forward primer, 0.8 µl reverse primer (See Table 1) and 3.4 µl nuclease free water. Reactions and data acquisition were carried out on CFX96 Real -Time Systems (Bio-Rad) following the protocol supplied by PCRBiosystem, including an initial Taq activation step at 95 °C for 2 min followed by 38 cycles of

denaturation (95 °C), annealing (the annealing temperatures are given in Table 1) and extension (63°C). In addition, an amplicon-specific melting temperature obtained in the above gradient test was applied to avoid any noise from smaller non-specific products, such as dimers, prior to the product acquisition. Concentrations of the sample amplicons were calculated using the standard curve and a semi-log regression built in the CFX Manager software package (Bio-Rad). The limit of quantification was 1×10^{-6} - 1×10^{-7}ng/ml for all tested genes.

Statistical data analysis

Among the four selected reference genes, only the expression of *GAPDH* was not significantly altered by the treatments (see Results below). The concentration of DNA for all the target genes was, therefore, normalised to the expression of *GAPDH* as described previously [44]. Statistical data analysis was carried out using SPSS V23 (Chicago, IL, USA). Logarithmical transform (*BST2, HERC5, ISG15, MX2, OAS1Y, RSAD2, SAMD9* and *USP18*) was applied where the data were not normally distributed. A linear mixed effect model was used for analysis of variance (ANOVA) with randomized block design, which included treatments (CONT, ncpBVDV, IFNT and their combination) as fixed effects and cow as random effect. The statistical level of significance was set to $P < 0.05$. Where ANOVA showed statistical significance, Fisher's LSD multiple comparisons based on the least square means were performed to identify the sources of differences.

RESULTS

The PCR test using the RNA extracted from the uterine tissues showed that the uteri used for the experiment were initially free of BVDV infection. An immunocytochemical procedure as described previously [31] was used to demonstrate that ncpBVDV antigen was detected in both the epithelial and stromal cells following experimental infection, with no cross-infection in the controls (data not shown). At the end of culture (day 9 in total and five days after ncpBVDV infection), ncpBVDV RNA was also detected by PCR in the endometrial cultures treated with ncpBVDV or ncpBVDV+IFNT

while it was not detectable in the CONT or IFNT-treated samples (Fig. 1). The MTS reduction assay demonstrated that viability was not affected by the individual or combined treatments at the doses used. After exposure of the cultured endometrial cells to ncpBVDV for 4 days and to IFNT at 0 or 100 ng/ml for 24 h, the absorbance values at 490 nm in a cell viability assay were 1.7 ± 0.07 for CONT, 1.8 ± 0.04 for IFNT, 1.7 ± 0.09 for ncpBVDV and 1.7 ± 0.06 for ncpBVDV+IFNT (mean ± SE, n=6/group, $P > 0.05$). These results are in accord with the strain of BVDV selected for use which was known to be non-cytopathic.

Effect of ncpBVDV, IFNT and their combination on expression of the selected reference genes by uterine endometrial cells

All four potential reference genes (*ACTB, RPL19, GAPDH* and *18SrRNA*) were highly expressed in the cultured bovine endometrial cells as measured in absolute units using qPCR (Table 2). Only *GAPDH* expression, however, remained stable following treatments ($P > 0.05$). *RPL19* expression was increased by the individual treatments with IFNT or ncpBVDV, whilst expression of *ACTB* and *18SrRNA* was significantly lower following the combined treatment ($P<0.05$-0.01). For subsequent comparison of the treatment effects the expression levels of each gene were, therefore, normalised against *GAPDH*. The absolute expression values of the candidate genes were also shown for the CONT cell cultures. All 18 candidate genes were detected with a large range in the basal levels of expression (Table 3).

Effect of ncpBVDV and IFNT alone on expression of candidate genes

ncpBVDV infection alone generally had little effect on gene expression (Fig. 2). Only one gene, *TRIM56*, was significantly up-regulated five days after infection ($P<0.001$) whereas expression of three ISGs (*ISG15, MX2, BST2*) together with *IL1B* was reduced ($P<0.05$). As anticipated, IFNT treatment alone significantly increased expression of all 17 candidate ISGs tested ($P < 0.05$-0.0001) but there was a tendency ($P = 0.06$) for IFNT to decrease expression of *IL1B* (Fig. 2).

Effect of IFNT on expression of candidate genes in BVDV infected endometrial cells

In contrast to the limited effect of ncpBVDV infection alone, the virus had a profound influence on the ability of IFNT to up-regulate the expression of the candidate ISGs. Of the 17 ISGs tested, the stimulatory effect of IFNT on expression of 15 was significantly inhibited ($P < 0.05$ - 0.001), generally to a value intermediate between that found in the CONT and IFN treated cells (Fig. 2). Genes responding in this way included *ISG15, HERC5, USP18, DDX58, IFIH1, IFIT1, IFIT3, BST2, MX1, MX2, RSAD2, OAS1Y, SAMD9, GBP4* and *PLAC8*. BVDV infection did not, however, alter IFNT-stimulated mRNA expression of two genes ($P > 0.05$), *TRIM56* or *IFI27*. Whereas ncpBVDV alone caused a small but significant reduction in *IL1B* ($P < 0.05$), expression of this gene was markedly reduced by the combined treatment ($P < 0.001$).

ISG15 protein assay

ISG15 is known to be secreted as a cytokine, so its protein concentration was measured by ELISA in the medium. This was nearly doubled following treatment with IFNT alone ($P<0.01$). BVDV infection alone did not change the concentration but in the combined treatment BVDV completely inhibited the IFNT stimulation (Fig. 3).

DISCUSSION

Many previous publications have described the key influence of IFNT in the maternal recognition of pregnancy in cows, which includes maintenance of the corpus luteum and development of a receptive environment in the uterus for successful implantation (Reviewed by [25, 27, 28]). During this process many ISG are up-regulated in the endometrial cells [22, 23, 25], and we confirmed here that expression of all the candidate ISGs increased significantly following treatment with IFNT alone. BVDV is a major cause of reduced fertility in many countries [2, 3]. The virus is known to have

evolved a variety of mechanisms to help it evade detection and eradication by host cells [13-15]. We confirmed here that ncpBDVD can become established and proliferate within epithelial and stromal cells of the uterine endometrium. While infection alone produced few changes in gene expression, we show here for the first time that the ability of endometrial cells to respond to IFNT by increased expression of a variety of ISGs was markedly inhibited in ncpBDVD infected cells. This is likely to facilitate the ability of the virus to survive within the uterine environment and may well contribute to the reduced fertility of infected cows.

The model system we developed was based on a mixed culture of epithelial and stromal cells as both can become infected with virus and the interaction between the two cell types is important in the maternal recognition of pregnancy. Both cell types are also known to exhibit innate immune activity [33]. The immune cell population may be important modulators of the response *in vivo* but were not included in our system [31], allowing us to demonstrate responses of the endometrial cells alone. We have shown previously that infection of these mixed endometrial cultures with ncpBVDV inhibits their immune response to a challenge with bacterial LPS [19]. Further validation of the system was provided by showing that IFNT treatment alone stimulated expression of *PTGS1* and synthesis of PGE_2 [29]. This is in accord with the suggested importance of PGE_2 as a key signalling molecule in early pregnancy [27, 30]. The cultured cells expressed *PGR*, *ESR1* and *OTR* as expected [29] in addition to the TLR receptors which are important for the detection of RNA viruses (TLR2, -3, -4, -7 and -8) [16, 45]. Progesterone is also known to play a key role in modulating the endometrial response to IFNT. The timing of the postovulatory progesterone rise co-ordinates the temporal changes in the endometrial transcriptome. Progesterone down regulates its own receptor while inducing a variety of genes whose expression is later increased further by IFNT. These include genes involved in transport of glucose and amino acids, cell proliferation and PG synthesis [28, 32]. Progesterone treatment was not included in the current experimental design as it would have introduced too many variables to test with adequate replication in the same batch of isolated cells. Its absence may, however, have altered some of the gene expression profiles in response to IFNT in comparison with the *in vivo* situation.

The cells were infected with ncpBVDV several days before the IFNT challenge. This was done to mimic one potential *in vivo* situation. Cows which have not previously been exposed to BVDV generally become infected following a herd breakdown, either directly from the persistently infected (PI) animal or from other cows which have recently become acutely infected. This may happen during the calving period when heifers and older cows are often mixed. Infection is also possible (although less likely) with semen from a PI bull or following intra-uterine transfer of infected embryos on d 7 of the oestrous cycle [9, 10, 46]. The length of time the virus can subsequently survive in the uterus has not been well established, but there is evidence that this can be for several weeks. Depending on the relative timing of infection and mating, this could impair fertilization and very early embryo development [47] or disrupt MRP as suggested by the present experiment.

One key mechanism by which BVDV can evade the host immune response is by the action of the viral protein N^{pro} which blocks production of Type 1 interferons [13, 19, 48]. On the other hand, there was up-regulation of type I interferon-induced genes in spleen and tracheo-bronchial lymph nodes of beef calves five days after BVDV infection [49] and in the blood of transiently infected mid-pregnant heifers and their fetuses [50]. There are a number of possible reasons behind these discrepancies. Different cell types may respond to BVDV infection differently and there are differences in virulence between strains of type 1 and type 2 ncp BVDV [51]. The absence of immune cells in most *in vitro* cultures is also a likely factor. Nevertheless, the lack of any rise in expression of ISGs with the single exception of *TRIM56* following the ncpBVDV treatment alone in the present study implies that the endometrial cells were not exposed to a significant increase in self-generated Type 1 IFN prior to the experimental treatment with IFNT.

When faced with a viral attack, the body mobilizes its' defence systems to restrict, neutralize and remove the virus. Gene targeting studies have revealed there are four main effector pathways of IFN-mediated antiviral responses, including an ISG15 ubiquitin-like pathway, an MxGTPase pathway, an OAS1-RNaseL pathway and a protein kinase R pathway [52]. Our present study demonstrated that IFNT challenge activated these pathways to develop a pro-immune and antiviral environment in the uterus by stimulating ISG expression. Inhibition of the stimulatory effect of IFN on uterine ISG

production may, however, facilitate the establishment of life-long persistent infection of bovine fetuses with BVDV following intra-uterine infection of the dam in the first trimester of pregnancy [53, 54], so leading to the birth of PI animals which are a major cause of disease spread within herds [4].

ISG15 is one of the most upregulated genes during pregnancy recognition in cows. This may facilitate successful conceptus attachment or act as a defence strategy against infection [22, 25, 55]. Its precise role in this respect has yet to be determined but studies in mice have shown increased embryo mortality in *Isg15*$^{-/-}$ dams [56]. In the present study BVDV infection markedly inhibited both basal and IFNT-induced *ISG15* expression. Free ISG15 is a cytokine to induce natural killer cell proliferation and IFN-γ production and to act as a chemotactic factor for neutrophils [57] and we confirmed here both its IFNT-stimulated secretion into the medium and the suppression of the stimulation by BVDV infection. ISG15 also acts intracellularly as an ubiquitin-like modifier of many target proteins. In a process known as ISGylation, the C-terminus of ISG15 is conjugated to lysine residues in the target protein following consecutive catalysis with three enzymes E1, E2 and E3 [58] (see also Supplementary Fig. 2). The modified protein may be targeted to lysosomes for destruction or used elsewhere. HERC5 and TRIM56 are both E3 ligases. HERC5 does not have substrate specificity so is able to block the IFN-mediated rise in the total level of ISGylated cellular proteins [58]. TRIM56 is a single-RING-finger E3 ligase which interacts specifically with the N-terminal protease of BVDV, N^{pro}, so exhibiting anti-viral activity [17]. It was interesting to note that *TRIM56* was the only ISG tested which was up-regulated by BVDV alone whereas *HERC5* followed the more common pattern in which ncpBVDV alone had no effect but the virus markedly inhibited IFNT-stimulated up-regulation.

USP18 is an ubiquitin-specific protease which removes ISG15 from the modified protein (De-ISGylation) and can also process the ISG15 precursor protein (Supplementary Fig 2) [34-36]. IFN-induced *USP18* expression was also inhibited by ncpBVDV. ISGylation affects many proteins with important biological functions, including regulation of 1) innate immunity and anti-viral/bacterial infections, 2) proteasome function for protein turnover, 3) apoptosis and tumorigenesis and 4) cell proliferation and remodelling [36]. The overall up-regulation of *ISG15*, *HERC5* and *USP18* by IFNT

supports the suggestion that the ISGylation pathway is an important regulator of protein processing in the endometrium during MRP [55], with the results of the present study implying that this system can be inhibited by ncpBVDV. On the other hand, up-regulation of *TRIM56* by ncpBVDV shows that the endometrial cells were still able to offer some immune response, even if this was much reduced.

DDX58 (RIGI), IFIH1 (MDA5), IFIT1 (ISG56), MX1 and MX2 are all potential targets of ISGylation. DDX58 and IFIH1 are both DEAD box proteins which are cytosolic sensors of viral RNA. They act as RNA helicases and are implicated in a number of cellular processes involving RNA binding and alteration of RNA secondary structure. DDX58 recognises ssRNA whereas IFIH1 targets dsRNA. As BVDV is a single stranded virus, it would only be recognised by IFIH1 during the RNA replication phase [50, 53, 59]. The virus may evade detection by rapid incorporation into the endosome which buds off to form multivesicular bodies [60, 61]. Although these can potentially target their contents to lysosomes for disposal, multivesicular bodies can also shield the viral protein from detection except when the viral ribonoprotein is released into the cytoplasm before uptake into the endoplasmic reticulum for replication (see Fig. 4). In contrast to the situation reported here, both *DDX58* and *IFIH1* were upregulated in fetal blood cells in response to persistent infection with ncpBVDV [50, 53, 59].

All the remaining ISGs studied apart from PLAC8 have known antiviral activity. *IFIT1* and *IFIT3* (also called retinoic acid-induced gene G protein, RIGG) both encode IFN-inducible proteins which can form a cytoplasmic complex to recognise and destroy viral RNA, so acting as inhibitors of viral replication [62, 63]. We found that *IFIT1* was much more highly expressed than *IFIT3* in bovine endometrial cells, by a factor of over 2,000. MX1 and MX2, the Mx dynamin-like GTPases, are key effectors in MxGTPase antiviral pathways. They can disturb the transport, transcription and translation of viruses within the cells [52, 64]. IFI27 (ISG12) is involved in promoting apoptosis via mitochondrial membrane destabilization that may influence the innate immune response of IFNs and cellular metabolism [65]. It and TRIM56 were the only ISGs tested in the present experiment in which up-regulation by IFNT was not affected by ncpBVDV infection. The OAS1 (including OAS1Y) gene family are the key effectors in the OAS1-RNaseL antiviral pathway [52]. They sense exogenous

nucleic acid and activate endoribonuclease L (RNAseL) which degrades viral RNA [66]. RSAD2 (Viperin) interacts with non-structural viral proteins to inhibit viral RNA replication and it can also limit egress of some viruses from the cell [67, 68]. GBP4 is an IFN-induced GTPase which disrupts the interaction of TNF receptor associated factor 6 (TRAF6) with IRF7, so reducing virus mediated induction of type 1 IFNs [18]. SAMD9 is a facilitator of endosome fusion which participates in the formation of granules with antiviral properties [69]. BST2 acts as the "tetherin" molecule to restrict the egress of many viruses, such as HIV-1 and Ebola virus, so reducing their spread within the host [70]. The down-regulation of the stimulatory effect of IFNT on all these genes by ncpBVDV is therefore clearly able to inhibit many of the mechanisms by which a cell normally combats a viral attack (Fig. 4A).

PLAC8 was originally identified in mouse placenta [71]. It has subsequently been detected in a variety of cell types including bovine endometrium [72] and was the gene with the highest basal expression value in the bovine endometrial cells studied in the present paper. It is upregulated by IFNT but its function appears variable according to cell type. It can inhibit growth of some cancers by attenuating cell-cycle progression, either induce or inhibit apoptosis and inhibit proliferation of endothelial colony-forming cells [38, 73], while PLAC8 deficient mice have defects in innate immunity [74]. The role of PLAC8 in bovine implantation remains to be determined.

The final gene investigated in the present study was the pro-inflammatory cytokine *IL1B.* In this case IFNT treatment alone tended to reduce expression whereas the combined treatment of ncpBVDV + IFN resulted in significant down-regulation in the endometrial cells. Its moderate inhibition by IFNT may be beneficial during implantation by preventing immune rejection [39], but the much greater inhibition in the presence of ncpBVDV may lead to decreased uterine immunity. In addition to the antiviral effects of uterine ISGs, many of the proteins encoded by these genes also possess antibacterial functions. Numerous immune genes, including all the ISGs examined here (*ISG15, HERC5, USP18, DDX58, IFIT1, IFIT3, MX2, RSAD2, GBP4, SAMD9, IFI27, OAS1Y* and *BST2*) were upregulated following treatment of bovine uterine endometrial cells with the bacterial endotoxin LPS [31]. This stimulatory effect was again significantly inhibited in the presence of ncpBVDV infection

[19, 31]. Infection of bovine uterine endometrial cells with ncpBVDV also induced an endocrine switch of PG production from $PGF_{2\alpha}$ to PGE_2 [29]. As $PGF_{2\alpha}$ is an immune enhancer and PGE_2 is an immune suppressor in the uterus [75, 76], this switch would also lead to decreased uterine immunity. These results support the suggestion that BVDV infection can predispose cows to develop endometritis due to bacterial infection following calving. This in turn may also contribute to early embryonic death and failure of pregnancy establishment [77].

In summary, IFNT challenge significantly stimulated the mRNA expression of many ISGs in uterine endometrial cells. These ISGs are clearly important for pregnancy recognition and implantation in the cow, although their precise actions in this respect generally remain to be established. They do, however, have more well defined roles in protecting the host from both viral and bacterial infections. We show here in an *in vitro* situation that the stimulatory effect of IFNT on 15 out of 17 ISGs tested was inhibited by prior infection with ncpBVDV, as summarised in Fig. 4. As outlined above, ncpBVDV normally evades detection in host cells by down regulation of Type 1 IFN synthesis. As it was still able to downregulate nearly all the ISGs in the face of exogenous IFNT treatment, the virus clearly has additional defence mechanisms available. Future work is warranted to determine whether these ISGs are also downregulated *in vivo* in the endometrium of recently mated cows transiently infected with BVDV. Previous studies show that if bovine conceptuses have not elongated sufficiently by day 16 of pregnancy, then their IFNT production will be inadequate to prevent onset of the normal luteolytic mechanism [21, 24]. This provides supporting evidence that a failure of the normal downstream effects of IFNT, as shown here, is also likely to result in pregnancy failure. Decreased uterine immunity against both viral and bacterial infection may provide another mechanism whereby ncpBVDV infection can cause early embryonic death and reduced fertility. Finally, the remarkable ability of ncpBVDV to inhibit bovine uterine defence systems may, at least in part, provide a mechanism whereby the reproductive system becomes a major site for ncpBVDV infection, facilitating maintenance and spread of this disease within the cattle population.

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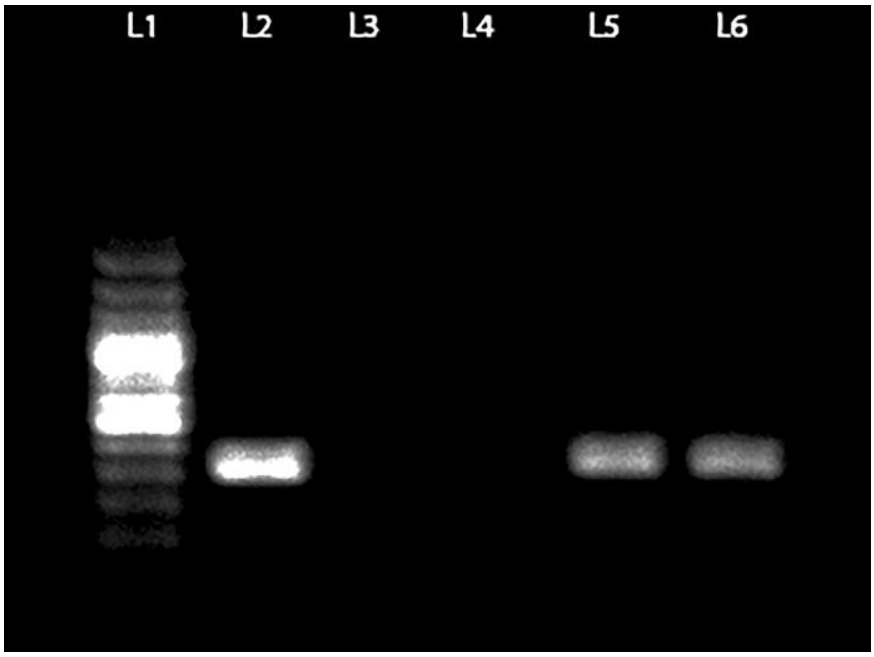

Fig. 1. Validation of ncpBVDV infection in bovine endometrial cell culture groups using PCR with RNA from the cells harvested at the end of the experiment. L1: DNA ladder, L2: ncpBVDV positive control, L3: CONT, L4: IFNT, L5: ncpBVDV and L6: ncpBVDV+IFNT.

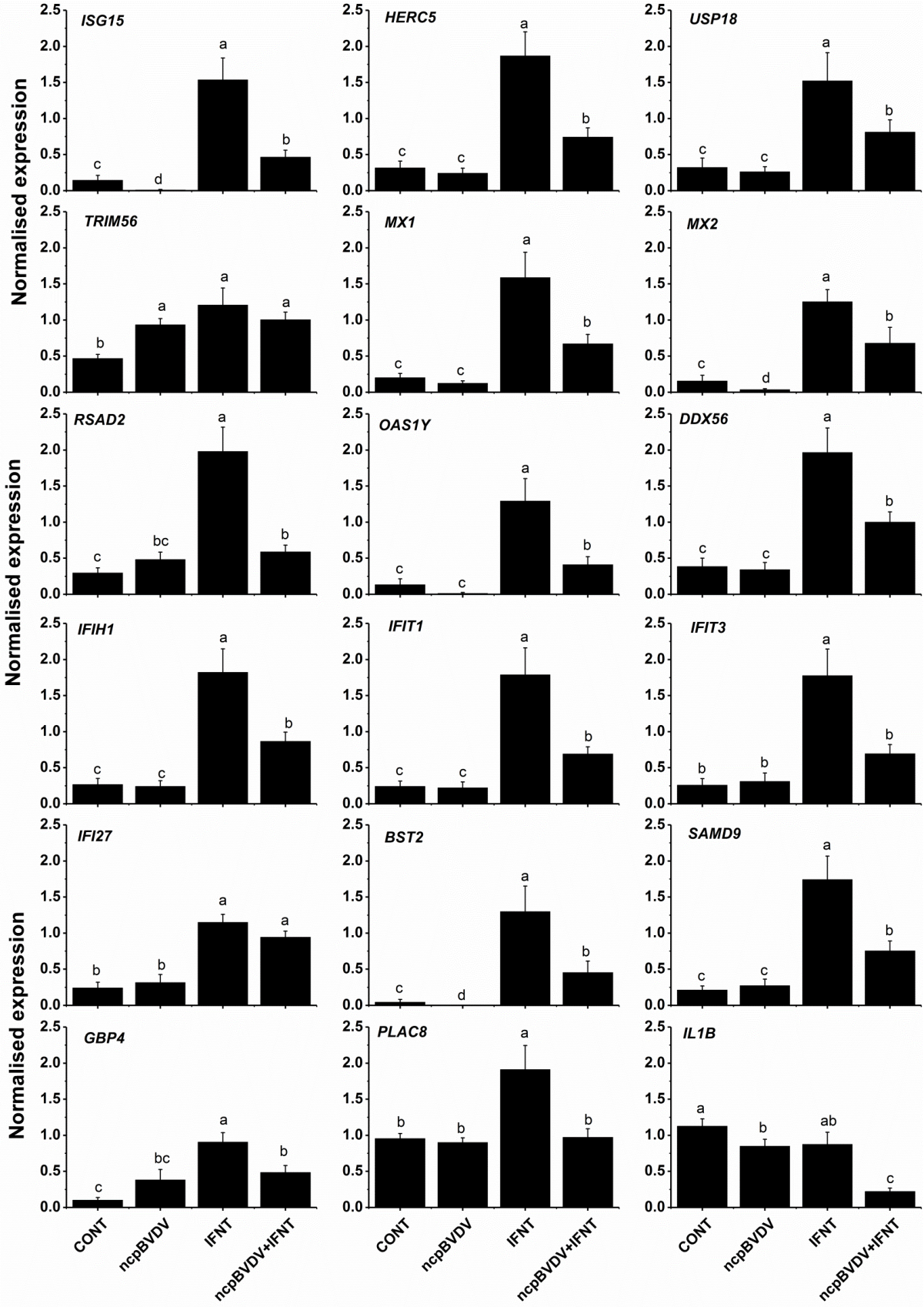
ISG15
HERC5
USP18
TRIM56
MX1
MX2
RSAD2
OAS1Y
DDX56
IFIH1
IFIT1
IFIT3
IFI27
BST2
SAMD9
GBP4
PLAC8
IL1B
Normalised expression
CONT
ncpBVDV
IFNT
ncpBVDV+IFNT

Fig. 2. Effect of CONT, ncpBVDV, IFNT and ncpBVDV+IFNT treatments on gene expression in bovine endometrial cells. Values are expressed as the ratios to *GAPDH* after an absolute gene quantification using qPCR and are presented as the mean ± SEM. The columns labelled with different letters were significantly different at P < 0.05-0.0001 (a > b > c > d).

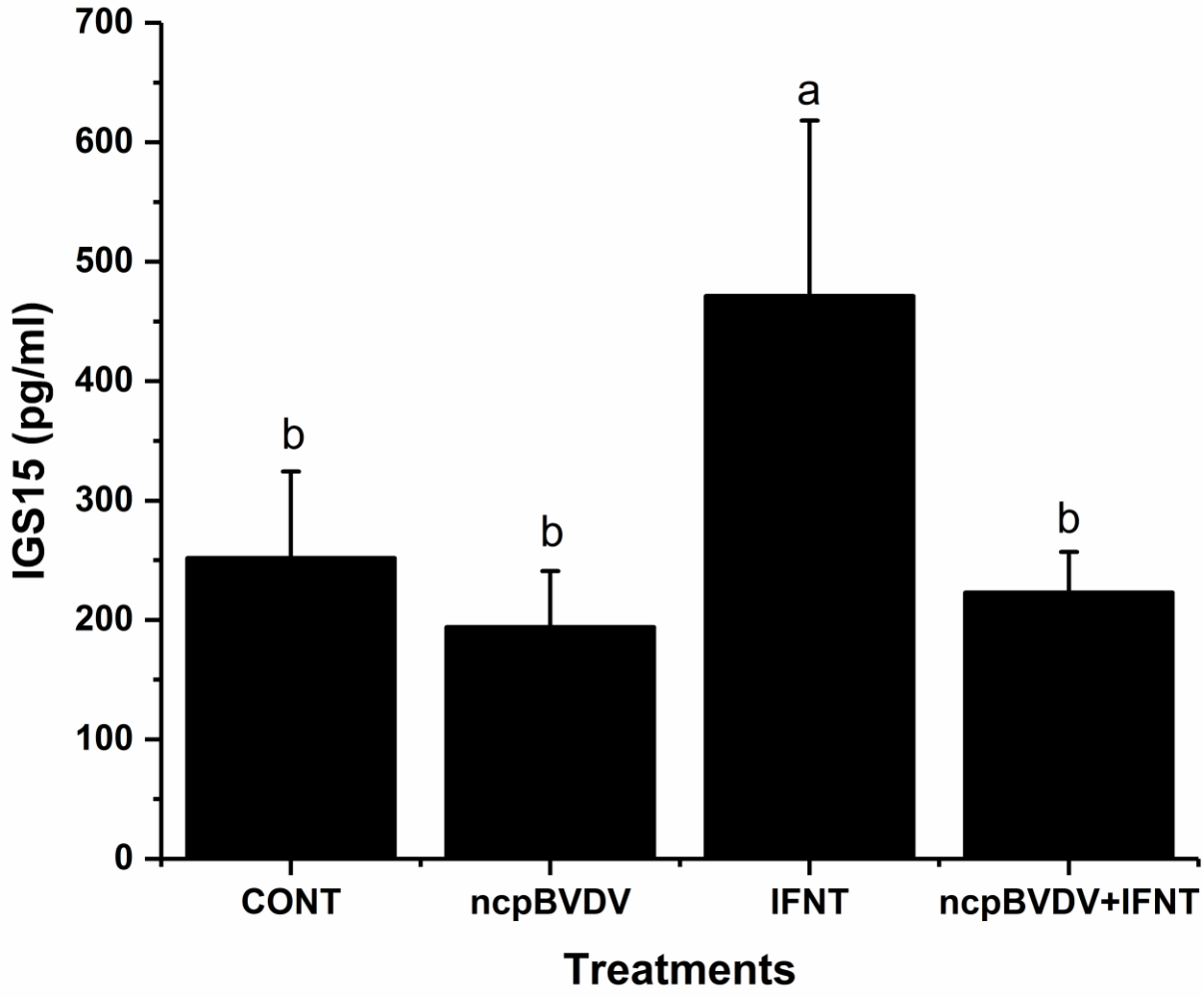

Fig. 3. Effect of CONT, ncpBVDV, IFNT and ncpBVDV+IFNT treatments on secretion of ISG15 into the culture medium. Results are presented as the mean ± SEM of 10 individual cow endometrial samples tested. The columns labelled with different letters were significantly different a > b at P < 0.05-0.01.

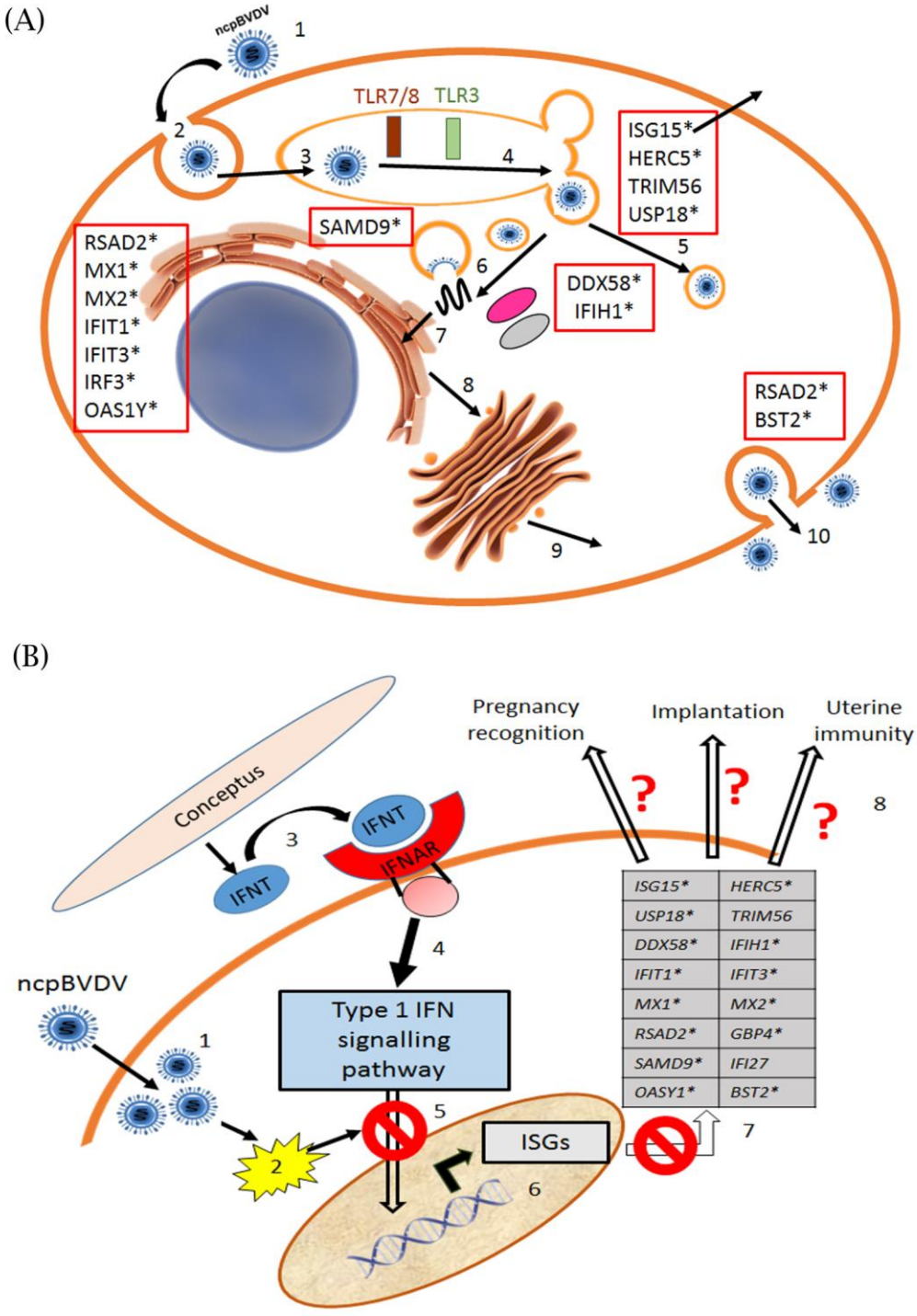
(A)
ncpBVDV
1
2
3
4
TLR7/8
TLR3
ISG15*
HERC5*
TRIM56
USP18*
5
SAMD9*
6
DDX58*
IFIH1*
7
RSAD2*
MX1*
MX2*
IFIT1*
IFIT3*
IRF3*
OAS1Y*
8
RSAD2*
BST2*
9
10
(B)
Conceptus
IFNT
3
IFNT
IFNAR
Pregnancy recognition
Implantation
Uterine immunity
?
?
?
8
ISG15* HERC5*
USP18* TRIM56
DDX58* IFIH1*
IFIT1* IFIT3*
MX1* MX2*
RSAD2* GBP4*
SAMD9* IFI27
OASY1* BST2*
4
ncpBVDV
1
Type 1 IFN signalling pathway
5
ISGs
7
2
6

Fig. 4. Diagrams illustrating the potential effects of an acute ncpBVDV infection on expression of uterine ISGs and pregnancy recognition in the cow. Panel (A) shows the points at which cells normally detect and deactivate the virus. (1) ncpBVDV reaches the reproductive tract following infection. (2) The virus attaches to the host cell membrane and is taken up by clathrin mediated endocytosis. (3) Virus is incorporated into the endosome, where it may be sensed by TLR3 or TLR7/8. (4) The endosome buds off multivesicular bodies. While in these ncpBVDV may be protected from detection by cytoplasmic pattern recognition receptors (PRR), in particular DDX58 which detects single stranded (ss)RNA. SAMD9 is a facilitator of endosome fusion. (5) Alternatively virus may be detected and targeted to lysosomes for disposal. This may involve ISGylation. ISG15 may also be secreted as a cytokine. (6) Viral ssRNA is released into the cytoplasm. (7) Viral mRNA is translated into a single polyprotein in the endoplasmic reticulum. This is cleaved into structural proteins (three envelope and one capsid protein) and about eight non-structural proteins, which are involved in replication and assembly. These include the protease N^{pro}. Viral ssRNA is replicated and transcribed. During this process double stranded (ds) RNA is produced, which can be detected by the PRR IFIH1. New virons are assembled. The ISGs RSAD2, MX1, MX2, IFIT1, IFIT3, IRF3 and OAS1Y are all able to disrupt viral transcription and/or translation (8) The ncpBVDV virons pass through the Golgi complex. (9) Virons are transported from the Golgi to the cell surface in small exocytotic vesicles. (10) New virons are released from the cell by exocytosis. Both RSAD2 and BST2 are able to inhibit viral egress. *IFNT-stimulated ISGs significantly suppressed by ncpBVDV. Panel (B) illustrates possible consequences of ncpBVDV infection for signalling by IFNT during the maternal recognition of pregnancy (MRP). (1) Infection of bovine endometrium with ncpBVDV and viral intracellular replication. (2) ncpBVDV produces viral factors which can counteract the host's immune response. (3) The early bovine conceptus subsequently secretes IFNT into the uterine lumen which initiates and establishes MRP, normally by day 16 of gestation in cows. (4) IFNT, a type I IFN, signals through binding to the interferon alpha receptors (IFNAR) to stimulate production of many ISGs crucial for MRP and uterine immunity. (5, 6, 7) Viral-derived factors inhibit the type I IFN signalling and transcription pathways, leading to decreased expression of many IFNT-stimulated ISGs. (8) ncpBVDV infection may, therefore, disrupt MRP and uterine immunity.

Supplementary Fig. 1. Summary diagram showing the experimental design used. ncpBVDV, non cytopathic bovine virus diarrhea virus; CONT, control; IFNT, interferon tau.

Supplementary Fig. 2. Illustration of the ISGylation pathway. Proteases cleave off the C-terminal extensions from ISG15 precursors to generate mature ISG15. This may be secreted as a cytokine or released during cell lysis. Alternatively, ISG15 may be activated by the sequential actions of enzymes E1 and E2. It is then linked to a target protein by the action of an ISG15 E3 ligase [58]. These include HERC5 and TRIM56. The modified protein may be either targeted to lysosomes for destruction or used elsewhere in the cell. USP18 (UBP43) reverses the ISGylation process by cleaving off ISG15 from the target protein. It can also act as one of the ISG processing proteases. The pathway is up-regulated by Type 1 interferons. These include IFNα, normally produced in response to a viral infection, and IFNT, produced by the ruminant conceptus during the maternal recognition of pregnancy.

Table 1. Oligonucleotide primer sequence information

Gene	Primer sequence (5'-3')	GenBank accession	Product length (bp)	Annealing (°C)
BST2	Forward: TGATCTACTTCGCTGTCATTGC	XM_871059.6	167	61.4
	Reverse: TGGGTCTGTTCCTTCTTCAGAG			
MX1	Forward: ACATGATCGTCAAGTGCCGT	NM_173940.2	113	61.4
	Reverse: AAGGTCCCTGAAATGTGCGT			
MX2	Forward: TATATGATCGTGAAGTGCCGGG	NM_173941.2	170	59.0
	Reverse: AGCTCGGTGGTAAGTCTTTCTG			
RSAD2	Forward: TATGCGCTTCCTGAACTGTAGA	NM_001045941.1	150	57.0
	Reverse: AGGTCTGCTTTGCTCCATACAT			
USP18	Forward: CCCTGAAAACGCTGGAGGAT	NM_001017940.1	133	61.4
	Reverse: GGCAGATGGGTCAGTGTCAA			
OAS1Y	Forward: CTCACAGAGTTCGGGTGTCC	NM_001040606.1	226	61.4
	Reverse: TGCCGTTTCTGGACCTCAAA			
ISG15	Forward: AGAAGATCAATGTGCCTGCTTT	NM_174366.1	161	61.4
	Reverse: CTTGTCGTTCCTCACCAGGAT			
GBP4	Forward: CCTACCTGATGAACCGCCTA	NM_001102261.2	197	61.4
	Reverse: CAGGGCAAAGATCCACAAGT			
IFI27	Forward: GAATCACTGCCTCCTCCTTG	NM_001038050.2	145	62
	Reverse: CCCACCAAGAGTTTGGATGA			
IFIT1	Forward: GGAACGTGCTGTGCAACTAA	XM_010819765.1	136	62
	Reverse: TTTGTCGAGTGCTTTCATGC			
IFIT3	Forward: TGCTGACAAGGTGAAACGAG	NM_001075414	111	63.2
	Reverse: TTTTTCCCACCGCACTTTAC			
DDX58	Forward: TCCGAACCAACAGAGACAGC	XM_002689480.4	137	64.2
	Reverse: TCTGCCTCTGGTCTGGATCA			
PLAC8	Forward: ACCCAGTTGTTTCACAGCCA	NM_001025325.2	135	62
	Reverse: GACATGAAAGGCACAGGGGA			
IFIH1	Forward: AGCCACTCCTTTTAGCCACG	XM_010802053.1	194	62
	Reverse: TCCCATGGTGCCTGAATCAC			
HERC5	Forward: GGTGATGGAAAGTACGGGCA	NM_001101995.1	193	62
	Reverse: CACCGTTTCCCAGTTGTCCT			
TRIM56	Forward: TTCAGACCCCAAATCAGGAC	NM_001206574.1	126	62
	Reverse: TCTGGGCTCTGCTCTCTTTC			
SAMD9	Forward: CGCTGGACATGCTAACAGAA	NM_001205781	181	64.2
	Reverse: TTAACTGCCACGTTCCCTTC			
IL1B	Forward: ACGAGTTTCTGTGTGACGCA	NM_174093.1	147	61.4
	Reverse: TGCAGAACACCACTTCTCGG			
GAPDH	Forward: GGTCACCAGGGCTGCTTTTA	NM_001034034.2	147	61.4
	Reverse: TTCCCGTTCTCTGCCTTGAC			
RPL19	Forward: TCGATGCCGGAAAAACAC	NM 001040516	119	59
	Reverse: ATTCTCATCCTCCTCATCCAG			
ACTB	Forward: GAAATCGTCCGTGACATCAA	NM_173979.3	182	61.4
	Reverse: AGGAAGGAAGGCTGGAAGAG			
18SrRNA	Forward: CGGCGACGACCCATTCGAAC	AY779625	99	64.5
	Reverse: GAATCGAACCCTGATTCCCCGTC			

Table 2. Effect of ncpBVDV, IFNT and their combination on expression of the selected reference genes in cultured mixed bovine endometrial cells#

Treatments	*ACTB*	*RPL19*	*GAPDH*	*18SrRNA*
CONT	81 ± 8.6[a]	118 ± 10.3[b]	159 ± 16.4	27,490 ± 2,301.3[a]
IFNT	99 ± 20.5[a]	166 ± 19.7[a]	165 ± 24.8	23,258 ± 2,270.5[a]
ncpBVDV	74 ± 11.7[ab]	151 ± 6.0[a]	170 ± 11.1	26,189 ± 3,490.3[a]
IFNT+ncpBVDV	48 ± 7.3[b]	138 ± 12.0[ab]	170 ± 17.7	15,665 ± 813.2[b]

Expressed in pg/ug RNA

Within columns, a>b, P<0.05-0.01.

Table 3. Basal expression values of the candidate genes measured in fg/µg RNA in the CONT cultures of mixed bovine endometrial cells using absolute qPCR[#]

High expression		Moderate expression		Low expression	
PLAC8	9240 ± 2002.7	*HERC5*	530 ± 90.9	*USP18*	0.63 ± 0.17
IFIT1	4634 ± 771.8	*GBP4*	306 ± 108.7	*OAS1Y*	0.14 ± 0.04
IFIH1	2144 ± 278.6	*IFI27*	155 ± 29.0	*BST2*	0.0014 ± 0.0004
SAMD9	1374 ± 329.7	*ISG15*	96 ± 19.4		
TRIM56	1094 ± 74.7	*RSAD2*	92 ± 16.9		
DDX58	984 ± 42.5	*MX1*	50 ± 4.8		
		IL1B	24 ± 5.7		
		MX2	5.2 ± 1.2		
		IFIT3	1.9 ± 0.9		

[#] Note that expression levels do not necessarily translate into similar levels of protein production due to further down-stream regulation in processing.