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Examining innate responses to Flaviviridae infection: how myeloid cells respond to and are modulated by bovine viral diarrhea virus in vivo and in vitro.

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Examining innate responses to Flaviviridae infection: how myeloid cells respond to and are modulated by bovine viral diarrhea virus in vivo and in vitro.

By

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILISOPHY

Major: Immunobiology

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Iowa State University
Ames, Iowa
2012
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CHAPTER ONE. INTRODUCTION

DISSERTATION ORGANIZATION

This dissertation follows the organizational format in which the experimental chapters of the thesis are prepared in manuscript format. The first chapter of the thesis outlines background information and serves as an introduction to the experimental chapters. Experimental chapters two, three, and four either have been submitted or will be submitted to peer reviewed journals for a total of three publications. The final chapter five, ties together the findings of the experimental chapters and discusses the results as it relates to the problems outlined in the first chapter.

LITERATURE REVIEW

Myeloid Lineage Immune Cells

Origination, Function and Characterization

Myeloid cells are derived from hematopoietic stem cells differentiated from the medulla ossium rubra or red marrow region within the bone marrow. Some of the hematopoietic cells of the red marrow develop into monocytes allowing them to leave the bone marrow barrier into the medullary blood vasculature entering circulation (39, 177). Granulocytes such as neutrophils, eosinophils, and basophils are generated through related but divergent developmental pathways (42). Although partially differentiated, peripheral blood monocytes are an immature immune cell that can further develop into various tissue macrophages (МΦ) and dendritic cells (DCs), which occurs after extravasation and infiltration into tissues (6). Monocytes arbitrate host antimicrobial defense by secreting cytokines and antimicrobial peptides through chemotaxis mediated by receptors CCR1,
CCR2, CCR4, CCR5, CX3CR; and ligands monocyte chemoattractant protein (MCP)-1 (CCL2), regulated on activation normal T expressed and secreted protein (RANTES) (CCL5), IL-8 (CXCL8), macrophage inflammatory protein (MIP)-1α/β (CCL3/4), MCP-2 (CCL8) (174). Additionally, monocytes can be recruited into tumor sites and potentially mediate some aspects of anti-tumor immune response through release of arginase or restricting vascular endothelial growth factor (VEGF) stimulation of vasculogenesis and angiogenesis (150).

The surface marker CD14 is highly expressed on monocytes, and has functional properties important in the binding of LPS in the context of LPS-binding protein and co-localization to the TLR4 signaling complex (20). CD16 is a low-affinity Fcγ receptor (FcγRIII), which is important in binding to and recognizing IgG and is expressed on some subsets of monocytes (212). Likewise expression of CX3CR1, a transmembrane molecule important in migration, and CCR2, the receptor for MCP-1, can be used to delineate peripheral blood monocyte subpopulations in the naïve animal (177). This leads to a classification system in humans and mice comprising three groups: classical, intermediate and nonclassical. Classical monocytes are CD14hi (or referred to as CD14++) absent in expression of CD16 (CD16–), CCR2hi, CX3CR1lo. These classical monocytes are pro-inflammatory and resemble LY6X monocytes in mice based on transcriptional analysis (63, 91, 186). Intermediate monocytes are CD14++, CD16+, CX3CR1hi, CCR2lo and contribute to a proinflammatory response (13, 218). Non-classical monocytes are CD14+, CD16++, CX3CR1hi, CCR2lo (174) and are important for anti-viral mediated responses and local surveillance of tissue (43). This classification system has been established for human and mice monocyte populations, although a bovine classification has not been delineated (86,
CD14+, CD16- monocytes are the predominant phenotypic blood monocyte of cattle and are believed to be homologous to classical monocytes in humans (86, 204, 207).

As monocytes infiltrate into tissues, they become monocyte-derived MΦ or MDMΦ. During infiltration and maturation these cells undergo phenotypic changes such as an increase in surface expression of CD14, CD11b (MAC-1), CD32 (FcγRII), and major histocompatibility complex (MHC) class II (207). MΦ can respond to extracellular cytokines to become activated. Activation signals of IFNγ and LPS will induce “classical” activated MΦ, which produce proinflammatory cytokines IL-12, IL-1β, IL-6, TNFα, proteolytic enzymes matrix metalloproteinase (MMP)-1, MMP-2, MMP-7, MMP-9, MMP-12, and an increase in arginase, reactive oxygen species (ROS) and nitric oxide (NO) production (209). Likewise, “alternative” activated macrophages respond to IL-4 or IL-13 signaling and produce an anti-inflammatory response by promoting repair of the extracellular matrix (ECM) and production of IL-10 and TGFβ. MDMΦ are important in recognizing innate recognition signals or pathogen associated molecular patters (PAMPs) to initiate an immune response (94, 137). One type of pattern recognition receptors (PRRs) are the toll like receptors (TLRs). As MDMΦ differentiate from monocytes, TLR expression increases and MDMΦ demonstrate an enhanced response to stimulation by an increase in IL-6, IL-8 and IL-1β expression compared to monocytes (72). These PRRs represent a target for infectious agents to elude the immune system. Likewise, this immune subversion can lead to increased susceptibility to secondary infection as other pathogens can go undetected since the innate abilities of the MΦ to recognize PAMPs is impaired. For instance, many Flaviviridae subspecies interact with these TLRs and signaling pathways such as hepatitis C virus
disruption of TLR3, IRF-3, and RIG-I signaling (101), yellow fever virus attenuation of TLR7 and TLR9 (131), and classical swine fever virus disruption of TLR7 activity (52).

MΦ are an important cell for antigen uptake and presentation. As such, MΦ are considered an antigen-presenting cell (APC), capable of processing peptides in the context of MHC class II. MΦ express fragment crystallizable receptors (FcR), which are important in receptor-mediated phagocytosis (16, 100). MΦ express FcγR CD16, CD32 and CD64 (high affinity Fcγ receptor), which when antigen is bound with antibody and complement (immune complex) will induce phagocytosis (4). Similarly, C3b complement opsonized particles can be phagocytosed by MΦ through complement receptor (CR) 3 (CD11b/CD18) (219). After internalization, the phagosome is acidified through action of vacuolar-type H⁺-ATPase (V-ATPase) via transport of protons into the phagosome (189). Fusion of the phagosome by the lysosome provides for additional hydrolytic enzymes and generation of a phagolysosome. As acidification, oxidation, lysis and degradation of phagocytized proteins occurs, small peptides are generated and may be loaded into MHC class II and expressed on the cell surface (171). Co-stimulatory molecules such as CD80/86, and CD40 are required in conjunction with MHC class II expression to potentiate CD4⁺ T cell receptor activation. Cytosolic infection of pathogens will activate the MHC class I pathway, which cytosolic peptides are processed through the immunoproteosome and be loaded into the MHC class I molecules located in the endoplasmic reticulum (ER) (30). Peptide loaded MHC class I will be trafficked from the ER to the cell surface where it will present antigen to CD8⁺ T cell receptor.

Alveolar macrophages (AMΦs) are a tissue-derived macrophage resident to the surfactant layer of the alveolar spaces of the lung. These cells are a primary innate immune
cell in the lung, and are one of the first cells to contact inhaled pathogens, antigens and toxins (49). AMΦ are responsible for pathogen clearance, antigen presentation and cytokine production (50). In response to pathogens, AMΦ can secrete inflammatory mediators such as IL-1α, IL-1β, TNFα, IFNα/β and IL-6 (11). AMΦ can produce granulocyte chemotactic factors such as IL-8, MIP-1 and leukotriene B4 which will recruit neutrophils to the lung (48, 50, 133, 158). However, lung inflammation is tightly regulated and AMΦ are limiting in their pro-inflammatory response as inflammation will restrict air exchange, induce bronchoconstriction or tightening of the smooth muscle airway lining and narrowing of the lumen of the bronchi (35). AMΦ may engulf infiltrating neutrophils to quell inflammation preventing the release of neutrophilic inflammatory mediators and granules (73). Likewise these AMΦ will secrete IL-10 and TGF-β to induce tissue regeneration and promote an anti-inflammatory response (35).

AMΦ play a vital role in pathogen clearance through abilities to phagocytize, process and present antigen. The cellular membrane of AMΦ is extremely dynamic, as antigen is captured by non-receptor mediated endocytosis or phagocytosis through membrane ruffling. Within 30 minutes after internalization, the entire membrane is replaced (68). Like MDMΦ, AMΦ can internalize antigen through receptor-mediated phagocytosis as FcyRs, FcεRs, FcαRs can recognize IgG, IgE and IgA opsonized molecules respectively (48). Similarly, AMΦ express CR1, CR3 and CR4 and can internalize complement opsonized microbes (64, 74, 166). Similar to MDMΦ, after internalization of proteins or pathogens, AMΦ can process and present antigen in the context of MHC class II, as well as cytosolic antigen through MHC class I cell surface expression.
Toll-Like Receptors

TLRs are essential for recognition of viral and bacterial components with many sharing common signaling pathway components. Within humans and cattle at least 10 TLRs have been determined with ligands and functions characterized. TLRs are not equally expressed across each cell type, however every cell expresses a repertoire of some TLRs. Monocytes, MΦ and some subsets of DCs each have the capabilities of expressing all 10 TLRs. TLR2 interacts with TLR1 to recognize peptidoglycan and (triacyl) lipoproteins (45). TLR2 can also interact with TLR6 to recognize diacyl lipoproteins as well as some forms of lipopolysaccharide (LPS) (142, 176). TLR3 recognizes double-stranded (ds)-RNA, as well as the synthetic nucleotide Poly I:C (15). TLR4 recognizes LPS as well as some mycobacterium cell membrane components (176). TLR5 recognizes flagellum (220). TLR7 and TLR8 recognize single-stranded (ss)-RNA (25). TLR9 recognizes CpG unmethylated DNA, which is a characteristic of bacteria (209). TLRs 2/1, 2/6, 4 and 5 are located on the surface plasma membrane of myeloid cells. TLRs 3, 7, 8, 9 are located within endosomal compartments or within the interior of the cell. After initial recognition by the TLRs, shared adapter proteins are required for propagation of signal. Signaling molecules myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) are the two known adapter molecules for TLR signaling which leads to a classification of MyD88-dependant or MyD88-independent (TRIF) associated signaling (175). These adapter proteins may be a beneficial target for pathogens to evade the innate immune response. Recently, it has been shown that vaccinia virus, human immunodeficiency virus and hepatitis B virus each target MyD88 or the MyD88 complex leading to disruption of innate signaling pathways potentially by ubiquination and
degradation of the MyD88 complex or disruption of interaction with TRAM and IRAK molecules (130, 181, 187, 198).

**Cytokines**

Cytokines are a class of molecules important in the initiation, regulation and control of the immune response. These molecules include the interleukin (IL) and interferon (IFN) families as well as some chemokines. Monocytes and MΦ induce a class of cytokines considered innate in their immune function. These cytokines include IL-1β, IL-15, IL-18, IFNα/β and TNFα (122). Cytokines such as IFNγ and IL-12 promote a cell mediated or T helper 1 response (Th1) (69). Cytokines IL-4, IL-5, IL-10, IL-13 promote a humoral or T helper 2 (Th2) response (113, 124).

The IL-1 family of cytokines includes IL-1, IL-18 and IL-33 (5). Unique to this family is the necessity of post-translational modifications required for mature protein production. A multi-protein complex or inflammasome is generated in conjunction with NOD-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and activity of caspase-1 (previously referred to interleukin-cleavage enzyme or ICE) to cleave immature IL-1 family members (pro-form) to an active form (90). This processing is required for the secretion of the IL-1 family members (132). Similar to TLR signaling, IL-1 and IL-18 receptor signaling is mediated through MyD88 (5, 184, 188).

TNFα is produced at nearly undetectable levels in quiescent MΦ, however after stimuli is an immediate early-transcribed cytokine. TNFα transcription increases sharply within 15-60 minutes after TLR stimulation, however TNFα mRNA is regulated post-transcriptionally (115). The 3’ region of TNFα contains multiple AU sequences, which in the
context of RNA-binding proteins help to stabilize translation or silence the gene. Similarly, extracellular levels of corticosteroids, prostanoids and anti-inflammatory cytokines downregulate TNFα expression and promote post-translational silencing (125).

IL-6 is a pleiotropic cytokine, which can exert T cell growth and differentiation, monocyte differentiation into macrophage, induce acute phase protein synthesis in the liver as well as development and differentiation of neuronal, osteoclast and hematopoietic cells. IL-6 is produced by TLR, IL-1R, and TNFR ligands in MΦ, and transcription factors NFκB, AP-1 and IRF induce transcription. Translational modifications in which histone deacetylase (HDAC) binds to the IL-6 promotor region will induce translation of IL-6. HDAC blocks histone protein H4 hyperacetylation which would turn off transcription and is a regulator of IL-6 transcription (115, 127). IL-6 has been shown to reduce viral replication by an early activation of STAT1 and STAT3 which induces IRF1/3/9 nuclear translocation and type I interferon production (141).

**MyD88 Dependent Signaling**

TLRs 2/1, 2/6, 4, 5, 7, 8, 9 are considered MyD88-dependent TLRs. Of note, TLR4 can be signal through either MyD88-dependent or MyD88-independent mechanisms. MyD88 signaling after initial TLR dimerization or recognition of PAMP will eventually lead to the activation of NF-κB, p38 MAPK or c-jun n-terminal kinase (JNK) (145). However, initially IL-1R-associated kinase (IRAK) are recruited immediately downstream of MyD88 (29). IRAK4 is the MyD88-peroximal kinase, which in turn recruits IRAK1. As a negative regulator of signaling, IRAK-M (105) appears to play an inhibitory role in activation as it dissociates IRAK1 and IRAK4 from MyD88. Downstream of IRAK1 is tumor necrosis-factor (TNF)-receptor-associated factor 6 (TRAF6) (103), which can activate upstream
kinases for p38 and JNK activation as well as initial degradation of inhibitor of NFκB kinase (IkB) complex. TRAF6 through the recruitment of transforming-growth-factor-β-activated-kinase 1 (TAK1) and TAK1-binding protein 2 (TAB2), the ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) becomes an activating complex by which contributes to the recruitment of the IKK complex, subsequent phosphorylation of the IKKαβ subunits by NFκB essential modulator (NEMO or IKKγ) which tags the IkB for ubiquitination and proteosomal degradation (208). Similarly the TRAF6/TAK1 complex can activate and recruit dual-specificity mitogen-activated protein kinase kinase (MKK) MKK3/MKK6 for p38 MAPK phosphorylation as well as MKK7 for JNK phosphorylation (41). Translocation of NFκB, p38 or JNK can lead to transcription of pro-inflammatory cytokines and mediators (145).

MyD88-Independent Signaling

MyD88-independent pathways are mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF). Only TLR3 and TLR4 are known to signal through TRIF (19, 32). Although, MyD88-independent, TRIF signaling does include some shared signaling molecules. For instance, TRIF can interact indirectly or directly with TRAF6 to initiate IKK signaling and downstream NFκB activation and subsequent pro-inflammatory cytokine transcription (104). However, TRIF can interact with TRAF3 and NAK-associated protein 1 (NAP1) to induce interferon regulatory factor (IRF)3 or IRF7 activation which in turn induces type-I interferon transcription (143). However, TRIF signaling can induce apoptosis by which receptor-interacting protein 1 (RIP1) activates pathways involving FAS-associated death domain (FADD) and subsequent apoptotic cascades (96).
c-Jun N-Terminal Kinase (JNK)

JNK is a member of the stress-activated protein kinase (SAPK) group of mitogen-activated protein kinases (MAPKs). Additionally included in the SAPK MAPKs are p38 MAPK families comprised of p38α, p38β, p38γ and p38δ (199). JNK includes three family members, JNK1, JNK2 and JNK3 that are differentially expressed depending on cell or tissue type (62, 134, 201, 205). JNK3 is primarily expressed in brain, heart, and testis; whereas JNK1/2 are widely and constitutively expressed among myeloid, lymphocyte and epithelial cells (165). Of note, JNK1/2 expression barely detectably in naïve T and B cells although will be induced after antigen stimulation (62). Four isoforms of JNK1 (JNK1α1, JNK1β2, JNK2α2, JNK1β2) and JNK2 (JNK2α1, JNK2β2, JNK2α2, JNK2β2) have been described dependent on tissue type; however functional characteristics of these isoforms have yet to be completely elucidated (21, 165).

In myeloid cells, JNK activation occurs by phosphorylation after an upstream multi-component cascade of kinase phosphorylation. TLR or IL-1R signaling triggers induction of this cascade (93). After receptor stimulation, a class of kinases, MAP3K (mitogen-activating kinase kinase kinase) will become phosphorylated and initiate phosphorylation of MAP2Ks, which in turn will phosphorylate MAPKs. 21 MAP3Ks have been identified with many of them having overlapping ability to provoke MAPK stimulation (165). The large number of MAP3Ks may suggest multiple MAP3K are required for activation of MAPK. Analyses of gene knockout studies have demonstrated JNK phosphorylation is greatly affected in cells lines lacking TPL-2, ASK1, TAK1, MLK3, MEKK1 or MEKK3 (205). JNKK1 (MAP2K4) and JNKK2 (MAP2K7) will phosphorylate and activate JNK at amino acid residues Thr^{183} and Tyr^{185} (89). Recently, JNK scaffolding proteins have become more characterized as these
proteins are essential in coordinating the formation of JNK signaling complexes (47, 210). Phosphorylation of JNK will allow it to translocate the nucleus to activate transcription factors c-Jun, ATF2, ELK1, SMAD4, p53 and HSF1 (144). Although some of the transcription factors activated by JNK can induce an inflammatory cytokine response, other are important for cell cycle regulation and initiation of apoptosis (71, 127, 217).

Transcription Factor Family NF-κB Activation

NF-κB is a family of transcription factors that arbitrates a multitude of cellular processes; however it plays an important role in the regulation of immune responses. In mammals, five members of the NF-κB family exist: RelA (p65), RelB, c-Rel, NF-κB1 (p105) and NF-κB2 (p100) (173). As overexpression of NF-κB can lead to inflammatory disorders, regulation is tightly controlled (98). Under inactive conditions, NF-κB dimerizes and is bound to inhibitory IκB proteins, which are localized in the cytoplasm (167). Under signal activation conditions, such as downstream of TLR or IL-1R activation, the IκB kinase (IKK) complex subunits IKKα/β become phosphorylated through the regulatory subunit IKKγ (NEMO) (179). These IKK catalytic subunits initiate the phosphorylation IκB, which is targeted for ubiquitination and degradation that releases NF-κB dimers to translocate to the nucleus and transcriptional regulation of genes can occur (146, 179). Generally NF-κB pathways are categorized into two groups, canonical and noncanonical. Canonical pathways are dependent on IKKβ and NEMO, which lead to phosphorylation of p65 containing NF-κB dimers (14). Noncanonical pathways depend on IKKα-mediated phosphorylation of the p100 associated with RelB, leading to creation of p52-RelB complexes (8, 14, 179). Activation of NF-κB can result in pro-inflammatory cytokine release, as regulation of IL-1α, IL-1β, IL-2,
IL-6, IL-8, IL-10, IL-12, IL-13, IL-23 and IFNβ are controlled, in part, by NF-κB (31, 83-85, 99, 111, 117, 126, 180).

**Bovine Viral Diarrhea Virus (BVDV)**

Bovine viral diarrhea virus (BVDV) is an enveloped single-stranded positive-sense RNA virus and is the etiological agent of bovine viral diarrhea (BVD). Disease associated with BVDV is multifaceted with animals suffering from severe acute, subclinical, or mucosal disease. Similarly, an immune tolerant persistently infected (PI) animal displaying no clinical symptoms is possible and these PI animals shed high amount of virus. Within the cattle industry, BVDV can be devastating leading to millions of dollars in loss either through BVD or secondary infection caused by increased susceptibility to disease (163).

**Unsolved Questions**

BVDV vaccines are widely available (54). However genetic variation within the subgenotypes of BVDV strains make is difficult to induce wide protection against various field strains (161). However, many feedlot managers do not vaccinate due to cost, lack of information on vaccines, or a generalized fear that vaccination may contribute to a severe condition deemed mucosal disease (33, 110, 139). As such, a primary focus of BVDV research has been on vaccination strategies. A few studies have suggested that BVDV induces an immunosuppressive host with modulation in T cell repertoire and response to secondary infection. For example, *in vivo* BVDV infection of calves induce a shift the immune response toward the T-helper 2 (Th2) response and avoid the production of cell-mediated immunity or T-helper 1 response (Th1) (36). Furthermore, the proliferative response of either allotypic or memory CD4⁺ T cells are greatly reduced when mixed with *in*
vitro infected monocytes (65). Similarly it has been suggested that lymphoid cytokine secretions (65) and disruption in chemotaxis (27) may be some contributing factors of immune suppression. The ability of APCs such as monocytes and МФ to potentiate T cell responses either in response to APC generated cytokines or cell surface marker expression is unknown in response to BVDV-2 infection. As such, the underlying mechanisms of immunomodulation after BVDV-2 infection are unknown.

**Economic Impact**

The yearly economic impact of BVDV is estimated to be in the millions of dollars in the United States. Reproductive costs are estimated at $376,000,000 to $1,500,000,000 per year, as these costs are associated with rebreeding animals due to abortion, stillbirths, or underweight calves (88, 160). Costs to milk production are estimated to be between $330,000,000 to $494,000,000, which is associated with decreased milk production from acute disease or underperforming PI cattle (88, 160). Feedlot sales associated with acute infection, exposure to PI animals, increased secondary infections, and production losses associated with diversion of resources to manage infected cattle lead to an estimated $360,000,000 in losses (88, 160). These yearly costs only refer to low virulence BVDV strains as losses due to high virulence strains may reach an estimated $100,000 per herd (120). Globally, BVDV monetary costs are within the equivalent of billions in US dollars (76, 79-81, 123, 157, 159, 183, 185, 191).

**Genome and Strains**

BVDV is in the *Pestivirus* genus, which includes *classical swine fever virus* (CSFV) and *border disease virus* (BDV). BVDV is also a member of the *Flaviviridae* family, and is closely related to *hepatitis C virus* (HCV). This close similarity to HCV has allowed for the
development of BVDV as a surrogate model of HCV (28). Members of Flaviviridae also include dengue virus (DV), West Nile virus (WNV), yellow fever virus (YFV) and St. Louis encephalitis virus (SLEV). The genome of BVDV, HCV and other viral family members are approximately 12.5kb in length. The BVDV genome is organized into 12 genes flanked by a 5’ nontranslated region (NTR) and a 3’ non-polyadenylated NTR. Within the 5’ nontranslated region is an internal ribosome entry site (IRES). This site drives cap-independent translation of the viral open reading frame, generating one single large polyprotein. This polyprotein is processed by a combination of viral and host proteases to produce the mature viral proteins. N-terminal protease (Npro) is the first gene transcribed off of the genome, which is a nonstructural gene and encodes an autoproteolytic subunit able to cleave its own C terminus (116, 168). The following genes are the structural genes of capsid (C), virion associated envelop glycoproteins (Erns, E1, E2) and P7, a small hydrophobic protein. The next six genes (NS2, NS3, NS4A, NS4B, NS5A, NS5B) comprise the majority of the BVDV genome and are nonstructural (NS) genes. This is a common feature of the NS genes throughout the Flaviviridae family and make up the amino terminal region of the genome (153). Interestingly, the BVDV genome can differ depending on the cytopathogenicity of the virus (23, 138). Cytopathogenicity is associated with the cleavage of the NS2/3 protein into the NS2 and NS3 proteins, whereas noncytopathic (ncp)-BVDV strains only express one whole protein referred to as NS2/3 and therefore ncp-BVDV contain only 11 functional genes (109, 138, 155, 196). This classification system of BVDV as cytopathic and non-cytopathic (cp/ncp) is determined by viral effect in epithelial cell culture. Biotypes of BVDV include types 1 and 2 which are determined by genetic variation and sequence identity (75). BVDV-2 genotypes can be further characterized as atypical or high
virulence (HV) and typical or low virulence (LV) as determined by viral titres *in vivo* and correlate to severity of acute disease (119, 120). In the United States biotypes of BVDV-1 and BVDV-2 are common (195). Recently, a newly characterized virus, the *Hobi-like* viruses have been isolated in South America and the Far East (169). The *Hobi-like* viruses are genetically and antigenically similar to BVDV 1 and 2, which may potentially classify these viruses as a BVDV genotype (10, 97, 182).

**Vaccines**

Within the United States, most BVDV vaccines consist of either a modified live viral vaccines (MLV) or heat killed vaccines to induce protective antibodies in the host (56). MLV BVDV vaccination is generally the preferred method of vaccination as the MLV vaccines induce broad protection to varying biotypes of BVDV and may provide protective immunity as early as 3 days post-vaccination (57, 58). MLV vaccination programs require one booster vaccination and yearly revaccination (55). As with most veterinary vaccination programs, BVDV is usually included as part of a multivalent vaccine against respiratory pathogens: *bovine respiratory syncytial virus* (bRSV), *bovine herpesvirus-1* (infectious bovine rhinotracheitis or infectious pustular vullovaginitis) (bHV-1.1, bHV1.2), *bovine parainfluenza-3 virus* (bPI-3) and bacterial pathogen *Mannheimia haemolytica* (57-61). However, vaccination of pregnant cows with MLV may induce infection and disease of the fetus (51, 118, 147, 213). Likewise vaccination is not always protective, cannot be used to treat persistently infected cattle and can potentially contribute to mucosal disease (22, 194).

**Mode of Transmission**

Transmission occurs via ingestion, inhalation and fomites (non-living sources such as boots and vehicles). Transmission may occur horizontally from cattle or through other
animals such as deer, alpacas, llamas and camels, which are host to BVDV (12, 34, 77, 128, 156, 215). Vertical transmission may also occur within an infected dam to fetus generating a persistently infected (PI) calf (7). PI animals display no clinical symptoms of disease, however are immune suppressed and shed high amount of virus (161). Superinfection of PI animals with cytopathic BVDV, may lead to a fatal syndrome called mucosal disease (23). Acute disease of non-PI animals may range from severe sickness, with bloody diarrhea, high fever, mouth ulcers, and pneumonia to mild sub-clinical acute; however disease does lead to an immune suppressed host (119-121). The majority of BVDV infections are believed to be transmitted via a PI animal, as these animals are non-symptomatic and difficult to clinically detect (151). Virus from PI animals is shed in all body secretions including nasal discharge, saliva, tears, milk, feces, urine and semen.

**Interaction of BVDV Proteins with Immune-associated Cellular Molecules**

There is little known about BVDV in context of either immune cell associated pathology or mechanisms of lasting immunological suppression. The N-terminal region of Npro of BVDV has been associated in vitro with the ubiquitination and subsequent proteosomal degradation of interferon regulatory factor-3 (IRF-3) (82, 140, 202). Likewise the Ems protein has been shown to also disrupt the IRF-3 signalling pathways by interacting directly with double stranded (ds)RNA and subsequently blocking recognition of the 3’-phosphates by retinoic acid-inducible gene-I (RIG-I) and induction of the type-2 interferons (IFNa/β) (92). Ems is also unique as it has been shown to be present in high concentrations within infected cells, secreted, and subsequently found extracellularly (203) which may potentially effect non-infected cells. The NS2 protein has been suggested to have similar function as HCV as an inhibitor of IFN-β (102, 178), although more recent studies have
indicated importance in virion morphology (114), and in coordination with expression of NS3/4A as an important mediator in induction of apoptosis (172). NS5A is one of the least characterized genes of BVDV, however there is evidence of alteration of NFκB transcription factor activation via blockade of phosphorylation of IKK-β. This disruption in activation is attained by interfering with the interaction of the IKK complex with NFκB-inducing-kinase (NIK) complex (216). This data fits with the observation from another study by which transcriptional dysregulation of the NFκB pathway is induced in BVDV infected cells (214).

**Cellular Entry and Tropisms**

BVDV is believed to enter cells through receptor-mediated internalization by CD46 (106, 136, 192), a co-receptor for the regulation of activation of the complement cascade. CD46 is expressed on all nucleated cells, which associates with the multitude of tissues and cell types in which BVDV has been isolated (17, 18, 65, 66, 119-121, 129, 149, 162). After initial attachment, BVDV is internalized via clatherin-mediated endocytosis (70). However, BVDV does not traffick to the endosome (108, 135), indicating that some modification of cellular compartmentalization may occur after internalization. Interestingly, cells resistant to infection with BVDV (CRIB) (53) have been generated in vitro, although expression of CD46 is present. Previous studies have believed the importance of low density lipoprotein (LDL) receptor as important in internalization of BVDV (2), as CRIB cells are deficient in expression of LDL receptors; however recent studies have suggested LDL receptor is not important for viral entry (107). As such, CD46 is believed to be important for viral entry, however the possibility persists that other cellular surface molecules may be important as co-receptors for viral entry. Likewise, the use of multiple receptors is important for HCV internalization (108, 193) and may be a commonality to BVDV and other Flaviviridae.
Impact on Circulating Lymphocytes

There is evidence of lymphopenia in sub-clinically acute and severe acute infected animals (119). Initially, both CD4+ and CD8+ T cells are depleted in the periphery with B cells and γδ-T cells maintaining circulation slightly longer (26, 149, 152, 164). This decrease in circulating lymphocytes has been hypothesized to be immune cell induced apoptosis although viral mediated necrosis may be occurring (36). Monocytes have been suggested as mediators of peripheral lymphopenia, as T cells initiate apoptosis when co-cultured with BVDV-infected monocytes (112). However evidence of potential tissue infiltration and subsequent immune mediated lesion development may be another explanation (17) for the decrease in circulating lymphocytes. Peripheral monocytes are not decreased in numbers and are potentially resistant to cytopenia induced by BVDV infection (119-121).

Impact on Monocytes

Monocytes have been identified \textit{in vitro} and \textit{in vivo} as supportive to BVDV replication (3, 18, 87, 129). With resilience to BVDV induced cytopenic effect (148) but not viral replication (65), monocytes potentially can be impaired in function by BVDV not induced by apoptosis or necrosis (3, 154).

An early study examined the effects of BVDV-1 on monocytes infected \textit{in vitro} in which no differences were seen between the intensities of the hybridization signals of IL-1 obtained using RNA extracted from LPS-stimulated BVDV-infected and uninfected monocytes. However there was a demonstration of inhibition of IL-1 activity in monocyte supernatants as supported by significant suppression of mouse thymocyte proliferation when compared to uninfected control supernatants (95). More recently, it has been shown \textit{in vitro} that BVDV-1 infection of monocytes resulted in reduced IL-1β and IL-6 mRNA transcription
and decreased IL-1β, IL-6 and TNFα secretion compared to uninfected controls (115). As there are few studies on the effects of BVDV on monocytes it is important to consider modifications of monocyte cytokine secretion induced by other Flaviviridae. Peripheral blood monocytes isolated from individuals chronically infected with HCV and stimulated with LPS exhibited enhanced expression of TNFα, IL-1β and IL-6 protein compared to aviremic or uninfected individuals (46, 211). Moreover, human monocytes infected with Dengue virus and stimulated with LPS were found to secrete high levels of IL-1β, TNFα, IFNα, IL-6, IL-8, IL-12, MIP-1α and RANTES compared to uninfected control cells (24, 37). Hypersensitivity of PRRs and enhanced expression of proinflammatory cytokines are contributing factors to severe clinical disease and a prolonged period of recovery (38, 190, 197, 200). Noteworthy is that in highly virulent infections of BVDV-2, recovery is prolonged compared to the typical or low virulent infections (119). Enhanced pro-inflammatory cytokine secretion from monocytes has been seen in cases of viral infection with associated peripheral lymphocyte depletion or increased induction of leukocyte apoptosis (67, 78, 170).

Impact on Monocyte Derived Macrophages

Few studies to date have examined the impacts of BVDV infection on the function of monocyte derived macrophages. Bone marrow derived macrophages (BMMΦ) infected with BVDV-1 in vitro have been shown to secrete less TNFα protein in response to LPS or bacterial infection compared to uninfected BMMΦs (1). As with other Flaviviridae TLR hyporesponsiveness has been demonstrated in MDMΦ from chronically infected HCV patients, which correlated to more severe disease and worse clinical outcome (40, 125).
Yellow fever virus and St. Louis encephalitis virus infection reduced IL-1β levels in LPS-stimulated MDMΦ compared to uninfected control cells (9).

Impact on Alveolar Macrophages

AMΦ are a terminally differentiated tissue macrophage resident to the alveolar spaces of the lung, which further play roles in lung homeostasis as well as tissue remodeling following lung damage (35). These cells respond to pathogens through production of proinflammatory cytokines (44) and are of interest to BVDV infection in the lung as these cells will be one of the first innate immune cells to encounter the virus. One study has shown that in vivo BVDV-1 infection with in vitro restimulation with BVDV-1 of AMΦ greatly reduced normal function of these cells, with a decline in their ability to secrete neutrophil chemotactic factors, a downregulation of Fcγ receptor and CR3 expression, and a reduction in phagocytic capability (206).

Specific Aims

Disease associated with BVDV infection can be devastating ranging to millions of dollars in loss to the cattle industry. As well, infection with BVDV leads to an immune-suppressed host though an unknown mechanism(s). Although there are vaccines available, they are not broadly protective and ineffective in PI cattle. The myeloid lineage cells such as monocytes and MΦ are important in recognizing BVDV as well as initiating an adaptive immune response. There is little information on the effects of BVDV in vivo on the functionality of these cells and a better understanding can lead to potential treatment. Investigating the effects on cytokine production, expression and response of TLRs and functional aspects of phagocytosis are critical. The specific aims in these studies utilize
neonatal calves for *in vivo* study and 1-year-old cattle for cell donors during *in vitro* investigation. BVDV-2 strain 1373 was utilized as it is a field strain, which induces severe acute disease and is a highly virulent. BVDV-2 strains RS886 and 28508 are subclinical acute disease inducing strains, which are lower in virulence and typical of field isolates from PI cattle or subclinical infections. BVDV-2 strain 296c is a cytopathic strain whereas BVDV-2 strain 296nc is an isogenic non-cytopathic strain. These isogenic strains were chosen as the only differences between the strains are the NS2/3 coding regions. BVDV-2 strains 1373 and RS886 were utilized for *in vivo* experimentation whereas BVDV-2 strains 1373, 28508, 296c and 296nc were used for *in vitro* experiments. Both *in vivo* and *in vitro* models were used to characterize viral effects on cytokine expression and secretion, TLR responsiveness, signaling events, and phagocytosis of myeloid cells after exposure to virus.

**Specific Aim 1.** Determine the effects of BVDV-2 virulence on cytokine expression of myeloid lineage cells isolated from high or low virulence BVDV-2 infected neonatal calves compared to cells from uninfected calves.

**S.A.1 Hypothesis.** Each of the myeloid lineage cell types isolated from BVDV-2 infected calves will exhibit a decrease in proinflammatory cytokine expression, especially in the calves infected with the highly virulent 1373 BVDV-2 strain, compared to cells isolated from uninfected calves.

There is limited information on the cytokine responses from myeloid lineage cells isolated from an *in vivo* infection with BVDV and none on BVDV-2 biotypes of varying virulence. Likewise, there are no studies comparing mRNA and protein from three different myeloid lineage cell types during an *in vivo* BVDV infection and none investigating the mechanisms of viral modulation of cell signaling molecules related to TLRs. Monocytes and
alveolar macrophages were isolated by plastic adherence and used for *ex vivo* analysis. Data collected from the proposed studies of this aim determine the functions of these cells were modulated by BVDV-2 infection. The following studies were utilized to elucidate the effects of BVDV on myeloid lineage cells isolated from infected neonatal calves:

1. Cytokines were quantified for mRNA expression by qPCR utilizing SYBR Green chemistry and bovine specific primers
2. Identified cytokine protein secretion by utilizing ELISA based assays.
3. Determined the viral effects on intracellular signaling molecules by western blot.
4. Identified a potential interaction between BVDV and host proteins by protein complex immunoprecipitation (Co-IP).

**Specific Aim 2.** Determine the effects of an *in vitro* infection of varying biotypes of BVDV-2 strains on MDMΦ and TLR responsiveness.

**S.A.2 Hypothesis.** The highly virulent and cytopathic strains of BVDV-2 will induce greater amounts of pro-inflammatory cytokines in myeloid lineage cells than the low virulence and non-cytopathic isogenic strains, but will induce a TLR hypo-responsiveness similar to that observed in *in vivo* MDMΦ from S.A.1.

There have been no *in vitro* studies comparing isogenic strains of cytopathic and noncytopathic BVDV-2 or strains of varying virulence on the effects of MDMΦ. Healthy BVDV-free cattle were used as blood donors and MDMΦ were generated from blood monocytes. TLR agonists were used in MDMΦ incubated for 48 h after viral inoculation. Data collected from the following studies determined the effects of BVDV-2 on the cytokine responses of MDMΦ prior to and after TLR ligation. The following studies were used to elucidate the effect of BVDV-2 on the MDMΦ:
1. Cytokines were quantified for mRNA expression by qPCR utilizing SYBR Green chemistry and bovine specific primers.

2. Identified cytokine protein secretion by utilizing ELISA based assays.

3. Determined the viral effects on intracellular signaling molecules by western blot.

**Specific Aim 3.** Determine the effects of BVDV-2 infection of varying virulence biotypes on MDMΦ antigen processing, presentation, phagocytosis and phagosome acidification and oxidative capacity.

**S.A.3 Hypothesis.** The high virulence and cytopathic BVDV-2 infected MDMΦ will decrease expression of cell surface markers associated with antigen presentation as well as decreasing the phagocytic capabilities and acidification of the phagosome compared to low virulence and isogenic non-cytopathic infected cells.

There have been no *in vivo* or *in vitro* studies to compare varying strains of BVDV-2 biotype effects on MDMΦ antigen presentation and phagosome functionality. For *in vivo* analysis, BVDV-2 strains 1373 or RS886 were used to inoculate neonatal calves and peripheral blood CD14+ cells were isolated. For *in vitro* experimentation healthy BVDV-free cattle were used as blood donors and MDMΦ were generated from blood monocytes. MDMΦ incubated for 48 h after viral inoculation prior to phagocytosis assay. Data collected from the following studies determined the effects of BVDV-2 on the expression of cell surface markers and phagosome processing. The following studies were used to elucidate the effect of BVDV-2 on the MDMΦ:

1. RNAseq analysis will be used to determine the transcriptome of the *in vivo* infected calves.
2. Cell surface markers from MDMΦ infected in vitro were quantified for mRNA expression by qPCR utilizing SYBR Green chemistry and bovine specific primers.

3. Flow cytometry will be used to analyze surface antigen expression.

4. Assess phagosome acidification and oxidative capacity by bead-based flow cytometry assay.


References


110. **Kvasnicka, B.** 2012. Bovine Virus Diarrhea: Mucosal Disease and Persistent Infection. In UCCE Livestock, Range and Natural Resources Fact Sheet No. 3.


137. **Medzhitov, R.** 2009. Approaching the asymptote: 20 years later. Immunity **30:**766-775.


**Figure 1.** Schematic diagram of Toll-like receptor pathways. TLRs 2/1, 2/6, 4, 5, and 7/8 signal through MyD88 dependent pathway. TLRs 2/1, 2/6 recognize bacterial cell membrane glycoproteins. TLR4 recognizes lipopolysaccharide with CD14 and MD-2. TLR 7/8 are located in the endosomal compartment and recognize single stranded viral RNA. This pathways leads to activation of NFκB family members, JNK and p38, which activate transcription of pro-inflammatory cytokines. TLRs 3 and 4 can signal though MyD88-independent pathways involving TRIF. TLR3 is located in the endosomal compartment and can recognize double stranded viral RNA. This can lead to activation of NFκB family members, JNK, p38, IRF and apoptosis.
Figure 2. Schematic diagram of Bovine Viral Diarrhea Virus (BVDV) genome. The BVDV genome is initially generated as one large polyprotein. For functional proteins to be generated, host peptidases, golgi proteases and the viral NS3 protease will cleave the polyprotein. The 5’ NTR of the BVDV genome is highly conserved followed by the nucleocapsid, capsid, and envelope proteins. The p7 is a small hydrophobic protein of unknown function, with the NS2-3 peptide a protease that cleaves NS4 and NS5. The envelope E1 and E2 proteins are antigenic; and antibodies against these proteins are protective.
CHAPTER TWO. BOVINE VIRAL DIARRHEA VIRUS GENOTYPE 2

\textit{IN VIVO} INFECTION MODULATES TLR4 RESPONSIVENESS IN
MYELOID CELLS VIA A DECREASE IN MYD88 EXPRESSION AND
IKKB PHOSPHORYLATION

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ABSTRACT

\textit{Bovine viral diarrhea virus} (BVDV) causes clinical signs in cattle ranging from mild to severe acute infection which can lead to increased susceptibility to secondary bacteria. In this study we examined the effects of BVDV genotype 2 (BVDV-2) infection on the ability of myeloid lineage cells derived from infected calves to produce proinflammatory cytokine mRNA and protein with or without bacterial LPS stimulation. Monocytes, monocyte-derived macrophages (MDM\(\Phi\)), and alveolar macrophages (AM\(\Phi\)), were chosen to represent differing stages of myeloid differentiation. It was found that monocytes isolated from infected calves produced higher levels of proinflammatory cytokine mRNA compared to uninfected calves and were hyperresponsive to LPS based on an increase in proinflammatory cytokine transcription and secretion. In contrast, MDM\(\Phi\) inflammatory cytokine gene expression and secretion were, in general, significantly reduced compared to cells isolated

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from control calves. There was a discordance between AMΦ cytokine gene expression and protein secretion, in that while mRNA levels were significantly reduced, protein secretion was similar to that of stimulated cells from control animals. The differential cytokine expression of these macrophage cells was attributed to the modulated expression of myeloid differentiation primary response gene 88 (MyD88) protein and phosphorylated inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ). Thus, BVDV-2 infection results in the modulation of LPS-induced myeloid pro-inflammatory cytokine expression by effects on two separate components of TLR4 signaling cascades involving MyD88 and transcription factor NFkB activation.

**INTRODUCTION**

*Bovine viral diarrhea viruses* (BVDV) are enveloped single-stranded positive-sense RNA viruses comprised of two species, BVDV-1 and BVDV-2, in the *Flaviviridae* family (19, 56). The BVDV genome is approximately 12.5kb in length and is organized into 12 genes flanked by a 5’ nontranslated region (NTR) and a 3’ non-polyadenylated NTR. The BVDV genome encodes a large polyprotein which is cleaved into varying polypeptides. Six genes (NS2, NS3, NS4A, NS4B, NS5A, NS5B), which comprise the majority of the BVDV genome, are nonstructural (NS) genes, which is a common feature throughout the *Flaviviridae* family and make up the amino terminal region of the genome (57).

Classification of BVDV as cytopathic (cp) or noncytopathic (ncp) biotypes is based on their cytopathogenicity in epithelial cell culture (55). Cytopathogenicity is associated with the cleavage of the NS2/3 protein into the NS2 and NS3 proteins, whereas ncp-BVDV strains only express one whole protein referred to as NS2/3 and therefore ncp-BVDV contains only
11 functional genes. NS5A is one of the least characterized genes of BVDV; however there is evidence of NS5A involvement in the alteration of the NFκB activation by blocking phosphorylation of the inhibitor of κB kinase binding protein-β (IKKβ) in vitro (82). BVDV-2 genotypes can be further characterized as atypical or high virulence (HV) and typical or low virulence (LV) as determined by viral titres in vivo and correlate to severity of acute disease (55).

Acute BVDV infections result in varying clinical syndromes in cattle ranging from subclinical to an acute, severe sickness, with bloody diarrhea, high fever, mouth ulcers, and pneumonia. Sub-clinically, there is evidence of lymphopenia in severely infected animals (39-42). 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 (14, 26). Secondary bacterial infection is the leading cause of morbidity and mortality among feedlot cattle (23, 51, 69). Mechanisms whereby BVDV predisposes cattle to secondary bacterial infection have not been completely elucidated.

In response to inflammatory signals, circulating monocytes rapidly disseminate into infected tissues and develop into antigen presenting cells. Previous research supports the idea that monocytes may be a vehicle for dissemination of BVDV throughout the host (24, 36). Studies have shown that BVDV replicates in monocytes (3, 6), however unlike lymphocytes, a reduction in the numbers of monocytes are not sustained following acute infection (40-43). While numbers of monocytes are not effected, studies have shown that in vitro BVDV may impair cellular functions of these cells including sugar metabolism,
interferon induction, apoptosis and migration (3, 52). Similarly, in vitro ncp BVDV-1 infection of monocytes downregulates TNFα, IL-1β and IL-6 gene expression (37). To date, there have been no in vivo studies of BVDV-2 impact on myeloid cell cytokine expression or mechanisms of immune modulation in these cells.

Monocytes may differentiate into macrophage cells (MΦ) or dendritic cells (DCs). MΦ are important in recognizing innate recognition signals or pathogen associated molecular patters (PAMPs) to initiate an immune response (30). These receptors represent a potential target for infectious agents to subvert the immune system. This 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 (47). Following activation, TLRs associate with cytosolic receptor-proximal proteins containing TIR domains, including myeloid differentiation primary response gene 88 (MyD88) or TRIF (TLR-associated activator of interferon). MyD88 recruits IRAK-1, which can complex with TRAF-6, leading to activation of transcription factor NFkB 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. Many Flaviviridae species modulate PRR signaling pathways, such as HCV disruption of TLR-3, IRF-3, and RIG-I signaling (34), Yellow Fever Virus (YFV) reduction of TLR7 and TLR9 signaling (46), and Classical Swine Fever Virus (CSFV) modulation of TLR7 activity (22). Recently, it has been shown that other viruses, including HCV, Vaccinia virus (VV), Human immunodeficiency virus (HIV) 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 mechanisms that have yet to be elucidated (45, 62, 64, 71).

The aim of this study was to examine cytokine mRNA and protein expression of myeloid lineage cells isolated during an in vivo BVDV-2 infection and investigate the ability of these cells to respond to subsequent bacterial LPS stimulation. Differential cytokine responses were observed for monocytes, MDMΦ and AMΦ from BVDV-2-infected calves. We found that the decrease in production of inflammatory cytokines in response to LPS may be due in part to the differences in MyD88 expression and phosphorylation of IKKβ in myeloid lineage cells from BVDV-2 infected calves.

MATERIALS AND METHODS

Animals. Eighteen clinically healthy, colostrum-deprived Holstein calves were used in two experiments. 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 BVDV 2 strain 1373 (13).

Viral inoculum. The ncp BVDV-2 strain 1373 was isolated during 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 tissue culture.

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 5mL of 10^{5.6} TCID_{50} of BVDV-2
strain 1373 (n=3/replicate) or RS886 (n=3/replicate). Additional calves (n=3/experiment) were mock inoculated and served as age-matched negative controls. Clinical signs were monitored twice per day. Blood samples were collected in anticoagulant tubes on days 4 and 12 post inoculation. Numbers of leukocytes and platelets were determined for each animal. 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.

**Cell Count Populations.** 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).

**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), 2mM L-glutamine, 1% antibiotic–antimycotic solution, and gentamicin sulfate (Gibco, Carlsbad, CA). Monocytes were isolated by plastic adherance. 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 5x10^6 cells per mL. Monocytes were plated in 96-well round bottom tissue culture plates in 100 μL medium. Monocytes selected for MDMΦ were cultured for 7 days with media change every 2-3 days prior to *in vitro* stimulation.
Monocytes or MDM\(\Phi\) were stimulated by addition of Escherichia coli LPS (055:B5 [1\(\mu\)g/mL], Sigma, St. Louis, MO).

**Alveolar macrophage isolation.** AM\(\Phi\) were isolated as described previously (21). Briefly, lungs of calves were aseptically removed at necropsy and lavaged with 500mL sterile PBS. The lavage fluid was centrifuged in 250mL conical tubes at 1000 x g (Beckman J-6B, Beckman Coulter, Indianapolis, IN) for 20 minutes at 4\(^{\circ}\)C. Cells were isolated by plastic adherence in 75mm petri dishes. Ice cold PBS was added to the dish and adherent cells were gently removed with a sterile cell scraper. Cells were centrifuged and resuspended in cRPMI 1640 at a concentration of 5x10\(^6\) cells per mL. AM\(\Phi\) were plated in 96-well round bottom tissue culture plates at 5 x10\(^5\)/ml and incubated 2 or 24 h with LPS.

**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\(^{\circ}\)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 DNasel digestion set (Qiagen) according to manufacturer’s instructions. Total RNA was eluted by the addition of 40 \(\mu\)L of DNase–RNase free water with addition of RNAse OUT (Invitrogen, 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 (Invitrogen) according to manufacturers instructions.

**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\(^{\circ}\)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-1β Forward 5’- TTC TGT GTG ACG CAC CCG TGC -3’, IL-1β Reverse 5’- AGC ACA CAT GGG CTA GCC AGC -3’, TNFα Forward 5’- CGG GGT AAT CGG CCC CCA GA -3’, TNFα 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 fluorescence 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 (44). Dissociation curves were analyzed for proper product amplification.

**Multiplex cytokine immunoassay.** 50 µL of monocyte or macrophage (MΦ) 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-1β, 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-1β=1.9, TNFα=2.4, IL-6=0.9, and IFNγ=0.5.

**Western blotting.** Lysates were generated for western blot analysis at 24 h post LPS stimulation. 50 µL 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 10ug 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 (20uL) and Benchmark Prestained Protein Ladder (Invitrogen) were loaded into a Novex Tris-Glycine 1.0mm 15 well gels (Invitrogen) and electrophoresed at 75V for approximately 1.5 h or until distinct ladder separation had occurred. Gels were transferred to nitrocellulose using an iBlot transfer system (Invitrogen) 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 IKKαβ, Anti-total IKKβ, 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-species 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 manufacturers 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).

**Production of BVDV NS5A antiserum.** An internal fragment of the NS5A protein of BVDV 2 strain 1373 (nucleotides 9296 through 9964) was amplified by PCR using primers that added BamHI (5’) and SalI (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 20mLs of antiserum was harvested from each animal.

**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. The IP lysate was loaded into a 10-20% Tris-Glycine gel (Invitrogen) 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.
Statistical Analysis. qPCR cytokine data was analyzed with the outcome variable (2^ {ΔΔCt} ) log transformed. ΔΔCt 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).

RESULTS

Circulating monocytes are not depleted in calves infected with BVDV-2 strains of high or low virulence. Previous studies have shown that peripheral blood monocytes are not depleted during BVDV-2 infection (40, 41). Lymphopenia was observed in calves infected with high or low virulence BVDV-2 strains (McGill et al., manuscript in preparation). Monocyte levels were not significantly reduced in circulation during the course of BVDV-2, although there was a reduction in monocytes isolated from the 1373 infected calves at day 12 post infection (Table 1). The decrease observed for day 12 was not seen at day 14 or observed at any other time point measured (data not shown). This data, as well as data from others, suggest that there is no sustained depletion of peripheral blood monocytes throughout the time course of BVDV-2 infection.

Monocytes isolated from BVDV-2-infected calves early in BVDV-2 infection display enhanced cytokine responses to LPS stimulation. Given that over the course of the
infection peripheral blood monocytes were not substantially depleted, we examined whether there was a modulation of cytokine responses of monocytes as a result of BVDV-2 infection. The basis for this idea was a previous study which demonstrated that *in vitro* ncp BVDV-1 infection downregulates TNFα, IL-1β and IL-6 gene expression in monocytes (37); however, the effects of *in vivo* BVDV-2 infection have not yet been examined with respect to monocytes and cytokine production. We investigated TNFα, IL-1β and IL-6 mRNA responses prior to and after LPS stimulation at 2 h or 24 h as these cytokines will have an early, sustained mRNA response (81). It is noteworthy that unstimulated monocytes from the HV BVDV-2-infected calves had significantly higher IL-1β and IL-6 RNA levels than monocytes from uninfected calves. Interestingly, at 2 h post LPS simulation monocytes isolated from HV and LV infected animals exhibited enhanced TNFα mRNA transcription compared to monocytes from uninfected animals (Fig 1A). Likewise monocytes isolated from HV BVDV-2-infected calves exhibited enhanced IL-1β and IL-6 compared to cells from uninfected controls (Fig. 1A). There was no increase in LPS-stimulated IL-1β or IL-6 mRNA in monocytes isolated from the LV BVDV-2-infected calves compared to the uninfected control monocytes. At 24 h (Fig. 1B), TNFα was greater in monocytes isolated from the HV BVDV-2 calves compared to uninfected controls, although there was no enhancement of mRNA following LPS stimulation. Upregulation of IL-1β and IL-6 mRNA was observed in monocytes isolated from the HV BVDV-2-infected group (Fig. 1B). Monocytes isolated from the LV BVDV-2 group exhibited enhanced IL-1β mRNA expression with LPS treatment. Furthermore, we examined levels of cytokine secretion as shown in Fig. 1C. Interestingly, monocytes isolated from the LV BVDV-2 group secreted significantly higher levels of TNFα and IL-1β following LPS stimulation compared to
monocytes from uninfected calves. Taken together, the results show that monocytes isolated early in BVDV-2 infection have upregulated proinflammatory cytokine mRNA prior to LPS stimulation and produce higher levels of cytokines in response to LPS than cells from uninfected controls.

**Monocytes isolated from BVDV-2-infected calves late in infection exhibit enhanced inflammatory cytokine responses to LPS.** An examination of day 12 post-infection was chosen as this represents the recovery phase following LV BVDV-2 infection (42), which is not observed for HV BVDV-2-infected calves (40). In unstimulated monocyte cultures there was an increased expression of TNFα, IL-1β and IL-6 in monocytes isolated from HV and LV BVDV-2-infected groups compared to cells from uninfected calves (Fig. 2A and B). At 2 h post LPS stimulation, monocytes from HV BVDV-2-infected calves exhibited enhanced mRNA for TNFα, IL-1β and IL-6 compared to monocytes from the controls. Increased TNFα and IL-1β gene expression was observed in monocytes isolated from the LV BVDV-2 group in response to LPS. Interestingly TNFα mRNA was not enhanced after 24 h LPS stimulation in monocytes isolated from either BVDV-2-infected group (Fig. 2B). Monocytes isolated from the HV BVDV-2 group with LPS treatment exhibited enhanced IL-1β and IL-6 at 24 h compared to cells from uninfected calves. Downregulation in IL-1β mRNA expression was seen after 24 h LPS stimulation in monocytes isolated from the LV BVDV-2-infected group compared to those from uninfected controls; however, the monocytes did exhibit enhanced IL-6 after 24 h LPS stimulation. Monocytes isolated from the HV BVDV-2-infected animals stimulated 24 h with LPS exhibited an enhanced TNFα, IL-1β and IL-6 secretion compared to monocytes from uninfected calves (Fig. 2C). Secretion of TNFα and IL-1β in response to LPS stimulation of
monocytes isolated from the LV BVDV-2 group was similar to monocytes from uninfected calves, while IL-6 was significantly higher. In summary, proinflammatory cytokine levels seen in monocytes isolated from HV BVDV-2-infected calves were higher than observed for monocytes isolated from LV BVDV-2-infected or control calves.

**MΦ derived from monocytes early in BVDV-2 infection display decreased cytokine mRNA and protein responses to LPS.** Monocytes differentiate into macrophages that play a pivotal role during infection via recognition of PAMPs and initiation of innate immune signaling pathways, and the effects of an *in vivo* BVDV-2 infection on this development of a MDMΦ has not been investigated. We examined the influence of BVDV-2 infection on the cytokine expression of monocytes differentiated into MΦ and the LPS responsiveness of these cells. Unstimulated MDMΦ from HV BVDV-2-infected calves had significantly enhanced TNFα mRNA compared to uninfected controls; however, MDMΦ isolated from HV or LV BVDV-2 groups did not upregulate TNFα mRNA expression in response to 2 h LPS stimulation (Fig. 3A). Interestingly, MDMΦ isolated from the HV BVDV-2 group exhibited a decrease in mRNA expression in response to 2 and 24 h LPS stimulation for IL-1β and IL-6 compared to cells from the uninfected group (Fig. 3A and B). MDMΦ from the LV BVDV-2 group exhibited an enhanced mRNA expression of TNFα in response to 24 h LPS stimulation compared to the controls. There was no significant difference in TNFα secretion between the HV or LV BVDV-2-infected groups compared to controls in response to LPS (Fig. 3C). However; secretion of IL-1β and IL-6 in response to LPS stimulation of MDMΦ from both HV and LV BVDV-2-infected groups was decreased compared to cells from the uninfected animals. In general, early in infection, LPS-stimulated
MDMϕ from HV BVDV-2-infected calves display reduced proinflammatory cytokine responses compared to cells from uninfected calves.

**MΦ derived from monocytes late in BVDV-2 infection have decreased levels of cytokine mRNA and protein secretion in response to LPS.** At day 12 post BVDV-2 infection unstimulated MDMϕs from both HV and LV infected groups exhibited a decrease in TNFα mRNA compared to the uninfected group (Fig. 4A and B). Likewise, MDMϕ isolated from the HV and LV BVDV-2-infected calves exhibited a downregulation in TNFα and IL-1β mRNA expression after 2 or 24 h LPS stimulation compared to cells from uninfected calves. However, IL-6 mRNA was enhanced after 2 or 24 h LPS stimulation in MDMϕ isolated from the HV BVDV-2 group compared to cells from the uninfected group (Fig 4A and B). Conversely, IL-6 mRNA was reduced in response to 2 or 24 h LPS stimulation from MDMϕ isolated from the LV BVDV-2-infected group compared to controls. TNFα protein secretion was decreased in MDMϕ isolated from the HV BVDV-2-infected group after LPS stimulation compared to MDMϕ from the uninfected group (Fig. 4C). MDMϕ from both HV and LV BVDV-2-infected groups exhibited decreased IL-1β protein secretion in response to LPS stimulation compared to cells from uninfected controls. IL-6 protein secretion was enhanced in MDMϕ isolated from the HV BVDV-2-infected group in response to LPS stimulation compared to the MDMϕ isolated from the uninfected group; although, MDMϕ from the LV BVDV-2-infected group demonstrated no change from controls. To summarize, MDMϕ from HV and LV BVDV-2 infected-calves were shown to have reduced expression of proinflammatory cytokines compared to MDMϕ 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.

**AMΦ from BVDV-2-infected calves exhibit a reduced proinflammatory cytokine mRNA response to LPS.** As we observed lung lesions in the HV and LV BVDV-2-infected calves (data not shown), we investigated the effect of BVDV-2 infection on the AMΦ and the LPS responsiveness of these cells. AMΦ are a tissue macrophage that can develop from circulating monocytes and are resident in the lung in which these cells act as sentinels to pathogens (15). Interestingly unstimulated AMΦ isolated from both HV and LV BVDV-2 groups exhibited decreased mRNA expression of IL-6 and after 2 h LPS stimulation compared to cells from uninfected controls (Fig 5A and B). TNFα and IL-1β mRNA expression was enhanced at 2 h in AMΦ isolated from the LV BVDV-2 group in response to LPS stimulation compared to that in AMΦ from the uninfected group. At 24 h post LPS stimulation, AMΦ isolated from the HV BVDV-2-infected calves exhibited reduced expression of TNFα mRNA compared to that of cells from the uninfected calves (Fig. 5B). Cytokines secreted by the AMΦ from HV and LV BVDV-2-infected groups in response to LPS did not significantly differ from uninfected control cells, except for the LV group which secreted significantly less IL-6 (Fig. 5C). In general, LPS-stimulated proinflammatory cytokine mRNA expression from AMΦ isolated from BVDV-2-infected calves is decreased compared to AMΦ control calves.

**Myeloid cells from BVDV-2 infected calves differentially express cytokine mRNA and protein.** To summarize the differences in cytokine expression in BVDV-2-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 BVDV-2 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, BVDV-2 alone induced an increase in each cytokine measured in the monocytes isolated from infected calves. However, TNFα was expression decreased in MDMΦ and IL-6 was decreased in AMΦ isolated from BVDV-2-infected calves. There was no change in protein expression of any of the cell types isolated from BVDV-2-infected calves compared to controls prior to LPS stimulation. After LPS stimulation, proinflammatory cytokine gene expression and protein was enhanced in monocytes isolated from BVDV-2-infected calves compared to controls. TNFα and IL-1β were reduced in expression after LPS stimulation in MDMΦ isolated from BVDV-2 infected calves, whereas IL-6 mRNA and protein was enhanced compared to controls. AMΦ isolated from BVDV-2-infected calves and stimulated with LPS exhibited increased mRNA expression of TNFα and IL-1β; however decreased expression of IL-6 mRNA was observed. Similarly, LPS-induced IL-6 secretion was lower in AMΦ isolated from infected calves compared to AMΦ from control calves.

**MyD88 protein expression is modulated in MDMΦ isolated from HV infected calves.** Bacterial LPS can signal through TLR4 which involves adapter protein MyD88-dependent or MyD88-independent TRIF/TRAM pathways (49). We examined expression of MyD88 and TRIF in myeloid lineage cells isolated at day 12 post BVDV-2 infection. Monocytes isolated from the HV infected calves (Fig. 7A) demonstrated reduced MyD88 expression after LPS treatment. MDMΦ isolated from the HV infected calves demonstrated the greatest amount of decrease in expression of MyD88 either with or without LPS stimulation (Fig. 7B). AMΦ from both the HV and LV infected groups exhibited a decrease in MyD88 after LPS stimulation (Fig. 7C). Further, we investigated expression of TRIF,
which can signal independently of MyD88 following LPS stimulation (74) and we did not observe any measurable difference between the \textit{in vivo} treatment groups (Fig. 7A, B and C).

To summarize, MyD88 expression in MDMΦ was downregulated prior to stimulation and after LPS treatment, myeloid cells isolated from BVDV-2-infected animals did not upregulate MyD88 to the same extent seen in cells from uninfected animals with the MDMΦ demonstrating the lowest expression of MyD88.

**BVDV NS5A interacts with MyD88.** We established that there was a modulation of MyD88 expression in myeloid lineage cells isolated from BVDV-2-infected calves. Moreover, it was reported that HCV NS5A colocalizes with MyD88 (1). Additionally, given that there are tertiary structural similarities between the NS5A proteins of BVDV and HCV (54), we examined whether BVDV-2 NS5A binds to adapter protein MyD88. We immunoprecipitated MDMΦ whole cell lysates with a BVDV NS5A antiserum and then blotted with an anti-MyD88 mAb (Fig 8A). In cells from HV or LV BVDV-2-infected animals, we determined that NS5A interacts with MyD88. The unbound NS5A flow-through fraction probed with the MyD88 mAb is shown in Fig 8B.

**Myeloid lineage cells from BVDV-2-infected calves differ in their expression of phosphorylated IKKβ in response to LPS.** TLR4 ligation upregulates transcription of NFκB family members which ultimately give rise to proinflammatory cytokines (27). A previous study demonstrated that BVDV-1 inhibited the phosphorylation of the IKKβ subunit of the IKK signaling complex to block NFκB signaling in an epithelial cell line (80). Monocytes isolated from BVDV-2-infected calves (Fig. 9A) exhibited increased expression of phosphorylated(p)-IKKβ in response to LPS to a similar extent as uninfected control cells. Interestingly, we observed a decrease in p-IKKβ in response to LPS in MDMΦ from animals
infected with BVDV-2 (Fig. 9B). Similarly, AMΦ (Fig. 9C) from the BVDV-2-infected animals were found to have a lower expression of p-IKKβ compared to cells isolated from control animals. These data show that MΦ from HV and LV BVDV-2-infected animals are unable to express phosphorylated IKKβ in response to LPS to the same extent as cells isolated from uninfected calves. The lack of increased p-IKKβ in MΦ stimulated with LPS may partially explain the observed downregulation of cytokine gene expression.

**DISCUSSION**

Experimental infection with LV BVDV-2 strain RS886 induced mild to no clinical signs as previously reported (41). By comparison, HV ncp BVDV-2 strain 1373-infected calves exhibited more severe clinical signs including high fever, diarrhea, signs of depression and recumbency (40). While lymphocytopenia was seen following challenge with these BVDV-2 strains, monocytes were not significantly depleted (Table 1) (40, 41). Given that circulating pools of monocytes are maintained during BVDV-2 infection, functional modifications may be targeted, as these cells differentiate into tissue macrophages following their exit from circulation and secrete inflammatory cytokines vital to innate host defenses. BVDV can be a contributing factor in bovine respiratory disease complex (BRDC) as it enhances susceptibility to secondary infection via immunosuppression (14). 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 BVDV-2 on subsequent TLR4 signaling in myeloid lineage cells we sought to unravel specific mechanisms whereby viral infection may alter host responses to secondary bacterial infections.
There have been few studies published on the effects of BVDV infection on the cytokine response of monocytes and these studies have examined BVDV-1 infection in vitro. An earlier study examined the effects of BVDV-1 on monocytes infected in vitro in which no differences were seen between the intensities of the hybridization signals of IL-1 obtained using RNA extracted from LPS-stimulated BVDV-infected and uninfected monocytes. However there was a demonstration of inhibition of IL-1 activity in monocyte supernatants as supported by significant suppression of mouse thymocyte proliferation when compared to uninfected control supernatants (31). More recently, it has been shown in vitro that BVDV-1 infection of monocytes resulted in reduced IL-1β and IL-6 mRNA transcription and decreased IL-1β, IL-6 and TNFα secretion compared to uninfected controls (37). To date, the effects of Flaviviral infection on LPS responsiveness of monocytes has been examined in studies with HCV or Dengue virus. Peripheral blood monocytes isolated from individuals chronically infected with HCV and stimulated with LPS exhibited enhanced expression of TNFα, IL-1β and IL-6 protein compared to aviremic or uninfected individuals (17, 76). Moreover, human monocytes infected with Dengue virus and stimulated with LPS were found to secrete high levels of IL-1β, TNFα, IFNα, IL-6, IL8, IL-12, MIP-1α and RANTES compared to uninfected control cells (7, 10).

We established that monocytes isolated from BVDV-2-infected animals generally exhibited enhanced TNFα, IL-1β and IL-6 mRNA compared to cells from uninfected animals. Similarly, monocytes isolated from BVDV-2-infected calves demonstrated an increase in expression of TNFα, IL-1β and IL-6 mRNA and protein secretion in response to LPS stimulation throughout the course of infection compared to monocytes isolated from uninfected calves. Furthermore, TNFα and IL-1β secretion in response to LPS stimulation
was significantly elevated in monocytes from HV BVDV-2 infected calves late in infection. Hypersensitivity of PRRs and enhanced expression of proinflammatory cytokines are contributing factors to severe clinical disease and a prolonged period of recovery (12, 68, 70, 72). Noteworthy is that in HV BVDV-2 infection, recovery is prolonged compared to LV BVDV-2 (40). The observed increase in proinflammatory cytokine secretion in response to LPS-stimulation by monocytes following BVDV infection would be predicted to contribute to more severe clinical BRDC. Enhanced pro-inflammatory cytokine secretion from monocytes has been seen in cases of viral infection with associated peripheral lymphocyte depletion or increased induction of leukocyte apoptosis (25, 28, 60). These data and those of others (7, 10, 17, 18, 76) indicate that monocytes from Flaviviral-infected hosts have an increase in expression of proinflammatory cytokine mRNA and are hyperresponsive to LPS stimulation as this may be contributory to inflammation following secondary infection.

Monocytes differentiate into macrophages and during maturation upregulate expression of TLRs as well as other PRRs (38). In response to TLR stimulation, macrophages become classically activated in which they secrete proinflammatory cytokines such as TNFα, IL-1β and IL-6, as well as produce reactive nitrogen and superoxide intermediates, promote TH1 responses, and increase microbicidal, tumoricidal and antiviral activity (48, 61). Previous studies have explored the impact of Flaviviradae on the TLR responsiveness of in vitro derived MΦ. Bone marrow derived macrophages (BMMΦ) infected with BVDV-1 in vitro have been shown to secrete less TNFα protein in response to LPS or bacterial infection compared to uninfected BMMΦs (2). TLR hyporesponsiveness has been demonstrated in MDMΦ from chronically infected HCV patients which correlated to more severe disease and worse clinical outcome (13, 43). Yellow fever virus (YFV) and St.
Louis Encephalitis Virus (SLEV) infection reduced IL-1β levels in LPS-stimulated MDMΦ compared to uninfected control cells (5). We found that MΦ derived during BVDV-2 infection generally have reduced inflammatory cytokine mRNA. Likewise, mRNA and protein secretion following LPS stimulation compared to uninfected cells was decreased in MDMΦ derived from BVDV-2-infected calves. However, MDMΦ derived late in infection from HV BVDV-2 infected calves produced enhanced IL-6 in response to LPS compared to cells from uninfected animals. A TH2 biased response has been implicated as a potentiating factor in the susceptibility to certain secondary pathogens observed in BRDC (35, 73). Given that IL-6 is a pleiomorphic cytokine which can contribute to TH2 responses, our observation of increased IL-6 from LPS-stimulated MDMΦ HV BVDV-2-infected calves may be one contributing factor to this bias. Together with previous studies (5, 13, 43), these results suggest that in vitro-derived terminally differentiated macrophages have a decreased proinflammatory cytokine mRNA profile and a reduced ability to respond to TLR4 ligation following infection with Flaviviruses.

Macrophages which reside in specific tissues of the host act as sentinels in the detection of invading pathogens (15). AMΦ are a terminally differentiated tissue macrophage resident to the alveolar spaces of the lung, which further play roles in lung homeostasis as well as tissue remodeling following lung damage (9). These cells respond to pathogens through production of proinflammatory cytokines (15) and are of interest to BVDV-2 infection in the lung as these cells will be one of the first innate immune cells to encounter the virus. One study has shown that in vivo BVDV-1 infection with in vitro restimulation with BVDV-1 of AMΦ greatly reduced normal function of these cells, with a decline in their ability to secrete neutrophil chemotactic factors, a downregulation of Fcg receptor and CR3
expression, and a reduction in phagocytic capability (75). Likewise, a study has demonstrated TLR4 responsiveness was decreased in Kupffer cells incubated with HCV viral proteins from HCV-infected patients as evidenced by a reduction in IL-1β secretion (29).

It is interesting to note that AMΦ isolated from BVDV-2-infected calves demonstrated a decrease in expression of IL-6 mRNA compared to cells isolated from control calves. On the other hand, our results indicate that AMΦ isolated from BVDV-2-infected calves are similar to AMΦ from control calves in their ability to secrete TNFa and IL-1b following LPS stimulation. It is of note that AMΦ isolated from LV BVDV-2-infected group had significantly decreased IL-6 secretion in response to LPS compared to AMΦ from uninfected controls. The implication of these observations is that proinflammatory cytokine production by AMΦ in response to LPS could play a role in increased susceptibility of the lung to secondary infection, as well as contribute to the pathogenesis resulting from these infections (4, 66, 77). As such, AMΦ-derived pro-inflammatory cytokines may be a contributing factor in BRDC.

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 of activation (20). These signaling pathways, with the exception of TLR 7/8, are critical to bacterial sensing. Worth noting is that there is a 2-3 fold decrease in expression of MyD88 in monocytes compared to matured MΦs, indicated that there are phenotypical changes occurring during culture of monocyte toward a MDMΦ (38). Similarly it has been shown that monocytes preferentially signal through TRIF mediated MyD88-independent signaling although a differentiated MΦ may be prone to MyD88-dependent signaling (65). 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. VV protein A46 interacts with MyD88 and Mal through electrostatic interactions blocking complex formation after activation of TLRs (45, 62). Likewise, it has been shown AMΦ from asymptomatic HIV+ individuals are impaired in their MyD88-dependent TLR4 cytokine response by blocking the MyD88-IRAK interaction, phosphorylation of IRAK and subsequent NFKβ translocation and proinflammatory cytokine secretion, which the authors suggested may be linked to increased bacterial pneumonia (64). Our studies have identified MyD88 as a target of BVDV-2 as MyD88 protein expression is decreased in LPS-stimulated myeloid cells isolated from the HV BVDV-2-infected groups. The greatest reduction in expression of MyD88 was in MDMΦ from BVDV-2-infected calves compared to MDMΦ from uninfected controls. Similarly, monocytes and AMΦ failed to upregulate MyD88 in response to LPS as observed in cells isolated from uninfected animals. TRIF was not significantly different between the myeloid cells isolated from the virally infected groups compared to the cells from uninfected controls. One explanation of how myeloid cells differ in responsiveness to LPS stimulation in induction of cytokine mRNA expression is through TRIF mediated MyD88-independent pathways (65). The data suggests that viral infections which lead to increased susceptibility to secondary bacterial infections may target TLR pathways or MyD88 adaptor molecules in myeloid cells (45, 62, 64).

NS5A is a polyprotein essential for BVDV replication as it is critical to the replicase complex and during viral replication NS5A can be found in abundance within an infected cell (8, 54). HCV and BVDV NS5A also share structural similarities such as a common essential zinc binding region (67) and association with cellular membranes via an amino-terminal helix (59). 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 (59). HCV and BVDV NS5A are also phosphorylated through cdc2 cyclin-dependent kinases (54, 79). These 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 \textit{in vitro} study (1, 18, 58). We have demonstrated that BVDV NS5A from an \textit{in vivo} infection interacts with MyD88. There have been no studies comparing sequences between the NS5A gene of varying BVDV-2 strains, although this may be a point in which genetic variability between the HV and LV viruses could effect expression of NS5A. However, it is plausible that BVDV-2 NS5A binding to MyD88 produces steric hindrance, inhibiting the interaction of this adapter protein with the IRAK and TRAF6 as described for VV (45, 62).

Downstream of the MyD88 adaptor protein are IKK complexes critical to the induction of transcription factors in the regulation of proinflammatory cytokine responses. After phosphorylation of the IKK complex, the inhibitor of NFκB, IκB (IκBα) is degraded, freeing NFκB to translocate into the nucleus of the cell and induce gene transcription. Previous research has demonstrated BVDV-1 can block IKKβ phosphorylation (80), which in turn inhibits transcription factor NFκB family members from becoming activated. Similarly, HCV has been shown to interfere with IKKβ kinase activity and IKK complex translocation via HCV core protein (32). Interestingly inhibition of IKKβ kinase activity in BVDV-1 (80) and HCV (53) increases viral replication through a mechanism yet to be elucidated. This suggests that IKKβ is an import factor in \textit{Flaviviral} pathogenesis. Furthermore, it has been observed that HCV-infected liver hepatocytes and DCs exhibit decreased NFκB activity (16, 33, 50, 63). We found that the expression of pIKKβ was reduced in MDMΦ from BVDV-2-
infected calves compared to cells from uninfected calves after LPS stimulation. This
depressed expression of pIKKβ in MDMΦ is similar to data from others showing a
downregulation of NFκB in BVDV-1-infected epithelial cells (11, 78, 80).

We demonstrated enhancement of proinflammatory cytokine mRNA transcription in
monocytes from BVDV-2-infected calves compared to control cells and in these cells
following LPS-stimulation. Conversely, we observed a deficiency in proinflammatory
cytokine production in MDMΦ from BVDV-2-infected calves compared to uninfected
MDMΦ and in their response to LPS-stimulation. Data from experiments with MDMΦ and
AMΦ show a differential response in macrophages derived in vitro compared to terminally
differentiated tissue macrophages. Interestingly, 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 BVDV-2 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.

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REFERENCES


**TABLE 1** Total cell counts of monocytes from experimental groups at days 4 and 12 post BVDV-2 infection. N=6 per group from two independent experiments

<table>
<thead>
<tr>
<th>Day 4 Total (Cells/μL)</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>SEM</th>
<th>Compared to Control</th>
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<tr>
<td>Control</td>
<td>1301</td>
<td>1259</td>
<td>476</td>
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<tr>
<td>HV 1373</td>
<td>987</td>
<td>678.8</td>
<td>240</td>
<td>p=0.5500</td>
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<tr>
<td>LV 28508</td>
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<td>1107</td>
<td>452</td>
<td>p=0.8538</td>
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<table>
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<th>Day 12 Total (Cells/μL)</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>SEM</th>
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<td>Control</td>
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<td>HV 1373</td>
<td>464</td>
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<td>p=0.0457</td>
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<tr>
<td>LV 28508</td>
<td>738</td>
<td>246.4</td>
<td>110</td>
<td>p=0.7847</td>
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</table>
**FIG 1** LPS-stimulated cytokine mRNA and protein expression in monocytes isolated at day 4 post BVDV-2 infection. (A and B) Cells were cultured in 96 well plates with or without LPS (1µg/mL) stimulation for 2 h or 24 h. 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 by SearchLight Array at 24 h post LPS stimulation. Data are means +/- SEM, n=6 per group from two independent experiments. * P < 0.05, ** P <0.001.
FIG 2 LPS-stimulated cytokine mRNA and protein expression from monocytes isolated at day 12 post BVDV-2 infection. (A and B) Cells were cultured in 96 well plates with or without LPS (1µg/mL) stimulation for 2 h or 24 h. 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 by SearchLight Array at 24 h post LPS stimulation. Data are means +/- SEM, n=6 per group from two independent experiments. * P < 0.05, ** P <0.001.
FIG 3 LPS-stimulated cytokine mRNA and protein expression from MDMΦ isolated at day 4 post BVDV-2 infection. (A and B) Cells were cultured in 96 well plates with or without LPS (1µg/mL) stimulation for 2 h or 24 h. 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 by SearchLight Array at 24 h post LPS stimulation. Data are means +/- SEM, n=6 per group from two independent experiments. * P < 0.05, ** P <0.001.
FIG 4 LPS-stimulated cytokine mRNA and protein expression from MDMΦ isolated at day 12 post BVDV-2 infection. (A and B) Cells were cultured in 96 well plates with or without LPS (1µg/mL) stimulation for 2 h or 24 h. 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 by SearchLight Array at 24 h post LPS stimulation. Data are means +/- SEM, n=6 per group from two independent experiments. * P < 0.05, ** P <0.001.
FIG 5 Cytokine mRNA and protein expression from AMΦ isolated from calves infected with BVDV-2. AMΦ were isolated from lung lavage of control or BVDV-2-infected calves upon time of necropsy (day 14). Cells were cultured with LPS (1µg/mL) or cell culture medium for 2 or 24 h. Cytokine mRNA (A and B) was measured by real time PCR and bar graphs represent mRNA expression relative to uninfected AMΦ in medium only after normalization to RPS9. (C) Cell supernatants were collected at 24 h post LPS and assayed for cytokine protein by Searchlight array. Data are means +/- SEM, n=6 per group from two independent experiments. * P < 0.05, ** P <0.001.
FIG 6 Summary of mRNA and protein expression data from myeloid cells isolated from BVDV-2 infected calves at day 12 post infection. Overall, monocytes from BVDV-2-infected calves exhibited increased proinflammatory cytokine mRNA expression compared to monocytes from control animals and enhanced mRNA and protein after LPS stimulation. MDMΦ isolated from BVDV-2-infected calves exhibited a decrease in cytokine mRNA expression prior to LPS stimulation and decreased cytokine mRNA and protein expression after LPS stimulation. AMΦ from BVDV-2 infected calves displayed a decrease in IL-6 expression compared to cells from control calves prior to LPS stimulation. There was an increase in cytokine mRNA, but no change in protein secretion after LPS stimulation except for a decrease in IL-6. ↑ indicates an increase in expression compared to uninfected control cells, ↓ indicates a decrease in expression compared to uninfected control cells, N/C indicates no change from control.
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<td>N/C</td>
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<td>N/C</td>
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<td>N/C</td>
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<td>↑</td>
<td>N/C</td>
<td>↓</td>
<td>N/C</td>
<td>↓</td>
<td>N/C</td>
</tr>
<tr>
<td>+ LPS</td>
<td>↑</td>
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|                  | ↑            | ↑       | ↑         | ↑       | ↑        | ↑       |
FIG 7 BVDV infection modulates the MyD88-dependent but not the MyD88-independent TRIF pathway in response to LPS. (A) Monocytes were isolated from peripheral blood of control or BVDV-2-infected calves day 12 post infection. Cells were cultured LPS (1µg/mL) or cell culture medium alone for 24 h. Lysates were harvested and 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) MDMΦ were isolated from peripheral blood of control or BVDV-2-infected calves day 12 post infection. After maturation, MDMΦs were cultured with LPS (1µg/mL) or cell culture medium for 24 hours. (C) AMΦ were isolated from lung lavage of control or BVDV-2 infected calves upon time of necropsy (day 14). AMΦs were cultured with LPS (1µg/mL) or cell culture medium for 24 h. Data are means +/- SEM, n=3 from one experiment.
**FIG 8** BVDV NS5A co-localizes with MyD88 with or without LPS stimulation. MDMΦ were isolated from peripheral blood of control or BVDV-2-infected calves day 12 post infection. Cells were stimulated with LPS (1µg/mL) or cell culture medium for 24 hours. Lysates were harvested and immunoprecipitated with beads coupled to anti-BVDV NS5A hyperimmunized serum. IPs were blotted with anti-MyD88 (A). The negative fraction from the flow through of the IP was blotted for MyD88 (B).
**FIG 9** Phosphorylation of IKKβ in response to LPS stimulation is reduced in myeloid lineage cells isolated from calves infected with a HV BVDV-2 strain. (A) Monocytes were isolated from peripheral blood of control or BVDV-2-infected calves day 12 post infection. Cells were cultured with LPS (1µg/mL) or cell culture medium alone for 24 h. Lysates were harvested and immunoblotted with antibodies against phosphorylated (phospho-) IKKβ, total IKKβ, or β-actin. Densitometry scans of phospho-IKKβ relative to total IKK are shown in the bar graphs. (B) MDMΦ were isolated from peripheral blood of control or BVDV-2-infected calves day 12 post infection. After maturation, MDMΦs were cultured with LPS (1µg/mL) or cell culture medium for 24 hours. (C) AMΦ were isolated from lung lavage of control or BVDV infected calves upon time of necropsy (day 14). AMΦs were cultured with 1µg/mL of LPS or cell culture medium for 24 h. Data are means +/- SEM, n=3 per group from one experiment.
ABSTRACT

*Bovine viral diarrhea virus* (BVDV) is a member of the *Flaviviridae* family and infection may induce severe acute disease followed by immunosuppression. As an immune suppressed condition may lead to enhanced susceptibility to secondary infection, it is possible that macrophages, which are critical in responding to pathogens, may be altered in their ability to respond via innate pathogen recognition receptors (PRRs). BVDV-2 has varying biotypes of virulence and cytopathogenicity, which may play a correlative role in modulating the immune response to PRR stimulation. As such, comparison of isogenic BVDV-2 cytopathic/noncytopathic strains can help to elucidate how cytopathogenicity could affect macrophage response. The current study demonstrates that infection of bovine monocyte-derived macrophages (MDMΦ) with cytopathic, noncytopathic, high and low virulence strains of BVDV-2 result in differential expression of pro-inflammatory cytokine mRNA compared to uninfected MDMΦ. However, there was not a significant increase in protein secretion by macrophages in response to BVDV-2 infection, except that cytopathic

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(cp)BVDV-2 induced IL-6. Furthermore after MDMΦ BVDV-2 infection, cytokine secretion in response to toll-like receptor (TLR) 2 or TLR4 stimulation was reduced compared to non-infected MDMΦ. Similarly, macrophages were hyporesponsive to viral TLR3 or TLR8 ligation. Instead, TLR7 stimulation of BVDV-2 infected MDMΦ induced JNK1/2 phosphorylation and a unique isoform p46 was observed. Together, these data suggest that BVDV-2 infection generally suppressed proinflammatory cytokine protein responses to bacterial sensing TLRs in MDMΦ. However infected MDMΦ responded to TLR7 and this may be an important pathway in viral infection.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a single stranded, positive sense RNA virus of the *Pestivirus* genus, in the *Flaviviridae* family. As such, it shares many similarities to related viruses such as *hepatitis C virus* (HCV) [1]. Infection with BVDV can range from mild to severe acute disease, which can be fatal. However after recovery, BVDV infection may predispose the host to lifelong susceptibility to secondary bacterial or viral infection, of which the mechanisms of immune modulation are unknown [2]. In cattle, BVDV is a contributing factor to bovine respiratory disease complex (BRDC), and may contribute secondary infection by *Mannheimia haemolytica*, or *Pasteurella multocida* and co-infection of viral agents such as *bovine respiratory syncytial virus* (bRSV), *bovine parainfluenza virus* (boPI-3) and *bovine herpes virus 1* (boHV-1) [3-6]. Thus, there is a need to understand the underlying mechanisms of disease and immune modulation to better develop anti-viral efforts toward BVDV and related *Flaviviridae* members.
BVDV can be categorized into genotypes BVDV-1 and BVDV-2 as determined by genetic analysis [7]. BVDV-2 is unique as it can be highly virulent in vivo and as such can be classified as atypical/high virulence (HV) or typical/low virulence (LV) with disease severity correlating to virulence [8]. Cytopathogenicity is an in vitro and genetic trait of BVDV in which cp strains produce 12 functional proteins including NS2 and NS3; however non-cytopathic (ncp) strains produce 11 genes in which NS2/3 is produced as one whole protein [9]. Interestingly cytopathogenicity does not always correlate with disease severity and almost all BVDV infections involve ncp strains [10]. Isogenic strains of cytopathic and non-cytopathic BVDV can be used to elucidate differences in immune response to genetic variation within the NS2/3 coding region. Furthermore, BVDV-2 has not been studied as well as BVDV-1 strains and potential differences in immune modulation between strains of differing virulence or cytopathogenicity is unknown.

Monocytes and monocyte-derived macrophages (MDMΦ) are essential cells for the innate immune response to pathogens. MDMΦ can recognize conserved molecules or components of microorganisms termed pathogen associated molecular patterns (PAMPs) [11]. PAMPs are recognized by pattern recognition receptors (PRRs), which will initiate proinflammatory and anti-viral immune responses. More specifically, Toll-like receptors (TLRs) are a class of PRRs, which are critical to viral and bacterial sensing. As TLRs are conserved over mammalian species, pathogens have developed similar ways to thwart immune recognition by these receptors [12-14], which in turn lead to a more successful infection with respect to the pathogen.

The initiation of the immune response, after recognition by TLRs, is characterized by cytokine secretion. MDMΦ are one of the critical cells that secrete IL-1β, TNFα and IL-6.
These pro-inflammatory cytokines have been shown to have an anti-viral role [13, 15-17] and as such the secretion and pathways involved in induction of these cytokines are targets of pathogens. BVDV-1 has been shown to downregulate pro-inflammatory cytokines in vitro however the cytokine response to BVDV-2 strains is unknown.

Induction of these pro-inflammatory cytokines are dependant on phosphorylation of signaling complexes such as mitogen-activated protein kinase p38 (p38 MAPK), c-Jun N-terminal kinases p46 and p54 (JNK1/2) and extracellular-signal-regulated kinases (ERK1/2) [18]. Blocking phosphorylation of these protein complexes are one way in which pathogens disrupt the signaling cascade induced by TLRs prior to cytokine transcription [19]. Interestingly, p38 MAPK phosphorylation is greatly induced in epithelial cells inoculation with cpBVDV-2 strains in vitro [20], however the other pathways have not been fully explored.

To date, there have been no comparative studies of BVDV-2 strains of cytopathogenicity or virulence. BVDV-2 strains 296c and 296nc are isogenic except for differences between the coding region of NS2/3 and allowed for the first reported study of differences in cytokine responses in MDMΦ to these strains. Likewise, there have been no comparative studies of the effects of any BVDV strains on TLR responsiveness across a multitude of TLRs. As such, TLRs are essential in recognition of both viral and bacterial pathogens and taken together with BVDVs effect of predisposition to secondary infection, it may be possible that these TLRs are severly disrupted in their function in an infection setting. In this study we investigated the effects of BVDV-2 infection of MDMΦ on cytokine expression prior to and after TLR stimulation and to the extent by which phosphorylation of JNK may play a roll in TLR pathway signaling.
MATERIALS AND METHODS

Animals

12 clinically healthy Holsteins of approximately 1-2 years of age were used for blood donors. Animals were negative for BVDV as measured by an immunohistochemistry (IHC) positive ear notch. Animal procedures employed in these studies were approved by the National Animal Disease Center Institutional Animal Care and Use Committee.

Monocyte Derived Macrophage Culture

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), 2mM 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 5x10^6 cells per mL. Monocytes were plated in 96-well round bottom tissue culture plates in 100 µL medium. Monocytes were cultured for 7 days with media change every 2-3 days to derive ΜΦ. Cells isolated from animals were tested for BVDV through reverse transcription (RT)-PCR with primers selective for BVDV genotypes 1a, 1b, and 2 and were negative prior to experimentation.
**Viral inoculum and TLR agonists**

BVDV-2 strains were propagated in MDBK cells with a viral titre determined by histological staining as follows: 296c [TCID\textsubscript{50} 6.8x10\textsuperscript{6}], 296nc [TCID\textsubscript{50} 3.8x10\textsuperscript{8}], 1373 [TCID\textsubscript{50} 6.22x10\textsuperscript{6}], 28508 [TCID\textsubscript{50} 2.37x10\textsuperscript{6}]. Cells were inoculated with an MOI of 1. For studies with TLR agonists, cells were inoculated with BVDV-2 strains for 48 h prior to TLR treatments. TLR agonists were used at the following concentrations: Pam3Cys [5µg/mL] (Invivogen, San Diego, CA), *Mannheimia haemolytica* LPS [10µg/mL] (produced in house), *Escherichia coli* LPS [1µg/mL] (055:B5 Sigma, St. Louis, MO), Poly I:C [50µg/mL, Sigma], Imiquimod [10µg/mL] (Invivogen), ssRNA40 LyoVec [10µg/mL] (Invivogen). Cell viability was measured by trypan blue exclusion and acridine orange staining prior to and after virus and TLR treatment.

**RNA extraction, cDNA synthesis and qPCR**

At 2, 6, 18, and 24 h post BVDV inoculation, 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) and genomic DNA was removed during RNA isolation using an on-column RNase-Free DNase I digestion set (Qiagen) per to manufacturer’s instructions. 500 ng of total RNA from each sample was reverse transcribed using random primers/hexamers and Superscript III (Invitrogen) to generate first strand cDNA. 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 [21]. Primer set sequences
are indicated in Table 1. 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 fluorescence 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 $2^{-\Delta\Delta\text{CT}}$ method [22].

**Searchlight Cytokine Multiplex Assay**

50 µL of macrophage (MΦ) supernatants or cytokine standards were incubated for 3 h in duplicate in 96 well, immobilized antibody 4-plex array plates (SearchLight, Aushon Biosystems, Billerica, MA). Wells of each plate were commercially coated with antibodies specific for bovine interleukin (IL)-1β, 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-1β=1.9, TNFα=2.4, IL-6=0.9, and IFNγ=0.5.

**Western Blots**

50 µL of lysis buffer (BD Pharmingen) with Halt Protease/Phosphatase and 0.5M EDTA (Thermo Scientific) was added directly to wells at specified timepoints and either analyzed immediately or stored at -80°C until analysis. For western blot analyses, lysates were
quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and standardized to 10µg 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 (20uL) and Benchmark Prestained Protein Ladder (Invitrogen) were loaded into a Novex Tris-Glycine 1.0mm 15 well gels (Invitrogen) and electrophoresed until distinct ladder separation had occurred. Gels were transferred to nitrocellulose using an iBlot transfer system (Invitrogen) 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-JNK, Anti-phospho JNK (Thr183/Tyr185), or 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. The blot was washed in Tris Buffered Saline containing 0.1% Tween-20 (TBST). HRP-secondary (anti-species 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, Bethesda MD). Restore Plus Buffer (Thermo Scientific) was used according to manufacturers instructions to strip blots prior to re-blotting. Western blot data were normalized to β-actin or JNK for semi-quantification using densitometry (ImageJ).
Statistical Analysis

Statistical Analysis. qPCR cytokine data was analyzed with the outcome variable ($2^{-\Delta\Delta CT}$) log transformed. $\Delta\Delta Ct$ values were analyzed using one-way ANOVA (Prism, GraphPad, LaJolla, CA). Bonferroni post test was used to compare replicate means by row to uninfected controls. Cytokine protein data was analyzed using one-way ANOVA (Prism, GraphPad). Results are expressed as means +/- standard errors of the means (SEM).

RESULTS

Cytopathic BVDV-2 induces higher levels of proinflammatory cytokine mRNA in bovine MDMΦ than a noncytopathic BVDV-2 of matched isogenic type

296c is a cp strain of BVDV-2 which is genetically identical to 296nc except for changes in the open reading frame of the NS2/3 gene allowing for both NS2 and NS3 to be expressed during 296c infections, whereas an NS2/3 read-through product is produced in 296nc [20]. The effects of these isogenic strains on MDMΦ cytokine expression have not been examined. Bovine MDMΦs were differentiated for 7 days in 96 well plates and inoculated with an MOI of 1 for each strain with collections of subsequent time points of 2h, 6h, 18h, and 24h post-inoculation collected for RNA isolation. Cytokines measured displayed similar kinetics throughout the duration of experiments with an initial induction of message followed by another induction of message at 18h post BVDV-2 inoculation (Fig. 1). IL-1β (Fig. 1A), IL-6 (Fig. 1C) and IL-12p40 (Fig. 1E) were induced to a greater extent in MDMΦs inoculated with 296c compared to 296nc at each time point measured. TNFα (Fig. 1B) and IL-10 (Fig. 1F) were induced to a greater extent in 296c inoculated MDMΦs compared to 296nc.
inoculation except for 6 h post treatment. 296c inoculated MDMΦs exhibited a greater amount of IL-8 (Fig. 1D) than 296nc inoculated MDMΦs except for 24 h post treatment.

Thus, MDMΦs inoculated with 296c exhibited greatly enhanced levels of proinflammatory cytokines. In contrast, 296nc inoculation of MDMΦs only modestly induced proinflammatory cytokines and was generally significantly (P<0.0001) less than the cytokines induced by 296c.

**High virulence BVDV-2 infection of MDMΦs induced greater proinflammatory cytokine mRNA responses compared to a low virulence strain**

BVDV-2 strains demonstrate vast differences in virulence *in vivo* and can be classified as either high/atypical or low/typical virulence strains [8]. BVDV-2 strain 1373 is a high virulence (HV) strain which causes severe acute disease in cattle; whereas strain 28508-5 is a low/typical (LV) BVDV-2 strain which causes mild to no clinical disease. Differences between virulence strains and the effects on cytokine expression in macrophages have not been explored *in vitro*, and we set forth to determine differences in proinflammatory cytokine expression which may be induced to varying levels depending on virulence. Interestingly, we found that HV 1373 induced greatly enhanced proinflammatory cytokine expression in inoculated cells compared to LV 28508 (Fig. 2). However, HV 1373 induced an initial mRNA expression of cytokines in MDMΦs at 6h post inoculation as opposed to the other BVDV-2 strains in which cytokine expression was beginning to be enhanced at 2 h post inoculation (Fig. 1&2). IL-1β (Fig. 2A), TNFα (Fig. 2B), IL-6 (Fig. 2C), IL-8 (Fig. 2D), and IL-12p40 (Fig2E) were enhanced to a greater extent in MDMΦs inoculated with HV 1373 at 6, 18, 24 h post inoculation compared to LV 28508 inoculated MDMΦs. Additionally, IL-10
(Fig. 2F) was increased in MDMΦs inoculated with HV 1373 at 18 and 24 h post inoculation compared to LV 28508 inoculated cells.

Overall, HV 1373 inoculated MDMΦs demonstrated a greater level of proinflammatory cytokine expression starting at 6h post inoculation compared to LV 28508 inoculated cells. Interestingly, HV 1373 did not induce a proinflammatory cytokine response at 2 h post inoculation compared to the other strains of BVDV-2 we used in our experiments.

**BVDV-2 strain 296c induced IL-6 secretion in infected MDMΦs, whereas all other strains did not induce secretion of proinflammatory cytokines**

Most studies of BVDV focus on mRNA expression in response to viral inoculation *in vitro* and are limited in scope of protein expression. To date, there have been no comparative studies of BVDV-2 strains *in vitro* in which cytokine protein is explored. As we observed an induction in cytokine mRNA, we investigated the protein secretion of MDMΦs in response to 296c, 296nc, 1373 and 28508. Interestingly, each of the viral strains did not induce cytokine secretion, except for 296c (Fig. 3). LPS was used as a positive control, and IFN-γ (Fig 3A), IL-1β (Fig. 3B), and TNFα (Fig. 3D) was not increased in the viral treated groups compared to control. IL-6 (Fig. 3C) was increased only in the 296c treated MDMΦs compared to both LPS stimulated and viral inoculated cells.

These results suggest that there is a disconnect between mRNA expression and cytokine secretion in BVDV-2 inoculated MDMΦs. It is noteworthy that although 296c did induce IL-6 secretion, which mirrors gene transcription, IL-1β and TNFα were not induced. Furthermore, it is possible that the cytopathic nature of 296c may be a factor in the induction
of IL-6. Additionally, later time points of 48 h and 72 h post inoculation did not yield any differences in expression of secreted cytokine protein (not shown).

**BVDV-2 infected MDMΦs stimulated with bacterial TLR agonists exhibited reduced proinflammatory cytokine secretions**

With BVDV-2 stimulation alone, most of the BVDV-2 strains we tested did not induce proinflammatory cytokine secretion. It would be interesting if this observation indicates a decrease in cytokine protein secretion in response to bacterial TLRs, as bacterial secondary infection can be common with BVDV-2 infection *in vivo*. Few studies have investigated *E. coli* LPS responsiveness in BVDV-1 inoculated MΦ derived *in vitro* [23-25] and there have been no studies comparing varying strain of BVDV-2 to multiple types of bacterial TLRs. Interestingly, TNFα protein secretion was decreased in viral inoculated MDMΦs in response to TLR stimulation compared to TLR-stimulated uninfected controls (**Fig. 4**). In MDMΦs inoculated with 296c or 296nc, there were no dramatically different levels of suppression between the groups (**Fig. 4A**). Noteworthy is that both *E. coli* and *M. haemolytica* LPS stimulation of MDMΦs inoculated with 296c or 296nc exhibited less TNFα protein secretion than ones stimulated with Pam3Cys. Similarly, MDMΦs inoculated with 1373 or 28508, there were no statistically different levels of suppression between the inoculated cells (**Fig. 4B**). Interestingly, *M. haemolytica* LPS stimulation was not as suppressed in BVDV-2 inoculated 1373 or 28508 MDMΦs as the 296c or 296nc inoculated cells.

In general, virulence levels and cytopathogenicity do not necessarily impact the level of suppression in BVDV-2 inoculated MDMΦs stimulated with bacterial TLRs, and thus are overall diminished in their cytokine response to bacterial TLR ligation. Similarly, IL-1β,
IFNγ and to some extent IL-6 were similar in the reduced levels of secreted cytokine from BVDV-2 inoculated and stimulated cells (Supplemental Fig. 1 & 2).

**BVDV-2 infected MDMΦs respond to TLR7 ligation; however other viral TLR agonists failed to induce similar cytokine responses**

As we observed a decrease in secreted cytokine protein expression from BVDV-2 inoculated MDMΦs in response to bacterial TLRs, it would be interesting to explore TLRs specific to viral PAMPs. Similarly as there is an increase in susceptibility to bacterial co-infection, BVDV infection *in vivo* does predispose an animal to secondary viral infection as well, and potentially a deficit in viral TLR responsiveness may contribute to this observation. Likewise, there have been no comparative studies of BVDV-2 strains and the effects of various viral TLRs *in vitro*. Interestingly TNFα protein was not suppressed in BVDV-2 inoculated MDMΦs in response to TLR7; however both TLR3 and TLR8 were suppressed in their responses ([Fig. 5](#)). There were no statistically different levels of suppression among TNFα expression in either cytopathic 296c or non-cytopathic 296nc BVDV-2-inoculated MDMΦs ([Fig. 5A](#)). Similarly, there were no differences in the amount of suppression of TNFα protein secretion between the MDMΦs inoculated with either HV 1373 or LV 28508 ([Fig. 5B](#)) and stimulated with TLR agonists; however TLR7 agonists were able to induce a response in each of the viral infected groups, with each of them statistically similar to agonist stimulation alone.

Similar to findings with bacterial TLR agonists, cytopathogenicity or virulence does not effect the level of suppression on BVDV-2 inoculated MDMΦs stimulated with viral TLR agonists and in which TLR3 and TLR8 were both diminished in their ability to respond
in virally inoculated cells. Likewise, IL-1β, IFNγ and IL-6 from virally inoculated cells with TLR3 and TL8 stimulation demonstrated a reduction in cytokine secretion (Supplemental Fig. 1 & 2). As our lab has found that MyD88 expression in vivo is modulated by BVDV-2 infection, there was a similar reduction in expression of MyD88 from BVDV-2 inoculated MDMΦs, which corresponds to the reduced TLR responsiveness (Supplemental Fig. 3). However, there were no differences in gene expression of TLRs with viral inoculation, indicating that viral modifications of TLR responsiveness is not necessarily being modulated at the level of gene expression (not shown).

**BVDV-2 infected MDMΦs response to TLR7 involves phosphorylation of JNK**

Previous research has suggested that although TLR7 and TLR8 demonstrate overlap in function, they can play distinct roles in anti-viral responses. TLR7 has been hypothesized to be JNK-dependent in its downstream signaling whereas TLR8 will be p38 MAPK-dependent. We investigated the aspect of JNK phosphorylation after TLR7 or TLR8 stimulation to elucidate the functionality of these TLRs in a BVDV-2 in vitro infection setting. TLR7 stimulation after BVDV-2 inoculation induced phosphorylation of p46 and p54 JNK with a unique isoform observed at p46 as opposed to TLR8 stimulation (Fig. 6). In MDMΦs uninfected with BVDV-2 and with TLR7 stimulation (Fig. 6A), there was an induction and increase in phosphorylation of p54 and p46 JNK over time beginning at 10 min post TLR7 stimulation and peaking at 120 min. Similarly, uninfected cells with TLR8 stimulation also induced phosphorylation of p46 JNK, although the p54 isoform was not phosphorylated to the same extent (Fig. 6B). Interestingly, the phosphorylation of p46 JNK after TLR8 stimulation was greater than TLR7 stimulation in the uninfected MDMΦs (Fig. 6C). After
BVDV-2 inoculation of 28508 and stimulation with TLR7 agonists, phosphorylation of p46 and p54 JNK was induced and increased over time, similar to uninfected cells; however a unique isoform was observed at p46 (Fig. 6D). After inoculation with 28508 and stimulation with TLR8 agonist, there was an induction of p46 JNK phosphorylation and there was no induction of p54 phosphorylation (Fig. 6E). The isoform observed with TLR7 stimulation and BVDV-2 inoculation was not present in the TLR8 stimulated cells (Fig. 6D and E). Additionally phosphorylation of p46 from the BVDV-2 inoculated MDMΦs with TLR8 stimulation was not as great at the TLR7 and BVDV-2 inoculated cells (Fig. 6F).

Likewise 296c- (Supplemental Fig. 4A-C), 296nc- (Supplemental Fig. 4D-F) or 1373- (Supplemental Fig. 4G-I) inoculated and TLR7 stimulated MDMΦs displayed similar phosphorylation and induction of a unique p46 isoform which was not observed with TLR8 stimulation. Thus, MDMΦs inoculated with BVDV-2 strains can respond to TLR7 ligation via phosphorylation of JNK p46 and p54 with a unique isoform present at p46, a phenomenon not observed with TLR8 ligation. It is interesting to note that p38 MAPK phosphorylation was induced by 296c only, 296c with either TLR7 or TLR8 ligation, and with TLR8 ligation alone; however other viral strains did not induce p38 phosphorylation and did not with TLR ligation (not shown).

**DISCUSSION**

This study demonstrates that a *Flaviviridae* infection of MDMΦ induces robust proinflammatory cytokine mRNA responses, which are dependent on cytopathogenicity and virulence of the viral strains. Conversely, there is no enhancement of TNFα, IL-1β, IFNγ or IL-6 protein secretion. Importantly, response to secondary bacterial or viral TLR stimuli
after *Flaviviridae* infection is generally reduced compared to uninfected cells. However, TLR7 signaling after BVDV infection is still intact; this response appears to be dependent on JNK phosphorylation. Furthermore, we observed phosphorylation of a unique isoform of p46 JNK.

Previous research has outlined some potential mechanisms of immune modulation in a BVDV infected host. For instance decreased lymphocyte proliferation [26], decreased chemotaxis and inhibition of leukotriene formation in monocytes [27, 28], decreased microbialic activity and random migration of neutrophils [29, 30], decreased expression of complement, Fc receptors and chemokine production of alveolar macrophages [31] each suggest a broad modulation within an infected host which can contribute to immunosuppression. Likewise, it has been suggested that monocytes or macrophage cells are critical in BVDV infection for disseminating virus [32, 33] and initiating apoptosis of T cells during infection [34, 35]. Interestingly, severe lymphopenia is observed in infected animals by means of an unknown mechanism; however monocytes are not reduced in the periphery [8, 36, 37]. This suggests that monocytes or macrophages may play a critical role in recovery from BVDV infection, as they are not depleted throughout the course of infection.

This is the first study to compare differences between isogenic or varying virulence strains of BVDV-2 and the impact on infected MDMΦ cytokine mRNA responses. It is noteworthy that we saw a robust cytokine mRNA response, which was more pronounced in the cp and hv BVDV-2 strains (Fig. 1 & 2). Of note, the work of others have demonstrated that TNFα mRNA transcription is enhanced in PBMCs infected with cp BVDV-1 strains, however comparison to non-cytopathic strains was not examined [38]. Similarly, persistently infected calves with either a cp or ncp BVDV-1 strain demonstrated enhanced TNFα
expression in cells isolated from the spleen compared to uninfected calves [39]. TNFα expression can lead to the induction of apoptosis and it should be considered a potential mediating factor in the cytopathic effect induced in epithelial cells by cytopathic strains of BVDV [38]. This suggests that cp strains of BVDV-2 would indeed induce greater proinflammatory cytokine responses and concurs with our finding in expression of mRNA from infected MDMΦ. Differences between induction of proinflammatory cytokines in MDMΦ infected with high or low virulence strains of BVDV-2 has not been explored; however our finding suggest that hv strains of BVDV-2 induce a more robust mRNA response (Fig. 2). Interestingly, this may correlate to disease severity as a greater amount of proinflammatory cytokines may be a contributing factor of host disease during BVDV infection [40] or severe Flaviviral infections in general [41-45], albeit we did not observe cytokine secretion. Noteworthy is the observation that hvBVDV-2 strains 1373 induced proinflammatory cytokines beginning at 6 h post inoculation as opposed to the MDMΦ response to the other strains which occurred beginning at 2 h post inoculation. As this is the first published observations of MDMΦ responses in vitro to hv BVDV-2 strain 1373, is it possible that this particular strain of BVDV-2 is unique compared to the other strains we studied, as it is either not recognized as early by the MDMΦ to induce cytokine mRNA at 2h or is recognized by differing innate mechanisms.

To date, expression of cytokine protein responses to BVDV-2 strains of varying virulence or isogenic cp/ncp strains have not been reported. Surprisingly, although BVDV-2 strains each induced cytokine mRNA, secreted protein was not significantly induced. However, the cytopathic 296c BVDV-2 strain did induce IL-6 which can help to induce apoptosis under certain conditions [46]. Similar to our finding, ncpBVDV-1 strains have
been shown to decrease intracellular protein expression of IL-1β and TNFα [47]. Not surprisingly, another Flaviviridae member, dengue virus demonstrated reduced expression of proinflammatory cytokines IL-1β, IL-8 and TNFα in whole blood from infected children; however this decrease in expression correlated to heightened clinical disease severity [48]. Overall, our research and the finding of others suggest that BVDV does limit cytokine protein secretion in infected cells.

As such, we demonstrated a disconnect between mRNA expression and cytokine protein production in each of the BVDV-2 inoculated MDMΦ. Previous studies have demonstrated a disassociation between mRNA expression and protein production [24, [49-51]. However, some difference between mRNA expression and protein production may be related to cellular physiological functions as post-translational modification that inhibit protein production [52, 53], sequestering of protein which block secretion [54] or production of inactive forms of cytokines which require secondary mechanisms for activation and secretion [55]. Nonetheless, it should be noted that the dissociation between cytokine mRNA expression and protein secretion may be a trait of BVDV-2 pathogenesis.

This is the only study to date in which cytokine responses of BVDV-2-infected MDMΦ to multiple TLR agonists have been explored. However, a previous study has demonstrated that bone marrow derived macrophages (BMMΦ) infected with BVDV-1 in vitro have been shown to secrete less TNFα protein in response to LPS or bacterial infection compared to uninfected BMMΦs [25]. Similarly, TLR4 hyporesponsiveness has been demonstrated in MDMΦ from chronically infected HCV patients whereas this observation correlated to more severe disease and worse clinical outcome [56, 57]. Likewise, Flaviviral infections including yellow fever virus (YFV) or St. Louis encephalitis virus (SLEV), reduced
IL-1\(\beta\) levels in LPS-stimulated MDM\(\Phi\) compared to uninfected control cells [58]. Similar to these findings, we demonstrated that varying TLR4 agonists as well as TLR2/1 agonists are diminished in their ability to promote pro-inflammatory cytokine secretion in MDM\(\Phi\) after BVDV-2 infection. Interestingly, each biotype suppressed MDM\(\Phi\) cytokine secretion in response to bacterial TLR stimulation.

As with bacterial TLR ligation, there is evidence that BVDV infection can decrease responsiveness to a double-stranded (ds)-RNA viral recognition receptor, TLR3 [47, 59]. The dampened TLR3 response can be attributed to the BVDV ubiquination and degradation of interferon regulatory factor (IRF)-3 directly downstream of TLR3 activation by means of interaction with Npro protein of BVDV [60-62]. Thus, we demonstrated a reduction in viral TLR responsiveness in MDM\(\Phi\) evidenced by a reduction in cytokine secretion by both TLR3 and TLR8 stimulation after BVDV-2 infection (Fig. 5). Intriguingly, TLR7 ligation was not dampened after BVDV-2 infection and was the only TLR tested that was statistically similar or enhanced in cytokine secretion compared to uninfected control cells. These findings along with the findings from this study of bacterial and viral TLR ligation (Fig. 4 & 5), demonstrate that there is a TLR hyporesponsivness induced by Flaviviradae including BVDV infection.

MyD88 is an adapter protein essential for IL-1 receptor and various TLR signaling [63]. Research in our lab has demonstrated BVDV-2 in vivo infection can modulate MyD88 expression in M\(\Phi\) (manuscript under review) and as such we did observe a decrease in expression of MyD88 from infected MDM\(\Phi\) (Supplemental Fig. 3). However, MyD88 expression was not completely ablated and some TLRs signal through complexes independent of MyD88 [64]. As such, viral means of manipulation of the TLR responses may not only be dependent on a reduction in MyD88 expression.
Furthermore, there have been no studies exploring JNK phosphorylation in response to BVDV-2 infection in MDMΦ. This is the first to demonstrate a difference between TLR7 or TLR8 stimulation and phosphorylation of p46 JNK isoforms in BVDV infected MDMΦ. TLR7 and TLR8 recognize similar viral components of single stranded (ss)-RNA and overlap in function. However, differences between the two TLR pathways are beginning to be explored. TLR7 tends to strongly phosphorylate JNK [65, 66] whereas TLR8 will phosphorylate p38 MAPK to a greater extent [65, 66]. Likewise, plasmacytoid DCs and B cells preferentially signal through TLR7 [67] whereas monocytes signal through TLR8 [68]; although contain both TLRs. TLR7 is believed to activate interferon regulatory factor (IRF) 3, 5 & 7 complexes and potentially mediate activation of cellular apoptosis [69, 70]. Recently, it is suggested that TLR7 may activate MyD88-independent pathways as some of these features of IRF activation as well as type-I interferon induction are homologous to TRIF signaling pathways [71]; however multiple studies have demonstrated reduced functionality of TLR7 with MyD88 knockout models [72-74]. Interestingly, JNK isoforms have been shown to be dependent on cell type although differences in functions of these isotypes are unknown [75-77]. Our study demonstrated that both TLR7 and TLR8 stimulation with BVDV infected did indeed induce phosphorylation of JNK 1/2 isoforms; although TLR7 stimulation phosphorylated JNK to a greater extent (Fig. 6 & Supplemental Fig. 4). Surprisingly, TLR7 stimulation in BVDV-2 infected MDMΦ produced a unique isoform at p46 not observed with TLR8 stimulation or in uninfected cells. This is the first study in which this isoform was observed in cattle, and as TLR7 functionality was not decreased with BVDV-2 infection is may be that the isoforms observed at p46 may be contributing to the observed cytokine response.
Taken together, the findings in this study demonstrate that the BVDV-2 strains do vary in their induction of cytokine mRNA response from MDMΦ. Although a robust mRNA response was observed, protein secretion was not. In addition, subsequent TLR stimulation of BVDV-2-infected MDMΦ resulted in a hyporesponsive cytokine response compared to uninfected MDMΦ. However, TLR7 ligation induced cytokine responses in infected MDMΦ. Furthermore, the cytokine response of TLR7 ligation involves JNK phosphorylation and phosphorylation of a unique p46 isoform. Interestingly, MDMΦ infected with isogenic strains of cp/ncp BVDV-2 did not differ in suppression of TLR stimulated cytokine responses, indicating that NS2, NS3 or NS2/3 may not be an explanation of this observation; however IL-6 secretion may be mediated by NS2 or NS3. These results demonstrate that BVDV-2 infection generally blocks TLR responsiveness and provide information on how this virus may contribute to susceptibility to secondary infection.

ACKNOWLEDGMENTS

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REFERENCES


by plasmacytoid dendritic cells is impaired in hepatitis C virus infection. *J. Immunol.* **178**, 4436-44.


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F=forward; R=reverse.
Figure 1. Expression of proinflammatory cytokine gene transcription in MDMΦs inoculated with cytopathic and non-cytopathic BVDV-2 strains. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains in duplicate at an MOI of 1 with RNA harvested at 2, 6, 18, and 24 h after inoculation. Cytokine mRNA was analyzed by qPCR using ribosomal protein s9 (RPS9) as an endogenous control with fold change expressed relative to uninfected control MDMΦs harvested at the corresponding time points. IL-1β (A), TNFα (B), IL-6 (C), IL-8 (D), IL-12p40 (E), IL-10 (F) were measured using primer sets specific for bovine genes using SYRB Green chemistry. Bars represent the mean value ± SEM from four different experiments from 9 different donor cattle. *** $P < 0.001$; ** $P < 0.005$. 
Figure 2. Expression of proinflammatory cytokine gene transcription in MDMΦs inoculated with high and low virulence BVDV-2 strains. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains in duplicate at an MOI of 1 with RNA harvested at 2, 6, 18, and 24 h after inoculation. Cytokine mRNA was analyzed by qPCR using ribosomal protein s9 (RPS9) as an endogenous control with fold change expressed relative to uninfected control MDMΦs harvested at the corresponding time points. IL-1β (A), TNFα (B), IL-6 (C), IL-8 (D), IL-12p40 (E), IL-10 (F) were measured using primer sets specific for bovine genes using SYRB Green chemistry. Bars represent the mean value ± SEM from four different experiments from 9 different donor cattle. *** P < 0.001.
Figure 3. Proinflammatory cytokine secretion of BVDV-2 inoculated or LPS stimulated MDMΦs 24 h after treatment. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains in duplicate at an MOI of 1 or 2ug/mL LPS with cell supernatants harvested at 24 h after treatment. Cytokine protein was analyzed by Searchlight Array using analytes specific for bovine IFNγ (A), IL-1β (B), IL-6 (C) and TNFα (D) with 50uL of cell supernatant analyzed in duplicate. Cytokines were quantified by generation of standard curves against recombinant bovine cytokines provided by the manufacturer of the Searchlight platform. Bars represent the mean value ± SEM from four different experiments from 9 different donor cattle. ** P < 0.001.
Figure 4. TNFα protein secretion from BVDV-2 infected monocyte derived macrophages after stimulation with bacterial TLR agonists. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Pam3Cys [5μg/mL], *M. haemolytica* LPS [10μg/mL], or *E. coli* (055:B5) LPS [1μg/mL]. Cell supernatants were analyzed for TNFα protein concentration 24 h after TLR stimulation and measured by Searchlight Array platform. Incubation with cytopathic and noncytopathic strains are in the top panel (A) or high and low virulence strains indicated in the lower panel (B). Bars represent the mean value ± SEM from four different experiments from 9 different donor cattle. ** P < 0.001 compared to uninfected, TLR stimulated cells.
Figure 5. TNFα protein secretion from BVDV-2 infected MDMΦ after stimulation with viral TLR agonists. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Poly I:C [50µg/mL], Imiquimod [10µg/mL], ssRNA40 LyoVec [10µg/mL]. Cell supernatants were analyzed for TNFα protein concentration 24 h after TLR stimulation and measured by Searchlight Array platform. Incubation with cytopathic and noncytopathic strains are in the top panel (A) or high and low virulence strains indicated in the lower panel (B). Bars represent the mean value ± SEM from four different experiments from 9 different donor cattle. ** P < 0.001 compared to uninfected, TLR stimulated cells.
Figure 6. Kinetics of JNK phosphorylation in response to TLR7 or TLR8 stimulation of MDMΦ either with or without BVDV-2 infection. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Imiquimod [10µg/mL] or ssRNA40 LyoVec [10µg/mL]. Cell lysates were analyzed for phosphorylated or total JNK through western blot after TLR stimulation at 10, 20, 30, 60 and 120 minutes. Lysates from uninfected MDMΦ stimulated with TLR7 agonists are in the top panel (A), stimulated with TLR8 agonists (B) and line graph representative of density ratio of phosphorylated:total JNK (C). Lysates from 28508 infected MDMΦ stimulated with TLR7 agonists are in the lower panels (D), stimulated with TLR8 agonists (E) and line graph representative of density ratio of phosphorylated:total JNK (F). Line graphs represent the mean value from four different experiments from 9 different donor cattle. Lanes 1 (10 min), 2 (20 min), 3 (30 min), 4 (60 min), 5 (120 min) are lysates harvested after TLR stimulation.
Supplemental Figure 1. IL-1β, IL-6 and IFNγ protein expression in response to TLR stimulation in MDMΦ infected with BVDV-2 strains 296c or 296nc. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Pam3Cys [5µg/mL], M. haemolytica LPS [10µg/mL], E. coli (055:B5) LPS [1µg/mL], Poly I:C [50µg/mL], Imiquimod [10µg/mL], or ssRNA40 LyoVec [10µg/mL]. Cell supernatants were analyzed for protein concentration 24 h after TLR stimulation and measured by Searchlight Array platform. Incubation with cytopathic and noncytopathic strains are indicated below each bar graph. Bars represent the mean value ± SEM from four different experiments from 9 different donors. ** P < 0.001, * P < 0.05 compared to uninfected, TLR stimulated cells.
TLR2/1 (Pam3Cys)

TLR 4 (E. coli)

TLR 4 (M. haemolytica)

TLR3 (Poly I:C)

TLR7 (Imiquimod)

TLR8 (ssRNA40)
Supplemental Figure 2. IL-1β, IL-6 and IFNγ protein expression in response to TLR stimulation in MDMΦ infected with BVDV-2 strains 1373 or 28508. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Pam3Cys [5µg/mL], M. haemolytica LPS [10µg/mL], E. coli (055:B5) LPS [1µg/mL], Poly I:C [50µg/mL], Imiquimod [10µg/mL], or ssRNA40 LyoVec [10µg/mL]. Cell supernatants were analyzed for protein concentration 24 h after TLR stimulation and measured by Searchlight Array platform. Incubation with hv1373 or lv28508 strains are indicated below each bar graph. Bars represent the mean value ± SEM from four different experiments from 9 different donors. ** P < 0.001, * P < 0.05 compared to uninfected, TLR stimulated cells.
Supplemental Figure 3. Expression of MyD88 in monocyte derived macrophages after 24 h TLR stimulation and 2 day pre-incubation with BVDV-2 strains. Bacterial TLRs (A), viral TLRs (B), representative blot from 296c pre-incubation (C), representative blot from 296nc pre-incubation (D), representative blot from 1373 pre-incubation (E), representative blot from 28508 pre-incubation (F). Treatments are represented in lanes 1 (TLR 8), 2 (TLR 2/1), 3 (e. coli TLR 4), 4 (m. haemolytica TLR 4), 5 (TLR 3), 6 (TLR 7).
Supplemental Figure 4. Kinetics of JNK phosphorylation in response to TLR7 or TLR8 stimulation in MDMΦ infected with BVDV-2. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains with an MOI of 1 for 48 h prior to stimulation with Imiquimod [10µg/mL] or ssRNA40 LyoVec [10µg/mL]. Cell lysates were analyzed for phosphorylated or total JNK through western blot after TLR stimulation at 10, 20, 30, 60 and 120 minutes. Lysates from 296c MDMΦ stimulated with TLR7 agonists are in the top panel (A), stimulated with TLR8 agonists (B) and line graph representative of density ratio of phosphorylated:total JNK (C). Lysates from 296nc infected MDMΦ stimulated with TLR7 agonists are in the middle panels (D), stimulated with TLR8 agonists (E) and line graph representative of density ratio of phosphorylated:total JNK (F). Lysates from 1373 infected MDMΦ stimulated with TLR7 agonists are in the lower panels (G), stimulated with TLR8 agonists (H) and line graph representative of density ratio of phosphorylated:total JNK (I). Line graphs represent the mean value from four different experiments from 9 different donors. Lanes 1 (10 min), 2 (20 min), 3 (30 min), 4 (60 min), 5 (120 min) are lysates harvested after TLR stimulation.
CHAPTER FOUR. BOVINE VIRAL DIARRHEA VIRUS MODULATES
EXPRESSION OF CELL SURFACE MARKERS AND PHAGOCYTIC
CAPACITY OF MONOCYTE DERIVED MACROPHAGES IN VIVO
AND IN VITRO

Manuscript to be submitted to Journal of Veterinary Immunology and Immunopathology

Robert G Schaut (a,b), Jodi McGill (b), David Lynn (c), Julia F Ridpath (b), Randy E Sacco (a,b)

Abstract

Macrophages are innate immune cells which can be important as antigen presenting cells (APCs) and are involved in the initiation of an adaptive immune response. This is the first study to investigate function of monocyte derived macrophages (MDMΦ) infected with isogenic strains of cytopathic bovine viral diarrhea virus genotype 2 (BVDV-2) and strains of varying virulence in vivo and in vitro. We isolated CD14+ cells from in vivo BVDV-2-infected calves and performed transcriptional sequencing analysis in which changes in endocytic and phagocytic pathways were observed compared to cells from uninfected controls. We recapitulated these findings in vitro and further demonstrated modulation of

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MHC class II and CD80 cell surface expression. Regardless of biotype, phagocytic capacity was diminished in BVDV2-infected MDMΦ. Together these results demonstrate that MDMΦ are impaired in their ability to upregulate essential cell surface markers for antigen presentation as well as take up and present antigen. These data support the evidence that BVDV-2 infection may alter antigen presentation capabilities of APCs that are critical for host responses to secondary infection.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a single stranded positive sense RNA virus in the Pestivirus genus which include classical swine fever virus and border disease virus. Pestiviral genomes are approximately 12.5kb in length and BVDV encodes for 11 or 12 genes depending on cytopathogenicity and as such can be classified as cytopathic (cp) or non-cytopathic (ncp) (Bendfeldt et al., 2007). BVDV is further categorized into genotypes of BVDV-1 and BVDV-2. BVDV-2 strains can cause severe disease, which can be related to atypical or high virulence (hv) strains (Liebler-Tenorio et al., 2002) as opposed to typical or low virulence (lv) strains (Liebler-Tenorio et al., 2003) that cause mild or subclinical disease.

BVDV infection predisposes the host to secondary bacterial and viral infections and contributes to bovine respiratory disease complex (BRDC) in feedlot cattle (Confer, 2009). The immunosupression induced by BVDV infection is not fully understood, although changes in lymphocyte populations (Brewoo et al., 2007), modulation of cytokine secretion (Glew et al., 2003) and disruption in chemotaxis (Brown et al., 1991) may be factors of an
immune suppressed host. However, study of varying virulence and isogenic cytopathic/noncytopathic biotypes of BVDV-2 strains on macrophage (MΦ) function has not been fully explored.

The MΦ is an important immune cell as it contributes to cytokine secretion in response to pathogenic challenge (Stow et al., 2009) and can present antigen to T cells to initiate an adaptive immune response (Unanue, 2002). To date, in vitro BVDV-1 infection has been shown to downregulate TNFα expression, modulate fluid uptake and endocytic pathways and decrease Fc receptor expression in MΦ (Boyd et al., 2004; Lee et al., 2008; Liu et al., 1999; Welsh and Adair, 1995); however the impacts of BVDV-2 on MΦ function is unknown. In this study we characterized changes of antigen presentation pathways in an in vivo BVDV-2 infection and recapitulated results in vitro.

2. Materials and Methods

2.1. In vivo experimental procedures and viral inoculum

Eighteen clinically healthy, colostrum-deprived Holstein calves were used in two experiments. 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 BVDV 2 strain 1373. 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 5mL of
10^{5.6} TCID_{50} of BVDV-2 strain 1373 (n=3/replicate) or RS886 (n=3/replicate). Additional calves (n=3/experiment) were mock inoculated and served as age-matched negative controls. Clinical signs were monitored twice per day. Blood samples were collected in anticoagulant tubes on days 5 and 14 post inoculation. Numbers of leukocytes and platelets were determined for each animal. 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.

2.2. RNAseq

RNAseq sample preparation and analysis was performed as previously described (Ridpath et al., 2012). Briefly, buffy coats were isolated at days 5 or 14 post BVDV inoculation. Buffy coats were sorted by magnetic separation using MiniMACS columns (Miltenyi Biotec, Auburn, CA) for T cells (CD3+), B cells (IgM+), or monocytes (CD14+) and lysed for RNA extraction. Samples were prepared using the TruSeq RNA sample prep kit v1 (Illumina, San Diego, CA). Clusters were generated using the cBot and TruSeq Cluster generation kits with sequencing performed on a HiSeq 2000 instrument using 50 Cycle Single Read approach with Tru Seq SBS v2 chemistries (Illumina).

2.3. In vitro experimental procedures, viral inoculum and MΦ isolation

12 clinically healthy Holsteins of approximately 1-2 years of age were used for blood donors. Animals were negative for BVDV as measured by an immunohistochemistry (IHC)
positive ear notch. 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), 2mM L-glutamine, 1% antibiotic–antimycotic solution, and gentamicin sulfate (Gibco, Carlsbad, CA). Monocytes were isolated by plastic adherance. 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 5x10^6 cells per mL. Monocytes were plated in 96-well round bottom tissue culture plates in 100 µL medium. Monocytes were cultured for 7 days with media change every 2-3 days to derive MΦ. Cells isolated from animals were tested for BVDV through reverse transcription (RT)-PCR with primers selective for BVDV genotypes 1a, 1b, and 2 and were negative prior to experimentation. BVDV-2 strains were propagated in MDBK cells with a viral titre determined by histological staining as follows: 296c [TCID\textsubscript{50} 6.8x10^6], 296nc [TCID\textsubscript{50} 3.8x10^8], 1373 [TCID\textsubscript{50} 6.22x10^6], 28508 [TCID\textsubscript{50} 2.37x10^6]. Cells were inoculated with an MOI of 1.

2.4. qPCR

At 2, 6, 18, and 24 h post BVDV inoculation, 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) and genomic DNA was removed during RNA isolation using an on-column RNase-Free DNase I digestion set (Qiagen) per to
manufacturer’s instructions. 500 ng of total RNA from each sample was reverse transcribed using random primers/hexamers and Superscript III (Invitrogen) to generate first strand cDNA. 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) (Nelson et al., 2010) was used as the endogenous control. Primer set sequences are indicated in Table 1. 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 fluorescence 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).

2.5. Flow cytometry

Cells were inoculated with BVDV-2 strains for 24h prior to staining for cell surface marker expression. Cells were blocked with goat serum and anti-FcγRI/III antibodies. MHC class I, MHC class II, CD14, CD80 were used at 1µg/µL concentrations with secondaries of 0.5 µg/µL.

2.6. Phagocytosis assays
Cells were inoculated with BVDV-2 strains at an MOI of 1 and allowed to incubate for 24 h prior to phagosome assays. Procedures for assays have been described previously (Savina et al., 2010). Briefly, amine-based latex beads were coated with FITC or Fluoprobe-678 for measurement of pH. Cells were allowed to phagocytose beads and samples collected at 10, 20, 30, 60, 120 and 180 min after phagocytosis. Amine-based latex beads were coated with Rhodamine or Fluoprobe-678 for oxidation assays. Cells were allowed to phagocytose beads and samples collected at 10, 60 and 120 minutes. Live cells were gated and cells which had phagocytosed beads were measured.

2.7. Statistical analysis

qPCR cytokine data was analyzed with the outcome variable \(2^{-\Delta\Delta CT}\) log transformed. \(\Delta\Delta Ct\) values were analyzed using one-way ANOVA (Prism, GraphPad, LaJolla, CA). Bonferroni post test was used to compare replicate means by row to uninfected controls. Flow cytometry data was analyzed using one-way ANOVA (Prism, GraphPad) with Bonferroni’s multiple comparison test was used to compare replicate means by row to all columns. Results are expressed as means +/- standard errors of the means (SEM).

3. Results

3.1. RNAseq analysis of CD14+ cells isolated from an in vivo infection with a high- or low-virulence BVDV-2 strain
To understand potential mechanisms of immune-modulation that may occur during BVDV-2 infection, deep sequencing analysis of CD14+ monocytes isolated from calves early in infection (day 3) and late (day 15) was performed. A total of 1033 genes was significantly different compared to uninfected CD14+ cells at day 3 post infection with a total of 1237 gene differentially expressed at day 15. Interestingly, when comparing the monocytes isolated from infected calves compared to cells isolated from uninfected animals differences in regulation of pathways involving phagocytosis, antigen processing, endocytosis and ubiquitin mediated proteolysis was impacted and these pathways were selected for further analysis (Table 2). Within these pathways, at day 3 post BVDV2 infection, there was an increase in gene expression of antigen peptide transporter 1, myristoylated alanine-rich protein kinase C and ubiquitin conjugating enzyme E2L6. These genes are important in the internalization, rearrangement of the cytoskeleton and proteolytic degradation of proteins. Conversely, at day 3 there was a decrease in gene expression of lysosomal lipase A, dynamin 1 and fragment crystalizable IgA receptor (FcαR). However, by day 15, only ubiquitin-like modifier activating protein enzyme 7 was increased, whereas macrophage receptor with collagenous structure (MARCO) and syntaxin 3 was decreased. Thus, as BVDV-1 can modulate endocytic pathways in vitro (Boyd et al., 2004), taken together with this data from in vivo BVDV-2 infection suggests that BVDV modulates the transcriptional pathways involved in the ability of MΦ to engulf and process antigens.

3.2. qPCR analysis of cell surface molecules related to antigen presentation
As phagocytic and antigen processing pathways were impacted in vivo, it is possible that surface molecule expression related to antigen presentation may be modulated at the transcriptional level. Cells were infected with varying strains of BVDV-2 and RNA was isolated at 2, 6, 18 and 24 h post inoculation. MHC class I expression in MDMΦ (Fig. 1A) was reduced at 2 h post 1373 and 28508 BVDV-2 inoculation compared to uninfected controls; however there was no significant changes in expression in any other timepoint or treatment groups. MHC class II (Fig. 1B) was reduced in expression from virally infected MDMΦ compared to controls at 24 h post 296c BVDV inoculation, as well as 2h post 1373 and 28508 BVDV-2 treatment. Conversely, CD80 (Fig. 1C) was heightened in expression from 296c infected MDMΦ response to at 2, 6, 18 and 24 h as well as MDMΦ infected with 296nc at 18 and 24 h after inoculation. 1373 and 28508 inoculated MDMΦ demonstrated increase mRNA expression of CD80 at 6, 18 and 24 h compared to expression from uninfected MDMΦ. CD86 (Fig. 1D) was not significantly up or down regulated after any viral treatment. Likewise it has been demonstrated that ncp or cpBVDV-1 can modulate CD80 or CD86 mRNA expression (Lee et al., 2008) in vitro and taken with these data suggest that BVDV-2 infection modulates mRNA expression of some cell surface markers.

3.3. Mean fluorescent intensity of cell surface marker expression as determined by flow cytometry

To develop a better understanding of the effects of BVDV on the cell surface marker expression, we used flow cytometry to measure expression of MHC class I, MHC class II and CD80 (Fig. 2). Interestingly, there was a significant decrease in expression of MHC
class II on the MDMΦ infected with BVDV-2 strains and stimulated with LPS compared to uninfected LPS-stimulated only. Likewise, there was a significant decrease in MFI expression between MDMΦ infected with BVDV2 strains and BVDV2 with LPS stimulation, indicating that BVDV2 can downregulate surface marker expression in response to LPS. Similarly, CD80 demonstrated similar findings to MHC class II surface expression. However, MHC class I was up regulated in some infected cells (Fig 2A). Overall, there was a significant decrease in expression of cell surface markers in response to LPS in BVDV2 infected MDMΦ (Fig. 2B).

3.4. Mean fluorescent intensity of pH sensitive FITC-labeled amino-latex beads as determined by flow cytometry

To determine if BVDV-2 infection would decrease the acidification processing of the phagosome, pH sensitive FITC-labeled beads were internalized by infected MDMΦ. Over a timecourse, the intensity of FITC was measured to allow for a determination in acidification and neutralization of the phagosome by gating on single bead internalized populations (Fig. 3A). However, there was no statistical difference in acidification, and the MDMΦ were able to acidify the phagosome in response to LPS (Fig. 3B).

3.5. Mean fluorescent intensity of reactive oxygen species-sensitive Rhodamine-labeled amino-latex beads as determined by flow cytometry
To determine if BVDV-2 infection would decrease the oxidative capacity of the phagosome, reactive oxygen species (ROS) sensitive rhodamine-PE-labeled beads were internalized by infected MDMΦ. Over a timecourse, the intensity of PE was measured to allow for a determination in oxidation of the bead by gating on single bead internalized populations. However, there was no statistical difference in oxidation, and the MDMΦ were able to oxidize the phagosome as well as in response to LPS (Fig. 4).

3.6. Analysis of phagocytic capacity as determined by flow cytometry

To determine if BVDV-2 infection of MDMΦ would alter the ability of the cells to internalize particles, infected MDMΦ were incubated with latex-amino-beads. The cells were then analyzed for percent positive or negative for internalized bead (Fig. 3A). Interestingly, BVDV-2 infection did decrease the ability of the cells to uptake beads. The 296c and 28508 infected MDMΦ demonstrated the greatest impact on phagocytosis; however each of the virally treated groups demonstrated a statistical decrease in phagocytic capacity (Fig. 5). To observe if LPS activation could recover any loss in phagocytic capacity, cells were stimulated with LPS prior to internalization of beads. Only 296nc-infected MDMΦ demonstrated recovery and were statistically similar to uninfected cells (Fig. 5 – right panels).

4. Discussion

Previous in vitro studies have explored transcriptional changes that may occur during infection with BVDV. For instance, MDBK cells infected in vitro with BVDV-2 strain 1373
demonstrated decreases in expression of α-Tubulin and β-Tubulin as well as genes associated with glycolysis, electron transport, ATP metabolism and elongation factors (Neill and Ridpath, 2003). These data suggest that BVDV-2 potentially modulates protein packaging and internalization or endocytosis mediated by tubulin molecules (Popova and Rasenick, 2004). Similarly bovine monocytes infected in vitro with BVDV-1 ncp strains NADL demonstrated a decrease in expression of MHC class I and MHC class II (Lee et al., 2009) which suggest a potential impairment in antigen presentation. As such, we decided to investigate the effects of BVDV-2 on CD14+ monocytes isolated from the blood from in vivo infected animals. Interestingly, we identified an early (day 3) increase in transcription of antigen presentation-associated genes; although endosomal associated genes were decreased in expression. This was sustained late in infection (day 15) in which we observed a decrease in expression of macrophage receptor with collagenous structure (MARCO), a non-specific receptor of endocytosis and phagocytosis. Taken together, these data suggest that BVDV infection may modulate endocytic or phagocytic pathways of MDMΦ.

Findings have suggested that monocytes infected with BVDV-1 strains are compromised in their gene expression of CD80, CD86, MHC class I and MHC class II (Lee et al., 2008). As there have been no comparative studies on the effects of BVDV-2 infection on expression of associated antigen presentation molecules in MDMΦ, we investigated the effects of viral infection to modulate cell surface marker gene expression. Interestingly, we did not observe a sustained decrease in expression of any of the molecules examined; however MHC class I expression in MDMΦ infected with 1373 and 28508 strains was reduced early in infection. Similarly MHC class II was reduced in MDMΦ infected with 1373 and 28508 early in infection. As our results are contrary to BVDV-1 studies, it is
possible that mechanisms of suppression in antigen presentation may differ between BVDV-2 genotypes. Infection of plasmacytoid dendritic cells with Dengue virus, a related Flaviviridae species, induces enhanced expression of CD80, CD86, and CD40; in turn, promote an enhanced TNFα and IFNα/β response potentially contributing to pathological outcome (Sun et al., 2009). However, BVDV-2 infection of MDMΦ may not necessarily be modulating antigen-presenting capabilities at the transcriptional level as there were no sustained significant reductions in expression of cell surface molecules associated with antigen presentation.

A previous study of BVDV-1 infection in monocytes or dendritic cells demonstrated only minimal differences in MFI of MHC class I, MHC class II, CD80 and CD86 between infected and mock treated cells. However, the authors demonstrated a deficiency in allogenic and ova specific induced responses of monocytes to induce T cell proliferation (Glew et al., 2003). Interestingly, an earlier study demonstrated that PBMCs isolated from persistently infected (PI) BVDV calves were able to induce a robust allogenic T cell response (Glew and Howard, 2001). As such, we demonstrated that varying genotypes of BVDV-2 infected and LPS stimulated MDMΦ have reduced expression of MHC class II and CD80 compared to LPS-only treated MDMΦ. Our studies suggest that there may be a decrease in the ability of BVDV-2 infected MDMΦ to drive T cell proliferation.

It is possible that a deficit in phagosomal function may contribute to the reduced antigen-presentation capabilities of BVDV-infected MDMΦ. Interestingly, alveolar macrophages from BVDV and BRSV infected cattle demonstrated a decrease in Fc receptor expression, phagosome-lysosome fusion and superoxide anion production (Liu et al., 1999). As phagosomal fusion is disrupted, is may be possible that pH is also impaired. However,
we found no differences in phagosome acidification between infected and uninfected MDMΦ with LPS treatment. Likewise, we observed no alterations in oxidative capacities of the phagosome between infected and non-infected groups. Thus our data suggests there are no modifications in acidification or oxidation in BVDV2-infected MDMΦ.

Previous studies have demonstrated that monocytes infected with BVDV strains have decreased expression of genes associated with Fc-mediated phagocytosis (Ammari et al., 2010; Pinchuk et al., 2008). Similarly, it has been demonstrated that monocytes infected with BVDV-1 strains have reduced mannose receptor-mediated endocytosis, macropinocytosis, or caveolae-mediated antigen uptake (Boyd et al., 2004). Likewise, alveolar macrophages isolated from BVDV infected calves and restimulated with BVDV demonstrated an impaired ability to phagocytose yeast particles (Welsh and Adair, 1995). Interestingly, we demonstrated cytopathic, noncytopathic, high or low virulence BVDV-2 infected MDMΦ suppressed the ability to phagocytose amine-latex-beads. In summary, our data and the data from others suggest BVDV-2 infection does impair phagocytosis function of antigen presenting cells.

Endocytosis and phagocytosis are important mediators in antigen internalization and processing. Equally critical are cell surface molecules required to interact with adaptive immune cells to promote lasting immunity. Changes in these cellular processes during infection may lead to a diminished immune response or potentially modulate disease progression. We have shown that BVDV-2 infected MDMΦ display a modulated cell surface expression of antigen presentation molecules. However, BVDV-2 infection of MDMΦ does not modulate acidification or oxidative capacity of the phagosome; although phagocytosis is impaired compared to uninfected cells. These changes in macrophage
antigen uptake and presentation may contribute to increased susceptibility to secondary infections.

Acknowledgements

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References


Glew, E.J., Howard, C.J., 2001. Antigen-presenting cells from calves persistently infected with bovine viral diarrhoea virus, a member of the Flaviviridae, are not compromised in their ability to present viral antigen. J. Gen. Virol. 82, 1677-1685.


Sun, P., Fernandez, S., Marovich, M.A., Palmer, D.R., Celluzzi, C.M., Boonnak, K., Liang,


### Table 1
Primer sequences for bovine target and control genes

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<thead>
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F=forward; R=reverse.
### Table 2
Summary of RNAseq analysis results.

**MΦ (CD14+ Cells) BVDVRS886 vs Control (Day 3)**

<table>
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<th>Pathway of Interest</th>
<th>P Value</th>
<th>Pathways Associated Genes of Interest</th>
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<td>Antigen processing and presentation</td>
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<td>Antigen peptide transporter 1</td>
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<td></td>
<td>Fc gamma R-mediated phagocytosis</td>
<td>5.72x10^{-20}</td>
<td>Myristoylated alanine-rich protein kinase C substrate (MARCKS)</td>
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<td></td>
<td>Ubiquitin mediated proteolysis</td>
<td>5.215x10^{-09}</td>
<td>Ubiquitin conjugating enzyme E2L6</td>
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<td></td>
<td>Lysosome</td>
<td>1.728x10^{-6}</td>
<td>Lysosomal lipase A</td>
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<td></td>
<td>Endocytosis</td>
<td>4.661x10^{-24}</td>
<td>Dynamin 1</td>
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<td></td>
<td>Phagosome</td>
<td>0.03</td>
<td>Receptor for Fc fragment of IgA</td>
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**MΦ (CD14+ Cells) BVDVRS886 vs Control (Day 14)**

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<th>P Value</th>
<th>Genes of Interest</th>
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<td></td>
<td>Phagosome</td>
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<td>Macrophage receptor with collagenous structure (MARCO)</td>
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<td></td>
<td>SNARE interactions in vesicular transport</td>
<td>0.012</td>
<td>Syntaxin 3</td>
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Figure 1. Expression of cell surface markers in MDMΦ after stimulation with BVDV-2 strains. MDMΦs were differentiated in 96 well plates for 7 days and inoculated with BVDV-2 strains in duplicate at an MOI of 1 with RNA harvested at 2, 6, 18, and 24 h after inoculation. Cytokine mRNA was analyzed by qPCR using ribosomal protein s9 (RPS9) as an endogenous control with fold change expressed relative to uninfected control MDMΦs harvested at the corresponding time points. MHC class I (A), MHC class II (B), CD80 (C), CD86 (D) were measured using primer sets specific for bovine genes using SYRB Green chemistry. Bars represent the mean value ± SEM from four different experiments from 9 different donors. ** P < 0.05.
Figure 2. MFI of cell surface antigens expression on MDMΦ as measured by flow cytometric analysis. MDMΦ were incubated for 7 days in culture, prior to BVDV-2 inoculation of an MOI of 1. Virally treated cells were incubated for 48 h prior to LPS stimulation. Histograms represent isotype controls (solid red), BVDV-2 + LPS treated MDMΦ (blue) and LPS only (solid green). Bar graphs represent SEM of ΔMFI (Treatment group MFI – isotype control MFI) from 6 different animals from 3 independent experiments. * P < 0.1.
Figure 3. Flow cytometric analysis of phagosome acidification in virally infected or uninfected MDMΦ. MDMΦ were incubated for 7 days in culture, prior to BVDV-2 inoculation of an MOI of 1. Virally treated cells were incubated for 48 h prior to LPS stimulation. Bead MFI was measured at 10, 20, 30, 60, 120 minutes post internalization. Cells were gated on one bead internalized (A) for pH analysis, and excluded cells which had not phagocytosed bead. Bar graphs represent MFI of viral or mock-infected treatment groups with or without LPS stimulation. pH standard curve was generated with known pH values for determination of MFI to pH ratio. Bar graphs represent SEM from 9 total animals from 3 independent experiments.
Figure 4. Flow cytometric analysis of phagosome oxidation in virally infected or uninfected MDMΦ. MDMΦ were incubated for 7 days in culture, prior to BVDV-2 inoculation of an MOI of 1. Virally treated cells were incubated for 48 h prior to LPS stimulation. Bead MFI was measured at 60 minutes post internalization. Cells were gated on one bead internalized for ROS analysis, and excluded cells which had not phagocytosed bead. Bar graphs represent SEM of MFI of viral or mock-infected treatment groups with or without LPS stimulation from 9 total animals from 3 independent experiments.
Oxidative Capacity

60 Minutes

MFI

Mock 296c 296nc 1373 25508

Treatment

60 Minutes w LPS

MFI

Mock 296c 296nc 1373 25508

Treatment
Figure 5. Flow cytometric analysis of phagocytic capacity in virally infected or uninfected MDMΦ. MDMΦ were incubated for 7 days in culture, prior to BVDV-2 inoculation of an MOI of 1. Cells were allowed to internalize amino-latex beads and were analyzed for percent positive or negative internalization. Bar graphs represent SEM from a total of 9 animals and three independent experiments. Bar graphs represent SEM 9 total animals from 3 independent experiments. ** P < 0.05
CHAPTER FIVE. GENERAL CONCLUSIONS

BVDV has a major annual economic impact on cattle, either through acute disease or susceptibility to secondary infection (11, 12). To date, vaccines are available although problems of efficacy, usage and cost are limiting factors. As BVDV is immunosuppressive, it contributes to BRDC in feedlot cattle predisposing the animals to a multitude of bacterial and viral pathogens (6, 7). There is a need to understand the underlying mechanism of viral suppression for developing treatment and helping to potentiate better vaccines. Given that monocytes are not depleted during \textit{in vivo} infection with BVDV (2, 3, 9, 10), they may be an important cell in the recovery of infection. Interestingly, during \textit{in vivo} BVDV-2 infection monocytes are hyperresponsive to LPS stimulation. This hypersensitivity may be contributory to increased levels of proinflammatory cytokines in circulation. This increase in inflammatory cytokine can contribute to induced apoptosis of peripheral blood cells as well as increased severity of disease (8, 13). Interestingly, we demonstrated that monocytes from infected calves that were differentiated into MDMΦ demonstrated hyporesponsive TLR4 cytokine secretion. Interestingly, this can be contributory to an increase in susceptibility to secondary infection, as TLR4 is critical in the sensing of bacterial LPS. When examining a tissue derived macrophage, the AMΦ, it was found that these cells isolated from infected calves are hyperresponsive to LPS in mRNA expression of IL-1β and TNFα; however conversely are reduced in expression and secretion of IL-6 compared to cells from uninfected animals. Taken together, these results suggest that myeloid lineage cells isolated from BVDV-2 infected calves are modulated in their TLR4 response; and potentially maturation state of these cells impact the viral effects on function. A mechanisms of reduction in cytokine expression and secretion is explained by the reduction in MyD88 and IKKβ
phosphorylation. We demonstrated that BVDV NS5A interacts with MyD88, although the effect of that interaction is unknown. Secondly, we determined that *in vitro* inoculation with varying strains of BVDV decreased TLR responsiveness across a multitude of TLRs. Interestingly, we found that TLR7 is responsive which may be explained by JNK phosphorylation and identification of a unique isoform of p46 JNK. Thirdly, we determined that the antigen presentation capability of BVDV-2 infected MDMΦ may be due to a decrease in expression of cell surface markers MHC class II and CD80 as well as a dramatic decrease in phagocytic capabilities. Taken together these studies provide information on the effects of BVDV-2 infection as well as identifying some potential aspects of underlying immunosuppression which can lead to secondary infection.

**Specific Aim 1.** These studies determined the effects of an *in vivo* infection of hv and lv BVDV-2 strains on myeloid cells isolated from infected calves. Although hv infected calves display severe acute disease whereas lv infection is sub-clinical acute, both strains demonstrated immunomodulation of myeloid cells isolated from infected calves.

2-6 week old colostrum-deprived calves, which were negative for BVDV and BVDV antibodies, were utilized for these studies. Monocytes were isolated throughout the course of infection and analyzed for pro-inflammatory cytokine transcription and secretion. As well, MDMΦ were generated as well as AMΦ harvested at necropsy. Interestingly, monocytes isolated from BVDV-2 infected calves were increased in mRNA transcription for proinflammatory cytokines and demonstrated a hyperresponse to LPS stimulation. Conversely, MDMΦ isolated from BVDV-2 infected calves were hyporesponse to LPS compared to cells isolated from uninfected calves. AMΦ enhanced IL-1β and TNFα mRNA expression; although IL-6 transcription and secretion was reduced compared to AMΦ from
control calves. Thus, MyD88 and IKKβ phosphorylation was decreased in cells isolated from infected calves, which may be a contributing factor of observed modulation in cytokines. Interestingly, we demonstrated that BVDV NS5A and MyD88 co-localize. Yet, the effect of this interaction is unknown although may be similar to the observed interaction demonstrated between HCV NS5A and MyD88 (1).

Specific Aim 2. These studies determined the effects of multiple biotypes of BVDV-2 strains on MDMΦ cytokine transcription and secretion. Additionally, TLR responsiveness was observed as well as downstream signaling molecule JNK. Interestingly, all BVDV-2 strains demonstrated an effect on MDMΦ by which cytokine response was reduced.

Approximately 1-year-old BVDV/BVDV antibody free cattle were used as blood donors for MDMΦ isolation and generation. These cells were inoculated with BVDV-2 strains and mRNA expression of pro-inflammatory cytokines was determined. Of note, a robust pro-inflammatory mRNA response was observed, although the cp and hv BVDV-2 strain induced the greatest levels of mRNA expression in the MDMΦ. Interestingly, protein was not secreted by the infected MDMΦ in response to BVDV-2 infection. The TLR response MDMΦ after BVDV-2 inoculation was diminished for TLR2/1, TLR4, TLR 3 and TLR8 stimulation compared to uninfected control cells. Indeed, TLR7 was responsive which was attributed to JNK phosphorylation and an observed isoform at p46. In summary, these results suggest that BVDV-2 infection will decrease the responsiveness to a multitude of TLRs, which can contribute to secondary infection.

Specific Aim 3. These studies determined the effects of BVDV-2 infection in vivo and in vitro on the antigen processing and presentation abilities of CD14+ or MDMΦ cells.
2-6 week old colostrum-deprived calves, which were negative for BVDV and BVDV antibodies, were utilized for the *in vivo* studies; whereas approximately 1-year-old BVDV/BVDV antibody free cattle were used as blood donors for MDMΦ isolation and generation. *In vivo* transcription of endocytic and phagocytic pathways were decreased in CD14+ cells isolated from infected animals compared to uninfected controls. Moreover, *in vitro* qPCR analysis suggest that BVDV-2 infected MDMΦ may slightly decrease MHC class I or MHC class II expression; although this observation is not sustained throughout the time course analyzed. When analyzing cell surface marker expression of MHC class II and CD80 by flow cytometry, MFI was decreased in BVDV-2 infected MDMΦ stimulated with LPS compared to LPS only treated cells. Likewise, phagocytic capability of these BVDV-2 infected cells was decreased compared to uninfected controls, although acidification and oxidation of the phagosome was not effected.

Taken together, these data reported in this dissertation studies support the immunesuppression observed in MDMΦ from BVDV infection *in vivo or in vitro* by a decrease in: expression of pro-inflammatory cytokines, reduction in expression of MyD88, a decrease in phosphorylation of IKKβ, hyporesponsive TLRs, decreased expression of antigen presentation associated molecules and reduction in phagocytic capabilities. Evidence suggests that BVDV contributes to secondary bacterial and viral infection (4, 6, 7) and as such, mediating factors in these infections may be the observed functional changes induced by BVDV infection of immune cells. This dissertation has demonstrated that a functionally deficit MDMΦ may be a contributing factor of BVDV disease and lasting immune suppression.
Recommendations for Future Work

The goal of this dissertation is to examine the changes induced by BVDV on the innate immune cells of the host. In vivo we were able to examine AMΦ at time of necropsy, however, this timepoint may not represent all of the changes which may have occurred during infection. As AMΦ are first responders to infection and would be one of the initial immune cells to initiate a response to BVDV infection, it would be interesting to examine the early response. Interestingly, within 24 hours after infection, changes within the peripheral blood leukocyte population occurred, which may indicate a cellular infiltration into the tissue such as the lung. As well, a timecourse by which AMΦ could be harvested throughout infection would be interesting to examine any functional changes which may occur.

As AMΦ are only one type of tissue MΦ, other MΦ such as splenic MΦ, peritoneal MΦ, or kupffer cells would be interesting to investigate cytokine responses as a comparison. We demonstrated that monocytes and terminally differentiated cells isolated from infected calves do indeed function differently, and potentially MΦ populations may as well. Likewise, in vitro derived BMMΦ may be another interesting cell as the bone marrow during BVDV infected undergoes dramatic change.

Although we conducted experiments to analyze cell surface markers of monocytes during in vivo BVDV infection, we were unable to determine changes in MHC class I, MHC class II, CD80, Fas or other potentially important maturation and effector molecules. Most likely, the blood we were able to collect and subsequent cells isolated was extremely limited, and future work could be planned to only harvest blood for flow analysis. Likewise, study of isolated monocytes and derived MΦ or DCs to stimulate an allogenic response may be another interesting aspect of BVDV induced modulation of function.
As we determined an interaction between NS5A and MyD88, it would be interesting to further characterize this interaction. By inserting point mutations within the NS5A sequence, we can modify the structure of this protein. *In vitro* analysis will allow us to determine potential binding regions and protein motifs which may be critical for NS5A-MyD88 interaction. Furthermore, it would be interesting to produce transgenic MyD88 knockout cell lines, such as with MDBKs, and observe potential changes this may have on viral propagation or cytokine response. As NS5A has been shown to be important in viral replication, sequencing analysis between virulence biotypes may provide further insight into how genetic differences of the NS5A gene could impact virulence. If localized regions in the NS5A gene demonstrate great genetic variability between strains, we could target those regions for mutagenesis and see if they are a critical aspect of virulence.

*In vitro* we found that TLR7 stimulation after BVDV-2 infection is still functional. Although we attributed this to cytokine expression in MDMΦ, JNK phosphorylation can lead to apoptosis. It is possible, certain cell types which express TLR7, may induce apoptosis in response to BVDV. For instance, lung epithelial cells have been demonstrated to express TLR7 (5), and as BVDV would interact with this cell type, TLR7 induction of apoptosis may play a role in disease. As we found TLR7 to be JNK mediated in response to BVDV, using JNK inhibitors can be used to confirm this aspect of the response.

References


