Bovine viral diarrhea virus infections affect professional antigen presentation in bovine monocytes

Sang-Ryul Lee

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BOVINE VIRAL DIARRHEA VIRUS INFECTIONS AFFECT PROFESSIONAL ANTIGEN PRESENTATION IN BOVINE MONOCYTES

By

Sang-Ryul Lee

A Dissertation
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Veterinary Medical Science in the College of Veterinary Medicine

Mississippi State, Mississippi

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BOVINE VIRAL DIARRHEA VIRUS INFECTIONS AFFECT PROFESSIONAL ANTIGEN PRESENTATION IN BOVINE MONOCYTES

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Monocytes are professional antigen presenting cells (APC). They serve as precursors of macrophages and dendritic cells (DC). We have used cytopathic (cp) and non-cytopathic (ncp) Bovine Viral Diarrhea Viruses (BVDV) to determine the genes and proteins expression levels in bovine monocytes.

Four specific aims were accomplished in this study. The first aim was to assess the baseline expression of the proteins involved in professional antigen presentation in bovine monocytes. The results showed that the differential detergent fractionation (DDF) approach can provide interpretable and meaningful functional information in bovine monocytes.

The second aim was to evaluate the role of in vitro cp and ncp BVDV infection in the expression of the selected bovine genes involved in professional antigen presentation.
The results showed that both BVDV could escape innate immune responses by modulating toll-like receptor (TLR) gene expression, followed by pro-inflammatory, type I interferon (IFN), Th1/Th2 type cytokine genes expression, and decreasing the expression levels of CD80/CD86 in professional APC.

The third objective was to determine how the two biotypes affect selective antigen uptake, receptor-mediated endocytosis and non-selective uptake, macropinocytosis in bovine monocytes. The results indicated that bovine monocytes use macropinocytosis for a bulk-flow uptake of soluble antigens.

The final aim was to characterize protein profiles in peripheral blood monocytes infected with cp BVDV isolate in vitro. Comparative profiling of the membrane and cytosolic proteins related to professional antigen presentation were assessed. The results showed that 47 bovine proteins, involved in immune function of professional APC have been significantly altered after cp BVDV infection. Overall, we hypothesize that by modulating expression levels of multiple proteins and genes related to immune responses BVDV could significantly compromise immune defense mechanisms resulting in uncontrolled immune activation or suppression.

Key words: monocytes, BVDV, proteomics, real time PCR, antigen uptake
DEDICATION

To my dearest father, Mr. Jae-Woo Lee who passed away in March 2007, I would like to dedicate this in the family vault.
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CHAPTER 1
INTRODUCTION

Bovine viral diarrhea virus (BVDV), a single-stranded RNA virus, in the family Flaviviridae, genus Pestivirus, infects a large proportion of cattle worldwide and causes a number of clinical forms of the disease ranging from transiently detectable mild clinical symptoms to a fatal diarrheic condition known as mucosal disease (MD). The BVDV are actually multiple strains with antigenic and genotypic differences, along with different growth patterns. According to their effect in cell cultures, BVDV is divided in two biotypes: noncytopathic (ncp), which is widely distributed, and cytopathic (cp), which is associated predominantly with animals that develop MD may be acute, resulting in death within a few days of onset, or chronic, persisting for weeks or months before the afflicted animal dies. Morbidity for either acute or chronic MD is low in most herds, but some outbreaks may involve 20% or more of cattle in a herd. The mortality rate for either acute or chronic MD is high. Ncp BVDV infection maintains and spreads itself in the cattle population by inducing immunotolerance following fetal infection, resulting in birth of calves persistently infected (PI) with the virus. PI cattle are the major source of virus spread both within and between farms. Therefore, reproductive losses are the most economically important consequence associated with BVDV infection and there are
indications that the incidence of BVDV related reproductive losses are increasing in the United States.

Both ncp and cp BVDV strains infect monocytes, macrophages and dendritic cells (DC) *in vitro* and *in vivo*. In contrast to cp, ncp biotypes of BVDV do not induce cell death and IFN production in response to the virus or synthetic double-stranded RNA. Monocytes, but not DC, infected with both cp and ncp BVDV *in vitro* are compromised in their ability to stimulate allogeneic and memory CD4$^+$ T cell responses.

Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells and produce cytokines, including tumor necrosis factor α (TNF-α) and interleukins 1 and 6 (IL-1, IL-6). Cytokine expression by monocytes is efficiently triggered by stimulation with bacterial products such as lipopolysaccharide (LPS) acting via the toll-like receptor (TLR). Bovine monocytes, as professional antigen presenting cells (APC), express relatively high levels of MHC class I and class II molecules, costimulatory molecules induced upon activation, receptors for endo- and phagocytosis, and adhesion molecules. Before entering the tissues, under the influence of certain cytokines, monocytes are capable of differentiating into macrophages or DC.

Professional APC antigen uptake and presentation of antigenic peptides on the relevant MHC molecules involve: phagocytosis, the upregulation of costimulatory and MHC molecules, a switch in chemokine receptor expression, and the secretion of cytokines. Furthermore, chemokines are regulated through the recognition of pathogens.
via receptors expressed by monocyte-derived DC leading to the generation of effector responses including T helper cell type 1 (Th1) and cytotoxic T cells (CTL) responses.

Followed by the antigen uptake, cytokine expression in monocytes is efficiently triggered by stimulation through the toll-like receptor (TLR). However, whether TLR3, 7, 8 and 9 mRNA are expressed differently in cp and ncp BVDV-infected monocytes remains unknown.

Multiple genes and proteins related to immune responses such as cell adhesion, apoptosis, antigen uptake, processing and presentation, and other acute phase response proteins are significantly compromised in BVDV infections. In this study we investigated how the two BVDV biotypes affect the expression levels of genes and proteins related to professional antigen presentation in bovine monocytes and evaluated the mechanisms of antigen uptake in BVDV-infected APC.
CHAPTER 2
REVIEW OF PERTINENT LITERATURE

2.1 Bovine Viral Diarrhea Virus

The cattle industry is a major economic force in the United States. As of July 2007, cattle totaled 104.8 million head [1]. The success of this industry depends on the control of significant disease causing agents, such as BVDV, a member of the Pestivirus genus of the Flaviviridae family. This group comprises economically important animal pathogens, such as classical swine fever virus, ovine border disease virus and BVDV. Pestiviruses are known for their ability to provoke transplacental infection, which may lead to fetal death, malformation, acute syndromes of the neonate, or immune tolerance and lifelong viral persistence, depending on the stage of gestation at which the infection takes place. Upon infection the severity of the clinical outcomes ranges from subclinical infection to acute fatal MD. Based on the presence or absence of visible cytopathological changes in the infected cell cultures BVDV is divided in two biotypes, cp and ncp [2,3].

While some of the pathogenic effects of BVDV are attributed to host responses, especially immunosuppression [4] and production of pro-inflammatory cytokines [5], other effects may be more directly caused by replicating BVDV. For example, bovine macrophages infected with a cytopathic BVDV strain were shown to release a factor
capable of priming other macrophages for apoptosis [6]. A BVDV cytopathic strain was also capable of inducing endoplasmic reticulum stress-mediated apoptosis in cells of a bovine kidney cell line, MDBK [7]. Lambot et al. showed that BVDV can directly induce apoptosis in bovine monocytes [8], and Schweizer and Peterhans demonstrated that BVDV can kill embryonic bovine turbinate cells by causing severe oxidative stress with subsequent apoptosis [9]. This is intriguing because oxidative damage is known to be a step in oncogenic transformation of cells by viruses [10].

2.2 Monocytes

Monocytes are circulating progenitors for tissue macrophages and DC. They originate in the bone marrow (BM) from a myeloid progenitor and are released in peripheral blood [11,12]. Monocytes vary in size and have different degrees of granularity [11]. Based on the differential expression of CD14 and CD16 humans monocytes are divided into two subsets: CD14hiCD16– cells and CD14+CD16+ cells [11,13]. Like in humans, two subsets in mice are subdivided according to their expression of CCR2, CD62L (L-selectin) and CX3CR1 [11,14]. The study of monocytes is extending to larger mammalian models because of the mice system limitations such as the availability of cells and the accessibility of physiological systems [11]. Monocyte heterogeneity was shown in rat and pig systems each of which also have two subsets according to the different phenotypes [15-18].
Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, express a range of chemokine receptors, present antigens to immunocompetent cells and produce cytokines, including tumor necrosis factor α (TNF-α) and interleukins 1 and 6 (IL-1, IL-6) [12,19-22]. As previously mentioned, monocytes are the precursor of myeloid DC. Experimental evidence showed the DC system comprises a large collection of subpopulations, each with their own functions, located in both lymphoid and non-lymphoid organs with their own functions [23]. DC are a unique population of APC, which initiate and control innate and specific T and B cell-mediated immune responses and serves as a link between innate and adaptive immunity [24]. Furthermore, a new population of DC that provides tight regulatory mechanisms for T cell activation was discovered recently [25]. DC are also important producers of IFN type 1, TNF-α, interleukin 1β (IL-1β), IL-12 and IL-2; the cytokines that provide early activation cues for NK cell activation, CD40-independent isotype switch in B cells, and Th1/Th2 type CD4+ T cell development [26-28]. Cells with phenotypic and functional characteristics of DC have been produced from fully differentiated bovine monocytes using granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 [28-29].

Interestingly, recent publication has shown that bone marrow-derived monocytes are also the precursor of the Langerhans cells (LC) that are the only DC of the epidermis [30].
2.3 Toll-like receptor (TLR)

Recent studies have provided important clues about the mechanisms of TLR-mediated control of innate and adaptive immunity [31-33]. TLR, which are pattern recognition receptors (PRR) members that recognize pathogen-associated molecular patterns (PAMP), are expressed in many different cell types including monocytes [32-24]. Several TLR, TLR3, 7, 8 and 9, participate in the recognition of viral components such as distinct types of virally-derived nucleic acids [33,35,36]. TLR3, 7, 8 and 9 also activate signaling cascades that result in the induction of type I IFN [33,36].

TLR 2 and TLR 4 mRNA transcripts have been detected in bovine macrophages and monocyte-derived DC by real time PCR [37]. Bovine gene, encoding the key pathogen recognition receptor, TLR 9 was characterized and mapped to bovine chromosome BTA22 and used to establish a real-time PCR quantification assay to measure the mRNA abundance of TLR9 in the bovine mammary gland [38]. No significant differences in mRNA level of TLR2, 3 and 4 were found in BVDV-infected and control macrophages [39]. However, it is still unknown whether TLR proteins are expressed differently in cp and ncp BVDV-infected antigen presenting cells (APC).

2.4 Immune response to BVDV

Both ncp and cp strains infect monocytes, macrophages and DC in vitro and in vivo [40-43]. In contrast to cp, ncp biotypes of BVDV do not induce cell death and IFN production in response to the virus or synthetic double-stranded RNA [44]. BVDV has
also been reported to modulate functions of immune cells after infection in vitro including phagocytic activity of alveolar macrophages [45]. Monocytes, but not DC, infected with both cp and ncp BVDV in vitro are compromised in their ability to stimulate allogeneic and memory CD4+ T cell responses [43]. Antigen uptake is affected in monocytes in the early stage of BVDV infection during the first 24 hrs, with both BVDV biotypes [46]. Furthermore, ncp BVDV promotes the release of IL-4 and inhibits IFN-γ and IL-2 cytokine gene expression in infected CD14+ monocytes [46]. In contrast, cp BVDV maintains relatively high levels of IFN-γ and IL-2 and low IL-4 gene expression. Contrary to the substantial body of evidence for the detrimental effects of BVDV on the immune system, studies assessing the effects of BVDV on DC, the most potent APC, are scarce [24]. Cells with phenotypic and functional characteristics of DC have been produced from fully differentiated bovine monocytes using granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 [28,29].

2.5 DDF and MudPIT

Recently, the bovine genome was sequenced and annotated (http://www.hgsc.bcm.tmc.edu/projects/bovine/). The complete annotation of the bovine genome allows reliable protein identification by MS² and greatly facilitates proteomics.

Once the proteins have been identified and characterized, it will then be possible to address the modulation in expression of these proteins in the process of conversion of monocytes to macrophages or DC by cytokines or infectious agents. DDF of whole cells
yields four electrophoretically distinct fractions enriched in cytosolic, membrane-organelle, nuclear membrane and cytoskeletal-matrix markers, respectively [47]. Furthermore, MudPIT couples two dimensional (2D) chromatography of peptides with tandem mass spectrometry (MS²), allowing for the identification of proteins from highly complex mixtures [48]. The combination of DDF-MudPIT was used to increase the proteome coverage over that of standard DDF and MudPIT [49,50].

Recently, we reported that DDF of bovine monocytes yielded four electrophoretically distinct fractions enriched in cytosolic, membrane-organelle, nuclear membrane and cytoskeletal-matrix markers, respectively [50]. Protein quantitation can be done by calculating a cross correlation (Xcorr) of experimental tandem mass spectra to in silico generated tandem mass spectra from sequence database [51]. This label-free method allows relative quantitation of proteins without compromising proteome coverage [51].
2.6 References


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CHAPTER 3
DIFFERENTIAL DETERGENT FRACTIONATION FOR NON-ELECTROPHORETIC
BOVINE PERIPHERAL BLOOD MONOCYTE PROTEOMICS REVEALS
PROTEINS INVOLVED IN PROFESSIONAL
ANTIGEN PRESENTATION

3.1 ABSTRACT

Professional antigen presenting cells (APC), dendritic cells (DC) and their
myeloid progenitors, monocytes/macrophages are critical controllers of innate and
adaptive immunity. Here we show that DDF analysis of bovine monocytes reveals
proteins related to antigen pattern recognition, uptake and presentation to
immunocompetent lymphocytes. We identify 53 bovine proteins involved in immune
function of professional APC. In particular, 13 adhesion molecules, 3 toll-like receptors
(TLR1, 6 and 8), 3 antigen uptake-related proteins (including mannose receptor [MR]
precursor), and 8 actin-like proteins involved in active endocytosis were identified.

In addition, MHC class I- and II- related proteins, cytokines, active substances and growth factors have been identified. We conclude that the DDF approach can provide interpretable and meaningful functional information concerning protein expression profiles associated with monocyte activation, transformation into macrophages and/or immature DC, and maturation of monocyte-derived DC in the presence of multiple bovine pathogens.

3.2 INTRODUCTION

Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells and produce cytokines, including tumor necrosis factor α (TNF-α) and interleukins 1 and 6 (IL-1, IL-6). Cytokine expression by monocytes is efficiently triggered by stimulation with bacterial products such as lipopolysaccharide (LPS) acting via the TLR [1-4]. Bovine monocytes, as professional APC, express relatively high levels of MHC class I and class II molecules, costimulatory molecules induced upon activation, receptors for endo- and phagocytosis, and adhesion molecules [5]. Recent data suggest that bovine monocytes are capable of directly inducing immunoglobulin (Ig) secretion in activated bovine peripheral blood B cells [5, 6] as was previously demonstrated in humans [7, 8]. Before entering the tissues, under the influence of certain cytokines, monocytes are capable of differentiating into macrophages or DC [9]. DC, highly specialized and the most powerful APC, are pivotal in immune responses, and are
considered to be terminally differentiated cells derived from hematopoietic progenitors or monocytes [10, 11]. Cells with phenotypic and functional characteristics of DC have been produced from fully differentiated bovine monocytes using granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 [12, 13].

Recent studies have provided important clues about the mechanisms of TLR-mediated control of adaptive immunity orchestrated by DC populations [14]. Professional APC antigen uptake and presentation of antigenic peptides on the relevant MHC molecules involve: phagocytosis, the upregulation of costimulatory and MHC molecules, a switch in chemokine receptor expression, and the secretion of cytokines [14-16]. Furthermore, chemokines are regulated through the recognition of pathogens via receptors expressed by monocyte-derived DC leading to the generation of effector responses including T helper cell type 1 (Th1) [15] and cytotoxic T cells (CTL) responses [16]. Activation of monocyte-derived DC through TLR modulated the expression of a common set of genes in response to different pathogens [14]. After pathogen recognition, DC downregulate mRNA levels for mannose receptor (MR) [17] and upregulate mRNA for the pro-inflammatory cytokines TNF-α, IL-1β and chemokines IL-8 and MIP-1α [18]. In the later stages of inflammation, the pathogen-induced differentiation and maturation of DC from monocytes becomes more apparent with the expression of chemokine receptors CCR7 and CXCR4, and cell-adhesion proteins integrin, LFA-1 and E-cadherin which are required for migration to secondary lymphoid tissues [19, 20]. Finally, in the secondary lymphoid tissues, mature DC express genes associated with T cell activation
and differentiation [19, 20]. The mRNA levels for CD80, MHC, as well as components of the proteosome Imp7 and Tap1 are upregulated at the mature DC stage [20-22].

Given the disconcordance between mRNA and protein levels [23], we have chosen to identify proteins in subcellular fractions of bovine monocytes using a proteomics-based approach. Once the proteins have been identified and characterized, it will then be possible to address the modulation in expression of these proteins during the conversion of monocytes to macrophages or DC by cytokines or infectious agents.

DDF of whole cells yields four electrophoretically distinct fractions enriched in cytosolic, membrane-organelle, nuclear membrane and cytoskeletal-matrix markers, respectively [24]. Furthermore, multidimensional protein identification technology (MudPIT) couples 2 dimensional (2D) chromatography of peptides with tandem mass spectrometry (MS²), allowing for the identification of proteins from highly complex mixtures [25]. The combination of DDF-MudPIT was used to increase the proteome coverage over that of standard DDF and MudPIT [26]. Here we provide the baseline information on expression of protein profiles related to immune function, in particular, professional antigen presentation, in bovine peripheral blood monocytes.
3.3 MATERIALS AND METHODS

3.3.1 Animals

Two conventionally reared and healthy Jersey cattle from the Mississippi State University Dairy Facility (Mississippi State, MS) were used. The Mississippi State University Institutional Animal Care and Use Committee approved all animal use.

3.3.2 Cell preparation

Bovine peripheral blood mononuclear cells (PBMC) were separated as described elsewhere [12, 13, 27]. Briefly, PBMC were isolated using Histopaque gradients (1.077g/ml, Amersham Biosciences) and resuspended in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Gibco Life Technologies), $5 \times 10^{-5}$M 2-mercaptoethanol and 100 IU/ml gentamicin (Gibco Life Technologies). Monocytes were separated from PBMC as described elsewhere [5]. Briefly, sheep red blood cells were used to separate E-rosette negative and positive fractions of PBMC. To isolate monocytes, the E-rosette negative fractions of PBMC were incubated for 2hr at 37°C and after removing non-adherent populations (mostly B cells), adherent cells were incubated with mAbs to CD14 (MM61A, VMRD) followed by the addition of magnetic beads conjugated with mouse anti-IgG1 (Miltenyi Biotech, Auburn, CA) [12, 27]. CD14$^+$ monocytes were positively selected by using magnetic cell separation technique according to the manufacturer’s instructions (Miltenyi Biotech). CD14$^+$ monocytes were
obtained from two animals and stored at –70°C separately. The bleeding procedures for the same animals have been repeated until the desirable number of $2 \times 10^7$ cells was achieved. Then all the samples were thawed and mixed followed by immediate protein separation procedure.

### 3.3.3 Protein extraction, trypsin digestion and 2D-LC ESI MS$^2$

Proteins were isolated using DDF as described [26]. Briefly, DDF sequentially extracts proteins from cellular compartments using a series of detergents. Cytosolic proteins were isolated by repeated washes in digitonin buffer. After the digitonin washes, the isolation of membrane, nuclear and cytoskeletal proteins were performed with triton X-100 (TX), deoxycholate (DOC), Tween 40, and SDS buffers, respectively. To evaluate the quality of isolated proteins, 1% of the protein samples were compared using 10% SDS-PAGE. For each of the detergent fractions, equal amounts of protein were precipitated with 25% trichloroacetic acid to remove salts and detergents. Protein pellets were solubilized and then digested with 100 ng of trypsin (50:1 ratio of substrate to enzyme) overnight at 37°C. Peptides were desalted using a peptide microtrap (Michrom BioResources, Inc.) and eluted by a 0.1% triflouroacetic acid, 95% acetonitrile solution. Desalted peptides were dried and resuspended in 0.1% formic acid.

2D LC ESI MS$^2$ was done as described elsewhere [26]. Briefly, LC analysis was accomplished by strong cation exchange (SCX) followed by reverse phase (RP) liquid chromatography (LC) coupled directly in line with electrospray (ESI) ion trap MS.
Samples were loaded into a LC gradient ion exchange system including a Thermo Separations P4000 quaternary gradient pump (ThermoElectron Corporation) coupled with a 0.32 x 100 mm BioBasic SCX column. A flow rate of 3µL/min was used for both SCX and RP columns. A salt gradient was applied in steps of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 57, 64, 71, 79, 90, 110, 300, and 700 mM ammonium acetate in 5% acetonitrile, 0.1% formic acid and the resultant peptides were loaded directly into the sample loop of a 0.18 x 100 mm BioBasic C18 RP LC column of a Proteome X workstation (ThermoElectron). The RP gradient used 0.1% formic acid in acetonitrile and increased the acetonitrile concentration in a linear gradient from 5% to 30% in 30 min and then 30% to 65% in 9 min followed by 95% for 5 min and 5% for 15 min. The LC peak ion chromatograms of 4 bovine monocyte DDF are presented as supplementary data on line (see Appendix A).

The spectrum collection time was 59 min for every SCX step. The LCQ Deca ion trap mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by alternating between a single full MS scan followed by three tandem MS scans on the three most intense precursor masses from full scan. The collision energy was normalized to 35%. Dynamic mass exclusion windows were set at 2 min, and all of the spectra were measured with an overall mass/charge (m/z) ration range of 200 – 2000. Shown in Figure 1 is one example of a MS chromatogram (Fig. 3.1 A) and MS² spectrum (Fig. 3.1 B) for MHC class I heavy chain peptide, GI# 1864812.
3.3.4 Protein identification and analysis

Proteins were identified and analyzed as previously described [26]. The non-redundant protein database (DB) downloaded from the National Center for Biotechnology Information (NCBI; 3/29/05) by TurboSEQUEST (Bioworks Browser 3.1; ThermoElectron) was used to create a bovine subset (bovine DB; search terms: *bos taurus* and *bos indicus*). Trypsin digestion was applied *in silico* to bovine DB and mass changes due to cysteine carbamidomethylation and methionine oxidation were included. The bovine DB was used to search tandem MS using peptide (MS precursor ion) mass tolerance of 1.5 Da and a fragment ion (MS²) mass tolerance of 1.0 Da. Peptide matches were considered genuine if they were ≥ 6 amino acids with a X correlation values of 1.5, 2.0 and 2.5 (+1, +2, and +3 ions respectively) and ∆Cn (≥ 0.1) values [28]. Each protein was classified with respect to its biological process using gene ontology (GO) annotation [29]. GO: biological process was determined for each protein by searching the UniProt database (http://www.pir.uniprot.org/).

3.4 RESULTS AND DISCUSSION

3.4.1 Subcellular fractionation of bovine CD14⁺ peripheral blood monocytes

A widely used 2D separation technique in proteomics has been 2D gel electrophoresis. However, this technique is limited by its inability to detect and identify low abundance proteins, proteins with extremes in isoelectric point and molecular weight
and very hydrophobic proteins [30]. To overcome these obstacles, several comprehensive
techniques have been developed [31], including MudPIT. The combination of DDF and
MudPIT allows obtaining a comprehensive cellular proteome as well as information
about a protein’s subcellular localization [26]. We identified a total of 284 proteins from
monocytes using this non-electrophoretic technique. In comparison, only 250 proteins
were identified from bovine neutrophils using 1 dimensional (1D) electrophoresis
coupled with MudPIT [32].

In our study proteins from four different subcellular fractions were obtained using
different detergent buffers. Different proteins are isolated from the different detergent
fractions, and after 8 washes with DDF buffer 1, most of the cytosolic proteins had been
extracted (Fig. 3.2).

In this study tandem mass spectra were analyzed using a bovine DB and the identified
proteins were classified according to their GO biological process (Table 3.1). We found a
total of 284 proteins of which 91 (32.0%) were not annotated and not related to the
immune function based on the available literature. Of the remaining proteins, 53 were
related to immune function (27.5%), including 13 unannotated bovine proteins that have
been assigned to this group based on the available literature in humans, mice and cattle
(Table 3.2). Proteins annotated to immune response were examined in more detail to
determine the specific biological process such as antigen uptake, processing and
presentation (Tables 3.2 and 3.3).
Information on the immunologically important proteins is summarized in Tables 3.2 and 3.3. We identified 8 categories of immune response proteins: antigen uptake; innate antigen recognition; immune response; cell proliferation; adhesion, integrin-mediated signal pathway; actin cytoskeleton organization and biogenesis; antigen presentation, immune response; response to unfolded protein (Tables 3.2 and 3.3). Most of the proteins involved in immune function were found in the TX fraction (62.3%) (Fig. 3.2).

Overall, 13 bovine proteins related to cell adhesion, 3 TLR proteins (bovine TLR1, 6, 8) involved in innate antigen pattern recognition, 3 proteins related to antigen uptake including the MR precursor, and 8 actin-related proteins involved in active endocytosis in monocytes/macrophages and immature DC were identified (Table 3.2). In addition, a total of 9 MHC class I- and II-related proteins were identified in bovine monocytes (Table 3.3). Finally, cytokines, including active substances and growth factor proteins were also found in bovine CD14⁺ monocytes. Namely, interferon-γ (IFN-γ), transforming growth factor beta 1 precursor, vascular endothelial growth factor C and heat shock protein (HSP) have been identified by DDF-MudPIT (Table 3.2).

3.4.2 Proteins involved in antigen uptake, processing and presentation identified by DDF

Different mechanisms of antigen uptake are used by different APC types and determine their relative efficiency in priming MHC restricted T cells [27, 33]. Capture of
antigens by surface receptors, such as IgR, FcR or MR allows efficient delivery of antigen to the processing compartment via receptor-mediated endocytosis [34]. Recently, we have identified MR as the important receptor involved in endocytosis in bovine monocytes [27]. We have also demonstrated that antigen uptake through MR-mediated endocytosis, an important APC function, is affected in monocytes in the early stage of Bovine Viral Diarrhea Virus (BVDV) infection during the first 24 hrs, with both BVDV biotypes [27]. Endocytosis can occur by several mechanisms, in particular, clathrin-, non-coated pit-, caveolar-, membrane ruffles-dependent and others [34]. In our research, we identified 3 proteins related to antigen uptake: the galactose binding lectin, MyD-1, and MR precursor (Table 3.2). In addition, we identified 8 proteins related to cytoskeleton such as actin, beta actin and gamma actin (Table 3.2). Previous data in humans suggest that lectins, in particular galectins (galactose binding lectins) and their ligands, regulate immune cell homeostasis whereas some members of this family, such as galectin-3, behave as amplifiers of the inflammatory cascade [35]. MyD-1, found in bovine monocytes, macrophages, granulocytes, and a subset of afferent lymph veiled cells, plays a role in regulation of the innate immunity in humans by pathogen products such as LPS, purified protein derivative and zymosan [36, 37]. MR, a C-type lectin containing carbohydrate recognition domains with broad specificity for sugars, mediates phagocytosis of mannose-coated particles, such as yeast and endocytosis of mannosylated glycoproteins in macrophages and immature DC [38- 41]. MR can be viewed as a pattern recognition molecule that can provide APC with some capacity of self non-self
discrimination at the level of antigen uptake on the basis of glycosylation and perhaps hydrophobicity [27, 38]. Antigens that fail to bind to cell surface receptors can still be taken up by micro- and macropinocytosis [42]. While micropinocytosis occurs constitutively in all cells, macropinocytosis is limited to few cell types, such as macrophages and endothelial cells stimulated by growth factors [43-45]. In this study, we found 2 growth factors that could function in antigen uptake: transforming growth factor beta 1 precursor and vascular endothelial growth factor C (Table 3.2).

One of the most important functions in professional APC is an innate form of antigen recognition [46]. TLR recognize specific molecular patterns that are present in microbial components, and trigger DC maturation. Mature human DC induce costimulatory molecule expression and increase antigen presenting capacity [46]. Therefore, TLR help to direct adaptive immune responses to antigens derived from microbial pathogens [46]. Stimulation of different TLR induces distinct patterns of gene expression which both lead to the activation of innate immunity and instruct the development of antigen specific acquired immunity in humans [47]. In this study, 3 bovine TLR were identified: TLR 1, 6 and 8 (Table 3.2). In addition, cathelicidin [48] and fetuin [49], which are involved in innate antigen recognition, were also identified (Table 3.2). Several reports that characterize different TLR are available. In particular, it is known that TLR 1 recognizes triacyl lipopeptides of bacteria and mycobacteria, and soluble factors of Neisseria meningitis [47]. Another report suggests that diacyl lipopeptides, lipoteichoic acid and zymosan, which are originally released from
**Mycoplasma**, gram positive bacteria and fungi, respectively, are recognized by TLR 6 [47]. Finally, TLR 8 recognizes imidazoquinoline, synthetic compounds, and single-stranded RNA of viruses [47]. At present, 11 TLR have been reported in mammals [47]. Complete and partial sequences of 10 bovine TLR are available in NCBI database. TLR 2 and TLR 6 mRNA transcripts have been detected in bovine macrophages and monocyte-derived DC [4]. The bovine gene, encoding the key pathogen recognition receptor, TLR 9 was characterized and mapped to *Bos taurus* chromosome A22 and used to establish real-time PCR quantification assays to measure the mRNA abundances of TLR 9 in bovine mammary gland [50]. In this study, we identified for the first time TLR 1, 6 and 8 proteins in bovine monocytes (Table 3.2), thus expanding the knowledge about innate immune responses in cattle.

MHC genes encode class I and II molecules. Recent data indicate that MHC class I molecules interact with a range of natural killer (NK) cell receptors, in addition to CD8⁺ T cells [51]. We found 9 proteins, including MHC class I, MHC class II DQ alpha and beta, DM and DR alpha, related to antigen presentation (Table 3.3). Each of the MHC genes exists in an extremely large number of allelic variations and the exact number of alleles is not known [52]. For example, GI# 2864812 and GI# 3688211 are both MHC class I heavy chain proteins, but they arose from two different MHC class I alleles (Fig. 3.3). Both proteins shared the same peptide sequence WAALVVPSSGEEQR (Fig. 3.3). However, both MHC class I proteins had unique peptide sequences to confirm that peptides identified by DDF-MudPIT experiments arose from two different MHC class I
alleles (Fig. 3.3). The most essential features of MHC gene regulation is that the expression of these molecules is controlled by the level of their transcription, and is responsive to a number of cytokines [52]. We found the cytokine IFN-γ protein, which enhances the expression of both MHC class I and II molecules [52].

A previous study demonstrated that human monocytes and DC induce CD40 independent Ig class switching through the B cell stimulator protein (BLyS) and a proliferation-inducing ligand (APRIL) in humans [8]. Furthermore, monocytes and DC upregulate BLyS and APRIL upon exposure to IFN-α, IFN-γ or CD40 ligand [8]. In this study, we were not able to identify the molecules involved in direct monocyte-B cell interactions in cattle. Although recent data suggest that bovine monocytes and monocyte-derived DC directly regulate Ig secretion in peripheral blood B cells [5, 6, 13], the possible bovine counterparts for APRIL and BLyS have yet to be identified.

CD80 but not CD86 proteins have been detected in the TX fraction of bovine monocytes (Table 3.2). Both CD80 and CD86 proteins are crucial for professional APC to provide costimulatory signals to naïve T cells [53]. At present there is no clear model for understanding which ligands promote or inhibit specific immune responses [54]. Surface expression of bovine CD80 and CD86 molecules have been characterized in cattle by flow cytometry with marker specific antibodies in monocytes and DC [5, 13, 55]. Low abundant molecules such as CD86 are difficult to detect at the protein level because the expression of the genes encoding costimulatory molecules is tightly regulated: they are often expressed at low levels and for the short periods of time [55]. Overall, DDF-
MudPIT was very useful to detect the low abundant cytosolic proteins in our study, suggesting high sensitivity of this approach.

Some of the anti-inflammatory proteins known to be important for signaling in monocytes have not been identified in our study. Cytokines such as TNF-α, IL-1, IL-6, and IFN-α and β are difficult to detect on the protein level because they are often expressed at low amounts and their production is efficiently triggered with bacterial products [9]. In our study, CD14+ monocytes were obtained from healthy animals that most likely had not been challenged with bacterial infection at the time of blood drawing. Therefore, we suggest that these soluble signaling proteins were downregulated below the detectable level under our experimental conditions.

### 3.4.3 Proteins related to cell adhesion

DC and their myeloid progenitors, monocytes and macrophages, serve as a bridge between the innate and adaptive immune systems and are critical in host protection against infectious agents. These professional APC can contribute to local sites of inflammation where, after being activated by pathogenic molecules, they uptake antigens and/or migrate to secondary lymphoid organs [52]. Adhesion molecules play an important role in the migration of monocytes from blood to the tissues and from tissues to the lymph nodes [56]. This migration is mediated by adhesive interactions between leukocytes and vascular or lymphatic endothelium [56]. We have identified 12 proteins related to adhesion: β integrins, CD18, 34, 44 and 46, metalloproteinase, vascular smooth
Integrins were named for their ability to link the extracellular and intracellular skeletons [57]. In our study two integrins, β1 and β2 have been identified in the detergent fractions (Table 3.2). In bovine system, β1 integrin was characterized in bovine trophoblasts by mRNA PCR and immunohistochemistry with anti-β1 integrin specific mAbs [58]. In addition, specific mAbs were used to detect bovine β2 integrin expressed in neutrophils [59]. Recent work has revealed that the integrin-actin cytoskeleton connection is highly dynamic and is a subject to many regulatory processes [60]. For example, loss of β1-integrin on keratinocytes leads to impaired as well as non-directed migration resulting in severely delayed re-epithelialization [60]. Importantly, the CD18 antigens and β2-integrins comprise a family of three closely related cell surface glycoproteins with CD11 α- and CD18 β-chains [61]. Four additional CD molecules related to cell adhesion were identified in this study (Table 3.2). One of them, CD34, a surface expressed glycoprotein [62], was detected in membrane fraction (Table 3.2). Bovine CD34 was characterized in bone marrow cells based on the CD34 mRNA level [63]. In this study, CD34 protein was detected and characterized in bovine monocytes for the first time. In humans CD34 plays a role in adhesion to the stromal microenvironment and may be involved in cell transformation [62]. Other members of the adhesion protein family identified in this study were CD44, CD46, and CD23 (Table 3.2). Previously,
CD44 cDNA was detected in bovine [64]. CD44 is a surface receptor that has been implicated in cell adhesion to a variety of matrix components and may be associated with cytoskeletal proteins and intracellular signal pathways [65]. CD46 is a complement regulatory protein that also functions as the cellular receptor in humans [66]. Recent studies indicate that CD46 may possess signaling functions that modulate cellular response [66]. In addition, cross-linking of CD46 with antibody or with the complement activation product C3b inhibited IL-12, which is critical for the generation of cell mediated and humoral immunity, by providing a regulatory link between the complement system and cellular immune responses [66]. Importantly, CD46 may act as a cellular receptor for the BVDV strains, in particular, the cytopathic strain NADL in cattle [67]. Finally, CD23 (FcεRII), the low-affinity receptor for IgE is expressed on the surface of B cells prior to the isotype switching as well as on monocytes, eosinophils and platelets [67]. In cattle, CD23 plays a role in IgE associated immune responses, including allergies [68]. However, it may also be important in endocytosis and adhesion because of its relation to the lectin family, which is involved in endocytosis and antigen presentation [69]. Interestingly, metalloproteinase was the one adhesion protein found in digitonin fraction (Table 3.2). Metalloproteinase is critical in pathological processes that depend on dysregulated cell-cell and cell-matrix interactions in monocytes [69]. Metalloproteinase was also assessed by Northern analysis and Western blotting in bovine brain [70]. Our study confirmed that bovine monocytes also expressed the metalloproteinase protein. Furthermore, two spondins in bovine system, involved in protein-protein interactions,
SCO-spondin [71] and VSGP/F-spondin [72], were identified in our study (Table 3.2). VSGP/F-spondin is a major factor for vascular SMC proliferation promoting factor, and vWF is a multimeric adhesive glycoprotein with cystein-rich domains [72, 73]. Finally, we found plakoglobin (γ-catenin), a close homolog of β-catenin, in the membrane protein fraction (Table 3.2). Plakoglobin shares some of the same functions of β-catenin [74]. Plakophilin, the member of plakoglobin family, was examined in various tissues and cell cultures including bovine with specific antibodies and cDNA probes [75].

In conclusion, the data presented in this manuscript are important because the identification of the proteins present in professional APC is instrumental to understanding the mechanisms involved in the function of monocytes/macrophages and monocyte-derived DC as controllers and stimulators of T and B cell specific immune responses. In particular, the transformation of monocytes into immature DC and their further maturation to become the most powerful APC is a very critical bridge between innate and adaptive immune responses. In future studies, comparative protein profiling could be used to elucidate the mechanisms involved in pathogen interference of DC maturation to avoid effective recognition and elimination by innate and adaptive immune responses which are controlled and regulated by the most powerful professional APC, DC. Finally, the unannotated proteins will be annotated in the future with database and literature searches to identify homologs that are publicly released through AgBase [76].
3.5 REFERENCES


MyD-1 (SIRP-1α) signaling pathway that inhibits LPS-induced TNFα production by monocytes. Blood 2003;102(7):2532-2540


Figure 3.1 MS chromatogram (A) and MS$^2$ spectrum (B) for the MHC class I heavy chain peptide sequence “YLENGKDTRL”.  

The MS chromatogram was obtained from a salt step gradient of 15mM ammonium acetate in acetonitrile, 0.1% formic acid in the TX fraction. The scan number and RT of the MS$^2$ spectrum were 430 and 10.5 min respectively. The arrow on the bottom of panel A indicates RT.
Figure 3.2 Distribution of immune-related proteins identified from bovine monocyte DDF.

The MS$^2$ data from each of the DDF samples were analyzed using the bovine DB subset and the resulting proteins were classified with the respect to their subcellular location.
<table>
<thead>
<tr>
<th>GI# 2864812</th>
<th>LTTETRAGSHLRFTAYASRPGLCEPEFSVGYVD 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI# 3688211</td>
<td>MGPRAFMMLLLGALVLTETRAGSHFLRYFHATAVSRPGRELPIFITYVGYVD 50</td>
</tr>
</tbody>
</table>

**Identical residues** * identical residues
**Conserved substitutions** * conserved substitutions
**Semi-conserved substitutions** - gaps were introduced to maximize homology

---

**Figure 3.3** The identification of different MHC class I protein alleles with peptide sequences detected in MHC class I α 1 domain.

Two MHC class I heavy chain alleles (GI# 2864812 and GI# 3688211) were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) to reveal the allelic differences between the proteins. MHC class I peptide sequences identified in bovine monocytes by DDF-MudPIT are marked with gray background (GI# 2864812 – dark gray, GI# 3688211 – light gray).

* identical residues
: conserved substitutions
. semi-conserved substitutions
- gaps were introduced to maximize homology
Table 3.1 Proteins related to major cellular functions identified in bovine monocytes by DDF-MudPIT.

<table>
<thead>
<tr>
<th>Biological functions</th>
<th>Number of protein</th>
<th>DDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins related to immune functions</td>
<td>53</td>
<td>D/T/TD/S</td>
</tr>
<tr>
<td>Sodium ion transport</td>
<td>2</td>
<td>T</td>
</tr>
<tr>
<td>Protein transport</td>
<td>1</td>
<td>T</td>
</tr>
<tr>
<td>Thyroid hormone generation; transport</td>
<td>1</td>
<td>T</td>
</tr>
<tr>
<td>ATP synthesis coupled proton transport</td>
<td>14</td>
<td>T/TD/S</td>
</tr>
<tr>
<td>Lipid transport</td>
<td>5</td>
<td>T</td>
</tr>
<tr>
<td>Oxygen transport</td>
<td>7</td>
<td>T</td>
</tr>
<tr>
<td>Electron transport</td>
<td>27</td>
<td>T/TD/S</td>
</tr>
<tr>
<td>Protein folding</td>
<td>9</td>
<td>T/TD</td>
</tr>
<tr>
<td>Lactose biosynthesis</td>
<td>14</td>
<td>D/T/TD</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
<td>12</td>
<td>T</td>
</tr>
<tr>
<td>Cytochrome-c oxidase activity</td>
<td>6</td>
<td>T/TD/S</td>
</tr>
<tr>
<td>Nucleosome assembly</td>
<td>5</td>
<td>T/TD/S</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>10</td>
<td>D/T/TD</td>
</tr>
<tr>
<td>Proteolysis and peptidolysis</td>
<td>2</td>
<td>T/TD</td>
</tr>
<tr>
<td>Gene regulation</td>
<td>7</td>
<td>T/TD</td>
</tr>
<tr>
<td>Blood coagulation</td>
<td>3</td>
<td>T/S</td>
</tr>
<tr>
<td>Protein amino acid dephosphorylation</td>
<td>4</td>
<td>D/T/TD/S</td>
</tr>
<tr>
<td>Regulation of apoptosis</td>
<td>1</td>
<td>T</td>
</tr>
<tr>
<td>Muscle contraction</td>
<td>3</td>
<td>T/TD/S</td>
</tr>
<tr>
<td>Metabolism</td>
<td>7</td>
<td>T</td>
</tr>
<tr>
<td>Unannotated</td>
<td>91</td>
<td>D/T/TD/S</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>284</strong></td>
<td></td>
</tr>
</tbody>
</table>

Key:
- DDF, Differential detergent fraction
- D = digitonin
- T = triton X-100
- TD = tween 40-deoxycholate
- S = sodium dodecyl sulfate
Table 3.2 Proteins related to professional antigen presentation identified in bovine monocytes by DDF-MudPIT.

<table>
<thead>
<tr>
<th>GI#</th>
<th>Protein</th>
<th>DDF</th>
<th># Peptides Identified</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>28189272*</td>
<td>Similar to galactose-binding lectin</td>
<td>T</td>
<td>1</td>
<td>Antigen uptake</td>
</tr>
<tr>
<td>2842388*</td>
<td>MyD-1 antigen</td>
<td>T/TTD</td>
<td>4</td>
<td>Antigen Uptake</td>
</tr>
<tr>
<td>163336</td>
<td>Mannose-6-phosphate receptor precursor</td>
<td>T</td>
<td>3</td>
<td>Antigen Uptake</td>
</tr>
<tr>
<td>48995341*</td>
<td>Toll-like receptor 1</td>
<td>T</td>
<td>1</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>44902491*</td>
<td>Toll-like receptor 6</td>
<td>T</td>
<td>1</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>61873594*</td>
<td>Similar to Toll-like receptor 8, partial</td>
<td>T/TTD/S</td>
<td>4</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>463</td>
<td>Cathelicidin</td>
<td>T</td>
<td>2</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>344</td>
<td>Fنم and light chain A</td>
<td>T</td>
<td>1</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>508</td>
<td>Clathrin light chain B</td>
<td>TD</td>
<td>1</td>
<td>Innate antigen recognition</td>
</tr>
<tr>
<td>28195120</td>
<td>Gamma interferon</td>
<td>T</td>
<td>1</td>
<td>Immune response</td>
</tr>
<tr>
<td>61864555</td>
<td>Predicted: Similar to CD80 antigen</td>
<td>T</td>
<td>1</td>
<td>Immune response</td>
</tr>
<tr>
<td>163748</td>
<td>Truncating growth factor-beta-1 precursor</td>
<td>S</td>
<td>1</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>4850314</td>
<td>Vascular endothelial growth factor C</td>
<td>TD</td>
<td>1</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>530521</td>
<td>Beta-1 integrin subunit</td>
<td>T</td>
<td>3</td>
<td>Adhesion, integrin-mediated signaling pathway</td>
</tr>
<tr>
<td>53680665</td>
<td>Integrin beta-2</td>
<td>T</td>
<td>3</td>
<td>Adhesion, integrin-mediated signaling pathway</td>
</tr>
<tr>
<td>162819</td>
<td>Antigen CD18</td>
<td>T</td>
<td>18</td>
<td>Adhesion, integrin-mediated signaling pathway</td>
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<tr>
<td>4996449</td>
<td>CD34</td>
<td>T</td>
<td>1</td>
<td>Adhesion, integrin-mediated signaling pathway</td>
</tr>
<tr>
<td>3006953*</td>
<td>Brain microvascular membrane cofactor protein CD46</td>
<td>T</td>
<td>2</td>
<td>Adhesion, integrin-mediated signaling pathway</td>
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<tr>
<td>37811992*</td>
<td>CD46 splice variant</td>
<td>T</td>
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<td>1048411</td>
<td>Metalloproteinase</td>
<td>D</td>
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<tr>
<td>1120818</td>
<td>VSGP/F-spondin</td>
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<td>20145864</td>
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<td>Adhesion, integrin-mediated signaling pathway</td>
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<td>6707484*</td>
<td>Low-affinity IgE receptor (CD23)</td>
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</tr>
<tr>
<td>162506</td>
<td>Actin</td>
<td>T/TTD/S</td>
<td>11</td>
<td>Actin cytoskeleton organization and biogenesis</td>
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<tr>
<td>2239063</td>
<td>Actin-binding protein CP3</td>
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Key:
GI#, Genbank accession number
DDF, Differential detergent fractionation
D = Digitonin
T = Triton X-100
TD = Tween 40-deoxycholate
S = Sodium dodecyl sulfate
* Proteins not annotated with GO biological process
Table 3.3 Proteins related to MHC class I and II alleles identified in bovine monocytes by DDF-MudPIT.

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<th>DDF</th>
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Key:
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- DDF, Differential detergent fractionation
- D = digitonin
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CHAPTER 4

BOVINE VIRAL DIARRHEA VIRUSES MODULATE TOLL-LIKE RECEPTOR, CYTOKINES AND CO-STIMULATORY MOLECULES GENES EXPRESSION IN BOVINE PERIPHERAL BLOOD MONOCYTES

4.1 ABSTRACT

We have used noncytopathic (ncp) and cytopathic (cp) Bovine Viral Diarrhea Viruses (BVDV) to determine the expression levels of TLR genes, type I IFN, pro-inflammatory and Th1/Th2 cytokine gene expression in bovine monocytes. In general, both BVDV strains had similar effects. However, we found some significant differences that could be due to biological differences between cp and ncp BVDV strains. TLR3 was significantly up-regulated in 1 h ncp, but not in cp BVDV- infected monocytes, whereas TLR7 expression dominated in 24 h infection with both BVDV strains. Type I IFN and IL-12 gene expression was also significantly up-regulated in 1 h ncp, but not cp BVDV.

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infection that correlated with the enhanced TLR3 gene expression. Both BVDV biotypes suppressed pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, co-stimulatory molecules CD80 and CD86, but did not change Th1 type cytokine IL-12 and INF-γ, gene expression after 24 h infection. We hypothesize that BVDV may escape immune responses by altering the expression of TLR 3 and 7 and their signaling pathways.

4.2 INTRODUCTION

Bovine viral diarrhea virus (BVDV), a single-stranded RNA virus, Pestivirus genus, and Flaviviridae family, infects a large proportion of cattle worldwide and causes a number of clinical forms of the disease ranging from transiently detectable mild clinical symptoms to a fatal diarrheic condition known as mucosal disease [1,2]. The BVDV are actually several viruses with antigenic and genotypic differences, along with different growth patterns [1,2]. According to their effect in cell cultures, BVDV is divided in two biotypes: noncytopathic (ncp), which is widely distributed, and cytopathic (cp), which is associated predominantly with animals that develop mucosal disease [3,4].

Recent studies have provided important clues about the mechanisms of toll-like receptor (TLR)-mediated control of innate and adaptive immunity [5-7]. The TLR function as pattern recognition receptors (PRR) and recognize a diverse array of pathogen-associated molecular patterns (PAMP). TLR are expressed in many different cell types, including monocytes and myeloid progenitors of dendritic cells (DC) [5,7,8]. Several TLR, such as TLR3, 7, 8 and 9, participate in the recognition of viral components.
and distinguish between different forms of viral nucleic acids [7,9,10]. In particular, TLR3 can recognize double-stranded RNA (dsRNA) produced in the course of a viral infection [11,12]. Signaling through TLR generally culminates in the production of pro-inflammatory cytokines, including type 1 IFN, chemokines and co-stimulatory molecules that activate adaptive T cell-mediated immune responses [7]. Importantly, TLR3, 7, 8 and 9 activate signaling cascades that result in the induction of type I IFN and inflammatory cytokines [7,10].

Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells, and produce cytokines. In previous studies we assessed selective (mannose receptor-mediated endocytosis) and non-selective (macropinocytosis) antigen uptake mechanisms in BVDV-infected monocytes and outlined some similarities and differences between cp and ncp BVDV biotypes [13]. Followed by the antigen uptake, cytokine expression in monocytes is efficiently triggered by stimulation through the TLR [14]. In a previous report evaluating the role of TLR in BVDV infection, no significant differences in mRNA levels of TLR2, 3 and 4 were found in BVDV-infected and control macrophages [15]. However, whether TLR7, 8 and 9 mRNA are expressed differently in cp and ncp BVDV-infected monocytes, remains unknown. In this study, we assessed the expression levels of several TLR genes that are able to promote signaling cascades in response to viruses, pro-inflammatory cytokine and Th1/Th2 cytokine genes that are regulated through TLR-dependent mechanisms in humans and mice [7-12].
4.3 MATERIALS AND METHODS

4.3.1 Animals

Four conventionally reared, healthy BVDV-free cows from a Holstein herd at the Mississippi State University Dairy Facility were used. The animals have been subjected to a comprehensive vaccination program, including Frontier 4 Plus Vaccine (IBR, BVD, PI3, RSV, Diamond Animal H, Inc). All animal use was approved by The Mississippi State University Institutional Animal Care and Use Committee.

4.3.2 Cell preparation

Blood samples (150 ml) were collected into Blood Collection Tubes (16 x 100 mm, Tyco Healthcare) by jugular venipuncture. Bovine peripheral blood mononuclear cells (PBMC) were separated as described elsewhere [13,16-18]. Briefly, PBMC were isolated using Histopaque gradients (1.077g/ml, Amersham Biosciences) and resuspended in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Invitrogen), 5 x 10^{-5}M 2-mercaptoethanol and 100 IU/ml gentamicin (Invitrogen). Monocytes were separated from PBMC as described elsewhere [16]. Briefly, 40 ml of PBMC suspension (5 x 10^8 cells) was added to Petri-dish (150 x 25 mm, BD sciences) for 2 h at 37 °C. Non-adherent cells were removed and the adherent cells were washed twice in PBS (Gibco Life Technologies). The yield of adherent cells was 20-30% of total PBMC number. After removing non-adherent populations (mostly B cells), adherent cells were incubated...
with mAbs to CD14 (MM61A, VMRD) followed by the addition of magnetic beads conjugated with mouse anti-IgG1 (Miltenyi Biotech, Auburn, CA) [13,16-18]. CD14+ monocytes were positively selected by using magnetic cell separation technique according to the manufacturer’s instructions (Miltenyi Biotech). The final yield of bovine monocytes was 2-3% of total PBMC number.

4.3.3 TLR agonists and stimulation

Lipopolysaccharide (LPS, 100ng/ml) from *Escherichia coli* strain 055:B5 (Sigma), Polyinosinic-polycytidylic acid potassium salt (Poly IC, 100µg/ml) (Sigma) and Peptidoglycan (PGN, 10µg/ml) from *Bacillus subtilis* (Sigma) were used as controls in the TLR gene expression experiments. 5 x 10⁶ monocytes were added to each well of a 6 well tissue culture plate and incubated with TLR ligands for 1 and 24 h at 37ºC.

4.3.4 BVDV stocks and infection

BVDV strains were prepared as described elsewhere [13]. Briefly, the NADL (cp) and New York 1 (NY, ncp) strains of BVDV were obtained from the American Type Culture Collection (ATCC) and amplified by growth in the bovine turbinate (BT) cell line (ATCC) according to the manufacturer’s handling procedures. For infection of BT cells, virus dilutions were made in DMEM with 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 10% horse serum. To measure the infectivity of the NADL strain, the quantal method of Reed and Muench was performed and the tissue culture infectious dose 50 (TCID₅₀) was determined. For the ncp BVDV strain NY, we
used the TCID$_{50}$ suggested by the manufacturer (ATCC). To select the dose of cp BVDV that did not have a cytopathic effect on monocytes cultured for 48 h we assessed the viability of the infected cells by using trypan blue and light microscopy. BVDV strain NADL at the multiplicity of infection (MOI) 0.002 had not affected the viability of bovine monocytes after 48 h of infection (data not shown). 5 x 10$^6$ monocytes were added to each well of a 6 well tissue culture plate and infected with cp and ncp BVDV at the same MOI of 0.002 for 1 and 24 h. The production of nitrite in supernatants was determined by the Griess Reagent System (Promega) according to the manufacturer’s instructions (Promega). Infected and control monocytes did not produce NO (data not shown).

4.3.5 Real time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and quantified using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.). RNA samples from each animal were evaluated for the expression of BVDV E2 transcripts as described previously [13]. Expression of TLR, type I IFN, and pro-inflammatory cytokine mRNA were determined by real time RT-PCR (iCycler iQ, Bio-Rad) using bovine specific primers and probes (Table 4.1). Reaction mixtures were assembled in optical 96 well plates using the SuperScript™ III Platinum One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). Amplifications were performed in an iCycler iQ (Bio-Rad, Hercules, CA) programmed for an initial step of 30 min at 50°C and 10 min at 95°C,
followed by 40 or 45 cycles at 95°C for 15s and 1min at 60°C. The Ct value of each gene was normalized for differences in amount of total RNA in the reaction using the GAPDH gene as an endogenous control [19]. All data were determined using samples from 4 animals, and measurements were performed in duplicate. Relative quantification of gene expression was determined by the standard curve method (ABI PRISM 7700 sequence detection system, user bulletin #2). Results were expressed as fold differences relative to the calibrator level of the uninfected group.

4.3.6 Western blot

Protein isolation and western blot analysis were performed as described elsewhere [20]. Briefly, bovine monocyte whole cell lysates were prepared immediately after 24 h of infection in radioimmunoprecipitation assay (RIPA) butter. The concentration of proteins was measured with 2-D Quant Kit (Amersham Biosciences) and 5µg samples were loaded on SDS-PAGE. Ready Gel™ blotting Sandwiches (Bio-Rad) was used for the transfer of proteins to the nitrocellulose membrane. After primary antibody labeling, goat anti-mouse Ig (H+L)-AP, human adsorbed (Southern Biotech) was used as secondary antibody. For developing the proteins labeled with antibodies, BCIP/NBT alkaline phosphatase substrate (Sigma) was used. All results were analyzed by ImageQuant TL (Amersham Biosciences, version 2005).
4.3.7 Statistical analysis

Each gene expression level normalized to internal standard was analyzed using ANOVA for a completely randomized design with sub-sampling using SAS version 8.2 (SAS institute). When significant differences among the samples were found, the means were separated using the Fisher’s Least Significance Difference (LSD). The level of significance for all tests of effects was \( P < 0.05 \).

4.4 RESULTS

4.4.1 TLR 3, 8 and 9 gene expressions are modulated with some TLR agonists in bovine monocytes

Some TLR agonists that induce potent up-regulation of TLR genes in mice and humans [11,12,21-25] were used as controls in this study (Figure 4.1). After 1 h stimulation, only the TLR8 gene was significantly induced in the presence of LPS and Poly I:C (Figure 4.1c). In addition, three different agonists showed the similar effect on the TLR gene expression after 24 h stimulation (Figure 4.1a, c, d). TLR3, 8 and 9 gene expression levels were significantly down-regulated (Figure 4.1a, c, d). However, TLR 7 mRNA level didn’t show any significant difference compared to controls (Figure 4.1b).
4.4.2 BVDV strains trigger differential TLR gene expression in peripheral blood monocyte

To evaluate TLR expression in response to BVDV, real time RT-PCR was performed with mRNA isolated from bovine monocytes (Figure 4.2). TLR 3 mRNA expression level was up-regulated differentially following exposure to cp and ncp BVDV (Figure 4.2a). In particular, there was a significant increase relative to the control at 1 h post-infection with ncp ($P < 0.05$), but the increase was not significant with the cp BVDV strain (Figure 4.2a). At 24 h post-infection, TLR3 mRNA was up-regulated in monocytes infected with both BVDV strains, although both increases were not significant (Figure 4.2a). In contrast to TLR3 gene expression, the TLR7 mRNA level was not different in infected and non-infected monocytes during early infection (Figure 4.2b). However, after 24 h infection, TLR7 mRNA was significantly up-regulated in monocytes infected with both strains ($P < 0.05$) (Figure 4.2b). Overall, TLR3 mRNA expression was dominant in early infection, but by 24 h it was replaced by increased expression of TLR7 mRNA (Figure 4.2a, b). TLR8 and 9 gene expression was not affected by BVDV infection (Figure 4.2c, d).

4.4.3 Ncp BVDV infection up-regulates type I IFN gene expression in bovine monocytes

In this study, we demonstrated that IFN-α and β gene expression in vitro are induced in monocytes infected with both cp and ncp BVDV strains (Figure 4.3a, b).
Particularly, 1 h ncp BVDV infection induced significantly higher type I IFN expression in monocytes compared to 1 h cp BVDV-infected and control cells (Figure 4.3a, b). There were no significant differences in the type I IFN gene expression in 24 h cp and ncp BVDV-infected monocytes (Figure 4.3a, b).

4.4.4 BVDV infection affects pro-inflammatory cytokine gene expression in bovine monocytes

TNF-α gene expression was up-regulated in monocytes infected with cp BVDV after 1 h post-infection (Figure 4.4a). However, 1 h ncp BVDV didn’t change TNF-α expression in monocytes compared to control cells (Figure 4.4a). After 24 h infection, the level of TNF-α expression was significantly decreased in both infected groups (Figure 4.4a). Unlike TNF-α, cytokine gene expression of IL-1β and IL-6 was marginally down-regulated after 1 h infection in both cp and ncp BVDV-infected monocytes (Figure 4.4b, c). However, after 24 h infection, IL-1β and IL-6 cytokine gene expression was significantly down-regulated in monocytes infected with cp and ncp BVDV strains \( (P < 0.05) \) (Figure 4.4b, c). In addition to pro-inflammatory cytokines, IL-10 production was also significantly decreased in BVDV-infected monocytes (Figure 4.5a).

The levels of TNF-α and IL-1β proteins in BVDV-infected monocytes were analyzed by western blotting. Both pro-inflammatory cytokine proteins were detected in control and BVDV-infected monocytes (Figure 4.6). Importantly, there were apparent
decreases in the protein levels of both cytokines after 24 h cp BVDV infection and TNF-α after 24 h ncp BVDV infection (Figures 4.4, 4.6).

4.4.5 BVDV strains modulate Th1/Th2 type cytokine gene expression

In this study, IL-10 gene expression was significantly down-regulated after 24 h infection in both cp and ncp BVDV infected monocytes (Figure 4.5a). However, the IL-4 gene showed background levels of expression in all experimental and control groups (data not shown). As was expected, the IL-15 gene expression level was also significantly down-regulated after 24 h infection (Figure 4.5c). In contrast to the expression levels of Th2 type cytokines, Th1 type cytokines, IL-12 and IFN-γ, were not affected by 24 h BVDV infection (Figure 4.5b, d). Interestingly, IL-12 gene expression was significantly up-regulated in monocytes infected with ncp BVDV (Figure 4.5b).

4.4.6 BVDV infection decrease CD80 and CD86 co-stimulatory molecules expression in bovine monocytes

To determine if BVDV strains affect the professional antigen presenting function of monocytes to activate T cells, we assessed the CD80 and 86 gene expressions levels in control and infected cells (Figure 4.7). In this study, both CD80 and 86 gene expression levels were significantly down-regulated after 24 h infection compared to control and 1 h post-infected groups (Figure 4.7a, b).
4.5 DISCUSSION

TLR are expressed in many different cell types, including monocytes and myeloid progenitors of dendritic cells (DC) [5,7,8]. Several TLR, such as TLR3, 7, 8 and 9, participate in the recognition of viral components and distinguish between different forms of viral nucleic acids [7,9,10]. TLR3 responds to dsRNA, a replication intermediate among many RNA viruses, and a byproduct of transcription among DNA viruses [8,11,12]. TLR7 recognizes ribonucleic acid homologs like imiquimod and resiquimod, and synthetic single-stranded RNA (ssRNA) oligonucleotides rich in guanosine or uridine derived from ssRNA viruses [21,22]. TLR8, like TLR7, also recognizes viral ssRNA and synthetic imidazoquinolines [22]. However, bacterial and viral DNA is recognized by TLR9 [23-25]. We confirmed that innate viral recognition in cattle depends on TLR3 and 7 as was demonstrated in humans and mice [11,12,21,22,25,26]. As shown in a previous report, in the bovine system, TLR2, 3 and 4 mRNA expression in macrophages infected with BVDV was not up-regulated [15]. Moreover, TLR4 expression in vivo was high in cattle infected with foot-and-mouse disease virus and in mastitis [27,28]. In addition, TLR2 but not TLR9 expression was increased in mastitis [27]. In our study, ncp infected monocytes tended to show higher gene expression of TLR3 and 7 levels than their cp infected counterparts. Moreover, TLR3 mRNA expression was dominant in early infection, but was replaced by increased expression of TLR7 mRNA at 24 h. The data obtained in our study in general, do not contradict the previously published observation by Franchini et al. [15]. Similarly to the data reported by Franchini and colleagues [15],
we did not observe changes in TLR3 after 1 h stimulation with TLR agonists. However, our data demonstrate that after 24 h stimulation, the TLR3 gene expression level was significantly down-regulated. In our study we also evaluated the expression levels of TLR 7, 8 and 9 genes in monocytes that have been in vitro cultured for 1 h or 24 h and did not produce NO. Franchini et al. did not find the differences in the expression of TLR 3 in fully differentiated NO-producing macrophages infected with BVDV and did not assess the TLR7, 8 and gene expression [15]. Therefore, the difference in the expression of TLR3 gene could be due to the differences in the differentiation levels of monocytes and the infectious doses of BVDV that have been used. Our data suggest that TLR signaling cascades could be triggered more efficiently with lower doses of BVDV (MOI 0.002) than with much higher viral doses (MOI 10) that have been used by Franchini et al. [15]. Our data are in agreement with the previous observation that the protein expression of TLR7 was induced, whereas the level of TLR3 declined beginning 8h after infection in human corneal epithelial cells (HCEC)-infected with Herpes Simplex Virus 1 (HSV-1) [29]. After recognition of viral components, TLR initiate the production of cytokines and stimulate inflammatory and adaptive immune responses through signal transduction [5,7,10]. Type I IFN is the most important cytokine in viral infection [25]. Plasmacytoid DC are considered to be the major source of type I IFN [30]. Unlike our study, previous in vivo and in vitro studies showed that cp BVDV induced type I IFN, such as IFN-α and IFN-β, whereas ncp BVDV failed to induce it in protein level in the bovine system [31-33]. Additionally, type I IFN protein expression was not identified in bovine monocytes
infected with ncp BVDV [34]. Interestingly, recently identified plasmacytoid DC-like populations produced IFN type I *in vivo* in response to both ncp and cp BVDV [33, 34].

In this study, as was mentioned above, type I IFN cytokine gene expression was significantly up-regulated in 1 h ncp, but not cp BVDV infection suggesting TLR3-mediated control of the type I IFN production in BVDV. There were also no significant differences in the type I IFN gene expression in 24 h cp and ncp BVDV-infected monocytes.

Signaling through TLR also induces pro-inflammatory cytokine gene expression, such as TNF-α, IL-1β, and IL-6 [7]. However, many viruses have mechanisms that inhibit inflammation and prevent apoptosis and are able to establish chronic infections [7, 10, 35, 36]. Our results indicate that pro-inflammatory cytokine gene expression was significantly decreased in monocytes infected with either cp or ncp BVDV strain for 24 h. The cytokine protein expression data supported our pro-inflammatory cytokine gene expression results. In particular, the decreases in the protein levels of TNF-a and IL-1β after 24 h cp BVDV infection and IL-1β after 24 h BVDV infection with both strains correlated with significant decreases in the corresponding cytokine gene expression levels.

Our results agree with a previous study which showed that the level of TNF-α protein expression in bovine bone marrow-derived macrophages was decreased after 24 h BVDV infection and this down-regulation was more significant in ncp infected macrophages [37]. Jensen et al. demonstrated that IL-1β was down-regulated in BVDV-infected bovine monocytes at the protein level, but not at the mRNA level [38]. We
confirmed this observation by showing that IL-1β protein levels were decreased in bovine monocytes after 1 and 24 h BVDV infection. However, in our study the IL-1β gene was significantly down-regulated in 24 h BVDV-infected monocytes. Furthermore, pro-inflammatory cytokines were not induced in macrophages or mature DC, stimulated with small anti-viral component, imidazoquinoline compounds imiquimod and R-848, from TLR7–deficient mice [39]. Moreover, Chase et al. (2004) demonstrated that down-regulation of TNF-α production could be achieved by a Th2 type cytokine response, such as IL-10 [40]. In this study, we also confirmed that expression of the Th2 type cytokine genes for IL-10 and IL-15 were significantly decreased in BVDV-infected monocytes [40]. Th1 type cytokines such as IFN-γ and IL-12 and co-stimulatory molecules CD80/CD86 are essential to stimulate Th1 type cell polarization [40]. We demonstrated that CD80/CD86 molecules were significantly down-regulated after 24 h in both cp and ncp BVDV infected bovine monocytes compared to uninfected controls suggesting an impaired antigen presentation to the specific T cells. However, IL-12 and IFN-γ mRNA levels were not significantly down-regulated by 24 h post-infection. Interestingly, IL-12 was significantly up-regulated in 1 h ncp BVDV infected group showing the positive correlation with TLR3 gene expression.

Therefore, our data is in agreement with the report that antigen presentation to T helper cells was decreased in bovine monocytes infected with BVDV [34]. We also confirmed the data of Archambault et al. that B7 molecules (CD80/CD86) were down-regulated in PBMC from cattle infected with the highly virulent ncp BVDV strain [41].
In both cp and ncp BVDV-infected bovine monocytes, CD80/86 molecules were significantly down-regulated after 24 h infection in this study. However, IL-12 and IFN-γ mRNA levels were not down-regulation in both 1 and 24 h post-infection. Particularly, IL-12 was a significantly up-regulated in 1 h ncp BVDV infected group suggesting TLR3-mediated control of the IL-12 production in BVDV.

Overall, our data demonstrate that both cp and ncp BVDV strains in general had similar effects on the TLR, type I IFN, pro-inflammatory, Th1/Th2 cytokine, and co-stimulatory molecules gene expression. However, we found some significant differences in their effects that could be due to biological differences between cp and ncp BVDV strains. In particular, TLR3 was significantly up-regulated in early ncp BVDV infection, whereas TLR7 represented the majority of TLR expression in late cp and ncp infection. Type I IFN cytokine gene expression was also significantly up-regulated in early ncp, but not cp BVDV infection suggesting TLR3-mediated control of type I IFN production in BVDV. In addition, IL-12 gene expression was significantly up-regulated in early ncp BVDV infection. We can not rule out the possibility that the differences in the effects of NY and NADL were also due to their genotype differences: NY is a BVDV1b strain and NADL is a BVDV1a strain.

In conclusion, persistently infected cattle or animals with sub-clinical symptoms are the source of maintaining BVDV in nature and/or the major source of virus for other cattle [42]. Therefore, we selected two BVDV viruses that cause persistent infection or mild symptoms in infected animals but differ in their cytopathic effects as a model
system to study the differences in the TLR-mediated control of type I IFN and pro-inflammatory cytokine gene expression in bovine monocytes. We hypothesize that both cp and ncp BVDV could escape innate immune responses by modulating TLR gene expression, followed by pro-inflammatory, type I IFN, Th1/Th2 type cytokine gene expression, and decreasing the expression levels of CD80/86 in professional APC.
4.6 REFERENCES


Table 4.1 Primer and probe sets used in this study for real time quantitative RT-PCR

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Figure 4.1 Changes in expression of TLR mRNA, TLR3 (a), TLR7 (b), TLR8 (c), and TLR9 (d) in monocytes stimulated with ligands relative to that of unstimulated monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$. 
Figure 4.2 Changes in expression of TLR mRNA, TLR3 (a), TLR7 (b), TLR8 (c), and TLR9 (d) in BVDV-infected monocytes relative to that of uninfected monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$. 
Figure 4.3 Expression of Type I IFN mRNA, IFN-α (a) and IFN-β (b), in BVDV-infected monocytes relative to that of uninfected monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$
Figure 4.4 Expression levels of pro-inflammatory cytokines, TNF-α (a), IL-1β (b) and IL-6 (c), in BVDV-infected monocytes relative to that of uninfected monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$
Figure 4.5 Expression levels of Th1/Th2 type cytokines in BVDV-infected monocytes relative to that of uninfected monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$
Figure 4.6 Western blot analysis of whole cellular proteins for β-actin as housekeeping protein, TNF-α and IL-1β expression in BVDV-infected monocytes relative to that of uninfected monocytes.

Lanes: 1 molecular weight marker (Prestained SDS-PAGE standards, low range, Bio-Rad); 2, control; 3, 1 h ncp; 4, 1 h cp; 5, 24 h ncp; 6, 24 h cp. The arrow indicates the position of the molecular weight marker in each group. Anti-bovine TNF-α mAb (E10011, Endogen), anti-bovine IL-1β mAb (VMRD), and anti-β-actin mAb (AC-15, Ambion) were used to label the proteins.
Figure 4.7 Expression levels of co-stimulatory molecules (CD80/86) in BVDV-infected monocytes relative to that of uninfected monocytes.

Data are expressed as fold difference of expression compared with values obtained for control monocytes. Samples were analyzed in duplicate from three different animals, and data are expressed as mean ± SD. *, $P < 0.05$
CHAPTER 5

CYTOPATHIC AND NON-CYTOPATHIC BVDV INFECTIONS DIFFERENTIALLY AFFECT SELECTIVE AND NON-SELECTIVE ANTIGEN UPTAKE IN BOVINE MONOCYTES

5.1 ABSTRACT

In this study, we selected cytopathic (cp) and non-cytopathic (ncp) bovine viral diarrhea virus (BVDV) viruses that cause persistent infection or mild symptoms in infected animals but differ in their cytopathic effects as a model system to study the active endocytosis in bovine monocytes. We have demonstrated for the first time by using flow cytometry that bovine monocytes use macropinocytosis for a bulk-flow uptake of soluble antigens. Furthermore, we have shown that selective mannose receptor (MR)-mediated endocytosis was significantly down-regulated in monocytes after 24h of infection with both BVDV biotypes; while macropinocytosis, a potent non-selective antigen uptake, was not affected in infected antigen presenting cells (APC). Moreover, only cp BVDV inhibited macropinocytosis in 1h post infected monocytes. In contrast,

1 Lee SR, Pinchuk LM. Cytopathic and non-cytopathic BVDV infections differentially affect selective and non-selective antigen uptake in bovine monocytes. Comparative Immunology, microbiology & infectious disease. Submitted.
after 24h infection, MR-mediated endocytosis was inhibited by ncp BVDV. Therefore, we conclude that cp and ncp BVDV differentially affect an early step of professional antigen presentation in bovine monocytes that might result in variable immune responses in BVDV infection.

5.2 INTRODUCTION

Bovine viral diarrhea virus (BVDV), a single-stranded RNA virus, Pestivirus genus, and Flaviviridae family, infects a large proportion of cattle worldwide and causes a number of clinical forms of the disease ranging from transiently detectable mild clinical symptoms to a fatal diarrheic condition known as mucosal disease [1,2]. The BVDV are actually several viruses with antigenic and genotypic differences, along with different growth patterns [1,2]. According to their effect in cell cultures, BVDV is divided in two biotypes: non-cytopathic (ncp), which is widely distributed, and cytopathic (cp), which is associated predominantly with animals that develop mucosal disease [3,4]. Persistently infected cattle or animals with subclinical symptoms are the source of maintaining BVDV in nature and/or the major source of virus for other cattle [5]. BVDV does not produce respiratory disease in its own right, but it has been incriminated as a component cause of bovine respiratory disease (BRD) [6]. Morbidity and mortality from BRD and associated losses in performance and carcass continue to plague the beef cattle industry.
Several viral/bacterial agents are responsible for BRD, and interactions occur among the agents. Viral agents often predispose animals to bacterial infections [7].

Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells, and produce cytokines [8]. Different mechanisms of antigen uptake are used by different cell types and determine their relative efficiency as APC for MHC class-restricted T cells [9]. Capture of antigens by surface receptors, such as the mannose receptor (MR) family, allows efficient delivery of antigen to the processing compartment via receptor-mediated endocytosis [9,10]. MR-dependent endocytosis can be inhibited by EDTA, anti-MR mAbs or mannann, a natural ligand of MR [11]. On the other hand, antigens that fail to bind to cell surface receptors can still be taken up by fluid phase endocytosis and are presented by APC, but with a lower efficiency [12]. Fluid phase uptake can occur via distinct mechanisms: micropinocytosis and macropinocytosis [12]. Specifically, macropinocytosis is a potent non-selective mechanism of antigen uptake limited to immature DC and their myeloid progenitors, monocytes/macrophages, and is activated by exogenous stimuli [13,14]. The internalization of solutes by macropinocytosis is much more effective than by other non-selective mechanisms, in particular, by micropinocytosis mediated by clathrin-coated vesicles [13]. Unlike monocytes/macrophages, immature DC constitutively macropinocytose large quantities of exogenous solute as part of their professional antigen presenting function [14]. Recently several reports demonstrated that cells of the monocyte/DC lineage are able to
endocytose via toll-like receptor-dependent mechanisms both microbial and apoptotic cells and discriminate between the two sources of antigens for their selective presentation by MHC molecules. These data suggested that in vivo DC progenitors, monocytes, could play an important role in the sampling of the tissue microenvironment [15-18]. In addition, Walzer et al. demonstrated that poxvirus semaphoring A39R did not affect MR-mediated endocytosis of FITC-DX, but induced cytoskeletal rearrangement, blocking cellular migration and phagocytosis strongly suggesting the inhibition of macropinocytosis [14,19,20]. Cytochalasin D (CCD) inhibits macropinocytosis by blocking the formation of microfilaments and microtubules, but has no significant effect on receptor-mediated endocytosis [12].

Little is known about the selective and non-selective mechanisms of antigen uptake in cattle APC and how these mechanisms are affected in BVDV infection. Werling et al. demonstrated that macropinocytosis did not occur in bovine immature monocyte-derived DC without exogenous activation or in peripheral blood monocytes [21]. Our previous report confirmed the finding of Werling et al. that control and infected bovine monocytes did not incorporate Lucifer Yellow (LY) by macropinocytosis [22]. Unlike a potent non-selective macropinocytosis, MR-mediated endocytosis occurred in both bovine myeloid DC and monocytes [21-23]. Particularly, our previous report suggested that MR-mediated antigen uptake in monocytes was affected during the first 24h of infection with both cp and ncp BVDV biotypes [22]. To further characterize the mechanisms of antigen uptake in BVDV infected monocytes, we have used a new
approach; magnetically separated CD14\(^+\) monocytes cultured for 12h and infected with ncp and cp BVDV to determine how two biotypes affect receptor-mediated endocytosis and macropinocytosis in bovine APC utilizing FITC-DX uptake to assess MR-dependent endocytosis and LY uptake to assess MR-independent endocytosis.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Conventionally reared, healthy BVDV-free cows from a Holstein herd at the Mississippi State University Dairy Facility were used. The animals have been subjected to a comprehensive vaccination program, including Frontier 4 Plus Vaccine (IBR, BVD, PI3, RSV, Diamond Animal H, Inc). All animal use was approved by The Mississippi State University Institutional Animal Care and Use Committee.

5.3.2 Cell preparation

Bovine peripheral blood mononuclear cells (PBMC) were separated as described elsewhere [24]. Monocytes were separated from PBMC as described elsewhere [25]. CD14\(^+\) monocytes were positively selected by using magnetic cell separation technique according to the manufacturer’s instructions (Miltenyi Biotech) and cultured in TCM (RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1, 5 x 10\(^{-5}\)M 2-mercaptoethanol and 100 IU/ml gentamicin) for 12h at 37\(^\circ\)C to decrease the separation procedure-related activation of peripheral blood monocytes, followed by BVDV infection.
5.3.3 BVDV stocks and infection

BVDV strains were prepared as described elsewhere [22]. Briefly, the NADL (cp) and New York 1 (NY, ncp) strains of BVDV were obtained from the American Type Culture Collection (ATCC) and amplified by growth in the bovine turbinate (BT) cell line (ATCC) according to the manufacturer’s handling procedures. For infection of BT cells, virus dilutions were made in DMEM with 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 10% horse serum. To measure the infectivity of the NADL strain, the quantal method of Reed and Muench was performed and the tissue culture infectious dose 50 (TCID$_{50}$) was determined. For the ncp BVDV strain NY, we used the TCID$_{50}$ suggested by the manufacturer (ATCC). In addition, reverse transcriptase-PCR (RT-PCR) was performed as described below (Fig. 5.1). To select the dose of cp BVDV that did not have a cytopathic effect on monocytes cultured for 48 hr we assessed the viability of the infected cells by using trypan blue and light microscopy. BVDV strain NADL at the multiplicity of infection (MOI) 0.002 did not affect the viability of bovine monocytes after 48 hr of infection (data not shown). Monocytes were infected with cp and ncp BVDV at the same MOI of 0.002 for 1 and 24 h.

5.3.4 PCR analysis

Previously, cp BVDV infection in primary cells and BT cell line was determined [22]. In this study, total RNA was isolated from BT cell line and monocytes or BT cell line
infected with cp and ncp BVDV using the RNeasy Mini Kit (Qiagen) including the recommended DNase treatment step and quantified using the NanoDrop® ND-1000 Spectrometer (NanoDrop Technologies) to determine cp and ncp BVDV infection (Fig. 1(A), (B)). PCR analysis was performed by using the OneStep Mini Kit System (Qiagen) and 1.2% agarose gels with low mass DNA ladder (Invitrogen). PCR primers amplifying a 508bp fragment of BVDV1 NY p125 gene (access number: L35850) were used to detect ncp BVDV NY strain infection. Primer sequences were as followed: Forward: 5’-AAG AAC GAG GAG TCC AAA CCC ACA-3’ and reverse: 5’-TAG CAG TCA TGG CAA CCA CCC TTA-3’. On the other hand, cp BVDV infection in bovine monocytes was detected as described elsewhere [26]. Finally, β actin-specific primers were used as a control for RT-PCR. The temperature profile used was 40 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min.

5.3.5 Endocytosis assay

The endocytic capacities of APC were determined by using classical antigens fluorescein isothiocyanate-labeled dextran (FITC-DX) and LY [11,21-23]. The ability of monocytes to endocytose FITC-DX and LY was measured as described elsewhere [21-23]. Briefly, Over 95% pure CD14+ cells were isolated using magnetic sorting procedure [25]. Isolated CD14+ monocytes from 3 different cows were treated with FITC-DX (1 mg/ml) or LY (100 µg/ml) for 30 min at 37 °C to measure active endocytosis or at 4 °C to determine background levels of endocytosis (negative control). Monocytes were
washed three times by centrifugation in cold PBS and analyzed using a FACS Calibur as follows. After setting a gate on CD14⁺ monocytes, the FITC-DX or LY incorporation was measured and analyzed. To selectively inhibit various pathways involved in antigen uptake, bovine monocytes were incubated for 5 min in the presence of mannan (500 µg/ml) and CCD (2.5 µg/ml) prior to the addition of FITC-DX or LY. Figure 5.2 shows that a substantial population of monocytes accumulated FITC-DX or LY when incubated at 37ºC (Fig. 5.2A and 5.2B). To characterize the mechanisms of the endocytic capacity in monocytes, FITC-DX and LY uptake were measured in the presence or absence of MR-mediated endocytosis or macropinocytosis inhibitors, mannan (Fig. 5.2A) and CCD (Fig. 5.2B), respectively [21-23].

5.3.6 Statistical analysis

The antigen uptake levels were analyzed using ANOVA for a completely randomized design with sub-sampling using SAS version 8.2 (SAS institute). When significant differences among the times were found, the means were separated using the Fisher’s Least Significance Difference (LSD). The level of significance for all tests of effects was \( P < 0.05 \).
5.4 RESULTS

5.4.1 Selective antigen uptake

This study confirmed our previous report that after 1 h infection the mechanism of endocytosis in bovine monocytes was MR-independent, since the addition of mannan did not inhibit FITC-DX uptake in infected and control monocytes (Fig. 5.3). While the levels of MR-mediated endocytosis were significantly down-regulated in bovine monocytes after 24 h by both BVDV biotypes, the LY incorporation was not affected in 24h infected APC (Fig. 5.3 and 5.4). Furthermore, in contrast to 1h infection, 24h cp BVDV-infected and control monocytes expressed the MR-dependent mechanism of antigen capture, since mannan significantly inhibited FITC-DX uptake in infected cells (Fig. 5.3). At the same time, 24h ncp BVDV did not significantly decrease the levels of FITC-DX uptake in monocytes (Fig. 5.3).

5.4.2 Non-selective antigen uptake

The addition of CCD significantly inhibited LY uptake in 1 h ncp BVDV-infected and control cells only, suggesting that antigen uptake in these APC was mediated by macropinocytosis (Fig. 5.4). 1h cp BVDV-infected monocytes did not show significant decreases in LY uptake in the presence of macropinocytosis inhibitor CCD (Figure 5.4). Macropinocytosis was not evident in bovine APC after 24h of infection since the LY uptake was not inhibited in the presence of CCD in infected and control cells (Fig. 5.4).
5.5 DISCUSSION

To further investigate the mechanisms of antigen uptake in BVDV-infected monocytes, we applied new monocyte isolation and BVDV infection techniques (see materials and methods). Briefly, we used magnetic sorting to purify bovine monocytes. In order to reduce the separating procedure-related cell activation we incubated CD14$^+$ monocytes for 12h at 37ºC prior to the infection procedure. We assumed that this method of monocyte isolation and BVDV infection in vitro could provide new evidence on the possibility of bovine monocytes to use the potent non-selective antigen uptake mechanism, macropinocytosis since we were not able to demonstrate that non-selective fluid phase uptake could occur in bovine monocytes in our previous report [22]. Importantly, for the first time we showed that bovine monocytes were able to capture the model antigen, LY by macropinocytosis as was previously demonstrated for human [11] and cattle [21,23] immature DC. Our current data indicate that, like cattle and human DC, bovine monocytes use macropinocytosis for a bulk-flow uptake of soluble antigens.

This study confirmed our previous report that after 1 h infection the mechanism of endocytosis in bovine monocytes was MR-independent. Our previous data demonstrated that endocytosis in cp and ncp infected bovine monocytes that were gated as CD14$^+$ PBMC by flow cytometry, was MR-independent in 1h post-infection [22]. In this study we confirm the MR-independent endocytosis in 1h infected monocytes, however, we were not able to confirm the MR-dependent FITC-DX uptake in control monocytes after
1h in culture [22]. We suggest that the differences may be explained by using different experimental conditions such as the monocyte separation and BVDV infection procedures. As was mentioned above, in this study, we separated CD14\(^+\) monocytes by magnetic sorting and incubated them for 12h at 37\(^\circ\)C prior to BVDV infection. Whereas in our previous report, we infected \textit{in vitro} freshly isolated bovine PBMC and gated CD14\(^+\) APC by flow cytometry [22]. Interestingly, for the first time we determined that 1 h ncp infected monocytes were able to capture the model antigen, LY by macropinocytosis, whereas in our previous report we were not able to induce macropinocytosis in BVDV-infected monocytes [22].

In the previous report we demonstrated that 24h BVDV infection promoted the MR-dependent endocytosis in cp and ncp BVDV infected monocytes [22]. However, in this study, endocytosis in ncp BVDV-infected monocytes was MR-independent after 24h of infection. At the same time, unlike in our previous report, the mechanism of antigen uptake in control monocytes after 24h of culture was MR-receptor mediated. Again, all these differences could be due to the differences in the monocyte purification and \textit{in vitro} BVDV infection procedures.

In conclusion, our report demonstrates that bovine monocytes are not limited to the selective fluid-phase antigen uptake mechanisms: they also use a potent non-selective mechanism of antigen capture such as macropinocytosis. Therefore, antigens that fail to bind to cell surface receptors can still be taken up by fluid phase endocytosis and presented by APC. Furthermore, we confirmed our previous observation
that both BVDV biotypes affected MR-mediated antigen uptake in peripheral blood
monocytes. In particular, cp and ncp BVDV infection significantly decreased the levels
of MR-mediated endocytosis after 24h infection in bovine APC. In addition, we
demonstrate that cp and ncp BVDV infections differentially affect antigen uptake in
monocytes: 1h cp but not ncp BVDV inhibited macropinocytosis, whereas 24h ncp but
not cp BVDV inhibited MR-mediated uptake in bovine APC. By influencing the early
antigen uptake mechanisms of APC, BVDV might alter the function of monocytes as
professional antigen presenting cells resulting in impaired innate and adaptive immune
responses that predispose animals to bacterial infection in BRD. In addition, by affecting
potent non-selective antigen uptake BVDV might disrupt the function of monocytes in
the self/non-self discrimination.
5.6 REFERENCES


Figure 5.1 Detection of BVDV strains infection in bovine monocytes by RT-PCR.

RNA was extracted, quantified, and semi-quantitative RT-PCR analysis for BVDV1 NY p125 gene, BVDV NADL E2 gene and β-actin (internal standard) performed as described in materials and methods section. A) Lane 1, 2, 3, 4, 5, 6, and 7 represent the ladder, monocytes 1 h NY, monocytes 24 h NY, negative control for BT cells, BT cells 1h NY, BT cells 24h NY, negative control without RNA, respectively. B) Lane 1, 2, 3, 4, and 5 represent the ladder, negative control without RNA, monocytes 1h NADL, monocytes 24h NADL, and vaccine, respectively. All internal standards were run separately on the same samples. BT cell line infected with ncp BVDV and Frontier 4 Plus Vaccine were used as positive controls for ncp and cp BVDV infection.
Figure 5.2 To inhibit various pathways involved in antigen uptake in bovine monocytes, cells were incubated with mannan (A) or CCD (B).

Active endocytosis or macropinocytosis at 37°C were assessed by measuring mean fluorescent intensity (MFI) in the 95% pure CD14+ bovine monocytes by flow cytometry. The fluorescent peaks indicate the active endocytosis or macropinocytosis. (A): black bold solid line indicates negative control at 4 °C; black solid line indicates positive control at 37 °C; black dot line indicates the active endocytosis in presence of mannan. (B): black bold solid line indicates negative control at 4 °C; black solid line indicates positive control at 37 °C; black dot line indicates the active macropinocytosis in presence of CCD.
Figure 5.3 Effects of cp and ncp BVDV infection on the mechanisms of active endocytosis in bovine monocytes.

Bovine monocytes were infected with cp and ncp BVDV strains for 1 and 24 h \textit{in vitro}. FITC-DX uptake was assessed at 37 °C to determine the active endocytosis in infected and uninfected (negative control) CD14\textsuperscript{+} bovine monocytes by flow cytometry in the presence or absence of the MR inhibitor, mannan (500 µg/ml). Endocytosis at 4 °C was measured to determine background levels of FITC-DX uptake. Samples were analyzed in three representative experiments, and data are expressed as fold change * (compared to inhibitors) and †(compared to control), \( P < 0.05 \).
Figure 5.4 Effects of cp and ncp BVDV infection on the mechanisms of macropinocytosis in bovine monocytes.

To investigate an active macropinocytosis, LY uptake at 37 °C was assessed in CD14+ monocytes by flow cytometry in the presence or absence of the macropinocytosis inhibitor CCD (2.5 µg/ml). Macropinocytosis at 4 °C was measured to determine background levels of LY uptake. Samples were analyzed in three representative experiments, and data are expressed as fold change. *, $P < 0.05$. 
CHAPTER 6

BOVINE VIRAL DIARRHEA VIRUS INFECTION AFFECTS THE EXPRESSION OF PROTEINS RELATED TO PROFESSIONAL ANTIGEN PRESENTATION IN BOVINE MONOCYTES

6.1 ABSTRACT

The complete annotation of the cattle genome allows reliable protein identification by tandem mass spectrometry (MS²) and greatly facilitates proteomics. Previously, we reported that differential detergent fractionation (DDF) analysis of bovine monocytes reveals proteins related to antigen pattern recognition, uptake and presentation to immunocompetent lymphocytes. Here we have identified 47 bovine proteins, involved in immune function of professional antigen presenting cells (APC) that have been significantly altered after cytopathic (cp) bovine viral diarrhea virus (BVDV) infection. In particular, proteins related to immune responses such as cell adhesion, apoptosis, antigen uptake, processing and presentation, acute phase response proteins, MHC

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¹ Lee SR, Nanduri B, Pharr GT, Pinchuk LM. Bovine Viral Diarrhea virus infection affects the expression of proteins related to professional antigen presentation in bovine monocytes. Developmental and Comparative Immunology. In review.
class I- and II- related proteins and other molecules involved in immune function of professional antigen presenting cells monocytes have been significantly altered after BVDV infection. We hypothesize that by modulating expression levels in multiple proteins related to immune responses BVDV could significantly compromise immune defense mechanisms resulting in uncontrolled immune activation or suppression.

6.2 INTRODUCTION

Professional antigen presenting cells (APC), dendritic cells (DC) and their myeloid progenitors, monocytes/macrophages are critical controllers of innate and adaptive immunity [1]. Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells and produce cytokines [1,2]. Bovine monocytes, as professional APC, express relatively high levels of MHC class I and class II molecules, costimulatory molecules induced upon activation, receptors for endo- and phagocytosis, and adhesion molecules [3]. Recent data suggest that bovine monocytes are capable of directly inducing immunoglobulin (Ig) secretion in activated bovine peripheral blood B cells [3,4] as was previously demonstrated in humans [5,6]. Before entering the tissues, under the influence of certain cytokines, monocytes are capable of differentiating into macrophages or DC [7].

Bovine viral diarrhea virus (BVDV), a single-stranded RNA virus, Pestivirus genus, and Flaviviridae family, infects a large proportion of cattle worldwide and causes
a number of clinical forms of the disease ranging from transiently detectable mild clinical symptoms to a fatal diarrheic condition known as mucosal disease [8,9]. The BVDV are actually multiple viruses with antigenic and genotypic differences, along with different growth patterns [8,9]. Previously, we demonstrated that bovine monocytes infected with cytopathic (cp) BVDV NADL for 24 h altered toll-like receptor (TLR), type I interferon, pro-inflammatory and Th1/Th2 type cytokine gene expression levels [10]. In detail, while TLR7 gene expression was significantly increased, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and co-stimulatory molecules (CD80, CD86) were down-regulated, Th1 type cytokine IL-12 and INF-γ gene expression did not change in infected monocytes compared to uninfected counterparts [10]. In addition, selective antigen uptake in bovine monocytes was significantly down-regulated after 24h of infection with cp BVDV (unpublished observation).

Recently we reported that differential detergent fractionation (DDF) of bovine monocytes yielded four electrophoretically distinct fractions enriched in cytosolic, membrane-organelle, nuclear membrane and cytoskeletal-matrix markers, respectively [11]. Furthermore, multidimensional protein identification technology (MudPIT) couples 2 dimensional (2D) chromatography of peptides with tandem mass spectrometry (MS²), allowing for the identification of proteins from highly complex mixtures of bovine monocytes and the combination of DDF-MudPIT was used to increase the proteome coverage in bovine monocytes [11]. Protein quantitation can be done by calculating a cross correlation (Xcorr) of experimental tandem mass spectra to in silico generated
tandem mass spectra from sequence database [12]. This label-free method allows relative quantitation of proteins without compromising proteome coverage [12].

Recently, the bovine genome was sequenced and annotated (http://www.hgsc.bcm.tmc.edu/projects/bovine/). The complete annotation of the bovine genome allows reliable protein identification by MS² and greatly facilitates proteomics. Here we used DDF-MudPIT analysis and relative quantitation of proteins expressed in bovine monocytes infected with cp BVDV strain NADL.

6.3 MATERIALS AND METHODS

6.3.1 Animals

Nine conventionally reared, healthy BVDV-free cows from a Holstein herd at the Mississippi State University Dairy Facility were used. The animals have been subjected to a comprehensive vaccination program, including Frontier 4 Plus Vaccine (IBR, BVD, PI3, RSV, Diamond Animal H, Inc). All animal use was approved by The Mississippi State University Institutional Animal Care and Use Committee. Peripheral blood mononuclear cells (PBMC) separated from the animals used in our study were tested for the expression of BVDV E2 transcripts with E2 BVDV specific primers by RT-PCR [13]. As we expected, all animals were BVDV mRNA-free (data not shown).
6.3.2 Cell preparation

Blood samples (150 ml) were collected into Blood Collection Tubes (16 x 100 mm, Tyco Healthcare) by jugular venipuncture. Bovine PBMC were separated as described elsewhere [3,11,13,14]. Briefly, PBMC were isolated using Histopaque gradients (1.077g/ml, Amersham Biosciences) and resuspended in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol and 100 IU/ml Gentamicin (Invitrogen). Monocytes were separated from PBMC as described elsewhere [3]. Briefly, 40 ml of PBMC suspension (5 x 10^8 cells) was added to Petri-dish (150 x 25 mm, BD sciences) for 2 h at 37 °C. Non-adherent cells were removed and the adherent cells were washed twice in PBS (Invitrogen). The yield of adherent cells was 20-30% of total PBMC number. After removing non-adherent populations (mostly B cells), adherent cells were incubated with mAbs to CD14 (MM61A, VMRD) followed by the addition of magnetic beads conjugated with mouse anti-IgG1 (Miltenyi Biotech, Auburn, CA) [11]. CD14^+ monocytes were positively selected by using magnetic cell separation technique according to the manufacturer’s instructions (Miltenyi Biotech). The final yield of bovine monocytes was 2-3% of total PBMC number.

6.3.3 BVDV stock and infection

BVDV strain was prepared as described elsewhere [13]. Briefly, the NADL (cp) strain of BVDV was obtained from the American Type Culture Collection (ATCC) and
amplified by growth in the bovine turbinate (BT) cell line (ATCC) according to the manufacturer’s handling procedures. For infection of BT cells, virus dilutions were made in DMEM with 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 10% horse serum. To measure the infectivity of the NADL strain, the quantal method of Reed and Muench was performed and the tissue culture infectious dose 50 (TCID$_{50}$) was determined. To select the dose of cp BVDV that did not have a cytopathic effect on monocytes cultured for 48 h we assessed the viability of the infected cells by using trypan blue and light microscopy. BVDV strain NADL at the multiplicity of infection (MOI) 0.002 did not affect the viability of bovine monocytes after 48 h of infection (data not shown). 5 x 10$^6$ monocytes were added to each well of a 6 well tissue culture plate and infected with cp BVDV at the same MOI of 0.002 for 24 h. After infection, at least 10$^7$ cells were pooled in one tube. All data were determined using triplicate monocyte cultures.

6.3.4 Protein extraction, trypsin digestion and 2D-LC ESI MS$^2$

Proteins were isolated using DDF as described [11,15]. Briefly, DDF sequentially extracts proteins from cellular compartments using a series of detergents. Cytosolic proteins were isolated by repeated washes in digitonin buffer. After the digitonin washes, the isolation of membrane, nuclear and cytoskeletal proteins were performed with triton X-100 (TX), deoxycholate (DOC), tween 40, and SDS buffers, respectively. To evaluate the quality of isolated proteins, 1% of the protein samples were compared using 10%
SDS-PAGE (data not shown). For each of the detergent fractions, equal amounts of protein were precipitated with 25% tricholoroacetic acid to remove salts and detergents. Protein pellets were solubilized and then digested with 100 ng of trypsin (50:1 ratio of substrate to enzyme) overnight at 37°C. Peptides were desalted using a peptide microtrap (Michrom BioResources, Inc.) and eluted by a 0.1% trifluoroacetic acid, 95% acetonitrile solution. Desalted peptides were dried and resuspended in 0.1% formic acid.

2D-LC ESI MS^2 was done as described elsewhere [11,15]. Briefly, LC analysis was accomplished by strong cation exchange (SCX) followed by reverse phase (RP) liquid chromatography (LC) coupled directly in line with electrospray (ESI) ion trap MS. Samples were loaded into a LC gradient ion exchange system including a Thermo Separations P4000 quaternary gradient pump (ThermoElectron Corporation) coupled with a 0.32 x 100 mm BioBasic SCX column.

6.3.5 Protein identification and analysis

Proteins were identified and analyzed as previously described [11]. The non-redundant protein database (DB) downloaded from the National Center for Biotechnology Information (NCBI; May 2006) by TurboSEQUEST (Bioworks Browser 3.2; ThermoElectron) was used to create a bovine subset (bovine DB; search terms: *bos taurus* and *bos indicus*). Trypsin digestion was applied *in silico* to bovine DB and mass changes due to cysteine carbamidomethylation and methionine oxidation were included. The bovine DB was used to search tandem MS using peptide (MS precursor ion) mass
tolerance of 1.5 Da and a fragment ion (MS²) mass tolerance of 1.0 Da. Peptide matches were considered genuine if they were ≥ 6 amino acids with Xcorr values of 1.5, 2.2 and 3.3 (+1, +2, and +3 ions respectively) and ∆Cn (≥ 0.1) values [16]. Each protein was classified by using Gene Ontology (GO) annotation. GO was obtained available GO annotation from UniProt database (http://www.pir.uniprot.org/) [17]. In addition, we used AgBase tool GOanna [18] to provide additional GO annotation for bovine gene products.

6.3.6 Statistical analysis

To determine significant changes in protein expression between the control and treated with BVDV-infected monocytes, proteins were analyzed as previously described [12]. Briefly, only proteins identified by at least three peptides in any dataset were considered. A custom program was used to calculate sum of Xcorr of all the identified peptides from all three replicates for each protein, and one way analysis of variance (ANOVA) (p < 0.05) was used to identify statistically significant differences in protein expression between treatments [19].

6.4 RESULTS

6.4.1 Protein identification

A total of 8272 known proteins were identified in bovine monocytes. Specifically, with three replicates 6470 proteins in control monocytes and 5459 proteins in cp BVDV-infected monocytes were identified using non gel-based proteomic 2D-LC ESI MS²
methods. The identified proteins have been distributed as follows: 34% (2813) in control, 21.8% (1802) in BVDV-infected and 44.2% (3672) in both groups (Fig. 6.1). Overall, BVDV infection significantly altered the expression of 445 proteins in bovine monocytes compared to uninfected controls (Supplementary Data). As expected 29 proteins identified in the BVDV-infected and control monocytes (Table 6.1) and all 18 MHC proteins (Table 6.2) were related to immunological function based on the biological process in GO category.

6.4.2 Comparative analysis of immunological proteins in control and BVDV-infected monocytes by GO categories

Bovine monocyte proteins that have been significantly altered by BVDV (445 proteins) were assigned function using known GO categories “Molecular Function”, “Biological Process” and “Cellular Component” (Supplementary Data). Importantly, 29 immunological proteins (6.5%) that have been significantly altered by BVDV infection were placed into known GO categories (Table 6.1). Furthermore, the percentage representation of identified proteins was determined for each GO biological process category (Fig. 6.2). Briefly, all 2 proteins related to acute phase response and 6 out of 9 proteins associated with cell adhesion were significantly increased in cp BVDV-infected bovine monocytes. In addition, CD14, CD68, peptidyl-prolyl cis-trans isomerase A, similar to apolipoprotein A-II precursor and myxovirus resistance 1 proteins were significantly increased in the cp BVDV-infected cells. On the other hand, desmoglein,
predicted: similar to desmoglein-2 precursor (HDGC), and predicted: similar to leukosialin precursor proteins, which are all related to cell adhesion, were significantly down-regulated in the cp infected group. Moreover, all proteins related to the negative regulation of apoptosis, invariant chain, and antigen uptake, processing and presentation were decreased in the BVDV-treated group. Proteins related to responses to unfolded protein were also significantly down-regulated in the infected group. Finally, 8 immune function proteins (27.6%) were identified as below detectable threshold (bdt) at our established cutoffs for Xcorr in each group (control and cp infected).

6.4.3 Analysis of MHC proteins in comparison between control and cp infected monocytes

In this study, 18 MHC proteins (4%) out of 445 were significantly altered in cp BVDV-infected bovine monocytes (Table 6.2). BVDV infection significantly altered the expression of 11 MHC class I and 7 MHC class II molecules in peripheral blood monocytes. Firstly, 9 of MHC class I proteins were significantly down-regulated in cp BVDV-infected bovine monocytes. Interestingly, two proteins, accession number AAZ73460 and ABA39524 were only detected in cp BVDV-infected group. Secondly, 6 of MHC class II proteins were significantly decreased in the infected monocytes, and one MHC class II DR-beta chain protein was increased in cp BVDV-infected monocytes only.
6.5 DISCUSSION

BVDV has been described as a group of multiple viruses affecting virtually all organs and systems in the body, including innate and adaptive immune responses [20]. However, the role of professional APC, in particular, monocytes/macrophages in BVDV infection is still unclear. Multiple studies assessed morphological and functional properties of the BVDV-infected monocytes/macrophages, including NO production, antigen uptake, T cell stimulatory capacities, cytokine production, cytokine and TLR gene expression [10,13,21-25]. Previously, we reported the identification of proteins involved in professional antigen presentation in bovine monocytes by using differential detergent fractionation (DDF) and multidimensional protein identification technology (MudPIT) [11]. Sequenced and annotated bovine genomes allow reliable protein identification using non-electrophoretic proteomics by 2D-LC ESI MS² method [11]. Compared with electrophoresis-based methods, non-electrophoretic proteomics is more comprehensive and is a high throughput.

However, comparative protein profiling of BVDV-infected APC, including quantitation of the relative immunologically important protein expression while maintaining comprehensive proteome coverage is still missing. Therefore, in this study, we applied DDF-MudPIT analysis and relative quantitation of proteins expressed in bovine monocytes infected with cp BVDV strain NADL.

With virus infection, acute phase response protein expressions are increased in human and porcine models [26-28]. Multiple reports demonstrated that acute phase
proteins in cattle were also elevated with several viral infections [29-31]. Especially, the acute phase proteins, such as serum amyloid A (SAA), were altered in BVDV-infected calves [29]. Moreover, apolipoprotein A-II is also related to SAA expression [32]. Here we demonstrate that acute phase response proteins, fetuins, SAA and apolipoprotein A-II were significantly up-regulated in bovine monocytes infected with cp BVDV.

Multiple studies demonstrated that uptake, processing and presentation of protein antigens by APC are down-regulated in some viral infections [33-36]. Especially, MHC class I or transporter associated with antigen processing (TAP) protein were down-regulated with varicella-zoster virus, bovine herpesvirus 1, and bovine papillomavirus E5 [33,36,37]. Moreover, BVDV-infected bovine monocytes decreased their ability to present antigens to the Th cells and their phagocytic activity [25,38,39]. In this study we demonstrate that most of the MHC-, endocytosis- and TAP- related proteins were significantly decreased in BVDV-infected monocytes.

Our data indicate that all proteins related to the negative regulation of apoptosis were decreased in cp BVDV-infected bovine monocytes. This evidence correlates with the previous observation that some viruses induce the apoptotic pathways of the host [40].

In order for circulating leukocytes to enter inflammatory tissue or peripheral lymphoid organs, the cells must adhere to and pass between the endothelial cells lining the walls and blood vessels [41]. Some of these surface molecules are expressed continuously, but others are only expressed in response to localized concentrations of cytokines produced during an inflammatory response and serve to increase the strength of
the functional interactions between cells of the immune system [41]. We report that most of the cell adhesion molecules expression was significantly increased in BVDV-infected monocytes. However, the desmoglein like proteins and the protein of negative regulation of cell adhesion were decreased in this study.

Two observations suggested that the BVDV group of viruses use integrin molecules as their receptors in the bovine system [42,43], BVDV use CD46 and low-density-lipoprotein receptor to enter host cells [44,45]. Although we have identified low-density-lipoprotein receptors in both control and infected monocytes, BVDV infection did not significantly alter their expression (data not shown). Interestingly, low-density-lipoprotein receptor-related molecules were significantly increased in cp BVDV- infected monocytes in our study.

Immune response proteins play an important role in professional APC function [46-48]. In particular, the CD14/TLR4 complex is involved in viral G protein-related production of type I IFN [49]. Moreover, Muller-Doblies et al. showed that Myxovirus resistance I (Mx) proteins induced by type I IFN were increased in ncp BVDV-infected bovine white blood cells [50]. Finally, our previous report demonstrated that the expression of type I IFN was numerically increased in bovine monocytes after infection with cp and ncp BVDV strains [10]. In this study we extend our previous findings and demonstrate that both proteins, CD14 and Mx were significantly increased in BVDV-infected monocytes.
In this study, the expression of invariant chains non-covalently associated with MHC class II complex was decreased unlike the findings reported on the expression of this molecule in viral infection previously [51-53].

It was shown previously that interleukin enhancer binding factor (ILF2, NF45) and NF90 (ILF3) regulate the IL-2 gene transcription via interaction with the antigen receptor response element [54]. Cp BVDV significantly down-regulated the expression levels of the protein similar to interleukin enhancer binding factor in our study.

Previous report showed that heat shock proteins (HSP) produced in response to unfolded proteins are highly conserved proteins that are strongly induced in physical and chemical stress in both prokaryotic and eukaryotic cells [55]. HSP are involved in tumor immunity mediated by APC, T cells and NK cells and enhance the development of innate and adaptive immune responses by interacting with viral proteins [56,57]. HSP make complexes with viral proteins that bind APC that could affect the cytokine expression, induction of adaptive immunity, and activation of CTL through antigen presentation with MHC molecules [57]. Previous report demonstrated that recombinant bovine HSP70 increased the antigen uptake, the expression of MHC molecules and CD40 in monocytes, monocyte-derived macrophages and DC [58]. In this study, cp BVDV-infected monocytes down-regulated the expression of molecules of response to unfolded proteins as well as MHC proteins suggesting possible correlation between the two groups of proteins in cp BVDV-infected bovine monocytes.
Calrecticulin is a major intracellular calcium-binding protein identified in skeletal muscle sarcoplasmic reticulum that modulates cell adhesion, integrin-dependent calcium signaling and steroid-sensitive gene expression [59,60]. Calrecticulin is related to the signaling pathway involving one of receptors for BVDV, lipoprotein receptor-related protein [44,61]. Our observation that calrecticulin expression was significantly decreased in bovine monocytes infected with cp BVDV strain confirms the involvement of this protein in BVDV pathogenesis.

It was previously demonstrated that CD163, a hemoglobin scavenger receptor exclusively expressed in the monocyte-macrophage system plays a major role in dampening the inflammatory response and in scavenging components of damaged cells [62]. It was also reported that CD163 was repressed in HIV infected human macrophages [63], however up-regulated in porcine monocyte/macrophages infected with African swine fever virus [64] and in cattle APC infected with tuberculosis [65]. In our study, CD163 and CD68, monocyte/macrophage differentiation marker [66] were significantly up-regulated in cp BVDV-infected monocytes.

Finally, several reports described peptidyl-prolyl cis-trans isomerases (PPIases) as ubiquitous foldases that contribute to the conformational changes during protein folding in both eukaryotes and prokaryotes [67], to promote HIV infectivity by facilitating virus uncoating in humans [68]. In contrast to humans, in non-human primates PPIases promote anti-HIV-1 restriction activity [69]. In this study, BVDV infection significantly
increased the PPLases expression levels suggesting their involvement in BVDV infectivity similar to HIV infection.

In conclusion, we hypothesize that by altering expression levels in multiple proteins related to immune responses such as cell adhesion, apoptosis, antigen uptake, processing and presentation, and other acute phase response proteins cp BVDV could significantly compromise immune defense mechanisms resulting in uncontrolled immune activation.
6.6 REFERENCES


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Figure 6.1 Distribution of total proteins identified from either control or BVDV infected bovine monocytes.

The MS$^2$ data from each of group were analyzed using the bovine DB subset and the resulting proteins were classified with the respect to their group.
Figure 6.2 Estimated percentage representation of the immunological proteins except MHC class molecules in each GO biological process category.

Biological process descriptions are as follows: actin cytoskeleton organization and biogenesis (1), acute phase response (2), antigen presentation (3), cell adhesion (4), endocytosis (5), immune response (6), negative regulation of apoptosis (7), regulation of interleukin-8 biosynthetic process (8), regulation of viral genome replication (9), response to unfolded protein (10), and function unknown (11).
Table 6.1 Immune function proteins in bovine monocytes with significantly altered expression after 24 h post-infection with cp BVDV strain

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a) Genbank accession number  
b) Sum of $X_{corr}$ values for all peptides identified from this protein in all three biological replicates  
c) GO: gene ontology  
d) Below detectable threshold at our established cutoffs for $X_{corr}$  
e) Not assigned to a GO category
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Table 6.2 Bovine monocytes MHC proteins with significantly altered expression after 24 h post-infection with cp BVDV strain

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a) Genbank accession number  
b) Sum of Xcorr values for all peptides identified from this protein in all three biological replicates  
c) GO: gene ontology  
d) Below detectable threshold at our established cutoffs for Xcorr  
Light gray: expressed in both groups  
Dark gray: expressed in cp infected monocytes only
Table 6.2 continued

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CHAPTER 7
CONCLUSION

Cells of the monocyte/macrophage lineage are important elements of the immune defense system because these cells can phagocytose foreign material, present antigens to immunocompetent cells and produce cytokines. Bovine monocytes, as professional APC, express relatively high levels of MHC class I and class II molecules, costimulatory molecules induced upon activation, receptors for endo- and phagocytosis, and adhesion molecules. Before entering the tissues, under the influence of certain cytokines, monocytes are capable of differentiating into macrophages or DC.

While some of the pathogenic effects of BVDV are attributed to host responses, especially immunosuppression and production of pro-inflammatory cytokines, other effects may be more directly caused by replicating BVDV. Both ncp and cp strains infect monocytes, macrophages and DC in vitro and in vivo.

A widely used 2D separation technique in proteomics has been 2D gel electrophoresis. However, this technique is limited by its inability to detect and identify low abundance proteins, proteins with extremes in isoelectric point and molecular weight and very hydrophobic proteins. To overcome these obstacles, several comprehensive techniques have been developed, including MudPIT. The combination of DDF and
MudPIT allows obtaining a comprehensive cellular proteome as well as information about a protein’s subcellular localization. Therefore, the data presented in the first part of this dissertation are important because the identification of the proteins present in professional APC is instrumental to understanding the mechanisms involved in the function of monocytes/macrophages and monocyte-derived DC as controllers and stimulators of T and B cell specific immune responses. In particular, the transformation of monocytes into immature DC and their further maturation to become the most powerful APC is a very critical bridge between innate and adaptive immune responses. In addition, comparative protein profiling could be used to elucidate the mechanisms involved in pathogen interference of DC maturation to avoid effective recognition and elimination by innate and adaptive immune responses which are controlled and regulated by the most powerful professional APC.

Persistently infected cattle or animals with sub-clinical symptoms are the source of maintaining BVDV in nature and/or the major source of virus for other cattle. Therefore, we selected two BVDV viruses that cause persistent infection or mild symptoms in infected animals but differ in their cytopathic effects as a model system to study the differences in the TLR-mediated control of type I IFN and pro-inflammatory cytokine gene expression in bovine monocytes. We hypothesize that both cp and ncp BVDV could escape innate immune responses by modulating TLR gene expression, followed by pro-inflammatory, type I IFN, Th1/Th2 type cytokine gene expression, and decreasing the expression levels of CD80/86 in professional APC. Particularly, gene
expression data in second chapter of this dissertation demonstrate that both cp and ncp BVDV strains in general had similar effects on the TLR, type I IFN, pro-inflammatory, Th1/Th2 cytokine, and co-stimulatory molecules gene expression. However, some significant differences in their effects that could be due to biological differences between cp and ncp BVDV strains. In particular, TLR3 was significantly up-regulated in early ncp BVDV infection, whereas TLR7 represented the majority of TLR expression in late cp BVDV and ncp infection. Type I IFN cytokine gene expression was also significantly up-regulated in early ncp, but not cp BVDV infection suggesting TLR3-mediated control of type I IFN production in BVDV. In addition, IL-12 gene expression was significantly up-regulated in early ncp BVDV infection.

The third topic in this dissertation demonstrates that bovine monocytes are not limited to the selective fluid-phase antigen uptake mechanisms: they also use a potent non-selective mechanism of antigen capture such as macropinocytosis. Therefore, antigens that fail to bind to cell surface receptors can still be taken up by fluid phase endocytosis and presented by APC. In addition, we demonstrate that cp and ncp BVDV infections differentially affect antigen uptake in monocytes: 1h cp but not ncp BVDV inhibited macropinocytosis, whereas 24h ncp but not cp BVDV inhibited MR-mediated uptake in bovine APC. By influencing the early antigen uptake mechanisms of APC, BVDV might alter the function of monocytes as professional antigen presenting cells resulting in impaired innate and adaptive immune responses that predispose animals to
bacterial infection in BRD. In addition, by affecting potent non-selective antigen uptake BVDV might disrupt the function of monocytes in the self/non-self discrimination.

Finally, protein expression in both control and cp BVDV infected monocytes were compared. Bovine monocyte proteins have been significantly altered by BVDV (445 proteins). Importantly, 47 immunological proteins that have been significantly altered by BVDV infection were placed into known GO categories. Briefly, all 2 proteins related to acute phase response and 6 out of 9 proteins associated with cell adhesion were significantly increased in cp BVDV-infected bovine monocytes. In addition, CD14, CD68, peptidyl-prolyl cis-trans isomerase A, similar to apolipoprotein A-II precursor and myxovirus resistance 1 proteins were significantly increased in the cp BVDV-infected cells. On the other hand, desmoglein, predicted: similar to desmoglein-2 precursor (HDGC), and predicted: similar to leukosialin precursor proteins, which are all related to cell adhesion, were significantly down-regulated in the cp infected group. Moreover, all proteins related to the negative regulation of apoptosis, invariant chain, and antigen uptake, processing and presentation were decreased in the BVDV-treated group. Particularly, BVDV infection significantly altered the expression of 11 MHC class I and 7 MHC class II molecules. Proteins related to responses to unfolded protein were also significantly down-regulated in the infected group. Finally, 8 immune function proteins (27.6%) were identified as below detectable threshold (bdt) at our established cutoffs for Xcorr in each group (control and cp infected). With this study we hypothesize that by altering expression levels in multiple proteins related to immune responses such as cell
adhesion, apoptosis, antigen uptake, processing and presentation, and other acute phase
time response proteins by BVDV could significantly compromise immune defense
mechanisms resulting in uncontrolled immune activation.

All together, this study has evaluated a new proteomic method as a useful and
alternative tool for protein analysis in bovine monocytes. This method can be used to aid
in monitoring the protein expression in different viral infection in bovine system.
Additionally, gene expressions and antigen uptake in bovine monocytes during the early
viral infection have been determined. This information helps to understand how bovine
monocytes respond to the BVDV infection *in vitro.*
APPENDIX A

SUPPLEMENTARY DATA FOR CHAPTER 3
SUPPLEMENTARY DATA 3.1 LC BASE PEAK ION CHROMATOGRAMS OF PERIPHERAL BLOOD BOVINE MONOCYTE DDF

Each DDF is presented with 19 different salt steps added. A salt gradient of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 57, 64, 71, 79, 90, 110, 300, and 700 mM of ammonium acetate in 5% acetonitrile, 0.1% formic acid was applied. The resultant peptides were loaded directly into the sample loop of a 0.18 x 100 mm BioBasic C18 RP LC column of a Proteome X workstation (ThermoElectron). Base Peak ion chromatograms of all the RP LC column fractions were collected and the numbers represent the salt steps as follows:

1: 0
2: 5
3: 10
4: 15
5: 20
6: 25
7: 30
8: 35
9: 40
10: 45
11: 50
12: 57
13: 64
14: 71
15: 79
16: 90
17: 110
18: 300
19: 700
Digitonin Fraction
TX Fraction
DOC Fraction
SDS Fraction
APPENDIX B

SUPPLEMENTARY DATA FOR CHAPTER 6
SUPPLEMENTARY DATA 6.1 Bovine monocytes proteins with significantly altered expression after 24h post-infection with cp BVDV strain

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<td>Isochorism dehydrogenase activity</td>
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<td>muscle contraction negative regulation of apoptosis</td>
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<td>protein binding</td>
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<td>74356373</td>
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<td>731</td>
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<td>actin binding</td>
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<td>75775158</td>
<td>Unknown (protein for MGC:128976)</td>
<td>450</td>
<td>30</td>
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<td>0.04</td>
<td>oxidoreductase activity</td>
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<td>76608815</td>
<td>PREDICTED: similar to Myosin heavy chain, cardiac muscle alpha isoform</td>
<td>2036</td>
<td>3</td>
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<td>0.00</td>
<td>motor activity</td>
<td>scavenger receptor activity</td>
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<td>76608883</td>
<td>PREDICTED: similar to CD163 antigen isoform a</td>
<td>1075</td>
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<td>0.00</td>
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<td>NA</td>
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<td>76610006</td>
<td>PREDICTED: similar to Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylgalactosaminyltransferase</td>
<td>739</td>
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<td>76610551</td>
<td>PREDICTED: similar to desmin isoform 4</td>
<td>489</td>
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<td>4</td>
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<td>structural molecule activity</td>
<td>NA</td>
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<td>598</td>
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<td>0.04</td>
<td>binding</td>
<td>NA</td>
<td>nucleus mouse</td>
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<td>binding</td>
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<td>76615580</td>
<td>PREDICTED: similar to myosin IG</td>
<td>1013</td>
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<td>myosin mouse</td>
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<td>1513</td>
<td>1</td>
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<td>76616887</td>
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<td>PREDICTED: similar to hematopoietic protein 1 isoform 2</td>
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<td>76617882</td>
<td>PREDICTED: similar to keratin 1 isoform 4</td>
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<td>76618754</td>
<td>PREDICTED: similar to family with sequence similarity 62 (C2 domain co</td>
<td>1106</td>
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<td>76619028</td>
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<td>PREDICTED: similar to Tripartite motif protein 47</td>
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<td>76620061</td>
<td>PREDICTED: similar to rap2 interacting protein x isoform 6</td>
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<td>76620232</td>
<td>PREDICTED: similar to heterogeneous nuclear ribonucleoprotein D isof</td>
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<td>0.01</td>
<td>61.1</td>
<td>nucleotide binding</td>
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<td>PREDICTED: similar to KH-type splicing regulatory protein (FUSE bindin</td>
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<td>0.00</td>
<td>17.5</td>
<td>nucleic acid binding</td>
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<td>nucleotide binding</td>
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<td>3</td>
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<td>20.6</td>
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<td>76623793</td>
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<td>76624010</td>
<td>PREDICTED: similar to nucleolin-related protein</td>
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<td>9.3 binding</td>
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<td>1018</td>
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<td>76628066</td>
<td>PREDICTED: similar to zinc finger and BTB domain containing 1 isoform</td>
<td>644</td>
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<td>bdt</td>
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<td>10.4</td>
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<td>76628721</td>
<td>PREDICTED: hypothetical protein</td>
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<td>8.4</td>
<td>0.0 NA molecular function unknown</td>
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<td>76629332</td>
<td>PREDICTED: similar to Reticulon 4</td>
<td>1207</td>
<td>11</td>
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<td>49.1</td>
<td>34.9 endoplasmic reticulum</td>
<td>NA</td>
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<td>76629785</td>
<td>PREDICTED: similar to chromosome 2 open reading frame 3 isoform</td>
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<td>10.8</td>
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<td>76630694</td>
<td>PREDICTED: similar to Spectrin alpha chain, brain (Spectrin, non-eryth)</td>
<td>1392</td>
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<td>22.0</td>
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<td>76630698</td>
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<td>37.0</td>
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<td>76630706</td>
<td>PREDICTED: similar to Spectrin alpha chain, brain (Spectrin, non-eryth)</td>
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<td>bdt</td>
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<td>15.0</td>
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<td>76630708</td>
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<td>0.00</td>
<td>34.8</td>
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<td>76631704</td>
<td>PREDICTED: similar to IMMUNE-RESPONSIVE PROTEIN 1</td>
<td>483</td>
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<td>23.3</td>
<td>12.8 NA NA calcium ion binding</td>
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<td>76631901</td>
<td>PREDICTED: similar to prohibitin</td>
<td>223</td>
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<td>bdt</td>
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<td>8.5</td>
<td>0.0 NA NA calcium ion binding</td>
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<tr>
<td>76633457</td>
<td>PREDICTED: similar to myosin, heavy polypeptide 7B, cardiac muscle, be</td>
<td>2083</td>
<td>bdt</td>
<td>3</td>
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<td>0.0</td>
<td>7.1 motor activity NA calcium ion binding</td>
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<td>76634569</td>
<td>PREDICTED: similar to Annexin A1 (Annexin I) (Lipocortin I) (Calpactin)</td>
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<td>13.6</td>
<td>0.0 NA NA calcium ion binding</td>
<td>NA</td>
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<td>76635820</td>
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<td>bdt</td>
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<td>24.7</td>
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<td>45.3</td>
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<td>0.04</td>
<td>12.9</td>
<td>2.7 receptor activity NA NA RNA binding</td>
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<td>76637153</td>
<td>PREDICTED: similar to heterogeneous nuclear ribonucleoprotein U isoform</td>
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<td>0.00</td>
<td>55.5</td>
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<td>Value 2</td>
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<td>76637895</td>
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<td>6</td>
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<td>76638241</td>
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<td>7.2</td>
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<td>76639219</td>
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<td>30.6</td>
<td>13.5</td>
<td>NA</td>
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<td>76639231</td>
<td>PREDICTED: similar to endoplasmic reticulum protein 29 precursor</td>
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<td>0.02</td>
<td>29.1</td>
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<td>76639809</td>
<td>PREDICTED: similar to Catechol O-methyltransferase</td>
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<td>0.04</td>
<td>16.2</td>
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<td>76642663</td>
<td>PREDICTED: similar to Myeloperoxidase precursor (MPO)</td>
<td>719</td>
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<td>2</td>
<td>0.03</td>
<td>17.4</td>
<td>6.5</td>
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<td>76645346</td>
<td>PREDICTED: similar to Probable RNA-dependent helicase p68</td>
<td>594</td>
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<td>bdt</td>
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<td>18.6</td>
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<td>76646771</td>
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<td>bdt</td>
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<td>9.5</td>
<td>0.0</td>
<td>NA</td>
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<td>76647057</td>
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<td>bdt</td>
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<td>9.9</td>
<td>0.0</td>
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<td>10</td>
<td>0.02</td>
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<td>76651508</td>
<td>PREDICTED: hypothetical protein XP_883400</td>
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<td>bdt</td>
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<td>0.0</td>
<td>7.7</td>
<td>NA</td>
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<td>76654387</td>
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<td>215</td>
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<td>0.00</td>
<td>17.7</td>
<td>4.4</td>
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<td>PREDICTED: similar to AHNAK nucleoprotein isoform 1 isof</td>
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<td>1</td>
<td>0.02</td>
<td>12.8</td>
<td>2.8</td>
<td>nucleotide binding</td>
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<td>PREDICTED: similar to heterogeneous nuclear ribonucleoprotein H3 isofo</td>
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<td>6.9</td>
<td>19.0</td>
<td>NA</td>
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<td>76657696</td>
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<td>0.01</td>
<td>23.4</td>
<td>7.4</td>
<td>DNA binding transferase activity</td>
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