**Bovine Viral Diarrhea Virus Type 2 *In Vivo* Infection Modulates TLR4
Responsiveness in Differentiated Myeloid Cells Which Is Associated With Decreased
MyD88 Expression**

Robert G. Schaut^{a,b,1}, Jodi McGill^{b,2}, John D Neill^b, Julia F Ridpath^b, Randy E Sacco^{a,b*}

^a Immunobiology Interdepartmental Graduate Program, Iowa State University, 2018

Molecular Biology Building, Ames, IA 50011, USA

^b Ruminant Diseases and Immunology Research Unit, National Animal Disease Center,

ARS, USDA, 1920 Dayton Avenue, Ames, IA 50010, USA

*Corresponding Author: National Animal Disease Center, 1920 Dayton Ave, Ames, IA

50010; phone: 515-337-7354; email: randy.sacco@ars.usda.gov

¹Present address: University of Iowa College of Public Health, Department of

Epidemiology, Center for Emerging Infectious Diseases, 2501 Crosspark Road,

Coralville, IA 52241, USA

²Present address: Kansas State University College of Veterinary Medicine, Department of

Diagnostic Medicine and Pathobiology, K-221 Mosier Hall, Manhattan, KS 66506, USA

20 **Keywords:**

21 **Bovine Viral Diarrhea Virus (BVDV 2), Bovine Respiratory Disease Complex**
22 **(BRDC), Myeloid Differentiation Primary Response Gene 88 (MyD88),**
23 **Nonstructural Gene 5A (NS5A), qPCR, Cytokine**

24

25 **Abbreviations:**

26 **BRDC – Bovine respiratory disease complex**

27 **BVDV – Bovine Viral Diarrhea Virus**

28 **CP – Cytopathic**

29 **HV – High Virulence**

30 **IFN γ – Interferon gamma**

31 **IL-6 – Interleukin 6**

32 **LPS – Lipopolysaccharide**

33 **LV – Low Virulence**

34 **MDM Φ – Monocyte derived macrophage**

35 **MyD88 - Myeloid Differentiation Primary Response Gene 88**

36 **NCP – Non-cytpathic**

37 **NS5A – Non-structural protein 5A**

38 **qPCR – Quantitative (Real-Time) Polymerase Chain Reaction**

39 **TLR – Toll like receptor**

40 **TNF α - Tumor necrosis factor alpha**

41 **TRIF - Toll-interleukin 1 receptor domain containing adapter-inducing interferon- β**

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Abstract

Symptoms of bovine viral diarrhea virus (BVDV) infection range from subclinical to severe, depending on strain virulence. Several *in vitro* studies showed BVDV infection impaired leukocyte function. Fewer studies have examined the effects of *in vivo* BVDV infection on monocyte/macrophage function, especially with strains of differing virulence. We characterized cytokine production by bovine myeloid cells isolated early or late in high (HV) or low virulence (LV) BVDV2 infection. Given BVDV infection may enhance susceptibility to secondary bacterial infection, LPS responses were examined as well. Monocytes from HV and LV infected calves produced higher levels of cytokines compared to cells from controls. In contrast, monocyte-derived macrophage cytokine levels were generally reduced. Modulated cytokine expression in HV BVDV2 macrophages was associated with decreased MyD88 expression, likely due to its interaction with viral NS5A. These data and those of others, suggest that certain *Flaviviridae* may have evolved strategies for subverting receptor signaling pathways involving MyD88.

1. Introduction

Bovine viral diarrhea viruses (BVDV) are enveloped single-stranded positive-sense RNA viruses comprised of two species, BVDV1 and BVDV2, in the *Flaviviridae* family (Driskell and Ridpath, 2006; Ridpath et al., 2011). The BVDV genome is approximately 12.5kb in length and is organized into 12 genes flanked by a 5'

85 nontranslated region (NTR) and a 3' non-polyadenylated NTR. The BVDV genome
 86 encodes a large polyprotein which is cleaved into varying polypeptides. The viral
 87 proteins from N terminus to C terminus, similar to other members of the *Flaviviridae*, are
 88 in the following order: N^{pro}, C, E^{ms}, E1, E2, p7, NS2-3 (NS2, NS3), NS4A, NS4B, NS5A,
 89 and NS5B (Becher et al., 2003; Gaunt et al., 2001). The structural proteins are the capsid
 90 protein, C, and envelope proteins E, while the reminder of the proteins are thought to be
 91 non-structural. Classification of BVDV as cytopathic (cp) or noncytopathic (ncp)
 92 biotypes is based on their cytopathogenicity in epithelial cell culture (Ridpath, 2005).
 93 Cytopathogenicity is associated with the cleavage of the NS2/3 protein into the NS2 and
 94 NS3 proteins. NS5A is one of the least characterized genes of BVDV; however there is
 95 evidence of NS5A involvement in the alteration of the NFκB activation by blocking
 96 phosphorylation of the inhibitor of κB kinase binding protein-β (IKKβ) *in vitro* making it
 97 a potential viral mediator of disease (Zahoor et al., 2010). BVDV2 can be further
 98 characterized as atypical or high virulence (HV) and typical or low virulence (LV) as
 99 determined by viral titers *in vivo* and based on severity of acute disease (Liebler-Tenorio
 100 et al., 2002).

101 Acute BVDV infection results in varying clinical syndromes in cattle ranging
 102 from subclinical to an acute, severe disease, with bloody diarrhea, high fever, mouth
 103 ulcers, and pneumonia (Driskell and Ridpath, 2006). There is evidence of lymphopenia in
 104 the periphery and tissues of severely infected animals (Liebler-Tenorio et al., 2002,
 105 2003a; Liebler-Tenorio et al., 2003b; Liebler-Tenorio et al., 2004). Infection with low
 106 virulence BVDV2 strains typically leads to a transient reduction in circulating leukocytes
 107 with recovery by day 9, while high virulence BVDV2 infection results in a more severe

leukocytopenia with a continual decline beyond 2 weeks post-infection. Furthermore, BVDV is believed to be a factor in potentiating bovine respiratory disease complex (BRDC) in transported cattle. Susceptibility to infection with bacterial pathogens such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* is greatly increased in cattle with BRDC (Confer, 2009; Griffin et al., 2010). As such, secondary bacterial infections have been associated with BVDV infections among feedlot cattle (Fulton, 2009; Peterhans et al., 2003; Van Campen, 2010). Mechanisms whereby BVDV predisposes cattle to secondary bacterial infection have not been completely elucidated.

In response to inflammatory chemokine signals, circulating monocytes rapidly disseminate into infected tissues and develop into antigen presenting cells (APCs). Studies have shown that BVDV replicates in monocytes, which may help to spread of the virus throughout the host (Ammari et al., 2010; Bielefeldt Ohmann et al., 1987). However unlike lymphocytes, a reduction in the numbers of monocytes is not sustained following acute infection (Liebler-Tenorio et al., 2003b; Liebler-Tenorio et al., 2004). While numbers of monocytes are not significantly reduced, studies have shown that *in vitro* BVDV may impair cellular functions of these cells including sugar metabolism, interferon induction, apoptosis and migration (Ammari et al., 2010; Pinchuk et al., 2008), although differences between cp and ncp strains of BVDV were observed indicating a potential strain dependent influence on function. In addition, *in vitro* both ncp and cp BVDV 1 infection of monocytes downregulates proinflammatory TNF α , IL-1 β and IL-6 gene expression (Lee et al., 2008), although protein expression of TNF α and IL-1 β differed between cp or ncp BVDV 1 responses.

Transcriptional regulation of proinflammatory cytokine gene expression is tightly controlled by a series of cellular signaling networks. Cell signaling networks have evolved to recognize innate recognition signals or pathogen associated molecular patterns (PAMPs) to initiate an immune response (Janeway, 1989). These cognate recognition receptor signaling pathways represent a potential target for viral agents to subvert the immune system. Such immune subversion can lead, in some cases, to increased susceptibility to secondary infection. One family of innate pattern recognition receptors (PRRs) are the toll-like receptors (TLRs). TLRs are essential for recognition of viral and bacterial PAMPs, with many sharing common signaling pathway components (Medzhitov, 2001). Following activation, TLRs associate with cytosolic receptor-proximal proteins containing Toll-IL-1 receptor (TIR) domains, including myeloid differentiation primary response gene 88 (MyD88) or TLR-associated activator of interferon (TRIF). MyD88 recruits IRAK-1, which can complex with TRAF-6, leading to activation of transcription factor NF κ B family members necessary for expression of proinflammatory cytokine genes. In contrast, the MyD88-independent, TRIF pathway induces activation of TBK-1, leading to phosphorylation of IRF-3, and cytokine gene induction. Several *Flaviviridae* species alter TLR signaling pathways, including *Hepatitis C virus* (HCV) blockade of TLR-3 via cleavage of TRIF to abrogate interaction with IRF3 (Katze et al., 2008) and *Classical Swine Fever Virus* (CSFV) modulation of TLR7 activation as demonstrated by mutations within the viral NS4B gene effecting TLR7 dependent gene transcription (Fernandez-Sainz et al., 2010). Recently, it has been shown that HCV, *Vaccinia virus* (VV) and *Hepatitis B virus* (HBV) specifically target the MyD88-dependant signaling pathways leading to disruption of TLR signaling through

electrostatic interactions between viral proteins and MyD88-TRAF adapter molecules or via mechanisms that have yet to be elucidated (Lysakova-Devine et al., 2010; Stack et al., 2005; Tachado et al., 2010; Vincent et al., 2011).

To date, there have been no studies comparing cytokine expression of myeloid lineage cells following *in vivo* infection with BVDV2 strains nor studies that have examined cytokine signaling pathways in these cells. The aim of this study was to characterize cytokine mRNA and protein expression of myeloid lineage cells isolated from calves during an *in vivo* BVDV2 infection with strains of high or low virulence. Moreover, since BVDV infection may predispose the host to secondary Gram-negative bacterial infections, we investigated the ability of these cells to respond to subsequent TLR4-ligation using bacterial LPS. Finally, we examined the effect of BVDV2 infection on the myeloid TLR pathway cytosolic receptor-proximal bifurcation involving MyD88 and TRIF.

2. Materials and Methods

2.1. Animals

Eighteen two- to five- week old clinically healthy, colostrum-deprived Holstein calves were used in two replications of the same experimental protocol. Calves were negative for BVDV and antibodies to BVDV as determined by virus isolation from buffy coat followed by polymerase chain reaction (PCR) and serum neutralization using BVDV 1 strain NY-1 and BVDV2 strain 1373 (Ridpath, 2005).

2.2. Viral inoculum

The ncp BVDV 2 strain 1373 was isolated from a clinically severe outbreak in Ontario, Canada in 1993. The ncp BVDV 2 strain RS886 was isolated from the tissues of a clinically normal, asymptomatic persistently infected calf. The viruses used for inoculation had been passed once in calves followed by 2 passages in bovine turbinate (BT) cells.

2.3. Experimental procedures

Experimental infections, run in duplicate, were conducted under BL2 containment. Calves were group housed by experimental group. Two- to five- week old calves were inoculated intranasally with 5 mL of 10^5 - 10^6 TCID of BVDV 2 strain 1373

(n=3/replicate) or RS886 (n=3/replicate) similar to that previously described (Liebler-Tenorio et al., 2003b; Liebler-Tenorio et al., 2004). Additional calves (n=3/experiment) were mock inoculated and served as age-matched negative controls. Clinical signs were scored twice per day based on the University of Madison-Wisconsin School of Veterinary Medicine calf health scoring criteria. Rectal temperatures, circulating leukocytes and platelet counts and viremia were monitored as previously described (Ridpath et al., 2013). Buffy coat was examined for BVDV by viral isolation and PCR for confirmation of infection. Animal procedures employed in these studies were approved by the National Animal Disease Center Institutional Animal Care and Use Committee.

2.4. Cell count and populations

Blood samples were collected in anticoagulant tubes on days 4 and 12 post inoculation. Cells were enumerated by using a Hemavet 950 (Drew Scientific, Dallas TX) calibrated to bovine blood samples and counted in duplicate. Monocyte counts were confirmed by flow cytometry using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA) labeled with an anti-CD14 antibody [MCA1568PB] (Serotec, Raleigh, NC).

2.5. Monocyte isolation

Peripheral blood mononuclear cells were isolated and red blood cells (RBC) lysed with buffered ammonium chloride salt solution. After RBC lysis, cells were washed with sterile phosphate-buffered saline (PBS). Cells were resuspended in complete RPMI 1640

(cRPMI) containing 10% fetal bovine serum (tested free of BVDV and antibodies against BVDV), 2 mM L-glutamine, 1% antibiotic–antimycotic solution, and gentamicin sulfate (Gibco, Carlsbad, CA). Monocytes were isolated by plastic adherence. Ice-cold PBS was added to the plate and adherent cells were removed with a cell scraper. Cells were centrifuged at 1180 x g (Sorvall RC3C Plus, Thermo Scientific, Waltham, MA), and resuspended in cRPMI 1640 at 5×10^6 cells/mL. Monocytes were plated in 96-well round bottom tissue culture plates in 100 μ L medium. M Φ were derived from monocytes following a 7-day culture period, as previously described (Düvel et al., 2012), with medium change every 2-3 days prior to *in vitro* stimulation. Monocytes or MDM Φ were then cultured in medium alone or stimulated with *Escherichia coli* LPS (055:B5 [1 μ g/mL], Sigma, St. Louis, MO).

2.6. Viral antigen staining and PCR confirmation

Antigen staining protocol has been described previously (Liebler-Tenorio et al., 2002). Briefly, BT cells in 96 well flat bottom plates were inoculated with cell supernatants from infected and non-infected control animals. BTs were allowed to incubate for 3-5 days and then fixed and permeabilized in -80°C chilled 1:1 methanol:acetone solution for 10 minutes. Plates were decanted and allowed to air dry. BVDV N2 antibody (Iowa State University Hybridoma Facility, Ames, IA) was added, plates washed and anti mouse-horse radish peroxidase (HRP) conjugate added and incubated for 1 hour. Anti-mouse-HRP was washed off and HRP color development was performed. Areas of cytosolic red indicate positive BVDV N2 antigen stain, whereas

cells staining dull pink/brown are considered antigen negative. Under similar conditions, monocytes were tested for BVDV2 N2 antigen *ex vivo*. Infection was confirmed, as described previously (Ridpath et al., 2013), by RNA extraction from cells with reverse-transcriptase PCR performed to amplify N^{pro} of the BVDV2 genome.

2.7. RNA extraction and cDNA synthesis

At 2 h or 24 h post LPS stimulation, cells were lysed with Buffer RLT containing 2-mercaptoethanol (Qiagen, Valencia, CA) and stored at -80°C . Total RNA was isolated using the RNeasy Mini RNA Isolation Kit (Qiagen) per manufacturer's instructions. Contaminating genomic DNA was removed during RNA isolation using an on-column RNase-Free DNaseI digestion set (Qiagen) according to manufacturer's instructions. Total RNA was eluted by the addition of 40 μL of DNase–RNase free water with addition of RNase OUT (Life Technologies, Carlsbad, CA) for increased RNA stability. RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). 500 ng of total RNA from each sample was reverse transcribed using random primers/hexamers and Superscript III (Life Technologies) according to manufacturer's instructions.

2.8. Real time PCR

Primers were designed specifically for SYBR Green chemistry with the use of Primer Express v 3.0 (Applied Biosystems, Foster City, CA) or NCBI Primer Blast.

Primer annealing temperature was set at 60°C with product size of 100-200 base pairs. Bovine ribosomal protein S9 (RPS9) was used as the endogenous control (15). Primer set sequence are as follows: RPS9 Forward 5'- CGC CTC GAC CAA GAG CTG AAG -3', RPS9 Reverse 5'- CCT CCA GAC CTC ACG TTT GTT CC -3'; IL-1 β Forward 5'- TTC TGT GTG ACG CAC CCG TGC -3', IL-1 β Reverse 5'- AGC ACA CAT GGG CTA GCC AGC -3'; TNF α Forward 5'- CGG GGT AAT CGG CCC CCA GA -3', TNF α Reverse 5'- GGC AGC CTT GGC CCC TGA AG -3'; IL-6 Forward 5'- CTG AAG CAA AAG ATC GCA GAT CTA-3', IL-6 Reverse 5'- CTC GTT TGA AGA CTG CAT CTT CTC -3'. An Applied Biosystems 7300 Real Time PCR Systems thermocycler was used. Amplification conditions for all genes were the same: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s 95°C and 1 min 60°C (measure florescence step) and a dissociation step of 15 s 95°C, 1 min 60°C, 15 s 95°C, 15 s 60°C. Ct values were calculated and normalized to the endogenous control and expressed relative to medium only treatment using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Dissociation curves were analyzed for proper product amplification.

2.9. Multiplex cytokine immunoassay

50 μ L of monocyte or macrophage (M Φ) supernatants or cytokine standards were incubated for 3 h in duplicate in 96 well, immobilized antibody 6-plex array plates (SearchLight, Aushon Biosystems, Billerica, MA). Wells of each plate were commercially coated with antibodies specific for bovine interleukin (IL)-2, IL-4, IL-1 β , tumor necrosis factor (TNF) α , IL-6 and interferon (IFN)- γ . The assay was conducted

according to manufacturer's instructions. After chemiluminescent substrate, plates were immediately read on a SearchLight Plate Reader (Aushon Biosystems). The concentrations of cytokines in each sample were determined from standard curves using SearchLight Array Analyst Software v 2.6.2.0 (Aushon Biosystems). The lower limits of detection for the cytokines were (pg/mL): IL-2=6.1, IL-4=0.8, IL-1 β =1.9, TNF α =2.4, IL-6=0.9, and IFN- γ =0.5.

2.10. Western blotting

Lysates were generated for western blot analysis at 24 h post LPS stimulation. 50 μ L of lysis buffer (BD Pharmingen) with Halt Protease/Phosphatase and 0.5M EDTA (Thermo Scientific) was added directly to wells. Lysates were stored at -80 °C until analysis. For western blot analyses, lysates were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and standardized to 10 μ g protein concentrations. Samples were diluted 1:1 with Laemmli Loading Buffer (Thermo Scientific) containing 2-mercaptoethanol. Samples were heated at 95 °C for 5 min. Heated samples (20 μ L) and Benchmark Prestained Protein Ladder (Life Technologies) were loaded into a Novex Tris-Glycine 1.0 mm 15 well gels (Life Technologies) and electrophoresed at 75 V for approximately 1.5 h or until distinct ladder separation had occurred. Gels were transferred to nitrocellulose using an iBlot transfer system (Life Technologies) per manufacturer's recommendations. Nitrocellulose was incubated in StartingBlock blocking buffer (Thermo Scientific) at room temperature for 30 min prior to addition of primary antibody. Rabbit mAb Anti-MyD88, Anti-TRIF and mouse mAb Anti-phospho

IKK α β , Anti-total IKK β , Anti- β -actin, (Cell Signaling Technologies) were diluted (1:2000) in StartingBlock blocking buffer with 0.05% Tween-20 (Sigma) and incubated on nitrocellulose blot overnight at 4°C with gentle agitation. The blot was washed in Tris Buffered Saline containing 0.1% Tween-20 (TBST). HRP-secondary (anti-mouse or rabbit specific to primary antibody) was used at 1:10000 in StartingBlock blocking buffer with 0.05% Tween-20 and incubated for 60 minutes with gentle agitation at room temperature. Blots were washed with TBST prior to addition of Dura Extended Signal Chemiluminescence Substrate (Thermo Scientific) and incubated for 5 min with gentle agitation. Blots were imaged using Searchlight Imager (Aushon Biosystems) and analyzed using ImageJ (NIH). Restore Plus Buffer (Thermo Scientific) was used according to manufacturer's instructions to strip blots prior to re-blotting. Western blot data were normalized to β -actin for semi-quantification using densitometry (ImageJ, NIH, Bethesda Maryland).

2.11. Production of BVDV NS5A antiserum

An internal fragment of the NS5A protein of BVDV2 strain 1373 (nucleotides 9296 through 9964) was amplified by PCR using primers that added BamHI (5') and Sall (3') restriction sites. The fragment was cloned into the prokaryotic expression vector pMAL-C1 (New England Biolabs, Beverly, MA) that produced a 24.5 kDa NS5a protein fused to maltose binding protein. The fusion protein was purified by standard procedures and used to immunize rabbits. Two rabbits were immunized and boosted once during

antiserum generation. Approximately 20 mLs of antiserum was harvested from each animal.

2.12. Co-immunoprecipitation

Immunoprecipitations were performed per manufacturers instruction (Thermo Scientific) using the IP Crosslinking Kit. BVDV NS5A antibodies were bound to Protein A/G beads of the column. Briefly, lysates were incubated overnight on column with collection of unbound lysate saved. A high pH elution for 5 minutes and regeneration of the columns was performed. 20 μ L IP lysate was loaded in equal concentrations [2 μ g/ μ L] into a 10-20% Tris-Glycine gel (Life Technologies) and protein separated at 120V. The proteins were transferred to nitrocellulose and blocked with StartingBlock Blocking Buffer (Thermo Scientific). The blots were probed using rabbit monoclonal anti-MyD88 antibody (1:1000 dilution; Cell Signaling Technologies) followed by anti-rabbit HRP secondary (1:500) (Thermo Scientific). Femto Signal Chemiluminescence Substrate (Thermo Scientific) was used for development.

2.13. Statistical analysis

qPCR cytokine data was analyzed with the outcome variable ($2^{-\Delta\Delta C_T}$) log transformed. $\Delta\Delta C_T$ values were analyzed using two-way ANOVA (Prism, GraphPad, LaJolla, CA) with sources of variation experimental infection and cell culture treatment. Bonferroni post test was used to compare replicate means by row to uninfected controls.

Cytokine protein data was analyzed using two-way ANOVA (Prism, GraphPad) with sources of variation experimental infection and cell culture treatment with Bonferroni post test was used to compare replicate means by row to uninfected controls. Results are expressed as means +/- standard errors of the means (SEM).

3. Results

3.1. Circulating monocytes are not depleted in calves infected with BVDV2 strains of high or low virulence

Previous studies have shown that peripheral blood monocytes are not appreciatively depleted during BVDV infection (Ellis et al., 1988; Gånheim et al., 2005; Molina et al., 2013). In the present study, lymphopenia was observed in calves infected with high or low virulence BVDV2 strains. However, monocyte levels were not significantly reduced in circulation during the course of BVDV2 infection, although there was a reduction in monocytes isolated from the HV 1373 infected calves at day 12 post infection (Fig 1). The decrease observed for day 12 was not seen at day 14 nor observed at any other time point examined. These data, as well those from other studies, suggest that there is not a sustained depletion of peripheral blood monocytes during the time course of BVDV2 infection, even though it has been known for sometime that monocytes support viral replication and infectious virus can be isolated from enriched cell populations (Bielefeldt Ohmann et al., 1987).

In spite of the fact that these monocytes were not significantly depleted, monocytes isolated from infected animals uniformly stained positive for BVDV2 N2 antigen at day 2 post infection and N2 antigen staining could be observed in monocytes isolated at several time points during the experimental infection (Supplemental Table S1). Further, there is evidence that BVDV2 virions were produced in MDM Φ through the time course of this study based on the presence of BVDV2 N2 antigen in fetal bovine turbinate cells inoculated with supernatants from these M Φ s (Supplemental Table S1). Thus, BVDV is capable of replicating in these myeloid lineage cells.

3.2. Monocytes isolated from BVDV2-infected calves early in BVDV2 infection display enhanced cytokine responses to LPS stimulation

While there was not a sustained reduction in peripheral blood monocyte numbers, it was of interest to determine whether there were cellular functional changes which occurred during the course of BVDV2 infection. To address this, we examined cytokine responses of monocytes isolated at two selected time points following BVDV2 *in vivo* infection. Sampling time points were chosen prior to the initiation of these experiments. LPS was used as a stimulus, as Gram-negative secondary bacterial infection is a common consequence of BVDV infection. Previously, *in vitro* ncp BVDV1 infection was shown to downregulate IL-1 β and IL-6 gene expression in monocytes (Lee et al., 2008). These cytokines typically have an early, sustained mRNA response (Zgair, 2012), thus at day 4 post BVDV infection, we investigated TNF α , IL-1 β and IL-6 mRNA responses in medium alone or in response to LPS stimulation at 2 h or 24 h. It is noteworthy that

unstimulated monocytes from the HV BVDV2-infected calves had significantly higher TNF α mRNA levels at 24 h whereas IL-1 β and IL-6 RNA levels were significantly increased at both time points compared to monocytes from uninfected calves (Fig. 2). Unstimulated monocytes isolated from LV BVDV2-infected calves displayed no significant change in cytokine mRNA levels compared to controls. However, there was no significant secretion of cytokine protein observed from monocytic cells cultured in medium only (Fig. 2). Interestingly, in response to LPS stimulation, monocytes isolated from HV and LV infected animals exhibited an early significant enhancement of TNF α mRNA transcripts, that did not differ significantly from controls by 24 h. (Fig 2A, left column). By comparison, monocytes from HV BVDV2-infected calves exhibited a sustained increase in IL-1 β mRNA levels compared to cells from uninfected controls (Fig. 2A, center column) in response to LPS whereas cells isolated from LV BVDV2-infected calves only demonstrated an increase in IL-1 β mRNA levels only at 24 h (Fig. 2B, center column). Only monocytes isolated from HV BVDV2-infected calves demonstrated an increase in IL-6 transcription in response to LPS at 2 or 24 h compared to control cells whereas monocytes isolated from LV BVDV2-infected calves indicated no significant change from controls.

Interestingly, monocytes isolated from the LV BVDV2 group released significantly higher levels of TNF α in response to LPS (Fig. 2C, left column). Similarly both monocytes isolated from HV or LV BVDV2-infected calves demonstrated an increase in IL-1 β secretion following LPS stimulation compared to monocytes from uninfected calves (Fig. 2C, center column). There was no significant difference in IL-6 secretion between the treatment groups and controls in response to LPS (Fig. 2C, right

column). Taken together, the results show that monocytes isolated early in BVDV2 infection exhibited an enhanced proinflammatory cytokine responsiveness to LPS stimulation.

3.3. Monocytes isolated from BVDV2-infected calves late in infection exhibit enhanced inflammatory cytokine responses to LPS

An examination of cells at day 12 post-infection was chosen as this represents a time point where there is a notable difference in circulating leukocyte numbers between calves infected with the LV or HV BVDV2. There is a recovery in leukocyte numbers evident at approximately day 9 following LV BVDV2 infection (Liebler-Tenorio et al., 2003b), which is not observed for HV BVDV2-infected calves (Liebler-Tenorio et al., 2002). In unstimulated monocyte cultures, there was an increased expression of TNF α , IL-1 β and IL-6 in monocytes isolated from HV and LV BVDV2-infected groups compared to cells from uninfected calves (Fig. 3A and B). In response to LPS stimulation, monocytes from HV and LV BVDV2-infected calves generally exhibited enhanced mRNA for TNF- α which was not sustained to 24 h (Fig. 3A, left column). However in response to LPS, monocytes isolated from HV BVDV2-infected calves demonstrated an increase in IL-1 β which was sustained from 2 to 24 h post treatment (Fig. 3A and B, center column). IL-6 transcription in response to LPS was increased in the monocytes isolated from HV BVDV2-infected calves at 2 h whereas both HV and LV isolated monocyte transcription was increased at 24 h compared to controls (Fig. 3A and B, right column).

Significantly enhanced secretion of $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 levels from HV BVDV monocytes in response to LPS were seen compared to control monocytes (Fig. 3C). The only cytokine to be significantly increased from monocytes isolated from LV BVDV2-infected calves in response to LPS compared to controls was IL-6 (Fig. 3C, right column). In summary, proinflammatory cytokines from monocytes isolated from HV BVDV2-infected calves were generally of greater magnitude than that observed for cells isolated from control or LV BVDV2-infected calves late in infection.

3.4. MΦ derived from monocytes early in HV BVDV2 infection display decreased $\text{IL-1}\beta$ and IL-6 cytokine mRNA and protein responses to LPS

Monocytes differentiate into macrophages and these cells play a pivotal role during infection via recognition of PAMPs and initiation of innate immune signaling pathways. Whether there are differential effects of an *in vivo* BVDV2 infection on cytokine production of monocytes compared to differentiated MΦ has not been investigated. In addition, how viral virulence might influence MΦ cytokine responsiveness is not known. To examine these questions, monocytes from HV or LV-infected calves were differentiated into MΦ and cytokine responses were characterized. At day 4 post BVDV infection, unstimulated MDMΦ from HV BVDV2-infected calves had significantly enhanced $\text{TNF}\alpha$ mRNA compared to uninfected controls (Fig 4A and B, left columns). Both MDMΦ isolated from HV and LV BVDV2-infected calves demonstrated enhanced $\text{IL-1}\beta$ mRNA (Fig. 4A center column) compared to uninfected controls. However, cells isolated from HV BVDV2-infected calves exhibited

significantly reduced IL-6 compared to controls (Fig. 4A, right column). Interestingly, MDM Φ isolated from the HV BVDV2 group exhibited a significant decrease in proinflammatory IL-1 β and IL-6 cytokine mRNA expression in response to LPS stimulation compared to cells from the uninfected group (Fig. 4A and B). MDM Φ from the LV BVDV2 group exhibited an enhanced mRNA expression of TNF α in response to 24 h LPS stimulation compared to the controls (Fig. 4B, left column). There were no significant differences in LPS-induced TNF α secretion between either HV or LV BVDV2-infected groups compared to controls (Fig. 4C, left column). However, secretion of IL-1 β and IL-6 in response to LPS stimulation of MDM Φ from both BVDV2-infected groups was decreased compared to cells from the uninfected animals (Fig. 4C, center and right columns, respectively). In general, macrophages derived from monocytes early in BVDV2 infection, display reduced LPS-induced IL-1 β and IL-6 cytokine expression compared to cells from uninfected calves.

3.5. M Φ derived from monocytes late in BVDV2 infection have decreased levels of cytokine mRNA and protein secretion in response to LPS

At day 12 post BVDV2 infection, unstimulated MDM Φ s from both HV and LV infected groups exhibited a decrease in TNF α mRNA, whereas levels of IL-1 β and IL-6 were similar to the uninfected group (Fig. 5A and B). Likewise, MDM Φ from the HV and LV BVDV2-infected calves exhibited a downregulation in TNF α and IL-1 β mRNA expression after LPS stimulation (Fig. 5A and B, left and center columns, respectively). However, following LPS stimulation, IL-6 mRNA was enhanced in MDM Φ from the HV

BVDV2 group, but was reduced in MDM Φ from the LV BVDV2-infected group (Fig 5A and B, right column).

LPS-induced TNF α protein secretion was decreased in MDM Φ isolated from HV BVDV2-infected calves compared to control MDM Φ (Fig. 5C, left column). MDM Φ from both BVDV2-infected groups exhibited decreased IL-1 β protein secretion in response to LPS (Fig. 5C, center column). However, it is interesting to note that following stimulation with LPS, IL-6 protein levels were significantly higher in MDM Φ from the HV BVDV2-infected calves (Fig. 5C, right column). To summarize, MDM Φ from HV and LV BVDV2 infected-calves were shown to have reduced expression of proinflammatory cytokines compared to MDM Φ isolated from control animals and similarly a decrease in LPS responsiveness as evidenced by a reduction in proinflammatory cytokine mRNA transcription and reduced cytokine secretion.

3.6. Myeloid cells from BVDV2 infected calves differentially express cytokine mRNA and protein

To summarize the differences in cytokine expression in BVDV2-infected calves prior to and after LPS stimulation, a diagram representative of each myeloid cell type is shown in Fig. 6. Representative results from day 12 post BVDV2 infection are shown with changes in mRNA and protein compared to uninfected cells as at this timepoint the greatest contrast in cytokine profiles were observed. Generally, BVDV2 induced an increase in cytokine expression in monocytes isolated from infected calves. However, TNF α expression was decreased in MDM Φ isolated from BVDV2-infected calves. There

was no difference in protein secretion for any of the myeloid cells from BVDV2-infected calves compared to controls. In response to LPS stimulation, proinflammatory cytokine gene expression and protein levels were enhanced in monocytes isolated from BVDV2-infected calves compared to controls. By comparison, expression of TNF α and IL-1 β were generally reduced in HV and LV BVDV2-infected MDM Φ following LPS stimulation. However, it is noteworthy that cells from HV BVDV2-infected calves exhibited higher IL-6 levels, whereas LV-infected MDM Φ showed lower IL-6 expression compared to controls.

3.7. Detection of MyD88 protein expression is reduced in MDM Φ isolated from HV infected calves.

Bacterial LPS can activate signal transduction cascades through TLR4 which involves adapter protein MyD88-dependent or MyD88-independent TRIF/TRAM pathways (O'Neill and Bowie, 2007). Further, it has been demonstrated that monocytes may preferentially signal through TRIF mediated MyD88-independent pathways (Tamassia et al., 2007). Given the differential modulation of proinflammatory cytokines in myeloid lineage cells observed in our previously described experiments, we examined MyD88 and TRIF expression in cells from day 12 post BVDV2 infection. MDM Φ isolated from the HV infected calves demonstrated the lowest detectable MyD88 expression either with or without LPS stimulation (Fig. 7B). In contrast, TRIF expression was not significantly different between the *in vivo* treatment groups (Fig. 7A and B). Overall, terminally differentiated MDM Φ from HV BVDV 2 infected calves failed to

upregulate MyD88 in response to LPS stimulation compared to levels detected in cells cultured in medium alone.

3.8. BVDV NS5A interacts with MyD88

We established that there was a reduction in MyD88 expression in macrophages from BVDV2-infected calves. Moreover, it was reported that HCV NS5A colocalizes with MyD88 and may be attributed to the disruption observed in cytokine responses to TLR agonists (Abe et al., 2007). Additionally, given that there are structural similarities between the NS5A proteins of BVDV and HCV either through amino acid sequence or predicted secondary structures of the N-terminal region of the protein as well as similarities in phosphorylation events produced by similar serine/threonine kinases (Reed et al., 1998), we examined whether the reduced detection of TLR adaptor protein MyD88 could be due to the binding of BVDV2 NS5A to MyD88. We immunoprecipitated MDM Φ whole cell lysates with a BVDV NS5A antiserum and then blotted with an anti-MyD88 mAb (Fig 8A). Using MDM Φ cells from HV or LV BVDV2-infected animals, we determined that NS5A interacts with MyD88, with higher levels observed in cells from HV BVDV2 infected animals. Likewise, macrophages isolated from HV BVDV infected calves demonstrated higher levels of NS5A than similar cells isolated from LV BVDV calves as determined by western blot (Supplemental Fig. S1). The unbound NS5A flow-through fraction probed with the MyD88 mAb demonstrate presence of MyD88 in the controls cells as well as unbound MyD88 present in the lysates of cells isolated from BVDV2-infected calves (Fig 8B).

4. Discussion

Experimental infection with LV BVDV2 strain RS886 induced a mild increase in body temperature with little to no other clinical signs as previously reported (Liebler-Tenorio et al., 2004). By comparison, HV BVDV2 strain 1373-infected calves exhibited more severe clinical signs including sustained, elevated body temperature, diarrhea, and progressive signs of depression, and recumbency as noted in an earlier study (Liebler-Tenorio et al., 2002). While lymphocytopenia was seen following challenge with these BVDV2 strains, monocytes were not significantly depleted (Fig. 1). Given that circulating pools of monocytes are maintained during BVDV2 infection, functional modifications may be targeted by the virus, as these cells differentiate into tissue macrophages and secrete inflammatory cytokines vital to innate host defenses. Therefore, we sought to determine whether infection with BVDV2 LV or HV strains modulated the functional capacity of myeloid lineage to produce proinflammatory cytokines. Time points of day 4 and day 12 post inoculation were chosen prior to initiation of these experiments to represent periods of acute infection followed by differences in recovery. Previous studies have utilized similar time points to demonstrate acute disease with BVDV1 or BVDV2 (Liebler-Tenorio et al., 2002, Palomarez et al., 2014). By day 12 post inoculation, LV RS886 calves are beginning to recover leukocyte pools, whereas HV 1373 infected calves do not demonstrate a similar recovery phase. This later time point has been described as a period with either partial or complete recovery from clinical disease for LV strains (Liebler-Tenorio et al., 2004, Müller-Doblies et al., 2004).

Furthermore, BVDV can be a contributing factor in BRDC as it enhances susceptibility to secondary infection via immunosuppression (Ridpath, 2010). Critical to the induction of inflammatory cytokines in response to bacterial infection are PRRs, such as TLRs, expressed on multiple cell types, which recognize evolutionarily conserved PAMPs. By studying the effects of BVDV2 on subsequent TLR4 signaling in myeloid lineage cells we further sought to unravel specific mechanisms whereby viral infection may alter host responses to secondary bacterial infections. Likewise some BVDV associated secondary infections are characterized by Gram negative bacteria (Confer, 2009) and for the purposes of this study, LPS was chosen as representative PAMP of Gram negative bacteria.

The studies published on the effects of BVDV infection on the cytokine response of monocytes have examined BVDV1 infection *in vitro*. One early study, in which the effect of *in vitro* BVDV1 infection of monocytes on IL-1 mRNA expression was examined, indicated there were no differences in IL-1 gene transcription (Jensen and Schultz, 1991). More recently, however, it has been shown *in vitro* that BVDV1 infection of monocytes resulted in reduced TNF α , IL-1 β and IL-6 mRNA transcription at 24 hr, as well as decreased IL-1 β protein levels, compared to uninfected controls (Lee et al., 2008). In contrast, in the present study, *ex vivo* monocytes isolated from BVDV2-infected calves exhibited an upregulation in cytokine mRNA; however, there was no corresponding significant increase in cytokine secretion detected either during early or late stages of infection (Fig. 2 and 3). Recently, similar to the data shown in this study, it has been reported that HV BVDV2 1373 infection induces an increase in TNF α and IL-1 β mRNA transcription in spleen and tracheobronchial lymph node tissue samples compared to a LV

BVDV1 strain (Palomares et al., 2014). However, in that study, individual cell populations were not analyzed to determine the cellular source of the proinflammatory cytokine gene mRNA.

The effect of *Flaviviridae* infection on responsiveness of monocytes to LPS stimulation has been examined in studies with HCV or CSFV. Peripheral blood monocytes isolated from individuals chronically infected with HCV and stimulated with LPS exhibited enhanced expression of TNF α protein compared to aviremic or uninfected individuals (Dolganiuc et al., 2007; Woitas et al., 2002). Monocytes infected with CSFV and stimulated with LPS demonstrated an increase in IL-1 bioactivity and transcription compared to mock-infected, LPS stimulated controls; however TNF α and IL-6 levels were similar to mock-infected controls (Knoetig et al., 1999). We have shown that LPS stimulation induced higher levels of cytokine mRNA and protein secretion in monocytes isolated from BVDV2-infected calves than in those cells isolated from controls. Taken together, these results suggest that monocytes from *Flaviviral*-infected hosts have an increased expression of proinflammatory cytokine mRNA and are hyperresponsive to LPS. Although an increase in TLR sensitivity has been demonstrated for several *Flaviviridae* species, the underlying mechanisms of this hyperresponsiveness are unknown.

LPS is a Gram-negative bacterial cell wall component that interacts with TLR4 resulting in the activation of a number of downstream signaling pathways, including NF κ B (Andreacos et al., 2004), MAPK (Wallet et al., 2012) and NFAT (Elloumi et al. 2012), that induce inflammatory cytokines. It is possible that following LPS stimulation, multiple proinflammatory signaling pathways become activated in monocytes leading to

secretion of proinflammatory cytokines. Furthermore, it has been reported that proinflammatory cytokine mRNAs induced by LPS are more stabilized than in the absence of LPS (Dai et al., 2003; Kishore et al., 1999). Hypersensitivity of PRRs in response to their cognate ligands and enhanced expression of proinflammatory cytokines are contributing factors to severe clinical disease and a prolonged period of recovery (Cheng et al., 2012; Totura and Baric, 2012; Villinger et al., 1999; Wahl-Jensen et al., 2011). Thus, in cases of BRDC that involve BVDV-bacterial co-infections, enhanced pro-inflammatory cytokine secretion in response to bacterial LPS could contribute to more severe clinical disease.

During differentiation of monocytes into MΦ, there are a multitude of transcriptional changes, as well as cell surface modifications, associated with this maturation process (Lehtonen et al., 2007). For instance, cell surface expression of CD14 is significantly increased after the development of monocytes into macrophages; CD14 is a GPI-anchored protein that forms a trimeric complex with MD-2 and TLR4. In response to TLR4 stimulation, macrophages become classically activated, wherein they secrete proinflammatory cytokines such as TNF α , IL-1 β and IL-6, as well as reactive nitrogen and superoxide intermediates, promote TH₁ responses, and increase microbicidal, tumoricidal, and antiviral activity (Mosser and Edwards, 2008; Sica and Mantovani, 2012). It is possible that BVDV2 infection modulates monocyte to macrophage maturation. However in this study, cellular CD14 expression was similar between MDMΦs isolated from BVDV2-infected calves compared to cells isolated from controls. In addition, MHC class II was only significantly upregulated on MDMΦs isolated from HV-infected calves at day 4 PI, as determined by flow cytometry (data not shown).

649 Several previous studies have explored the impact of *Flaviviridae* on the TLR
 650 responsiveness of *in vitro* derived MΦ. Peripheral blood MDMΦs pre-incubated with
 651 ncp BVDV exhibited increased NO production in response to LPS (Franchini et al.,
 652 2006). However, these authors did not examine modulation of LPS-stimulated
 653 proinflammatory cytokine secretion following BVDV infection of MDMΦs. Bone
 654 marrow derived macrophages (BMMΦ) infected with BVDV1 *in vitro* have been shown
 655 to secrete less TNFα protein in response to LPS or killed whole bacterial stimulation
 656 compared to uninfected BMMΦs (Adler et al., 1996). TLR hyporesponsiveness has been
 657 demonstrated in PBMCs isolated from chronically infected HCV patients which
 658 correlated to more severe disease and worse clinical outcome (Chung et al., 2011; Liu et
 659 al., 2011). *Yellow fever virus* and *St. Louis encephalitis virus* infection reduced IL-1β
 660 levels in LPS-stimulated MDMΦ compared to uninfected control cells (Barros et al.,
 661 2009). We found that MΦ derived during BVDV2 infection generally have reduced
 662 inflammatory cytokine mRNA. Likewise, mRNA and protein secretion following LPS
 663 stimulation compared to uninfected cells was decreased in MDMΦ derived from
 664 BVDV2-infected calves. However, MDMΦ derived late in infection from HV BVDV2-
 665 infected calves produced enhanced IL-6 in response to LPS compared to cells from
 666 uninfected animals. Given that proinflammatory cytokine production can be induced via
 667 several signaling pathways, it is plausible that not all are affected in the same manner.
 668 Nonetheless, these results, along with those in previous studies (Barros et al., 2009;
 669 Chung et al., 2011; Liu et al., 2011), suggest that *in vitro*-derived terminally
 670 differentiated macrophages generally have a decreased proinflammatory cytokine mRNA

profile and a reduced ability to respond to TLR4 ligation following infection with *Flaviviridae*.

MyD88 is an adapter protein important for TLR1/2, 2/6, 4, 7/8, 9 as well as IL-1 receptor signaling, as it bridges the cytosolic-exposed tails of these receptors to signaling molecules downstream (El Kasmi et al., 2008). These signaling pathways, with the exception of TLR 7/8, are critical to bacterial sensing. Worth noting is that there is a decrease in expression of MyD88 in monocytes compared to mature MΦs, as a result of transcriptional changes which occur during the differentiation of monocytes into MDMΦ (Lehtonen et al., 2007). Similarly it has been shown that monocytes preferentially signal through TRIF-mediated or MyD88-independent signaling (Tamassia et al., 2007).

Together these data suggest a differentiated MΦ may be more dependent on MyD88 signaling. There are no studies exploring the impact of BVDV infection on MyD88 expression, however some viruses are known to interact with the TIR domains of MyD88 (Stack et al., 2005). VV protein A46 interacts with MyD88 and Mal through electrostatic interactions blocking complex formation after activation of TLRs (Lysakova-Devine et al., 2010; Stack et al., 2005). The experiments outlined in this study have identified MyD88 as a potential target of BVDV2 dysregulation, as MyD88 is decreased in LPS-stimulated macrophage cells isolated from the HV BVDV2-infected groups. This reduction in MyD88 expression may occur as a result of its co-localization with the BVDV NS5A protein. Conversely, TRIF expression was not significantly different between the myeloid cells isolated from the BVDV2-infected groups compared to the cells from uninfected controls. As such, this may partially explain how monocyte and macrophage cells differ in responsiveness to LPS stimulation, as induction of cytokine

mRNA expression is though TRIF mediated MyD88-independent pathways in monocytes and thought to be directed toward MyD88-dependent pathways in MΦ. The data suggest that BVDV infections which are known to lead to increased susceptibility to secondary bacterial infections may target TLR pathways or MyD88 adaptor molecules in myeloid cells, similar to what has been observed in other *Flaviviridae* species (Lysakova-Devine et al., 2010; Stack et al., 2005; Tachado et al., 2010).

NS5A is a polyprotein essential for BVDV replication as it is critical to the replicase complex (Buckwold et al., 2003; Reed et al., 1998). HCV and BVDV NS5A share structural similarities such as a common essential zinc binding region (Tellinghuisen et al., 2006) and association with cellular membranes via an amino-terminal helix (Sapay et al., 2006). As the BVDV NS5A membrane anchor has been determined, molecular dynamic simulations suggest that this helix interacts with membranes in a manner similar to that of the HCV NS5A anchor (Sapay et al., 2006). HCV and BVDV NS5A are also phosphorylated through cdc2 cyclin-dependent kinases (Reed et al., 1998). Those data suggest that BVDV and HCV NS5A proteins are similar in both structure and function. Interestingly, HCV NS5A has been shown to interact with MyD88 in an *in vitro* study (Abe et al., 2007; Dolganiuc et al., 2004; Ryan et al., 2011). We have demonstrated that a HV BVDV 2 NS5A interacts with MyD88 in MΦ derived from monocytes from an *in vivo* infection. In cells isolated from HV BVDV infected calves, there was a greater interaction between viral NS5A and cellular MyD88 than what was observed in cells isolated from LV BVDV infected calves. It was determined by western blot densitometry that NS5A is expressed to a greater extent in the cells isolated from HV BVDV infected calves than LV BVDV infected calves (Supplemental Fig. S1)

which may be reflective of the differences in virulence between the viral strains. Site mutations directed toward the HCV NS5A/NS5B polypeptide have indicated an increase in both cytopathic effect and an early increase in levels of core protein expression suggesting a link between HCV NS5A and virulence; however any increase in viral replication was limited to the acute stages of disease *in vivo* (Mishima et al., 2010). The data presented herein suggests higher levels of NS5A and therefore greater interaction between MyD88 and NS5A may be a hallmark of the highly virulent 1373 BVDV2. Although the mechanism of MyD88-NS5A downstream interactions were not examined in this current study, it is plausible that BVDV2 NS5A binding to MyD88 TIR domains results in steric hindrance, inhibiting the interaction of this adapter protein with IRAK and TRAF6 as described for VV A46 (Lysakova-Devine et al.; Stack et al., 2005). On the other hand, kinases involved in NF κ B signaling pathways have been shown to interact with NS5A and are an additional potential target of dysregulation observed during BVDV infection (Zahoor et al., 2010). Therefore, BVDV may employ multiple strategies to interfere with NF κ B signaling.

We demonstrated enhancement of proinflammatory cytokine mRNA transcription in monocytes from BVDV2-infected calves compared to control cells and in these cells following LPS-stimulation. Conversely, we observed a reduction in proinflammatory cytokine expression in MDM Φ from BVDV2-infected calves compared to uninfected MDM Φ and in their response to LPS-stimulation. Overall, however, there was not a distinctly different cytokine profile between cells isolated from HV and LV infected animals. Importantly, the data in this study suggest there are important differences between myeloid lineage cells in cytokine expression following LPS stimulation which

may be partially explained through the differences in the utilization of MyD88-dependent and MyD88-independent signaling pathways. During BVDV2 infection, immature monocytes may initially exhibit a hyperresponsive proinflammatory response to TLR4 ligation via TRIF-mediated MyD88-independent pathways, however after differentiation into macrophages, these cells may become less responsive possibly due to an interaction between BVDV NS5A and MyD88.

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Figure Legends

Figure 1. Circulating levels of CD14⁺ monocytes as a percent of total live cells isolated and total number of monocytes from experimental groups during the course

of BVDV2 infection. CD14⁺ cell percentages and total numbers were determined by flow cytometry. n=6 per treatment group from two independent experiments.

Figure 2. Cytokine mRNA and protein expression in monocytes isolated at day 4 post BVDV2 infection. Cells were cultured in 96 well plates with or without LPS (1µg/mL) stimulation for 2 h (A) or 24 h (B) and cytokine mRNA levels measured by real time PCR. Data shown are the fold changes in monocyte cytokine mRNA expression relative to uninfected monocytes in medium only after normalizing to RPS9. (C) Secreted cytokine levels were measured in supernatants collected at 24 hr from cells cultured in medium only or with LPS using a SearchLight multiplex assay. Data are means +/- SEM, n=6 per treatment group from two independent experiments. * P < 0.05, ** P < 0.001.

Figure 3. Cytokine mRNA and protein expression from monocytes isolated at day 12 post BVDV2 infection. Cells were cultured in 96 well plates with or without LPS (1 µg/mL) stimulation for 2 h (A) or 24 h (B) and cytokine mRNA levels measured by real time PCR. Data shown are the fold changes in monocyte cytokine mRNA expression relative to uninfected monocytes in medium only after normalizing to RPS9. (C) Secreted cytokine levels were measured in supernatants collected at 24 hr from cells cultured in medium only or with LPS using a SearchLight multiplex assay. Data are means +/- SEM, n=6 per treatment group from two independent experiments. * P < 0.05, ** P < 0.001.

Figure 4. Cytokine mRNA and protein expression from MDMΦ isolated at day 4

post BVDV2 infection. Cells were cultured in 96 well plates with or without LPS (1 µg/mL) stimulation for 2 h (A) or 24 h (B) and cytokine mRNA levels measured by real time PCR. Data shown are the fold changes in monocyte cytokine mRNA expression relative to uninfected monocytes in medium only after normalizing to RPS9. (C) Secreted cytokine levels were measured in supernatants collected at 24 hr from cells cultured in medium only or with LPS using a SearchLight multiplex assay. Data are means +/- SEM, n=6 per treatment group from two independent experiments. * P < 0.05, ** P < 0.001.

Figure 5. Cytokine mRNA and protein expression from MDMΦ isolated at day 12

post BVDV2 infection. Cells were cultured in 96 well plates with or without LPS (1 µg/mL) stimulation for 2 h (A) or 24 h (B). Cytokine mRNA levels were measured by real time PCR. Data shown are the fold changes in monocyte cytokine mRNA expression relative to uninfected monocytes in medium only after normalizing to RPS9. (C) Secreted cytokine levels were measured in supernatants collected at 24 hr from cells cultured in medium only or with LPS using a SearchLight multiplex assay. Data are means +/- SEM, n=6 per treatment group from two independent experiments. * P < 0.05, ** P < 0.001.

Figure 6. Summary of mRNA and protein expression data from myeloid cells

isolated from BVDV2 HV or LV-infected calves at day 12. Overall, monocytes from BVDV2-infected calves exhibited increased proinflammatory cytokine mRNA levels

compared to monocytes from control animals. Following LPS stimulation, monocytes from calves infected with HV BVDV2 were found to have enhanced cytokine gene expression and protein secretion; whereas, monocytes from calves infected with LV BVDV2 exhibited similarly enhanced mRNA and increased IL-6 secretion. Overall, cytokine gene expression and protein secretion for MDM Φ isolated from BVDV2-infected calves did not differ from MDM Φ isolated from control calves. However, LPS-stimulated MDM Φ generally showed decreased levels of cytokine mRNA and protein, with the exception that IL-6 secretion was significantly increased for cells from the HV group. $\uparrow$ increased expression compared to control cells, $\downarrow$ decreased expression, NS not significant.

Figure 7. HV BVDV infection modulates the MyD88-dependent but not the MyD88-independent TRIF pathway. (A) Monocytes were isolated from peripheral blood of control or BVDV2-infected calves day 12 post infection. Cells were cultured with or without LPS (1 μ g/mL) in cell culture medium for 24 h. Lysates were immunoblotted with antibodies against MyD88, TRIF or β -actin. Densitometry scans of MyD88 or TRIF relative to β -actin are shown in the bar graphs. (B) MDM Φ were isolated from peripheral blood of control or BVDV2-infected calves day 12 post infection. After maturation, MDM Φ s were cultured with or without LPS (1 μ g/mL) in cell culture medium for 24 hours.

Figure 8. BVDV NS5A co-localizes with MyD88. MDM Φ were derived from peripheral blood monocytes of control or BVDV2-infected calves day 12 post

1060 **infection.** Cells were stimulated with LPS (1 µg/mL) or cell culture medium for 24 hours.
1061 Equivalent protein concentrations of lysates were immunoprecipitated with beads
1062 coupled to anti-BVDV NS5A hyperimmunized serum. Immunoprecipitates were blotted
1063 with anti-MyD88 (A). The negative fraction from the flow through of the IP was
1064 immunoblotted using anti-MyD88 (B).

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Figure(s)

Figure 1

Figure 2

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Figure 4

Figure 5

Figure 6

Monocyte			MDMΦ		
		mRNA	Protein	mRNA	Protein
HV BVDV2 Alone	TNFα	↑	NS	↓	NS
	IL-1β	↑	NS	NS	NS
	IL-6	↑	NS	NS	NS
+ LPS	TNFα	↑	↑	↓	↓
	IL-1β	↑	↑	↓	↓
	IL-6	↑	↑	↑	↑
<hr/>					
		mRNA	Protein	mRNA	Protein
LV BVDV2 Alone	TNFα	↑	NS	↓	NS
	IL-1β	↑	NS	NS	NS
	IL-6	↑	NS	NS	NS
+ LPS	TNFα	↑	NS	↓	NS
	IL-1β	↑	NS	↓	↓
	IL-6	↑	↑	↓	↓

Figure 7

Figure 8