2018

The Effects of Bovine Viral Diarrhea Virus and Bovine Herpesvirus Type 1 on Monocyte-Derived Dendritic Cells

Jacob Sobraske
South Dakota State University

Recommended Citation

THE EFFECTS OF BOVINE VIRAL DIARRHEA VIRUS AND BOVINE

HERPESVIRUS TYPE 1 ON MONOCYTE-DERIVED DENDRITIC CELLS

BY

JACOB SOBRASKE

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Science

Specialization in Microbiology

South Dakota State University

2018

THE EFFECTS OF BOVINE VIRAL DIARRHEA VIRUS AND BOVINE

HERPESVIRUS TYPE 1 ON MONOCYTE-DERIVED DENDRITIC CELLS

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Christopher C. L. Chase, DVM, Ph.D.   Date

Major Advisor

Jane Christopher-Hennings, DVM, M.S.   Date

Head, Department of Veterinary
and Biomedical Sciences

Kinchel Doerner, Ph.D.   Date

Dean, Graduate School

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my parents, brother, grandparents, and friends for all of the support they have given me during graduate school. They kept me grounded during the times experiments didn't work out and encouraged me to keep trying.

I can't thank Dr. Christopher Chase enough for all of the patience he had and the guidance he supplied me with. He taught me to be a better scientist, a better writer, and a more mature person. I grew a lot under his tutelage.

A special thanks to Mr. Lyle Braun who helped train me in the basic lab protocols.

A special thanks to Karim Abdelsalam who managed Dr. Chase's lab and helped when I needed it.

I'd like to thank the undergraduate students who helped out on my experiments and a crucial part of Dr. Chase's lab.

I'd like to thank my committee members: Dr. Eric Nelson, Dr. Alan Young, and Dr. Michael Nagy.

I'd like to thank the Department of Veterinary and Biomedical Sciences for providing a place for my research.

I'd like to thank the Dairy Research and Training Facility for the generous donation of blood samples from calves at their facility.

I'd like to thank the office staff, serology, and PCR for additional assistance.

Finally, I would like to thank the College of Agriculture and Biological Sciences, specifically Dr. Volker Brozel, for granting me a graduate teaching assistantship in the Biology and Microbiology Department. Additionally, I would like to thank Dr. Scott

Pedersen and Andy Ellis for allowing me to teach anatomy for my GTA position. I

couldn't have asked for a better course to teach. I would also like to thank all of the

interns I worked with for all their hard work. They made teaching more enjoyable with

their dedication to the anatomy program.

CONTENTS

LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

BHV-1: Bovine Herpesvirus type 1

BVDV: Bovine Viral Diarrhea Virus

CD: Cluster of Differentiation

CP: Cytopathic

cRPMI: Complete Roswell Park Memorial Institute Medium

DC: Dendritic Cell

FBS: Fetal Bovine Serum

FITC: Fluorescent Isothiocyanate

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

IFN: Interferon

IL: Interleukin

IPA: Immunoperoxidase Assay

LPS: Lipopolysaccharide

mAb: Monoclonal Antibody

MDBK cells: Madin-Darby Bovine Kidney Epithelial Cells

MDDC: Monocyte-Derived Dendritic Cell

MHC: Major Histocompatibility Complex

MOI: Multiplicity of Infection

NCP: Noncytopathic

NS: Nonstructural

PBMC: Peripheral Blood Mononuclear Cell

qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction

PBS: Phosphate-Buffered Saline

RBC: Red Blood Cell

RPMI: Roswell Park Memorial Institute Medium

TCID$_{50}$: 50% Tissue Culture Infective Dose

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

THE EFFECTS OF BOVINE VIRAL DIARRHEA VIRUS AND BOVINE

HERPESVIRUS TYPE 1 ON MONOCYTE-DERIVED DENDRITIC CELLS

JACOB SOBRASKE

2018

Bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BHV-1) are important pathogens that affect cattle. In this *in vitro* study, the main goal was determining the direct effects of BVDV and BHV-1 infections of monocyte-derived dendritic cells (MDDC) on cytokine mRNA expression. For the BVDV infected MDDC experiments, Holstein Friesian and Brown Swiss calves were included with a breed comparison with cytokine expression. Surface marker expression was also measured in BHV-1-infected MDDCs. For both viruses, a high virulent strain (1373 for BVDV and Cooper for BHV-1) and lower virulent strains (20508 for BVDV and Los Angeles for BHV-1) were used. Ten cytokines: interferon-alpha (IFN-alpha), interferon-beta (IFN-beta), interferon-gamma (IFN-gamma), interleukin-1a (IL-1a), interleukin-1b (IL-1b), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), and tumor necrosis factor (TNF) were measured. BVDV infection of MDDCs down regulated all cytokine mRNA compared to the control. IL-1b had the greatest down regulation with more than a 40-fold decrease. The three IFN cytokines had less than a 2-fold decrease. The other IL cytokines had less than 10-fold decreases. Five cytokines expressed by BHV-1 infected MDDCs were down regulated. The IFN family were up regulated. IFN-alpha had greater than a 10-fold increase, IFN-beta had greater than a 3-fold increase, and IFN-gamma had less than a 1-fold increase. The other 5 IL cytokines

were down regulated with less than a 4-fold decrease. For both BVDV and BHV-1, IFN-alpha and IL-8 showed statistical significance between the two strains. There was a difference for the 2 BVDV strains for 2 cytokines: BVDV 1373 down regulated both IFN-Alpha and IL-8 more than 28508. BHV-1 Cooper strain up regulated IFN-Alpha and down regulated IL-8 as compared to the Los Angeles strain. For the breed comparison study with BVDV, Brown Swiss calves had higher MDDC yields than Holstein Friesian calves after MDDC preparation. Cytokine mRNA expression had a greater up regulation and less down regulation in Holstein Friesian calves. Finally, BHV-1 infection of MDDC resulted in decreased MHCI, MHCII, and CD86 surface marker expression. Future studies will analyze the effect of infection on antigen presentation and stimulation of naïve T cells.

CHAPTER 1: RESEARCH OBJECTIVES AND LITERATURE REVIEW

INTRODUCTION

BVDV and BHV-1 are viral pathogens of cattle that infect both dairy and beef cattle. BVDV and BHV-1 infections cause severe economic losses to the industry. Both viruses are associated with respiratory and reproductive disease. The effect that cytokines have on the immune system are an important area of study. Cytokines play important roles in regulation of cells and infections. The study of the effect of two common cattle viruses on monocyte-derived dendritic cells (MDDC) and the production of cytokines is important. Dendritic cells are the major cells required for the immune response as they present antigens and stimulate the adaptive immune system. Studying the *in vitro* effect of BVDV or BHV-1 on MDDCs will help in understanding the immune responses in infected cattle or cattle vaccinated with modified live BVDV and/or BHV-1 vaccines.

RESEARCH GOAL AND OBJECTIVES

The overall goal of this research was to provide new information regarding cytokine expression and surface marker expression of virally infected MDDCs. Specific goals were:

1. Optimizing the *in vitro* MDDC yields.

2. Determining the effect of BVDV or BHV-1 infection on MDDCs mRNA cytokine expression.

3. Determining the effect of breed MDDC response to BVDV infection on cytokine expression.

4. Determining the effect of BHV-1 virally infected MDDC surface marker expression.

LITERATURE REVIEW

1. Bovine Viral Diarrhea Virus

1.1 BVDV Viral Structure and Replication

Bovine Viral Diarrhea Virus (BVDV) is a small, enveloped, single stranded RNA virus. The BVDV genome is only about 12.3 Kbp in length (Baule, *et al*., 1997) (Fig 1.1.). It comes from an unassigned order, the family *Flaviviridae* and the genus *Pestivirus*. This family also includes the genera *Flavivirus* and *Hepacivirus* that are similar to *Pestivirus* (Nettleton and Entrican, 1995; Thiel, 1996). Each of the three genera, have different viruses associated with them. The genus *Flavivirus* includes dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, West Nile virus, and yellow fever virus. The genus *Hepacivirus* contains only the hepatitis C virus. Finally, the genus *Pestivirus* contains border disease virus, classical swine fever virus, bovine viral diarrhea virus 1, and bovine viral diarrhea virus 2. All of the *Pestivirus* genus are non-zoonotic (Mayo, 2002). The genome of BVDV contains one single open reading frame (ORF) containing approximately 4,000 codons, which yields 10 or 11 posttranslational cleaved proteins. At both 5' and 3' ends, there are untranslated regions (Donis, 1995). The 5' untranslated region (5' UTR) is essential for viral replication, while the 3' untranslated region (3' UTR) is for the viral replication termination. The translation

occurs when a CAP independent mechanism is able to enter the internal ribosomal entry site (IRES) located in the 5' UTR (Frolov, *et al.*, 1998; Isken, *et al.*, 2004). The structural proteins and the nonstructural proteins are encoded in different regions. The 5' 1/3 of the genome encodes structural proteins and the 3' 2/3 of the genome encodes non-structural proteins (Collet, *et al.*, 1988). There are four structural proteins: the capsid protein (C), and three surface glycoproteins expressed in the envelope; Erns (envelope protein with RNAse activity), E1, and E2 (Steck, *et al.*, 1980). There are eight non-structural (NS) proteins: Npro (non-structural protease), NS2, NS3, NS4A, NS4B, NS5A, NS5B, and NS23 (Tamura, *et al.*, 1993). NS23 is a protein that differs between cytopathic and noncytopathic strains of BVDV. The protein is cleaved in cytopathic strains becoming NS2 and NS3, but remains NS23 in noncytopathic strains (Ridpath and Bolin, 1995). In the genome, the proteins are organized 5' to 3': Npro, C, Erns, E1, E2, p7, NS23, NS4A, NS4B, NS5A, NS5B (Pocock *et al.*, 1987) (Fig. 1.2.). Each of the proteins serve a specific purpose. Npro is an autoprotease, a protease that cleaves itself, causing the C protein to be cleaved (Wiskerchen and Collett, 1991). Erns degrades the double stranded RNA (dsRNA) structures that accumulate during infection using its RNase domain. This is important as the lack of dsRNA prevents toll-like receptor 3 and RIG-1-like receptors from detecting the viral infection (Meyers *et al.*, 2007). The E2 protein is the primary antigenic protein, while E1 is secondary (Steck, *et al.*, 1980). E1 and E2 are surface glycoproteins that are in the viral envelope which is the outermost layer of the virion as dimers (Fig 1.1.) (Thiel, 1996). Viral infection is prevented by incubating BVDV with anti-E2 antibodies. This suggests that viral infection occurs through an E2-mediated mechanism (Cardoso *et al.*, 2016). The p7 protein serves as an ion channel for

transportation (Griffin, *et al.*, 2003). NS3 has two important functions. The N terminal

encodes a serine protease which functions to assist NS4A that mediates the processing of

the downstream proteins (Tautz, *et al.*, 1997; Xu, *et al.*, 1997). The C terminal encodes a

RNA helicase that is vital in RNA replication (Warrener and Collett. 1995). NS5A

contains a serine phosphoprotein that allows interaction with cellular kinases (Reed *et al.*,

1998). Finally, NS5B is an RNA polymerase that is viral RNA-dependent (Zhong, *et al.*,

1998). NS2 and NS4B proteins are important in viral replication (Behrens, *et al.*, 1998).

After replication has occurred, BVDV takes the lipid envelope from the host cell that also

contains E1 and E2 viral glycoproteins (Thiel, 1996).

1.2 BVDV Genetics and Immunology

Bovine viral diarrhea virus strains are classified into two different biotypes and

two different species. The biotypes are cytopathic (cp) and noncytopathic (ncp). The cp

strains cause pathological changes in an infected cell, while the ncp strains do not cause

pathological changes in an infected cell (Lee and Gillespie, 1957). Persistent infection

(PI) results only when ncp strains but not cp infect the fetus early in pregnancy (Bolin, *et

al.*, 1985). Cp strains are associated with mucosal lesions. Cp strains stimulate the

immune system, up regulating inflammatory cytokines and causing apoptosis while ncp

strains down regulate inflammatory cytokines (Brock, 1995; Hoff and Donis, 1997;

Peterhans *et al.*, 2003; Vivier and Malissen, 2005). The two species of BVDV are type-1

and type-2. They are based on the 5' UTR region, which differs between the two types,

and is also used for classification into subgroups (Ridpath, *et al.*, 2000). Type-1 strains

were the first BVDV strains identified and tend to be less virulent than type-2 (Corapi, *et

al.*, 1990). Type-2 strains occur in the most virulent BVDV outbreaks, but can also be

found causing less virulent disease (Hamers, *et al.*, 2001). The strains are also broken into subgroups. Type-1 has 16 subgroups. Type-2 only has 3 subgroups (Peterhans, *et al.*, 2010).

1.3 BVDV Symptoms

There are a number of diseases and symptoms associated with BVDV. These diseases and symptoms depend on the strain, the age, and condition of the host. Diseases caused by BVDV are either acute, chronic, or persistent infection (PI) (Fig 1.3.). A PI animal is an animal that will shed the virus for life. Acute symptoms are relatively short, lasting less than 2 weeks, chronic symptoms last for months, and persistent infections can last the life of the animal. Additionally, mucosal disease and spontaneous abortion are associated with BVDV (Radostits, 1985; Brownlie *et al.*, 2000). Mucosal disease is a serious pathological complication that can be caused by BVDV. It only develops in an animal if the animal is persistently infected with a ncp strain, and becomes superinfected with a cytopathic strain of BVDV that is similar to the PI ncp strain. Mucosal disease has three forms: acute, chronic, and delayed onset (Bolin, 1995). In the case of acute mucosal disease, the disease develops when a cp BVDV strain that is homologous to the PI ncp BVDV strain is present. As the cp strain is homologous to a ncp strain, the cp strain is able to grow without alerting the immune system (Bolin, 1995; Tautz *et al.*, 1994). Incubation for an acute infection is one to two weeks and is first observed around 6 to 18 months of age. There have been cases observed in younger animals, but these are not the norm (Laureyns, *et al.*, 2011). Symptoms of acute mucosal disease include: anorexia, bloody watery diarrhea, decreased milk production, depression, drooling, oral erosions, and pyrexia. Chronic mucosal disease animals usually die. If the animal

recovers, the PI animal will continue to be viremic with the original ncp BVDV strain. However, the cp BVDV infection will be cleared. Chronic mucosal disease symptoms include: alopecia, anorexia, bloating, diarrhea, oral erosions, lameness, and weight loss. With both acute and chronic mucosal disease, oral erosions are only seen in the most severe of cases (Baker, 1995). The final form of mucosal disease is delayed onset. Delayed onset mucosal disease is similar to acute mucosal disease, but is seen after the inoculation of the PI animal with a heterologous cp BVDV strain. This heterologous strain is often a modified live cp BVDV vaccine and is seen weeks to months after the inoculation (Westenbrink, *et al.*, 1989; Ridpath and Bolin, 1995).

The age of the fetus plays an important role in outcome of the BVDV fetal infection. If a fetus is infected in the first 60 days of pregnancy, the pregnancy will likely end in an abortion or fetal death. If the fetus is infected between days 60 and 150, the pregnancy will likely yield a PI calf. It is possible that the fetus does clear the virus. A PI calf may have decreased birth weight, stunted growth, and weight gain, but also may appear normal (Fredriksen, *et al.*, 1999; Moennig, *et al.*, 2005). If the fetus becomes infected after 150 days of pregnancy, the immune system of the fetus has developed enough to mount an immune response to the BVDV infection. However, the calves may be born with congenital birth defects (Brownlie, *et al.*, 2000). In addition, congenital infection will result in no significant birth defects but may result in increased neonatal morbidity and reproductive delay (Muñoz-Zanzi *et al.*, 2003; Muñoz-Zanzi *et al.*, 2004).

2. Bovine Herpesvirus-1

<u>2.1 BHV-1 Viral Structure and Replication</u>

Bovine herpesvirus-1 (BHV-1) is a large, enveloped double stranded DNA virus. It belongs to the order *Herpesvirales*, the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*, and the genus *Varicellovirus* (Kurjogi, *et al*., 2012) (Fig 1.4.). There are a number of viruses that are closely related to BHV-1. These include BHV-2, BHV-4, BHV-5, equine herpesvirus types 1, 4, and 9 (EHV-1, EHV-4, and EHV-9), pseudorabies virus (PRV), and varicella zoster virus (VZV). Herpes simplex virus (HSV) is a distantly related alphaherpesvirus. BHV-1 is a major virus in the cattle industry and is associated with a variety of acute diseases. Diseases include: abortion, conjunctivitis, dermatitis, enteritis, encephalitis, infectious balanoposthitis (IPB) in bulls, infertility, infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) in cows, mastitis, and shipping fever (Straub, 2001; Raaperi *et al*., 2014; Jones, 2003; Tikoo *et al*., 1995; Fiorito *et al*., 2013). BHV-1 infects the host through the conjunctiva, the mucosa of the upper respiratory tract, or the reproductive tract. The virus is able to destroy the epithelium layer, and enter the blood where it multiples to cause viremia. This viremia can spread to additional organs of the body, particularly the central nervous system (CNS) and reproductive system (Straub, 1990; Renjifo *et al*., 1999). The virus infects cells by binding to the cell surface and entry is by using viral glycoproteins (gB, gC, gD, gE, gH, gK, gL). Not all of the glycoproteins are essential. Glycoproteins gB and gC are important to bind heparin sulfate on the host cell and tethers the virus to the cell. Next, BHV-1 gD binds to a virus specific receptor allowing successful infection of a cell (Li *et at*., 1995; Dasika and Letchworth, 1999; Hanon *et al*., 1999; Schroder and Keil, 1999;

Hanon *et al*., 1998; Tyler and Nathanson, 2001; Li *et al*., 1996; Biswas *et al*., 2013; Chowdhury and Sharma, 2012; Whitbeck *et al*., 1996). After the virus has entered the cell, the viral nucleocapsid is transported to the nucleus where it uses the host cell machinery for DNA replication. Gene expression is completed in three phases: immediate-early (IE), early (E), and late (L). In IE gene expression, stimulation occurs by virion component bTIF. There are two transcription units in IE transcription. IE transcription unit 1 encodes function homologues of ICP0 and ICP4, while IE transcription unit 2 encodes a protein similar to ICP22. The IE proteins activate E gene expression causing viral DNA replication to begin. The protein bICP0 activates L gene expression allowing virion assembly and release (Jones, 2003). The virus takes the host's nuclear envelope, and is then released from the cell. The virus infected cell infects other cells as early as 8 hours post infection (p.i.) (Hunter, 2001; Knipe *et al*., 2001; Biswas *et al*., 2013). An important property of herpesviruses is their ability to establish a latent infection. The virus can be reactivated by stressors such as transportation, weaning, parturition and then cause symptoms again. The route of infection for BHV-1 determines the site of virus latency. It may be latent in the dorsal root ganglia which is most common, peripheral blood cells, or the tonsils. Additionally, BHV-1 is able to predispose animals to secondary bacterial infections. The virus can also be shed when an animal is going through immunosuppression (Fiorito *et al*., 2013; Winkler *et al*., 2000; Biswas *et al*., 2013).

2.2 BHV-1 Genetics and Immunology

The *Herpesviridae* is a very large family of viruses. There are many different herpesviruses that infect many different species of mammals. Latent infection is a feature

of all viruses of this family. Latency results in a lifelong possibility that the virus will reactivate from its latent state and then spread to other animals of the same species. Three areas of biology are of particular importance in learning about herpesviruses: basic immunology, latency, and treatments. Comparative studies are important because excessive proinflammatory cytokine production, route of infection, and latency are conserved among herpesviruses (Compton *et al*., 2003; Kurt-Jones *et al*., 2004; Aravalli *et al*., 2005; Glaser *et al*., 2006; Waldman *et al*., 2008; Ariza *et al*., 2009, 2013; Gregory *et al*., 2012; Ariza *et al*., 2014).

Herpesviridae is further divided into subfamilies for classification according to the genetics and tropism of the virus. Subfamilies are alpha (α), beta (β), and gamma (λ) (Ariza *et al*., 2014). BHV-1 is an α-herpesvirus. Additionally, HSV-1, HSV-2, and VZV are also α-herpesviruses. The alpha subfamily is associated with establishing latency in neurons (Sloan *et al*., 2011). Cytomegalovirus (CMV) and human herpesvirus (HHV) 6 and 7 are β-herpesviruses. This subfamily establishes latency in leukocytes and the virus multiples slower than the other two subfamilies. This is consistent longer incubation period these viruses take to show symptoms. Finally, λ -herpesviruses establishes latency in immune cells. This subfamily includes the Epstein Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). As noted, each subfamily has differences, but share a similarity in dUTPase enzymatic activity. The dUTPase genes have different sizes and functions, likely indicating evolution played a role in the viruses' ability to interact with the host immune system (Ariza, 2014). By classifying the different herpesviruses, it helps to identify the different clinical outcomes. Within the α-herpesviruses subfamily, is the *Varicellovirus* genera, which includes BHV-1. A number

of viruses are related to BHV-1. These include BHV-2, BHV-4, BHV-5, pseudorabies

virus (PRV), equine herpesvirus types 1, 4, and 9 (EHV-1, EHV-4, and EHV-9), and

VZV. The most similarly related virus to BHV-1 is BHV-5 which was originally known

as BHV-1.3. This virus has an 82% amino acid homology with BHV-1. In addition, it

establishes latency in sensory ganglia and induces fatal meningoencephalitis in calves 18

months or younger (Fan *et al*., 2012; Vogel *et al*., 2003). BHV-5 infections occur more

frequently in South America, but are reported sporadically in Australia. The similarity

between BHV-1 and BHV-5 is close enough that BHV-1 vaccines have cross protection

against BHV-5 (Delhon *et al*., 2003; Del Medico *et al*., 2006; Fan *et al*., 2012). Another

closely related virus, PRV, is a swine virus that causes Aujeszky's disease and abortions.

For swine, PRV infection is most commonly found only in the peripheral nervous system

(PNS), which can cause mortality of the animal (Lyman *et al*., 2009). It is also seen in

cattle, and is known as mad itch (Li *et al*., 2014) because it causes rabies-like symptoms.

These clinical signs include excessive salivation and neurological symptoms. In cattle,

the infection doesn't stop at the PNS, but continues from the PNS to the central nervous

system (CNS) causing the death of the animal (Lyman *et al*., 2009). PRV is extensively

studied in the laboratory as a model for neuronal transmission (Enquist and Card, 2003;

Lyman *et al*., 2009). In horses, EHV-1 is very similar to BHV-1 and causes abortion,

equine herpesvirus myeloencephalopathy (EHM) and respiratory problems (Pusterla *et

al*., 2009; Pusterla and Hussey, 2014). Interestingly enough, EHV-4 has an 80% nucleic

acid similarity, but EHV-4 is less severe than EHV-1. The virus, EHV-4, only causes

fever, mild upper respiratory infections and general malaise (Spiesschaert *et al*., 2015).

The other similar equine virus, EHV-9, is a neurotropic strain of EHV. Neurotropic

indicates that the virus can cause nervous system issues (El-Habashi *et al*., 2014). For

human viruses in this family, VZV causes chickenpox (varicella) in children or shingles

(herpes zoster) in adults that have a latent infection (Sloan *et al*., 2011). Finally, HSV-1

and HSV-2 are the most common herpes-viruses in humans. (Koelle and Corey, 2008;

Bedoui and Greyer, 2014).

2.3 BHV-1 Symptoms

BHV-1 infection symptoms are heavily influenced by the subtype of the virus.

There are two types of BHV-1: BHV-1.1 and BHV-1.2. BHV-1.3 is now classified, as

previously mentioned, as BHV-5. The most common syndrome associated with BHV-1

is respiratory disease, which is usually associated with BHV-1.1. Respiratory symptoms

include: serous to mucopurulent discharge from the mouth and nose, fever, and severe

breathing problems. If the infected animal becomes infected with bacteria, the symptoms

can become more severe. This is seen in bovine respiratory disease complex, which is

often the result of stress, in combination with a viral infection followed by secondary

bacterial pneumonia (Biswas *et al.*, 2013). The other subtype, BHV-1.2, is associated

with localized genital infections, balanoposthitis in the bull and vulvovaginitis in heifers.

Interestingly both 1.1 and 1.2 can cause abortion after the fifth month in the gestation

cycle. Since BHV-1 causes viremia, the reproductive tract is at risk regardless of the

subtype and the initial route of infection. These viruses also infect the ovaries and result

in necrosis of the corpus luteum. Additionally, BHV-1.2 can cause frequent urination

and pustules (Biswas e*t al.*, 2013; Brower *et al.*, 2008). Infection of BHV-1 is hard to

detect if no symptoms are present. When symptoms are observed, it is around the fourth

day post infection (p.i.). Symptoms will usually last no more than ten days, but

additional infection can cause an increase and worsen the infection. Having an infected

animal in a herd can cause the virus to spread, so it is important to monitor conditions

closely (Biswas *et al.*, 2013).

3. Monocyte-Derived Dendritic Cell Biology

3.1 MDDC Biology

Due to the link between the adaptive and innate immune system, dendritic cells

(DC) are an important area of interest in studying immunology and viral effects on the

host. A dendritic cell is the most important antigen presenting cell (APC). Classification

of a DC consists is based on morphology, phenotype, location and function (Summerfield

et al., 2015). There are a variety of subtypes. These include conventional DCs (cDC;

previously known as myeloid dendritic cells), plasmacytoid DCs (pDC) and the *in vitro*

monocyte-derived dendritic cells (MDDC). Each of the DCs have a different function;

cDCs are involved in T cell activation and antigen presentation and pDCs produce large

quantities of Type I interferon when stimulated with nucleic acids (Summerfield *et al*.,

2015). Production of DCs isn't the same for each subtype. Both cDCs and pDCs derive

from common dendritic cell precursors, whereas MDDCs are derived from monocyte

precursors (Merad *et al*., 2013). When looking at the location of *in vivo* DCs, they are

regulated by monocytes that are circulating throughout the body and can be found in non-

lymphoid organs (Merad *et al*., 2013). Studying differentiated DCs is difficult because

they can undergo phenotypic changes as they translocate *in vivo*. This can alter cell

surface markers and functions (Summerfield *et al*., 2015). Due to the standardized of *in*

vitro culture methods for MDDCs, they are an easier DC type for investigation. Bovine

MDDCs are isolated *in vitro* and used for immunological studies. The easiest way to

characterize an immunological cell is to identify surface marker expression. Each surface

marker has specific cellular functions and are only found on those cell types. MDDCs

are recognized as a DC subset that has the following surface markers: MHCI, MHCII,

CD1a, CD1b, CD11b, low amounts of CD14, CD86, CD172a, CD205 (Dec 205), and

CD206 on their surfaces. No CD4, CD11c, CD13, CD21 and CD26 surface markers are

present (Summerfield *et al*., 2015). For these studies, MHCI, MHCII, CD14, and CD86

were measured. DC subsets express different cell surface markers compared to each

other. This helps classify subsets. MHCI is found on the cell surfaces of all nucleated

cells. MHCI is important because of the interaction with CD8 found on cytotoxic T cells.

MHCII is expressed on APCs and is important because of the interaction with CD4 also

found on T cells. B cells, monocytes and other APCs also express MHCII on their

surfaces (Benoist and Mathis, 1990; Taylor *et al*., 1993). CD86 is a co-stimulatory

molecule that is up-regulated on the surface of activated DCs (Summerfield *et al*., 2015).

CD80 and CD86 are co-receptors found on DCs. These provide an activation signal

following MHCII presentation to T lymphocytes to complete DC maturation and

macrophage and B cell activation (Teichmann *et al*., 2000; Palomares *et al*., 2014).

Another important surface marker is CD14. CD14 is used to distinguish the level of

differentiation for monocytes, macrophages, and dendritic cells. CD14 is a co-receptor

that interacts with TLR 4 for bacterial LPS (Rajput *et al*., 2014) and is absent or at low

levels on DCs. Dec 205 is a specific marker for DCs (Rajput *et al*., 2014). Dec 205

introduces antigens to the MHCI and MHCII pathways following antigen uptake. This

allows presentation to CD4 and CD8 T cells (Shrimpton, 2008). The phenotype of

MDDCs is defined by these cell surface markers (MHCI$^+$, MHCII$^+$, CD86$^+$, CD14^{low}, DEC205$^+$).

When a DC activates, cell surface marker expression changes. As MDDCs originate as monocytes, there is the expectation to see overlapping of phenotypes. Identifying the correct phenotype of bovine DCs is important since different surface markers on the cells can cause different outcomes (Rajput *et al*., 2014). MDDCs are distinguishable from macrophages by lower levels of CD14. Macrophages have high levels of CD14 (Mirkovitch *et al*., 2006; Summerfield *et al*., 2015). By using GM-CSF and IL4, MDDCs differentiate, causing a change in response to LPS stimulation. This is seen with increased expression of MHCI, MHCII, CD80 and CD86, and a decrease in CD14 (Summerfield *et al*., 2015).

4. Cytokines

4.1 Cytokines

A cytokine is a signaling protein produced by many cells of the immune system. Cytokines serve to regulate immune responses. Small cytokines are called chemokines, and are also known as chemotatic cytokines (Stenken and Poschenrieder, 2015). Classification of cytokines has been done in multiple ways over the years. Classification has been based on both cytokine three-dimensional structure and function, but classification is now based only on cytokine receptor three-dimensional structures. The families include: the hemopoietic growth factor (type 1) family, the interferon (type 2) family, the tumor necrosis factors (type 3) family, the interleukin-1 receptor family, and the interleukin-17 receptor family (Schwartz *et al.*, 2016). Interferon-alpha (IFN-alpha),

Interferon-beta (IFN-beta), Interferon-gamma (IFN-gamma), Tumor Necrosis Factor-alpha (TNF-alpha), Interleukin-1a (IL-1a), Interleukin-1b (IL-1b), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), and Interleukin-12 (IL-12) were measured in MDDCs in these experiments. Both IFN-alpha and IFN-beta are part of the type 2 cytokine family and type 1 interferon subgroup. IFN-alpha is produced by most cells of the body and is primarily involved in innate immune responses against a viral infection. There are 13 subtypes of IFN-alpha. IFN-beta is produced by fibroblasts and are primarily involved in innate immune responses against a viral infection. There are 2 subtypes of IFN-beta (Stenken and Poschenrieder, 2015). There are also six other IFN types: IFN-kappa, IFN-delta, IFN-Epsilon, IFN-tau, IFN-omega, and IFN-zeta (Hardy *et al*., 2004). IFN-gamma is also part of the type 2 family, but is a type 2 interferon subgroup and is produced by leukocytes - particularly NK cells and T cells. IFN-gamma is involved in regulating immune and inflammatory responses, but also has some weak antiviral and antitumor effects. IFN-gamma is able to potentiate the effects of interferon type 1. TNF-alpha is part of the type 3 family. TNF-alpha is produced by a variety of cells, but is primarily produced by macrophages. It is involved in systemic inflammation and is involved in the acute phase reaction. TNF-alpha has the primary role of immune cell regulation. It is an endogenous pyrogen, a substance that induces fever, but causes apoptosis and inhibits tumorigenesis and viral replication. Both IL-1a and IL-b are part of the interleukin-1 family. They are both produced by a variety of cells such as macrophages and dendritic cells. They are 2 of the 11 cytokines found in this family. Both IL-1a and IL-1b are also endogenous pyrogens, but are also able to cause vasodilatation, hypotension, and hyperalgesia (Stenken and Poschenrieder, 2015).

Additionally, unique to this family, IL-1a and IL-1b are synthesized as a precursor

protein. IL-1b is synthesized as precursor protein only after stimulation, but IL-1a does

not require stimulation (Contassot *et al.*, 2012). IL-6 and IL-12 are both part of the type

1 family. IL-6 is secreted by T-cells and macrophages to help stimulate the immune

response during infections where it is an important mediator of the acute immune

response and fever. It also has an anti-inflammatory activity when it inhibits the effects

of TNF-alpha, IL-1 and activates IL-10. IL-12 is produced by a variety of cells including

macrophages and dendritic cells. Macrophages produce IL-12 only after antigenic

stimulation (Stenken and Poschenrieder, 2015). However, dendritic cells naturally

produce IL-12 (Kaliński *et al.*, 1997). IL-12 is a T cell-stimulating factor that is able to

differentiate naive T cells into Th1 cells (Hsieh, *et al.*, 1993). IL-12 also stimulates the

production of IFN-gamma and TNF-alpha from T cells and natural killer cells.

Additionally, IL-12 is able to enhance the cytotoxic activity of natural killer cells and T

lymphocytes. IL-8 is a chemokine that is produced by macrophages. It is part of the

CXC chemokine family (Hedges *et al.*, 2000). IL-8 has two main functions. These are

inducing neutrophil chemotaxis and then inducing neutrophil phagocytosis at the target

site. The final cytokine is IL-10 is also part of the type 2 family. It is primarily produced

by monocytes, and has anti-inflammatory and immunoregulatory effects (Stenken and

Poschenrieder, 2015).

SUMMARY

BVDV and BHV-1 play important roles as disease agents in the cattle industry.

Both viruses have significant economic consequences. MDDC studies will hopefully

give a better understanding of how cytokine production affects the relationship between the innate and adaptive immune systems. This literature review has covered the major areas regarding BVDV, BHV-1, DCs, and cytokines. The chapters that follow will examine each of these categories in more detail. Chapter 2 covers the optimization of MDDC yields and the affect BVDV has on cytokine production of infected MDDCs. Chapter 3 covers the affect BHV-1 has on cytokine production on infected MDDCs and surface marker expression of infected MDDCs. Chapter 4 is a general summary of the results from the experiments.

Fig 1.1. Structure of BVDV: ViralZone 2010, Swiss Institute of Bioinformatics.

Fig 1.2. BVDV encoding proteins: ViralZone 2008, Swiss Institute of Bioinformatics.

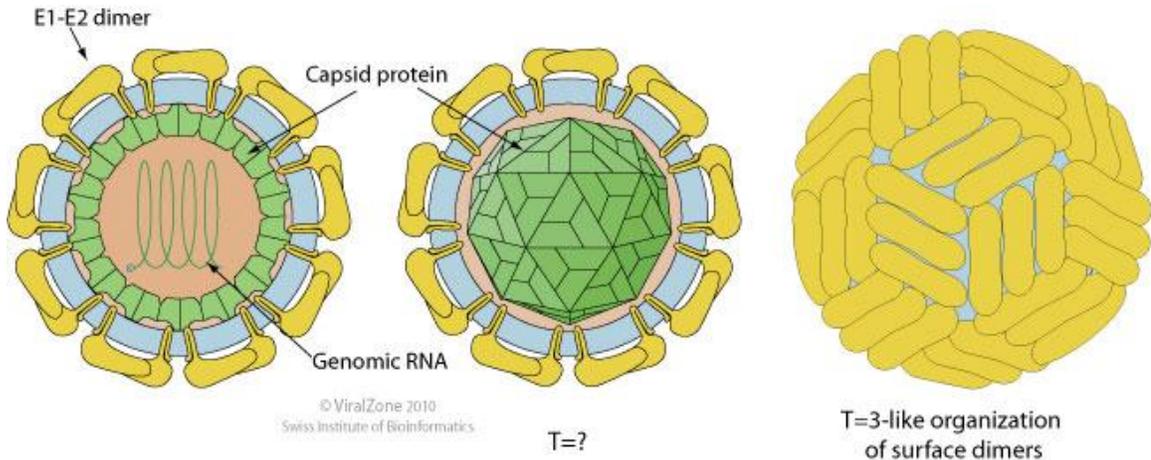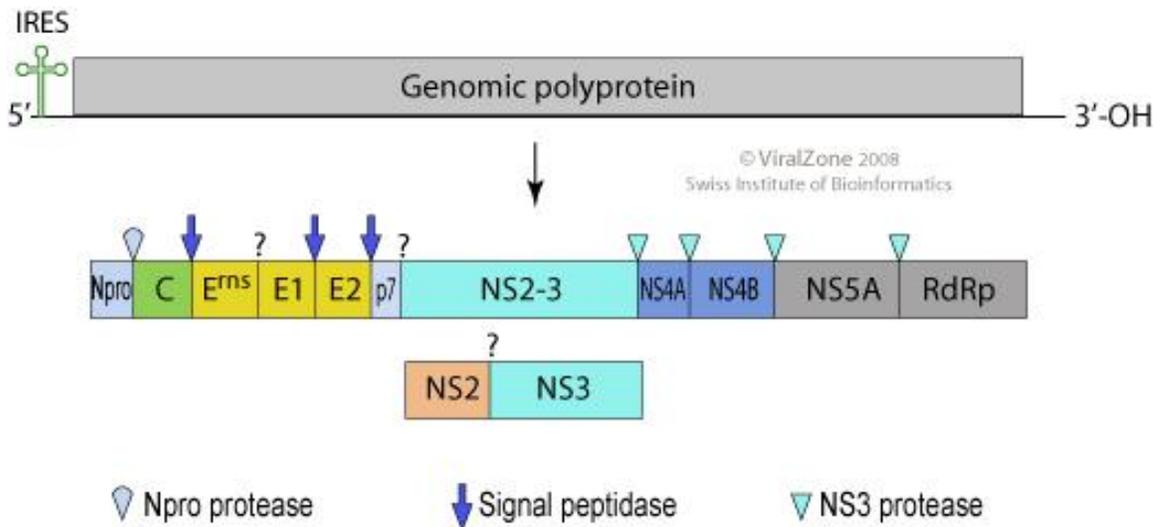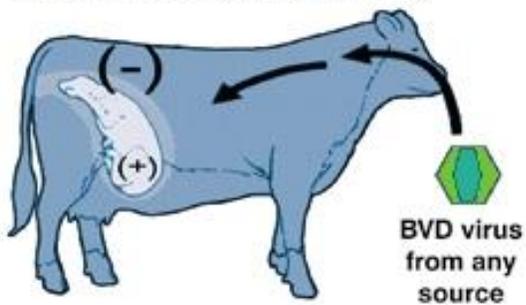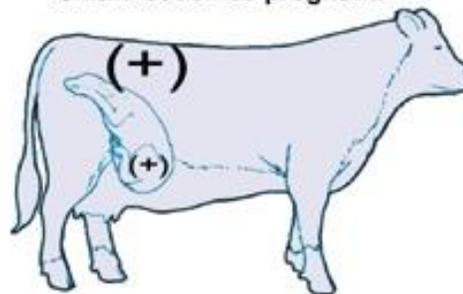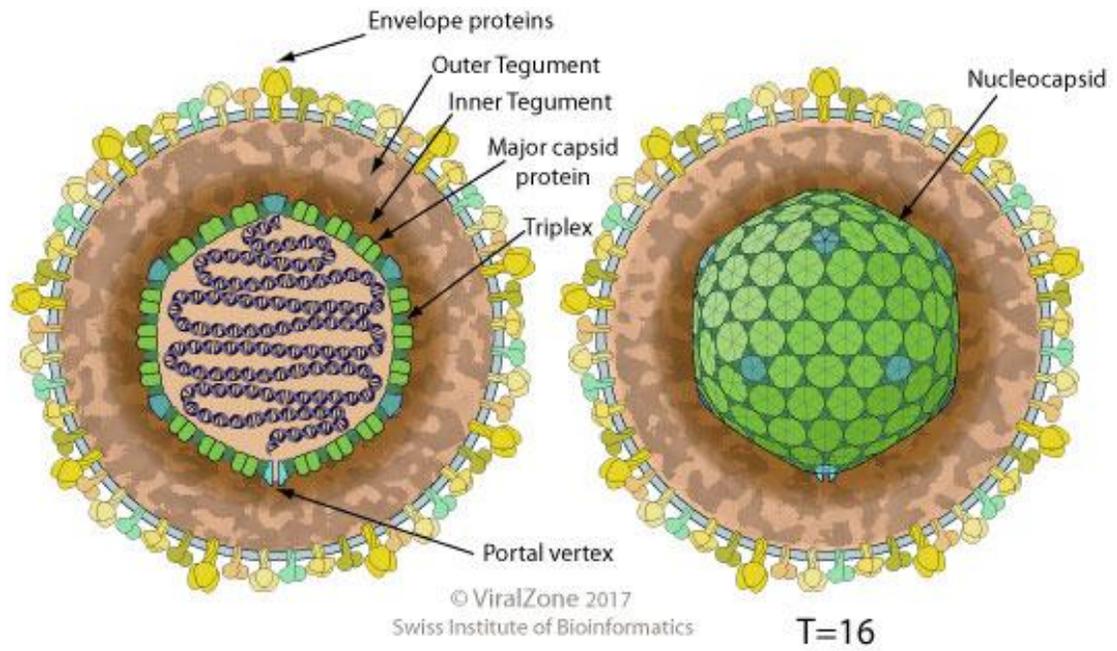**Fig 1.3. BVDV PI infection:** The routes of PI infection in a calf.

Fig 1.4. Structure of BHV-1: ViralZone 2017, Swiss Institute of Bioinformatics.

CHAPTER 2: MONOCYTE-DERIVED DENDRITIC CELL TECHNIQUE

OPTIMIZATION AND BOVINE VIRAL DIARRHEA VIRUS CYTOKINE

ANALYSIS

ABSTRACT

Dendritic cells serve as vital cells in the innate immune system. When functioning normally, dendritic cells produce cytokines that impact other cells in the body. An *in vitro* study was conducted to determine cytokine production in monocyte-derived dendritic cells to determine the influence BVDV has on cytokine production. Monocytes were first isolated from calf blood and differentiated into monocyte-derived dendritic cells. This was done over a 5-day time period after using IL-4 and GM-CSF to stimulate monocyte-derived dendritic cell differentiation. An optimization process was done to maximize the yield of viable monocyte-derived dendritic cells for the experiments. The optimization resulted in around $1x10^6$ MDDCs per mL from the starting value of $1x10^5$ per mL. This increased the yield by 10 times the original value. After the optimization was finished, the monocyte-derived dendritic cells were then infected with either BVDV strains 1373 or 28508. The following cytokines were measured: IFN-alpha, IFN-beta, IFN-gamma, IL-1a, IL-1b, IL-6, IL-8, IL-10, IL-12, and TNF. Time points were investigated for cytokine up regulation or down regulation at 0h, 6h, 12h, 24h, and 48h post infection. BVDV infection of monocyte-derived dendritic cells down regulated all cytokine mRNA compared to the control. IL-1b had the greatest down regulation with more than a 40-fold reduction. The three IFNs had less than a 2-fold decrease. The remainder of the IL cytokines and TNF had less than a 10-fold decrease. Both 1373 and 28508 down regulated IFN-alpha, IFN-beta, IFN-gamma, IL-a,

IL-1b, IL-6, IL-8, IL-10, IL-12, and TNF with 1373 having a greater down regulation

than 28508. Of the cytokines investigated, IL-8 and IFN-alpha had statistically

significant differences between the two strains. The remainder of the cytokines tested

had reduced expression compared to the housekeeping gene controls, but no statistical

significance between the two strains. This shows that both viruses interfere with normal

cytokine production of monocyte-derived dendritic cells.

INTRODUCTION

Dendritic cells (DC) are the main antigen presenting cell (APC) in the body. A

DC serves to connect the innate immune system to the adaptive immune system by

presenting antigens to naive T-cells to start the adaptive immune response (Summerfield

et al., 2015). Normally DCs are difficult to study due to being *in vivo*. By using isolated

monocytes and adding IL-4 and GM-CSF, monocytes are able to differentiate into

monocyte-derived dendritic cells (MDDC) *in vitro* (Rajput *et al.*, 2014). DCs have

MHCI, MHCII, CD86, and a decrease in CD14 surface markers that differentiates them

from other cells (Summerfield *et al.*, 2015).

Bovine Viral Diarrhea Virus (BVDV) is a small, enveloped, single stranded RNA

virus (Baule, *et al.*, 1997). BVDV strains are classified into two different biotypes and

two different species. The biotypes are cytopathic (cp) and noncytopathic (ncp). A cp

strain causes pathological changes in cells, while ncp strains don't causes pathological

changes (Lee and Gillespie, 1957). The two different species are type 1 and type 2. Type

2 are generally thought to be more virulent. Within each species there are also strain

differences. Within the type 2 ncp viruses, 1373 strain is a highly virulent virus (Liebler-Tenorio *et al.*, 2002) resulting in high morbidity and high mortality while the 28508 strain has low morbidity and no mortality (Liebler-Tenorio *et al.*, 2003). BVDV is associated with a number of diseases and symptoms, with persistent infection (PI) of the virus being a large problem. A PI animal will shed the virus for life causing infection of other animals in the herd. Mucosal disease and spontaneous abortion are two diseases that have large economic impact that are caused by BVDV (Radostits, 1985; Brownlie *et al.*, 2000).

Monocytes and MDDCs differ in their interaction with BVDV. With ncp BVDV, monocytes were compromised in allogenic stimulation and CD4+ T-cell responses. No effect was noticed with MDDCs. Monocytes are killed apoptotically by cp BVDV infection, but MDDCs are unaffected. Stimulation of gene expression of IFN-1 is predominantly seen in MDDCs (Reid *et al.*, 2016). Interferon type 1 (IFN-1) is produced in response to BVDV in both *in vivo* and *in vitro*. IFN-Alpha and IFN-Beta are produced in cp BVDV-infected cells (Glew, *et al.*, 2003).   Some strains of ncp BVDV have a decrease in IFN-Alpha (Alkheraif, *et al.*, 2017).

This study was designed to determine the effect of BVDV *in vitro* infection on MDDCs cytokine mRNA expression. The susceptibility of MDDCs to BVDV strains 1373 and 28508 were tested and the cytokine expression was measured for both strains.

MATERIALS AND METHODS

Animals

Healthy calves between the ages of 6-8 months housed at the South Dakota State University (SDSU) Dairy Farm located in Brookings, SD, USA were used in this study as blood donors. Four calves were used: two Holstein Friesian and two Brown Swiss calves were used. The SDSU Institutional Animal Care and Use Committee approved the handling of animals and blood collection. Blood (~ 250 mL per draw) was collected via jugular venipuncture using 60 mL heparinized plastic syringes with 16 gauge 1-1/2 needles. All of the calves were confirmed to be BVDV-free using virus isolation confirmed with a BVDV immunoperoxidase assay (IPA) (Fulton et al., 1997).

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Rajput *et al.*, 2014) with modifications. Two (2) mL of heparin sulfate was added to each of 4-60 mL syringes. The heparin sulfate (Sigma, Sigma Chemicals, St. Louis, MO, USA) was made by adding 1 mL of 1000 U/L stock solution to 99 mL of phosphate-buffered saline (PBS) to make 100 mL of liquid heparin sulfate at 1000 U/mL per mL of PBS. The syringes were used to collect 250 mL of blood by jugular venipuncture from the calves. After collection, the heparinized blood was layered over lymphoprep in SepMate™ lymphoprep 50 mL tubes (Stemcell Technologies, Cambridge, MA, USA). Fifteen (15) mL lymphoprep was added to each SepMate™ tube, to slightly cover the insert in the tube. Around 20 mL of heparinized blood was then layered on top of the lymphoprep. Sixteen (16) SepMate™ tubes were used for each blood collection. Each SepMate™ tube containing 35 mL of heparinized blood and lymphoprep were

centrifuged at 1200xg for 20 minutes at room temperature using a Beckman J6-MI

centrifuge. After centrifugation, with the red blood cells below the insert in the

SepMate™ tube, the liquid above the insert that contained the buffy coat was poured into

50 mL conical tubes (Falcon, Oxnard, CA, USA). After combining all of the buffy coats,

from the SepMate™ tubes, the tubes were centrifuged at 120xg for 10 minutes at room

temperature to pellet the cells. When the cells were pelleted, the supernatant was poured

into new 50 mL conical tubes. The pellets were then resuspended in 1 mL of PBS and

combined into a single conical tube. This tube was then filled to a total of 50 mL of PBS.

The tube with the cells from the pellets and the tubes with the liquid poured off were then

centrifuged at 120xg for 10 minutes at room temperature to wash the cells. The

supernatant was disposed and the pellets were all suspended in 1 mL of PBS. The pellets

were then all added to a single conical tube. This conical tube was filled with PBS to 50

mL and centrifuged at 120xg for 10 minutes at room temperature. The supernatant was

discarded and the cells were washed 2 or 3 more times to remove the platelets. When the

washing was finished, the viability of the PBMCs was determined using trypan blue

exclusion assay with 0.4% trypan blue stain (Strober, 2001). The viability of the

monocytes was calculated using the formula: cell viability % = number of viable cells X

100/total counted cells. The viable cells were the cells not stained by trypan blue, while

the non-viable cells were stained by trypan blue. The pellet of PBMCs was suspended in

1 mL of a mixture containing Roswell Park Memorial Institute (RPMI) 1640 medium

(GE Healthcare, Hyclone Laboratories, Logan, UT, USA), penicillin (100 U/mL) and

streptomycin (100 μg/mL). The cells were then divided into 4 conical tubes with 4 mL of

FBS (Fetal bovine serum) and 36 mL of RPMI to create complete Roswell Park

Memorial Institute (cRPMI) medium with 10% FBS into each conical tube. Twenty (20)

mL of the cells and medium was added to each T_{175} Flask (Corning, NY, USA) and

incubated in a CO_2 incubator for 2 hours and 30 minutes at 37°C.

Monocyte Isolation Using Plastic Adherence Technique

Monocytes were isolated using a modified version of the plastic adhesion method

(Rajput, 2014). After the 2-1/2 hour incubation, the floating cells were removed from the

flasks by decanting the media, removing by pipetting any additional media in the flasks

and then discarded. Two-hundred and fifty (250) mL of warm PBS was poured into each

flask at 50 mL intervals for a total of 5 washes. Each wash consisted of rocking the flask

back and forth for 1 minute and then the liquid was disposed of. After a flask had been

washed, 6 mL of Accutase™ (eBioscience, San Diego, CA, USA) was added to the flask

and the flask was incubated in a CO_2 incubator for 10 minutes at 37°C. When incubation

was finished, the detached cells were washed and pelleted through centrifugation at

120xg for 10 minutes at room temperature. The pellet was resuspended and washed and

pelleted a second time. Before culturing the monocytes into MDDCs, the cells were

verified to be monocytes using the phenotypic markers MHCI, MHCII, and DC86.

Monocyte-Derived Dendritic Cell (MDDC) Culture

MDDCs were cultured following the protocol in Rajput, 2014 with procedural

modifications. RPMI 1640 medium (GE Healthcare, Hyclone Laboratories, Logan, UT,

USA) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) was

added. Twenty mL (4 mL of FBS and 16 mL of RPMI; final concentration of 20% FBS)

were added to the monocytes. Additionally, 1 mL of IL-4 (10 μg/mL) and 100 μL of

GM-CSF (10 μg/mL) were added to the 20 mL mixture. Two, 6-well plates were used

for the MDDC growth. Into each well, ~3 mL of cell mixture were added. The two

plates were placed into a CO_2 incubator at 37˚C on a plate shaker for 12 hours. After 12

hours, the plates were removed from the shakers and allowed to grow for 4 days and 12

hours longer. Every other day, each well containing cells was feed with 1 mL of RPMI

1640 medium containing 20% FBS, IL-4, and GM-CSF. After the 5 days of growth, the

DCs were examined for surface markers and virally infected.

Monocyte-Derived Dendritic Cell (MDDC) Surface Marker Expression

Before the DCs were infected with virus, the DCs were characterized

phenotypically as MHC1, MHCI positive, and CD14 low using the Rajput, 2014

technique. The DCs were first split into a 96-well plate at a $1x10^5$ concentration per well.

The DCs were then washed with PBS by centrifugation for 10 minutes at 120xg. The

DCs were labeled using the primary mouse monoclonal antibodies (mAbs) MHCI

(H58A), MHCII (H42A), and CD14 (CAM36A) (Monoclonal Antibody Center, WSU,

Pullman, WA, USA). MHCI, MHCII, and CD14 antibodies were diluted 1:100 in PBS.

Controls of DCs not treated with antibodies and not treated with secondary antibodies

were used. Additionally, 1% FBS was added to each diluted primary antibody. For each

of the three antibodies, 50 µL of the diluted primary antibodies were added to the DCs

and incubated for 10 min at 4°C followed by washing with PBS. Following the wash, the

DCs were stained with 50 µL of a 1:1000 diluted in PBS fluorescein isothiocyanate

(FITC) labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA)

containing 1% FBS for 10 minutes at 4°C. Following the secondary antibody staining,

the DCs were resuspended in 200 µL of 1% paraformaldehyde. Finally, the DCs were

analyzed for surface marker expression using a BD Accuri™ C6 Plus Flow Cytometer

(BD Biosciences, CA, USA).

Viral Growth and Titration

For each virus, 5 mL of $5x10^5$ MDBK cells/mL were grown in 5 T25 flasks. The

cells were grown to around 60-70% confluency. At inoculation, the RPMI growth media

was removed and 0.75 mL of the specific BVDV virus was added to each flask using an

MOI of 1. The cells were incubated for 1h at 37°C. After incubation, the virus was

removed and the cells were washed using sterile PBS. After washing, 5 mL of RPMI was

added to each flask and then incubated for 4 days at 37°C. The cultures were harvested

using two freeze thaw cycles. The freeze thaw cycles consisted of -80°C exposure for 15

minutes and then thawed using 25°C. The cell debris was pelleted using centrifugation at

1200xg for 10 minutes at 4°C. The supernatant containing the virus was then titrated at

$1x10^5$ and stored at -80°C.

Viral titration was done using the method developed by Reed and Muench (Reed

and Muench, 1938). MDBK cells were grown prior, detached, and adjusted to $5x10^5$

cells/mL. One hundred-eighty (180) μL, of the MDBK cell suspension, was added to

each well in a 96-well plate. Twenty (20) μL of virus stock was added to the first row,

mixed, and then 20 μL was taken out from each well and added to the well below. This

process was repeated until the bottom 2 rows as they were used for negative control.

Four replications were used for each dilution. The plate was incubated at 37°C for 4

days. An immunoperoxidase (horseradish peroxidase, HRP) assay was conducted to

determine the endpoint to calculate virus titer (Fulton *et al.*, 1997) using the BVDV E2

Mab 15C5. The endpoint was determined by cellular red precipitate formed by HRP labeled BVDV antibody causing a reaction with the HRP substrate 3-amino-9-ethylcarbazole indicating the presence of BVDV protein.

Viral Infection

MDDCs were infected with one of 2 different BVDV strains, 1373 or 28508. The viral strains were diluted from the original titration to a multiplicity of infection (MOI) of 1 using RPMI 1640 medium. After an incubation period of 5 days, the MDDCs were washed with PBS to remove old media and resuspended in RPMI 1640 medium with $1x10^6$ MDDCs per mL. A 48-well plate was used for the viral infection with 100 μL of MDDCs and 100 μL of diluted virus added to each infected well. In the control wells, 100 μL of MDDCs were added and 100 μL of RPMI 1640 medium was used instead of virus. BVDV strains 1373, 20508, and the control were all done in triplicates for each time point. After infection, incubation was done at 37°C. Cells were collected at 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours for a total of 9 wells for each strain and control. Cells were collected at each time point by removing the supernatant and then centrifuging each sample at 120xg for 10 minutes. The supernatant was then removed and frozen at -80°C, and 100 μL of PBS was added to the pellet. The pellet was resuspended and was used for qRT-PCR.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The BVDV infected cells, noninfected cells (cell control), and DNase RNase free water (negative control) (Invitrogen, Life Technologies, Thermo Fisher Scientific, PA, USA) had nucleic acid extracted using a RNeasy extraction kit (Qiagen, Valencia, CA,

USA). The extracted nucleic acid from the infected cells and noninfected cells were normalized using the lowest ng/μL which was determined using Nanodrop ND-2000 Spectrophotometer (Fisher Scientific, NH, USA). Next, qRT-PCR was conducted for expression of the following cytokines: IFN-alpha, IFN-beta, IFN-gamma, IL-1a, IL-1b, IL-6, IL-8, IL-10, IL-12, and TNF. Beta-actin was used for the housekeeping gene to ensure PCR efficiency and standardization. The primers used are included in table 2.1. For qRT-PCR, reaction sizes of 25 μL were used in each well of a standard 96-well PCR plate. For qRT-PCR, a Power SYBR® Green RNA-to-Ct™ 1-Step Kit was used for the master mix (Thermo Fisher Scientific, PA, USA). For the tested samples 2 μL of the normalized samples, DNase RNase free water, for the negative control, and LPS for the positive control were used. The quantification of the cellular mRNA was conducted using an ABI 7900HT High-Throughput Real-Time Thermocycler (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, PA, USA). Results from the qRT-PCR were given in cycle threshold (CT) values. The CT value refers to the number of PCR cycles before threshold detection is found. The CT values were analyzed using the relative expression software tool (REST©2009 software) (Pfaffl *et al.*, 2002).

Statistical Analysis

Data was analyzed using a student's t-test to determine the significance of the differences between the mean values of the treated samples and the control samples at the time points. A statistical significance was seen at a P-value <0.5 and a biological significance was seen at a P-value <0.1 but >0.5. All of the experiments were conducted using at least 3 animals, breed depended on the experiment, and 3 repeats for each animal. Standard deviation was used for each time point. The cytokine analysis was

based on the analysis of variance (ANOVA) using the REST© 2009 program (Pfaffl *et al.*, 2002).

RESULTS

Monocyte-Derived Dendritic Cell Technique Optimization

Prior to these optimization experiments the yield of MDDCs (MHCI$^+$, MHCII$^+$, CD86$^+$, CD14^{low}) was ~ 1x10^4 MDDCs per well in a 48-well plate. After the protocol optimization, the MDDCs increased 10-fold to 1x10^5 per well in a 48-well plate. A number of adjustments were made to the starting protocol to increase yield. The first change made from the original protocol was using SepMate™ tubes instead of normal conical tubes. The total amount of buffy coat collected increased by about 40% using the SepMate™ tubes as opposed to the conical tubes from the original protocol. Before the yield increased, the SepMate™ tubes were optimized with various volume ratios of blood and lymphoprep. With the centrifuge speed, 1200xg, used by Rajput, *et al.*, 15 mL of lymphoprep and 20 mL of blood was the optimal ratio. Another problem that caused repeated trials to optimize yield was the large number of platelets with the PBMC. Centrifuge speeds were tested starting at 80xg to 160xg. The speed of 120xg was the optimal speed to separate platelets in the conical tubes from the pellets. This decreased the number of washes needed by 1 or 2 depending on the starting platelet count. The number of cells lost during each washing step was small, around 10%, and the total number of cells retained increased. Originally, 6-well plates were used for attachment separation but was switched to T$_{175}$ flasks. When using the 6-well plates, yield was

$1.5\text{x}10^5$ MDDCs per mL, but when flasks were used the yield of MDDCs averaged about

$3.5\text{x}10^5$, >200% increase. Originally, the attachment incubation time was 3 hours. When

decreased to 2 hours and 30 minutes of incubation time, the monocytes were easily

detachable. A checkerboard was done to determine the optimal amount of IL-4 and GM-

CSF. An increase of 0-150% was found depending on the combinations of these two

cytokines. The best combination increased the number of MDDCs from $3.5\text{x}10^5$ per mL

to around $5\text{x}10^5$ MDDCs per mL with the previous optimization steps included in the

MDDC totals. Shaking the cells during the first 12 hours of monocyte differentiation

increased the number of MDDCs from $5\text{x}10^5$ per mL to around $1\text{x}10^6$-$1.2\text{x}10^6$ per mL.

Brown Swiss cattle have a higher number of monocytes resulting in a higher number of

MDDCs than Holstein Friesian. On average, a Brown Swiss would yield about $1\text{x}10^5$-

$2\text{x}10^5$ more MDDCs per mL than a Holstein Friesian. A summary can be found in table

2.2.

BVDV Infected MDDC Cytokine Expression

 MDDC cytokine expression was analyzed for IFN-alpha, IFN-beta, IFN-gamma,

IL-1b, IL-1b, IL-6, IL-8, IL-10, IL-12 and TNF. Each of the IFN cytokines were similar

to each other, all being under a 2-fold increase (Figs 2.1, 2.4, and 2.5). The IL cytokines

were broken into two groupings. First, IL-1b had > 40-fold decrease (Fig 2.7). Second,

the remainder of the IL cytokines had a 5-10-fold decrease (Figs 2.6, 2.8, 2.9, 2.12, and

2.13). Finally, TNF had a 2-7-fold decrease (Fig 2.14). Only IFN-alpha and IL-8

showed statistical significance between the strains with a p-value <0.05, while the other 8

cytokines mRNA had neither statistical significance nor biological significance

(biological significance is, a p-value between >0.05 and <0.1). While there was

statistical significance between 1373 and 28508 for IFN-alpha, the down regulation was

small and likely would not have a large biological effect. IFN-alpha was down regulated

in 1373-infected MDDCs at every time point after infection. Only at the 48h time point

was IFN-alpha down regulated by 28508 (Fig 2.1). When analyzed by breed, IFN-alpha

was down regulated by 1373 -0.6±0.0-fold for Brown Swiss at 6h compared to

-1.1±0.1-fold for Holstein Friesian. At 12h p.i., the IFN-alpha levels for the Brown Swiss

was at -1.0±0.1-fold compared to the Holstein Friesian at -1.9±0.0-fold. At 24h p.i., IFN-

alpha for the Brown Swiss was down regulated -1.6±0.1-fold and the Holstein Friesian

was down regulated -2.2±0.1-fold. At the final time point of 48h, the IFN-alpha down

regulation was -1.4±0.0-fold for the Brown Swiss compared to -1.7±0.0-fold for the

Holstein Friesian. For IFN-alpha with 28508, only the Holstein Friesian calves had down

regulation with a value of -1.0±0.0-fold at the 48h time point. A cattle breed comparison

for IFN-alpha was done for the 1373 (Fig 2.2) and 28508 (Fig 2.3) strains. IFN-beta

decreased in 1373-infected MDDCs from 6h to 48h and 24h to 48h for 28508. The

largest decrease was at -1.3-fold for 1373 and -0.4-fold for 28508 (Fig 2.4). No cytokine

expression was seen until 12 hours for 1373 and 24 hours for 28508 with IFN-gamma.

The largest decrease was seen for 1373 at -0.3-fold at 24h and -0.2-fold at 48h (Fig 2.5).

No cytokine expression was seen until 6h for IL-1a. Both viruses had the largest

decrease at 48h with 1373 at -8.5-fold and 28508 at -6.9-fold (Fig 2.6). No cytokine

expression was seen until 6h for IL-1b. Both viruses had the largest decrease at 24h with

1373 at -46.1-fold and 28508 at -44.7-fold. A large difference was seen at the 48h time

point (Fig 2.7). No cytokine expression was seen until 6h for IL-6 for both viruses. For

1373-infected MDDCs, had the largest decrease of -6.4-fold and -3.4-fold for 28508 at

6h. The cytokine expression varied at the other time points (Fig 2.8). At the 6h time

point, 1373 down regulated IL-8 -3.9±0.5-fold while 28508 down regulated IL-8

-1.4±0.2-fold (Fig 2.9). For the 12h time points, 1373 down regulated -5.6±0.5-fold and

28508 was -2.2±0.2-fold. At 24h, IL-8 was down regulated by 1373 -7.9±1.2-fold and

28508 was decreased -4.3±0.6-fold. At the 48h time point, the down regulation

decreased, with 1373 at -6.8±1.1-fold and 28508 at -5.6±1.0-fold. A comparison of the

two cattle breeds expression of IL-8 for 1373 (Fig 2.10) and 28508 (Fig 2.11) was also

done. At 6h p.i., the IL-8 expression in Brown Swiss calves was down regulated -3.4-

fold while the Holstein Friesian was down regulated -4.4-fold. At 12h p.i., the IL-8

mRNA in Brown Swiss MDDC averaged -5.4-fold down regulation while Holstein

Friesian averaged -5.9-fold down regulation. The largest down regulation for IL-8 was

seen at 24h p.i., with Brown Swiss averaging -7.4-fold and Holstein Friesian at -8.4-fold.

Down regulation of IL-8 decreased at 48h p.i. with an average of -6.1-fold for Brown

Swiss and -7.6-fold for Holstein Friesian. For IL-10 expression was first seen at 6h for

both viruses. The greatest decrease for 1373 was seen at 24h with -6.9-fold and the

greatest decrease for 28508 at 48h with -5.5-fold. A decrease started after 24h for 1373

(Fig 2.12). Expression was first seen at 6h for IL-12. A peak was seen at 24h with -9.2-

fold for 1373 and 48h with -6.7-fold for 28508. A decrease started after 24 for 1373 (Fig

2.13). Finally, for TNF peaks were seen at 48h for 1373 at -6.5-fold and 28508 at -4.5-

fold. The 24h time point for 1373 was higher than the peak of 28508 (Fig 2.14).

DISCUSSION

Monocyte-Derived Dendritic Cell Technique Optimization

The MDDC protocol that was developed by Rajput, 2013 was further optimized to increase MDDC yield. When first starting the experiments and culturing MDDCs, about 500 mL of blood was collected from each calf. Using less blood gave too little MDDCs to work with. This amount of blood took a large amount of resources and time to isolate monocytes and culture them into MDDCs. While the original procedure worked, a less expensive and less time consuming process was developed. SepMate™ tubes made a large difference in obtaining the buffy coat. The original way of layering blood without the insert that is in a SepMate™ tube caused problems in either obtaining all of the buffy coat to avoid collecting any RBCs or RBCs would be collected in the attempt to collect as much buffy coat as possible. With the insert in the SepMate™ tubes the RBCs that can cause problems in buffy coat collection are trapped underneath the insert. This made the chance of contaminating the buffy coat with RBCs less likely and also decreased the time required for removing the buffy coat. With the addition of using SepMate™ tubes the problem of centrifugation speed had to optimized with them. The speed of 1200xg was found to be ideal as the speed prevented any buffy coat from going beneath the insert and any RBCs to come above the insert. Before finding the optimal speed for the amount of blood and lymphoprep used in each SepMate™ tube, the tubes actually proved to be worse. In the case of centrifugation at higher speeds, more buffy coat was usually lost under the insert than would have been lost using the original separation method. After starting with a speed that was too high, the speed was reduced to 800xg and increased in increments of 100xg. Using a spin time of 20 minutes for the

entire time period of allowed for an easy way to keep testing the speed until all of the

RBCs went underneath the insert while leaving the buffy coat on top. Finding the

optimal speed for the blood separation increased the number of MDDCs at the end by

$4x10^4$. The other time the centrifuge speed was optimized was when attempting to wash

the pellet and remove as many platelets as possible. Supernatant above the insert was

poured off, an excess number of platelets had to removed. The goal was to do as few

washes a possible, while removing the platelets. At a speed of 120xg, the PBMCs were

able to pellet while retaining very few platelets after removing the supernatant.

Spinning any faster than 120xg saw a drastic increase in platelets that were part of the

pellet. This required more washes, which caused the loss of MDDCs with each

additional wash. Next, the separation of monocytes from the rest of the cells found in the

pellet was optimized. Originally 6 well plates were used, but the surface area wasn't

large enough and many monocytes were washed off after the incubation period because

they didn't stick to the plastic. Using cell culture flasks rather than 6-well plates, resulted

in more viable cells to grow in a large, single layered sheet than in smaller wells. With

the 6-well plates, the cells would attach to the sides or more frequently adhere to each

other. In both cases the cells were usually lost when washing after incubation. Using a

flask eliminated this problem; a specific number of cells was added to each flask to allow

the formation of a monolayer of cells during the incubation. An increase of $2x10^5$ cells

was seen from the original protocol by using a flask. Trying to find the optimal ratio of

IL-4 and GM-CSF proved to be the most challenging part of the optimization phase.

Interestingly enough, nearly every ratio that was tried had monocytes differentiate into

MDDCs just at different amounts. Using no GM-CSF, but only IL-4 produced MDDCs

in a rather low number. This was seen even with low concentrations of IL-4. When

using just GM-CSF, no MDDCs were observed regardless of how much GM-CSF was

used. Using equal ratios of both cytokines produced more MDDCs than using only one.

After showing that both cytokines were needed, the ratios of both cytokines were

increased or decreased. A 10:1 ratio of IL-4 to GM-CSF produced the highest numbers

of MDDCs. Around 1.5×10^5 more MDDCs were produced when using 100 μL IL-4 (10

μg/mL) and 10 μL of GM-CSF (10 μg/mL). The biggest improvement to the MDDC

yield came through the use of a cell shaker. A major problem that was encountered when

differentiating monocytes into MDDCs was the monocytes would settle at the bottom of

the wells even after adding cytokines. When the monocytes attached to the wells, they

would differentiate into macrophages instead of MDDCs. This problem was remedied by

using a cell shaker on low during the first 12 hours of MDDC growth. The cell shaker

caused a majority of the monocytes that weren't differentiating to remain unattached,

increasing the MDDCs yield to 5×10^5. At first 24 hours was used, but more macrophages

were seen after 24 hours than 12 hours. This can be explained by the monocytes being

stimulated due to aggravation, which caused the monocytes to differentiate into

macrophages instead of MDDCs. At 12 hours, macrophages were seen in small

quantities. Finally, cow breed plays an important role in the number of monocytes. The

breed difference between Brown Swiss and Holstein Friesian differed largely by the

number of circulating monocytes. The Brown Swiss calves had between 1×10^5 - 2×10^5

more MDDCs after growth than the Holstein Friesians. While it was to be expected that

a different breed of cattle would have some differences in immune cells, the large

difference in the two breeds studied provided the Brown Swiss an ideal breed to use for future studies of MDDCs.

<u>BVDV Infected MDDC Cytokine Expression</u>

For IL-1b, down regulation was seen starting at 6h for both viruses. A large decrease was seen between the control for both viruses and between both viruses at 48h. At 48h a decrease in cytokine expression was seen for 1373 and 28508. This coincides with a study conducted in mice where IL-1b was down regulated with the neuroadapted Sindbis virus (NSV) (Liang *et al.*, 1998), but differs in a study on HCV infected macrophages in humans (Shrivastava *et al.*, 2013). In the study (Liang *et al.*, 1998) conducted with mice, a lack or decrease in IL-1b provides protection against NSV-induced disease but not infection. A decrease in IL-1b could help in decreasing innate immunity against BVDV which would aid in immunosuppression and increase in bacterial secondary infections as IL-8 is a neutrophil chemotaxic factor. In HCV infections, induction of IL-1b synthesis occurs by the NF-kB signaling pathway. The secretion then occurs by HCV contacting the macrophages (Shrivastava *et al.*, 2013). The IL-1b cytokine down regulation in MDDCs is consistent with the Shrivastava study as macrophages will want up regulation, but DCs won't because of the increased mobility issues inflammation causes for DCs *in vivo*. In another study, IL-1b and IL-8 mRNA were up regulated in MDBK cells using a low virulence cytopathic BVDV-1 type 1b strain (Fredericksen *et al.*, 2015). Epithelial cells like MDBK do not produce IL-8. IFN-alpha is involved in inflammation with the down regulation of IFN-alpha BVDV by strain 1373 likely decreasing the inflammation process. It is well established *in vitro* that ncp BVDV evades the host's IFN defense in a complex way: (i) it avoids induction of

IFN, (ii) once infection is established, the virus ("self") is resistant to the action of IFN and (iii), it does not interfere with IFN action against unrelated viruses ("nonself") replicating in the same host cells (Peterhans, 2013). There is additional data that interferon is measured in the serum 3-5 days post ncp BVDV infection (Reid *et al.*, 2016). A study conducted with either ncp or cp BVDV obtained from PI animals with an enriched population of plasmacytoid dendritic cells (pDCs) from lymph nodes induce type III IFNs and low levels of type I IFNs like IFN-alpha (Reid *et al.*, 2016). Although, pDCs were not the cell type used in the current study. This study also infected animals with the same viruses and demonstrated that all of the serum IFN was acid labile, which indicated that it was not type 1 IFN (IFN-alpha or IFN-beta) consistent with the results from our study (Reid *et al.*, 2016). In MDDCs infected by 28508, no change in cytokine was seen until the 48h time points. Yet by the time 1373 had reached 48h p.i., the MDDCs were only shown to have down regulation equal to the 6h time point for 1373. Additionally, the Holstein Friesian calves were down regulated while Brown Swiss calves had no down regulation. With the results obtained, breed seems to play an important role in how easily MDDCs are infected. With IFN-beta, 1373 infection resulted in significant decrease compared to both the control and 28508. On the other hand, 28508 had no significant difference from the control. With IFN-gamma, a small difference was observed between viruses and control. A decrease in expression started after 24h for 1373. For IL-1a, the decrease was less than IL-b by a large amount. The largest down regulation was seen in 1373 for all time points. For IL-6, there was a significant decrease between the two viruses at the 6h and 24h time points. The cytokine expression was odd as a pattern like the other cytokines was not seen, with 1373

increasing and decreasing and 28508 decreasing until 24h then increasing after. Because

of the immune suppression and secondary bacterial infection associated with BVDV,

down regulation was expected for IL-8 due to its importance in chemotaxis to attract

neutrophils. Down regulation was more prominent in 1373 infected MDDCs, the higher

virulence strain than 28508, the lower virulence strain. Both strains had an increase in

cytokine down regulation up until the 24h time point. Interestingly, the 48h time point

for 1373 infected MDDCs saw a decrease in the down regulation. This can be explained

by the loss of MDDCs by the 48h time point. With 28508 this decrease was not seen at

the 48h time point, but rather saw a continual down regulation of cytokine expression. It

is likely that enough cells would die after 28508 infection that a decrease in down

regulation would be seen. Cattle breed played a more important role than originally

expected in cytokine expression. At all time points with both viruses, Brown Swiss had

less down regulation than Holstein Friesian. This indicates, at least in the case of

MDDCs, Brown Swiss have a higher quality of MDDCs that are not as easily infected.

Also, the possibility of an overall better immune system that fights off the viruses to

prevent more infection is a plausible conclusion. Both IL-10 and IL-12 were similar in

the cytokine expression, but IL-12 had more down regulation. Both cytokines had

significant decreases at 6h, peaked at 24h for 1373 and 48h for 28508, and decreased

after the 24h time point for 1373. Another BVDV study in 1373 infected calves showed

that pro-inflammatory (TNF, IL-1b, IFN-gamma, and IL-12) and anti-inflammatory (IL-

10) were up regulated in lymph nodes cells, i.e. B and T cells (Palomares *et al.*, 2014).

Finally, TNF was down regulated at similar levels as the non-IL-1 cytokines. Strain 1373

infected MDDCs down regulated TNF-alpha more than 28508, with the 24h time point of

1373 being higher than the 48h time point for 28508. These TNF results correlate with a study on ncp infected monocytes. Both BVDV-1 and BVDV-2 down regulated TNF compared to the control (Seong *et al.*, 2013). For the majority of the cytokines, cytokine expression increased or decreased the most at the later time periods. Depending on the cytokine, the highest reductions were at the 24h or 48h time points with the exception of IL-6 that had the highest reduction at the 6h time point.

<u>Summary</u>

The greatest impact to improve MDDC yield came from shaking the monocytes for 12 hours at the start of incubation. This prevented the monocytes from sticking to the plates and becoming macrophages instead. The breed of calf indicated that there was a difference in MDDC yield. This was because monocytes were found at a higher level in the blood for Brown Swiss calves than for Holstein Friesian calves. IL-1b had the greatest down regulation of more than 40-fold. The IFN cytokines all had less than 2-fold decrease. The remainder of the IL cytokines and TNF had less than 10-fold decrease. Down regulation was higher for 1373 than 28508.
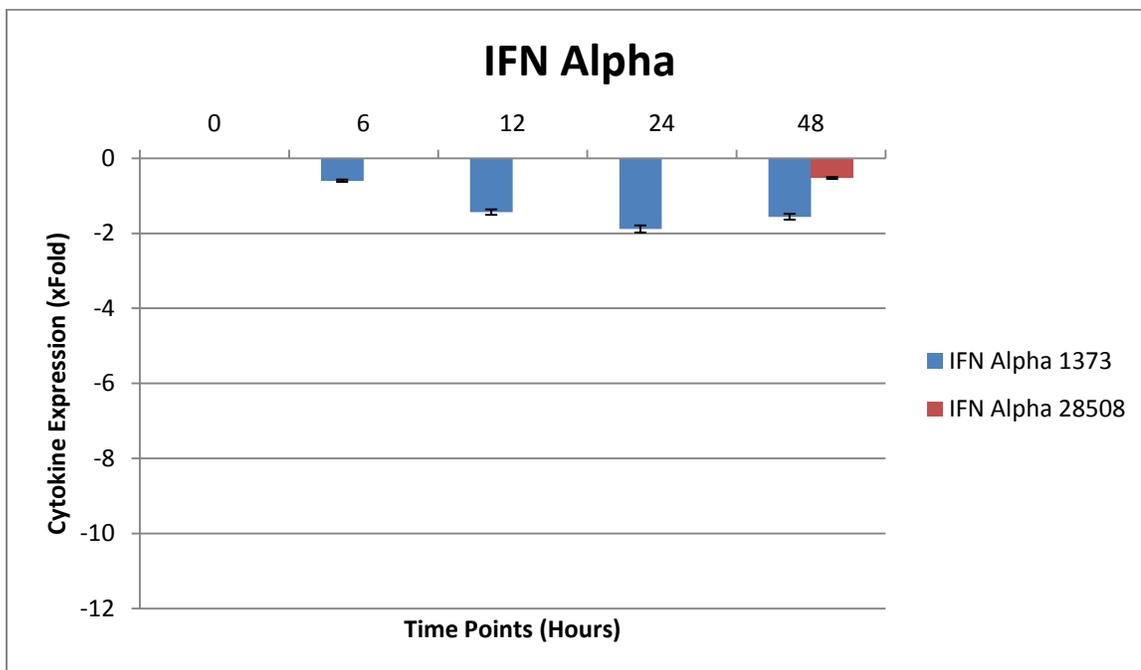
Fig 2.1. BVDV IFN-Alpha down regulation: BVDV 1373 and 28508 strains IFN-Alpha down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
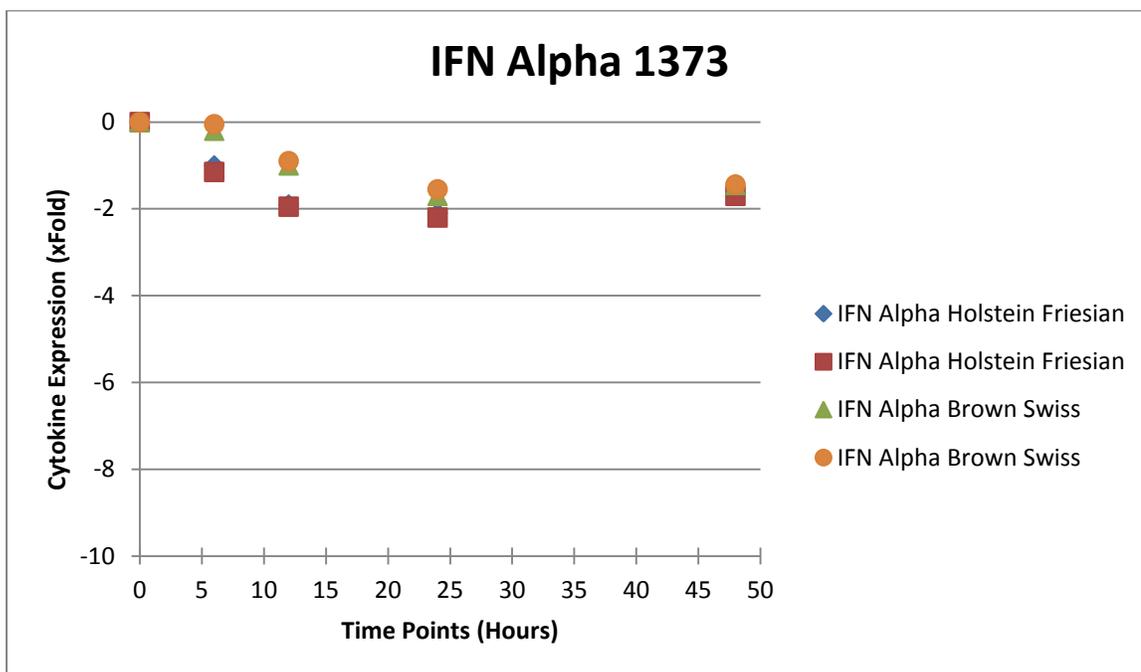

Fig 2.2. BVDV strain 1373 IFN-Alpha down regulation: BVDV 1373 strain IFN-Alpha down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
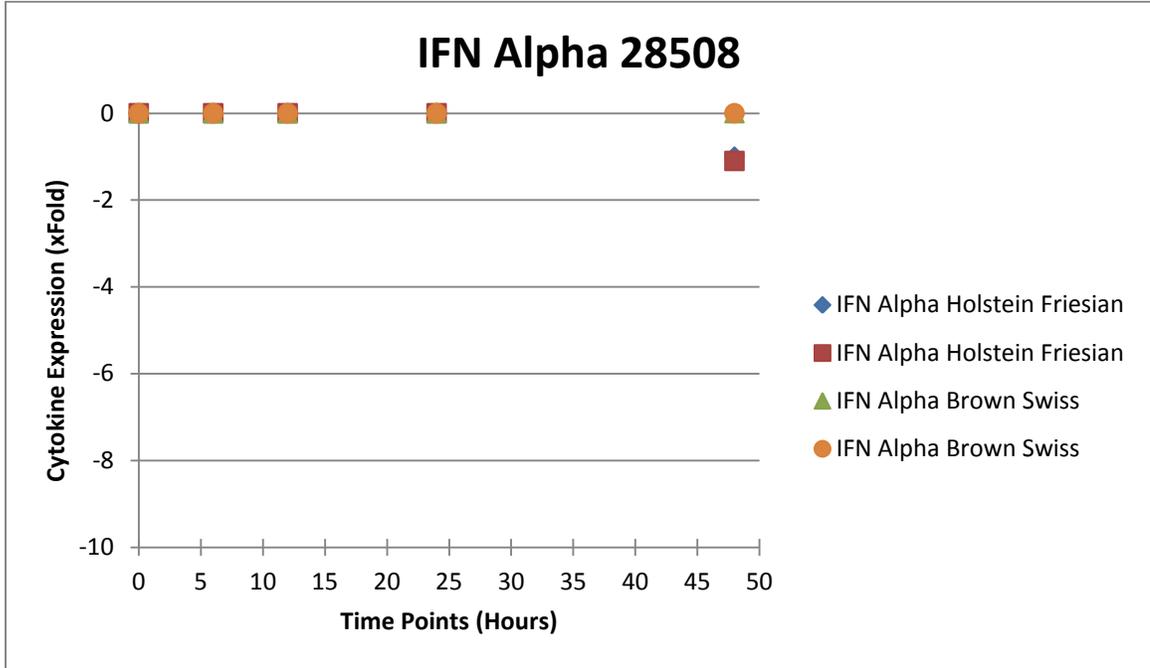
Fig 2.3. BVDV strain 28508 IL-8 down regulation: BVDV 28508 strain IFN-Alpha down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
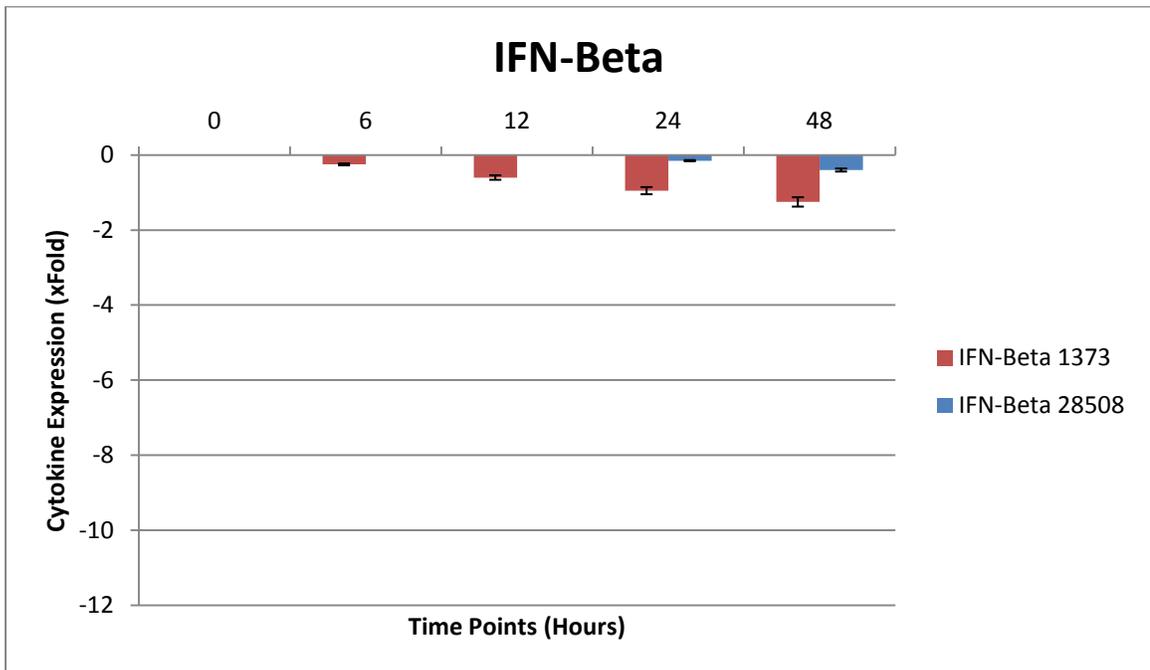

Fig 2.4. BVDV IFN-Beta down regulation: BVDV 1373 and 28508 strains IFN-Beta down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
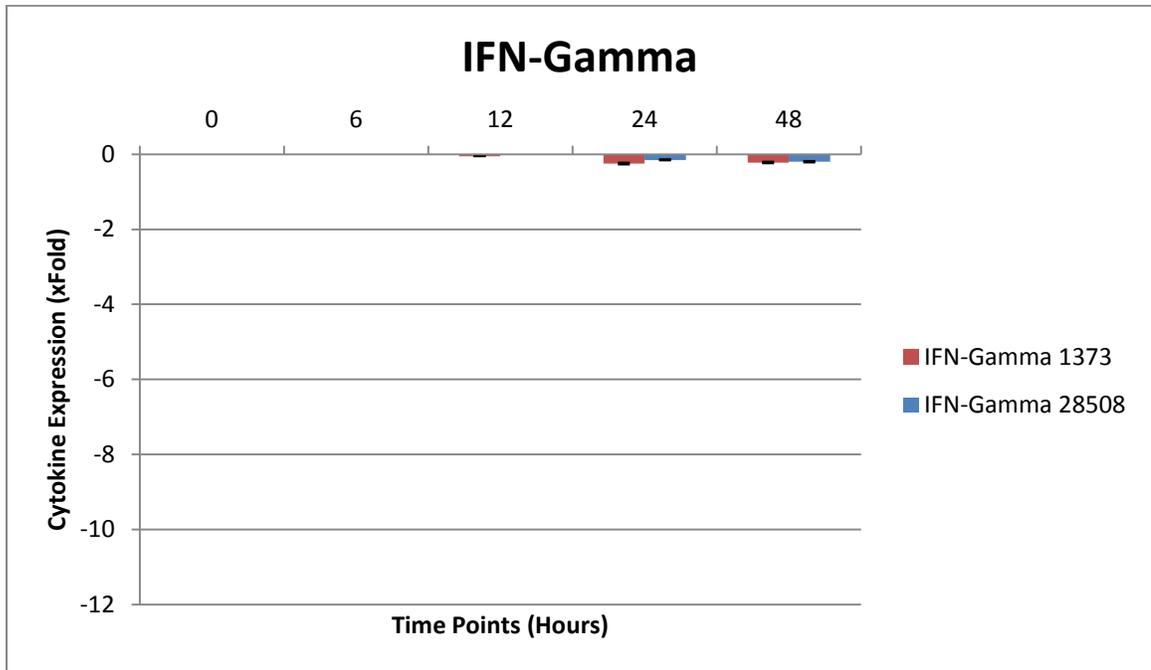
Fig 2.5. BVDV IFN-Gamma down regulation: BVDV 1373 and 28508 strains IFN-Gamma down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
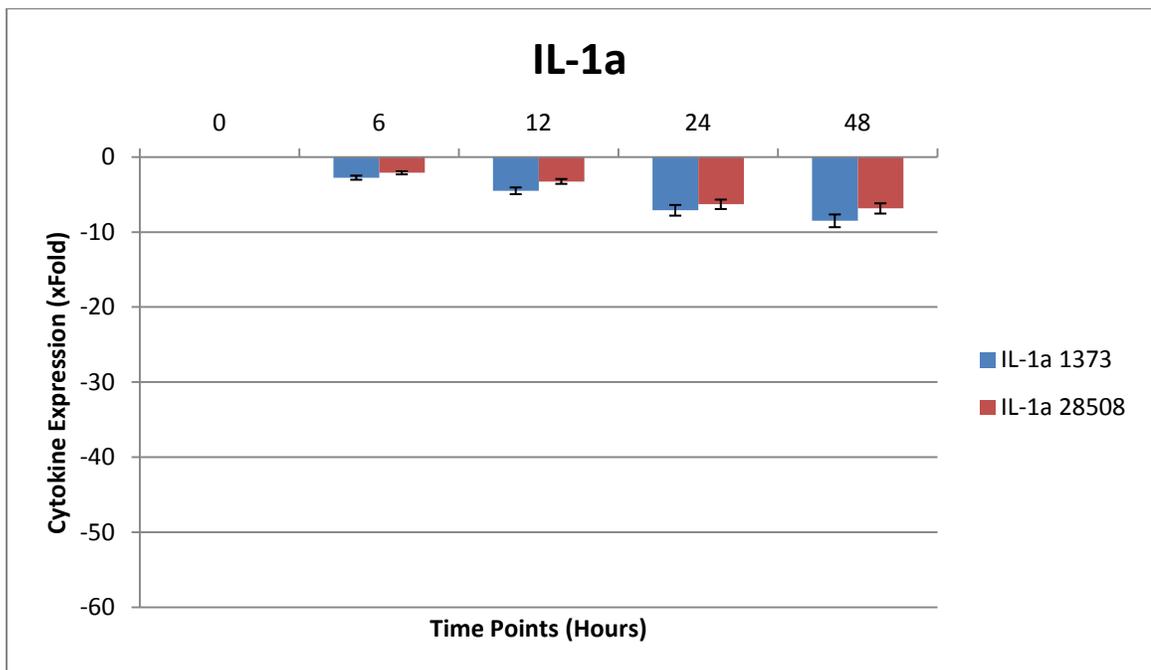

Fig 2.6. BVDV IL-1a down regulation: BVDV 1373 and 28508 strains IL-1a down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 2.7. BVDV IL-1b down regulation: BVDV 1373 and 28508 strains IL-1b down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 2.8. BVDV IL-6 down regulation: BVDV 1373 and 28508 strains IL-6 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
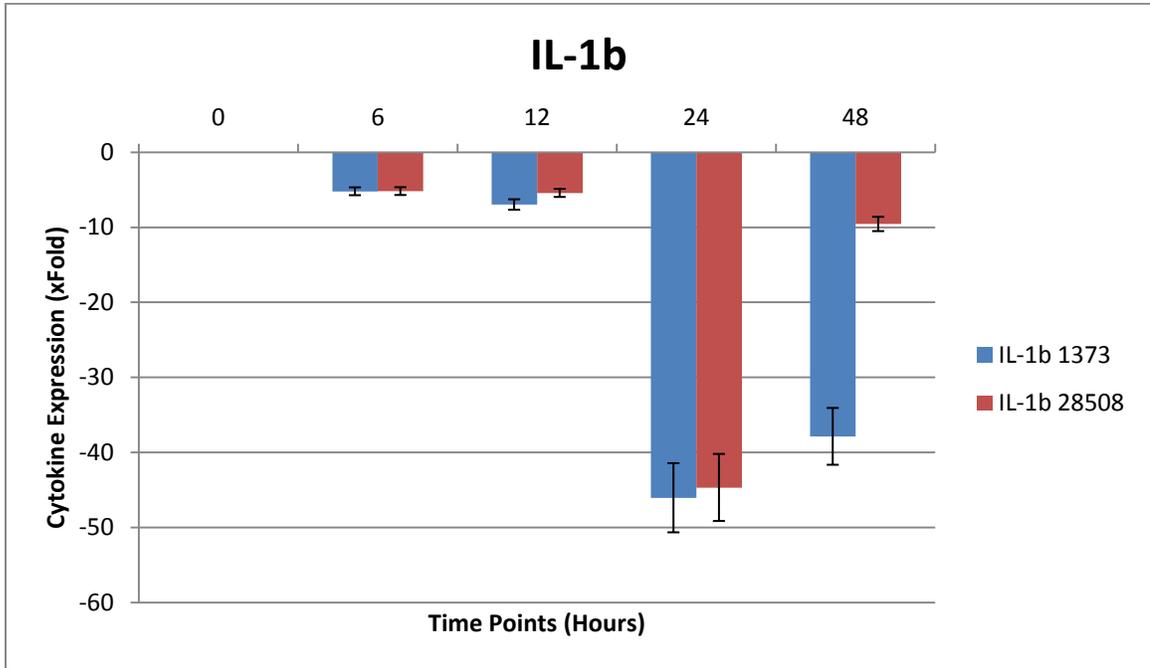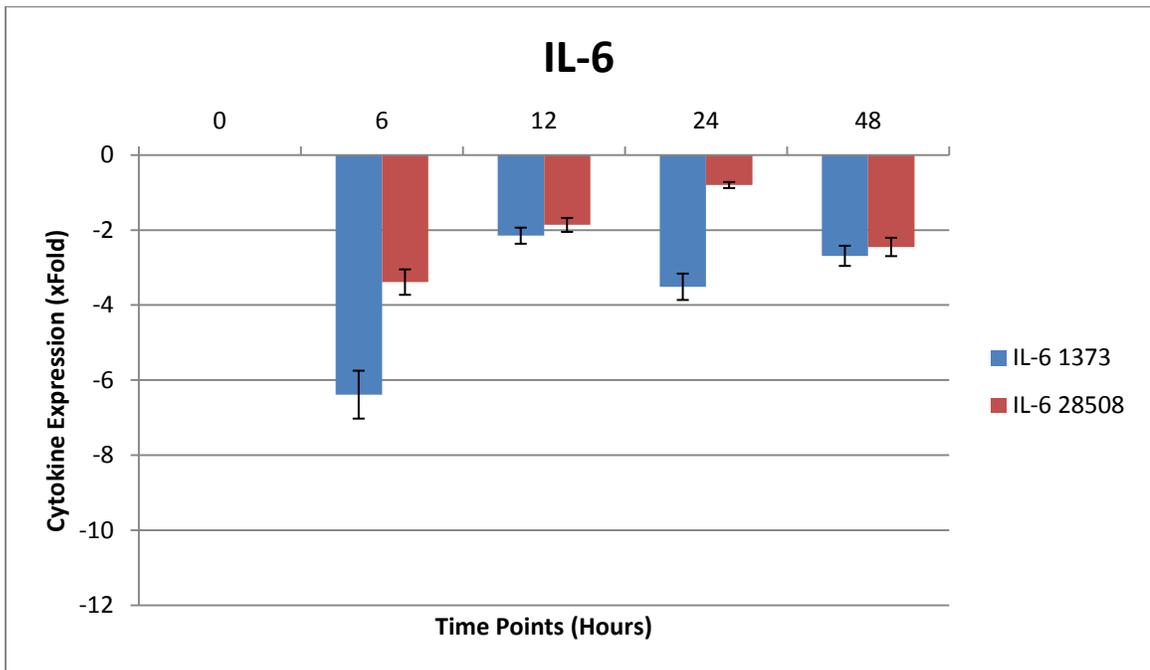
Fig 2.9. BVDV IL-8 down regulation: BVDV 1373 and 28508 strains IL-8 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
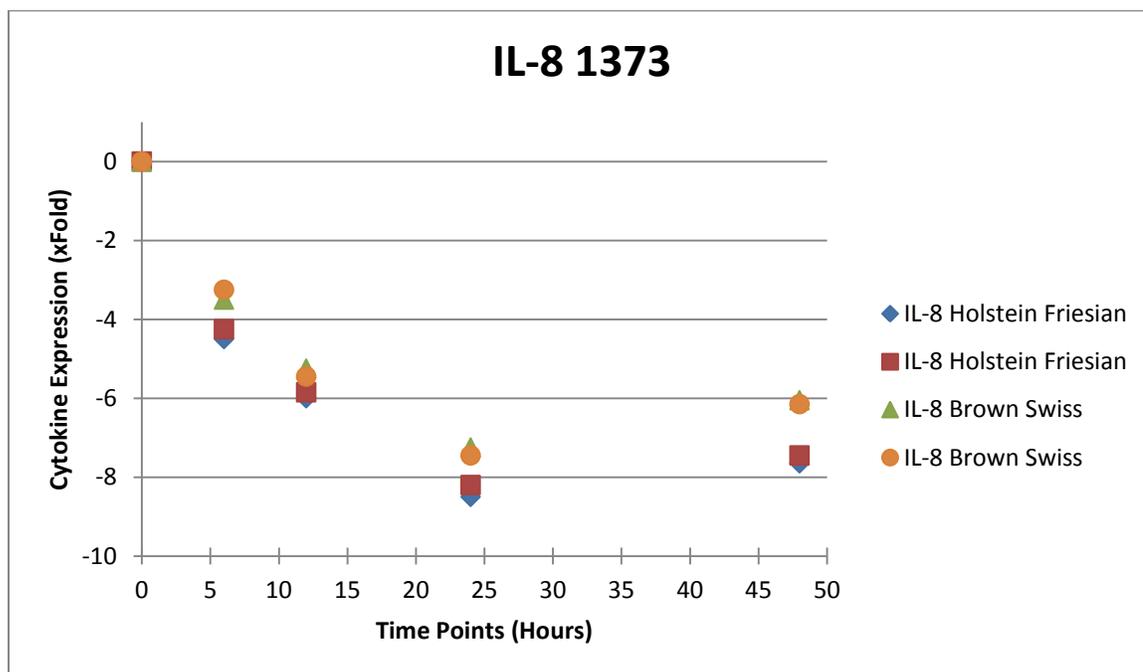
Fig 2.10. BVDV strain 1373 IL-8 down regulation: BVDV 1373 strain IL-8 down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
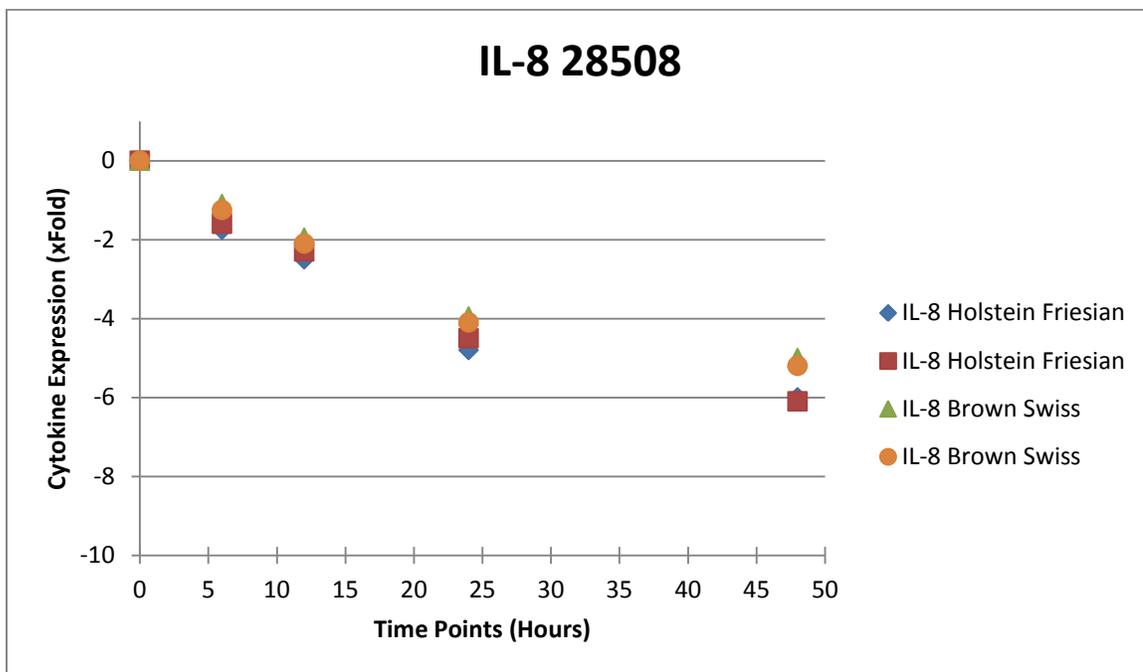
Fig 2.11. BVDV strain 28508 IL-8 down regulation: BVDV 28508 strain IL-8 down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
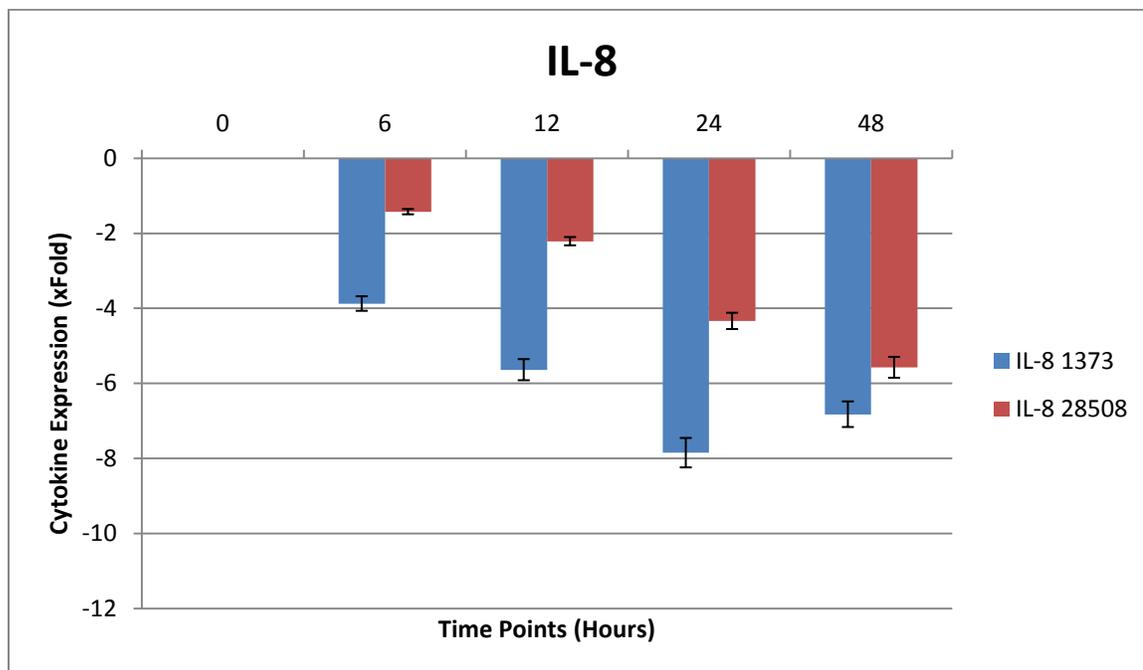

Fig 2.12. BVDV IL-10 down regulation: BVDV 1373 and 28508 strains IL-10 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 2.13. BVDV IL-12 down regulation: BVDV 1373 and 28508 strains IL-12 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 2.14. BVDV TNF down regulation: BVDV 1373 and 28508 strains TNF down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
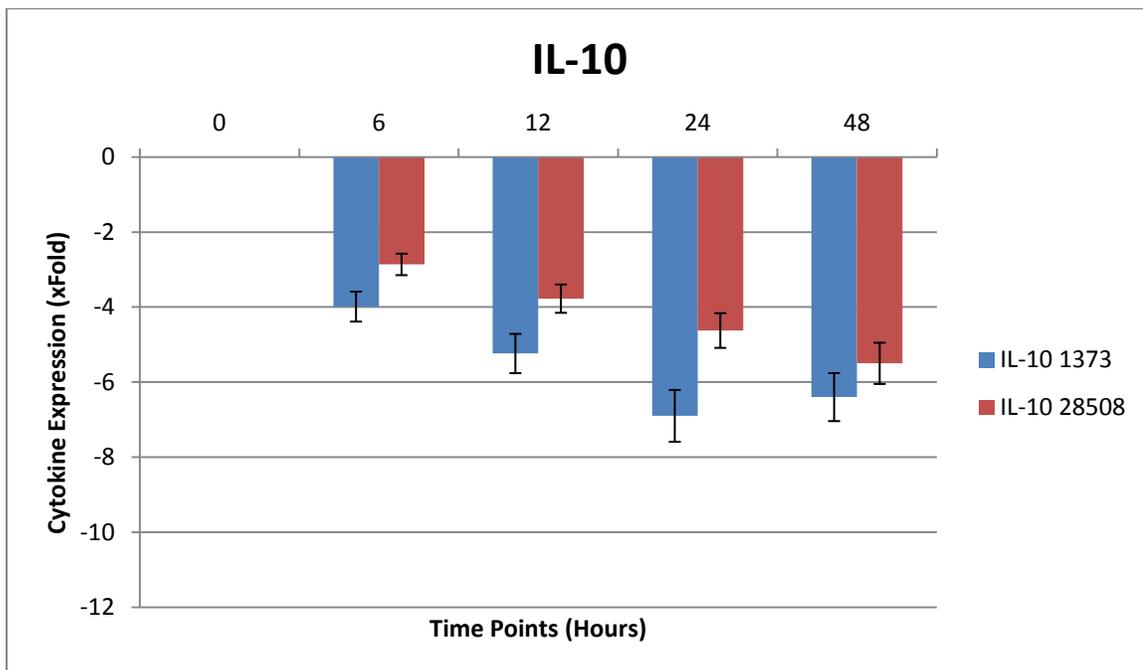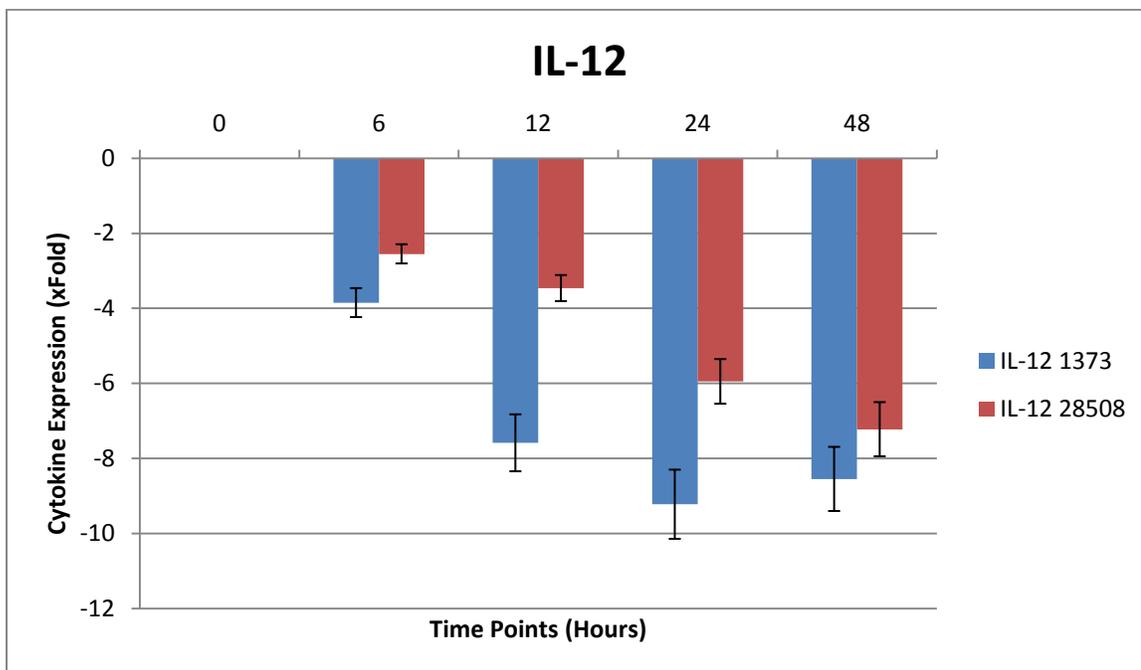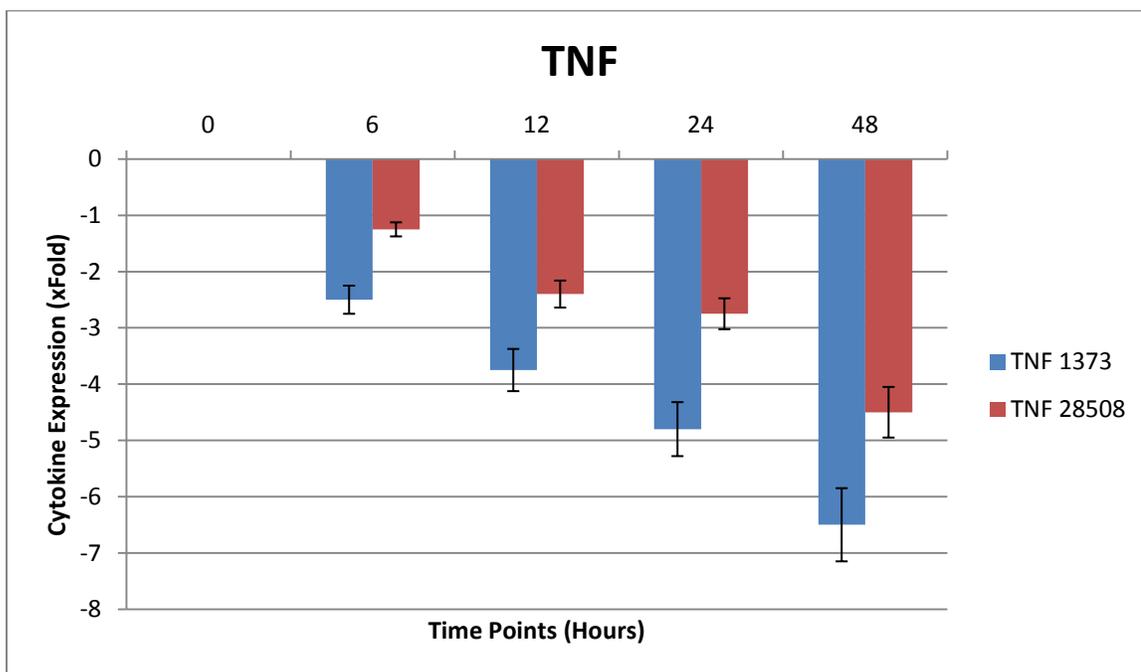
Cytokine	Forward Primer 5'-3'	Reverse Primer 5'-3'
Beta-Actin	CGC ACC ACT GGC ATT GTC AT	TCC AAG GCG ACG TAG CAG AG
IFN-Gamma	CAG ATC ATC CAC C	GGA ATT TGA ATC AGC
IL-1a	GAT GCC TGA GAC ACC CAA	GAA AGT CAG TGA TCG AGG G
IL-1b	CAA GGA GAG GAA AGA GAC A	TGA GAA GTG CTG ATG TAC CA
IL-6	TCC AGA ACG AGT ATG AGG	CAT CCG AAT AGC TCT CAG
IL-8	AAT GGA AAC GAG GTC	TGC TTA AAC CCC AAG
IL-10	TGC TGG ATG ACT TTA AGG G	AGG GCA GAA AGC GAT GAC A
IL-12	AAC CTG CAA CTG AGA CCA TT	ATC CTT GTG GCA TGT GAC TT
TNF	AGC CCA CGT TGT A	GCC GAC ATC AAC TCC

Table 2.1 Primer Summary: The primers used in qRT-PCR.

Optimization Technique	MDDC % Increase	MDDCs per mL
Original Protocol	N/A	1×10^5
SepMate™ Tubes	40%	4×10^4
Centrifuge Speed	10%	1×10^4
Plate Adhesion	200%	2×10^5
Optimal Cytokine Amounts	150%	1.5×10^5
Cell Shaker	500%	5×10^5
Cow Breed	100%-200%	1×10^5 - 2×10^5
Total	900%	1×10^6

Table 2.2. Summary of MDDC Optimization Techniques: The techniques that were used in optimization with the effect on MDDCs yield is summarized. Cattle breed wasn't factored into the total increase.

CHAPTER 3: BOVINE HERPESVIRUS TYPE 1 CYTOKINE AND SURFACE MARKER ANALYSIS

ABSTRACT

Bovine herpesvirus type 1 (BHV-1) is an important virus in the cattle industry that leads to a variety of important symptoms. It is important to develop a better understanding of BHV-1 and the effect it plays on dendritic cells to better understand the relationship between the innate and adaptive immune systems. Dendritic cells are the main antigen presenting cells that serve to link the two immune systems together. A dendritic cell will present an antigen to a naive T cell and cause activation into a mature T cell. A virally infected dendritic cell may cause problems between the innate and adaptive immune system link by having an adverse effect on the antigen presenting response dendritic cells have. BHV-1 was used in this study and the effects the virus had on dendritic cell cytokine production and surface marker expression were observed. Monocytes were collected from calf blood and differentiated into monocyte-derived dendritic cells. This was done over a 5-day time period using IL-4 and GM-CSF. The monocyte-derived dendritic cells were then infected with the Cooper strain or the LA strain, two BHV-1 strains. Five (5) of the 8 measured cytokines expressed by BHV-1 infected monocyte-derived dendritic cells were down regulated. The IFN family, IFN-alpha, IFN-beta, and IFN-gamma were up regulated. IFN-alpha had a more than a 10-fold increase, IFN-beta had a more than a 3-fold increase, and IFN-gamma had less than a 1-fold increase. The other 5 IL cytokines were down regulated with less than a 4-fold decrease. The Cooper strain significantly down regulated cytokine IL-1b, IL-6, IL-8, IL-10, and IL-12 mRNAs more than the LA strain compared to the control. The Cooper

strain significantly up regulated IFN-alpha, IFN-beta, and IFN-gamma mRNA. The cytokine fold differences between Cooper and LA strains, were: 0.25 for IL-1b, 0.25 for IL-6, 0.25 for IL-8, 0.50 for IL-10, 0.10 for IL-12, 5 for IFN-alpha, 0.5 for IFN-beta, and 0.25 for IFN-gamma. Statistically significant differences between the Cooper and LA strains were only seen for IL-8 and IFN-alpha. Surface marker expression was also observed following BHV-1 infection of MDDC. From the first time point observed, 6h, to the last time point observed, 48h, mean fluorescence intensity decreased in MHCI, MHCII, and CD86. This was seen primarily in the Cooper strain infected MDDCs, but CD86 also decreased with the LA strain. No change was seen in CD14 due to the very low amount of CD14. Both Cooper and LA strains interfered with normal MDDC cytokine and surface marker expression.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is a major viral pathogen that infects cattle and causes symptoms such as: abortion, conjunctivitis, genital infections, and respiratory disease (Jones, 2003, Tikoo *et al.*, 1995). Bovine respiratory disease, which BHV-1 is a contributing factor, is also known as shipping fever and plays an important role in large financial loses each year in the cattle industry. BHV-1 infections can cause immunosuppression and increase the possibility of secondary bacterial infections (Biswas *et al.*, 2013, Jones, 2003). BHV-1 symptoms depend on the subtype of the virus. Respiratory diseases arise from BHV-1.1. BHV-1.2 results in genital infections. Both substrains can cause abortions (Biswas *et al.*, 2013).

BHV-1 is able to gain access to the host through the conjunctiva, the mucosa of the upper respiratory tract, or the reproductive tract. BHV-1 destroys the epithelium layer and enters the blood to cause viremia. Through viremia, the virus is able to spread to additional organs of the body, specifically the central nervous system and the reproductive system (Straub, 1990; Renjifo *et al*., 1999). Symptoms are observed around the fourth day of post infection and generally last no more than ten days. Having an infected animal in the heard is problematic due to the ease of the spread of BHV-1 and the animal should be monitored closely (Biswas *et al.*, 2013). Two BHV-1 strains, Cooper (Colorado) and LA, were isolated from severe respiratory disease outbreaks in Colorado and California in the 1950's (Madin, 1956).

This study was designed to determine the effect of BHV-1 on MDDC cytokine mRNA and surface marker expression through *in vitro* studies. The susceptibility of MDDCs to BHV-1 strains Cooper and LA were tested and the cytokine expression and surface marker expression was measured for both strains.

MATERIALS AND METHODS

<u>Animals</u>

Healthy calves between the ages of 6-8 months housed at the South Dakota State University (SDSU) Dairy Farm located in Brookings, SD, USA were used as blood donors in this study. Three Holstein Friesian calves were used. The SDSU Institutional Animal Care and Use Committee approved the handling of animals and blood collection. All of the calves were confirmed to be BVDV-free using virus isolation following a test

by immunoperoxidase assay (IPA) (Fulton et al., 1997). Blood (~ 250 ml per draw) was collected via jugular venipuncture using 60 ml heparinized plastic syringes with 16 gauge 1-1/2 needles.

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated, cultured and characterized as described in the Materials and Methods Section "Peripheral Blood Mononuclear Cell Isolation" in Chapter 2.

Monocyte Isolation Using Plastic Adherence Technique

Monocytes were isolated and characterized as described in the Materials and Methods Section "Monocyte Isolation Using Plastic Adherence Technique" in Chapter 2.

Monocyte-Derived Dendritic Cell (MDDC) Culture

MDDCs were cultured as described in the Materials and Methods Section "Monocyte-Derived Dendritic Cell (MDDC) Culture" in Chapter 2.

Monocyte-Derived Dendritic Cell (MDDC) Surface Marker Expression

Before the DCs were infected with virus, the DCs were characterized phenotypically as MHC1, MHCII, CD86 positive, and CD14 low using the Rajput, 2014 technique as described in the Materials and Methods Section "Monocyte-Derived Dendritic Cell (MDDC) Surface Marker Expression" in Chapter 2.

Viral Growth and Titration

For each virus, 5 mL of $5x10^5$ MDBK cells/mL were grown in 5 T25 flasks. The culture was grown to around 60-70% confluency. At inoculation, the RPMI growth

media was removed and 0.75 mL of the specified BHV-1 virus was added to each flask using an MOI of 1. The cells were incubated for 1h at 37°C. After incubation, the virus was removed, and the cells were washed using sterile PBS. After washing, 5 mL of RPMI was added to each flask and then incubated for 4 days at 37°C. The cultures were harvested using two freeze thaw cycles. The freeze thaw cycles consisted of -80°C exposure for 15 minutes and then thawed at 25°C. The cell debris was pelleted using centrifugation at 1200xg for 10 minutes at 4°C. The supernatant containing the virus was then titrated at $1x10^5$ and stored at -80°C.

Viral titration was done using the method developed by Reed and Muench (Reed and Muench, 1938). MDBK cells were grown prior, detached, and adjusted to $5x10^5$ cells/mL. One hundred-eighty (180) μL, of the MDBK cell suspension, was added to each well in a 96-well plate. Twenty (20) μL of virus stock was added to the first row, mixed, and then 20 μL was taken out from each well and added to the well below. This process was repeated until the bottom 2 rows as they were used for negative control. Four replications were used for each dilution. The plate was incubated at 37°C for 4 days. The highest dilution showing a cytopathic effect was used as the end point for calculating the proportionate distance which was then used for determining the viral concentration (Reed and Muench, 1938).

<u>Viral Infection</u>

MDDCs were infected with one of 2 different strains of BHV-1, Cooper or LA . The viral strains were diluted to a multiplicity of infection (MOI) of 1 by using RPMI 1640 medium. After an incubation period of 5 days, the MDDCs were washed with PBS to remove old media and resuspended in RPMI 1640 medium with $1x10^6$ MDDCs per

mL. A 48-well plate was used for the viral infection with 100 μL of MDDCs and 100 μL of diluted virus added to each infected well. In the control wells, 100 μL of MDDCs were added and 100 μL of RPMI 1640 medium was used instead of virus. The Cooper strain, the LA strain, and the control were all done in triplicates for each time point. After infection, incubation was done at 37°C. MDDCs were collected at 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours for a total of 9 wells for each strain and control. MDDC collection consisted of removing the supernatant and then centrifuging each sample at 120xg for 10 minutes. The supernatant was then removed and frozen at -80°C. The pellet was resuspended in 100 uL of PBS and was used for analyzing surface marker expression or for qRT-PCR.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Quantitative Reverse Transcriptase Polymerase Chain Reaction was conducted as described in the Materials and Methods Section "Quantitative Reverse Transcriptase Polymerase Chain Reaction" in Chapter 2.

Monocyte-Derived Dendritic Cell (MDDC) Surface Marker Expression Following BHV-1 Infection

Before the DCs were infected with virus, the DCs were characterized phenotypically as MHC1, MHCII, CD86 positive and CD14 low using the Rajput, 2014 technique. The DCs were first split into a 96 well plate at a $1\text{x}10^5$ concentration per well. The DCs were then washed with PBS by centrifugation for 10 minutes at 120xg. The DCs were labeled using the primary mouse monoclonal antibodies (mAbs) MHCI (H58A), MHCII (H42A), CD14 (CAM36A), and CD86 (ILA190A) (Monoclonal

Antibody Center, WSU, Pullman, WA, USA). MHCI, MHCII, and CD14 antibodies were diluted 1:100 in PBS while CD86 was diluted 1:50 in PBS. Controls of DCs not treated with antibodies and not treated with secondary antibodies were used. Additionally, 1% FBS was added to each diluted primary antibody.   For each of the four antibodies, 50 µL of the diluted primary antibodies were added to the DCs and incubated for 10 min at 4°C followed by washing with PBS. Following the wash, the DCs were stained with 50 µL of a 1:1000 diluted in PBS fluorescein isothiocyanate (FITC) labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA) containing 1% FBS for 10 minutes at 4°C. Following the secondary antibody staining, the DCs were resuspended in 200 µL of 1% paraformaldehyde. Finally, the DCs were analyzed for surface marker expression using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, CA, USA).

Viral infection of MDDCs was examined for the Cooper or LA strains and a non-infected control. The same protocol as detailed in the above paragraph was used with a few additions. Before the primary antibody labeling, MDDCs were infected with the Cooper strain, LA strain, or left non infected. Viral infection was conducted using a MOI of 1 and MDDCs were infected for 6 hours, 12 hours, or 24 hours. After each period of infection, the MDDCs were labeled and their surface markers were analyzed.

Statistical Analysis

Data was analyzed using a student's t-test to determine the significance of the differences between the mean values of the treated samples and the control samples at the time points. A statistical significance was seen at a P-value <0.5 and a biological

significance was seen at a P-value <0.1 but >0.5. All of the experiments were conducted using at least 3 animals, breed depended on the experiment, and 3 repeats for each animal. Standard deviation was used for each time point. The cytokine analysis was based on the analysis of variance (AVOVA) using the REST© 2009 program.

RESULTS

TCID$_{50}$ Assay

A TCID$_{50}$ assay was done for both virally infected MDDC with the Cooper strain or LA strain (Fig 3.1 A) and a control with MDBK cells (Fig 3.1 B). Both experiments were broken into measuring the lysate and supernatant. Following BHV-1 infection of the virus control, MDBK cells, virus was present in the lysate and supernatant at the 6h time point. Both the lysate and supernatant steadily increase in viral titer until the 48h time point when both viruses had the same viral titers for both lysate and supernatant. Following BHV-1 infection of MDDCs lysate, infectious virus was present in both the cell lysate and supernatant as early as 6h. The viral titers was 6.5log$_{10}$ was seen early on, but slightly increased to a peak of 8.5 for Cooper lysate and 7.8 for Cooper supernatant. Both supernatant and lysate peaked at 7 logs for the LA strain. Compared to the control, MDBK cells, MDDCs had a lower virus growth curve and lower virus yields. The viral growth curve for Cooper infected MDDCs was 3.5 logs less than Cooper infected MDBK cells. The viral growth curve for LA infected MDDCs was 6.5 logs less than LA infected MDBK cells. The viral yield for Cooper infected MDDCs was 1.5 logs less than Cooper

infected MDBK cells. The viral yield for LA infected MDDCs was 3 logs less than LA

infected MDBK cells.

<u>BHV-1 Infected MDDC Cytokine Expression</u>

      MDDC cytokine mRNA expression was analyzed for IFN-alpha, IFN-beta, IFN-

gamma, IL-1b, IL-6, IL-8, IL-10, and IL-12. IFN-alpha had > 10-fold increase (Fig 3.2),

IFN-beta had approximately a 3-fold increase (Fig 3.5), and IFN-gamma had

approximately a 0.5-fold increase (Fig 3.6). The IL cytokines all had a decrease of less

than 4-fold (Fig 3.7-3.9, 3.12, and 3.13). Only IFN-alpha and IL-8 had statistical

significance, with a p-value <0.05, while the other 6 cytokines showed neither statistical

significance nor biological significance (biological significance is a p-value between

>0.05 and <0.1). For the Cooper strain at the 6h time point, the data was not statistically

significant. The LA strain had an average of 4.0±0.1-fold increase in IFN-alpha mRNA

expression. At the 12h time point, both the Cooper strain and the LA strain were similar

with the Cooper strain having 6.0±0.3-fold up regulation and the LA strain having

6.0±0.2-fold up regulation. At 24h, the Cooper strain increased to 10.0±0.5-fold up

regulation while the LA strain remained at 6.0±0.2-fold. The final time point at 48h with

the Cooper strain had an increase of 12.0±0.5-fold while the LA strain slightly increased

to 7.0±0.3-fold (Fig 3.2). Calf #1 and #2 were similar up regulation for all 3 statistically

significant time points (Fig 3.3). Calves #1-3 were also similar up regulation for all 4

time points (Fig 3.4). IFN-beta had an increase for both the Cooper and the LA strains

from 6h to 48h. There was a 3.6-fold increase for the Cooper strain and 2.9-fold increase

for the LA strain (Fig 3.5). No IFN gamma cytokine expression was seen until 12 hours

for the Cooper strain and 24 hours for the LA strain. IFN gamma mRNA peaked for

Cooper strain at 0.5-fold increase at 48h and 0.3-fold increase for the LA strain at 48h

(Fig 3.6). Both viruses had peak mRNA levels for IL-1b at 48h with the Cooper strain at

-2.9-fold and the LA strain at -2.6-fold (Fig 3.7). No IL-6 cytokine expression was seen

until 12h for Cooper. The Cooper strain had the greatest IL-6 mRNA levels decrease of

-2.5-fold at 48h and -2.3-fold for the LA strain (Fig 3.8). IL-8 was down regulated by

BHV-1 (Fig 3.9). Both Cooper and LA strains down regulated IL-8 at the 6h time point

with the Cooper strain having a slightly larger down regulatory effect. There was a

-1.0±0.0-fold decrease in IL-8 mRNA expression by the Cooper strain and -0.8±0.0-fold

for the LA strain. At the 12h time point, both the Cooper strain and the LA strain IL-8

expression was similar. The Cooper strain down regulated -1.0±0.0-fold while the LA

strain had a -1.0±0.0-fold decrease. IL-8 down regulation increased at the 24h time

points with the Cooper strain decreasing -3.5±0.1-fold and the LA strain a -2.0±0.1-fold.

At the 48h time point, the Cooper strain down regulated IL-8 mRNA expression

-3.8±0.2-fold and the LA strain a value of -3.5±0.151 fold. The calves used in the study

had similar results. For the Cooper infected MDDCs, only 2 calves had viable MDDCs.

The 3rd calf's MDDCs were not statistically nor biologically significant and was not

included in the results. The two calves showed similar down regulatory effects for IL-8

(Fig 3.10). For the LA infected MDDCs, all 3 calves had statistically significant results.

For the first time point at 6hs, all 3 calves had similar down regulation. Calf #3 had a

slightly larger down regulation than the other two calves at the 12h time point. At the

48h time point calf #1 was slightly less in down regulation than the other two calves (Fig

3.11). For IL-10, a peak for the Cooper strain was seen at 48h with -1.5-fold and a peak

for the LA strain at 48h with -2.0-fold (Fig 3.12). For IL-12 a peak was seen at 48h with

-2.0-fold for the Cooper strain and 48h with -1.9-fold for the LA strain (Fig 3.13).

<u>BHV-1 Infected MDDC Surface Marker Expression</u>

The Cooper strain decreased MHCI expression in mean fluorescence intensity

(MFI) by 50,000 between the 6h to 24h time points. No decrease in MFI was seen

following the LA strain infection (Fig 3.14 A). The Cooper strain decreased MHCII MFI

by 250,000, while the LA strain didn't affect expression (Fig 3.14 B). Finally, the Cooper

strain infected MDDCs had a slight decrease in CD86 MFI going from about 25,000 to

20,000 from 6h to 24h time points. The LA strain had a larger decrease in CD86 MFI

with about 10,000 less MFI compared to the Cooper strain (Fig 3.14 C). The controls

were consistent for all 3 surface markers. For MHCI (Fig 3.14 A) there was about a

25,000 difference in MFI for the 3 control time points. MHCII (Fig 3.14 B) had around a

100,000 MFI difference between controls. For CD86 (Fig 3.14 C) there was around a

10,000 MFI difference between time points.

DISCUSSION

<u>TCID$_{50}$ Assay</u>

For the production of BHV-1 in the highly susceptible MDBK cells, the TCID$_{50}$,

viral titer was seen in both the lysate and supernatant at the 6h time point. This was true

for both viruses. The Cooper strain had a higher viral titer than the LA strain in both the

lysate and supernatant. The presence of BHV-1 in the supernatant indicated that viral

infection, replication, and release is occurring as soon as 6h p.i. At each time point, the

virus titer in the supernatant at each time point was greater than the virus titer in the

lysate up to 48h. At 48h the titer was similar in both supernatant and lysate. Prior to this

study, the susceptibility of MDDC to BHV-1 viral infection and replication was

unknown. Comparing the MDDC $TCID_{50}$ to the MBDK control, the results indicated

that viral infection, replication, and release occurred in MDDCs. This occurred at 6h p.i.

for both the Cooper strain and the LA strain. The supernatant viral titer was at 6.5 log_{10}

for both the Cooper strain and the LA strain at 6h. There wasn't little difference between

the 6h time point and 48h time point indicating that the virus replication remains

consistent between the supernatant and lysate. These results indicate that MDDCs are

susceptible to BHV-1, produce less virus than MDBK cells.

BHV-1 Infected MDDC Cytokine Expression

IFN-alpha up regulation had the largest increase in MDDC cytokine expression

following BHV-1 infection. This was consistent with previous studies with BHV-1 *in*

vivo and *in vitro* in other cell types. One study (Höhle *et al.*, 2005) indicated the

importance IFN-alpha plays in BHV-1 infected animals. An artificial open reading frame

for IFN-alpha was created and tested with BHV-1. The study showed that infected cells

secreted substantial amounts of IFN-alpha causing an inhibition of BHV-1 replication.

The antiviral effect was rapid and sustained and showed little or no direct spreading.

Compared to the other cytokines tested, IFN-alpha had the greatest change in cytokine

expression. While the 6h time point wasn't significant for Cooper, a 3.5-fold increase

was seen by the 48h time point. The LA strain had an increase starting at 6h, but only a

2.8-fold increase was seen by the 48h time point. Interestingly, the calves that had the

most down regulation for IL-8 had the most up regulation for IFN-alpha. This shows a

trend that the calves' immune systems remained either better or worse between the compared animals for all time points with both viruses and cytokines examined. The rest of the cytokines tested didn't have statistical significance, but still showed significance or difference from the control and other virus. With IFN-beta, an up regulation was observed, but much less than seen in IFN-alpha. The Cooper strain showed significance from both the control and the LA strain. A steady up regulation was seen for both viruses with the most significance seen at 6h between the Cooper and LA strains. With IFN-gamma, a difference was observed between viruses and control at 6h for the Cooper strain and 12h for the LA strain, but little significance was seen. Significance was observed between the two viruses at 48h. Less expression was seen for IFN-gamma than both IFN-alpha and IFN-beta. Human herpesvirus 8 (HHV-8) targets IL-8, IL-1b, and IL-6 of B lymphocytes. An up regulation of these cytokines occurred (Knowlton, *et al.*, 2014) in infected B lymphocytes. Due to the inverse relationship between the inflammatory action of cytokines produced by B lymphocytes and DCs, the down regulation of these cytokines for MDDCs is consistent with the data. A down regulation of inflammatory cytokines for MDDCs during an infection allows easier movement of virus throughout the body. With less inflammation present, more space is available for MDDC movement. A down regulatory effect for IL-8 was expected due to the role of recruiting neutrophils through chemotaxis. Because chemotaxis helps in virus removal, down regulation of IL-8 is important for the Cooper and LA strains. While neither virus had a large down regulation until 24h, down-regulation was seen. At the 24h time points both viruses had a down regulation that was at least 1-fold. The Cooper strain had almost a 2-fold down regulatory effect, but the LA strain had a 1-fold down regulatory effect.

Both calves studied with Cooper infected MDDCs were similar which can be explained as having biologically similar immune systems because of the same breed. Calf #1 had less of a down regulatory effect than Calf #2 for all time points indicating that it has a slightly better MDDC immune response. With the LA infected MDDCs, a similar trend was observed. Each of the 3 calves were consistent in their down regulation, with Calf #1 having the least down regulation of IL-8 and Calf #3 having the most down regulation of IL-8. Down regulation for the Cooper strain was more prominent until the 48h time point when both viruses were about equally down regulation IL-8 production. For IL-1b, slight significance in down regulation was seen starting at 6h for both viruses. At the 24h time point, the LA strain had more down regulation than was seen in the Cooper strain, but only slightly. For IL-6, no difference was observed for the Cooper strain, and the LA strain showed a slight difference. Significance was best seen between the two viruses at the 12h time point. With IL-10, significance was seen at all time points. The interesting observation is that the Cooper strain had less down regulation for all time points than the LA strain. The down regulation followed a relatively steady decline for both viruses. The 6h time point for IL-12 only showed difference from the control and the viruses, Significance was first seen at 12h in IL-12 compared to the control, but both viruses had no significance between them. A slight significance was observed for 24h and 48h time points, but the LA strain was slightly more down regulated at 24h and the Cooper strain was slightly more down regulated at 48h. Cytokines are necessary in a proper immune response. If an infected MDDC isn't producing the proper cytokines in adequate amounts certain responses may not be elicited and part of the immune system might not work. The viral infection doesn't want the immune response to work properly causing cytokine

expression changes. This study indicates that cytokine expression is affected by viral

infection. However, the MDDCs are able to up or down regulate cytokine production in

infected cells to help the rest of the immune system fight off the infection.

BHV-1 Infected MDDC Surface Marker Expression

The decrease in surface marker expression in the controls was likely due to the

lower viability of cells over time. The decrease in the control MDDCs does not skew the

results, as the virally infected cells had a larger decrease than the control MDDCs did

over the time points. MHCI and MHCII surface markers were expected to be higher than

CD86 surface markers due to only MDDCs containing CD86 surface markers. The

Cooper strain had effects on both MHCI and MHCII surface makers while the LA strain

didn't. MHCI and MHCII are crucial for immune responses, and having a decrease can

cause problems in the immune system. Both MHCI and MHCII were significantly down

regulated starting at 2 hours after cell infection using Northern blot (Hinkley *et al*, 2000.).

The BHV-1 LA strain is less virulent and may have little effect on marker expression.

With the CD86 surface marker, the Cooper strain down regulated expression earlier. The

Cooper strain didn't have much of a decrease throughout the rest of the experiment. This

may indicate that Cooper affects earlier with CD86 than MHCI or MHCII. The LA strain

had an effect on CD86 surface marker expression that wasn't measured until the 24h time

point was roughly equal between the two strains. This study indicates that there is a

decrease in virally infected cells. This is important because without the necessary surface

markers present, cells are not able to interact with other cells. Due to this decrease in

surface markers, an infected MDDC might not elicit as strong as an interaction with other

cells or no interaction at all. If no interaction occurs T and B cells may not activate in infections preventing the body to surmount a response.

Summary

IFN-alpha had the greatest up regulation at approximately 12-fold. IFN-beta had a slight up regulation of 2-fold, but IFN-gamma had a negligible up regulation. The IL cytokines all had down regulations around 4-fold. The Cooper strain had a greater overall regulatory compared to the LA strain. For surface markers, a decrease in MFI was observed for MHCI, MHCII, and CD86. In the TCID$_{50}$ tests MDDCs were shown to be not as susceptible to the Cooper strain and the LA strain than MDBK cells.
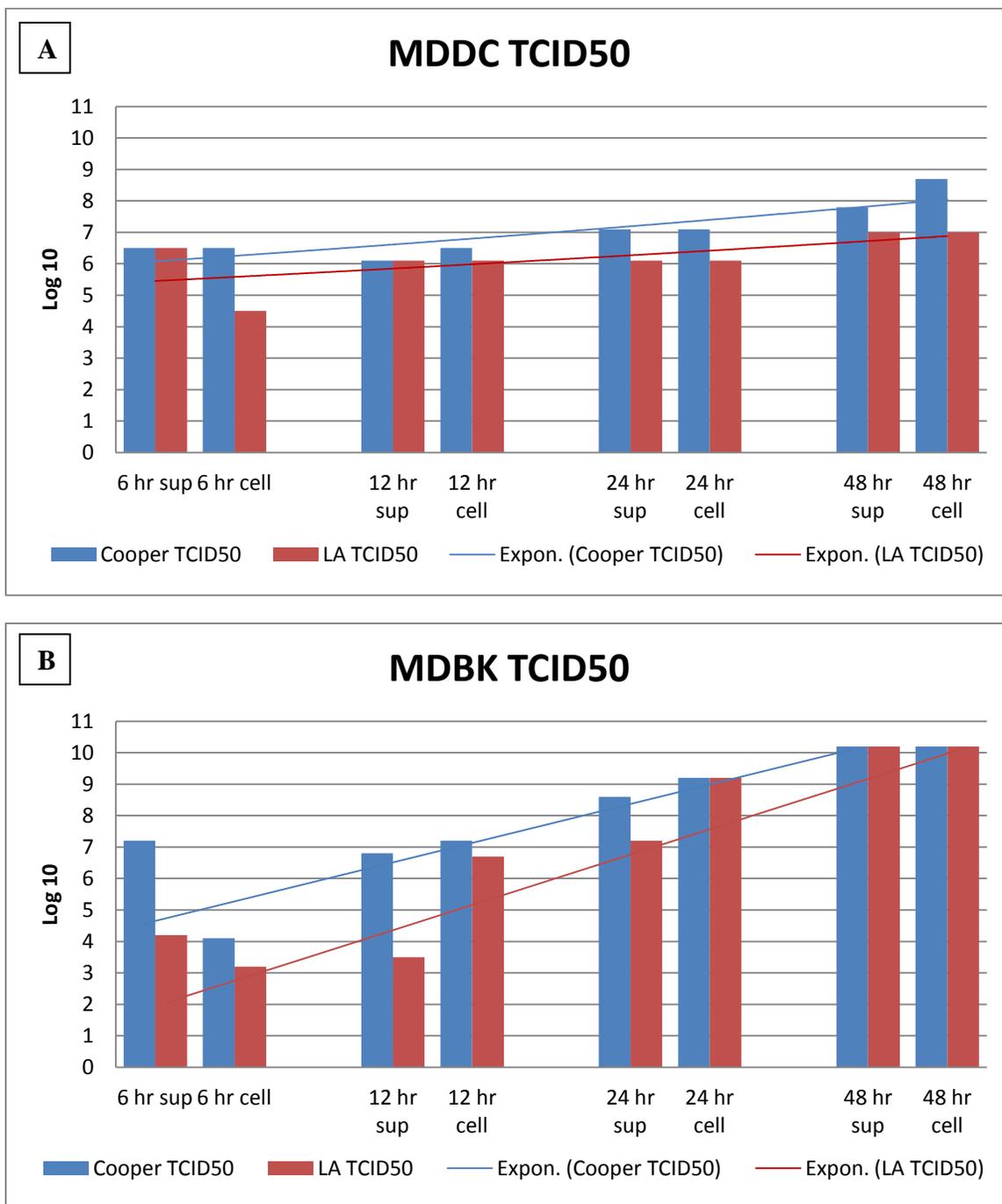
Fig 3.1. BHV-1 virus production in MDDC and MDBK infected cells: Results of a TCID$_{50}$ assay for MDDCs (A) and MDBK cells (B) showing supernatant titer and cell titer.
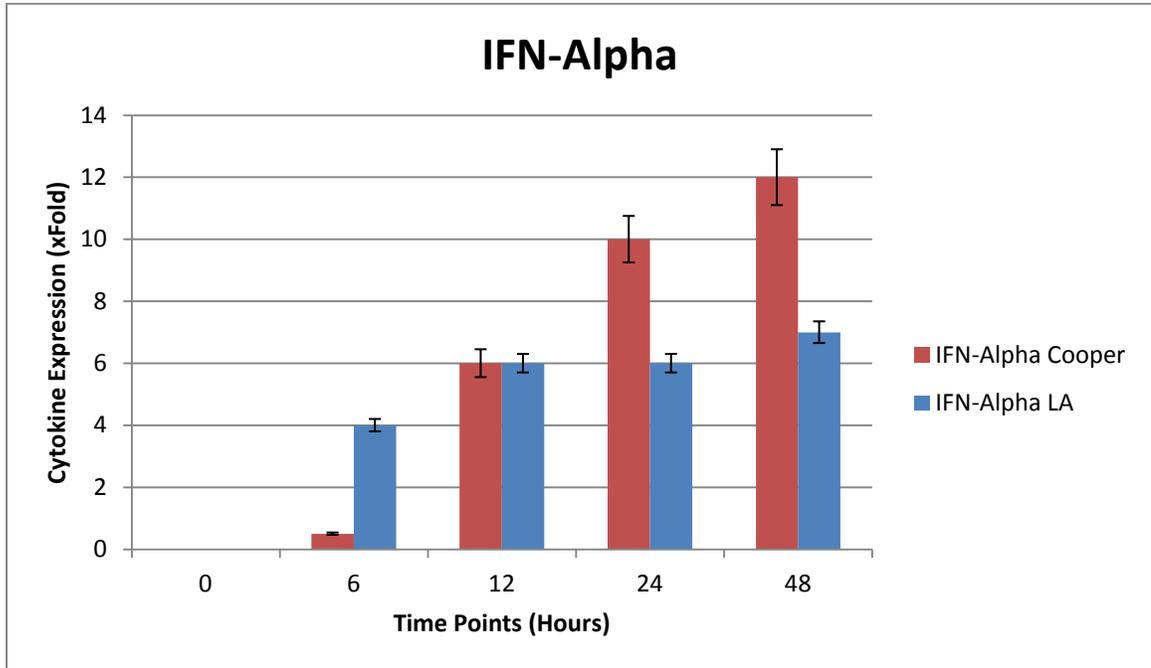
Fig 3.2. BHV-1 IFN-Alpha up regulation: BHV-1 Cooper and LA strains IFN-Alpha up regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
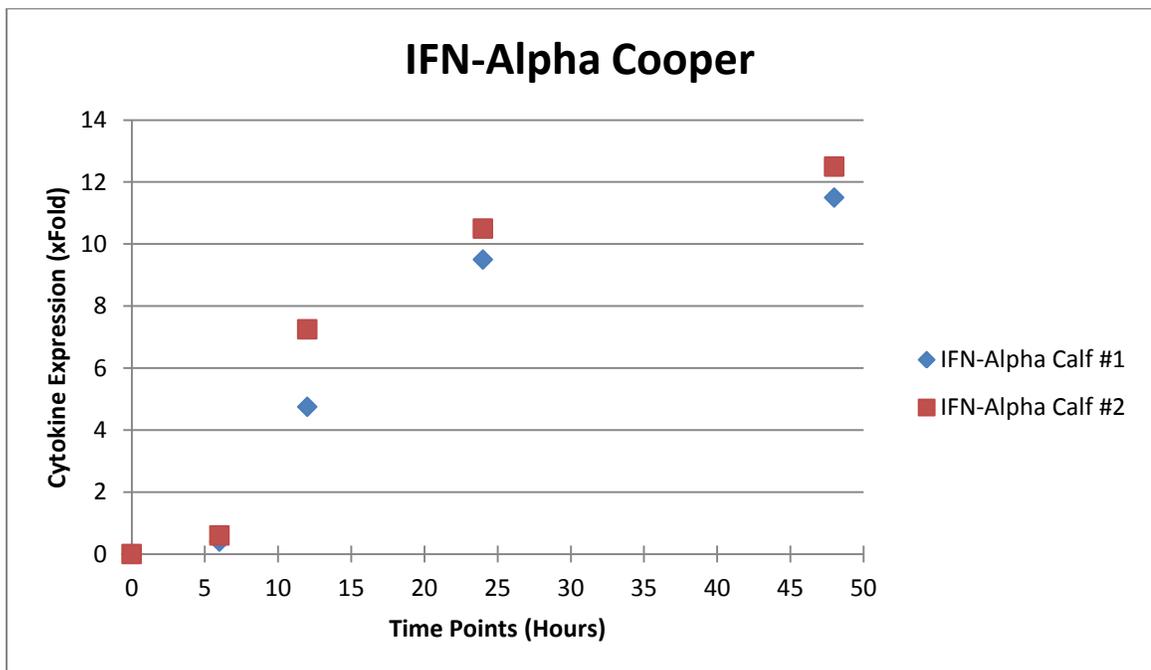

Fig 3.3. BHV-1 Cooper strain IFN-Alpha up regulation: BHV-1 Cooper strain IFN-Alpha up regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
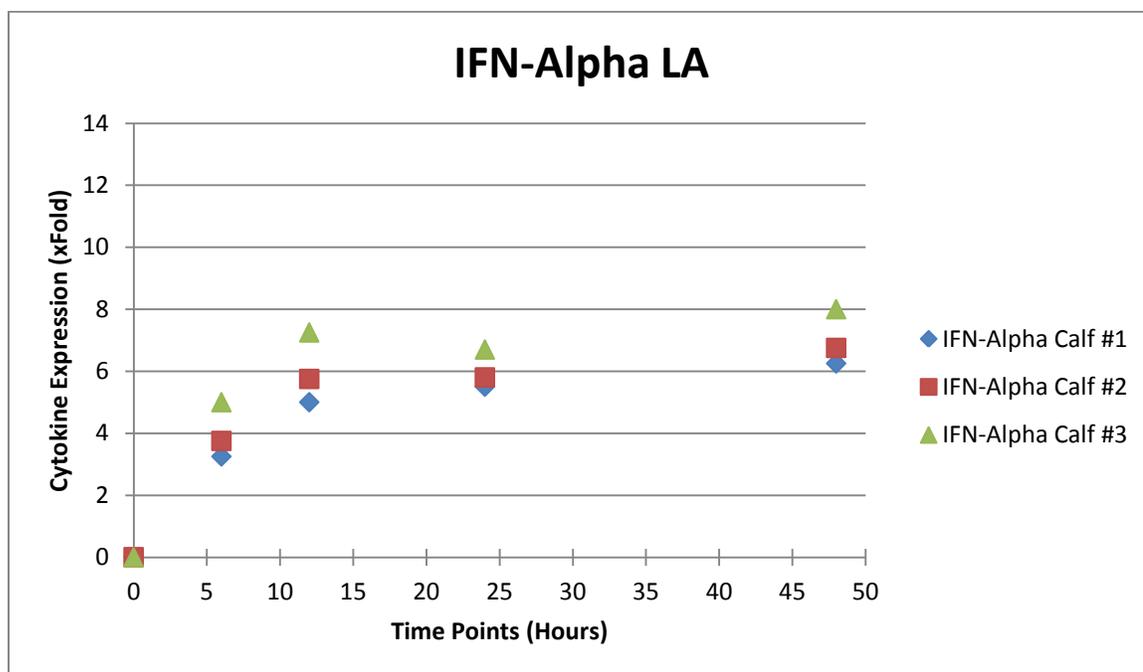
Fig 3.4. BHV-1 LA strain IFN-Alpha up regulation: BHV-1 LA strain IFN-Alpha up regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
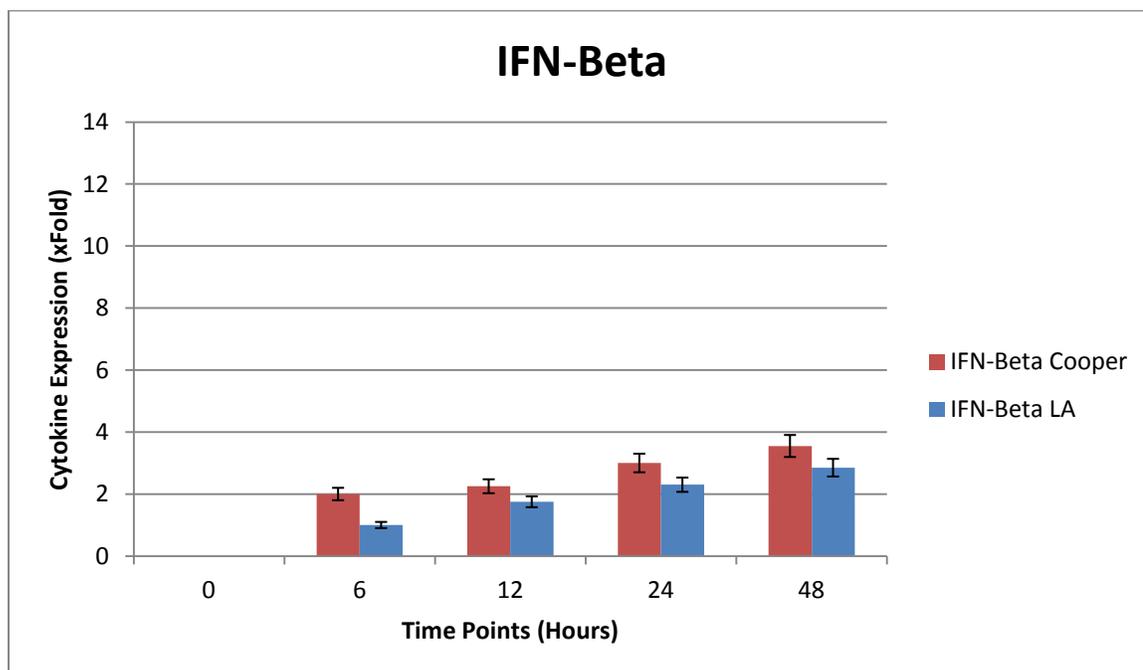

Fig 3.5. BHV-1 IFN-Beta up regulation: BHV-1 Cooper and LA strains IFN-Beta up regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
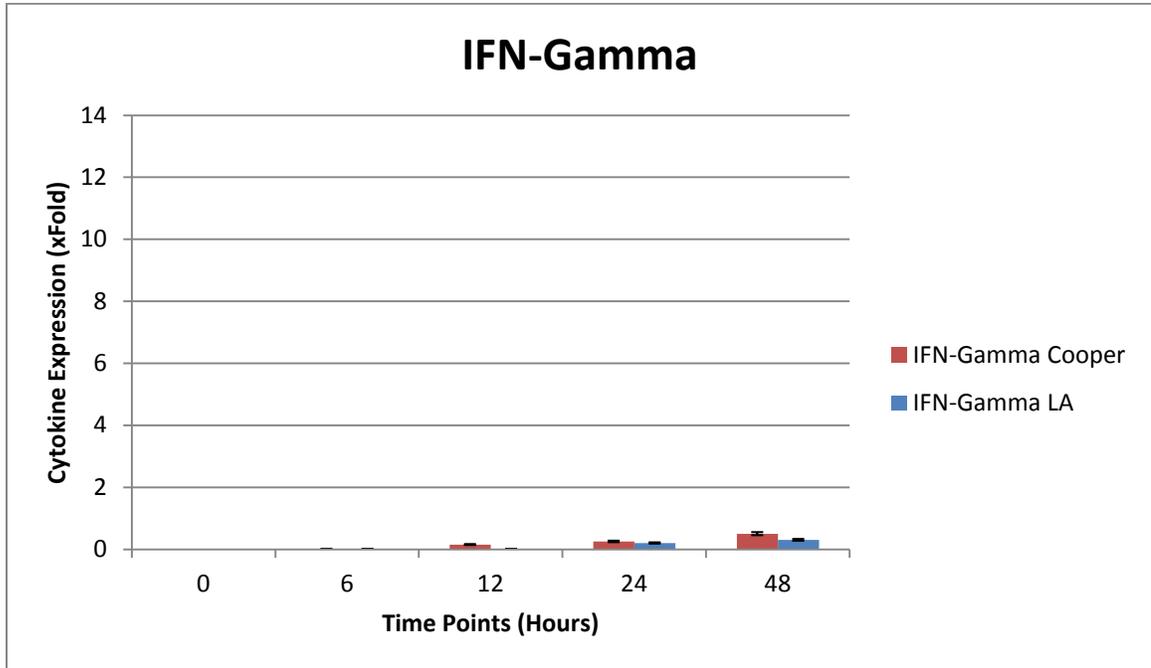
Fig 3.6. BHV-1 IFN-Gamma up regulation: BHV-1 Cooper and LA strains IFN-Gamma up regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
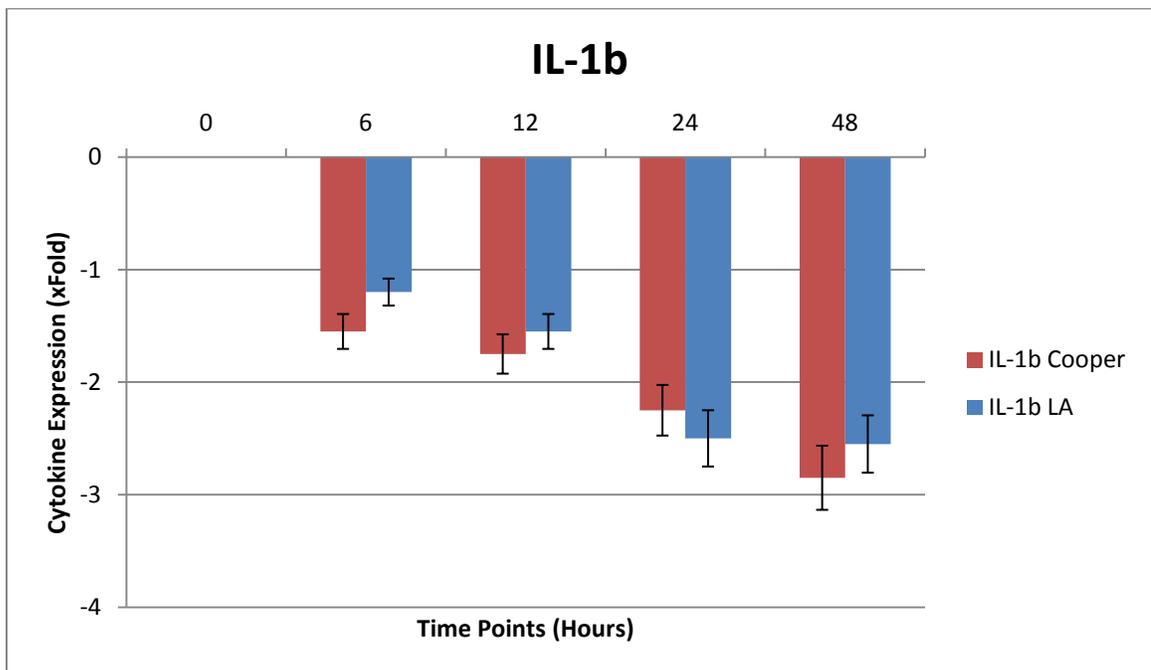

Fig 3.7. BHV-1 IL-1b down regulation: BHV-1 Cooper and LA strains IL-1b down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
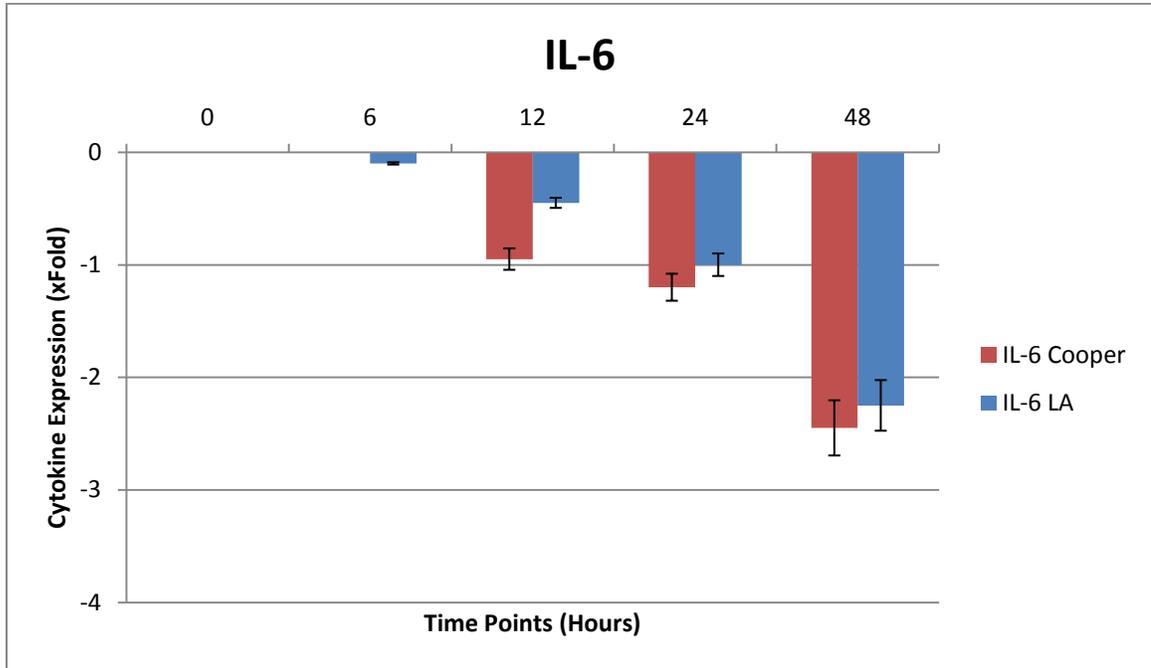
Fig 3.8. BHV-1 IL-6 down regulation: BHV-1 Cooper and LA strains IL-6 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 3.9. BHV-1 IL-8 down regulation: BHV-1 Cooper and LA strains IL-8 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 3.10. BHV-1 Cooper strain IL-8 down regulation: BHV-1 Cooper strain IL-8 down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.

Fig 3.11. BHV-1 IL-8 down regulation: BHV-1 LA strain IL-8 down regulation by calf in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
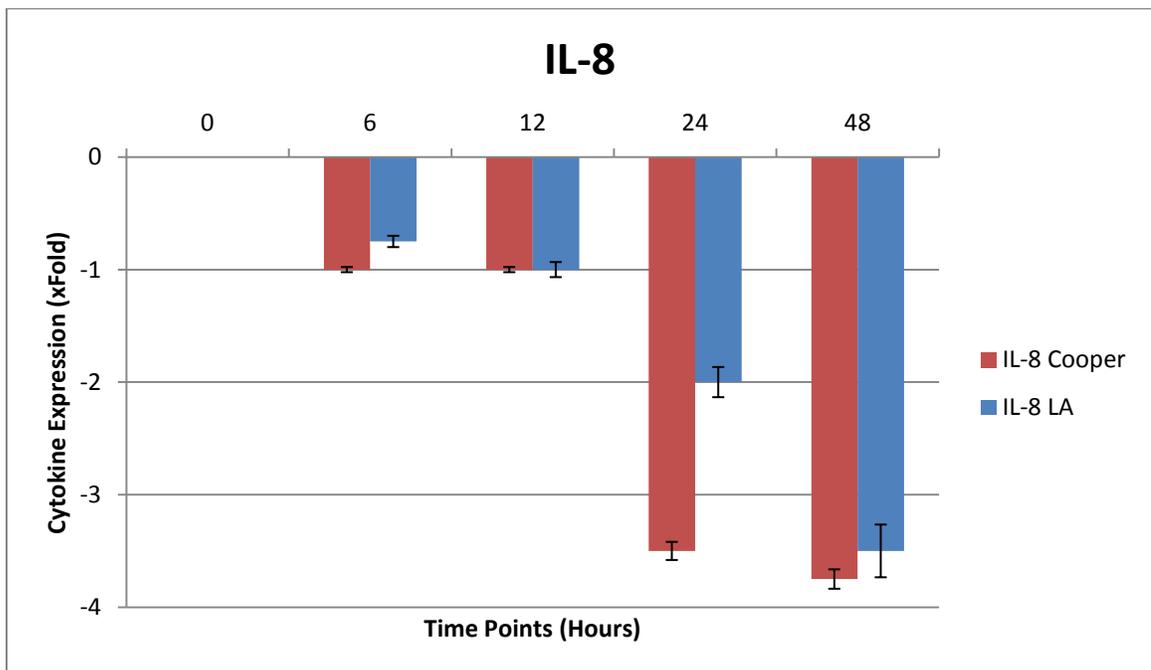
Fig 3.12. BHV-1 IL-10 down regulation: BHV-1 Cooper and LA strains IL-10 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
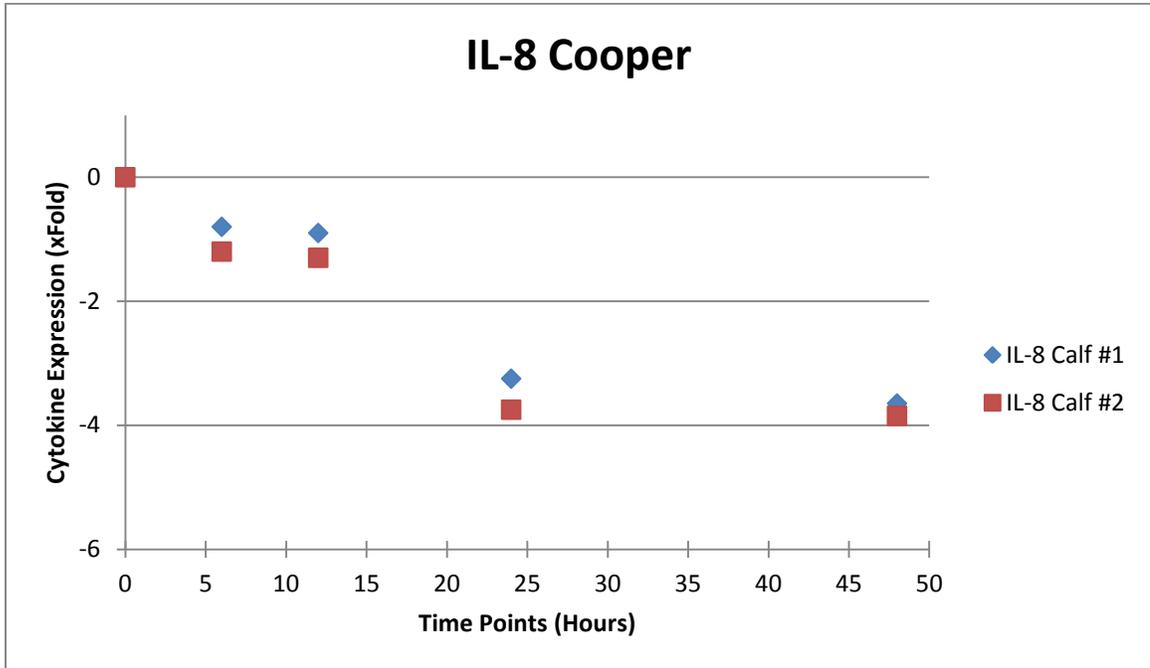

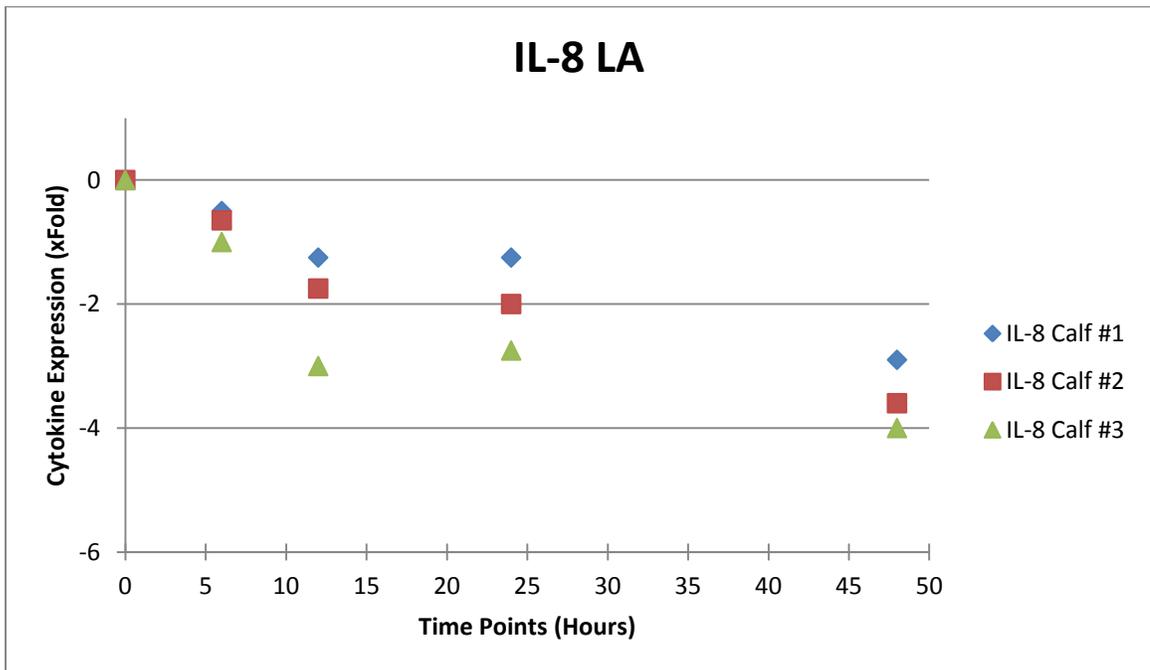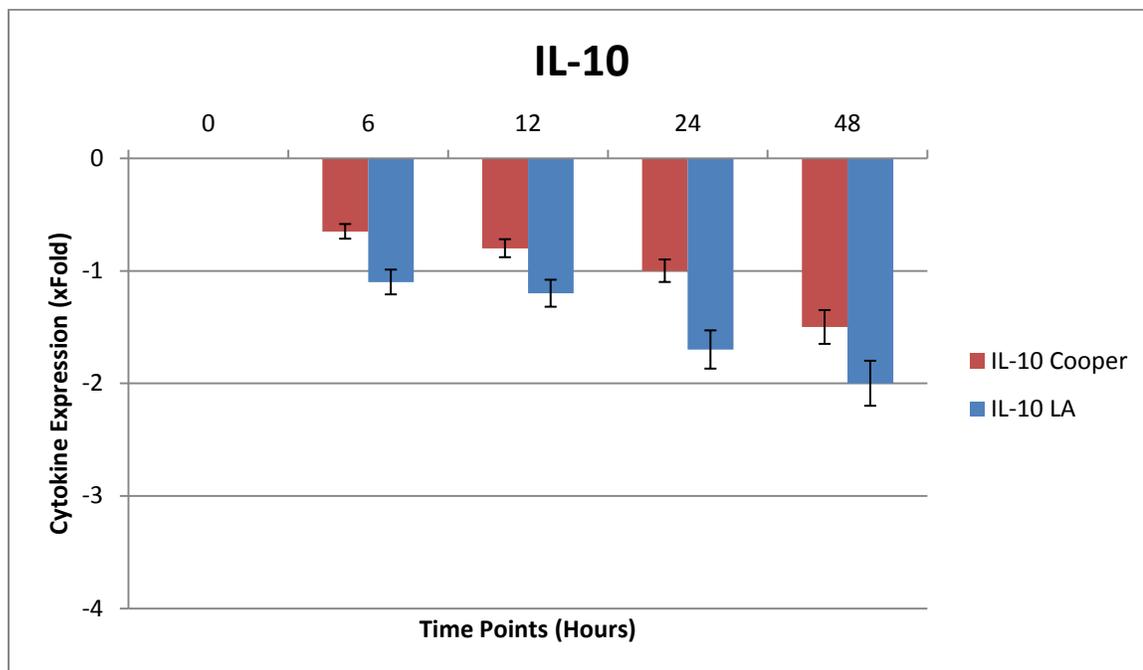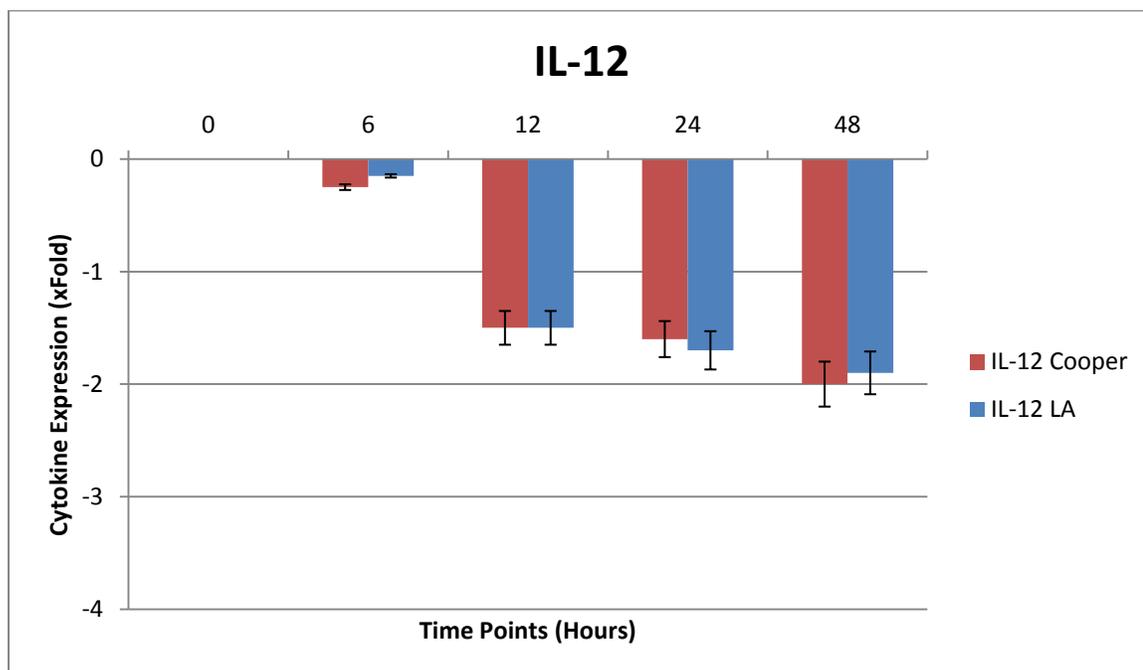**Fig 3.13. BHV-1 IL-12 down regulation:** BHV-1 Cooper and LA strains IL-12 down regulation in MDDCs at 0h, 6h, 12h, 24h, and 48h time points.
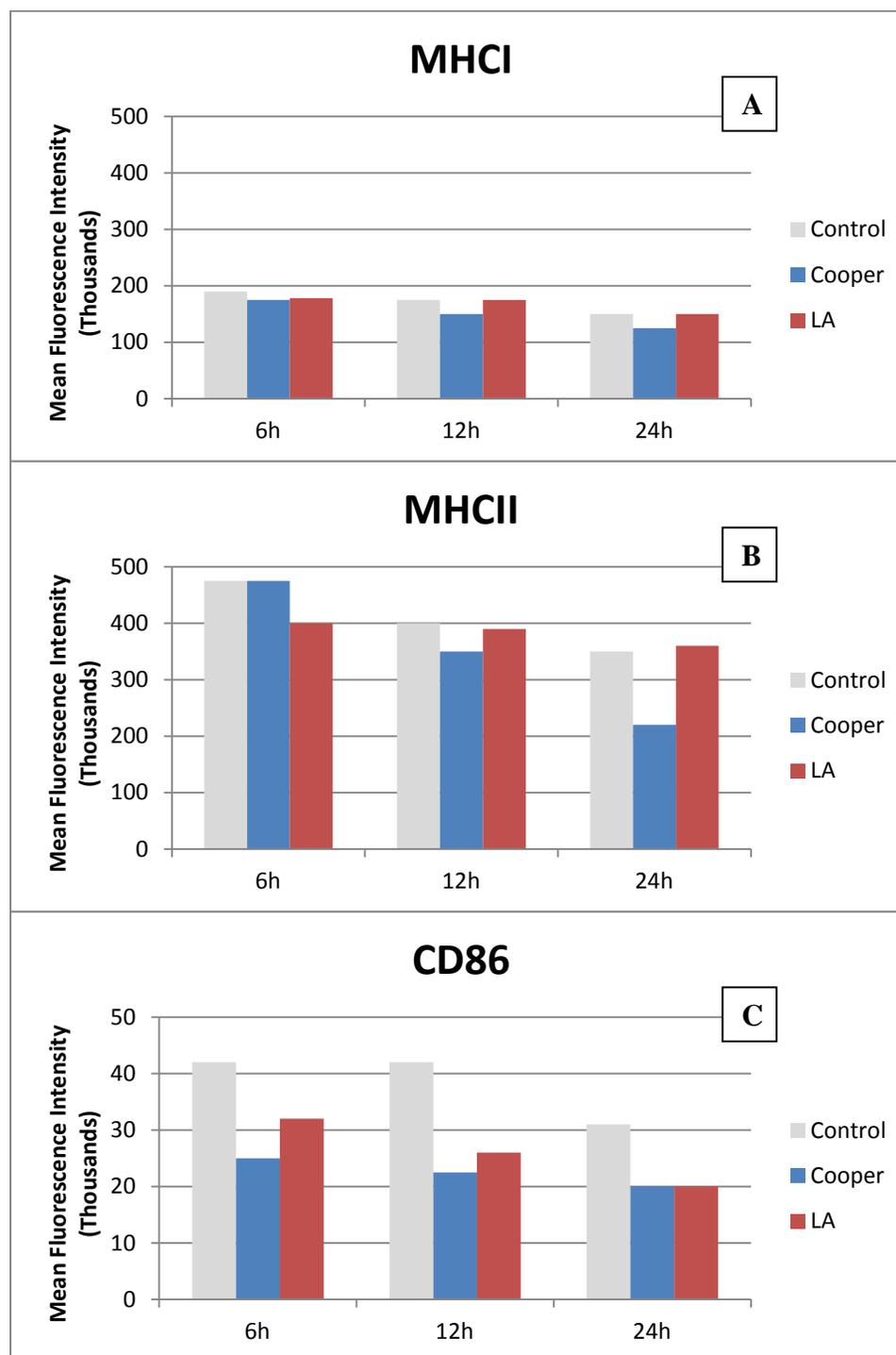
Fig 3.14. The effect of BHV-1 infection on expression of MHCI, MHCII, and CD86 on MDDC: Mean fluorescence intensity of surface marker expression per cell at 6h, 12h, and 24h time points after viral infection of MDDCs for MHCI (A), MHCII (B), and CD86 (C).

CHAPTER 4: GENERAL CONCLUSION

BVDV and BHV-1 are two of the costliest viral diseases in the cattle industry. This study was important to show the effects BVDV and BHV-1 have on cytokine production. Prior to this study there was little information on the effect of BVDV and BHV-1 infection on MDDC cytokine production. Both BVDV and BHV-1 affected cytokine production with all the cytokines measured, but varied by virus and strains. For BVDV, down regulation of cytokine production of IFN-alpha, IFN-beta, and IFN-gamma was shown. Additionally, down regulation of IL-1a and TNF was shown. These results suggested that a higher virulent strain, 1373, has a greater impact on cytokine regulation than a lower virulent strain, 28508. The cytokines affected are likely to play a more important role in BVDV viral infection. For BHV-1, up regulation of the same cytokines was observed. The rest of the cytokines: IL-1b, IL-6, IL-8, IL-10, and IL-12 were down regulated for both viruses. Similar observations can be made between BVDV and BHV-1. The results suggested that a higher virulent BHV-1 strain, Cooper, has a greater impact on cytokine regulation than a lower virulent strain, Los Angeles. More cytokines were affected by BHV-1, implying that BHV-1 will affect cytokine regulation more than BVDV. A summary of the cytokine mRNA expression is shown in Fig 4.1. Furthermore, in the BVDV study, cattle breed played a role in cytokine regulation. Brown Swiss calves are less susceptible to cytokine regulation than Holstein Friesian. This may be due to Brown Swiss calves having greater monocyte quantities in their blood. This led to the hypothesis that both viral virulence and cattle breed can impact cytokine regulation. Finally, in the BHV-1 study, the MFI of MHCI, MHCII, and CD86 decreased over the time points observed. The results suggested that a highly virulent

strain has the greatest effect on MHCI and MHCII expression. MFI for CD86 was not affected by a high virulent strain, but by a low virulent strain instead.

Further investigation of different strains of BVDV and BHV, such as BHV-4 are worth investigation. Both BVDV and BHV have received little investigation on the effects different strains have on MDDCs. Investigating more strains will give a better idea on how the viruses interact with MDDCs. The observations could be drastically different compared to other strains in the same viral grouping. Additionally, a comparative study on MDDCs and DCs infected with BVDV and BHV is also worth investigating. The more knowledge on these two economically challenging viruses could lead to better methods of treatment and prevention.

Additional experiments could focus on T cell interaction between infected MDDCs or DCs. As DCs serve to stimulate naive T cells and activate them, a virally infected DC could have a different effect on the naive T cells. The presentation of the antigens to the T cells might not yield as strong of a stimulus as uninfected DC. Perhaps this won't allow the naive T cells to activate. Furthermore, a virally infected DC might change the T cell cytokine yield after activation. If the naive T cell isn't stimulated as well by a virally infected DC, the cytokines produced might be decreased. Observing T cell interaction with virally infected MDDCs or DCs is an good next step to look at how different MDDCs and DCs are when infected.

Cytokine	IFN Alpha	IFN Beta	IFN Gamma	IL-1a	IL-1b	IL-6	IL-8	IL-10	IL-12	TNF
BVDV				🟥	🟥	🟥	🟥	🟥	🟥	🟥
BHV-1	🟩	🟩			🟥	🟥	🟥	🟥	🟥	

Table 4.1. BVDV and BHV-1 Cytokine Summary: A summary of cytokine regulation with white cells indicating minimal change, red indicating down regulation, dark red indicating significant down regulation, green indicating up regulation, and dark green indicating significant up regulation.

REFERENCES

Aravalli, R.N., Hu, S., Rowen, T.N., Palmquist, J.M., Lokensgard, J.R. (2005). "TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus." J Immunol 175:4189-4193. doi: 10.4049/jimmunol.175.7.4189.

Ariza, M.E., Glaser, R., Kaumaya, P.T.P., Jones, C., Williams, M. (2009). "The Epstein-Barr Virus (EBV)-encoded dUTPase activates FN-kB through the TLR2 and MyD88-dependent signaling pathway." J Immunol 182:851-859. doi: 10.4049/jimmunol.182.2.851.

Ariza, M.E., Rivailler, P., Glaser, R., Chen, M., Williams, M.V. (2013) "Epstein-Barr virus encoded dUTPase containing exosomes modulate innate and adaptive immune responses in human dendritic cells and peripheral blood mononuclear cells." PLoS One 8:e69827. doi: 10.1371/journal.pone.0069827.

Ariza, M., Glaser, R., Williams, M.V. (2014). "Human herpesviruses-encoded dUTPases: a family of proteins that modulate dendritic cell function and innate immunity." Front Microbiol 5(504). doi: 10.3389/fmicb.2014.00504.

Baker, J.C. (1995). "The clinical manifestations of bovine viral diarrhea infection." Vet Clin North Am Food Anim Pract 11: 425-445.

Baule, C., van Vuuren, M., Lowings, J.P., Belak, S. (1997). "Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa." Virus Res 52(2): 205-220.

Bedoui, S., Greyer, M. (2014). "The role of dendritic cells in immunity against primary herpes simplex virus infections." Front Microbiol 5(533). doi: 10.3389/fmicb.2014.00533.

Benoist, C., Mathis, D. (1990). "Regulation of major histocompatibility complex class-II genes: X, Y and other letters of the alphabet." Annu Rev Immunol 8: 681-715.

Behrens, S.E., Grassman C.W., Thiel, H.J., Meyers, G., Tautz, N. (1998). "Characterization of an autonomous subgenomic pestivirus RNA replicon." J Virol 72: 2364-2372.

Biswas, S., Bandyopadhyay, S., Dimri, U., Patra, P. (2013). "Bovine herpesvirus-1 (BHV-1)-a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis." Vet Q 33(2): 68-81.

Bolin, S.R., McClurkin, A.W., Cutlip, R.C., Coria, M.F. (1985). "Response of cattle persistently infected with noncytopathic bovine viral diarrhea virus to vaccination for bovine viral diarrhea and to subsequent challenge exposure with cytopathic bovine viral diarrhea virus." Am J Vet Res 46: 2467-2470.

Bolin, S.R. (1995). "The pathogenesis of mucosal disease." Vet Clin North Am Food Anim Pract 11(3): 489-500.

Brock, K.V. (1995). "Diagnosis of bovine viral diarrhea virus infections." Vet Clin North Am Food Anim Pract 11: 549-561.

Brower, A., Homb, K.M., Bochsler, P., Porter, R., Woods, K., Ubl, S., Krueger, D., Cigel, F., Toohey-Kurth, K. (2008). "Encephalitis in aborted bovine fetuses associated with Bovine herpesvirus 1 infection." J Vet Diagn Invest 20: 297-303.

Brownlie, J., Thompson, I., Curwen, A. (2000). "Bovine virus diarrhoea virus - strategic decisions for diagnosis and control." In Practice 22(4): 176-187.

Cardoso, N., Franco-Mahecha, O.L., Czepluch, W., Quintana, M.E., Malacari, D.A., Trotta M.V., Mansilla F.C., Capozzo, A.V. (2016). "Bovine viral diarrhea virus infects monocyte-derived bovine dendritic cells by an E2-glycoprotein-mediated mechanism and transiently impairs antigen presentation." Viral Immunol 29(7): 417-429. doi:10.1089/vim.2016.0047.

Chowdhury, S., Sharma, B. (2012). "Transcription of gD and gI genes in BHV1-infected cells." J Biosci 37(6): 971-977.

Collett, M.S., Larson, R., Gold, C., Strick, D., Anderson, D.K., Purchio, A.F. (1988). "Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus." Virology 165(1): 191-199.

Compton, T., Kurt-Jones, E.A., Boehme, K.W., Belko, J., Latz, E., Golenbock, B.T., et al. (2003). "Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2." J Virol 77:4588-4596. doi: 10.1128/JVI.77.8.4588-4596.2003.

Contassot, E., Beer, H.D., French, L.E. (2012). "Interleukin-1, inflammasomes, autoinflammation and the skin." Swiss Med Wkly 142: w13590. doi: 10.4414/smw.2012.13590.

Corapi, W.V., Donis, R.O., Dubovi, E.J. (1990). "Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhea virus." Am J Vet Res 51: 1388-1394.

Dasika, G.K., Letchworth, G.J. (1999). "Cellular expression of bovine herpesvirus 1 gD inhibits cell-to-cell spread of two closely related viruses without blocking their primary infection." Virology 254: 24-36.

Del Medico, Z.M.P., Puntel, M., Zamorano, P.I., et al. (2006). "BHV-1 vaccine induces

cross-protection against BHV-5 disease in cattle." Res Vet Sci 81: 327-334.

Delhon, G., Moraes, M.P., Lu, Z., et al. (2003). "Genome of bovine herpesvirus 5." J Virol 77: 10339-10347.

Donis, R.O. (1995). "Molecular biology of bovine viral diarrhea virus and its interactions with the host." Vet Clin North Am Food Anim Pract 11: 393-423.

El-Habashi, N., El-Nahass, E., Haridy, M., Nayel, M., Abdelaziz, A.A., Fukushi, H., Kuroda, K., Sakai, H., Yanai, T. (2014). "Pathological Findings in Equine Herpesvirus 9-Induced Abortion in Rats." J Comp Pathol 151(4): 400-409.

Enquist, L.W., Card, J.P. (2003). "Recent advances in the use of neurotropic viruses for circuit analysis." Curr Opin Neurobiol 13: 603-606.

Fan, Q., Yao, L., Ding, M., Wang, D.J., Chen, H.C., Liu, Z.F. (2012). "Development of latex agglutination test for rapid detection of antibodies against Bovine herpesvirus 1 and Bovine herpesvirus 5 in cattle." J Vet Diagn Invest 24(6): 1162-1165.

Fiorito, F., Irace, C., Di Pascale, A., Colonna, A., Iovane, G., et al. (2013). "2,3,7,8-Tetrachlorodibenzo-p-Dioxin Promotes BHV-1 Infection in Mammalian Cells by Interfering with Iron Homeostasis Regulation." PLOS One 8(3): e58845. doi: 10.1371/journal.pone.0058845.

Fredriksen, B., Press, C.M., Loken, T., Odegaard, S.A. (1999). "Distribution of viral antigen in uterus, placenta and foetus of cattle persistently infected with bovine viral diarrhaea virus." Vet Microbiol 64(2-3): 109-122.

Fredericksen, F., Carrasco, G., Villalba, M., Olavarría, V.H. (2015). " Cytopathic BVDV-1 strain induces immune marker production in bovine cells through the NF-κB signaling pathway." Mol Immunol 62(2 pt A): 213-22. doi: 10.1016/j.molimm.2015.08.010.

Frolov, I., McBride, M.S., Rice, C.M. (1998). "cis-acting RNA elements required for replication of bovine viral diarrhea virus-hepatitis C virus 5' nontranslated region chimeras." RNA 4: 1418-1435.

Fulton, R. W., Saliki, J. T., Burge, L. J., d'Offay, J. M., Bolin, S. R., Maes, R. K., ... & Frey, M. L. (1997). Neutralizing antibodies to type 1 and 2 bovine viral diarrhea viruses: detection by inhibition of viral cytopathology and infectivity by immunoperoxidase assay. Clin Diagn Lab Immunol 4(3): 380-383.

Glaser, R., Litsky, M.L., Padget, D.A., Baiocchi, R.A., Yang, E.V., Chen, M., et al. (2006). "The EBV-encoded dUTPase induces immune dysregulation: implications for the pathophysiology of EBV-associated disease. Virology 346: 205-218. doi: 10.1016/j.virol.2005.10.034.

Gregory, S.M., Wang, L., West, J.A., Dittmer, D.P., Damania, B. (2012). "Latent Kaposi's sarcoma-associated herpesvirus infection of monocytes down-regulates expression of adaptive immune response costimulatory receptors and proinflammatory cytokines." J Virol 86: 3916-3923. doi: 10.1128/JVI.06437-11.

Griffin, S.D.C., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., Jaeger, J., Harris, M.P.G., Rowlands, D.J. (2003). "The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine." FEBS Letters 535(1-3): 34-38.

Hamers, C., Dehan, P., Couvreur, B., Letellier, C., Kerkhofs, P., Pastoret, P.P. (2001). "Diversity among bovine pestiviruses." Vet J 161: 112-122.

Haniffa, M., Collin, M., Ginhoux, F. (2013). "Ontogeny and functional specialization of dendritic cells in human and mouse." Adv Immunol 120: 1-49.

Hanon, E., Keil, G., van Drunen Little-van den Hurk, Griebel, P., Vanderplasschen, A., Rijsewijk, F.A., Babiuk, L., Pastoret, P.P. (1999). "Bovine herpesvirus 1-induced apoptotic cell death: role of glycoprotein D." Virology 257: 191-197.

Hanon, E., Meyer, G., Vanderplasschen, A., Dessy-Doize, C., Thiry, E., Pastoret, P.P. (1998). "Attachment but not penetration of bovine herpesvirus 1 is necessary to induce apoptosis in target cells." J Virol 72: 7638-7641.

Hardy, M.P., Owczarek, C.M., Jermiin, L.S., Ejdebäck, M., Hertzog, P.J. (2004). "Characterization of the type 1 interferon locus and identification of novel genes." Genomics 2: 331-45. doi: 10.1016/j.ygeno.2004.03.003.

Hedges, J.C., Singer, C.A., Gerthoffer, W.T., (2000). "Mitogen-activated protein kinases regulate kytokine gene expression in human airway myocytes." Am J Respir Cell Mol Biol. 23: 86-94. doi: 10.1165/ajrcmb.23.1.4014.

Hinkley, S., Ambagala, A.P., Jones, C.J., Srikumaran, S. (2000). "A vhs-like activity of bovine herpesvirus-1." Arch Virol 145(10): 2027-46.

Hoff, H.S., Donis, R.O. (1997). "Induction of apoptosis and cleavage of poly(ADP-ribose) polymerase by cytopathic bovine viral diarrhea virus infection." Virus Res 49: 101-113.

Höhle, C., Karger, A., König, P., Giesow, K., Keil, G.M. (2005). "High-level expression of biologically active bovine alpha interferon by Bovine herpesvirus 1 interferes only marginally with recombinant virus replication in vitro." J Gen Virol 86(Pt 10): 2685-95.

Hsieh C.S., Macatonia S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., Murphy, K.M. (1993). "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages." Science 260: 547-9. doi: 10.1126/science.8097338.

Hunter, E. (2001). "Virus assembly." Chapter 8 in Knipe DM, Howley PM, editors. Fields' virology. 4th ed. Volume 1. Philadelphia: Lippincott Williams & Wilkins; p. 171-197.

Isken, O., Grassmann, C.W., Yu, H., Behrens, S.-E. (2004). "Complex signals in the genomic 3' nontranslated region of bovine viral diarrhea virus coordinate translation and replication of the viral RNA." RNA 10: 1637-1652.

Jones, C. (2003). "Herpes simplex virus type 1 and bovine herpesvirus 1 latency." Clin Microbiol Rev 16: 79-95.

Kaliński, P., Hilkens, C.M., Snijders, A., Snijdewint, F.G., Kapsenberg, M.L. (1997). "IL-12 deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells." J Immunol 159: 28-35.

Knipe, D.M., Samuel, C.E., Palese, P. (2001). "Virus-host cell interactions." Chapter 7. In: Knipe DM, Howley PM, editors. Fields' virology. 4th ed. Volume 1. Philadelphia: Lippincott Williams & Wilkins; p. 133-170.

Knowlton, E.R., Rappocciolo, G., Piazza, P., Lepone, L.M., Nadgir, S.V., Bullotta, A., Berendam, S.J., Li, J., Reinhart, T.A., Jenkins, F.J., Rinaldo, C.R. (2014). "Human herpesvirus 8 induces polyfunctional B lymphocytes that drive Kaposi's sarcoma." MBio 5(5): e01277-14. doi: 10.1128/mBio.01277-14.

Keolle, D.M., Corey, L. (2008). "Herpes simplex: insights on pathogenesis and possible vaccines." Annu Rev Med 59: 381-395.

Kurjogi, M.M., Sanakal, R.D., Kaliwal, B.B. (2012). "Identification and analysis of putative promoter motifs in bovine herpes virus." Bioinformation 8(23): 1167-70. doi: 10.6026/97320630081167.

Kurt-Jones, E.A., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., et al. (2004). " Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis." Proc Natl Acad Sci USA 101: 1315-1320. doi: 10.1073/pnas.0308057100.

Laureyns, J., Letellier, C., Maganck, V., Pardon, B., Deprez, P., de Kruif, A. (2011). "Severe disease in neonatal calves with detection of cytopathic BVDV." Vet Rec 169(4): 101.

Lee, K.M., Gillespie, J.H. (1957). "Propagation of virus diarrhea virus of cattle in tissue culture." Am J Vet Res 18: 952-953.

Liebler-Tenorio, E.M., Ridpath, J.E., Neill, J.D. (2002). "Distribution of viral antigen and development of lesions after experimental infection with highly virulent bovine viral diarrhea virus type 2 in calves." Am J Vet Res 63(11): 1573-1584. doi: 10.2460/ajvr.2002.63.1575.

Liebler-Tenorio, E.M., Ridpath, J.E., Neill, J.D. (2003). "Distribution of viral antigen and development of lesions after experimental infections of calves with a BVDV 2 strain of low virulence." J Vet Diagn Invest 15(3): 221-232.

Li, M.L., Zhao, Z.Y., Cui, W., Mo, C.C., Wang, J.L. Cai, M.S. (2014) "Molecular cloning and characterization of the pseudorabies virus UL31 gene." Genet Mol Res 13(1): 1832-1847.

Li, Y., Kiang, X., van Drunen Little-van den Hurk, Attah-Poku, S., Babiuk, L.A. (1996). "Glycoprotein Bb, the N-terminal subunit of bovine herpesvirus 1 gB, can bind to heparan sulfate on the surfaces of Madin-Darby bovine kidney cells." J Virol 70: 2032-2037.

Li, Y., van Drunen Little-van den Hurk, Babiuk, L.A., Liang, X. (1995). "Characterization of cell-binding properties of bovine herpesvirus 1 glycoproteins B, C, and D: idenification of a dual cell-binding function of gB." J Virol 69: 4758-4768.

Lyman, M.G., Kemp, C.D., Taylor, M.P., Enquist, L.W. (2009). "Comparison of the pseudorabies virus Us9 protein with homologs from other veterinary and human alphaherpesviruses." J Virol 83(14): 6978-6986.

Mayo, M.A. (2002). "A summary of taxonomic changes recently approved by ICTV." Arch Virol 147(8): 1655-1663.

Merad, M., Sathe, P., Helft, J., Miller, J., Mortha, A. (2013). "The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting." Annu Rev Immunol 31: 563-604.

Meyers, G., Ege, A., Fetzer, C., von Freyburg, M., Elbers, K., Carr, V., Prentice, H., Charleston, B., Schürmann, E.M. (2007). "Bovine viral diarrhea virus: prevention of persistent fetal infection by a combination of two mutations affecting Erns RNase and Npro protease." J Virol 81(7): 3327-3338.

Mirkovitch, J., Konig, A., Sauter, K.S., Brcic, M., Hope, J.C., et al. (2006). "Single-cell analysis divides bovine monocyte-derived dendritic cells into subsets expressing either high or low levels of inducible nitric oxide synthase." Vet Immunol Immunopathol 95: 21-31.

Moennig, V., Eiken, K., Flebbe, U., Frey, H.R., Grummer, B., Haas, L., Greiser-Wilke, I., Liess, B. (2005). "Implementation of two-step vaccination in the control of bovine viral diarrhoea (BVD)." Prev Vet Med 72: 109-114.

Muñoz-Zanzi C.A., Hietala S.K., Thurmond M.C., Johnson W.O. (2003). "Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves." Am J Vet Res 64(3): 358-365.

Muñoz-Zanzi C.A., Thurmond M.C., Hietala S.K. (2004). "Effect of bovine viral diarrhea virus infection on fertility of dairy heifers." Theriogenology 61(6): 1085-1099. doi:10.1016/j.theriogenology.2003.06.003.

Nettleton, P.F., Entrican, G. (1995). "Ruminant pestiviruses." Br Vet J 151(6): 615-642.

Palomares, R.A., Brock, K.V., Walz. P.H. (2014). "Differential expression of pro-inflammatory and anti-inflammatory cytokines during experimental infection with low or high virulence bovine viral diarrhea virus in beef calves." Vet Immunol Immunopathol 15(3-4): 149-154. doi: 10.1016/j.vetimm.2013.12.002.

Peterhans, E., Jungi, T.W., Schweizer, M. (2003). "BVDV and innate immunity." Biologicals 31: 107-112.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M. (2010). "Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction." Vet Res 41(6): 44. doi: 10.1051/vetres/2010016.

Peterhans, E., Schweizer, M. (2013). "BVDV: a pestivirus inducing tolerance of the innate immune response." Biologicals 41(1): 39-51. doi: 10.1016/j.biologicals.2012.07.006.

Pfaffl, M.W., Horgan, G.W., Dempfle, L. (2002). "Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR." Nucleic Acids Res 30: e36.

Pocock, D.H., Howard, C.J., Clarke, M.C. Brownlie, J. (1987). "Variation in the intracellular polypeptide profiles from different isolates of bovine virus diarrhoea virus." Arch Virol 94(1-2).: 43-53.

Pusterla, N., Wilson, D.W., Madigan, J.E., Ferraro, G.L. (2009). "Equine herpesvirus-1 myeloencephalopathy: A review of recent developments." Vet J 180: 279-289.

Pusterla, N., Hussey, G.S. (2014). "Equine herpesvirus 1 myeloencephalopathy." Vet Clin North Am Equine Pract 30(3): 489-506.

Raaperi, K., Orro, T., Viltrop, A. (2014). "Epidemiology and control of bovine herpesvirus 1 infection in Europe." Vet J 201: 249-256.

Radostits, O.M. (1985). "Bovine virus diarrhoea - Mucosal disease complex. A clinician's review of the history and current status of a fascinating disease." Aust Vet J 62: 84-92.

Rajput, M.K.S., Darweesh, M.F., Park, K., Braun, L.J., Mwangi, W., Young, A.J., Chase, C.C.L. (2014). "The effect of bovine viral diarrhea virus (BVDV) strains on bovine monocyte-derived dendritic cells (Mo-DC) phenotype and capacity to produce BVDV." Virol J 11: 44.

Reed, K.E., Gorbalenya, A.E., Rice, C.M. (1998). "The NS5A/NS5 proteins of viruses from three genera of the family flaviviridae are phosphorylated by associated serine/threonine kinases." J Virol 72(7): 6199-6206.

Renjifo, X., Letellier, C., Keil, G.M., Ismaili, J., Vanderplasschen, A., et al. (1999). "Susceptibility of bovine antigen-presenting cells to infection by bovine herpesvirus 1 and in vitro presentation to T cells: Two independent events." J Pathol 73(6): 4840-4846.

Ridpath, J.F., Bolin, S.R. (1995). "Delayed onset postvaccinal mucosal disease as a result of genetic recombination between genotype 1 and genotype 2 BVDV." Virology 212(1): 259-262.

Ridpath, J.F., Neill, J.D., Frey, M., Landgraf, J.G. (2000). "Phylogenetic, antigenic and clinical characterization of type 2 BVDV from North America." Vet Microbiol 77: 145-155.

Schroder, C., Keil, G.M. (1999). "Bovine herpesvirus 1 requires glycoprotein H for infectivity and direct spreading and glycoproteins gH(W450) and gB for glycoprotein D-independent cell-to-cell spread." J Gen Virol 80: 57-61.

Schwartz, D.M., Bonelli, M., Gadina, M., O'Shea, J.J. (2016). "Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases." Nat Rev Rheumatology 12: 25-36. doi: 10.1038/nrrheum.2015.167.

Shrimpton, R.E., Butler, M., Morel, A.S., Eren, E., Hue, S.S., Ritter, M.A. (2008). "CD205 (DEC-205): A recognition receptor for apoptotic and necrotic self." Mol Immunol 46(6): 1229-1239.

Sloan, E., Henriquez, R., Kinchington, P.R., Slobedman, B., Abendroth, A. (2011). "Varicella-Zoster Virus Inhibition of the NF-kB Pathway during Infection of Human Dendritic Cells: Role for Open Reading Frame 61 as a Modulator of NF-kB Activity." J Virol 86(2): 1193-1202.

Spiesschaert, B., Osterrieder, N., Azab, W. (2015). "Comparative Analysis of Glycoprotein B (gB) of Equine Herpesvirus Type 1 and Type 4 (EHV-1 and EHV-4) in Cellular Tropism and Cell-to-Cell Transmission." Viruses 7: 522-542.

Steck, F., Lazary, S., Fey, H., Wandeler, A., Huggler, C., Oppliger, G., Baumberger, H., Kaderli, R., Martig, J. (1980). "immune responsiveness in cattle fatally affected by bovine virus diarrhea-mucosal disease." Zetralbl Veterinarmed B27: 429-445.

Stenken, J.A., Poschenrieder, A.J. (2015). "Bioanalytical chemistry of cytokines-a review." Anal Chim Acta 853: 95-115. doi: 10.1016/j.aca.2014.10.009.

Straub, O.C. (1990). "Infectious bovine rhinotracheitis virus." p. 71-108 in Z. Dinter and B. Morein (ed.), Virus infections of ruminants. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Straub, O.C. (2001). "Advances in BHV (IBR) research." Deutsche Tierarztliche Wochenschrift 108: 419-422.

Strober, W. (2001). Trypan blue exclusion test of cell viability. Current protocols in immunology, A3-B.

Summerfield, A., Auray, G., Ricklin, M. (2015). "Comparative Dendritic Cell Biology of Veterinary Mammals." Annu Rev Anim Biosci 3: 533-57.

Tamura, J.K., Warrener, P., Collett, M.S. (1993). "RNA-stimulated NTPase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus." Virology 193(8382392): 1-10.

Tautz, N., Thiel, H.J., Dubovi, E.J., Meyers, G. (1994). "Pathogenesis of mucosal disease: a cytopathogenic pestivirus generated by an internal deletion." J Virol 68: 3289-3297.

Tautz, N., Elbers, K., Stoll, D., Meyers, G., Thiel, H.J. (1997). "Serine protease of pestiviruses: determination of clevage sites." J Virol 71: 5415-5422.

Taylor, B.C., Choi, K.Y., Schibienski, R.J., Moore, P.F., Scott, J.L. (1993). "Differential expression of bovine MHC class II antigens identified by monoclonal antibodies." J Leukoc Biol 53: 479-489.

Teichmann, U., Liebler-Tenorio, E.M., Pohlenz, J.F. (2000). "Ultrastructural changes in follicles of small-intestinal aggregated lymphoid nodules in early and advanced phases of experimentally induced mucosal disease in calves." Am J Vet Res 61: 174-182.

Thiel, H.-J., Plagmann, P.G.W., et al. (1996). Philadelphia, Lippincott-Raven.

Tikoo, S.K., Campos, M., Babiuk, L.A. (1995). "Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control." Adv Virus Res 45: 191-223.

Tyler, K.L., Nathanson, N. (2001). "Pathogenesis of viral infections." In: Knipe DM, Howley PM, editors. Fields' virology. 4th ed. Volume 1. Philadelphia: Lippincott Williams & Wilkins; p. 199-243.

Vivier, E., Malissen, B. (2005). "Innate and adaptive immunity: specificities and signaling hierarchies revisted." Nat Immunol 6(1): 17-21.

Vogel, F.S., Caron, L., Flores, E.F., et al. (2003). "Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves." J Clin Microbiol 41: 4512-4520.

Waldman, W.J., Williams, M.V., Lemeshow, S.A., Binkley, P., Guttridge, D., Kiecolt-Glaser, J.K., et al. (2008). "Epstein-Barr virus-encoded dUTPase enhances proinflammatory cytokine production by macrophages in contact with endothelial cells: evidence for depression-induced atherosclerotic risk. Brain Behav Immun 22: 215-223. doi: 10.1016/j.bbi.2007.07.007.

Warrener, P., Collett, M.S. (1995). "Pestivirus NS3 (p80) protein processes RNA helicase activity." J Virol 69: 1720-1726.

Westenbrink, F., Staver, P., Kimman, T., De Leeuw, P. (1989). "Development of a neutralizing antibody response to an inoculated cytopathic strain of bovine virus diarrhoea virus." Vet Rec 125(10): 262-265.

Whitbeck, J.C., Knapp, A.C., Enquist, L.W., Lawrence, W.C., Bello, L.J. (1996). "Synthesis, processing and oligomerization of ovine herpes virus 1 gE and gI membrane proteins." J Virol 70: 7878-7884.

Winkler, M.T.C., Doster, A., Jones, C. (2000). "Persistence and reactivation of bovine herpesvirus 1 in the tonsil of latently infected calves." J Virol 74: 5337-5346.

Wiskerchen, M., Collett, M.S. (1991). "Pestivirus gene expression: protein p80 of bovine viral diarrhea virus is a proteinase involved in polyprotein processing." Virology 184: 341-350.

Xu, J., Mendez, E., Caron, P.R., Lin, C., Murcko, M.A., Collett M.S., Rice C.M. (1997). "Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication." J Virol 71: 5312-5322.

Zhong, W., Gutshall, L.L., Del Vecchio, A.M. (1998). "Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus." J Virol 72(11): 9365-9369.