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Understanding the Effect of BVDV on Innate Immune Response of Neutrophils

Neelu Singh Thakur

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UNDERSTANDING THE EFFECT OF BVDV ON INNATE IMMUNE RESPONSE
OF NEUTROPHILS

BY
NEELU SINGH THAKUR

A thesis submitted in partial fulfillment of the requirements for
Master of Science
Major in Biological Sciences
South Dakota State University
2017
UNDERSTANDING THE EFFECT OF BVDV ON ANTIGEN PRESENTING CELLS
AND CYTOPLASMIC TRAFFICKING

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

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Abstract

Introduction

Materials and Methods

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Chapter 4: General Discussion

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LIST OF ABBREVIATIONS

BBMM: Bone marrow-derived macrophages
BMMCs: Bone marrow mononuclear cells
BRDC: Bovine respiratory disease complex
BVDV: Bovine Viral Diarrhea Viruses
C5a: Component 5a
CD: Cluster of differentiation
CO2: Carbon dioxide
Cp: Cytopathic
CPE: Cytopathogenic effect
DAPI: 4,6-diamidino-2-phenylindole
°C: Degree centigrade
DTH: Delayed type hypersensitivity
DNA: Deoxyribonucleic acid
DHR 123: Dihydrorhodamine 123
ER: Endoplasmic reticulum
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal Bovine Serum
FITC: Fluorescein isothiocyanate
Gp: Glycoprotein
HCV: Hepatitis C virus
hr: Hour
IL: Interleukin
IL-1: Interleukin-1
IL-2: Interleukin-2
IP-10: Interferon inducible protein 10
IFN: Interferon
IACUC: Institutional Animal Care and Use Committees
LDH: Lactate dehydrogenase
LTB4: Leukotriene B4
LPS: Lipopolysaccharide
LDL: Low-density lipoprotein
MDBK: Madin Darby bovine kidney cells
MIP-1α: Macrophage inflammatory protein
MFI: Mean fluorescent intensity
μg: Microgram
μl: Microliter
μm: Micrometer
MEM: Minimal essential medium
min: Minute
ml: Milliliter
MLV: Modified live vaccine
MDM: Monocyte-derived macrophages
MD: Mucosal disease
MOI:Multiplicity of infection
MPO: Myeloperoxidase
fMLP: N-formylmethionyl-leucyl-phenylalanine

ng: Nanogram

nm: Nanometer

nM: Nanomolar

NET: Neutrophil extracellular traps

NCP: Noncytopathic

NS: Nonstructural protein

NS23: Non-structural 23

NOD: Nucleotide-binding oligomerization domain-like receptors

C: nucleocapsid protein

OD: Optical density

PFA: Paraformaldehyde

PAMP: Pathogen associated molecular pattern

PRR: Pathogen recognition receptors

%: Percentage

PBMC: Peripheral blood mononuclear cells

PI: Persistent infection

PBS: Phosphate buffered saline

PMA: Phorbol 12-myristate 13-acetate

PAF: Platelet activating factor

p.i: post infection

RIG-I: Retinoic acid-inducible gene-I-like receptors

ROS: Reactive oxygen species
RNA: Ribonucleic acid
RPNI: Roswell Park Memorial Institute (Medium) TNF-α, GM-CSF
TGAC: Tifton Georgia Cytopathic
TGAN: Tifton Georgia Non-cytopathic
TLR: Toll-like receptors
UTR: Untranslated region
UV: Ultraviolet
VEGF: Vascular endothelial growth factor
VMRD: Veterinary Medical Research & Development
WBCs: White blood cells
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ABSTRACT

UNDERSTANDING THE EFFECT OF BVDV ON INNATE IMMUNE RESPONSE THROUGH NEUTROPHILS

NEELU SINGH THAKUR

2017

Bovine viral diarrhea virus (BVDV) is one of the highly prevalent and economically important diseases of cattle industry worldwide. The two major consequences of this disease are persistent infection and immunosuppression. Several studies have been done to determine the underline mechanisms of BVDV-induced immunosuppression targeting antigen presenting cells, adaptive immune system cells and cytokine gene expression. However, very little research has been done to determine the effect of BVDV on neutrophils.

Neutrophils are one of the most abundant while blood cells (WBC) in the peripheral blood, which play a critical role in the innate as well as adaptive immune response. The current study measured the effect of BVDV infection on viability of neutrophils, their surface marker expression and functional abilities including migration/chemoattraction, phagocytosis, reactive oxygen species production (oxidative burst) and neutrophil extracellular trap (NET) formation. These studies revealed that none of BVDV strains affected the viability of neutrophils in vitro. BVDV infection did affect surface marker expression. TGAC and TGAN reduced the expression of CD18 and L-selectin while increasing CD14 expression. All ncp BVDV strains used enhanced neutrophil migration while the cp BVDV strain reduced neutrophil migration as compared to mock-infected control treatment. Among the BVDV strains used in current
study, highly virulent 1373 significantly enhanced neutrophil migration. The
enhancement in neutrophil migration by 1373 was approximately 55% higher as
compared to LPS-treated positive control macrophages.

BVDV infection significantly enhanced neutrophil phagocytosis activity for 0.2
µm microsphere beads as compared to mock infection. Neutrophil phagocytic activity for
rhodamine-labeled *E. coli* was reduced by BVDV infection as compared to LPS-control.
TGAC, TGAN, 1373 or 28508 had 23%, 6%, 19% or 12% less phagocytic activity
respectively as compared to mock-infected rhodamine-labeled *E. coli*-treated neutrophils.
All BVDV strains used in the current study also reduced oxidative burst by
approximately 50% as compared to positive control (p<0.05). In contrast, the four strains
increased the neutrophil NET formation.

The current study revealed that BVDV infection modulates neutrophil activity in
a strain dependent manner. This effect may result in different disease outcomes, e.g.
enhanced neutrophil migration by highly virulent 1373 may be the reason for severe
neutropenia and hemorrhagic lesions in *in vivo* infection. In addition to pathogenesis,
进一步 studies need to be done to determine the role of neutrophils in shaping adaptive
immune system following BVDV infection.
INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the major devastating problems in the cattle industry. BVDV infections produce variable and complicated symptoms including reproductive disorders, abortion, growth retardation, diarrhea, and lethal mucosal disease. The greatest impact is likely from immunosuppression, which makes animals more susceptible to a vast variety of other pathogens including bacteria and viruses. Several studies have been conducted to evaluate the underline mechanisms of BVDV-induced immunosuppression and have targeted mainly adaptive immune cells.

The current study was designed to investigate the effect of BVDV on a key cell of the innate immune system, the neutrophil. Neutrophils are the first line of defense in the innate immune system along with macrophages and represent the largest population of circulating white blood cells (WBCs). Neutrophils not only phagocytize and destroy the invading pathogen but also secrete a wide variety of cytokines and chemical mediators that help in shaping the adaptive immune response. Viral infection to neutrophils may significantly affect their number (quantity) and functional activity. This can lead to sub-optimal innate immune defense as well as reduced stimulation to generate an effective adaptive immune response.
To address these questions, the major objective of current study was to investigate the effect of different strains of BVDV on neutrophils. Our hypotheses were:

1) BVDV infection to neutrophils significantly affects neutrophil viability in a strain dependent manner; 2) BVDV infection changes the phenotypic characteristics of neutrophils; 3) BVDV infection alters the functional activity of neutrophils leading to immunomodulation.

RESEARCH OBJECTIVES

1) To isolate and culture bovine neutrophils as an *in vitro* model to determine effect of BVDV on innate immune system. With this objective, three approaches were taken:
   
   a) Optimize methods for neutrophil isolation from bovine peripheral blood to achieve high yields and viability.
   
   b) Optimize morphological and phenotypic characterization of the neutrophils.
   
   c) Optimize culture conditions for the neutrophils.

2) Investigate the effect of BVDV on neutrophil viability and phenotype. The approaches for this objective were:

   a) Study the effect of different biotypes and virulent strains of BVDV on neutrophils viability as well as surface marker expression (CD14, CD18 and L-selectin).

3) Investigate the effect of different strains of BVDV on neutrophil functional activity. Under this objective, four major functions of neutrophils were investigated:

   a) Phagocytic activity
   
   b) Chemotaxic ability
   
   c) Oxidative burst
   
   d) Neutrophil extracellular trap formation
LITERATURE REVIEW

1.1 BVDV disease:
Bovine viral diarrhea virus (BVDV) is a major problem for the cattle industry worldwide. BVDV infections cause multiple forms of disease ranging from inapparent to lethal mucosal disease (MD) (Chase et al. 2015). Its ability to establish lifelong persistent infection in animals, rank this disease as the most insidious and devastating viral pathogen of the cattle industry (Brackenbury et al. 2003, Chase et al. 2004, Chase 2013, Chase et al. 2015).

Despite 60 years of vaccination, BVDV infections remain a source of significant economic loss for producers in the United States (Van Campen 2010, Ridpath 2012) and the world (Houe 1999, Mockeliuniene et al. 2004). The economic importance of BVDV increased with emergence of more virulent strains during early 1980s and 1990s (Goens 2002). A study conducted in 1999, estimated that farmers lose about US$20 million per million calvings when animals were infected with a low-virulence BVDV strain, however it increased to US$57 million per million calvings with a highly-virulent BVDV strain (Houe 1999). The recent appearance of a new putative pestivirus species, tentatively called "HoBi-like" or "BVDV-3" or "atypical pestiviruses," which was first identified in Europe in a fetal bovine serum (FBS) imported from Brazil (Bauermann et al. 2013), may further increase these losses.

BVDV infections produce variable and complicated symptoms, that vary from growth retardation, persistent infection, hemorrhagic symptoms, respiratory and enteric infections, reproductive disease to lethal mucosal disease (MD) (Chase et al. 2015). On the basis of severity and duration, BVDV infection can be divided into various forms:
inapparent infections, acute disease, *in utero* or congenital infection, mucosal disease and chronic disease.

The inapparent form of disease is characterized by a slow rise in antibody levels over the first 10-12 weeks after infection and an inability to recover virus. The failure to isolate virus from either nasal swabs or blood and the slow development of detectable antibody in serum may be due to antibody is still developing and in an undetectable range and the virus may be sequestered in lymphoid tissues (Ohmann 1983).

Acute infection is most commonly caused by the noncytopathic (ncp) strain of BVDV (Lanyon et al. 2014), characterized by reduced general health condition, respiratory distress, increased body temperature, sporadic coughing, nasal discharge and elevated body temperature (Muller-Doblies, Arquint et al. 2004). Viremia can be observed as early as 2 days post infection and usually peaks at 7 days post infection (Smirnova et al. 2008), with the highest body temperature reaching values above 39°C. The rise in body temperature is generally biphasic, with one peak at day 4 and a second peak at day 8. These clinical signs last from 3 to 15 days with a full recovery by the end of three weeks (Muller-Doblies, et al. 2004). An acute infection has different outcomes depending upon physiological status of the infected animals (Figure 1.1).
**Figure 1.1. Clinical forms of BVDV.** BVDV can cause a spectrum of diseases depending on the virulence of the strain and the immune state of the host. Adapted from Elmowalid, G (2003). *Unmasking the effect of bovine viral diarrhea virus on macrophage inflammation.* Ph.D. Thesis. South Dakota State University, Brookings, SD 57007, U.S.A (Pt): 73

BVDV infection of pregnant animal results in a placentome infection, leading to an *in utero* or congenital infection. The *in utero* or congenital infection early in pregnancy generally results in early embryonic death and infertility (Van Oirschot 1983). BVDV infection of pregnant cows significantly reduced conception rates (Virakul et al. 1988, McGowan et al. 1993) and resulted in death, abortion or mummification of the fetus (Kendrick 1971, Sprecher et al. 1991, Barr and Anderson 1993). Fetal death during that period may be due to extensive damage of placenta that interferes with the oxygen and nutrient supply to fetus (Murray 1991). However, experimental infections with the cytopathic (cp) strain at a similar stage of pregnancy did not affect conception or fetal viability, indicating the importance of different biotypes in disease outcomes (Brownlie et al. 1989).
In addition to death, abortion or mummification of the fetus, BVDV infection to pregnant cows may result in birth of anatomically abnormal calves. Congenital defects result when BVDV establishes a stable infection during early organogenesis in the developing fetus (Van Oirschot 1983, Karakaya et al. 2013, Brown et al. 1973, Done et al. 1980).

The most important fetal infection outcome is persistent infection (PI). PI generally occurs in calves, which are infected with ncp BVDV during the first 40-120 days of pregnancy. This occurs because the fetal immune system is not fully developed and cannot distinguish self and non-self-antigen (Brackenbury et al. 2003, Chase et al. 2004, Chase et al. 2015).

In PI, the BVDV infected animals immune system treat BVDV as self and are tolerazied. This results in the lack of a BVDV immune response including neutralizing antibody. PI animals may look apparently health but they serve as a continuous source of BVDV infection to other animals. In these PI animals, any stress may cause mutations in the ncp BVDV strain to become a homologous cp strain (Tautz et al. 1998, Darweesh et al. 2015) and the superinfection of antigenically homologous ncp and cp BVDV strain results in fatal mucosal disease (Brownlie et al. 1984, Sentsui et al. 2001, Kane et al. 2015). Mucosal disease is characterized by high mortality and extensive lesions in the gastrointestinal tract (Huck 1957).

1.2 Bovine Viral Diarrhea Virus:

Bovine viral diarrhea virus (BVDV) is a single-stranded, positive-sense RNA virus belonging to the Pestivirus genus and the family Flaviviridae (Ostachuk 2016). The virus shares similarities to other genera of Flaviviridae, which includes viruses of human
importance including hepatitis C virus, yellow fever virus, Dengue fever virus, Japanese encephalitis virus, West Nile virus, St. Louis encephalitis virus and the recently reemerging Zika virus (Burd and Griffin 2016). The Pestivus genus also contains two other viruses of animal importance: classic swine fever virus and border disease virus in sheep (Nettleton et al. 1998).

BVDV has a genome of approximately 12.5 kb that encodes a single open reading frame. This single open reading frame encodes a polyprotein of about 4000 amino acids. This polyprotein is then cleaved into 11-12 individual viral proteins either by host cell and/or viral proteases (Tautz et al. 1996, Xie et al. 2014) to make the complete virion. (Figure 1.2).

![Figure 1.2. The schematic representation of the BVDV encoded proteins. BVDV is a single-stranded RNA virus comprised of 12,300 nucleotides. BVDV consists of a single open reading frame between two untranslated regions (5’ and 3’). The polyprotein is autoprocessed into both structural and non-structural proteins. In cytopathic biotype viruses, NS23 protein cleaves into NS2 and NS3 proteins (CP-lower panel) while in noncytopathic biotype viruses, NS23 does not cleave into two proteins (NCP upper panel). from: Morarie, S.E. (2012). “Unraveling the biology of bovine viral diarrhea virus (BVDV) persistent infections: integrating field and laboratory studies.” Ph.D. Thesis. South Dakota State University, Brookings, SD 57007, U.S.A (Pt): 9.](image-url)
BVDV strains can be classified in two ways: 1) comparison of sequences from the 5' untranslated region (UTR) of the viral genome, which divide BVDV into two genotypes Type 1 (BVDV1) or Type 2 (BVDV2) and subgenotypes, 1a, 2a, etc. (Ridpath et al. 1994) and 2) their cytopathogenicity in cell culture—where BVDV is divided into two biotypes; cytopathic (cp) or non-cytopathic (ncp) BVDV (Weiss et al. 1994, Deregt and Loewen 1995).

The phylogenetic analyses of BVDV isolates using the least conserved portion of the BVDV E2 glycoprotein gene (sequence of 420 nucleotides) also can be used to identify the two genotypes (BVDV1 and BVDV2) classification based on 5' UTR sequence (Tajima and Dubovi 2005). At the time of this writing, the BVDV1 genotype has at least 15 subtypes (1a-1o) with complete genomic sequences of subtype BVDV-1a, 1b, 1d, 1e, 1k and 1m while BVDV2 genotype has only two subtypes, BVDV2a and 2b (Xie et al. 2014).

The cp biotypes of BVDV are generated by mutation with a few nucleotides inserted in their homologous ncp biotypes, that results in nonstructural protein NS23 being cleaved into NS2 and NS3 (Tautz et al. 1996, Balint et al. 2005, Darweesh, et al. 2015) (Figure 1.2)

1.3 Structure of BVDV virion and its replication:

BVDV is a relatively small oval to pleomorphic enveloped viral particle, 40-60 nm in diameter, with numerous projecting knobs, 4 to 5 nm in diameter in its envelope (Chu and Zee 1984, Ohmann 1990). BVDV genome (12.5 kb) is packed with C (capsid) protein and finally by envelope glycoproteins Erns, E1 and E2. E2 (Wang et al. 2014).
The binding of glycoproteins with various cell surface receptors including cluster of differentiation 46 (CD46), heparan sulfate, glycosaminoglycans and/or the low-density lipoprotein (LDL) receptor help virus attachment to host cell (Iqbal et al. 2000, Krey et al. 2006, Krey et al. 2006) followed by clathrin-dependent endocytosis (Krey et al. 2006). The low pH of the endosomes in the cytoplasm induces fusion of viral and endosomal membrane, causing release of the genome.

Viral RNA replicates and is translated into structural and non-structural proteins. The virions assemble in association with the endoplasmic membrane (Jordan et al. 2002) and autophagosomes (Ohmann 1990, Fu et al. 2014, Rajput, 2013) and are subsequently released either by cell lyses and/or via exocytosis. (Figure 1.3)

**Figure 1.3. BVDV replication cycle.** BVDV virion binds to specific cellular receptors: cluster of differentiation 46 (CD46), heparan sulfate, glycosaminoglycans and/or the low-density lipoprotein (LDL) receptor through their envelope proteins. Following attachment, BVDV virion are internalized through receptor-mediated endocytosis. The virion replicates in the cytoplasm and assembles in the endoplasmic reticulum. Mature BVDV virions are released through virion-containing vesicles that fusion to the host cell plasma membrane. Adopted from Lindenbach and Rice, 2001. Flaviviridae: the viruses and their replication, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. (Eds.), Fields Virology, 4th ed., Vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 991–1041.
During replication, the BVDV polyprotein is cleaved into 11-12 individual viral proteins: Npro, capsid/nucleocapsid (C), Erns, envelop 1 (E1), E2, P7, non-structural 23 (NS23) [NS2 and NS3 in cp BVDV], NS4A, NS4B, NS5A and NS5B, respectively by host cell and viral proteases. The processing of the polyprotein starts with cleavage of nucleocapsid protein (C) by Npro, which contains autoprotease activity (Rumenapf et al. 1993, Stark et al. 1993).

This event is followed by cleavage of Erns12 protein at the C terminus of E2 protein separated from Erns12, resulting Erns1 and E2 proteins. After E2 (gp53) is released from the precursor, E01 is processed into Erns (gp48), E1 (gp25) by endoplasmic reticulum (ER)-resident host cell proteases (Rumenapf et al. 1993, Bintintan and Meyers, 2010).

A protease located in the N-terminal region of nonstructural (NS) protein, NS3, catalyzes the cleavages and release of NS4A, NS4B, NS5A, and NS5B (Tautz et al. 2000). Each processed protein in BVDV has its unique role in virus replication. The C protein play an important role in genomic RNA packaging (Reimann et al. 2007) and provides structure for the virion envelope. The Erns, E1, and E2 have important roles in virus binding and cell entry as well as for immunologic recognition by the host.

The E2 protein contains the major recognition sites for BVDV neutralizing antibodies. The neutralizing epitopes of E2 are important targets for BVDV vaccine efficacy (Donofrio et al. 2006, Chimeno Zoth et al. 2007). Unlike E1 and E2, Erns is dispensable for cellular entry (Iqbal et al. 2004, Ronecker et al. 2008). The E1 protein is
predicted to have various functions including a membrane anchor for E2. The E1 and E2 form E1-E2 heterodimers.

The E1-E2 heterodimers appear to be essential for cell entry of BVDV (Ronecker et al. 2008). Among the BVDV nonstructural proteins, the Npro (p20) is the first protein produced from the open reading frame. It has papain-like protease activities (Rumenapf et al. 1998), which initiates BVDV polyprotein processing (Stark et al. 1993). NS23 (p125) mediates multiple functions including zinc-finger, a protease, and a helicase (Wiskerchen and Collett 1991, Warrener and Collett 1995, Xu et al. 1997). However, for full serine protease activity, NS3 requires NS4A (p10) as a cofactor (Lattwein et al. 2012). The cleavage of NS23 protein in NS2 (p54) and NS3 (p80) results in cytopathic BVDV strains (Balint et al. 2005, Darweesh et al. 2015). Protease activity of NS3 cleaves NS4A, NS4B (p32), NS5A (p58) and NS5B (p75).

The NS4B protein plays an important role in the BVDV replication complex (Weiskircher et al. 2009), while NS5A contains an essential zinc-binding site (Tellinghuisen et al. 2006). The NS5B (p75) acts as RNA-dependent-RNA polymerase and is needed to replicate the viral genome (Zhong et al. 1998).

1.4 BVDV-induced Immunosuppression:

BVDV infection is an immunosuppressive infection. It affects both the adaptive as well as the innate immune system (Chase 2013). BVDV infection reduced the number and functional ability of both granulocytes as well as monocytes (Brewoo et al. 2007). An acute BVDV infection or vaccination with modified live virus vaccine (MLV) of BVDV resulted in decreased delayed type hypersensitivity (DTH) to Mycobacterium avium
subspecies (Thoen and Waite 1990). Similarly, animals with an established immune response to attenuated
*Mycobacterium bovis* (BCG) had a transient immune suppression following acute ncp BVDV infection (Charleston et al. 2001). Immunosuppression caused by BVDV facilitated secondary infections from bovine respiratory syncytial virus, parainfluenza virus, reovirus, bovine adenovirus and bovine herpesvirus-1 infection (Richer et al. 1988, Risalde et al. 2011).

BVDV is an important part of the bovine respiratory disease complex (BRDC) (Hay et al. 2016). BRDC is one of the most economically significant disease of the cattle feedlot industry (Larson 2015), which further enhances the economic impact of BVDV in a country’s agricultural economy

1.4.1 Effect of BVDV on Innate immune response:

The pattern recognition receptors (PRR) including Toll-like receptors (TLR), retinoic acid-inducible gene-I-like receptors (RIG-I-like receptor) and nucleotide-binding oligomerization domain-like receptors (NOD like receptors) play an important role in innate immune system. These receptors activate the innate immune system and shapes an effective adaptive immune response (Kawai and Akira 2009, Kumar et al. 2011).

The interaction of TLR with their respective ligands leads to downstream pathway changes to cytokines and chemokines (Schaefer et al. 2004). Both cp and ncp BVDV altered the TLR3, TLR7, TLR8 and TLR9 expression (Lee et al. 2008). Both biotypes upregulated TLR7 expression while, only ncp BVDV enhanced the expression of TLR3, while TLR3 was down regulated by cp BVDV. In addition, both these laboratory biotypes suppressed pro-inflammatory cytokines, TNF-alpha, IL-1beta, IL-6 and co-
stimulatory molecules, CD80 and CD86, following infection (Lee et al. 2008). More virulent NCP strains enhance pro-inflammatory cytokines (Chase personal communication). Ncp BVDV did not induce type 1 interferons in vitro (Diderholm and Dinter 1966). However, a strong type 1 interferon response was observed in an in vivo experiment with similar ncp strain (Charleston et al. 2002), indicating that BVDV biotypes behave differently in different environments.

Ncp BVDV infection reduced IFNγ and IL-12 production (Risalde et al. 2011). IL-12 acts as a growth factor for NK cells and cytotoxic T cells (Trinchieri 1995), indicating that BVDV infection can indirectly affect NK cells and T cells activity through IL-12. In addition to cytokines, various antimicrobial peptides and superoxides also modulate the innate immune response (Ganz 2003, Break et al. 2012). Both cp TGAC and ncp TGAN BVDV reduced phorbol-12-myristate-13 acetate (PMA)-induced superoxide production following infection, while only ncp BVDV primed bone marrow-derived macrophages (BBMM) enhanced reactive nitrogen production in response to Salmonella dublin (Adler et al. 1994). Similarly, only BVDV2, not BVDV1 inhibited the LPS-induced upregulation of tracheal antimicrobial peptide (TAP) mRNA (Al-Haddawi et al. 2007). Another study showed that BVDV (unidentified strain) inhibited phytohemagglutinin- (PHA), PHA plus phorbol-12-myristate-13 acetate- (PMA) or PHA plus calcium ionophore (A23187)-stimulated bovine peripheral blood mononuclear cell (PBMC) proliferation. Further, BVDV inhibited A23187stimulated leukotriene B4 (LTB4) synthesis in the culture supernatants (Atluru et al. 1992).

1.4.2 BVDV, neutrophils and immune response:
Neutrophils are one of the predominant white blood cells in circulating blood and are considered the first line of defense in the innate immune system (Kobayashi and DeLeo 2009) along with macrophages. Neutrophils play an important role against invading bacteria and, initiate innate as well as adaptive immune responses. Neutrophils activate innate immune response through various mediators including interleukin-8 (IL-8), platelet activating factor (PAF), leukotriene B4 (LTB4) or complement fragment 5a (C5a) (Guo et al. 2003, Mitchell et al. 2003, Mantovani et al. 2011, Mitchell et al. 2014). The ability of neutrophils to migrate and destroy the invading microorganism depends upon their expression of surface markers including cluster of differentiation (CD) -14 (CD14), CD-18 and L-selectin (Yoshitake et al. 2002) Virus affecting the neutrophil surface markers can significantly affect the host immune defense mechanism.

Normally, neutrophils are present in circulation in a resting state, which ensures that their oxidative intracellular contents are not released to damage the host tissue (Wright et al. 2010). During acute inflammation, neutrophils become activated and kill the invading organism (mainly bacteria) and help in shaping adaptive immune response (Kasama et al. 2005, Jaillon et al. 2013). Neutrophils activate the adaptive immune system through pro-inflammatory cytokines and chemokines including tumor necrosis factor alpha (TNF α), interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), interferon inducible protein 10 (IP-10) and macrophage inflammatory protein (MIP)-1α (Altstaedt et al. 1996, Kasama et al. 2005).

Neutrophils become activated via a two-stage process: 1) priming and 2) mobilization. Priming occurs by exposure to invading bacterial products or the cytokines/chemokines, TNF-α, GM-CSF, IL-8, IFN-γ, and /or MIP-1 (Hallett and Lloyds
1995, Mercer-Jones et al. 1999). This rapid priming occurs within minutes after receiving the signals. The primed neutrophils have an extended life span. Activated neutrophils are mobilized to the site of infection by chemoattractants $N$-formylmethionyl-leucyl-phenylalanine (fMLP) or complement component 5a (C5a). These chemoattractants induce cellular polarization to make actin-rich pseudopodia, which helps in their movement (Servant et al. 2000).

Formation of pseudopodia also helps in phagocytosis and destroying invading bacteria through intracellular phagosome and reactive oxygen species (ROS) (Lee et al. 2003). In addition to these strategies, neutrophils also use neutrophil extracellular traps (NET) to destroy larger pathogens which cannot be phagocytized (Segal 2005, Halverson et al. 2015). The process of NET formation is NETosis. NETosis is distinct from apoptosis and necrosis and defined as the release of nuclear DNA, DNA associated proteins and lactate dehydrogenase (LDH) from an activated neutrophil into the extracellular environment (Kawasaki and Iwamuro 2008, Urban et al. 2009, Brinkmann and Zychlinsky 2012). NET also contains primary, secondary, and tertiary granular components that include neutrophil elastase, cathepsin G, and myeloperoxidase (MPO) (Urban et al. 2009, Brinkmann and Zychlinsky 2012), lactoferrin, and gelatinase (Borregaard 2010). Among all the components, histones proteins are the most abundant component and act as a potent antimicrobial agent (Kawasaki and Iwamuro 2008, Urban et al. 2009).

During NET formation, the nuclear envelope degrades and the mixing of nuclear DNA with cytosolic proteins takes place, which then extrude from the cell to trap the invading pathogen. NET can be induced by the treatment of neutrophils with interleukin-
phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), bacteria, fungi or activated platelets. There are numerous viruses which also induce the NET formation including influenza A virus, HIV-1 virus, myxoma virus, encephalomyocarditis virus and respiratory syncytial virus (Narasaraju et al. 2011, Saitoh et al. 2012, Jenne et al. 2013, Funchal et al. 2015). There is no information on the effect of BVDV on neutrophil NET formation. There are few studies on the effect of BVDV on neutrophils. Most of the information is from histological examination characterizing BVDV lesions or through immunocytochemical/flow cytometric procedures (Bolin and Ridpath 1990) characterizing population dynamics but only a few studies have looked at the effect of BVDV on neutrophil function. BVDV infection significantly reduced the number of circulating neutrophils (Roth et al. 1981, Brown et al. 1991, Ganheim et al. 2005) with reduced degranulation and impaired myeloperoxidase, hydrogen peroxide, halide antibacterial activity (Roth et al. 1981). Similarly, vaccination with BVDV modified live virus vaccine (MLV) significantly reduced circulating neutrophils, suppressed iodination, and antibody-dependent cell-mediated cytotoxicity (ADCC) activity (Roth and Kaeberle 1983). High (HV24515) and low virulent (LV11Q) BVDV infection induced a severe neutropenia after viral inoculation. The number of neutrophils returned to normal but recovery was delayed in calves infected with high virulence virus as compared to low virulence (Keller et al. 2006).

Several studies have been done to study the effect of BVDV on chemokines (Burr et al. 2012) and pro-inflammatory cytokines (Schweizer and Peterhans 2001, Fredericksen et al. 2015). IL-1β, IL-8, IL-15, IL-18, Mx-1, IFN gamma, which recruit or activate neutrophils, have all been studied (Hammond et al. 1995, Leung et al. 2001,
Verri et al. 2007). However, a study to determine the effect of BVDV on neutrophil function with its underline mechanism is needed.

The current study was designed to understand the effect of BVDV infection on neutrophil phenotype as well as functional activities of migration/chemotaxis, phagocytosis, oxidative burst and NETs formation. There are over 200 identified strains of BVDV that are either type 1 or type 2 genotype, or cp or ncp biotypes. The current study used BVDV strains from both genotypes as well as biotypes to study their effect on neutrophils. The BVDV strains used in current study were a virus pair (biotypes) of cp BVDV1b TGAC and ncp BVDV1b TGAN from type 1a genotypes and a highly pathogenic ncp BVDV2a 1373 and moderate pathogenic ncp BVDV2a 28508 type 2a genotypes.
REFERENCES


CHAPTER 2
EFFECT OF BVDV ON VIABILITY OF BOVINE NEUTROPHILS AND ITS CELL
SURFACE MARKERS EXPRESSION

ABSTRACT

Infection with bovine viral diarrhea virus (BVDV) is one of the most important infectious causes of immunosuppressive in ruminants. One of the hallmarks of immunosuppression is reduced cell surface markers expression on antigen presenting cells. However, very little known is about the effect of BVDV infection on neutrophil viability and surface marker expression. In this chapter, the effect of BVDV infection on viability of neutrophil and surface marker expression of cluster of differentiation -14 (CD14), CD-18 and L-selectin were examined. Bovine neutrophils were isolated by gradient centrifugation followed by red blood cells (RBCs) lysis and neutrophil restoration. Isolated neutrophils were confirmed morphologically and phenotypically and, further examined for viability, purity and yield. The neutrophils had characteristic polymorphic nucleus with 99.80±0.1% purity and 98.86±0.90 % viability. Isolated neutrophils had high expression for CD18\(^+\) (99.77±0.13\%) and L-selectin (97.05±2.41\%) and low CD14\(^+\) (11.34±3.89\%) expression. The typical yield was 20.64±1.89 10\(^6\) neutrophils from 50 ml of peripheral blood (e.g. A 10 ml cell suspension with concentration of 2.064±1.89x10\(^6\)/ ml was obtained from 50 ml blood)

To determine the effect of BVDV biotype on neutrophil viability and phenotypes, neutrophils were infected with either Tifton Georgia cytopathic (TGAC) or Tifton Georgia noncytopathic (TGAN) strains recovered from an animal that died of mucosal disease. Neither of the biotypes (TGAC or TGAN) used in the study affected the viability
of neutrophils \textit{in vitro}. Interestingly TGAC and TGAN reduced the expression of CD18 and L-selectin while increasing CD14 expression. This effect on surface marker expression by TGAC or TGAN infection likely reduces neutrophil migration and extravasation.

\textbf{INTRODUCTION}

Bovine viral diarrhea virus (BVDV) is one of the most insidious and devastating viral pathogens of the cattle industry around the world (Ridpath, 2012; Van Campen, 2010). The main hurdle in BVDV control is immunosuppression, which reduces vaccine efficacy and places animals in a greater risk of secondary infections (Hay et al., 2016; Richer et al., 1988; Risalde et al., 2011). Several studies have demonstrated the effect of BVDV on immune cells that influence adaptive immune system including monocytes (Glew et al., 2003), macrophages (Chase et al., 2004), dendritic cells (Glew et al., 2003; Rajput et al., 2014) and lymphocytes (Rypula, 2003). However, very little is known about the effect of BVDV on the granulocytic cells of the innate immune system. Neutrophils are one of the predominant white blood cells and considered a first line of defense in the innate immune system (Kobayashi and DeLeo, 2009). Neutrophils play an important role against invading bacteria and help initiate the adaptive immune response. Neutrophils phagocytize invading microorganisms and activate innate as well as adaptive immune response through various mediators including interleukin-8 (IL-8), platelet activating factor (PAF), leukotriene B$_4$ (LTB$_4$) or complement fragment 5a (C5a) (Guo et al., 2003; Mantovani et al., 2011; Mitchell et al., 2003; Mitchell et al., 2014). However, the functional ability of neutrophils is dependent on its cell surface maker expression of
CD14, CD-18 and L-selectin. CD14 helps in bacterial recognition (McAvoy et al., 2011), while CD18 and L-selectin helps in migration (receptor mediated homing) (Gao and Issekutz, 1996; Gomez and Doerschuk, 2010) as well as in adhesion (von Andrian et al., 1993) respectively. Finally, those surface markers help in activating the neutrophils. Activated neutrophils not only destroy the invading pathogens but also activate the adaptive immune system (Jaillon et al., 2013).

In the current study, we optimized a simple, reproducible method to isolate bovine neutrophils with high purity, yield and viability. Isolated neutrophils were confirmed morphologically as well as phenotypically. Freshly isolated neutrophils were used to measure the effect of BVDV biotypes on viability and surface marker expression (CD14, CD18 and L-selectin).

**MATERIALS AND METHODS**

**Cell and Virus Strains and Virus Propagation**

A homologous pair of ncp and cp type 1b viruses, Tifton GeorgiA Non-cytopathic (ncp BVDV1b TGAN) and Tifton GeorgiA Cytopathic (cp BVDV1b TGAC) were isolated from an animal that died of mucosal disease (Brownlie et al., 1984; Ridpath et al., 1991) were used. Virus stocks of each BVDV strain was prepared in BVDV-free Madin Darby bovine kidney (MDBK) cells. BVDV-free MDBK cells (passages 98-112) were grown in minimal essential medium (MEM, Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% BVDV-free fetal calf serum (FCS) (PPA, Pasching, Austria), penicillin (100 U/ml) and streptomycin (100 μg/ml). A five (5) ml of 5x10⁵ MDBK cells/ml were seeded in T25 flasks using minimal essential medium (MEM, Gibco BRL, Grand Island, NY) supplemented with 10% FBS, penicillin (100 U/ml) and
streptomycin (100μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown to 60-70% confluency and infected with either of each virus. At the time of infection, the media was removed from T25 flasks and 0.75 ml of virus inoculum with a multiplicity of infection [MOI] of one was added to each flask. Virus was adsorbed for 1 hr at 37°C in a humidified CO2 incubator with gentle rotation at every 15 minutes. After one hr incubation, unabsorbed virus was removed and the cells were washed with sterile PBS. After washing, 5 ml MEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml) was added to each flask. The cells were incubated at 37°C in a humidified CO2 incubator for 4-5 days or up to 70-80% cytopathic effect for cp BVDV1b TGAC. After 4-5 days of incubation, cells were frozen at (-80°C for 15 minutes) and thawed in ice for two cycles. The cell debris were pelleted by centrifugation at 3000 rpm for 10 min at 4°C in 15 ml conical tubes. The supernatants, containing virus were carefully collected. Supernatants were titrated for virus concentration and then aliquoted and stored at -80 °C for further use.

The viral titers were determined by serially inoculating 1:10 dilutions of supernatants on MDBK cells as per method described earlier (Reed and Muench, 1938). Briefly MDBK cells were detached from tissue culture flask using 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA). The number of cells was adjusted to 5x10^5 cells/ml. One hundred eighty (180) µl cell suspension was added to each well of 96-well plate. Twenty (20) µl of virus was added to the first row of the plate. The virus was then mixed with MDBK cells and 20 µl of this dilution was added to next row to achieve 10-fold dilutions. The last two rows were treated as negative controls with no virus. The plates were incubated at 37°C in humidified incubator for next 4 days. The plate was examined
every day for cytopathic effect (CPE). The highest dilution showing CPE was used as end
to calculate the proportionate distance (PD). The PD was then used to determine the viral
concentration (TCID50) as per formula as described earlier (Reed and Muench, 1938).

| 1. Proportionate distance (PD) = (\% CPE at dilution above 50\%) – (50\%)/ (\% CPE
| at dilution above 50\%)-(\% CPE at dilution below 50\%) (e.g. 60-50/60-0= 0.166
| 2. Calculation of endpoint just next to 50\% CPE and conversion into – Log (e.g.10^{-6}
dilution would be -6)
| 3. Calculation of TCID_{50}.
| 4. TCID_{50} for 20 \mu l= (PD+ - Log dilution above 50\%) (e.g. 1x10^{6.166})

For ncp BVDV, the same procedures were done except the endpoint for ncp
BVDV was determined by staining the MDBK cells with anti-BVDV antibody (IDEXX
Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti-mouse IgG
(Zymed, Invitrogen Corporation, Frederick, MD, USA), streptavidin-HRP (Invitrogen
Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole)
(Sigma-Aldrich, St. Louis, MO, USA). The endpoint for ncp BVDV was determined by
the presence of red stained cells showing BVDV protein.

**Animals**

Sixteen (16) healthy cattle including Holstein Friesian (n=9) and Brown Swiss calves
(n=7) (8-12 months of age), housed at the Department of Dairy Science Dairy Farm,
South Dakota State University (SDSU), Brookings, SD, USA were used in this study.
The SDSU Institutional Animal Care and Use Committee approved animal handling and
blood collection.

**Neutrophil Isolation and Viability**
To isolate neutrophils, fifty (50) ml of peripheral blood was collected in 10 ml heparinized vacutainer tubes (BD, Franklin Lakes, New Jersey, USA). The neutrophils along with red blood cells (RBCs) were separated by centrifuging the blood at 1,000 X g for 30 minutes at 25°C. The plasma and buffy coat were removed and discarded. The 3-ml cell pellet (neutrophils+ RBCs) was divided among eight (8), 15 mL conical tubes. To each 15-mL conical tube, 10 mL of RBC lysing solution was added. Each tube was gently inverted several times for 10 minutes to lyse the RBCs. After gently inverting the tubes, the tubes were centrifuged at 1,000 X g for 5 minutes at 25°C. Supernatants were discarded and cells pellets were washed 3 times using 10 mL of HBSS (the tubes were centrifuged at 1,000 X g for 5 minutes at 25°C). After each wash, cell pellets from two tubes were combined to one, leaving 4 tubes, 2 tubes and 1 tube at the end of each wash (after three wash, cells from eight tubes were combined to one tube). The final cell pellet was suspended in 10 mL RPMI 1640 medium (MEM, Gibco BRL, Grand Island, NY) supplemented with 10% BVDV-free fetal calf serum (FCS) (PPA, Pasching, Austria), sodium pyruvate, penicillin (100 U/ml) and streptomycin (100μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). Freshly collected neutrophils were examined for purity through morphological examination using 4’,6-diamidino-2-phenylindole (DAPI) as well as hematoxylin and eosin (H & E) staining as described below.

Freshly collected neutrophils were also examined for yield. Neutrophil yield was calculated using trypan blue staining as described above. The total number of neutrophil/mL was measured using a hemocytometer. The calculation was multiplied by diluting factors (eg. 100) and total number of cells calculated using the following formula.
Total neutrophil yield (from 50 mL blood) = Total number of cells calculated through
hemocytometer/mL X dilution factor (eg. 100) X Total volume of cells (eg. 10 mL).

The typical yield from 50 ml of peripheral blood was 20.64±1.89×10⁶ neutrophils (e.g. A 10 ml cell suspension with concentration of 2.064±1.89×10⁶/ml was obtained from 50 ml blood)

Neutrophil viability was examined through trypan blue exclusion assay as well as by apoptosis using Annexin V Apoptosis Detection Kit (eBiosciences, San Diego, CA). The trypan blue exclusion assay was used to determine the cell viability by staining the neutrophils with 0.4% trypan blue stain as described (Strober, 2001). Briefly, in 20 µl freshly isolated neutrophils suspension [after 1:100 dilution in 1x phosphate buffered saline (PBS)] was mixed with 20 µl of 0.4% trypan blue. The cells were incubated for 2 minutes at room temperature and examined under microscope. The non-stained viable cells were counted and cell viability was calculated using the following formula.

\[
\text{Cell viability percentage} = \frac{\text{Number of viable cells (none trypan blue stained cells)}}{\text{Total counted cells}} \times 100
\]

Freshly collected neutrophils were also examined for apoptosis using Annexin V Apoptosis Detection Kit (eBiosciences, San Diego, CA). Briefly, 50 ul neutrophils (a 1.0×10⁶ neutrophils in 50 ul) were suspended in equal volume of 1X Binding Buffer [(BB) provided with the Kit]. One hundred (100ul) of suspended-1X BB neutrophils were then added to each round bottom 96 well plates in triplicates. Five microliters (5 µl) of fluorochrome-conjugated Annexin V (provided with Kit) was added to each well and
the plates were incubated for 10-15 minutes at room temperature in the dark. Cells were washed with 1X 200 µl binding buffer by centrifugation at 200xg for 4 minutes at room temperature and analyzed by flow cytometry using a FACScan (Becton-Dickson, Mountain View, CA).

To determine the effect of BVDV biotypes on neutrophil viability, neutrophils were infected with either cp BVDV1b TGAC or ncp BVDV1n TGAN at a MOI 6 for 0 hr, 1 hr or 6 hr while mock-infected or LPS-treated (10 ng/ml) neutrophils were used as negative or positive controls respectively. Neutrophil viability was examined through trypan blue exclusion assay and Annexin V Apoptosis Detection Kit (eBioscience, San Diego, CA) as described above. For apoptosis, 10µl stauosporine (STS) was used as a positive apoptotic control in respective wells (Belmokhtar et al., 2001).

**Characterization of neutrophils**

Morphological characterization

The freshly collected neutrophils were stained with 4’, 6-diamidino-2-phenylindole (DAPI) (Chazotte, 2011) or H&E stain as per method described earlier (Bleyer et al., 2016). Neutrophils were cytospun at 1000x for 10 minutes at room temperature. After cytopspinning, cells were either stained directly with DAPI stain or fixed with methanol for H&E staining. For H&E staining, fixed cells were immersed in hematoxylin stain for 10 seconds followed by washing with tap water. During washing, slides were immersed in water until slides were clear of stain. The slides were then immersed in eosin dye for 30 seconds followed by washing with tap water. Finally, H&E slides were air dried and examined under compound microscope at 20x (Olympus, PA,
USA) while the DAPI-stained cells were examined under florescent microscope with 358/461nm excitation/emission filter at 20x (Olympus, PA, USA).

**Phenotypic characterization using flow cytometry.**

The phenotypic characterization of freshly collected neutrophils as well as neutrophils after BVDV infection was done by measuring CD14, CD18 or L-selectin surface marker expression. Briefly, freshly isolated neutrophils were collected by gentle pipetting and centrifugation for 5 min at 1500 rpm at room temperature. Cell numbers were adjusted to 1x10⁶/ml in PBS containing 1% FBS. A 100 µl cell suspension were added to each well of round bottom 96-well plates in triplicate. Fifty (50) µl of primary antibodies of anti-CD14 antibody (clone M-M9), VMRD Inc, Pullman, WA.; anti-CD18 antibody (clone BAQ30A; Kingfisher Biotechnology, St Paul, MN) or anti-L selectin antibody (clone FMC46; Novus Biological, Littleton, CO) were added to respective wells. The primary antibodies used in staining were pre-diluted 1:100 in PBS containing 1% FBS. After adding primary antibodies, cells were incubated at 4°C for 10 min followed by 2X washes with 200 µl PBS containing 1% FBS. After washing, cells were stained with FITC-labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA) with 1:1000 dilution in PBS containing 1% FBS at 4°C for 10 min. After secondary antibody straining, cells were washed again for 2X as described above. Finally, stained cells were fixed with 200 µl of 1% paraformaldehyde and analyzed by FACScan (Becton-Dickson, Mountain View, CA).

**Virus infection of neutrophils**

Freshly collected neutrophils were suspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) to
achieve final concentration $1 \times 10^6$ /ml. One (1) ml of the cell suspension was added to each well of the 12-well plates. Cells were infected with either cp TGAC or ncp TGAN strains of BVDV with a multiplicity of infection (MOI) of 6. Mock-infected neutrophils or lipopolysaccharide-treated (LPS; 10 ng/ml, Sigma-Aldrich, St. Louis, MO, USA) neutrophils were used as negative or positive controls respectively. BVDV-, mock-infected or LPS-treated neutrophils were collected at 0 hr, 1 hr or 6 hr, post infection (p.i.) and examined for their viability and cell surface marker expression of CD14, CD18 or L-selectin as described above. These time points were chosen because beyond 6 hr, the short half of neutrophils resulted in high back ground apoptosis in the mock-infected cells so differences could not be measured.

**Statistical analysis**

The significance of BVDV infection on neutrophil viability and cell surface marker expression was calculated by paired t-test (Glantz, 2002).

**RESULTS**

*Neutrophil morphology, viability and yield*

The isolated neutrophils had characteristic polymorphic nucleus (Figure 2.1), with 99.80±0.1% purity. H & E staining of neutrophils showed purple colored multiple lobulated nucleus (polymorphic nucleus) (Figure 2.1A). Similar multiple lobulated nuclei (polymorphic nucleus) were observed following DAPI staining (Figure 2.1B). Neutrophil purity was determined and confirmed through morphological examination, and differential leukocyte count (Chung et al., 2005) by the Veterinary Clinical Pathology Section, Animal Disease Research and Diagnostic Laboratory (ADRDL), South Dakota State University (SDSU), Brookings, SD.
Isolated neutrophil viability was 98.86±0.90% as measured by the trypan blue exclusion assay. The yield of was 20.64±1.89X10^6 neutrophils from 50 mL of peripheral blood.

*Phenotypic characterization of neutrophils*

Freshly isolated neutrophils were stained for CD14, CD18 or L-selectin. Freshly isolated neutrophils were low in CD14^+ (11.34±3.89), and high for CD18^+ (99.77±0.13) and L-selectin (97.05±2.41) (Figure 2.2).

*The effect of BVDV infection on neutrophil viability*

Neutrophil viability did not change during BVDV infection (p<0.05). Neutrophil viability was 99.17± 1.41%, 98.13± 2.65% in unstimulated control cell while LPS-treated neutrophils was 98.25± 1.82% and 93.43± 3.08%, TGAC- and TGAN-infected neutrophil viability was 98.08± 1.26%, 97.33± 2.08% and 99.25±1.27%, 97.10± 1.09% at 1hr or 6 hr post infection respectively, which were not significantly different from the unstimulated control cells (p<0.05) (Figure 2.3, Table 2.1).

The effect of BVDV infection on neutrophil apoptosis was determined. TGAN induced significant apoptosis at 1 hr post infection as compared to unstimulated control neutrophils (p<0.05). At 1 and 6 hr post infection, TGAN induced around 7% and 1% higher apoptosis as compared to time point unstimulated control neutrophils respectively (Figure 2.4, Table 2.2). TGAC induced approximately 3% and 4% more apoptosis at 1 and 6 hr post infection as compared to unstimulated control neutrophils respectively (Figure 2.4, Table 2.2). LPS treatment did not induce any apoptosis in neutrophils at 1 hr post infection while it caused significant apoptosis (around 6% higher as compared to time point unstimulated control neutrophils) at 6 hr post infection (p<0.05) (Figure 2.4,
Table 2.2). However, the staurosporine (STS) (positive control) induced around 13% and 11% apoptosis in neutrophils at 1 hr and 6 hr post treatment, which were significantly higher as compared to the unstimulated control neutrophils (p<0.05)(Figure 2.4, Table 2.2).

**Effect of BVDV on neutrophil surface marker expression**

BVDV infection with either TGAC or TGAN had no effect on neutrophil CD14 expression at 1 hr p.i. In contrast at 6 hr, CD14 expression increased approximately 12% and 27% following TGAC or TGAN infection as compared to their unstimulated time point control neutrophils (Table 2.3, Figure 2.5). The positive control, LPS, a well-known inducer of CD14, significantly enhanced neutrophil CD14 expression at 1 hr as well as 6 hr post treatment (p<0.05). This was approximately 200% and 300% higher at 1 hr and 6 hr post treatment respectively as compared to its time point control (Table 2.3, Figure 2.5).

Both TGAC and TGAN infection significantly reduced CD18 expression at 1 hr post infection, which was approximately 17% and 13% less than their unstimulated time point control neutrophils respectively (p<0.05) (Table 2.3, Figure 2.6). At 6 hr p.i. TGAN further reduced CD18 expression. The reduction in CD18 expression at 6 hr p.i. by TGAN was significant and was approximately 24% less as compared to unstimulated time point control neutrophils (p<0.05) (Table 2.3, Figure 2.6). However, homologous TGAC increased CD18 expression at 6 hr p.i. by approximately 8% as compared to unstimulated control neutrophils. Neutrophils treated with LPS increased CD18 expression by 2% and 15% higher at 1hr and 6 hr p.i. respectively as compared to unstimulated control neutrophils (Table 2.3, Figure 2.6).
LPS treatment also reduced the L-selectin expression by 4% and 29% at 1 and 6 hr p.i. respectively, as compared to their time point control (Table 2.3, Figure 2.7). The reduction in L-selectin expression by LPS at 6 hr p.i. was significant as compared to their time point control (p<0.05)(Table 2.3, Figure 2.7).

DISCUSSION

The current study was conducted to standardize neutrophil isolation protocol with high yield, viability and purity. The effect of BVDV biotypes on neutrophil viability and phenotypic characteristics was measured. The optimized centrifugation, RBCs lysis and neutrophil restoration method in current study yielded 20.64±1.89 10^6 neutrophils from 50 ml peripheral blood. Isolated neutrophils were 99.80±0.1% pure with 98.86±0.90 % viability. Freshly isolated neutrophils had high expression of CD18^+ (99.77±0.13%), L-selectin (97.05±2.41%), and low expression of CD14^+ (11.34±3.89%) markers.

Neither biotypes (eg. TGAC or TGAN) had any effect on neutrophil viability (p<0.05). A previous in vivo study showed that BVDV infection cause neutropenia in vivo (Roth et al., 1981) but they did not measure the effect BVDV on neutrophil viability. Such changes in neutrophil population could be due to reduced neutrophil production, margination or other hemodynamic mechanisms. However, the current study showed that BVDV infection significantly reduced CD18 and L-selectin expression on neutrophils. Both surface makers (e.g. CD14 or L-selectin) are needed for neutrophil margination and extravasation (Ley et al., 1995, Simon et al., 1995, Walzog et al., 1999, Kolaczkowska and Kubes, 2013). Additionally, CD18 helped in inducing neutrophil maturation and release to the circulation from bone marrow (Gomez and Doerschuk, 2010). The crosslinking of L-selectin to its ligands upregulated CD18 expression (Green et al.,
Thus, reduced expression of L-selectin and CD18 may result in neutropenia due to reduced neutrophil production from the bone marrow.

In addition to surface marker expression, BVDV could affect neutrophil production through direct effect on hematopoietic cells. Studies with the highly virulent ncp BVDV2-890 and comparatively less virulent TGAN revealed that highly virulent ncp BVDV2-890 has higher association with lymphocytes and platelets as compared to the less virulent TGAN. The higher ability to infect and replicate in lymphocytes and in platelets by ncp BVDV2-890 may be a reason for lymphopenia, neutropenia and thrombocytopenia caused by ncp BVDV2-890 as compared to TGAN (Bolin and Ridpath, 1992).

Similarly, an in vivo study with high virulent ncp BVDV2-HV24515 and low virulent ncp cp BVDV2-LV11Q revealed that both viruses cause neutropenia. However, the number of neutrophils rebounded earlier in low virulent ncp cp BVDV2-LV11Q infected animals as compared to high virulent ncp BVDV2-HV24515 infected animals. Rebound of neutrophil numbers was associated with bone marrow mononuclear cell (BMMCs) proliferation, which were isolated from each group. Results of that study suggested that BVDV induced neutropenia by affecting proliferative capacity of bone marrow progenitor cells (Keller et al., 2006).

Another virus of the Flaviviridae family, dengue virus type 4, infected immature human bone marrow progenitor cells resulting in bone marrow failure and neutropenia (Nakao et al., 1989). Similarly, hepatitis C virus (HCV), another member of the Flaviviridae family, infected pluripotent hematopoietic CD34+ cells (Sansonno et al., 1998), suggesting that HCV cause neutropenia (Sheehan et al., 2014) this neutropenia...
could be the results of affecting pluripotent hematopoietic CD34+ cell (Sansonno et al., 1998) or through direct neutrophil apoptosis through caspases 3 and 10 (Aref et al., 2011), or by both.

In another in vivo study, neutrophils isolated from a BVDV persistently infected cattle had impaired neutrophil activity (Brown et al., 1991). However, this study in vivo which was a complete different environment then the current in vitro study. Additionally, that study did not measured the effect of BVDV on neutrophil surface marker expression, which helps determines the neutrophil functional ability.

The current study measured the effect of two different BVDV biotypes, TGAC or TGAN, on neutrophil cell surface marker expressions. Both, TGAC or TGAN increased CD14 expression approximately 12% and 27% at 6 hr p.i. respectively, as compared to their unstimulated time point control neutrophils. LPS that was used as positive control in assay significantly enhanced neutrophil CD14 expression at 1 hr as well as 6 hr post treatment (p<0.05). Upregulation of CD14 expression by LPS-positive control was approximately 200% and 300% higher at 1 hr and 6 hr post treatment respectively as compared to its time point control. (Jersmann et al., 1998; Landmann et al., 1996).

The current study also revealed that TGAN infection significantly reduced CD18 expression (p<0.05) while TGAC slightly enhanced the expression. Reduction in CD18 expression by TGAN was about 24% while increment in CD18 expression by TGAC was about 8% at 6 hr p.i. as compared to their time point control neutrophil. However, both TGAC and TGAN significantly reduced L-selectin expression at 6 hr p.i. as compared to their time point control neutrophil (p<0.05). The reduction in L-selectin at 6 hr p.i. by both biotypes was approximately 11% as compared to their time point control.
neutrophils. Downregulation of CD18 and L-selectin on neutrophils following BVDV infection indicated that BVDV infection could significantly affect neutrophil functional ability (Yoshitake et al., 2002). CD18 plays an important role in neutrophil maturation and its release into the circulation from bone marrow (Gomez and Doerschuk, 2010) as well as neutrophil extravasation from blood to tissues (Walzog et al., 1999).

Similarly, L-selectin has adhesive properties to endothelium, which helped in extravasation of neutrophils from blood to tissues (Smolen et al., 2000). Additionally, crosslinking of L-selectin to its ligands was required for phosphorylation of mitogen-activated protein kinases (MAPKs) leading activation of CD18 (Green et al., 2003). Thus, downregulation of L-selectin could further reduce the CD18 expression on neutrophils. Therefore, reduced L-selectin and CD18 could lead to reduced neutrophil production resulting neutropenia. This neutropenia and impaired neutrophil function was reported earlier in in vivo studies (Brown et al., 1991; Ganheim et al., 2005; Roth et al., 1981).

In brief, the results of this study suggested that BVDV infection does not affect the viability of neutrophils. However, it reduced the L-selectin and CD18 expression leading to neutropenia and impaired neutrophil function. Further studies need to be done with other BVDV strain inducing highly pathogenic and moderate pathogenic to evaluate the effect of BVDV pathogenicity of neutrophil surface marker expression.
REFERENCES


Figure 2.1. Morphologic characterization of isolated bovine neutrophils.

Freshly isolated bovine neutrophils were cytospun and fixed with methanol followed by H and E staining or stained with DAPI. H and E staining of neutrophils showed purple colored multiple lobes in nucleus (polymorphic nucleus-red arrow) (A). Similar multiple lobes in nucleus (polymorphic nucleus) with blue color (shown with red arrow) were observed following DAPI staining. Cells were examined at 20X using compound microscope (Olympus, PA, USA) (A) or fluorescent microscope (Olympus, PA, USA) (B)
Figure 2.2. Phenotype of bovine neutrophils. Bovine neutrophils were stained with anti-CD18, anti-L-selectin and anti-CD14 antibodies followed by FITC-labeled secondary antibody. The percentage of cells expressing CD18 (99.77±0.13) (A), L-selectin (97.05±2.41) (B) and CD14 (11.34±3.89) (C) in M1 gating, shown by red arrow were measured using FACScan flow cytometer (Becton-Dickson, Mountain View, CA).
Figure 2.3. Neutrophils viability following BVDV infection. Neutrophils were infected with TGAC or TGAN at a MOI 6 for 0 hr, 1 hr and 6 hr. Mock-infected or LPS (10ng/ml) treated neutrophils were used as negative or positive controls respectively. Neutrophil viability was examined for through trypan blue exclusion assay and examined for significant differences using paired T test (p<0.05).
Figure 2.4. Effect of BVDV in inducing apoptosis in neutrophils. Neutrophils were infected with TGAC or TGAN at a MOI 6 for 0 hr, 1 hr and 6 hr. Mock-infected, or staurosporine (STS) 10nM treated neutrophil were used as negative or positive controls respectively. LPS (10ng/ml) also used for neutrophil stimulation. Neutrophil apoptosis was measured through Annexin V Apoptosis Detection Kit (eBiosciences, San Diego, CA). Significant differences from unstimulated control cells was calculated using paired T-test (p<0.05). Significant differences in treatment are shown with asterisk (*)
Figure 2.5. Effect of BVDV infection of CD14 expression on neutrophils.
Freshly isolated neutrophils were infected with TGAC or TGAN at a MOI of 6 or treated with LPS (10ng/ml). Neutrophils were collected at 0 hr, 1 hr or 6 hr post infection/treatment. Neutrophils were then stained with anti-CD14 antibodies. Followed by FITC-labeled secondary antibody. After secondary antibody staining, cells were analyzed through FACScan (Becton-Dickson, Mountain View, CA). Significant difference in surface marker expression following BVDV infection or LPS-treatment then its time point control was calculated through paired T-test (p<0.05) and indicated with an asterisk (*).
Figure 2.6. Effect of BVDV infection of CD18 expression on neutrophils.

Freshly isolated neutrophils were infected with TGAC or TGAN at a MOI of 6 or treated with LPS (10ng/ml). Neutrophils were collected at 0 hr, 1 hr or 6 hr post infection/treatment. Neutrophils were then stained with anti-CD18 antibodies. Cells were analyzed using a FACScan flow cytometer (Becton-Dickson, Mountain View, CA). Significant difference in surface marker expression following BVDV infection or LPS-treatment at each time point were calculated using a paired T-test (p<0.05) and are shown with an (*).
Figure 2.7. Effect of BVDV infection of L-selectin expression on neutrophil. Freshly isolated neutrophils were infected with TGAC or TGAN at a MOI of 6 or treated with LPS (10ng/ml). Neutrophils were collected at 0 hr, 1 hr or 6 hr post infection/treatment. Neutrophils were then stained with anti-L-selectin antibodies and using flow cytometry. Significant differences in surface marker expression following BVDV infection vs. LPS-treatment at each time point were determined using a paired T-test (p<0.05) and statistical differences were indicated with an asterisk sign (*).
TABLES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 hr</th>
<th>1 hr</th>
<th>6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Unstimulated neutrophils)</td>
<td>99.39±0.93%</td>
<td>99.17± 1.41%</td>
<td>98.13± 2.65%</td>
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<tr>
<td>Neutrophils treated with LPS (10ng/ml)</td>
<td>99.39±0.93%</td>
<td>98.25± 1.82</td>
<td>93.43± 3.08</td>
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<td>Neutrophils infected with TGAC (6 MOI)</td>
<td>99.39±0.93%</td>
<td>98.08± 1.26</td>
<td>97.33± 2.08</td>
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<td>Neutrophils infected with TGAN (6 MOI)</td>
<td>99.39±0.93%</td>
<td>99.25± 1.27</td>
<td>97.10± 1.09</td>
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**Table 2.1. Neutrophils viability following BVDV infection.** Neutrophils were infected with TGAC or TGAN at a MOI 6 for 0 hr, 1 hr and 6 hr. Mock-infected or LPS-treated neutrophils were used as negative or positive controls respectively. Neutrophil viability was examined for through trypan blue exclusion assay and examined for significant differences using a paired T test (p<0.05).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 hr</th>
<th>1 hr</th>
<th>6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Unstimulated neutrophils)</td>
<td>5.39±0.48%</td>
<td>20.17± 5.58%</td>
<td>37.11± 2.41%</td>
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<td>Neutrophils treated with LPS (10ng/ml)</td>
<td>5.39±0.48%</td>
<td>18.55± 3.43</td>
<td>43.49±2.53*</td>
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<td>Neutrophils treated with staurosporine (STS) 10nM (positive control)</td>
<td>5.39±0.48%</td>
<td>33.82±0.00*</td>
<td>48.62±2.82*</td>
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<tr>
<td>Neutrophils infected with TGAC (6 MOI)</td>
<td>5.39±0.48%</td>
<td>23.23±6.94</td>
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<td>Neutrophils infected with TGAN (6 MOI)</td>
<td>5.39±0.48%</td>
<td>27.49± 1.97*</td>
<td>38.72± 1.18</td>
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</table>

**Table 2.2. Effect of BVDV in inducing apoptosis in neutrophils.** Neutrophils were infected with TGAC or TGAN at a MOI 6 for 0 hr, 1 hr and 6 hr. Mock-infected, or staurosporine (STS) 10nM treated neutrophil were used as negative or positive controls respectively. LPS (10ng/ml) also was used for neutrophil stimulation. Apoptosis in neutrophil was measured with Annexin V Apoptosis Detection Kit (eBiosciences, San Diego, CA). Significant differences from unstimulated control cells was calculated using a paired T-test (p<0.05). Significant differences in treatment are indicated with an asterisk sign (*).
<table>
<thead>
<tr>
<th></th>
<th>CD14</th>
<th>CD18</th>
<th>L-selectin</th>
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<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>1 hr</td>
<td>6 hr</td>
</tr>
<tr>
<td>Control (Unstimulated Neutrophils)</td>
<td>100 ±0.00</td>
<td>103.91 ±27.83</td>
<td>118.93 ±34.20</td>
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<tr>
<td>Neutrophils treated with LPS (10ng/ml)</td>
<td>100 ±0.00</td>
<td>204.41 ±6.25*</td>
<td>360.52 ±26.30*</td>
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<tr>
<td>Neutrophils infected with TGAC (6 MOI)</td>
<td>100 ±0.00</td>
<td>104.54 ±35.02</td>
<td>133.97 ±43.17</td>
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<tr>
<td>Neutrophils infected with TGAN (6 MOI)</td>
<td>100 ±0.00</td>
<td>100.81 ±27.99</td>
<td>151.19 ±43.23</td>
</tr>
</tbody>
</table>

Table 2.3. Effect of BVDV infection of cell surface marker expression CD14, CD18 and L-selectin on neutrophils. Freshly isolated neutrophils were infected with TGAC or TGAN at a MOI of 6 or treated with LPS (10ng/ml). Neutrophils were collected at 0 hr, 1 hr or 6 hr post infection/treatment. Neutrophils were then stained with anti-CD14, anti-CD18 and anti-L-selectin antibodies, followed by FITC-labeled secondary antibody.

After secondary antibody staining, cells were analyzed using flow cytometer. Significant differences in surface marker expression following BVDV infection or LPS-treatment then its time point control was calculated with a paired T-test (p<0.05) and are shown with an asterisk (*).
CHAPTER 3.
EFFECT OF BVDV ON FUNCTIONAL ABILITY OF BOVINE NEUTROPHILS

ABSTRACT

Bovine viral diarrhea virus (BVDV) is one of the highly prevalent and economically devastating diseases of the cattle industry worldwide. The two major consequences of BVDV infection are persistent infection and immunosuppression. Several studies have been done to determine the underline mechanisms for BVDV-induced immunosuppression, targeting antigen presenting cells, cells of adaptive immune system and cytokine gene expression. However, very little research has focused on the effect of BVDV on neutrophils.

Neutrophils are one of the most abundant white blood cell (WBC) in the peripheral blood and play a critical role in innate as well as adaptive immune response. During injury or pathogen invasion, neutrophils become activated and migrate to the site of infection. At the site of infection, neutrophils phagocytize and destroy the invading pathogen through intracellular phagosomes and/or reactive oxygen species. In some cases, neutrophils also use extracellular traps to eliminate the invading pathogen.

The effect of BVDV infection on neutrophil functions, migration/chemoattraction, phagocytosis, oxidative burst and NET formation, were measured. The three ncp strains enhanced migration while the cp strain reduced neutrophil migration as compared to the mock-infected control treatment. The number of neutrophil that reached the lower chamber were 139%, 350% or 44% higher in TGAN, 1373 or 28508-infected macrophages as compared to mock-infected macrophage control, respectively. The number of neutrophils that migrated to TGAC-infected macrophages was 10% less as
compared to mock-infected control neutrophils. Compared with LPS-treated positive macrophage, all strains infection except the highly virulent 1373, reduced neutrophil migration. The enhancement in neutrophil migration by 1373 was approximately 55% higher as compared to LPS-treated positive control macrophages.

BVDV infection by any of the four strains significantly enhanced neutrophil phagocytosis activity of 0.2 \( \mu \)m microsphere beads as compared to mock infection (p<0.05). Phagocytosis activity following TGAC, TGAN, 28508 or 1373 infection was approximately 159%, 816%, 384% and 741% higher as compared to mock-infected control neutrophils respectively. Compared with LPS-treated positive control neutrophils, the 4 BVDV strains significantly reduced the phagocytosis of microsphere beads (p<0.05). Neutrophils infected with TGAC, TGAN, 1373 or 28508 reduced phagocytosis activity as 78.9%, 25.7%, 60% or 31.8% respectively, as compared to LPS-treated neutrophils.

Neutrophil phagocytic activity of rhodamine-labeled-\( E. \) coli was significantly reduced by BVDV infection. TGAC, TGAN, 1373 or 28508 had 23%, 6%, 19% or 12% less phagocytic activity as compared to mock-infected rhodamine-labeled-\( E. \) coli-treated neutrophils (p<0.05).

BVDV significantly enhanced the NET formation as compare to mock-infected negative control neutrophils (p<0.05). NET formation following TGAC, TGAN, 1373 or 28508 infection was observed at 102.82±4.83%, 106.91±7.58%, 107.04±6.72 % and 111.01±10.86% respectively as compared to the LPS positive control. NET formation was dramatic higher 179%, 190%, 190% and 201% higher as compared to mock-infected negative control neutrophils (37%)
These results indicated that BVDV infection modulates neutrophil activity of migration, phagocytosis and oxidative burst in a strain-dependent manner. All strains reduced neutrophil phagocytosis and oxidative burst activity, indicating that BVDV infection likely enhanced the susceptibility of secondary bacterial infection.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most widespread viruses in the cattle industry. It is a single-stranded, positive-sense RNA virus with a genome of approximately 12.5 kb (Renard et al., 1985). The BVDV genome encodes a single open reading frame flanking with a 5′-nontranslated region (5′-NTR) and a 3′-untranslated region (3′-NTR) (Fan and Bird, 2012; Wiskerchen et al., 1991). The polyprotein is cleaved into 11-12 structural or non-structural viral proteins either by host or viral proteases (Kummerer et al., 1998; Wiskerchen et al., 1991). The genetic diversity between different strains of BVDV has resulted in the classification of BVDV into two genotypes based on highly conserved 5’UTR region: BVDV Type I and BVDV Type II (Ridpath, 2003). BVDV can also be divided into two biotypes: cytopathic (cp) BVDV and noncytopathic (ncp) BVDV based on their effect in cell culture (Baker, 1987).

BVDV produces a wide range of clinical symptoms. The severity of the disease can range from mild acute infection to severe infection depending on the virulence of strain and health of infected host (Chase et al., 2015). A common feature of all BVDV infections are immunosuppression. Several studies determined the effect of BVDV on the adaptive response from antigen presenting to T cell activation and B cell responses (Chase, 2013; Chase et al., 2015). However, few studies have determined the effect of
BVDV on neutrophils, specifically the comparative effect of BVDV biotypes and strains with different virulence. Neutrophils are the major white blood cell in circulation, which play an important role innate immune response as well as activating and shaping the adaptive immune response (Jaillon et al., 2013). This study was designed to measure the effect of BVDV on neutrophil functional activity using a variety of BVDV strains: homologous pair of ncp and cp viruses (e.g. ncp BVDV1b TGAN and cp BVDV1b TGAC) recovered from an animal that died of mucosal disease as well as highly virulent, ncp BVDV2a 1373 and moderate virulent ncp BVDV2a 28508-5 strains.

MATERIALS AND METHODS

Virus Strains and Preparation

A homologous pair of ncp and cp type 1b viruses, ncp BVDV1b TGAN and cp BVDV1b TGAC (Brownlie et al., 1984), highly virulent ncp BVDV2a 1373 and moderate virulent ncp BVDV2a 28508-5 strains were used. Virus stocks of each strain was prepared in BVDV-free Madin Darby bovine kidney (MDBK) cells as described in chapter two. Briefly, BVDV-free MDBK cells (passage 98-112) were grown in minimal essential medium (MEM, Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% BVDV-free fetal calf serum (FCS) (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 μg /ml). Five (5) ml of 5x10^5 MDBK cells/ml were seeded in T25 flasks using minimal essential medium (MEM, Gibco BRL, Grand Island, NY) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). MDBK cells with 60-70% confluency were infected with either viral strain. During viral infection, the media from each flask was replaced with and 0.75 ml of virus inoculum with a multiplicity of infection [MOI] of
one. Virus was adsorbed for 1 hr at 37°C in a humidified CO₂ incubator with gentle rotation at every 15 minutes. After one hr incubation, unabsorbed virus was removed and the cells were washed with sterile PBS. After washing, 5 ml MEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml) was added to each flask. The cells were incubated at 37°C in a humidified CO₂ incubator for 4-5 days or up to 70-80% cytopathic effect for TGAC. After 4-5 days of incubation, cells were frozen at (-80°C for 15 minutes) and thawed on ice for two cycles. The cell debris were pelleted by centrifugation at 3000 rpm for 10 min at 4°C in 15 ml conical tubes. The supernatants, containing virus, were carefully collected. Supernatants were titrated for virus concentration and then aliquoted and stored at -80°C for further use.

The viral titer was determined by serially inoculating 1:10 dilutions in MEM containing MDBK cells as per the method described earlier (Reed and Muench, 1938). Briefly MDBK cells were detached from tissue culture flask using 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA). The number of cells was adjusted to 5x10^5 cells/ml. One hundred eighty (180) µl cell suspension was added to each well of 96-well plate. Twenty (20) µl of virus was added to the first row of the plate. The virus was then mixed with MDBK cells and 20 µl of this dilution was added to next row to achieve 10-fold dilutions. The last two rows were treated as negative controls with no virus. The plates were incubated at 37°C in humidified incubator for next 4 days. The plate was examined every day for cytopathic effect (CPE). The highest dilution showing CPE was used as endpoint to calculate the proportionate distance (PD). The PD was then used to determine the viral concentration (TCID50) as per formula as described earlier (Reed and Muench, 1938).
5. Proportionate distance (PD) = (% CPE at dilution above 50%) – (50%)/ (% CPE at dilution above 50%)- (% CPE at dilution below 50%) (e.g. 60-50/60-0= 0.166)

6. Calculation of endpoint just next to 50% CPE and conversion into – Log (e.g.10^-6 dilution would be -6)

7. Calculation of TCID<sub>50</sub>.

8. TCID<sub>50</sub> for 20 µl = (PD+ - Log dilution above 50%) (e.g. 1x10<sup>-6.166</sup>)

For ncp BVDV, the same procedures were done except the endpoint for ncp BVDV was determined by staining the MDBK cells with anti-BVDV antibody (mAb 16C6: IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti-mouse IgG (Zymed, Invitrogen Corporation, Frederick, MD, USA), streptavidin-HRP (Invitrogen Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA). The endpoint for ncp BVDV was determined by the presence of BVDV positive red stained cells.

**Animals**

Sixteen (14) healthy cattle (Holstein Friesian (n=9) and Brown Swiss calves (n=5) (8-12 months of age) housed at Department of Dairy Science’s Dairy Farm, South Dakota State University (SDSU), Brookings, SD, USA were used in this study. The SDSU Institutional Animal Care and Use Committee approved animal handling and blood collection.

**Neutrophil Isolation and Viability**

To isolate neutrophils, fifty (50) ml of peripheral blood was collected in 10 ml heparinized vacutainer tubes (BD, Franklin Lakes, New Jersey, USA) as described in chapter two. Briefly, neutrophils along with red blood cells (RBCs) were separated by centrifuging the blood at 1,000 X g for 30 minutes at 25°C. The plasma and buffy coat
were removed and discarded. The 3-ml cell pellet (neutrophils+ RBCs) was placed into eight (8)-15 ml conical tubes. To each 15-ml conical tube, 10 ml of RBC lysing solution was added. Each tube was gently inverted several times for 10 minutes to lyse the RBCs. After gentle inverting, tubes were centrifuged at 1,000 X g for 5 minutes at 25°C. Supernatants were discarded and cells pellets were washed 3 times using 10 ml of HBSS (for each tube, then were centrifuged at 1,000 X g for 5 minutes at 25°C). After each wash, cell pellets from two tubes were combined to one, leaving 4 tubes, 2 tubes and 1 tube at the end of each wash (after three wash, cells from eight tubes were combined to one tube). The final cell pellet was suspended in 10 ml RPMI 1640 medium (MEM, Gibco BRL, Grand Island, NY) supplemented with 10% BVDV free fetal calf serum (FCS) (PPA, Pasching, Austria), sodium pyruvate, penicillin (100 U/ml) and streptomycin (100μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). A 2.06±1.89 10^6/ml (in a final volume of 10 ml) neutrophils with 98.86±0.90% viability.

**Effect of BVDV on neutrophil migration ability**

The effect of BVDV on the neutrophil migration was measured using collagen-coated 6.5 mm transwell plates (Corning life Sciences, Corning, NY). Briefly, monocyte derived macrophages (MDM) were seeded and infected with BVDV in a 24-well plate (lower part of the transwell plate) while freshly collected neutrophils were added to upper chamber of the transwell. The number of neutrophils migrating to the lower well after one hour was used to estimate neutrophil migration ability.

The neutrophil migration assay was conducted as follows:

A. **Generation of monocyte derived macrophage (MDM):**
Sixty (60) ml heparinized venous blood was collected using 6-10 ml heparinized
vacutainers tubes through adopter needles from healthy calves. Freshly collected
heparinized blood was centrifuged at 2000 rpm for 30 minutes at 4°C to collected buffy
layers. Cells from buffy layer were suspended into heparinized (10 U/ml) PBS in 1:3
ratio. The 10 ml of diluted cells were overlaid on a 3 ml, 65% Percoll (GE Healthcare
Biosciences, Pittsburgh, PA, USA) in 15 ml conical tubes (Falcon, Oxnard, CA, USA).
After the cells were overlaid, the cells were centrifuged for at 2000 rpm, 4°C for 30
minutes. The white cell layer containing PBMC were aspirated by pipette from the
interphase. The collected PBMC were washed two times using heparinized PBS (10
U/mL) by centrifugation at 1700 rpm for 15 min at 4°C. Finally, PBMC were
resuspended in complete RPMI medium [RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml)] to achieve a final concentration of 1X 10⁶ cells/ ml. Three (3) ml of the cell suspension was added to each well of a 6-
well plate (Falcon, Oxnard, CA, USA) and incubated at 37°C in a humidified CO₂
incubator for 3 hour. After 3-hour attachment, non-adherent cells were washed away
using pre-warmed PBS. Adherent monocytes were cultured for 5 days by changing one-
half of the media every other day.

B. BVDV infection to monocyte derived macrophages (MDM):

On the 5th day of culture, MDM were detached with 0.5 ml/well Accutase
(eBioscience, San Diego, CA, USA) and dissolved in complete RPMI medium to achieve
a final concentration of 5x10⁵ cell/ml. One (1) ml of MDM cells was seeded to each well
of 24 well plates and allowed to attach overnight at 37 °C. The following day, MDM
were infected with either TGAC, TGAN, 1373 or 28508 at a MOI of 6. Mock-infected or
LPS–treated (10ng/ml) MDM were used as negative and positive controls respectively. After one-hour adsorption/treatment, MDM were washed with PBS and each well was supplemented with fresh 0.6 ml complete RPMI medium (Figure 3.1).

C. Neutrophil transwell migration assay

Freshly collected neutrophils were resuspended in complete RPMI medium to a final concentration of 1x10^6 cell/ml. One hundred (100) µl of the cell suspension was placed in the upper chamber of the transwell and the plate was incubated at 37°C for one hour (Figure 3.1). After one-hour incubation, a digital image of neutrophils migrated to lower chamber were taken. After migration, activated neutrophils were tightly attached to the lower chamber of transwell and Accutase/trypsin was unable to detach all cells. For better accuracy, cells were counted using ImageJ software. At least six images from different fields at 20X magnification were taken through a microscope (Olympus, Center Valley, PA) and number of cells in each field was counted using ImageJ software (Schneider et al., 2012).

Effect of BVDV on phagocytic activity on neutrophils

A. Effect of BVDV on phagocytic activity on neutrophils using fluorescent beads as target

The effect of BVDV on neutrophil phagocytic activity was measured using a homologous pair, TGAC and TGAN, highly virulent BVDV2a 1373 or moderate virulent BVDV2a 28508. Briefly, freshly collected bovine neutrophils were infected with either strain at a MOI of 6 for 1 hour. Mock-infected or LPS-treated (10ng/ml) neutrophils were used as negative and positive controls respectively. After one-hour infection/treatment, neutrophils were washed with PBS once. During washing, neutrophils from respective well of 6-well plates were transferred to 15 ml conical tubes. Tubes were centrifuged at
500xg for 10 minutes at 25°C. After centrifugation, supernatant was discarded and the pel-let was resuspended in 5 ml PBS by gentle pipetting. Neutrophils were centrifuged at 500xg for 10 minutes at 25°C and resuspended in RPMI media supplemented with 10% FBS and antibiotics to achieve final concentration 5x10^5/ml. One (1) ml neutrophils from each treatment was transferred to 24-well plates. To these plates, 0.2 µm red (580/605) carboxylated-modified microsphere beads (Thermo Fisher, Waltham, MA) were added at 1:10 target effector ratio. The neutrophil-bead mixture was incubated at 37 °C for one hour and then washed with PBS by centrifugation at 1000xg for 10 minutes at 4°C. The neutrophil-bead pellet was resuspended and fixed with 200µl 1% paraformaldehyde in PBS. Fixed cells were transferred to individual well of 24-well plates and examined under fluorescent microscope (Olympus, Center Valley, PA). The percent phagocytosis was calculated with following formula

\[
\text{Percent phagocytosis} = \frac{\text{Number of neutrophils with phagocytized beads} \times 100}{\text{Total number of neutrophils}}
\]

B. Effect of BVDV on phagocytic activity on neutrophils using rhodamine-labeled E. coli as target

The effect of BVDV infection on phagocytic activity of neutrophils was further examined through rhodamine-labeled-E. coli. Briefly, E. coli (DH5-Alpha), was streaked on nutrient agar plate and incubated overnight at 37°C in humidified incubator. After overnight incubation, a single colony of E. coli was collected and propagated in Luria-Bertani (LB) broth for 8 hours at 37°C in shaking incubator. After 8 hours incubation,
the LB broth was centrifuged at 2000X g for 15 minutes at 4 °C to obtain *E. coli* pellet. *E. coli* were washed one time with PBS by centrifugation at 2000X g for 15 minutes at 4°C and, then inactivated by autoclaving at 121°C for 20 minutes. *E. coli* inactivation was confirmed by absence of its growth on nutrient agar plate up to 48 hours following incubation at 37°C, while integrity of the *E. coli* cells was confirmed by microscopic examination (Olympus, Center Valley, PA).

To label *E. coli* with rhodamine, 2-gram of rhodamine was dissolved in 100 ml carbonate bicarbonate buffer (pH 9.2). Then the *E. coli* pellet was dissolved in rhodamine-carbonate bicarbonate buffer and incubated for 1 hour at room temperature. After the 1-hour incubation, rhodamine-labeled *E. coli* was washed five times with PBS by centrifugation at 2000X g for 15 minutes at 4 °C. Finally, rhodamine labeling to *E. coli* was confirmed using fluorescent microscopy (Olympus, Center Valley, PA).

To measure the effect of BVDV on neutrophil phagocytosis of *E. coli*, freshly collected bovine neutrophils were infected with either TGAC, TGAN, 1373 or BVDV2a 28508 at a MOI of 6. Following a 1-hour incubation, BVDV or mock-infected neutrophils were washed with PBS and resuspended in RPMI media supplemented with 10% FBS and antibiotics to achieve final concentration 5x10⁵/ml. One ml of neutrophils from each treatment was transferred to 24-well plates, to those wells, rhodamine-labeled-*E. coli* were added at 1:10 target effector ratio. Neutrophils were incubated at 37°C for hour and washed with PBS as described above. Neutrophils were then fixed with 200 µl, 1% paraformaldehyde suspended in PBS. Fixed neutrophils were either transferred to individual wells in 24-well plates to determine phagocytosis by fluorescence microscopy (Olympus, Center Valley, PA) using the following formula
Percent phagocytosis = \( \frac{\text{Number of neutrophils with phagocytized beads} \times 100}{\text{Total number of neutrophils}} \)

or examined using the FACS Calibur (Becton-Dickson, Mountain View, CA, USA).

**Effect of BVDV on neutrophil oxidative burst activity**

Freshly collected neutrophils were infected with either TGAC, TGAN, 1373 or 28508 at a MOI of 6 to measure the effect of BVDV on neutrophil oxidative burst activity. After one-hour of infection, BVDV or mock-infected neutrophils were washed with PBS by centrifugation at 500X g for 10 minutes at 25°C. After washing, the neutrophils were suspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) to achieve final concentration as 1x10⁶/ml. The neutrophil suspension (200 µl) from each treatment was then transferred to an individual well in a round bottom 96-well plate. Mock-infected neutrophils were divided into three groups: 1) positive control (mock-infected neutrophils treated with oxidative burst inducer phorbol 12-myristate 13-acetate: PMA); 2) negative control (mock-infected neutrophils with no PMA; and 3) DHR 123-negative control [neutrophils with no treatment and no dihydrorhodamine 123 (DHR 123)]. The DHR 123-negative control was used to set the fluorescence gates on the FACS Calibur flow cytometer.

Twenty (20) µl of 10 µm dihydrorhodamine 123 (DHR 123) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, except no DHR123 was added to the negative control cells. DHR 123 is colorless dye, which passively enters the cell and produces green fluorescent in the presence of reactive oxygen species. After adding DHR 123, plates were incubated at 37°C for 15 minutes. Following the incubation, 50 µl Phorbol
12-myristate 13-acetate (PMA) 10nM was added to each well (except DHR123-negative control) and the plate was incubated for 15 minutes at 37°C. After 15 minutes of incubation, the plates were washed with PBS and fixed with 1% paraformaldehyde in PBS. The oxidative burst activity in fixed cells were measured using the flow cytometer (Becton-Dickson, Mountain View, CA, USA).

**Effect of BVDV on Neutrophil extracellular traps (NETs) formation**

Effect of BVDV on neutrophil extracellular traps (NETs) formation was measured through NETosis assay kit (Cayman Chemical, Ann Harbor, MI). Briefly, freshly collected bovine neutrophils were infected with one of the four strains at a MOI of 6 for 1 hr.

BVDV or mock-infected neutrophils were centrifuged at 500X g for 10 minutes at 25 °C to obtain cell pellet. The neutrophil pellet was resuspended in pre-warmed NET assay buffer (prepared by combining 500 ml RPMI medium with 5 grams of bovine serum albumin and 500 µl calcium chloride) to achieve final concentration 1x10⁶/ml. A 900 µl cell suspension was transferred to individual wells of 24-well plate. One hundred (100) µl 1X phorbol 12-myristate 13-acetate: PMA (provided with the kit) was added to each well and plate was incubated at 37 °C for four hours to induce NET formation. The mock-infected neutrophils treated with PMA were treated as positive control while mock-infected neutrophils with no PMA treatment was used as a negative control.

After the four-hour incubation, the supernatant was aspirated and discarded carefully without disturbing the NET-cells. The NET-cells were washed gently with one ml NET assay buffer twice. Five hundred (500) µl of S7 nuclease (1:1000 dilution: provided with kit) was added to each well and the plate was incubated for 15 minutes at
37°C to digest NET DNA and release neutrophil elastase. After 15 minutes of incubation, the supernatant was collected and placed into a new 1.5 ml tube. The 10 µl 500Mm ethylenediaminetetraacetic acid (provided with kit) was added to each tube and incubated for one minutes at room temperature to inactivate nuclease. The tubes were inverted gently a few times and then centrifuged at 300Xg for 5 minutes at room temperature. One hundred (100) µl of supernatant from each tube was transferred to a respective well of flat bottom 96-well plate. The 100 µl neutrophil elastase substrate containing 15 mM N-methoxysuccinyl-Ala-Ala-Pro-val-p nitroanilide (provided with kit) was added to cell supernatant. The plate was covered with aluminum foil and incubated at 1 hour for 37°C. After the 1-hour incubation, absorbance from each well was measured at 405nm using Bioteck ELX808 ELISA plate reader (Thermo Fisher Scientific Inc., MA, USA). Each experiment was repeated in at least three different animals to determine the effect of BVDV on neutrophil extracellular traps (NETs) formation.

Statistical analysis:

A paired T test at 5% level of significance was used to determine the significant effect of BVDV on neutrophil migration/chemoattraction, phagocytosis, reactive oxygen species production (oxidative burst) or NET formation (Glantz, 2002).

RESULTS

Effect of BVDV on neutrophil chemotaxis/ migration ability

The effect of BVDV on neutrophil chemotaxis activity was measured using transwells (Corning life Sciences, Corning, NY). The number of neutrophil that reached the lower chamber were 1509±117.97 for TGAN-infected macrophages, 2836.4±560.97 for 1373-infected macrophages and 913.2±85.26 for 28508-infected macrophages
respectively, which was approximately 139%, 350% and 44% higher as compared to mock-infected control neutrophils respectively (Figure 3.3) (p<0.05). The number of neutrophils that migrated after TGAC-infected macrophages were 568.4±208.02 was 10% less as compared to mock-infected control neutrophils (Figure 3.3) (p<0.05). Mock infection or LPS treatment of macrophage (10ng/ml) resulted in migration of 630±143.94 and 1818±184.83 neutrophils, respectively (Figure 3.2 and 3.3).

TGAC, TGAN or 28508 infection reduced the neutrophil migration by 68%, 16% and 50% respectively, while 1373 significantly enhanced neutrophil migration by approximately 55% as compared to LPS-treated positive control neutrophils (Figure 3.2 and 3.3) (p<0.05).

**Effect of BVDV on neutrophil phagocytic capacity.**

To measure the effect of BVDV on neutrophil phagocytic activity, freshly collected neutrophils were infected with one of the 4 BVDV strains at a MOI of 6 for one hour. LPS-treated (10ng/ml) or mock-infected neutrophils were used as positive or negative controls respectively. After the one-hour incubation, either 0.2 µm red carboxylated-modified microsphere beads or rhodamine-labeled *E. coli* at 1:10 target effector ratio was added to the treated or infected neutrophils and incubated an additional hour. After the one-hour incubation, phagocytic activity of neutrophil for was measured by counting the number of fluorescent beads or rhodamine-labeled-*E. coli* by fluorescent microscope (Olympus, Center Valley, PA) internalized in the neutrophils. Additionally, the phagocytic activity of neutrophils for rhodamine-labeled *E. coli* was also measured using flow cytometry.
BVDV infection by any of the four strains significantly enhanced neutrophil phagocytic activity of 0.2 µm microsphere beads (p<0.05) as compared to mock infection (Figure 3.4). Phagocytosis activity following TGAC, TGAN, 28508 or 1373 infection was approximately 159%, 816%, 384% and 741% higher as compared to mock-infected control neutrophils respectively (Figure 3.4).

Compared to LPS-treated positive control neutrophils, infection with any of the 4 BVDV strains significantly reduced the phagocytosis of microsphere beads (p<0.05). Indicating that BVDV infection compromise the neutrophils’ phagocytic activity during bacterial infection as gram negative bacteria contain LPS. Neutrophils infected with TGAC, TGAN, 373 and 28508 had phagocytic activity for microsphere beads of 13.83±4.16%, 48.83±3.34%, 25.83±6.58%, and 44.85±5.77% respectively, which was approximately 78.9%, 25.7%, 60% and 31.8% less as compared to LPS-treated neutrophils (Figure 3.4). The phagocytic activity of LPS-treated and mock-infected neutrophils was 65.83±6.14% and 5.33±0.52% respectively (Figure 3.4).

Neutrophil phagocytic activity of rhodamine-labeled *E. coli* was significantly reduced by infection with TGAC, 1373 or 28508 as compared to mock-infected rhodamine-labeled *E. coli*-treated neutrophils (p<0.05). Neutrophils infected with cp BVDV1b TGAC, ncp BVDV2a 1373 or ncp BVDV2a 28508 had less significantly phagocytic activity [62.65±7.44% (23% less), 65.92±9.76% (19% less) and 69.92±6.09% (12% less) respectively] as compared to mock-infected, rhodamine-labeled-*E. coli*-treated neutrophils with phagocytic activity of 81.29±5.54% (p<0.05) (Figure 3.5). Neutrophils infected with TGAN had less phagocytic activity (76.60±4.59%, 6% less) but not significantly lower than the mock-infected control neutrophils (Figure 3.5). In the current
assay, mock-infected-rhodamine-labeled *E. coli*-treated control neutrophils could also be considered as LPS-treated positive control as *E. coli* contain LPS.

Additional neutrophil phagocytic experiments were done with rhodamine-labeled *E. coli* using flow cytometry. The percent geometric mean fluorescence from rhodamine-labeled *E. coli* in neutrophils infected with TGAC, TGAN, 1373 or 28508 was reduced by all 4 strains (71.45±16.39, 27.8% reduction; 91.03±5.93, 8% reduction; 84.21±10.49, 15% reduction and 87.98±11.70, and 11% reduction, respectively) as compared to the mock-infected rhodamine-labeled *E. coli*-treated neutrophils that had a geometric mean of 99.00±13.22 (Figure 3.6). Among the four strains, cp TGAC infection reduced neutrophil phagocytic activity significantly (p<0.05) as compared to mock-infected rhodamine-labeled *E. coli*-treated neutrophils.

**Effect of BVDV infection on neutrophil oxidative burst**

To measure the effect of BVDV infection on neutrophil oxidative burst activity, freshly collected neutrophils were infected with one of the four BVDV strains at a MOI of 6. BVDV or mock-infected neutrophils were treated with PMA oxidative burst inducer positive control. The neutrophil oxidative burst activity was measured using flow cytometry (Figure 3.7). All strains of BVDV significantly reduced the oxidative burst activity in neutrophils (p<0.05). The neutrophil oxidative burst activity was 57.33±3.38% (50% less) 55.89±7.32% (51% less) 61.20±4.44% (46% less) and 58.96±5.16% (48%) following infection with TGAC, TGAN, 1373 and 28508 respectively (Figure 3.8, Table 3.1). Mock-infected-PMA-treated neutrophils (positive control) oxidative burst activity was 115.72±3.08%, while mock-infected negative control (no PMA treatment) oxidative burst activity was 0.96± 0.02 % (Figure 3.7, Figure 3.8, Table 3.1).
**Effect of BVDV infection on neutrophil extracellular trap (NETs) activity**

The effect of BVDV infection on neutrophil extracellular traps (NETs) activity was measured through NETosis assay kit (Cayman Chemical, Ann Harbor, MI). Freshly collected neutrophils were infected with one of the four BVDV strains at a MOI of 6. BVDV- or mock-infected neutrophils were treated with PMA as a positive control NET inducer. Mock-infected neutrophils treated with PMA were used as a positive control while mock-infected neutrophils with no PMA treatment were used as a negative control. The four BVDV strains significantly enhanced the NET formation as compared to mock-infected negative control neutrophils (p>0.05). NET formation following TGAC, TGAN, 1373 and 28508 was observed as 102.82±4.83%, 106.91±7.58%, 107.04±6.72 % and 111.01±10.86% respectively which were approximately 179%, 190%, 190% and 201% higher as compared to mock-infected negative control neutrophils which showed NET formation as 36.87± 0.83% (Figure 3.9, Table 3.1).

**DISCUSSION**

This study was conducted to measure the effect of BVDV infection on neutrophil functional activities of migration/chemotaxis, phagocytosis, oxidative burst and NETs formation. The ncp BVDV, TGAN, 1373 or 28508, significantly enhanced neutrophil migration by 139%, 350% and 44% higher while cp TGAC reduced neutrophil migration as 10% as compared to mock-infected control respectively. While comparing with LPS-treated positive control, cp TGAN (68% reduction) and moderately virulent ncp 28508 (50% reduction) significantly reduced the migration ability of neutrophils (p<0.05). TGAN infection reduced neutrophil migration by 16%. A surprising finding was that the highly virulent 1373 enhanced the neutrophil migration by approximately 55%. The cp
biotype (cp TGAC) significantly reduced the migration of neutrophils as compared to its homologous ncp biotype (TGAN) (p<0.05). The reduction in neutrophil migration by cp TGAC was 62% less than its homologous ncp biotype (TGAN). However, there was no significant difference between BVDV type 1 and type 2 on neutrophil phagocytic activity. Surprisingly, the moderately virulent ncp BVDV2a 28508 significantly reduced neutrophil migration as compared to the highly virulent BVDV (ncp BVDV2a 1373). Higher neutrophil migration following highly virulent 1373 strain infection may be the reason of severe leucopenia and hemorrhagic syndrome caused by this virus in vivo (Ridpath et al. 2006a, Ridpath et al. 2006b).

All four strains significantly enhanced neutrophil phagocytic activity for 0.2 µm microsphere beads (p<0.05) as compared to mock infection. Phagocytosis activity following TGAC, TGAN, 28508 or 1373 infection was approximately 159%, 816%, 384% and 741% higher as compared to mock-infected control neutrophils respectively.

Compared with LPS-treated positive control, which simulates gram-negative bacterial infection, the four BVDV strains significantly reduced neutrophil phagocytic activity of microsphere beads. Similarly, BVDV infection with all four strains (TGAC, TGAN, 1373 and 28508) reduced neutrophil phagocytic activity for rhodamine-labeled E. coli and there was little difference between the microscopy and flow cytometry (23% vs.27.8, 6% vs 8%, 19% vs 15% and 12% vs 11%). The higher magnitude of reduction in the microsphere assay (TGAC ~3-fold higher; TGAN ~4X fold higher; 1373 ~4X fold higher and 28508 ~3X fold higher) between may be due to the difference in targets between the two different phagocytosis assays and the high response of the LPS-positive control in the microsphere assay. The microsphere beads used in the phagocytosis assay
were not coated with any other ligand, and the LPS was added independently to the LPS-positive control. However, rhodamine-labeled *E. coli* had endogenous LPS and LPS has been shown to increase neutrophil phagocytosis in a dose-dependent manner (Bohmer et al., 1992). Additionally, the size of E. coli (2µm) is 10 times larger than microsphere beads (0.2µm).

A previous study with neutrophils isolated from cp NADL la-infected animals also showed decreased paraffin oil uptake as compared to neutrophils isolated from non-infected control cattle (Roth et al., 1981) supporting the findings of the current study. However, *in vivo* studies did not compare BVDV biotypes or genotypes.

Previously, our lab also showed that there was a BVDV strain effect on phagocytosis with monocyte-derived macrophages (MDM), another phagocytic cell. In that study, both cp and ncp BVDV infection significantly reduced the phagocytic activity of MDM for *Candida albicans* (Elmowalid, 2003; Chase et al., 2004), and such phagocytic activity was reduced with course of infection (e.g. 12 hr p.i., 24 hr p.i. and 48 hr p.i.). That study also showed that moderately virulent 28508, BJ or PA131 did not have any effect on MDM phagocytic activity for *Candida albicans*. While highly virulent 1373, BVDV2-890, Singer, NY-1 or A125 significantly reduced phagocytic activity of MDM for *Candida albicans* at 48 hr p.i. by 51.6%, 54.0%, 59.3%, 72.3% and 73.2% respectively as compared to mock-infected MDM that had phagocytic activity of more than 95%. (Elmowalid, 2003).

The study showed that all four strains used in study significantly reduced oxidative burst activity (p<0.05) by approximately 50% as compared to positive control (p<0.05). BVDV from both biotypes (cp or ncp), genotypes (type 1 or type 2) or
virulence (high virulence or moderate virulence) had similar effect on neutrophil oxidative burst activity. There was no significant difference in neutrophil oxidative burst activity between BVDV biotypes, genotypes or virulence (p<0.05). Reduced neutrophil functional activity following BVDV infected was also observed in a previous in vivo experiment. Neutrophils isolated from MLV (cp BVDV1a Singer) vaccinated animal had reduced neutrophil iodination as well as antibody-dependent cell-mediated cytotoxicity (ADCC) (Roth and Kaeberle, 1983).

Our study also showed that BVDV infection significantly enhanced neutrophil extracellular trap (NET) formation (p<0.05). NET formation following TGAC, TGAN, 1373 and 28508 was approximately 179% (102.82±4.83%), 190% (106.91±7.58%), 190% (107.04±6.72 %) and 201% (111.01±10.86%) higher than mock-infected control neutrophils respectively which showed NET formation as 36.87± 0.83%.

In conclusion, the study measured the strain effect of BVDV on major functions of neutrophils. The study used four strains from both genotypes (type 1 and type 2), biotypes (cp and ncp), including highly virulent and moderate virulent strains of BVDV. Our results indicated that BVDV infection has a significant impact on neutrophil functional activity phagocytosis, migration, oxidative burst and NET formation. However, in vitro effects can be different than in vivo. Further in vivo studies need to be done to measure the effect of BVDV strains on neutrophil functional activity and overall innate immune response. However, it is very difficult to infect neutrophils alone in an in vivo system as BVDV infects almost all types host cell, host ranging from epithelial cells to cells of innate as well as adaptive immune system.
REFERENCES


**Figure 3.1. Neutrophil transwell migration assay.** MDM were seeded and infected with BVDV in a 24-well plate (lower part of the transwell plate). Following incubation with the virus, neutrophils were added to the upper chamber of the transwell. The number of neutrophils that migrated to the lower well after one hour was used to estimate the neutrophil migration ability. Picture adapted from [https://www.corning.com/worldwide/en/products/life-sciences/products/permeable-supports.html](https://www.corning.com/worldwide/en/products/life-sciences/products/permeable-supports.html).
Figure 3.2. Effect of BVDV infection on neutrophil chemotaxis/migration. Five day cultured MDM were seeded in the lower chamber of transwell and infected with either TGAC (A), TGAN(B), 1373 (C) or 28508 (D) at a MOI of 6 for 1 hour. Mock-infected (F) or LPS-treated (10ng/ml) (E) MDM were used as negative or positive controls respectively. A 100µl (1x10^6 cell/ml) of freshly collected neutrophils were seeded in the upper chamber of the transwell and allowed to migrate for 1 hour at 37°C. After an one-hour incubation, the number of neutrophils that migrated to the lower chamber was counted by ImageJ software.
Five day cultured MDM were seeded in the lower chamber of transwell and infected with either cp TGAC (A), TGAN(B), 1373 (C) or 28508 (D) at a MOI of 6 for 1 hour. Mock-infected (F) or LPS-treated MDM (10ng/ml)(E) were used as negative or positive controls respectively. One hundred (100 µl) (1x10^6 cell/ml) freshly collected neutrophils were seeded at upper chamber of transwell and allowed to migrate for 1 hour at 37°C. After an one-hour migration, the number of neutrophils reached at lower chamber were counted by ImageJ software. Significant difference from LPS treatment was calculated through paired T-test (p<0.05) and shown with asterisk sign (*)
Figure 3.4. Effect of BVDV infection on neutrophil phagocytosis of 0.2µm beads.

Freshly collected neutrophils were infected with TGAC, TGAN, 1373 or 28508 at a MOI of 6 for 1 hour. Mock-infected or LPS-treated (10ng/ml) neutrophils were used as negative or positive controls. After one hour of infection/treatment, neutrophils were added to 0.2µm beads with 1:10 target effector ratio. The neutrophil-bead mixture was further incubated for one hour and examined for phagocytic activity and the number of cells counted using fluorescence microscopy. Significant difference in neutrophil phagocytosis activity from LPS treatment was calculated through paired T-test (p<0.05) and shown with asterisk (*)
Figure 3.5 The effect of BVDV infection on neutrophil phagocytosis of rhodamine-labeled E. coli as compared to mock-infected neutrophils. Freshly collected neutrophils were infected with TGAC, TGAN, 1373 or 28508 at a MOI of 6 for 1 hour while mock-infected neutrophils were used as negative controls. After one hour of infection neutrophils were added with rhodamine-labeled E. coli with a 1:10 target effector ratio. The neutrophil-E. coli mixture was further incubated for one hour and examined for phagocytic activity using fluorescent microscope (Olympus, Center Valley, PA). Significant differences in neutrophil phagocytosis was calculated with a paired T-test (p<0.05) and indicated with an asterisk (*)
Figure 3.6. The effect of BVDV on neutrophil phagocytosis of rhodamine-labeled *E. coli* using flow cytometry. Freshly collected neutrophils were infected with cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV2a 28508 at a MOI of 6 for 1 hour while mock-infected neutrophils were used as negative controls. After one hour of infection, neutrophils were added to rhodamine-labeled *E. coli* with 1:10 target effector ratio. The neutrophil-*E. coli* mixture was further incubated for one hour and fixed with 1% paraformaldehyde. Fixed cells were examined for phagocytic activity using flow cytometry. Significant differences in neutrophil phagocytosis activity from mock infection was calculated using a paired T-test (p<0.05) and indicated with an asterisk (*)
Figure 3.7. Flow cytometry histograms of neutrophil oxidative burst activity following BVDV infection. Neutrophils were infected with TGAC (C), TGAN (D), 1373 (E) or 28508 (F) at a MOI 6 for 1 hr. BVDV or mock-infected (B: positive control) neutrophils were treated with PMA. Mock-infected neutrophils with no PMA treatment were used as negative control (A). The neutrophil oxidative burst activity in all treatment was measured using flow cytometer.
Figure 3.8. Neutrophil oxidative burst activity following BVDV infection.

Neutrophils were infected with TGAC, TGAN, 1373 or 28508 at a MOI 6 for 1 hr. BVDV or mock-infected (positive control) neutrophils were treated with PMA. While mock-infected and no PMA-treated neutrophils were used as negative control. The neutrophil oxidative burst activity in all treatments was measured through FACS Caliber (Becton Dickson, Mountain View, CA, USA). Significant reduction in oxidative burst activity from positive control cell was calculated using a paired T test (p<0.05) and indicated with an asterisk (*).
Figure 3.9. Neutrophil extracellular trap (NET) formation following BVDV infection. Freshly collected neutrophils were infected with TGAC, TGAN, 1373 or 28508 at a MOI 6 for 1 hr. BVDV or mock-infected neutrophils were treated with NET inducer, PMA. Mock-infected and PMA-treated neutrophils were used as positive control while mock-infected with no PMA treated neutrophils were used as negative control. NET formation was analyzed through enzyme substrate reaction and measured at 405nm using a Bioteck ELX808 ELISA plate reader (Thermo Fisher Scientific Inc., MA, USA).
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<th>Oxidative burst activity (%)</th>
<th>Neutrophil extracellular traps (NETs) (%)</th>
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<tr>
<td>Positive control neutrophils</td>
<td>115.72±3.08</td>
<td>100.00± 4.05%*</td>
</tr>
<tr>
<td>Mock-infected negative control neutrophils</td>
<td>0.96±0.02%*</td>
<td>36.87± 0.83</td>
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<tr>
<td>Neutrophils infected with cp TGAC (6 MOI)</td>
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<td>102.82± 4.83*</td>
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<tr>
<td>Neutrophils infected with TGAN (6 MOI)</td>
<td>55.89±7.32%*</td>
<td>106.91± 7.58*</td>
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<tr>
<td>Neutrophils infected with 1373 (6 MOI)</td>
<td>61.20±4.44%*</td>
<td>107.04± 6.72*</td>
</tr>
<tr>
<td>Neutrophils infected with 28508 (6 MOI)</td>
<td>58.96±5.16%*</td>
<td>111.01± 10.86*</td>
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Table 3.1. Neutrophil oxidative burst and Neutrophil extracellular traps (NETs) formation ability following BVDV infection. Freshly collected neutrophils were infected with cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV 28508 at a MOI 6 for 1 hr. BVDV-infected or mock-infected neutrophils were treated with the PMA which induced both oxidative burst and/or NET inducer. Mock-infected and PMA-treated neutrophils were used as positive control while mock-infected with no PMA-treated neutrophils were used as negative control. Neutrophil oxidative burst activity was measured through FACS Calibur (Becton-Dickson, Mountain View, CA, USA) while NET formation was analyzed through enzyme substrate reaction at 405nm using Bioteck ELX808 ELISA plate reader (Thermo Fisher Scientific Inc., MA, USA). Significant reduction in oxidative burst activity or NET formation from positive control cell was calculated using a paired T test (p<0.05) and indicated with an asterisk (*).
Neutrophils are the one of the major white blood cells in circulating blood. Neutrophils are considered the first line in the innate immune system (Kobayashi and DeLeo, 2009) along with macrophages. Neutrophils play an important role against invading bacteria and initiates the adaptive immune responses. They phagocytize the invading microorganisms and activate the innate as well as adaptive immune response. Neutrophils activate the immune response through various mediators including interleukin-8 (IL-8), platelet activating factor (PAF), leukotriene B₄ (LTB₄) or complement fragment 5a (C5a) (Guo et al., 2003; Mantovani et al., 2011; Mitchell et al., 2003; Mitchell et al., 2014). The ability of neutrophils to migrate and destroy the invading microorganism depends upon surface marker expression, phagocytic ability and oxidative enzymes. Among the surface markers, the cluster of differentiation (CD)-14 (CD14), CD18 and L-selectin, play an important role against invading organism recognition and initiating the immune response (Yoshitake et al., 2002). A pathogen that infects neutrophils could alter its surface marker expression, functional ability and significantly affect innate as well as adaptive immune response as discussed in review (Drescher and Bai, 2013)

In the current study, we had three hypotheses: 1) BVDV infection to neutrophils significantly affects neutrophil viability in a strain dependent manner; 2) neutrophils infected with BVDV have impaired cell surface marker expression of CD14, CD18 and
L-selectin; and, 3) altered surface marker expression can result in abnormal functional ability including improper migration, phagocytosis activity, chemotaxis ability, oxidative burst and/or, altered neutrophil extracellular trap formation. Such changes in neutrophils could vary depending on infection with different BVDV biotypes, genotypes or virulence types. To address this question, we used four different viruses: a virus pair (biotypes) of cp BVDV1b TGAC and ncp BVDV1b TGAN, highly pathogenic ncp BVDV2a 1373 and moderately pathogenic ncp BVDV2a 28508.

The hypothesis of the current study was tested using the following objectives:

1) To isolate and use bovine neutrophils as an in vitro model to determine effect of BVDV on innate immune system. Under this objective three approaches were taken:
   a) Optimize the method for neutrophil isolation from bovine peripheral blood with high yields and viability.
   b) Morphological and phenotypic characterization of neutrophils.
   c) Optimize culture conditions for neutrophils.

2) To investigate the effect of BVDV on neutrophil viability and phenotype. The approaches for that objective were:
   a) Study the effect of different biotypes and virulent strains of BVDV on neutrophils viability as well as surface marker expression (CD14, CD18 and L-selectin).

   To investigate the effect of different strains of BVDV on neutrophil functional activities of phagocytosis activity, chemotaxis ability, oxidative burst and neutrophil extracellular trap formation

   Bovine neutrophils were isolated by gradient centrifugation followed by red blood cells (RBCs) lysis and neutrophil restoration. Isolated neutrophils were confirmed
morphologically and phenotypically and, further examined for viability, purity and yield. The neutrophils had characteristic polymorphic nucleus with 99.80±0.1% purity and 98.86±0.90% viability.

Neutrophils were CD18+ (99.77±0.13%), L-selectin (97.05±2.41%), and CD14+ (11.34±3.89%) positive. A total 20.64±1.89 x 10^6 neutrophils were obtained from 50 mL peripheral blood (e.g. 10 ml of 2.064±1.89 x 10^6/ml neutrophils). None of the BVDV strains used in current study cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV2a 28508 affected the viability of neutrophils in vitro. Trypan blue exclusion assay showed neutrophils viability at 98.08±1.26%, 97.33±2.08% and 99.25±1.27%, 97.10±1.09% and 98.50±2.14%, 94.48±0.87% and 98.12±2.22%, 93.97±5.79% at 1hr and 6 hr post infection with cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV2a 28508 respectively. While unstimulated control cell or LPS-treated neutrophils showed viability of 99.17±1.41%, 98.13±2.65% and 98.25±1.82% and 93.43±3.08% at 1hr and 6 hr post infection respectively.

Surface marker expression was measured following BVDV infection revealed that both cp BVDV1b TGAC and ncp BVDV1b TGAN increased CD14 expression approximately 12% and 27% at 6 hr p.i., respectively, as compared to their unstimulated time point control neutrophils. The current study also revealed that ncp BVDV1b TGAN infection significantly reduced the CD18 expression (p<0.05) while cp BVDV1b TGAC slightly enhanced the expression. Reduction in CD18 expression by ncp BVDV1b TGAN was about 24% while increment in CD18 expression by cp BVDV1b TGAC was about 8% at 6 hr p.i. as compared to their time point control neutrophils.
However, both cp BVDV1b TGAC and ncp BVDV1b TGAN significantly reduced L-selectin expression at 6 hr p.i. as compared to their time point control neutrophils (p<0.05). The reduction in L-selectin at 6 hr p.i. by both biotypes was approximately 11% as compared to their time point control neutrophils.

The effect of BVDV infection on neutrophil functional activity migration/chemotaxis, phagocytosis, oxidative burst and NETs formation was measured. The neutrophil migration assay was designed to measure the effect of BVDV-infected macrophages on neutrophil migration mechanisms (Zec, et al. 2016). Macrophages acted as tissue resident sentinels and attract circulating neutrophils to the site of infection (Kolaczkowska and Kubes 2013, Schiwon et al. 2014, Zec et al. 2016). This indirect effect of BVDV-infected macrophages on neutrophil migration was measured.

Cp BVDV1b TGAC infection to macrophage, significantly reduced migration ability of neutrophils as compared to mock infected negative control macrophage (p<0.05). The reduction in neutrophil migration following cp BVDV1b TGAC was approximately 10% less as compared to mock-infected control. While ncp BVDV strains BVDV1b TGAN, 1373 and 28508 enhanced neutrophil migration. The number of neutrophil that reached the lower chamber were 1509±117.97 for TGAN-infected macrophages, 2836.4±560.97 for 1373-infected macrophages and 913.2±85.26 for 28508-infected macrophages respectively, which was approximately 139%, 350% and 44% higher as compared to mock-infected control neutrophils respectively.

The comparative effect of biotypes and virulence revealed that cp biotype (cp BVDV1b TGAC) significantly reduced the migration ability of neutrophils as compared to its homologous ncp (ncp BVDV1b TGAN) (p<0.05). The reduction in neutrophil
migration by cp BVDV1b TGAC was about 62% as compared to its homologous ncp (ncp BVDV1b TGAN). Surprisingly, highly virulent BVDV (ncp BVDV2a 1373) significantly enhanced neutrophil migration as compared to moderate virulent BVDV (ncp BVDV2a 28508) (p<0.05). Enhancement of neutrophil migration by highly virulent BVDV (ncp BVDV2a 1373) was about 210% higher as compared to moderate virulent BVDV (ncp BVDV2a 28508). Higher neutrophil migration following ncp BVDV2a 1373 may be the reason for excess tissue injury caused by this virus (Jaeschke and Hasegawa 2006, Ridpath et al. 2006, Ramaiah and Jaeschke 2007, Kruger et al. 2015).

Phagocytic activity was also studied. All BVDV strains used in the current study significantly reduced the phagocytic activity of neutrophils for microsphere beads as compared to LPS-treated neutrophils (p<0.05). Neutrophils infected with cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV2a 28508 reduced phagocytic activity by 78.9%, 25.7%, 60% and 31.8% respectively for microsphere beads as compared to LPS-treated neutrophils.

While comparing with mock-infected control neutrophils, BVDV (e.g. cp BVDV1b TGAC, ncp BVDV1b TGAN ncp BVDV2a 1373 or ncp BVDV2a 28508) infection significantly enhanced the neutrophil phagocytic activity (p<0.05), which was approximately 159%, 816%, 384% and 741% higher as compared to mock-infected control neutrophils respectively.

Similar results were observed with rhodamine-labeled *E. coli* (*E. coli* gram negative bacteria which contain LPS) was used as target. Neutrophils infected with cp BVDV1b TGAC, ncp BVDV2a 1373 or ncp BVDV2a 28508 had a reduction in
phagocytic activity of 22.8%, 18.9%, and 13.9% respectively for rhodamine-labeled E. coli as compared to mock-infected neutrophils.

Similar to migration and phagocytic activity, BVDV infection also affected the oxidative burst activity in neutrophils. Neutrophils infected with cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV 28508 showed approximately 50%, 51%, 46% and 48% reduction in oxidative burst activity respectively as compared to mock-infected-PMA treated neutrophils - after 1 hr of infection (p<0.05). BVDV from both biotypes (cp or ncp), genotypes (type 1 or type 2) or virulence (high virulence or moderate virulence) had similar effect on neutrophil oxidative burst activity.

The PMA-treated BVDV-infected (e.g. cp BVDV1b TGAC, ncp BVDV1b TGAN, ncp BVDV2a 1373 or ncp BVDV 28508) neutrophils, increased the oxidative burst by 57%, 55%, 61% and 58% respectively as compared with mock-infected neutrophils.

All four of the BVDV significantly enhanced the NET formation as compared to mock-infected negative control neutrophils (p>0.05). NET formation following TGAC, TGAN, 1373 and 28508 was observed as 102.82±4.83%, 106.91±7.58%, 107.04±6.72 % and 111.01±10.86% respectively which were approximately 179%, 190%, 190% and 201% higher as compared to mock-infected negative control neutrophils, which showed NET formation as 36.87± 0.83%.

There are very limited studies have been done to determine the effect of BVDV on neutrophils. In an in vivo study, neutrophils isolated from experimentally BVDV infected cattle showed decreased phagocytic activity with reduced paraffin oil uptake as compared to neutrophils isolated from non-infected control cattle (Roth et al., 1981).
Similarly, neutrophils isolated from persistently infected cattle also showed reduced number as well as its functional activity (Brown et al., 1991). Animals experimentally vaccinated with live modified BVDV vaccines also showed similar results with reduced neutrophil numbers as well as functional activity (Roth and Kaeberle, 1983). However, these studies did not evaluate neutrophil functional activities like migration, phagocytosis, oxidative burst, pathogen killing ability or cytokine production. In addition, there are no studies, which compared BVDV biotypes, genotypes and virulence on neutrophil function making this study unique.

Another flavivirus, hepatitis C virus (HCV), also cause neutropenia (Yu et al. 2011, Sheehan et al. 2013) and altered neutrophil function (Hassoba et al. 2010). Neutropenia caused by HCV may be due to its infection to pluripotent hematopoietic CD34+ cell (Sansonno et al., 1998), resulting reduced neutrophil production or direct neutrophil apoptosis through caspases 3 and 10 pathways (Aref et al., 2011), or by both. Study with 24 HCV patients with hemodialysis had significantly reduced neutrophil oxidative burst activity before as well as after hemodialysis (Hassoba et al. 2010). Measurement of oxidative burst in that study after hemodialysis was performed to avoid any effect of uric acid.

West Nile virus (WNV), another flavivirus behaved differently with neutrophils. WNV increased peripheral neutrophil count as observed in case report (Lustig et al. 2016), with rapid recruitment of neutrophils at site of infection (Bai et al. 2010). WNV infected and used neutrophils to transport virus to the brain (Wang et al. 2012). Depletion of neutrophils or knockdown of Cxcr2 gene (neutrophil chemokine gene) prior to WNV resulted in reduced viremia and enhanced host survival (Bai et al.
Depletion of neutrophils after WNV infections in mice resulted in higher viremia with enhanced host mortality (Bai et al. 2010). Results of this study further strengthening that WNV used neutrophils for virus transport and disease pathogenesis.

Although in the same family, HCV and WNV infections result in different outcomes in neutrophils. Therefore, a study on effect of BVDV on neutrophil viability was done to better understand BVDV pathogenesis. The current study tried to choose the most representative BVDV strains: a virus pair (biotypes) of cp BVDV1b TGAC and ncp BVDV1b TGAN from type 1a genotypes and the highly pathogenic ncp BVDV2a 1373 and moderate pathogenic ncp BVDV2a 28508 were chosen from type 2a genotypes. However, there are still number of questions which need to be answered including the effects of BVDV on neutrophil toll-like receptors (TLR) and cytokine production, which ultimately shapes the adaptive immune response. The current study showed that highly virulent ncp BVDV2a 1373 induced more neutrophil migration, which can result in excessive tissue damage. However, the oxidative burst assay revealed that ncp BVDV2a 1373 reduced neutrophil oxidative activity. There is further need to determine the effect of BVDV on other underline mechanisms, which may lead to tissue damage from neutrophil protease activity (e.g. elastase, proteinase-3, and cathepsin G). Among the various antimicrobial neutrophil products serine proteases plays an important role in microbial killing as well as tissue damage (Kruger et al. 2015).

Additionally, in the current study, we infected macrophages and observed the neutrophils migration. It will be interesting, if we can infect neutrophils or both neutrophils and macrophages and then see the combined effect on neutrophil migration and other neutrophil functional activity.
REFERENCES


