INNATE HOST RESPONSES TO BOVINE VIRAL DIARRHEA VIRUS

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a pestivirus that suppresses the innate and adaptive host immune responses. Each of the two classified genotypes (BVDV1 and BVDV2) has two distinct biotypes – cytopathic (cp) and non-cytopathic (ncp) – and evidence has suggested that cytopathic strains may disrupt host interferon (IFN) synthesis and IFN-mediated responses. However, inconsistent results examining ncpBVDV strains have generated controversy regarding whether they also exhibit this capability. The purpose for this study was to determine the occurrence and functionality of IFN-induced responses within the serum cattle infected with ncpBVDV2-1373. Specifically, this involved analysing the changes in both the serum levels of IFN-α and IFN-γ and the expression of genes that are classically regulated by these cytokines. Serum analysis showed that the infected cattle induced both serum IFN-α and IFN-γ during BVDV infection while PBMC analysis showed increased expression of genes that classically respond to IFN-α – Mx-1, OAS-1, and STAT-1 – and IFN-γ – SOCS-1 and SOCS-3. These findings are supported by temporal kinome analysis, which verified activation of the JAK-STAT signalling network within the PBMCs of the virus-infected animals. In addition to establishing evidence for its synthesis, results from this challenge identified IFN-γ as a possible indicator of animal mortality as analysis of its change within the non-surviving, infected animals was statistically greater than the levels of the surviving, infected animals. Collectively, these results demonstrate 1373-mediated induction of, and host cell response to, both IFN-α and IFN–γ, and the potential for IFN-γ to be a predictive marker for mortality during BVDV infection.

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ABBREVIATIONS

$\gamma^{32}P$	Gamma phosphorus-32 isotope
ABVD	Acute bovine viral diarrhea
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhea virus
CARD	Caspase activation and recruitment domain
CIS-1	Cytokine-inducible SH2 protein
CPI	Congenital persistent infection
CSFV	Classical swine fever virus
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
FBS	Fetal bovine serum
HAX-1	HCLS1-associated protein X-1
HEPES	4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IKK	IκB kinase
IRAK	Interleukin-1 receptor-associated kinase 1
IRES	Internal ribosome entry sequence
IRF	Interferon regulatory factor
JAK	Janus kinase
LGP2	Laboratory of genetics and physiology 2
log2	Logarithm to base 2
logFC	Logarithm fold change (Base 2)
LPS	Lipopolysaccharide

LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MD	Mucosal disease
Mda-5	Melanoma differentiation-associated protein 5
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MYD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NK	Natural killer
OAS-1	2',5'-oligoadenylate synthetase 1
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIIKA	Platform for integrated, intelligent kinome
PKR	Protein kinase R
PNPP	Para-nitrophenylphosphate
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptor
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
Tbk-1	TANK-binding kinase 1
TBST	Tris-buffered saline and tween 20
TBST-g	Tris-buffered saline and tween 20 + 0.1% gelatin
TCV	Threshold cycle value

T_H	Helper T lymphocyte
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
UTR	Untranslated region
VSN	Variance stabilization
WBC	White blood cell
WRST	Wilcoxon rank sum test

1. INTRODUCTION

1.1 OVERVIEW OF BOVINE VIRAL DIARRHEA VIRUS (BVDV)

1.1.1 Taxonomy

Bovine Viral Diarrhea Virus (BVDV) is one of the most economically devastating pathogens in the global cattle industry. This is due to its role as a main contributor to loss and poor reproductive performance of cattle (Smirnova *et al*., 2008). BVDV is a pestivirus within the Flaviviridae family, which, based on common structural and genetic traits, is grouped among several well-known viruses including West Nile Virus, hepatitis C virus (HCV), dengue virus, and yellow fever virus. The common structural and genetic traits between these viruses include i) their non-polyadenylated, positive-sense, single-stranded RNA (+ssRNA) genome that translates into a single, precursor polyprotein; ii) the need to cleave this protein into multiple, mature structural and non-structural proteins using viral and cellular proteases; and iii) encompassing itself in an outer, glycoprotein-rich, lipid membrane obtained via budding through host cell, organelle membranes during the maturation step of its lifecycle (Neill, 2013).

Pestiviruses are currently organized into the following categories: classical swine fever virus (CSFV), border disease virus (BDV), and BVDV. BVDV is further separated into two genotypically-based groups – BVDV1 and BVDV2 – which are distinguished by the differences in their E2 and N^{pro} proteins, as well as their 5' untranslated genomic regions. Strains within BVDV2 are more virulent and tend to initiate acute infections more often than BVDV1 strains. Strains within these two genotypes can be phenotypically categorized into two biotypes on the basis of their lytic ability on epithelial tissue cultures *in vitro*: cytopathic (lytic) strains and non-cytopathic (non-lytic) strains (Smirnova *et al*., 2008; Gil *et al*., 2006). Research has further lead to the putative inclusion of several other pestiviruses, including one that initiates a clinically similar response to the other two BVDV genotypes: "Hobi-like" virus or BVDV3 (Pellerin *et al*., 1994; Bauermann *et al*., 2014, Bauermann *et al*., 2013).

The non-cytopathic and cytopathic biotypes are antigenically similar and have a nearly indistinguishable genomic sequence. Due to this similarity, cytopathic strains are thought to develop from pre-existing non-cytopathic strains via a variety of mutations that affect the

processing of the NS2/3 protein (Agapov *et al.*, 2004). Additionally, recent evidence from a thorough examination of its genome has suggested that the strains of BVDV3 are distinguishable by differences within a specific 3'-domain sequence of the non-structural protein, NS2 (Decaro *et al*., 2012).

1.1.2 Physical Description of BVDV

The structure of BVDV is comprised of the core region, which includes the viral genome; the capsid, which encompasses this genome; and the outer envelope consisting of a glycoprotein-rich lipid bilayer, which is obtained by the virus during the assembly and maturation processes of its lifecycle. This envelope makes BVDV susceptible to viral inactivation by heat, organic solvents, and detergents (Rumenapf *et al*., 1991). Despite its pleiomorphic structure, BVDV has an estimated diameter between 40 and 60 nm.

1.1.2.1 BVDV Genome

BVDV houses a 12.3-kb, +ssRNA genome that is subject to high rates of mutation, which, in addition to the high recombination rate of genotypically-different strains, is due to the poor proofreading ability of the viral replicase enzyme (Brackenbury *et al*., 2003; Smirnova *et al*., 2008). High mutation rates are evolutionarily-beneficial for BVDV as this leads to heterogeneity, a characteristic that is essential for adaptation and evasion of host immune responses (Ridpath *et al*., 2003). Structurally, the genome has a large open reading frame (ORF) that encodes all of its structural and non-structural proteins. Additionally, its 3' and 5' termini do not encode proteins, and are aptly classified as untranslated regions (UTRs) (Brock *et al*., 1992). These UTRs tend to form secondary structures that are involved in a three-way interaction with certain viral and cellular proteins, which collectively regulate the replication and transcription of the genome, as well as the translation of the ORF (Yu *et al*., 2000).

Translation is often promoted by the interaction of the 5' mRNA cap with the ribosome at the rough endoplasmic reticulum. However, the BVDV genome does not have a cap; therefore, its translation begins at an internal ribosome entry site (IRES) located within the 5' UTR. This IRES is characterised as a tertiary structure that forms a series of smaller distinct shapes known as pseudoknots that are identified as A, B, C, D1, D2, D3, D4, and D5, all of

which are recognized by cellular translational proteins. These proteins lead the ribosome to the viral +ssRNA so that it can bind and initiate translation at the correct AUG in a cap-independent fashion. Translation ultimately synthesizes a single, precursor polyprotein, which is further cleaved into twelve smaller (structural and non-structural) proteins by several cellular and viral proteases (Deng and Brock, 1992; Isken *et al*., 2004, Moes and Wirth, 2007).

1.1.2.2 BVDV Proteins and their Functions

The ORF of the BVDV ssRNA encodes four structural and eight non-structural proteins. The structural proteins include the core protein (Cap) and three envelope glycoproteins – E^{rns}, E1, and E2. The non-structural proteins include p7, NS2, NS3, NS4a, NS4b, NS5a, NS5b, and N^{pro}. As mentioned, the regions encoding NS2 and NS3 may be translated separately or as a single protein (Neill, 2013). Figure 1.1 shows the order in which these proteins are encoded on the BVDV genome from the 5' end to the 3' end.

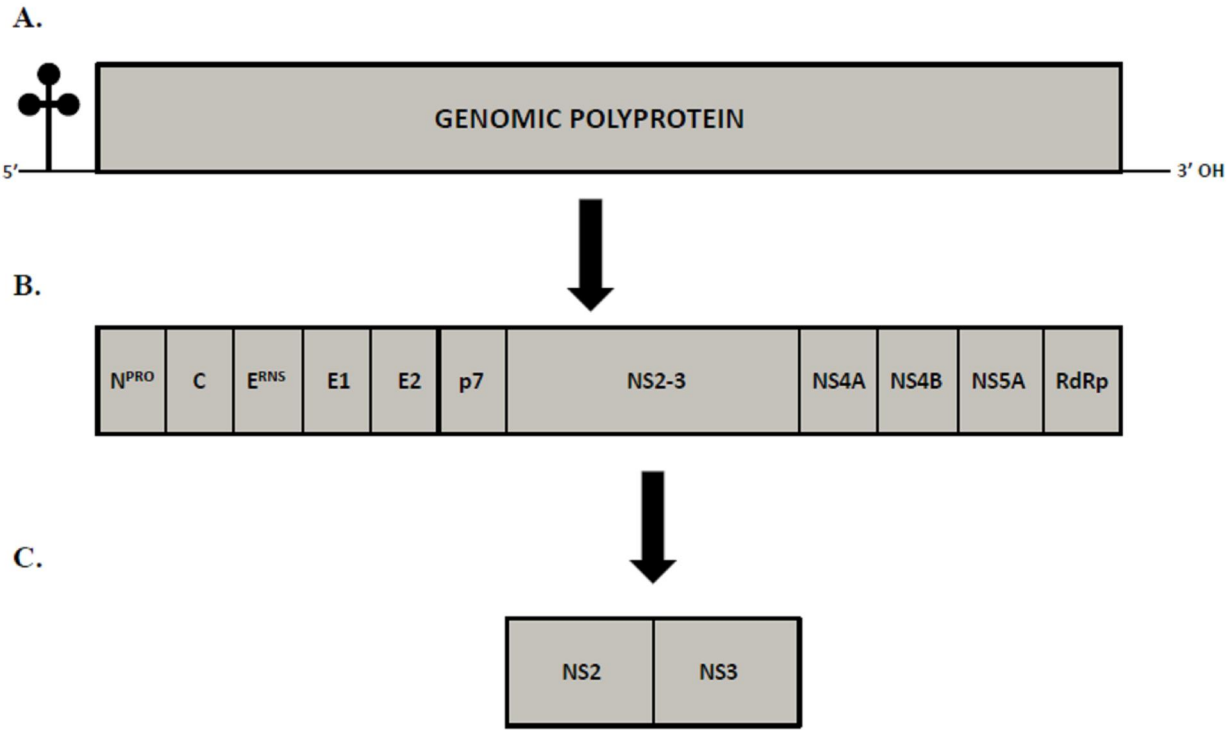

Figure 1.1: Structure and organization of BVDV genome. **A.** Physical structure illustrating the precursor polyprotein is translated from the BVDV genome. **B.** The order in which BVDV proteins are translated from the genome, including the uncleaved NS2-3 that is seen in ncpBVDV strains. **C.** The separation of NS2 and NS3 as seen in cpBVDV strains.

The structural proteins are incorporated into the virion and collectively comprise the capsid and the envelope. Cap is the main core protein that comprises the protective structure encompassing the genome. E^{rns} is a loosely-attached, glycosylated protein that is expressed as a homodimer. While it is a component of the viral envelope, E^{rns} can also be secreted as a soluble protein that likely functions in countering host immune response, a role that will be discussed in more detail later (Schneider *et al*., 1993). E1 and E2 are integral membrane glycoproteins. Little is known about E1, but recent evidence has shown that its heterodimerisation with E2 plays a crucial role in virion entry (Ronecker *et al*., 2008). Additionally, E2 is one of the main proteins which host antibodies (soluble, antigen-binding proteins) target. Evidence has shown that antibodies can efficiently halt infection by just targeting E2 (Deregt *et al*., 1998).

The non-structural proteins encompass a variety of roles that are related to BVDV's infectivity. N^{pro} is a self-protease that separates itself from the polyprotein immediately after translation and has been shown to block IRF3, a major role in interferon (IFN) suppression that will be detailed later (Chen *et al*., 2007). p7 is thought to form an ion channel; thereby, facilitate viral movement between cells, which is similar to its role in HCV. However, this suggestion is mainly based on the p7 function of HCV. Little evidence has been gathered to confirm that its function is similar in BVDV (Griffin *et al*., 2004; Harada *et al*., 2000; Neill, 2013). As mentioned, NS2 and NS3 are the major determinants in BVDV biotype formation. They are mainly unprocessed – remain fused as NS2/3 – in non-cytopathic BVDV strains, while normally being distinct in cytopathic strains. However, recent evidence suggests that NS2/3 cleavage is also required for early, non-cytopathic, viral replication; this fission rapidly dropping once sufficient viral replication has occurred (Lackner *et al*., 2004). NS4a is involved in helping to cleave NS3 while NS4b functions in reconfiguring the membranes of infected cells and associating with the RNA replication complex (Xu *et al*., 1997; Weiskircher *et al*., 2009). The function of NS5a is largely unknown, but its accumulation is thought to inhibit IRES-dependent translation (Ridpath, 2005a). Finally, NS5b is the virus's RNA-dependent RNA polymerase, which catalyzes replication of the viral genome (Gong *et al*., 1996).

1.1.3 BVDV Lifecycle

BVDV follows a stepwise lifecycle that is similar to most viruses: i) receptor-binding, ii) entry, iii) uncoating, iv) transcription, v) translation, vi) replication, vii) assembly, and viii) maturation and release.

1.1.3.1 Entry to Translation

As mentioned, E1-E2 heterodimerisation is critical for virion entry. This heterodimer initiates entry by binding to CD46, a receptor that promotes pH-dependent fusion of the viral envelope with the host cell membrane. This ultimately enables the virus to cross the host cell membrane via clathrin-dependent, receptor-mediated endocytosis. BVDV is immediately brought into the lysosome, where it uncoats and releases its genetic material into the cell to be translated (Grummer *et al*., 2004; Maurer *et al*., 2004; Krey *et al*., 2006; Ronecker *et al*., 2008; Xue *et al*., 1997; Zurcher *et al*., 2014).

1.1.3.2 Replication

Like other +ssRNA viruses, BVDV often replicates following an initial round of transcription and translation. Its genome serves as the template on which replication is initiated. A secondary structural motif located within the 5' UTR serves to transfer the function from transcription to replication (Yu *et al*., 2000). As mentioned, the increase in NS5A and NS5B proteins additionally serves to prevent further translation. A viral replicase complex is assembled to mediate replication and requires the use of multiple, non-structural proteins (Ridpath, 2005). Following translation, these proteins form into this replicase structure at the 3' genome terminus in order to catalyze the transcription of the genome into a –ssRNA structure, which is used as the template off of which to replicate the BVDV genome, catalyzed via RNA-dependent RNA polymerase; occurring within a membrane structure (Gong *et al*., 1998).

1.1.3.3 Assembly and Maturation

Following replication, assembly and maturation take place within intracellular vesicles at either the endoplasmic reticulum or the Golgi apparatus. The lipid-based envelope is gathered via

vesicle budding at one of these organelles. Ultimately, virions are released via exocytosis, nearly eight hours post-infection (Grummer *et al*., 2001; Nuttall, 1980).

As protein synthesis precedes replication, BVDV stimulates expression of host genes that function in translation and post-translational modification as a mechanism that favours its replication and continued presence in the host. Additionally, it reduces expression of host genes involved in cell structure and energy production; therefore, prevents the host from responding to the virus before it can amplify its genome (Neill and Ridpath, 2003). BVDV can be identified within infected cell cultures between 4 and 6 hours post-infection, with peak titres between 12 and 24 hours post-infection (Gong *et al*., 1996).

1.1.4 Mechanisms of Infection

BVDV maintains its status as a successful global, infectious pathogen due to its ability to utilize a variety of mechanisms to infect its host. Like other viruses, BVDV's primary objectives are to evade the host immune response, establish persistence within the host, and potentiate its lifecycle. To successfully spread and persist within a host population, viruses must be able to alter their infection mechanisms in response to altering host factors that include the host's generation time, population, and mobility (Schweizer *et al*., 2006). BVDV's global success is primarily due to its ability to adjust its infectious mechanism in response to these ever-changing factors. These mechanisms are organized within two general strategies of host infection: the hit-and-run strategy and the infect-and-persist strategy (Peterhans and Schweizer, 2012).

The hit-and-run (or transient infection) strategy is used for the rapid synthesis of new viral particles and is commonly observed in such viruses as Ebola and influenza. It is epidemiologically-characterised by the rapid dissemination of infection within a population during a short period of time and is commonly utilized by viruses that do not have efficient mechanisms to evade the host's immune response. Hit-and-run is adequate if the host population is large, the host has a short generation time, or the host is mobile. However, it is inadequate if the virus cannot infect a new host following the death of the original host; therefore, cannot continue its lifecycle. This strategy will end in either the death of the host or the elimination of the virus and acquisition of host immunity (Schweizer *et al*, 2006). Cytopathic BVDV strains are more likely to initiate a hit-and-run infection, the host's survival

against which will depend on the strength of its immune system. Immunocompetent cattle can usually eliminate a strain establishing hit-and-run within 10 – 14 days while those cattle with undeveloped immune systems – including fetal calves – will more likely die when BVDV infects in this fashion (Desport *et al*., 1998).

To avoid the risk of being unable to spread to a new host, viruses may employ an infect-and-persist strategy, which is commonly utilized by such pathogens as herpesviruses and lentiviruses. For this mechanism to succeed, viruses must evade the host's immune system, often invoking elaborate mechanisms to do this to ensure they are not eliminated from the host (Alcami and Koszinowski, 2000; Finlay and McFadden, 2006). The known mechanisms by which BVDV avoids the immune system – which will be discussed later – are mainly based on how it successfully prevents IFN from initiating an anti-viral state. BVDV mainly enters its hosts via the infect-and-persist strategy, the non-cytopathic strains being those most likely to establish a persistent infection, which accounts for the greatest number of clinical cases (Peterhans *et al*., 2003; Peterhans and Schweizer, 2013). Non-cytopathic BVDV strains establish the greatest population of reservoirs by infecting fetal calves. When a pregnant dam is infected, her immune system may eliminate the infection, but if a virus infects the fetus prior to day 150 – prior to the fetus developing a competent immune system – the fetus will likely gain immunotolerance to the virus. As such, it will not eliminate BVDV even after it develops its immune system; therefore, it will be born a viral reservoir, continuously shedding viral progeny throughout its life (Desport *et al*., 1998; Smirnova *et al*., 2009; Stokstad and Loken, 2002).

Currently, the best viral management practice in preventing the entry of infected calves into livestock herds is to remove infected, pregnant animals. Future development of therapeutics against BVDV may focus on eliminating the virus from infected fetal calves. However, methods to effectively identify infected calves within dams that have already developed immunity to the virus need to be improved.

1.1.5 Transmission

BVDV transmission occurs in one of two general patterns: vertical and horizontal. Vertical transmission (transmission between mother and calf) often initiates the infect-and-persist strategy while horizontal transmission (transmission between animals of the same generation)

generally follows a hit-and-run strategy. Four primary components affect transmission: infectivity (dosage and infectious route); form of external contact; period of infection; and the number of susceptible animals, which declines as hosts develop immunity to the virus (Haloran, 1998; Thurmond, 2005).

1.1.5.1 Forms of Transmission

Vertical transmission often occurs when a dam that's persistently infected by a non-cytopathic strain, passes the virus to the fetus when she experiences an acute infection. When fetuses are infected prior to day 150 of gestation, which is before their immune systems have developed, they will normally survive. However, this is not because they eliminate the virus but because they often just become immunotolerant to the virus (Brock *et al*., 2005). Fetal infections that occur before day 150 of gestation will often result in abortion, stillbirth, congenital defections, or stunted growth; but in some cases, the calf will be born with a normal, healthy appearance (Thurmond, 2005). Upon birth, persistently infected animals are known to intermittently shed high quantities of virus throughout their lifetime. This is in contrast to fetal infection after day 150 of gestation: the fetus typically has a competent immune system that is strong enough to eliminate the virus (Brock *et al*., 1991). Horizontal transmission often leads to an acute infection, the severity of which can range from asymptomatic to fatal. Animals acutely infected will temporarily release BVDV for roughly 7 days post-infection (Niskanen *et al*., 2000).

1.1.5.2 Means of Transmission

Infected animals can shed the virus via tears, saliva, urine, feces, nasal discharges, semen, and milk (Brock *et al*., 1991; Thurmond, 2005). Susceptible cattle may also become infected via fomites released by BVDV-infected cattle undergoing a respiratory infection (Baule *et al*., 2001). While these natural direct routes are prevalent, transmission may also occur via other sources, such as insect vectors and people. Flies can become BVDV reservoirs if they bite an infected cow (Tarry *et al*., 1991). Additionally, researchers and clinicians contribute to transmission via iatrogenesis – infection during medical examination or treatment. This often occurs when cattle are exposed to contaminated medical equipment (Houe, 1995).

1.1.6 Clinical Presentation

Clinical responses to BVDV widely vary and often depend on the time at which infection occurred and the biotype. Common symptoms include anorexia, pyrexia, diarrhea, enteric disease, upper respiratory disease, transient immune suppression, and fetal abortion (Bauermann *et al*., 2014; Smirnova *et al*., 2009). Several, few, or none of these symptoms may appear following infection, which will lead to one of three outcomes – congenital persistent infection (CPI), mucosal disease (MD), or acute bovine viral diarrhea (ABVD); the severity of any of these outcomes is dependent on the viral strain, environmental stresses, and the presence of opportunistic pathogens (Desport *et al*., 1998; Smirnova *et al*., 2009)

CPI manifests postnatally following a non-cytopathic, prenatal infection of a calf prior to day 150 of gestation. Some newborns exhibit growth retardation and a general inability to function normally while others remain asymptomatic and healthy for years. MD also manifests postnatally, but as a result of a prenatal infection of both non-cytopathic and cytopathic strains, which is termed a "mixed" infection. If MD manifests acutely, the infected animal will likely exhibit many of the aforementioned symptoms; notably exude excessive saliva and mucous due to the formation of ulcers within its nose and mouth. In severe cases, the animal will die within days of clinical onset. However, MD may also manifest chronically; in which case, animals often exhibit lameness, persistent weight loss, intermittent bouts of diarrhea, and respiratory disease. These animals will often succumb to opportunistic pathogens following the weakening of their immune systems instead of the virus itself. Finally, ABVD results from a postnatal infection by either a non-cytopathic or cytopathic strain. ABVD often results from a hit-and-run infection during which the animal will exhibit a potent and notable clinical response. The animal will either recover within a two-week period or succumb to the infection (Bolin, 2002).

1.1.7 BVDV Entry and Cellular Targets

BVDV is naturally transmitted via the oronasal passages. It replicates within both the mucous and the tonsils and disseminates through the body via leukocyte circulation (Bruschke *et al*., 1998). Leukopenia (decrease in white blood cells), lymphoid reduction, and immunosuppression are hallmarks of acute BVDV infection; as mentioned, increase the susceptibility of the animal to secondary infections. Immunosuppression is partially mediated

via reducing the total leukocyte number, but also by inhibiting various functions of leukocytes – typically lymphocytes and macrophages. These functions include reducing their chemotaxis and impairing both their bacterial killing and their responsiveness of innate immune sensory systems (Baigent *et al.,* 2004; Charleston *et al.,* 2001; Glew *et al.,* 2003; Jensen and Schultz, 1991; Kapil *et al*., 2005; Lamontagne *et al*., 1989; Liu *et al*., 1999; Potgieter, 1995; Schweizer *et al*., 2006).

While BVDV often infects during a point in fetal gestation prior to immune system development, it must overcome immune barriers in order to successfully infect calves after the immune system matures. Considerable research has been devoted to characterising the interactions between BVDV and the host; in particular, on interferon (IFN)-mediated defenses. As viruses generally induce IFN and an IFN-mediated response, this focus is not surprising, but there has been ongoing debate regarding the extent to which BVDV influences this response, particularly with respect to non-cytopathic BVDV strains.

The immune system is an interactive network of cells, chemicals, and barriers that is involved in protecting the host from foreign substances. It is separated into two components: the ever-present, unlearned, nonspecific, innate immune system and the learned, specific, adaptive immune system (Chaplin, 2010; Parkin and Cohen, 2001). Before discussing the current knowledge of BVDV-host interaction, it is important to first review the immune system and discuss the role IFNs play in host defense.

1.2 IMMUNE RESPONSE

1.2.1 Overview of the Immune Response

The innate immune system is the first line of defense and is comprised of physical and microbiological barriers; chemical effector molecules; and a variety of cells that collectively mediate inflammation, directly kill pathogens via phagocytosis, and coordinate additional immune responses through the release of cytokines (chemical messengers). Neutrophils, macrophages, and dendritic cells (DCs) are the primary cellular mediators of the innate immune system, which collectively phagocytose foreign substances and infected cells, release pro-inflammatory cytokines, and activate the adaptive immune system through antigen presentation.

DCs liaise between the two major forms of immunity by stimulating adaptive immune cells using degraded antigens originally processed by innate immune cells. DCs present degraded antigens via surface identity markers – major histocompatibility complexes (MHCs) – to T lymphocytes, a class of immune cells that facilitate the destruction of intracellular pathogens in a response known as "cellular immunity". Distinct subclasses of T lymphocytes perform different functions. While more exist, cytotoxic T lymphocytes (CTLs) and helper T (T_H) lymphocytes are the main agents of cellular immunity. CTLs eliminate pathogen reservoirs – virus- or bacteria-infected cells – particularly those that evade macrophage destruction. T_H lymphocytes stimulate further immune responses by releasing a series of cytokines that activate additional T lymphocytes, additional macrophages, and B lymphocytes (a population of adaptive immune cells) that mediate elimination of extracellular antigens through the release of antibodies. IFN-γ is a pivotal cytokine released by T_H lymphocytes that is involved in eliminating intracellular pathogens (Chaplin, 2010; Parkin and Cohen, 2001).

1.2.2 Activation of Innate Immunity

1.2.2.1 Innate Immunity – Detecting Foreign Substances

The innate immune system utilizes several structures within and on the surfaces of cells to detect pathogens and other foreign substances. These structures – pattern recognition receptors (PRRs) – detect common structural elements that are associated with one or more groups of pathogens – pathogen-associated molecular patterns (PAMPs). Effectively, PRRs do not specifically identify microbial, viral, or fungal pathogens, but rather identify structures to which these pathogens would commonly exhibit or release. Upon identification, PRRs act as alarms for host cells and signal the host cells to initiate an immune response.

There are four known categories of PRRs. The first two are the Toll-like receptors (TLRs) and the C-type-lectin receptors (CLRs), both of which are bound to extracellular or intracellular host cell membranes. The other two are the RIG-I-like receptors (RLRs) and the NOD-like receptors (NLRs), both of which are located within the cytoplasm. When these receptors detect a foreign pattern – a potential host cell threat – they initiate signalling pathways that lead to the synthesis and release of cytokines that respond to the detected threat. As TLRs and RLRs are essential for viral detection, it will be these structures on which this thesis will

focus (Daigle *et al*., 2014; Kumagai and Akira, 2010; Medzhitov and Janeway, 2000; Mogensen, 2009; Takeuchi and Akira, 2010; Vance *et al*., 2009).

1.2.2.2 Toll-like Receptors

TLRs are primarily located on the surfaces of the extracellular plasma membrane and the internal endosomal membranes of monocytes, macrophages, DCs, and murine B cells. Their distribution across cellular locations and types ensures that the immune system maintains constant surveillance for extracellular and intracellular threats (Meylan *et al*., 2006). Researchers have so far identified 13 TLRs within mammalian species and up to 20 when including non-mammalian species (Hansen *et al*., 2011).

Structurally, TLRs are type-I-membrane glycoproteins that are divided into an extracellular, leucine-rich repeat (LRR) domain; a transmembrane domain; and a cytosolic, Toll-interleukin-1 receptor (TIR) homology domain. The LRR interacts with and identifies PAMPs of both viruses and bacteria. This interaction triggers the TLR to send a signal through to its TIR domain, which transmits this signal via of a kinase-mediated pathway through the cytoplasm to ultimately induce the transcription of genes that are involved in mediating an immune response. When the initial host cell detects a viral PAMP, most of the responsive genes are translated into proteins that are involved in mediating inflammation of the currently infected cell, as well as the neighbouring cells, in a process called "anti-viral state initiation" (García-Sastre and Birion, 2006; Peterhans and Schweizer, 2013).

As mentioned, several TLRs have been identified, which collectively respond to viral and bacterial PAMPs. The classic TLR4 recognizes bacterial lipopolysaccharide (LPS) at the extracellular plasma membrane. Other bacterial-recognizing PAMPs include TLR-1, -2, and -6, which recognize lipoteichoic acid and bacterial lipoproteins; TLR-5, which recognizes bacterial flagella; and TLR-9, which recognizes hypomethylated CpG DNA of both bacteria and viruses. TLRs that primarily recognize viral patterns include TLR-3, which recognizes dsRNA; and both TLR-7 and -8, which recognize ssRNA. As such, TLR-7 and -8 would be the most important receptors in identifying BVDV (Aderem and Ulevitch, 2000; Kumar *et al*., 2009; Mogensen, 2009).

TLRs -1, -2, -4, -5, and -6 are expressed at the extracellular plasma membrane while TLRs -7, -8, and -9 are expressed on the internal endosomal membrane. TLR-3 may be found

at both sites. While the TLRs that detect bacterial components recognize patterns that would not be found within the host, the nucleic-acid detecting TLRs identify patterns that the host cell might produce, such as RNA. This conflict in identifying which ssRNA structures are associated with the host or a pathogen is primarily reduced by the endosomal location of the TLRs. Host cell nucleic acid is not normally found within compartmentalized organelles, except for the nucleus. Furthermore, host nucleic acids are often methylated, which discriminates them from non-host nucleic acids (Bauer, 2006; Hornung *et al.*, 2008; Ishii and Akira, 2005; Iwasaki and Medzhitov, 2004; Karikó *et al*., 2005; Vercammen *et al*., 2008). Despite its source, TLR stimulation by ssRNA initiates a strong cytokine response, particularly an IFN response that further activates secondary immune responses via a well-defined signalling network that exists in both natural killer (NK) cells and T lymphocytes, the details to which will be discussed later (Bekeredjian-Ding *et al.*, 2005).

1.2.2.3 RIG-I-like Receptors

Working along with TLRs are the RLRs, RNA helicases that exist within the cytoplasm to observe and identify viral ssRNA and dsRNA (Kawai and Akira, 2009). Three RLRs have so far been identified and include retinoic acid-inducible gene (RIG-1), melanoma differentiation associated factor 5 (mda-5), and laboratory of genetics and physiology 2 (LGP2). Structurally, they all have PAMP-binding, C-terminal repressor domains; RNA helicase domains; and with the exception of LGP2, two caspase recruitment domains (CARDs). RLRs broadly exist at low levels but particularly within myeloid, epithelial, and central nervous tissues. Expression of these molecules drastically increases upon stimulation by viral nucleic acids or IFN (Mogensen, 2009; Wilkins and Gale, 2010; Andrejeva *et al*., 2004; Yoneyama *et al*., 2004).

While these three receptors recognize non-self RNA, they have their own "preferences" in that they often interact with RNA based on the RNA's characteristics. RIG-I preferentially identifies foreign ssRNA that is 5'-triphosphosphorylated (5'ppp) at the ends. Furthermore, RIG-I preferentially interacts with shorter dsRNA pieces with respect to mda-5 (Kato *et al*., 2008). This makes RIG-I comparatively more important than mda-5 in identifying BVDV. Finally, LGP2 recognizes the terminal ends of dsRNA, interacting with the foreign nucleic acid in a similar manner to RIG-I and mda-5. While further research still needs to be performed to confirm its role, current understanding suggests LGP2 changes the structural conformation of

foreign RNA in such a manner that when the altered RNA subsequently interacts with the other two RLRs, it enhances the activation of their signalling functions (Reikine *et al*., 2014). As with TLRs, RLR stimulation activates a strong cytokine response, which is initiated when the PAMP signal is carried through a well-defined signalling pathway that upregulates a series of inflammatory and IFN-stimulated genes (ISGs).

1.2.2.4 From PRR to Immune Response

While they signal through different intermediary proteins in their cascades, they target the same downstream transcription factors: AP-1, IRF3/7, and NF-κB (Peterhans and Schweizer, 2013). For this section, the TLR signalling discussion will be restricted to TLR7/8 due to its relevance to BVDV.

When foreign RNA interacts with TLR7/8, their extracellular domains homo- or heterodimerise and then send an initial signal through the TIR domain. This initiates recruitment of the universal adaptor protein, myeloid differentiation primary response gene 88 (MyD88), to that site. After recruitment, MyD88 interacts with downstream interleukin receptor-associated kinases (IRAKs) 1 and 4 to form a large "signalling tower". This tower amplifies the initial signal by sending it to numerous downstream proteins (ligases, proteases, and kinases) to ultimately activate the previously mentioned transcription factors (Baccala *et al*., 2009; Kumar *et al*., 2009; Lin *et al*., 2010; Mogensen, 2009; Wasserman, 2010).

RLRs activate these transcription factors through a signalling cascade independent of TLRs 7 and 8. While further research is still needed to confirm the role of LGP2, RIG-I and mda-5 have been shown to initiate a signalling cascade following interaction with foreign RNA. Upon stimulation, the CARD domains of RIG-I and mda-5 interact with the adaptor protein, mitochondrial antiviral signalling protein (MAVS), located on the extracellular mitochondrial membrane, as well as on cellular peroxisomes. While the mechanism by which peroxisomal MAVS mediates downstream signalling is still unknown, mitochondrial MAVS aggregates following this RLR-interaction and potentiates a downstream signalling cascade. The first set of kinases to be signalled are MAPK, IKK, and Tbk-1 (or IKKε), which respectively activate AP-1, NF-κB, and IRF3/7 (Moresco and Beutler, 2010; Satoh *et al*., 2010; Seth *et al*., 2005; Kawai *et al*., 2005; Dixi *et al*., 2010; Sharma and Fitzgerald, 2010; Hou *et al*., 2011).

Whether BVDV ssRNA is sensed by TLRs, RLRs, or both types of PRRs, the three common transcription factors promote the expression of important genes that are involved in the initial immune response. These include an assortment of pro-inflammatory cytokines, as well as cytokines that initiate an antiviral state: the type I interferons. Figure 1.2 shows the TLR7/8 and RLR pathways, with reference to stimulation by BVDV ssRNA.

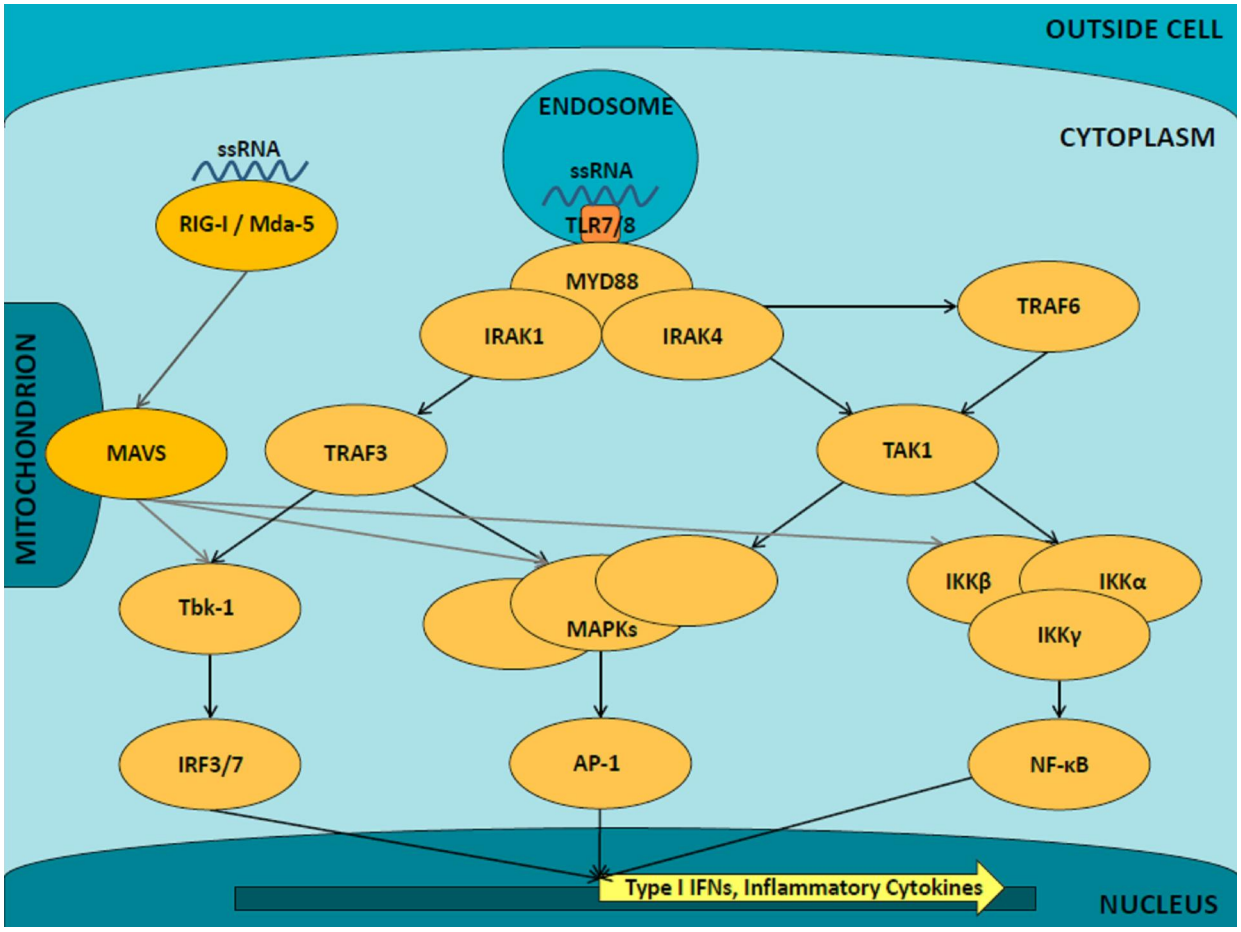

Figure 1.2: Signalling cascades initiated by TLR7/8 and RLRs (RIG-1 and Mda-5). When ssRNA interacts with an RLR, the receptor will activate mitochondrial MAVS, which stimulates multiple kinase signalling intermediates that can activate transcription factors IRF3/7, AP-1, and NF-κB to transcribe type I IFNs or inflammatory cytokines. When ssRNA interacts with TLR7/8, it initiates the formation of a "signalling tower" that's comprised of MYD88, IRAK1, and IRAK4, which activates TRAF3, TRAF6, and TAK1 to stimulate the same kinase intermediates to activate IRF3/7, AP-1, and NF-κB and transcribe the same genes as the RLR signalling cascade.

1.2.3 Cytokines and the Interferons

Cytokines are essential components of the immune system and ensure efficient immune cell interaction. These molecules are mainly first messengers, which bind to cell surface receptors and initiate a signalling cascade. This cascade ultimately leads to the stimulation of genes, which translate to effector proteins, including additional cytokines. As mentioned, viral PAMPs also stimulate surface or internal receptors that lead to increased expression of many of these genes, but also the initial cytokines that coordinate the innate immune response (García-Sastre and Biron, 2006; McNab *et al*., 2015; Schneider *et al*., 2014). The most influential cytokines involved in the response to BVDV and other viruses are the interferons.

"Interferon" is the name for a group of cytokines that, as the name suggests, interfere with or reduce the ability of viral pathogens to potentiate their infections. Three groups of IFNs – type I, type II, and type III – have so far been discovered. Type I IFNs – the best defined of which are IFN-α and IFN-β – are classically synthesized by virus-infected cells. Type II IFN – also called IFN-γ – is synthesized by select immune cells involved in cellular immunity. Type III IFNs – the IFN-λ cytokines – are also synthesized by virus-infected cells, but by a more limited cell population than type I IFNs. This thesis will focus on the synthesis and the action of type I and type II IFNs.

1.2.3.1 Type I IFNs

Type I IFNs are often the first cytokines synthesized during viral infection. As mentioned, viral nucleic acids stimulate TLRs and RLRs, which then activate AP-1, NF-κB, and IRF3/7. These transcription factors cross the nuclear membrane and initiate type I IFN up-regulation. Upon translation, type I IFNs initiate a series of biologic actions that are meant to establish an "antiviral state". This antiviral state is not established because these cytokines are directly antiviral, but rather because they activate genes that initiate an antiviral response. Following their syntheses, type I IFNs are released by the infected cell and bind to receptors on the same infected cell and on adjacent cells to activate these genes in an autocrine and paracrine manner, respectively (Abbas and Lithman, 2005; Bonjardim, 2005; García-Sastre and Biron, 2006; Goodbourn *et al*., 2000; McNab *et al*., 2015; Randall and Goodbourn, 2008, Samuel, 2001). The signalling kinases through which these genes are activated are within the JAK-STAT

pathway, the details of which will be discussed later. Researchers have identified more than 100 genes that respond to type I IFNs. Of these, the most well-known include myxovirus-1 (Mx-1), protein kinase R (PKR), and 2',5'-oligoadenylate synthetase (OAS)-1. These interferon-stimulated genes (ISGs), once synthesized, are released to initiate autocrine and paracrine signalling, which collectively limits the spread of infection by inhibiting viral replication and translation (García-Sastre and Biron, 2006; Schneider *et al*., 2014; Taniguchi and Takaoka, 2002). While their role in inhibiting the viral lifecycle is paramount, type I IFNs have other functions that strengthen immune response by linking both innate and adaptive immune components.

Type I IFNs exert their effects on well-known immune cell populations, including myeloid cells, DCs, B lymphocytes, T lymphocytes, and NK cells. Their contribution to enhancement of the adaptive immune response begins by activating immature DCs. Type I IFNs increase the expression of class I MHC molecules and such co-stimulatory molecules as CD80 and CD86. Collectively, this increases the DCs' abilities to stimulate CTLs, which increases the ability of the latter cell population to destroy virus-infected cells (Hahm *et al*., 2005; Ito *et al*., 2001; Montoya *et al*., 2002). Furthermore, DCs promote both CTL and T_H lymphocyte responses. Type I IFNs enhance the ability of T_H lymphocytes to increase the survival and the clonal expansion of B lymphocytes, as well as promote their differentiation into T_H1 lymphocytes and the survival of NK cells, both of which produce IFN-γ (Brinkmann *et al*., 1993; Le Bon *et al*., 2006; McNab *et al*., 2015). Finally, IFN-α mediates the accumulation of lymphocytes around the lymph nodes, thereby increasing the likelihood of lymphocyte stimulation by antigens within the nodes (Welsh *et al*., 2012).

Overall, type I IFN responses consist of two components: induction and activity. As these cytokines are so important in both preventing virus spread and bolstering immune responses, viruses must develop ways to prevent either their induction or their biologic actions. Evidence has demonstrated that BVDV invokes various mechanisms to prevent both, methods which will be further discussed. In addition to type I IFNs, another important IFN is involved in countering viral infection by promoting adaptive immune responses and bolstering innate immune responses – IFN-γ.

1.2.3.2 IFN-γ

IFN-γ is a homodimeric protein that is an important mediator of innate immunity and (adaptive) cellular immunity. It is not specifically an antiviral cytokine, but its biologic actions are crucial to antiviral immune response. This cytokine is primarily released by CTLs, and T_H1 lymphocytes, but tends to first be released by NK cells. The main status of IFN-γ is as the immune system's primary macrophage-activating messenger, increasing the microbicidal ability of macrophages by stimulating them to transcribe genes that encode the enzymes responsible for activating nitric oxide and various reactive oxygen intermediates (Bonjardim, 2005; Goodbourn *et al*., 2000; Lin and Young, 2013; Schroder *et al*., 2004).

IFN-γ stimulates a variety of immune cells to promote various functions. Firstly, IFN-γ promotes the expression of both MHC molecules and co-stimulators on DCs to improve their ability to present antigens to naïve T lymphocytes; thereby, hasten adaptive immune response initiation (Cook *et al*., 1992, Schroder *et al*., 2004). Upon antigen recognition, IFN-γ enhances the activities of both T and B lymphocytes. More important to the viral response, IFN-γ induces differentiation of T cells to T_H1 cells, a move that's enhanced by the release of IL-12 – another T_H1 promoting agent – by IFN-γ-activated macrophages (Schmitt *et al*., 1994). Studies using murine cell lines have shown that IFN-γ also increases expression of the IL-12 receptor (Yoshida *et al*., 1994). Furthermore, IFN-γ promotes isotype switching of B cells to specific subclasses that activate the complement cascade (a series of successively activated proteins that surround and bind (opsonise) infected cells to promote their phagocytosis) (Hasbold *et al*., 1999). These effector functions collectively promote the cell-mediated destruction of virus-infected cells.

Like type I IFNs, IFN-γ signals through the JAK-STAT pathway to activate a variety of gamma-activated sequence (GAS) genes, one of the most well-known being IRF1, a transcription factor responsible for, among other things, further activating Type I IFNs. Additional genes transcribed include signal transducer and activator of transcription (STAT)-1, a component of the JAK-STAT pathway; and suppressors of cytokine signalling (SOCS), proteins that repress IFN-γ signalling through a negative feedback loop (Lehtonen *et al*., 1997). Given their collective importance in the viral immune response, suppression of both type I IFNs and IFN–γ would be pivotal in order for BVDV to successfully infect its host. In addition to

preventing their interaction with immune cell receptors, BVDV could also target components of the JAK-STAT pathway to restrict cytokine signalling.

1.2.4 JAK-STAT Signalling Pathway

1.2.4.1 Components of IFN Signalling

IFNs transfer their signals through a series of STAT proteins that reside within the cytoplasm. They are phosphorylated by *Janus* kinases (JAKs) following cytokine-receptor interaction. The four known JAKs include JAK-1, JAK-2, TYK-2, and JAK-3, and the seven known STATs include STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b, and STAT-6. Collectively, JAK-1, JAK-2, and TYK-2 are involved in mediating IFN-α- and IFN-γ-dependent responses, utilizing STAT-1 and STAT-2 to further transmit the cytokines' signals (Shuai *et al*., 2003).

IFN-α binds to a receptor that consists of two subunits, IFNAR1 and IFNAR2, which cross-phosphorylate and then interact with TYK-2 and JAK-1, respectively. The JAKs phosphorylate STAT-1 and STAT-2, which dimerise and translocate into the nucleus to activate ISGs (Welsh *et al*., 2012). IFN-γ exerts a similar response. The receptor to which the cytokine binds consists of IFNGR1 and IFNGR2, which cross-phosphorylate and then interact with JAK-1 and JAK-2, respectively. The JAKs phosphorylate two STAT-1 proteins, which dimerise and translocate to the nucleus to activate GAS genes (Hilton *et al*., 1998; Samuel, 2001). Figure 1.3 shows the specific pathways within the JAK-STAT network that are utilized following the binding of IFN-α or –γ.

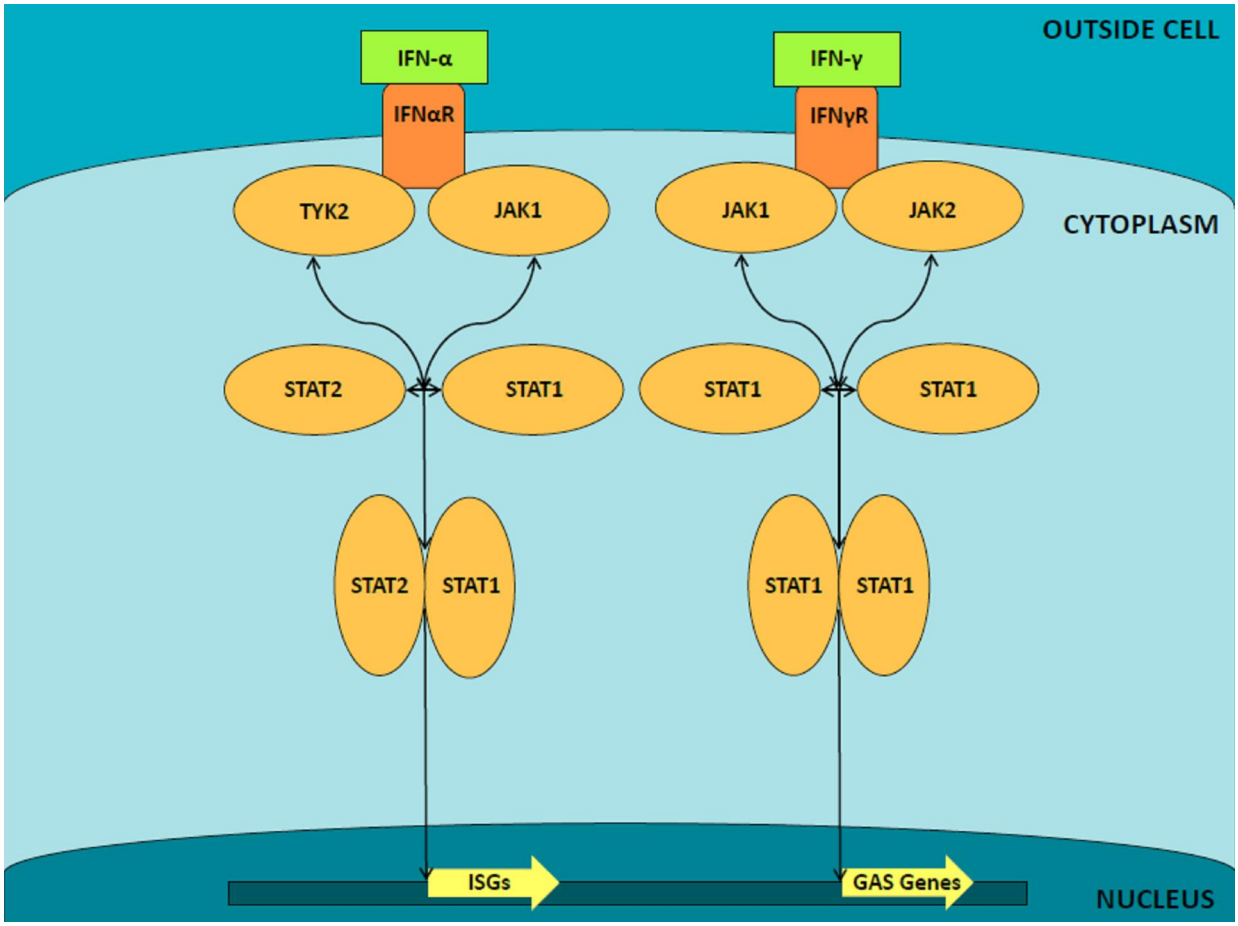

Figure 1.3: IFN-α and IFN-γ signalling through the JAK-STAT signalling network. IFN-α binds to two receptors that cross-phosphorylate and then interact with TYK-2 and JAK-1, which phosphorylate and initiate the dimerisation of STAT-1 and STAT-2, two transcription factors that stimulate the up-regulation of various ISGs. IFN-γ binds to two receptors that interact with JAK-1 and JAK-2, which phosphorylate and initiate the dimerisation of two STAT-1 proteins that stimulate the up-regulation of various GAS genes.

1.2.4.2 Negative Regulation of IFN Signalling: SOCS

Several cellular and viral proteins have been identified as JAK-STAT pathway negative feedback regulators. A well-studied group includes SOCS, the roles of which are to reduce the likelihood of uncontrolled inflammation. There are eight members of the SOCS family: cytokine-inducible SH2 protein (CIS) and SOCS-1 through -7. These inhibitory proteins all share two common features: a central SH2 domain and a conserved, 40-mer, C-terminal motif, which is known as the "SOCS box" (Hilton *et al*., 1998; Samuel, 2001).

SOCS-1 and -3 are classic negative feedback regulators, repressing IFN-γ signalling after their expression is increased by IFN-γ. Evidence suggests that both SOCS-1 and -3 repress the JAK-STAT pathway at the intracellular domain of the cytokine's receptor (Samuel, 2001; Larsen and Ropke, 2002). As mentioned, JAK-1 and JAK-2 phosphorylate the two intracellular IFNGR domains after IFN-γ binds; the intracellular domains phosphorylate two STAT-1 proteins, which dimerise and cross the nuclear membrane to initiate transcription of SOCS-1 and -3, which after translation, bind at or near the intracellular side of the receptor and prevent the IFN-γ signal from initiating further STAT-phosphorylation. SOCS-1 interacts directly with JAK-2 while SOCS-3 interacts with the IFN-γ receptor at the phosphorylation site. Evidence has suggested that SOCS-3 may also interact with STAT-1 to reduce JAK-STAT signalling.

Given their role in inhibiting the JAK-STAT pathway, manipulation of SOCS could be advantageous to a viral pathogen. A virus that can control the expression of SOCS could also control the host cell immune response. In fact, recent studies have shown that hepatitis C virus (HCV) – a pathogen within the same family as BVDV – independently induced the expression of SOCS-3 (Persico *et al*., 2007). These results were gathered from experiments in which the HCV core protein was overexpressed in a hepatocyte cell line. Overexpression of these viral proteins caused increased SOCS-3 production, which inhibited IFN-α-signalled-STAT-1 activation, STAT-1 nuclear translocation, and STAT-1 DNA-binding (El-Saadany *et al*., 2013).

Extensive evidence has shown that other viruses also initiate mechanisms that induce strong SOCS expression and inhibit antiviral immune response (Akhtar and Benveniste, 2011). As such, analysing both SOCS-1 and -3 is important to see if BVDV utilizes these proteins to reduce antiviral immune responses.

1.3 BVDV-HOST INNATE IMMUNE INTERACTION

BVDV's global success is credited to its ability in altering the mechanisms through which it establishes infection. While more virulent strains commonly initiate acute infections, most strains, particularly non-cytopathic strains, typically establish persistent infections during fetal development. BVDV has a unique and remarkable ability to bypass the adaptive immune system by gaining immunotolerance to both B and T lymphocytes, which is in addition to its ability to subvert components of the innate immune system. Importantly, its subversion of the innate immune system involves inhibiting the induction of IFN (Schweizer and Peterhans, 2001).

The IFN response is separated into two components: i) induction of IFN synthesis; and ii) activity of IFN. This is an important distinction as IFN primarily guards uninfected cells against viral infection. Most viruses, particularly those that primarily establish persistent infections, have developed mechanisms to inhibit induction of IFN; by extension, induction of an antiviral state. These range from inhibiting the production of IFN, the activity of IFN, or the activity or expression of ISGs and their effector proteins. Research has elucidated distinct mechanisms through which BVDV is able to avoid inducing IFN-α.

1.3.1 Controversy Regarding IFN Induction

Extensive debate has been generated regarding BVDV's influence on the IFN defense system. In particular, while there is consensus on the tendency of cpBVDV strains to activate IFN responses, there are conflicting reports on whether similar activation of IFN-mediated responses occurs during infection by non-cytopathic strains. Early *in vitro* infection models indicated that ncpBVDV isolates did not induce type 1 IFN (Adler *et al*., 1997; Baigent *et al*., 2002; Diderholm *et al*., 1966; Glew *et al*., 2003; Nakamura *et al*., 1995; Schweiser *et al*., 2006) or block the induction of IFN by double-stranded RNA or other viruses (Baigent *et al*., 2004; Rossi *et al*., 1980; Schweiser *et al*., 2001). Furthermore, it was demonstrated that fetal challenge with cpBVDV results in type I IFN production, whereas ncpBVDV does not, suggesting the ability of ncpBVDV to inhibit the induction of type I IFN to enable the virus to establish persistent infection early in fetal gestation (Charleston *et al*., 2001). In contrast, both *in vitro* (Weiner *et al*., 2012) and *in vivo* (Brackenbury *et al*., 2005; Charleston *et al*., 2002;

Müller-Doblies *et al*., 2004; Smirnova *et al*., 2008; Palomares *et al*., 2013; Palomares *et al*., 2014) acute infections with ncpBVDV induced a type I IFN response in cattle.

While this debate regarding induction of IFN has persisted, there seems to be a consensus that ncpBVDV does not interfere with the biologic activity of IFN. This was assessed by treating uninfected cells with IFN prior to challenging them with ncpBVDV. As IFN primarily protects uninfected cells from viral infection, the result of this challenge was that the IFN-treated cells were resistant to the virus (Müller-Doblies *et al*., 2002; Peterhans and Schweizer, 2013). Conversely, when cells previously-infected with ncpBVDV were treated with IFN, and then further infected with other viruses (i.e. EMCV, VSV, or Sendai virus), they were guarded against challenge by the additional viruses. However, the treatment with IFN was unable to clear the original BVDV (Schweizer *et al*., 2006). This evidence suggests ncpBVDV avoids IFN response using a mechanism to i) avoid IFN induction; ii) resist IFN action; or iii) avoid interacting with IFN that's targeting unrelated viruses. This is novel as this suggests BVDV is able to establish tolerance to the innate immune system in addition to the adaptive immune system. Furthermore, this suggests that persistently infected animals maintain their health during infection because BVDV has established tolerance to the host; not because the host resists the virus (Peterhans and Schweizer, 2013).

1.3.2 BVDV: Known Methods of IFN Evasion

Within the classified *Flaviviridae* genera, N^{pro} and E^{rns} are distinct to the pestiviruses and are thought to be involved in evasion of innate immunity, particularly in early IFN response.

1.3.2.1 N^{pro}: Reduction of Innate Immune Response

In BVDV, N^{pro} is needed for the separation of its N-terminus from the rest of the precursor polyprotein (Szymanski *et al*., 2009). Evidence has shown that N^{pro} has two functions that maintain viral presence and mediate infection: i) inhibit dsRNA-initiated host cell apoptosis, and ii) stimulate degradation – either directly or indirectly – of IRF-3, a main gene involved in type I IFN activation (Schneider *et al*., 2014).

While it has even been hypothesized that BVDV's N^{pro} actually stimulates rather than degrades IRF3, this hypothesis further suggests that the ability of N^{pro} to bind to host cell DNA

is reduced, at least during the early stages of infection, following stimulation. As such, BVDV would have at least some effect on the ability of IRF3 to activate innate immunity (Baigent *et al*., 2002; Hilton *et al*., 2006). Furthermore, results gathered from one study have been used to suggest one mechanism by which BVDV regulates IRF3. N^{pro} was shown to interact with IRF3 via virus-induced phosphorylation, an interaction that lead to polyubiquitination and subsequent proteasomal degradation of the protein (Chen *et al*., 2007). Based on results gathered from studying CSFV, researchers have hypothesized that BVDV's N^{pro} may also target other host proteins. In CSFV, N^{pro} was shown to interact with IκB-α – a kinase involved in NF-κB activation – and the anti-apoptotic HCLS1-associated protein X-1 (HAX-1) protein (Doceul *et al*., 2008; Johns *et al*., 2010). This has yet to be confirmed in BVDV though.

1.3.2.2 E^{rns}: RNA Degradation

Recent research has shown E^{rns} to be much more important in establishing immunotolerance than N^{pro}. As mentioned, E^{rns} acts as both a structural protein and a viral RNase. This protein is expressed as an envelope-attached glycoprotein, but is also secreted as a soluble protein that is thought to function against host immunity (Schneider *et al*., 1993). E^{rns} is capable of degrading both ssRNA and dsRNA molecules. This degradative ability makes E^{rns} a powerful IFN antagonist, not only because it can prevent activation of the TLR network, but because its secretion enables it to expand its IFN inhibition to neighbouring cells. E^{rns} is thought to cross adjacent cell membranes via receptor-mediated endocytosis. This protein can then remain active for several days, degrading any RNA that would otherwise stimulate IFN. This role as a decoy protein appears pivotal in establishing BVDV's immunotolerance (Zurcher *et al*., 2014).

1.3.3 Inducing and Responding to IFN

As the IFNs are major mediators of initial immune responses against viral pathogens, elucidations of IFN evasion and repression by BVDV, particularly by non-cytopathic strains, are not surprising. While evidence is further accumulating in favour of ncpBVDV establishing immunotolerance via bypassing this early immune response, it does not yet dispel the controversy regarding whether or not ncpBVDV induces IFN synthesis or response.

As mentioned, while it is still debatable whether ncpBVDV induces type I IFN, current evidence suggests host cell ISGs respond within ncpBVDV-infected cells. In one *in vivo* study, two groups of cattle were challenged with one of two non-cytopathic strains – the low virulence, SD-1, strain; and the high virulence, 1373, strain. Expression of type I IFN genes and ISGs – Mx-1, OAS-1, PKR, and ISG-15 – was examined from splenic and tracheo-bronchial lymph nodes samples. Fold changes on day 5 of the challenge showed a statistically significant increase in all four genes within both challenge groups relative to the uninfected group. Interestingly, no significant increase in type I IFN expression was apparent in the low virulence challenge group relative to the uninfected group, a result contrasted in the high virulence group, which only showed a significant increase in IFN-β expression. These results collectively demonstrate a response by ISGs, but not a significant induction of IFN-α (Palomares *et al*., 2013).

If ISGs are responding during ncpBVDV infection, then it would seem logical that IFNs are signalling this response. However, despite evidence showing transcriptional up-regulation of IFN-β in cells infected with the high virulence ncpBVDV strain, the study did not demonstrate transcriptional induction of type I IFNs in the low virulence strain. Even if transcriptional IFN increase was established, further data showing increased translational IFN would have to be gathered in order to establish a proper link between induction and response.

While information regarding type I IFN induction is still poor, some information has been gathered regarding IFN-γ induction. However, information on host cell response to IFN-γ is scant. Transcriptional analyses in one study has demonstrated increased IFN-γ expression within the PBMCs isolated from BVDV-infected cattle, but the animals in that study were already immune to the virus; therefore, increased IFN-γ is anticipated (Waldvogel *et al*., 2000). Another study summarized results from experiments that examined the presence of IFN-γ within the amniotic fluid and blood, in addition to downstream activation of IFN-γ pathways within lymphoid tissues of uninfected, persistently-infected, and transiently-infected fetal calves. The results showed that IFN-γ was synthesised within both the persistently and transiently infected fetuses. This report became the first to identify IFN-γ increases during establishment of ncpBVDV persistent infection (Smirnova *et al*., 2014).

Attempting to answer some of the more puzzling questions, this thesis examined the responses of ncpBVDV2-1373-infected, pregnant cattle, with priority on defining induction of, and responses to IFN-α and IFN-γ. Serum IFN-α and –γ were assessed to examine induction of immune response while expression of genes that classically respond to either cytokine, along with signalling behaviour of the JAK-STAT network, were examined within peripheral blood mononuclear cells (PBMCs) to determine the patterns of response to the synthesised IFNs. Furthermore, correlations between the magnitude of these responses and disease outcomes were performed in order to determine if the animals were responding to the IFNs. Overall, these assessments were correlated with outcomes of disease to see what may have led to or what may have characterised a severe infection and a manageable infection.

1.4 RATIONALE, HYPOTHESIS, AND OBJECTIVES

1.4.1 Hypothesis

Infection by ncpBVDV2-1373 will initiate both the syntheses and biologic activities of IFN-α and IFN-γ.

1.4.2 Overall Objective

To examine the induction of IFN-α and -γ and the expression of select genes that classically respond to these cytokines during the first 10 days of an *in vivo* ncpBVDV2-1373 challenge of pregnant cattle.

1.4.3 Specific Objectives

1. Challenge animals and monitor the induction of IFN-α and -γ, as well as the induction of clinical responses.
2. Monitor the induction of functional IFN-α and -γ responses, including the JAK-STAT signalling pathway and classic interferon-stimulated genes (ISGs).
3. Identify correlations between immune system induction and response in both cattle that are severely and non-severely infected by BVDV.

2. MATERIALS AND METHODS

2.1 Reagents

The names of the materials used in this project, their suppliers, and the suppliers' addresses are listed in Table 2.1.

Table 2.1: List of materials and suppliers

Reagent/Chemical	Supplier
Acetic Acid	VWR
Acetonitrile	EMD Biosciences, VWR
Adenosine triphosphate (ATP)	New England Biolabs
Streptavidin alkaline phosphatase	Jackson ImmunoResearch
Aprotinin	Sigma-Aldrich
Beta-glycerophosphate	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich
Brij-35	ICN Biomedicals
Chloroform, with amylene stabilizer, ≥ 99%	Sigma-Aldrich
Diethanolamine	Sigma-Aldrich
Ethyl alcohol, 95%	Greenfield Specialty Alcohols Inc.
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum	Life Technologies
Gelatin from cold water skin fish	Sigma-Aldrich
Gentamycin	Bio Basic Canada Inc.
Glycerol, 50%	GE Healthcare
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Life Technologies
Horse serum	Life Technologies
iCycler iQ PCR plates	Bio-Rad Laboratories Inc.

IFN-α	VIDO-InterVac
IFN-γ	Novartis
Immulon 2 96U plate	Thermo Fisher Scientific
Isopropyl alcohol	Sigma-Aldrich
Lab-Tek chamber slide (4-well)	Thermo Fisher Scientific
Leupeptin	Sigma-Aldrich
Magnesium chloride hexahydrate	EMD Biosciences
Microseal B plates	Bio-Rad Laboratories Inc.
Minimal Essential Media (MEM)	Sigma-Aldrich
Non-essential amino acids	Life Technologies
Para-nitrophenylphosphate (PNPP)	Sigma-Aldrich
Percoll	GE Healthcare
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich
Phosphate-buffered saline (PBS)	VIDO-InterVac
PNPP buffer	VIDO-InterVac
Sodium acetate	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium fluoride	Sigma-Aldrich
Sodium pyrophosphate	Sigma-Aldrich
Sodium pyruvate	Life Technologies
Sodium vanadate	Sigma-Aldrich
Sterile Water	Life Technologies
Streptavidin alkaline phosphatase	Jackson ImmunoResearch
SYBR Green Supermix	Bio-Rad Laboratories Inc.
Tris	Sigma-Aldrich
Triton-X	Sigma-Aldrich
Trizol LS	Life Technologies
Trypsin	Bio Basic Canada Inc.
Tween-20	Sigma-Aldrich

Versene	VIDO-InterVac
Cell Lines	**Supplier**
Madin-Darby Bovine Kidney (MDBK) cell line	VIDO-InterVac
Antibodies	**Supplier**
Biotin-Goat Anti-Rabbit IgG (H+L) DS Grade	Life Technologies
Mouse anti rBoIFN-α monoclonal antibody, E2-1C6	VIDO-InterVac
Mouse anti rBoIFN-α monoclonal antibody, D5-1D10	VIDO-InterVac
Mouse anti rBoIFN-γ monoclonal antibody, 2-2-1A	VIDO-InterVac
Rabbit anti BoIFN-α	VIDO-InterVac
Rabbit anti BoIFN-γ	VIDO-InterVac
Rabbit BVDV type 2 E2 antibody	VIDO-InterVac
ReserveAP™ phosphatase-labeled goat anti-rabbit IgG (H+L)	KPL
Kits	**Supplier**
Alkaline-Phosphatase Conjugate Substrate Kit	Bio-Rad Laboratories Inc.
Superscript® III SuperMix for qRT-PCR	Life Technologies
Supplier	**Supplier Address**
Bio-Rad Laboratories Inc.	Mississauga, ON, Canada
Bio Basic Canada Inc.	Markham, ON, Canada
EMD Biosciences	Mississauga, ON, Canada
GE Healthcare	Baie d'Urfé, PQ, Canada
Greenfield Specialty Alcohols Inc.	Brampton, ON, Canada
ICN Biomedicals	St. Laurent, PQ, Canada
Jackson ImmunoResearch	West Grove, PA, USA
KPL	Guelph, ON, Canada
Life Technologies	Burlington, ON, Canada

New England Biolabs	Pickering, ON, Canada
Novartis	Mississauga, ON, Canada
Sigma-Aldrich	Oakville, ON, Canada
Thermo Fisher Scientific	Whitby, ON, Canada
VIDO-InterVac	Saskatoon, SK, Canada
VWR	St. Catharines, ON, Canada

2.2 BVDV Challenge and Clinical Evaluation

Two groups of Angus-Aberdeen cross-bred heifers, identified as Group A (n = 8) and Group B (n = 14), were screened via ear notch extract and ELISA (Idexx) to confirm negative results for BVDV, bovine herpes virus, and bovine respiratory syncytial virus. The heifers were inseminated at one-year-old and confirmed pregnant via ultrasound. At Day 80 of pregnancy, Group B cows were intranasally infected with ncpBVDV2-1373 ($TCID_{50} = 10^{7.2}$) while Group A cows were kept uninfected during the duration of the trial.

2.3 Body Weight and Temperature

Body weight and temperature measurements of the group B animals were taken daily during the challenge period between 9 AM and 12 PM by the Animal Care staff of VIDO-InterVac.

2.4 Serum and Peripheral Blood Mononuclear Cell (PBMC) Isolation

For the following isolation, all PBS that was used contained 140 mM NaCl, 2.7 mM KCl, 7.0 mM Na_3PO_4, and 1.5 mM KH_2PO_4, and was set to a pH of 7.3. 50 mL of blood were extracted from each animal using a 60-mL syringe that contained 2 mL of 7.5% EDTA (Sigma-Aldrich). The blood was transferred to a 50-mL polypropylene tube and centrifuged at 1400 x g, at 20°C, for 20 minutes. The PBMC layer from the centrifuged sample was collected and combined with room temperature 0.1% EDTA in PBS up to a volume of 35 mL. This volume was layered onto 15 mL of 54% Percoll (GE Healthcare) in PBS, which was then centrifuged at 2000 x g, at 20°C, for 20 minutes. The PBMC layer was collected and combined with cold PBS. The cells were centrifuged at 300 x g, at 4°C, for 8 minutes; after which, the supernatant was removed,

and the cells were resuspended. This step was repeated twice, with the third centrifugation being run at 150 x g. Before the third centrifugation, the pellet was resuspended in 10 mL of PBS, 100 µL of which was removed for cell counting via the trypan blue exclusion method (Strober, 1997). After the third centrifugation, the supernatant was removed and 5 x 10^6 cells were resuspended in 250 µL of PBS. This was combined with 750 µL of Trizol LS (Life Technologies) and frozen at -80°C for future gene expression analyses. After centrifugation at 1400 x g within the previous steps, 800 µL of serum was transferred to a 96 deep-well plate, which was stored at -20°C for future ELISAs.

2.5 White Blood Cell (WBC) Isolation

5 mL of blood from each cow was combined with 9.5 mL of a lysis buffer (124 mM NH_4Cl (Sigma-Aldrich) and 34.6 mM Tris (Sigma-Aldrich), pH 7.2). The red blood cells were lysed for three minutes before the samples were centrifuged. After removing the supernatant, the pellet was resuspended with 10 mL of PBS and centrifuged. The supernatant was poured off and the pellet was resuspended in 1 mL of 1% HEPES in minimum essential medium (MEM) (Sigma-Aldrich, Life Technologies). All centrifugations were performed at 300 x g, at 4°C, for 10 minutes. The samples were stored at -80°C for future analyses.

2.6 Viral Titration

For this immunocytochemistry protocol, the following conditions were applied unless otherwise specified. All reagents were added at 100 µL per well to a round-bottomed plate (Thermo Fisher Scientific). All incubations occurred at room temperature on the benchtop.

2.6.1 Growth and Infection of Madin-Darby Bovine Kidney (MDBK) Cells

Nasal and WBC samples from each challenged animal were assessed for viral presence on days 3, 7, and 10 post-challenge, as well as day -1 pre-challenge. Madin-Darby Bovine Kidney (MDBK) cells were grown in 10% fetal bovine serum (FBS) in MEM (Life Technologies, Sigma Aldrich) in T150 flasks, counted using a haemocytometer, and diluted to a suspension of 2.5 x 10^5 cells/mL. 100 µL of cells were then added to each well of a 96-well flat-bottomed plate at 2.5 x 10^4 cells per well. The cells were grown overnight at 37°C. Serial 2-fold

dilutions of the nasal and WBC samples were made in a separate plate. Dilutions of pure virus stock served as positive controls. The medium was removed from the cells and replaced with 100 µL of sample from the corresponding wells of the dilution plates. The now-infected cells were incubated at 37°C for 1.5 hours; after which, 100 µL of 2% FBS in MEM was added to each well. They were then incubated at 37°C for 96 hours.

2.6.2 Immunocytochemistry

Following the 96-hour incubation, the cells were stained to identify the presence of BVDV. The cells were first blocked with 10% horse serum in MEM and incubated for 10 minutes. The medium was then removed and the cells were fixed with a mixture of 75% ethanol (Greenfield Alcohols Inc.) and 25% acetic acid (VWR) for 10 minutes. After fixation, the cells were washed thrice with 0.05% Tween 20 in PBS (PBST) (Sigma-Aldrich) and then permeabilized in 0.01% Triton X (Sigma-Aldrich) in PBS for 20 minutes. The wash was then repeated with PBST. Rabbit BVDV Type 2 E2 antibody (VIDO-InterVac) was diluted 1:500 in PBS, and added to each well. The plates were incubated for 1.5 hours and then washed. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (KPL) was diluted 1:500 in PBS and added to the plates, which were incubated. The cells were washed and then stained using an AP staining kit (BioRad). Staining was indicative of viral infection; therefore, of a positive result.

2.7 ELISAs

For the following ELISA protocols, the following conditions were applied unless otherwise specified. All reagents were added at 100 µL per well to an Immulon 2 96U plate (Thermo Fisher Scientific). All incubations occurred at room temperature on the benchtop for one hour. 0.05% Tween 20 in Tris-buffered saline (TBST) (Sigma-Aldrich) was used 5 x for each washing step. All reagent dilutions were made in 0.1% fish gelatin in TBST (Sigma-Aldrich).

2.7.1 IFN ELISAs

ELISAs for IFN-γ (Baca-Estrada *et al*., 1995) and IFN-α (Hughes *et al*., 1994) were carried out as previously described. To quantify IFN-γ, a plate was coated with mouse anti-rBoIFN-γ monoclonal antibody (Clone 2-2-1A) (VIDO-InterVac), diluted 1:8000 in 50.0 mM bicarbonate

buffer (Sigma-Aldrich), incubated at 4°C overnight, and then washed. Serially-diluted bovine IFN-γ standards (Novartis AG), starting from 1 ng/mL, as well as undiluted serum samples, were added to the plate and incubated for two hours. Rabbit anti-BoIFN-γ (Clone 92-132) (VIDO-InterVac) was diluted 1:5000, added to the plate, and incubated, followed by biotin-goat anti-rabbit IgG (H + L) (Life Technologies) diluted 1:10000. Streptavidin alkaline phosphatase (Jackson ImmunoResearch) in 50% glycerol was diluted 1:5000, added to the plate, which was then incubated. Para-nitrophenylphosphate (PNPP) (Sigma-Aldrich) was diluted in a buffered solution comprised of 105 mM diethanolamine (Sigma-Aldrich) and 0.5 mM $MgCl_2 \cdot 6H_2O$ (EMD Biosciences) to a final density of 1 mg/mL and added to the plate, which was incubated for 20 minutes. The plates were washed with PBST between each incubation step. Optical density readings (405 nm read; 490 nm reference) of the standards were used to construct a standard curve from which the concentrations of IFN-γ in the serum samples were calculated. IFN-α was detected according to the same protocol, but by using the following reagents: anti-rBoIFN-α (Clones E2-1C6 and D5-1D10) (VIDO-InterVac) monoclonal antibodies to coat the plate, bovine IFN-α standards (VIDO-InterVac) to construct the standard curve, and rabbit anti-BoIFN-α (Clone 92-133) (VIDO-InterVac) as the primary antibody.

2.8 Gene Expression Analysis

2.8.1 RNA Extraction

All incubations took place at room temperature, unless otherwise specified. The Trizol-frozen cell samples were thawed in their Eppendorf tubes. 200 µL of chloroform (Sigma-Aldrich) were added to the samples. They were then mixed by inversion for 30 seconds and subsequently incubated for 3 minutes. This mixture was centrifuged at 12 000 x g for 15 minutes in order to separate the Trizol layer from the aqueous layer. After centrifugation, the aqueous layer was collected and combined with 500 µL of isopropyl alcohol (Sigma-Aldrich). The sample was mixed by inversion and incubated for 10 minutes. After incubation, the sample was centrifuged at 12 000 x g for 10 minutes to pellet the RNA. After centrifugation, the supernatant was removed; 1 mL of 70% ethanol (Greenfield Specialty Alcohols, Inc.) was added to the pellet. The pellet was suspended, mixed by inversion, and then centrifuged at 7

500 x g for 5 minutes. After centrifugation, the supernatant was poured off, the Eppendorf tube was inverted, and the pellet was allowed to air dry for at least 20 minutes. After air drying, 40 µL of sterile water (Life Technologies) was added to the pellet; the tube was placed in a water bath at 55°C for 5 minutes. After 5 minutes, the dissolved RNA was mixed via pipetting before being placed back into the water bath for another 5 minutes. Afterwards, 2 µL of dissolved RNA was used to determine the concentration (ng/µL) via bioanalysis while the remaining sample was frozen at -80°C until cDNA synthesis was performed.

2.8.2 cDNA Synthesis

Using solutions from the Superscript III SuperMix kit (Life Technologies), RNA samples were reverse-transcribed to cDNA at a quantity of 500 ng. The RNA sample was thawed from -80°C. Using the concentration assessed in 2.8.1, 8 µL of sterile, water-diluted RNA was combined with 10 µL of Reaction Mix (Buffer) and 2 µL of Enzyme Mix to a final volume of 20 µL in a PCR Thin Wall Tube (BioRad). The tube was placed in a thermocycler; cDNA synthesis commenced for 80 minutes under the following conditions: 25°C for the first 5 minutes, 60°C for the following 60 minutes, and 70°C for the final 15 minutes. Following synthesis, the cDNA was diluted 3-fold in sterile water to a final volume of 60 µL. The sample was then stored at -20°C until qRT-PCR was performed.

2.8.3 qRT-PCR

3 µL of cDNA was combined with 9 µL of iQ SYBR green reaction mix (BioRad) and 3 µL of 3.3 µM of forward-and-reverse primer pair mix to a final volume of 15 µL. Each sample at this volume was added to one well of a 96-well PCR plate (BioRad) and covered with a Microseal cover (BioRad). The plate was placed in a BioRad iCycler; the qRT-PCR amplification is set to the following parameters in Table 2.2:

Table 2.2: Amplification cycle for cDNA samples.

Cycle	Step	Temperature (°C)	Time (min.)
1 (x1)	1	55	2:00
2 (x1)	1	95	8:30
3 (x45)	1	95	0:15
	2	58	0:30
	3	72	0:30
4 (x45)	1	55	0:10

Duplicate threshold cycle values (TCVs) for each gene of a particular sample were averaged and assessed against the average, duplicated β-actin TCVs of the same sample using the following series of steps.

Firstly, the difference between the average TCV of a particular gene against the average TCV of β-actin was calculated on Day -1. This same calculation was performed on day 3, day 7, and day 10 of the challenge. Afterwards, the TCV difference calculated on day -1 was subtracted separately from each of the TCV differences of days 3, 7, and 10. Finally, fold change values for each gene were calculated by setting the negative value of these double-TCV differences to a base-2 exponent. A fold change of 1 indicated no difference in gene expression relative to day -1. Fold changes less than 1 indicated reduced gene expression relative to day -1. Fold changes greater than 1 indicated an increased gene expression relative to day -1.

2.9 Variance Analysis

To assess the statistical significance of the variation in temperatures between the surviving and the perishing Group B animals, a non-parametric variance test was used to complement the median data: the Wilcoxon Rank Sum Test (WRST). Firstly, values for temperature change from days 1 to 10 were summed for all animals. Following summation, each animal was given a rank within a range of 1 through 14, as there are 14 animals under analysis. The animal with the lowest rank had the highest temperature sum across the 10 days while the animal with the highest rank had the lowest temperature sum. WRST was then performed.

2.10 Kinome Analysis

All reagents are from Sigma-Aldrich and all incubations occurred at room temperature unless otherwise specified. Experiments involving staining of the peptide arrays were all performed on the same day to minimise technical variance.

2.10.1 Peptide Array Design and Construction

Design, construction and application of the peptide arrays were performed using a modified version of a previously described protocol (Jalal *et al*., 2009). The arrays were fabricated by a commercial provider (JPT Peptide Technologies, Berlin, Germany; http://www.jpt.com) and were designed to include peptides representing phosphorylation events from a wide variety of signaling pathways. Specifically, the array included nine technical replicates of each of the 297 unique peptides giving a total of 2 673 spots on the array. Each peptide was 15 amino acids in length and had a central residue known to be phosphorylated *in vivo*. A complete description of the array, including the coordinates of the spots and the sequence of each peptide is described elsewhere (Jalal *et al*., 2009).

2.10.2 Peptide Array Staining

Isolated PBMCs obtained on days 3, 7, and 10 post-challenge, as well as day -1 pre-challenge, were thawed and then lysed using 100 µL of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% TRITON® X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 µg/mL leupeptin, 1 g/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were incubated on ice for 10 minutes and then centrifuged at 1000 x g, at 4°C, for 10 minutes. A 70-µL aliquot of the resulting supernatant was combined with 10 µL of activation mix (50% glycerol (GE Healthcare), 50 µM adenosine triphosphate (ATP) (New England Biolabs), 60 mM magnesium chloride, 0.05% v/v Brij-35 (ICN Biomedicals), 0.25 mg/mL bovine serum albumin (BSA), 2 Ci/L γ^{32}P-ATP) and incubated on a custom-designed bovine peptide array (JPT) at 37°C for 2 hours. The arrays were then washed once with Tris-buffered saline (TBS), pH 7.3 containing 1% TRITON® X-100. Following this wash, the arrays were submerged in Pro-Q Diamond Phosphoprotein gel

stain (Life Technologies) and agitated for one hour. Following the wash, the arrays were washed twice with 50 mL of destain (20% acetonitrile (EMD Biosciences) and 50 mM sodium acetate at pH 4.0) for 10 minutes each. New destain was used for each wash. The arrays were then washed with ddH_2O before being dried.

2.10.3 Peptide Array Scanning

After drying, the arrays were read using a GenePix Professional 4200A microarray scanner (MDS Analytical Technologies, Toronto, ON) at 532 nm. Array images were taken with a camera that was controlled using GenePix 6.0 software (MDS). For each array, the signal intensity for each peptide was collected. The parameters at which each intensity value was set include the following: 65535 scanner saturation level, local feature background set to calculate the background, analysis using signal mean and background mean intensity values, local background features excluding 2 pixels, and background width set to three feature diameters.

2.10.4 Statistical Analysis: PIIKA 2

Using a program called Platform for Intelligent, Integrated Kinome Analysis (PIIKA) 2 (Trost *et al*., 2013), a variety of statistics were generated following a previously developed pipeline (Li, *et al.*, 2012).

2.10.4.1 Data Pre-processing

For all peptide spots on a particular array, actual intensity values were calculated by subtracting the background intensity values from the foreground intensity values. This intensity difference was converted using a variance stabilization (VSN) method (Huber, *et al*., 2002), which maintained the structure of the data while adjusting it to a positive scale. This alleviated mean-dependent variance. Additionally, cross-experimental data were assembled on the same scale in order to compare arrays between different cell treatments; in this case, control and infected. To make subsequent analyses easier to examine, the dataset was reassembled such that each row consisted all of the replicates related to a specific peptide. The R software environment (www.r-project.org) function *vsn2* from the *vsn* package was used for the VSN method (Huber,

et al., 2003). Replicates were kept as separate values in all subsequent analyses, but averaged for clustering analysis (Everitt, 1974; Hardigan, 1975).

2.10.4.2 Spot-Spot Variability Analysis

A chi-squared (χ^2) test was performed to assess the variability of replicates for each specific peptide on the array: the technical replicates (Draghici, 2003). The null hypothesis (H_o) for each peptide stated that there was no difference in the intensities of the technical replicates, while an alternative hypothesis (H_A) claimed that there was significant variation between the technical replicates. The peptides with *p*-values greater than 0.1 were considered inconsistently phosphorylated across the array replicates. A confidence level of 90% was used to keep as much data as possible. The *p*-value was calculated using the R function, *pchisq*, from the *stats* software package.

2.10.4.3 Subject-Subject Variability Analysis

Following background subtractions of biological data, subject-subject variability analysis was performed and applied to datasets. For this, an F test was performed on each peptide to determine if there were significant differences between infected cattle. Data for inconsistently phosphorylated peptides were removed from further analyses.

2.10.4.4 Treatment-Treatment Variability Analysis

Those peptides identified as having consistent response patterns to multiple treatments across the same replicates were analysed via one-sided paired t-tests. T-tests were performed to compare the intensities of the peptides under treatment to the intensities of the same peptides under control conditions.

As a result, two *p*-values were calculated for each peptide: one related to the differential phosphorylation of each peptide and the other to the dephosphorylation of each peptide. Those peptides having a *p*-value lower than the defined cut-off (i.e. 0.1) were identified as differentially dephosphorylated and used for additional analyses. To keep as much data as possible, the *p*-value was not adjusted.

A paired t-test was conducted in order to acknowledge the interdependence between the peptides of both the control and treatment conditions, and was performed via the R environmental function *t-test* of the *stats* package, in which *paired* = *True*. Of significance was that the t-test could account for the variability across the peptide replicates; therefore, those replicates with a significant *p*-value assessed via χ^2 tests had insignificant *p*-values from the t-test automatically. However, this was not applicable to datasets with numerous subjects as significant variation for a specific peptide across these subjects under a given treatment might have been biologically relevant. As such, the analysis may have been confounded if the peptides were statistically examined as if they were all from the same source.

2.10.5 Pathway Analysis

Peptides that are differentially phosphorylated can be utilized within a software platform called InnateDB (Lynn *et al.*, 2008). As this software uses fold changes as input values, fold changes were calculated as the differences between variance-stabilized normalization-transformed intensities of the control and the treatment.

Fold change, in addition to one of the *p*-values obtained from the one-sided t-test for each peptide on the array (with the exception of those peptides that were removed previously: Section 2.10.4.3), represented the complete InnateDB input. If fold change was positive, then the *p*-value associated with "phosphorylation" was used. If the fold change was negative, then the *p*-value associated with "dephosphorylation" was used.

Thresholds (cut-off values) associated with fold changes and *p*-values were set prior to calculation: fold change was set > 1 and *p*-value was set < 0.10. Peptides that were respectively below or above these thresholds were removed from InnateDB analysis.

3. RESULTS

The immune response of cattle to BVDV was examined. These included identifying the relative levels of virus in their WBCs and nasal passages, tracking the changes in their temperatures and body weights, examining the levels of IFN-γ and IFN-α in their sera, and analysing the expression of several genes, including STAT-1, OAS-1, Mx-1, SOCS-1, and SOCS-3, within their PBMCs.

Some of the (the infected) Group B animals died during the challenge while others survived. As such, the animals that died and those that survived were classified into subgroups of group B animals, which were labelled Group B(D) and Group B(S), respectively. Throughout the results, trends between (the uninfected) Group A, Group B, Group B(S), and Group B(D) were compared and assessed for statistical difference.

3.1 Viral Presence

To determine the efficiency of viral infection, a semi-quantitative assessment of BVDV presence in both nasal samples and WBCs was performed, the results of which are displayed in Table 3.1. For each well of the eight rows in which the sample was diluted, presence was assessed as "+", "++", or "+++", while lack of presence was assessed as "–". If wells on the plate were stained within rows 1 – 3, that sample was given a "+" or low rating. If wells on the plate were stained up to rows 1 – 6, that sample was given a "++" or moderate rating. If wells on the plate were stained up to rows 7 – 8, that sample was given a "+++" or high rating.

Table 3.1: Semi-quantitative assessment of viral presence in WBC and nasal samples obtained from all animals on days -1, 3, 7, and 10. No viral presence (–). Low viral presence (+). Moderate viral presence (++). High viral presence (+++). For ease of distinction, low, moderate, and high presence are coloured blue, green, and red, respectively.

Group	Animal	WBC Viral Loads				Nasal Viral Loads			
		Day -1	Day 3	Day 7	Day 10	Day -1	Day 3	Day 7	Day 10
A	24	–	–	–	–	–	–	–	–
A	28	–	–	–	–	–	–	–	–
A	33	–	–	–	–	–	–	–	–
A	37	–	–	–	–	–	–	–	–
A	41	–	–	–	–	–	–	–	–
A	46	–	–	–	–	–	–	–	–
A	51	–	–	–	–	–	–	+	–
B (S)	27	–	–	++	+++	–	–	+++	+++
B (S)	30	–	–	++	–	–	+	++	++
B (S)	32	–	–	+	–	–	–	–	+++
B (S)	42	–	–	+	–	–	–	++	++
B (S)	43	–	–	++	+	–	–	++	++
B (S)	44	–	–	+	+	–	+	++	++
B (S)	48	–	–	+	+	–	–	+++	+++
B (S)	49	–	–	++	–	–	+	++	+
B (D)	23	–	–	++	+	–	–	++	+++
B (D)	26	–	–	++	–	–	+	++	++
B (D)	31	–	–	+++	+	–	–	+++	+++
B (D)	36	–	–	++	+	–	–	+	+++
B (D)	45	–	–	++	++	–	+	+++	+++
B (D)	50	–	–	+++	+++	–	–	+++	+++

This temporal virus titration confirmed that all Group B animals were successfully infected with ncpBVDV2-1373. The virus was first detected in the nasal samples, with five of the fourteen group B animals exhibiting positive results on day 3 and all group B WBC samples exhibiting viral presence by day 7.

Of the animals that eventually died, there is a trend of increased viral presence in their nasal samples. On day 7, 25% of the group B(S) animals reached the highest score, while 50% of the group B(D) animals reached the highest score. Furthermore, on day 10, 38% of the group B(S), in contrast to 83% of group B(D), reached the highest score.

3.2 Mortality

During the challenge, six of fourteen group B animals died as a result of infection while all uninfected, group A animals survived. Other reports have also shown that challenging with other virulent BVDV2 strains led to severe clinical disease with a high mortality rate (Bolin and Ridpath, 1992; Brock *et al*., 2007; Ellis *et al*., 1998; Liebler-Tenorio *et al*., 2003a,b).

3.3 Clinical Scores

Temperature and body weight data were only available for the group B animals during the challenge. It was assumed that the temperatures of the group A animals would remain relatively constant across the 10-day period; possibly deviating by a maximum of 0.5°C as a result of normal circadian rhythm temperature fluctuation (Peedel *et al*., 2012). As the animals were pregnant, it was also assumed that their body weights would continually increase over this same period.

3.3.1 Temperature Change

To more accurately track the influence of the virus during the course of infection, differences in temperature were taken relative to day 0. The value for each day represents the subtraction of the actual temperature on that day by the actual temperature on day 0.

There was a notable spike in temperature between 2 and 3 days post-challenge followed by a second period of increased temperature roughly 7 days post-infection. The magnitude and temporal aspects of these elevated periods of temperature changes have been previously demonstrated in other BVDV challenges (Ellis *et al*., 1998; Liang *et al*., 2006; Snider *et al*. 2014; van Drunen Littel-van den Hurk *et al*., 2013). Temperature changes in both the group B(S) and group B(D) animals were separated and presented individually in Figures 3.1A and 3.1B, respectively. Additionally, the median trends of each of these groups are shown in Figure 3.1C. While there was an apparent trend toward higher temperatures among animals that did not survive infection, the differences between groups B(S) and B(D) were not statistically different, i.e the *p*-value obtained from WRST was greater than 0.05.

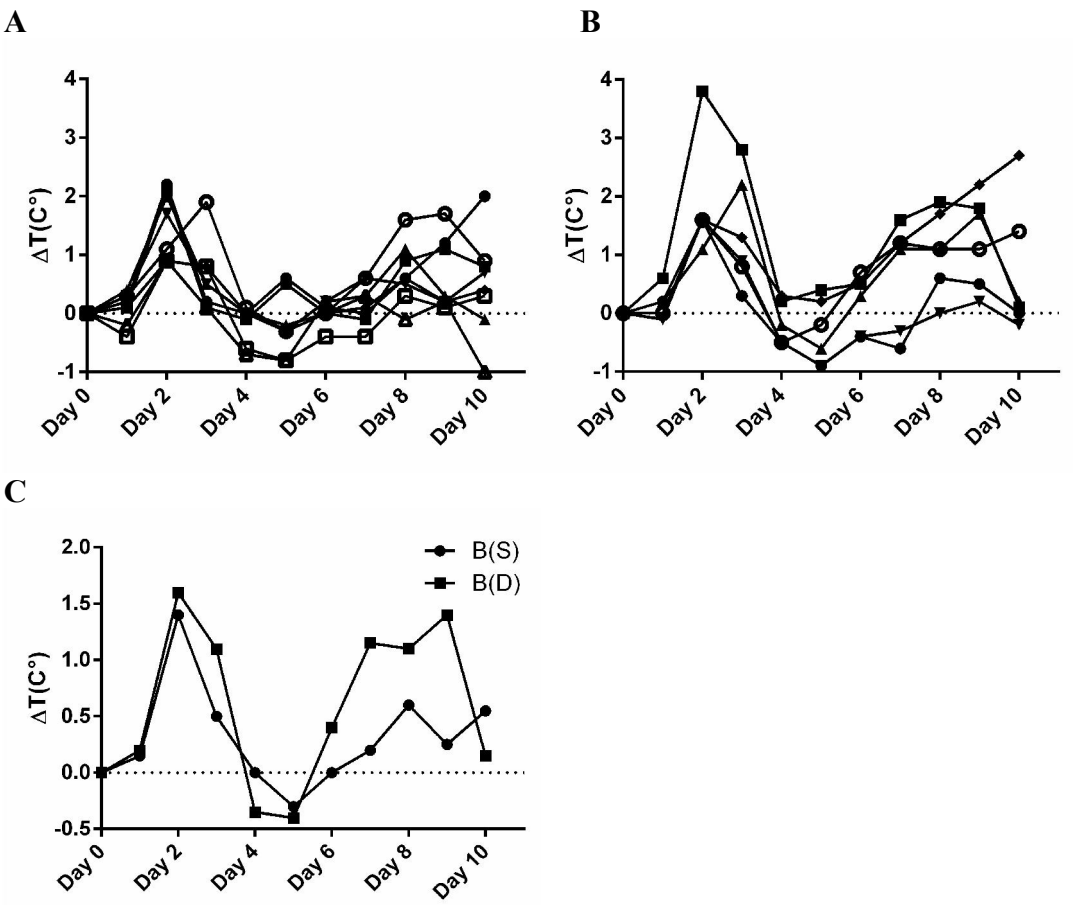

Figure 3.1: Temperature changes in the challenged cattle with respect to day 0. **A.** Individual data for group B(S) animals (n = 8). **B.** Individual data for group B(D) animals (n = 6). **C.** Median data for group B(S) and group B(D) animals.

Table 3.2 provides a visual representation of the temperature ranks that were used to compute the *p*-value for the animals in Group B and shows that increased temperature favours increased likelihood of mortality. The top two temperature responders and four of the top five temperature responders were within group B(D), illustrating the trend towards higher temperature of animals that succumb to acute, BVDV infection.

Table 3.2: Temperature change ranks for all group B animals based on the summation of the temperatures (i.e. rank sum analysis) from days 1 to 10 of the challenge. **1** = highest temperature summation. **14** = lowest temperature summation. Animals were assigned ranks **(1 – 7)** if their sums were in the top half of this ranking order. Animals were assigned ranks **(8 – 14)** if their sums were in the bottom half of this ranking order.

Group	Animal Label	Rank
B(S)	27	5
B(S)	30	7
B(S)	32	9
B(S)	42	8
B(S)	43	10
B(S)	44	3
B(S)	48	13
B(S)	49	14
B(D)	23	11
B(D)	26	1
B(D)	31	5
B(D)	36	12
B(D)	45	2
B(D)	50	4

The results in Table 3.2 illustrate that animals with high temperature ranks generally survived the challenge while animals with low temperature ranks generally did not survive the challenge.

3.3.2 Body Weight Change

As with temperature change, differences in body weight were determined relative to day 0. Within all animals, there was a consistent trend for reduction in body weight relative to the day of challenge. Body weight changes of both group B(S) and group B(D) animals were separated and presented individually in Figures 3.2A and 3.2B, respectively. Additionally, median trends of these groups were presented in Figure 3.2C. Despite a noticeable trend distinguishing the body weight changes between groups B(S) and B(D), the two subgroups were not statistically distinct, i.e. the *p*-value obtained from WRST was greater than 0.05. However, there was one animal that notably stood out in group B(S), exhibiting substantial weight loss relative to the other animals, as seen in Figure 3.2A. Additionally, there was one notable animal within group B(D) that didn't follow the trend of weight loss observed in the other animals, as seen in Figure 3.2B.

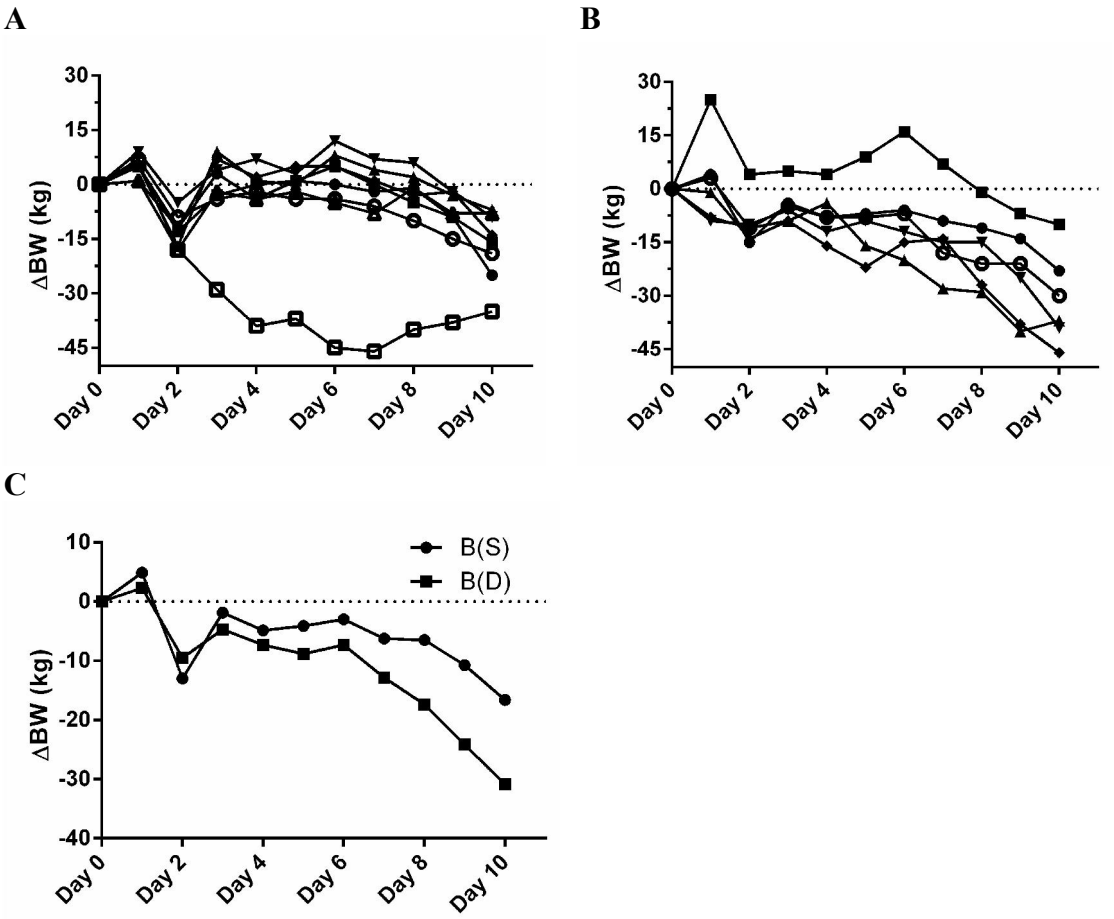

Figure 3.2: Body weight changes in the challenged cattle with respect to day 0. **A.** Group B(S) animals (n = 8). **B**. Group B(D) animals (n = 6). **C.** Median data for group B(S) and group B(D) animals.

Collectively, there was a consistent weight reduction post-challenge. Given the age and pregnancy status of all animals, significant weight gain over the 10-day period would be expected when uninfected. While the group B(D) animals trended towards greater weight loss, the differences in the trends between the two groups were not statistically significant.

As with temperature, ranks were assigned to each animal before statistical variance was assessed. Table 3.3 provides a visual representation of the body weight change ranks. Despite the lack of statistical difference between the two subgroups, five of the six group B(D) animals exhibited the greatest weight loss.

Table 3.3: Body weight change ranks for all group B animals based on the summation of the body weight changes (i.e. rank sum analysis) from days 1 to 10 of the challenge. **1** = highest body weight change summation. **14** = lowest body weight change summation. Animals were assigned ranks **(1 – 7)** if their sums were in the top half of this ranking order. Animals were assigned ranks **(8 – 14)** if their sums were in the bottom half of this ranking order.

Group	Animal Label	Rank
B(S)	27	9
B(S)	30	10
B(S)	32	12
B(S)	42	13
B(S)	43	11
B(S)	44	7
B(S)	48	1
B(S)	49	8
B(D)	23	6
B(D)	26	14
B(D)	31	3
B(D)	36	4
B(D)	45	2
B(D)	50	5

Similar to the temperature ranking trends, animals with high body weight ranks tended to survive the challenge while those with low body weight ranks generally did not survive the challenge.

3.4 Induction of and Response to Interferon

As results from previous studies on induction and response to IFNs during BVDV infection have been conflicting, one major goal of this challenge was to characterise IFN trends. Levels for both IFN-α and IFN–γ were assessed on select days pre- and post-challenge to characterise IFN induction. Additionally, expression of genes known to be induced by IFN was examined to characterise the cellular IFN response.

3.4.1 IFN-α

3.4.1.1 IFN-α Induction

Serum IFN-α levels were examined on days -1, 3, 7, and 10 of the challenge. Table 3.4 displays the raw serum IFN-α levels for all animals in the challenge.

Table 3.4: Serum IFN-α levels (pg/mL) on days -1, 3, 7, and 10 of all group A and group B animals. "U" indicates that insufficient serum was available to perform the assay on this day.

Group	Animal	Day			
		-1	3	7	10
A	24	0.00	0.00	0.00	0.00
A	28	8.91	7.40	8.36	11.10
A	33	0.00	0.00	0.00	0.00
A	37	0.00	2.13	0.00	0.00
A	41	4.88	4.45	3.56	4.01
A	46	0.00	0.00	0.00	2.73
A	51	0.00	0.00	0.00	0.00
B(S)	27	0.00	6.44	5.99	0.00
B(S)	30	0.00	5.65	5.54	5.99
B(S)	32	0.00	7.58	3.59	0.00
B(S)	42	5.20	11.14	9.23	13.08
B(S)	43	3.47	12.03	6.20	2.88
B(S)	44	6.71	12.47	17.63	10.55
B(S)	48	3.71	17.63	11.10	4.88
B(S)	49	0.00	13.54	4.34	2.88
B(D)	23	6.56	17.53	10.33	5.54
B(D)	26	4.85	15.80	8.49	7.12
B(D)	31	0.00	6.90	5.08	U
B(D)	36	0.00	10.25	8.07	5.77
B(D)	45	7.78	71.50	32.08	20.27
B(D)	50	9.45	47.75	19.92	10.55

Responding to infection, there was an immediate increase in serum IFN-α levels in the group B animals by day 3, the day by which levels peaked in most animals. These levels sharply fell by day 7 and returned to near baseline levels by day 10. The duration and extent of IFN-α elevation is consistent with results gathered from similar BVDV challenge models using similar strains (Charleston *et al*., 2002; Palomares *et al*., 2013).

As with the clinical measurements, IFN-α levels on days 3, 7, and 10 of the challenge were evaluated as a difference in IFN-α with respect to day -1. Figures 3.3A, 3.3B, and 3.3C show the individual changes within groups A, B(S), and B(D), respectively. Additionally, Figure 3.3D shows the median trends of groups A, B, B(S) and B(D). Animal 31 was removed from median and variance calculations as insufficient serum was collected to make obtain a reading from being obtained at that time point.

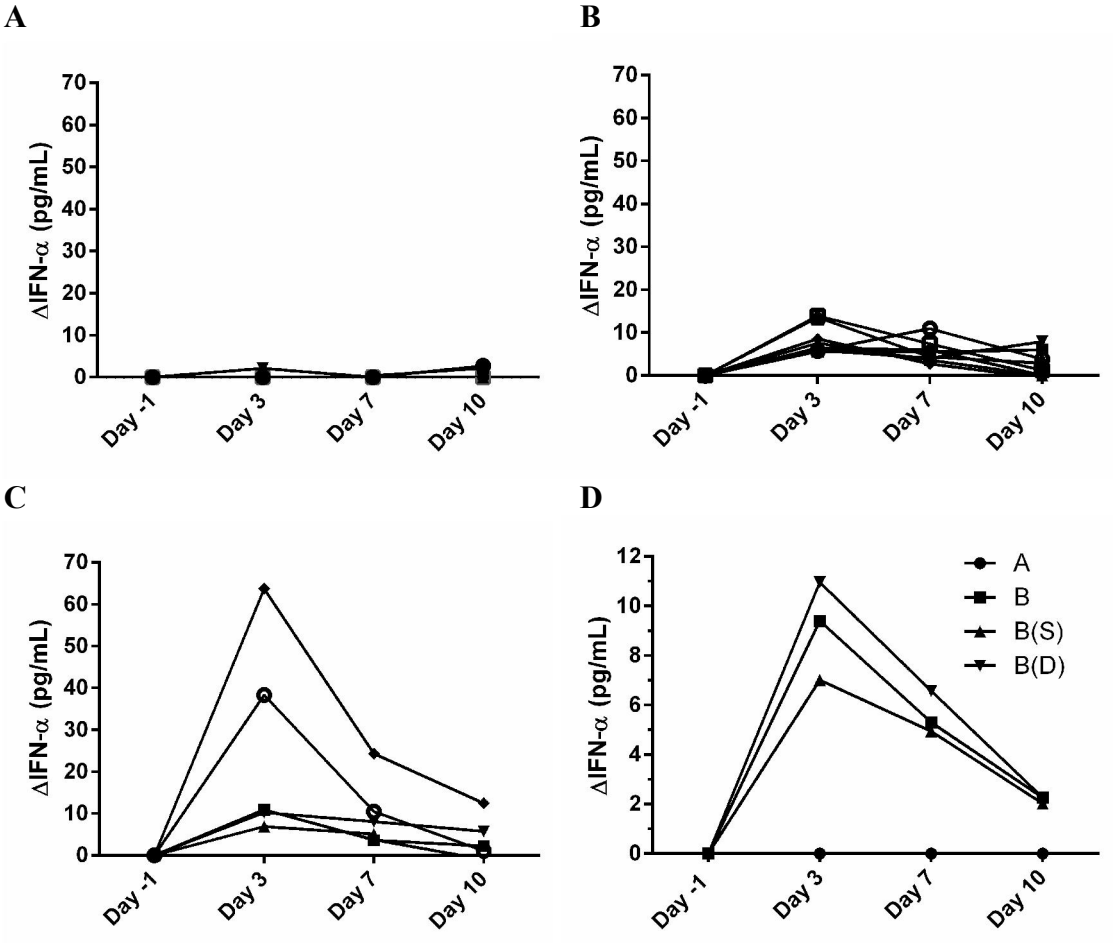

Figure 3.3: Serum IFN-α differences of cattle on days 3, 7, and 10, with respect to day -1. **A.** Group A animals (n = 7). **B.** Group B(S) animals (n = 8). **C.** Group B(D) animals (n = 6). **D.** Median data for groups A, B, B(S), and B(D).

There was a significant difference ($p \leq 0.05$) in the serum IFN-α between groups A and B based on WRST. Additionally, both groups B(S) and B(D) exhibited significant induction of serum IFN-α relative to group A. Furthermore, animals in group B(D) tended to exhibit higher serum IFN-α relative to group B(S), particularly on day 3. However, the difference was not statistically significant. Table 3.5 shows the *p*-values between the various groups on days 3, 7, and 10, as well as summed through all three days.

Table 3.5: *P*-values computed from serum IFN-α level comparisons of various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	**Group Comparisons**			
	A vs. B	**A vs. B(S)**	**A vs. B(D)**	**B(S) vs. B(D)**
3	**<0.01**	**<0.01**	**<0.01**	0.08
7	**<0.01**	**<0.01**	**<0.01**	0.49
10	0.12	0.16	0.24	0.91
All	**<0.01**	**<0.01**	**<0.01**	0.34

As was compiled for the clinical measurements, ranks based on the summation of serum IFN-α on days 3, 7, and 10 were displayed to illustrate the qualitative trend, particularly between group B(S) and B(D) animals. Table 3.6 displays these ranks**.**

Table 3.6: IFN-α change ranks for all animals based on the summation of the IFN-γ changes (i.e. rank sum analysis) on days 3, 7, and 10 of the challenge. **1** = highest IFN-α sum. **21** = lowest IFN-α sum. Animals were assigned ranks **(1 – 7)** if their sums were in the top third of this ranking order. Animals were assigned ranks **(8 – 14)** if their sums were in the middle third of this ranking order. Animals were assigned ranks **(15 – 21)** if their sums were in the bottom third of this ranking order.

Group	Animal Label	Rank
A	24	18
A	28	17
A	33	18
A	37	16
A	41	21
A	46	15
A	51	18
B(S)	27	11
B(S)	30	8
B(S)	32	13
B(S)	42	7
B(S)	43	14
B(S)	44	6
B(S)	48	4
B(S)	49	5
B(D)	23	10
B(D)	26	9
B(D)	31	12
B(D)	36	3
B(D)	45	1
B(D)	50	2

While the three lowest ranking animals perished during the challenge, no clear trend was observed regarding whether low or medium ranking IFN-α levels predicted an animal's mortality. All group A animals were clustered in the highest ranks while all group B animals were within the low or medium ranks. This suggests that, regardless of mortality outcome, BVDV stimulated some level of IFN-α synthesis.

3.4.1.2 IFN-α Response: OAS-1, Mx-1, and STAT-1

While evidence demonstrates that serum IFN-γ is induced in response to BVDV infection, there exists potential for disconnect between production of and response to this cytokine as other pathogens have been shown to prevent host cells from responding to it (Schweizer *et al*., 2001).

As mentioned, there are multiple well-characterised genes that respond to IFN-α, including OAS-1, MX-1 and STAT1 (Sadler *et al.*, 2008).

Expression of each of these genes within the group B animals spiked at three days post-infection and was followed by a gradual decline towards the levels of group A animals. OAS-1, Mx-1, and STAT-1 median trends are shown in Figures 3.4A, 3.4B, and 3.4C, respectively.

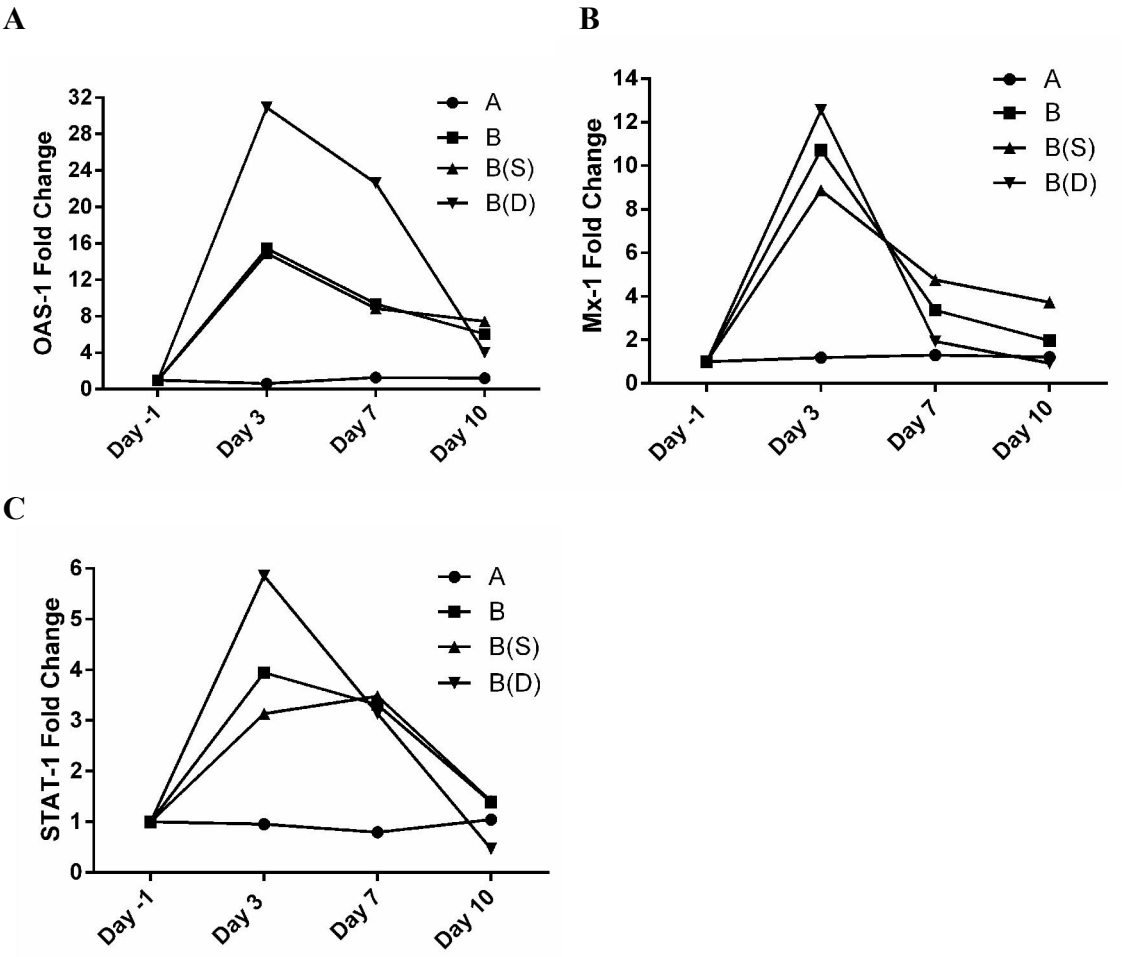

Figure 3.4: Expression of IFN-α responsive genes. **A.** Expression of OAS-1. **B.** Expression of Mx-1. **C.** Expression of STAT-1.

For each of these genes, there was a significant difference in the levels of expression by the group B animals relative to the group A animals at days 3 and 7 post-infection, the *p*-values for which are presented in Tables 3.7 – 3.9.

Table 3.7: *P*-values computed from serum OAS-1 level comparisons of various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	Group Comparisons			
	A vs. B	A vs. B(S)	A vs. B(D)	B(S) vs. B(D)
3	**<0.01**	**<0.01**	**<0.01**	0.41
7	**<0.01**	**<0.01**	**<0.01**	0.33
10	**<0.01**	**<0.01**	**<0.01**	0.63
All	**<0.01**	**<0.01**	**<0.01**	0.41

Table 3.8: *P*-values computed from serum Mx-1 level comparisons of various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	Group Comparisons			
	A vs. B	A vs. B(S)	A vs. B(D)	B(S) vs. B(D)
3	**<0.01**	**<0.01**	**<0.01**	0.86
7	**<0.01**	**<0.01**	**0.02**	0.20
10	0.12	**<0.01**	0.52	**<0.01**
All	**<0.01**	**<0.01**	**<0.01**	0.53

Table 3.9: *P*-values computed from serum STAT-1 level comparisons of various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	Group Comparisons			
	A vs. B	A vs. B(S)	A vs. B(D)	B(S) vs. B(D)
3	**<0.01**	**<0.01**	0.18	0.60
7	**<0.01**	**<0.01**	0.13	0.91
10	0.75	0.36	0.65	0.74
All	**0.02**	**0.01**	0.24	0.82

OAS-1 exhibited the greatest response to IFN-α stimulation when compared to pre-challenge levels, with statistical distinction calculated between group A and groups B, B(S), B(D). A similar trend was seen in Mx-1. This gene exhibited its highest response to IFN-α on day 3, but which rapidly fell by day 7. The temporal kinetics of STAT-1 exhibited the least distinction between the various groups. While there was statistical distinction in comparing groups A with B and B(S), this was only significant on days 3 and 7. Group B(D) animals did not statistically separate from the group A animals and neither did the group B subgroups.

While the level of statistical distinction between groups A and B was different, there was an apparent correlation between IFN-α increase and gene response: early induction and subsequent return to baseline levels. Additionally, there appeared to be a direct relationship

between the extent of serum IFN-α increase and the degree of induction of IFN-α-responsiveness, as seen in Figure 3.5.

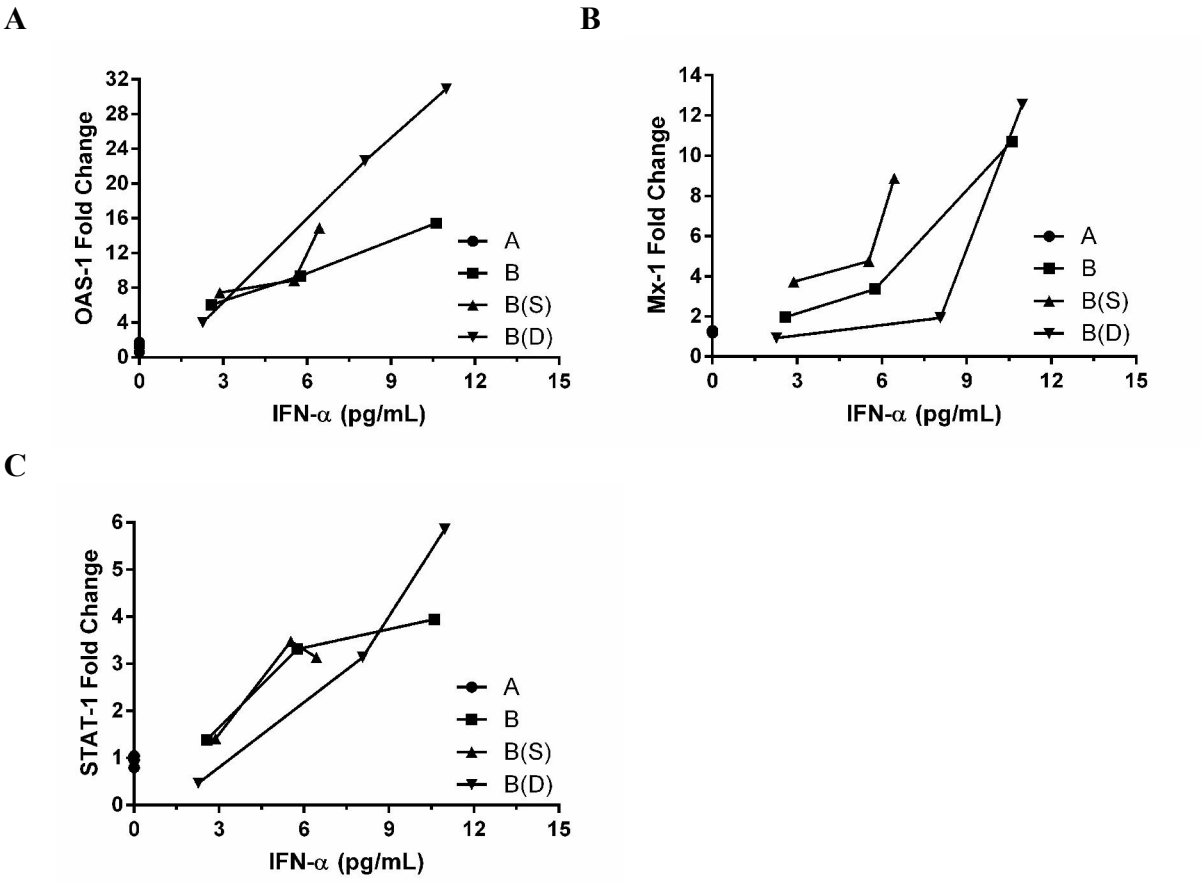

Figure 3.5: Correlation between concentrations of serum IFN-α and expression of ISGs. **A.** OAS-1. **B.** Mx-1. **C.** STAT-1.

3.4.2 IFN-γ

3.4.2.1 IFN-γ Induction

IFN-γ levels were analyzed in serum samples obtained from the animals on days -1, 3, 7, and 10 of infection, the values for which are displayed in Table 3.10.

Table 3.10: Serum IFN-γ levels (pg/mL) on days -1, 3, 7, and 10 of all group A and group B animals.

Group	Animal	Day			
		-1	3	7	10
A	24	0.00	0.00	1.60	2.04
A	28	0.00	0.00	0.00	5.67
A	33	0.00	0.00	0.00	0.00
A	37	1.29	0.00	1.60	3.13
A	41	0.00	0.00	0.00	0.00
A	46	1.60	2.02	2.66	2.48
A	51	0.00	1.22	1.49	3.29
B(S)	27	1.18	2.96	5.79	97.23
B(S)	30	0.54	1.03	9.22	12.57
B(S)	32	0.83	5.05	13.87	17.40
B(S)	42	0.00	2.84	7.30	8.37
B(S)	43	0.00	5.27	6.71	8.37
B(S)	44	0.00	1.11	7.67	20.40
B(S)	48	0.00	3.65	2.96	12.70
B(S)	49	0.00	15.71	6.12	2.65
B(D)	23	0.95	8.94	11.49	29.51
B(D)	26	0.69	4.04	16.40	12.06
B(D)	31	0.00	1.22	42.66	236.87
B(D)	36	0.00	2.58	6.04	14.87
B(D)	45	6.25	10.17	18.35	140.31
B(D)	50	0.59	16.90	40.80	74.00

As with the clinical measurements and serum IFN-α levels, IFN-γ levels on days 3, 7, and 10 of the challenge were evaluated as a difference in IFN-γ with respect to day -1. Figures 3.6A, 3.6B, and 3.6C show the individual changes within groups A, B(S), and B(D), respectively. Additionally, Figure 3.6D shows the median trends of groups A, B, B(S) and B(D).

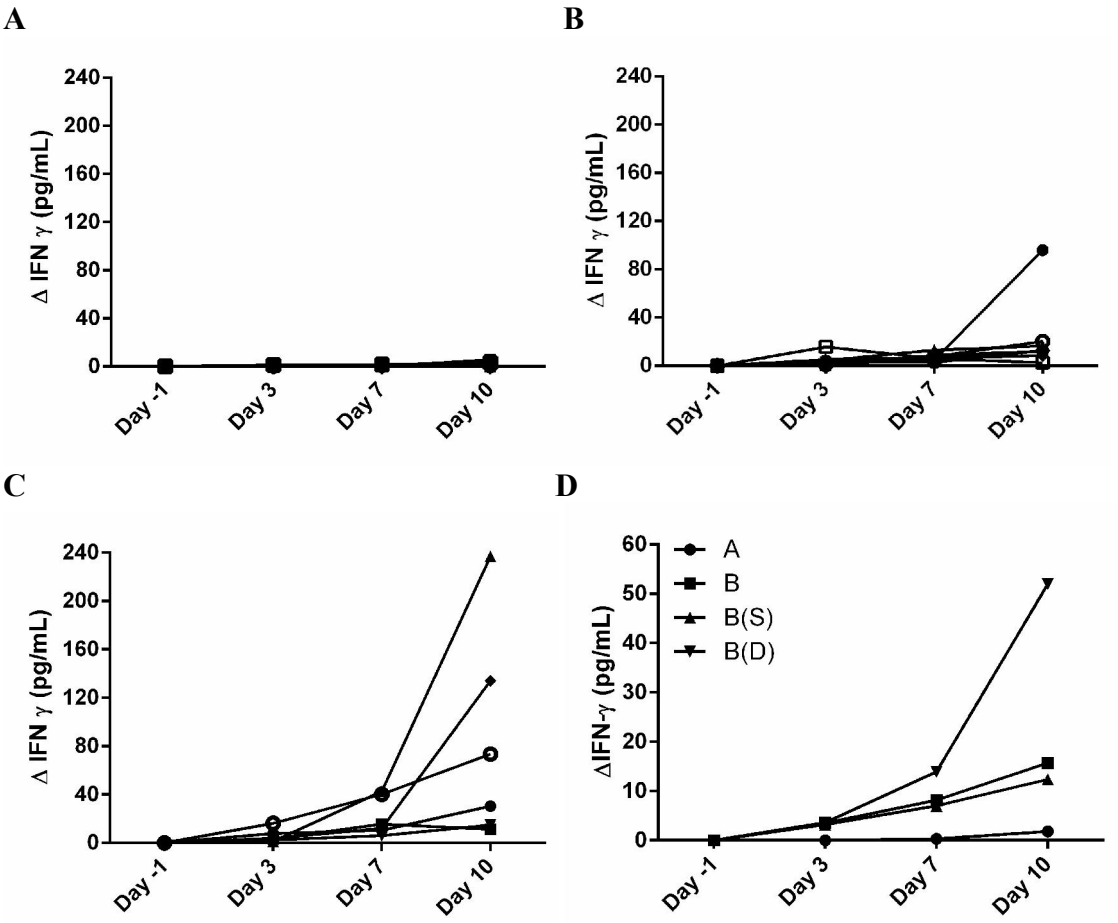

Figure 3.6: Serum IFN-γ differences of cattle with respect to day -1. **A.** Group A animals (n = 7). **B.** Group B(S) animals (n = 8). **C.** Group B(D) animals (n = 6). **D.** Median Data for groups A, B, B(S), B(D).

The difference in serum IFN-γ levels between groups A and B, as well as between group A and the two group B subgroups, was significant ($p \leq 0.05$). Significant distinction was also established between group B(S) and group B(D) on day 7 and when all of the days were summed, the latter exhibiting substantially higher levels of IFN-γ. Table 3.11 shows the *p*-values between the various groups on days 3, 7, and 10, as well as summed through all three days.

Table 3.11: *P*-values computed from serum IFN-γ level comparisons of various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	Group Comparisons			
	A vs. B	A vs. B(S)	A vs. B(D)	B(S) vs. B(D)
3	**<0.01**	**<0.01**	**<0.01**	0.57
7	**<0.01**	**<0.01**	**<0.01**	**0.04**
10	**<0.01**	**<0.01**	**<0.01**	0.08
All	**<0.01**	**<0.01**	**<0.01**	**0.03**

While distinction between the two subgroups was insignificant on days 3 and 10, there was an apparent trend towards increased likelihood of mortality in those animals that exhibited high levels of serum IFN-γ. In addition to the *p*-values between the various groups, Table 3.12 shows the ranks assigned to each animal based on IFN-γ serum changes.

Table 3.12: IFN-γ change ranks for all animals based on the summation of the IFN-γ changes (i.e. rank sum analysis) on days 3, 7, and 10 of the challenge. **1** = highest IFN-γ sum. **21** = lowest IFN-γ sum. Animals were assigned ranks **(1 – 7)** if their sums were in the top third of this ranking order. Animals were assigned ranks **(8 – 14)** if their sums were in the middle third of this ranking order. Animals were assigned ranks **(15 – 21)** if their sums were in the bottom third of this ranking order.

Group	Animal Label	Rank
A	**24**	**18**
A	**28**	**16**
A	**33**	**20**
A	**37**	**19**
A	**41**	**20**
A	**46**	**17**
A	**51**	**15**
B(S)	**27**	**4**
B(S)	**30**	**11**
B(S)	**32**	**6**
B(S)	**42**	**14**
B(S)	**43**	**12**
B(S)	**44**	**8**
B(S)	**48**	**13**
B(S)	**49**	**9**
B(D)	**23**	**5**
B(D)	**26**	**7**
B(D)	**31**	**1**
B(D)	**36**	**10**
B(D)	**45**	**2**
B(D)	**50**	**3**

A trend was observed within the data. Group B(S) animals tended to rank between the group A and the group B(D) animals while the group B(S) animals tended to rank low. All group A animals were clustered in the highest ranks while all group B animals were within the low or medium ranks. This suggests that, regardless of mortality outcome, BVDV stimulated some level of IFN-γ synthesis.

3.4.2.2 IFN-γ Response: SOCS1 and SOCS3

As mentioned, many characterised genes – GAS genes – respond to IFN-γ stimulus. Additionally, several negative feedback inhibitors – the SOCS genes – are up-regulated as cytokine stimulation persists. SOCS-1 and SOCS-3 are members of this gene family and are well-characterised negative feedback inhibitors of IFN-γ stimulus (Larsen and Ropke, 2002). Response of these genes to IFN-γ was explored in this challenge. While evidence demonstrates that serum IFN-γ is induced in response to BVDV infection, there exists potential for disconnect between production of and response to this cytokine as other pathogens have been shown to prevent host cells from responding to it (Hussain *et al*., 1999; Kierszenbaum *et al*., 1995; Ray *et al*., 2000).

SOCS-1 expression notably increased in group B on day 3 relative to day -1. This increase was short-lived as it drastically fell to near the pre-challenge levels by day 7, as seen in Figure 3.7A. This expression pattern did not mirror the serum IFN-γ trend, which slowly increased within the first 10 days (Figure 3.6). Additionally, there appears to be an indirect relationship between serum IFN-γ stimulus and SOCS-1 response, as seen in Figure 3.7B. SOCS-1 increased upon initial stimulus, but gradually declined as serum IFN-γ increased.

A **B**

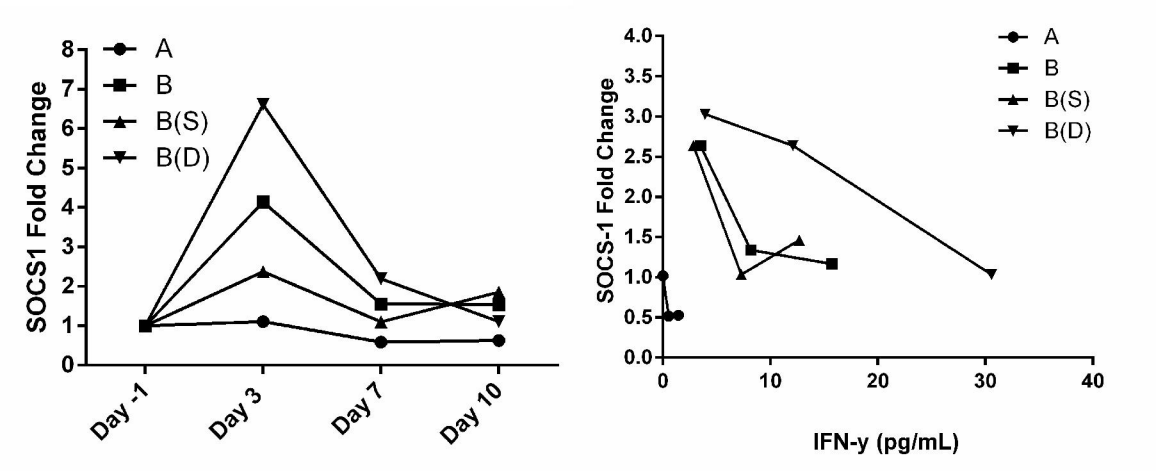

Figure 3.7: Expression of SOCS-1 in the PBMCs of cattle infected with BVDV. **A.** Median expression of SOCS-1. **B.** *P*-Values for each compared group.

SOCS-1 expression was statistically distinct between groups A and B; however, this difference was only maintained on days 3 and 7 when group A was compared individually compared with the two subgroups. Additionally, there was no difference between the two subgroups temporally or in summation. The *p*-values corresponding to these comparisons are listed in Table 3.13.

Table 3.13: *P*-values computed from comparisons of SOCS-1 expression levels between various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	**Group Comparisons**			
	A vs. B	**A vs. B(S)**	**A vs. B(D)**	**B(S) vs. B(D)**
3	**<0.01**	**0.03**	**0.03**	0.45
7	**0.01**	**0.04**	**0.03**	0.15
10	**0.04**	0.06	0.18	0.61
All	**<0.01**	**0.01**	**<0.01**	0.60

SOCS-3 expression was significantly increased on days 7 and 10 post-challenge in group B relative to group A, as shown in Figure 3.8A. This increased expression mirrored the serum IFN-γ levels, in that expression of both gene and cytokine remained elevated until day 10. While there was an apparent correlation between serum IFN-γ levels and SOCS-3 expression

levels, SOCS-3 induction appeared to plateau by day 7 while serum IFN-γ production continued to increase, as seen in Figure 3.8B.

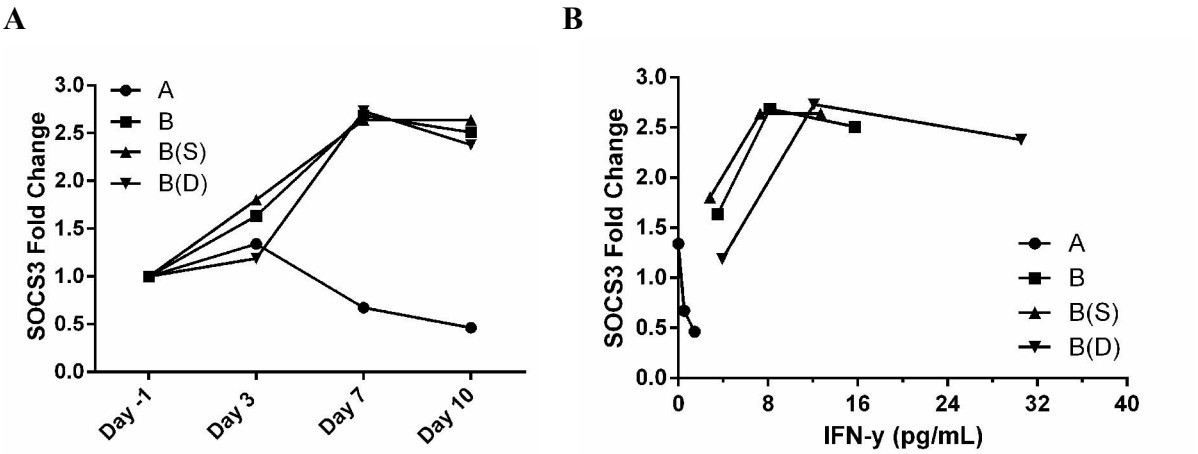

Figure 3.8: Expression of SOCS-3 in the PBMCs of cattle infected with BVDV. **A.** Median expression of SOCS-3. **B.** *P*-Values for each compared group.

While serum IFN-γ levels were much higher in group B(D), SOCS-3 expression levels between this group and group B(S) were not statistically different, as seen by the WRST-calculated *p*-values listed in Table 3.14.

Table 3.14: *P*-values computed from comparisons of SOCS-3 expression levels between various groups on days 3, 7, and 10, as well as across all three days. $P \leq 0.05$ is considered statistically different.

Days	**Group Comparisons**			
	A vs. B	**A vs. B(S)**	**A vs. B(D)**	**B(S) vs. B(D)**
3	0.21	0.18	0.50	0.64
7	**<0.01**	**<0.01**	**0.01**	0.84
10	**0.01**	0.03	**0.05**	0.84
All	**<0.01**	**<0.01**	**0.05**	0.86

3.5 Summary of Clinical and Cytokine Ranks

Trends were observed in nearly every experiment used to assess ncpBVDV infection. To identify collective trends in the viral presence, clinical, and cytokine data, their ranks were compiled together in Table 3.15.

Table 3.15: Rank summary for WBC viral presence, nasal fluid viral presence, temperature change, body weight change, serum IFN-γ change, and serum IFN-α change. Animals were assigned ranks **(1 – 7)** if their sums were in the top third of this ranking order. Animals were assigned ranks **(8 – 14)** if their sums were in the middle third of this ranking order. Animals were assigned ranks **(15 – 21)** if their sums were in the bottom third of this ranking order.

Group	Animal Label	WBC Viral Presence Sum Ranks	Nasal Viral Presence Sum Ranks	Temp. Sum Ranks	Body Weight Change Sum Ranks	IFN-γ Sum Ranks	IFN-α Sum Ranks
A	24	15	15	N/A	N/A	18	18
A	28	15	15	N/A	N/A	16	17
A	33	15	15	N/A	N/A	20	18
A	37	15	15	N/A	N/A	19	16
A	41	15	15	N/A	N/A	20	21
A	46	15	15	N/A	N/A	17	15
A	51	15	15	N/A	N/A	15	18
B (S)	27	2	2	5	9	4	11
B (S)	30	8	8	7	10	11	8
B (S)	32	9	14	9	12	6	13
B (S)	42	9	10	8	13	14	7
B (S)	43	5	10	10	11	12	14
B (S)	44	8	8	3	7	8	6
B (S)	48	8	2	13	1	13	4
B (S)	49	8	10	14	8	9	5
B (D)	23	5	6	11	6	5	10
B (D)	26	8	6	1	14	7	9
B (D)	31	3	2	5	3	1	12
B (D)	36	5	10	12	4	10	3
B (D)	45	3	1	2	2	2	1
B (D)	50	1	2	4	5	3	2

In addition to confirming that the group A animals were appropriate controls in all analyses, the results in Table 3.15 suggest that ncpBVDV2-1373 affected animals in groups B(S) and B(D) differently. In four of the six categories, five of the six group B(D) animals are high responders and only in one instance do groups B(S) and B(D) have an equivalent 1:1 ratio of high and medium responders. Additionally, the top three responders are group B(D) animals in five of the six categories.

3.6 Kinome Analysis: JAK-STAT Signalling in PBMCs

Species-specific peptide arrays have shown to be informative tools for detailing kinome (global, cellular kinase) activity in multiple species, including cattle (Jalal *et al*., 2009). Recently, bovine-specific peptide arrays have highlighted mechanisms by which pathogens guide and alter signalling in both monocytes and gut tissues (Arsenault *et al*., 2012; Arsenault *et al*., 2013; Maattanen *et al*., 2013).

In this investigation, kinome analysis was used on PBMCs obtained from two group A animals and two group B animals – one each from groups B(S) and B(D) – on days -1, 3, 7 and 10. Individual animals possess unique kinotypes (individual patterns of cellular signalling); as with the clinical, cytokine, and gene expression analyses, the signalling profile of each animal was assessed against its signalling profile at a pre-challenge time point – day -1 (Trost *et al*., 2013). While the arrays can provide information on global cell signalling patterns, the focus within this challenge was on the activity of the JAK-STAT pathway, which mediates signalling by both IFN-α and -γ (Shuai *et al*., 2003).

There was no evidence of JAK-STAT signalling in either of the group A animals at any of the three time points, as shown in Table 3.16. Conversely, activation of the JAK-STAT pathway was apparent in both of the group B animals at all three of the time points.

Table 3.16: *P*-values related to kinase activity of the JAK-STAT Pathway (KEGG 568) in PBMCs from representatives of both group A and group B.

Group	**Animal**	**Day**		
		3	**7**	**10**
A	**28**	0.22	0.15	0.52
A	**41**	0.13	0.49	0.60
B(S)	**48**	**0.04**	**0.02**	**0.09**
B(D)	**50**	**0.09**	**0.09**	**0.06**

*Based on levels of differential expression or phosphorylation, InnateDB, a publically available pathway analysis tool (Lynn *et al*., 2008), predicted pathways that are consistent with the experimental data. Pathways were assigned *p*-values based on the number of proteins present for a particular pathway. For this investigation, fold change cut-offs were set at a *p*-value of 0.1 for confidence of difference between post-challenge and pre-challenge days.

Table 3.16 shows that there was no significant change in JAK-STAT expression of the two group A animals relative to day -1 on any of their assessed challenge days. However, the two group B animals showed a significant increase in JAK-STAT phosphorylation across days 3, 7, and 10, with *p*-values remaining below 0.10; below 0.05 on days 3 and 7 in animal 48.

4. DISCUSSION

4.1 Interferon: Barrier to Infection

Many pathogens, particularly viruses, establish persistent infections to maintain host presence and potentiate their lifecycles. To be successful, these pathogens must counter responses initiated by the host's immune system. BVDV's continued prevalence in the environment is owed to its ability to manage host cell defenses; even more importantly, establish presence within fetal hosts prior to the development of their immune systems. A major, early barrier to establishing host presence is the IFN response. Most viruses have developed mechanisms that downplay immune responses; the first and most important mechanism is often one that stymies IFN response. Viruses may employ a single- or multi-faceted approach; mechanisms of which include disabling IFN production, down-regulating IFN receptors, interfering with IFN-mediated signalling, or altering the expression and/or activity of IFN-induced effectors (Bonjardin *et al*., 2005; Bowie *et al*., 2008; García-Sastre *et al*., 2006; Goodbourn *et al*., 2000; Haller *et al*., 2006; Randall *et al*., 2008; Versteeg *et al*., 2010).

Substantial research has been performed to identify the tools and mechanisms through which BVDV interacts with the host's immune system. Numerous studies have been published highlighting experiments that have examined host IFN responses using a variety of scenarios that have altered the host, the BVDV strain, and the experimental tools that have directly and indirectly transcriptionally measured the IFNs – mainly IFN-α – and the levels of IFN-regulated gene expression, in order to understand the complete host IFN response to this virus. Following these studies, BVDV's influence on host IFN responses has been shown to be a complex, multi-component interaction, which is even more characteristic of non-cytopathic BVDV strains. A previous hypothesis was made that suggested BVDV has three objectives in responding to the host immune system and establishing either a transient or a persistent infection: i) prevent IFN induction, ii) counter IFN response upon establishment of infection, and iii) avoid interfering with IFN action that targets unrelated viruses (Peterhans and Schweizer, 2013). Such a mechanism in establishing self-tolerance requires such complex, multi-component interactions with the host in order to avoid viral elimination by IFN processes.

Researchers have generally agreed that the cpBVDV strains stimulate IFN responses but there is contention regarding whether ncpBVDV strains also stimulate IFN responses. There are multiple possible reasons for the conflicting conclusions regarding the influence of ncpBVDV on host IFN – particularly IFN-α – responses. These reasons can mainly be summarized in the fact that there exists a breadth of genetic and phenotypic variation within BVDV strains that probably influences the extent and the mechanism to which the host is immunosuppressed (Palomares *et al*., 2013). It is important to understand that there is a distinction between the induction of IFN and the activity of IFN. Therefore, to properly understand BVDV's influence, this discussion will separate the information related to the induction and the action of IFN.

4.1.1 Induction of IFN

Conclusions from past studies suggest that ncpBVDV is capable of infecting host cells when type I IFNs have not been induced (Schweizer *et al*., 2006). Additionally, several specific mechanisms and effectors by which BVDV uses to subvert host type I IFN responses have been defined, particularly the degradation of IRF-3 via N^{pro} activation, which limits transcriptional activation of the IFN-β gene (Seago, *et al*., 2007). Furthermore, E^{rns} degrades viral RNA to limit host responses to BVDV (Rumenapf *et al*., 1993; Schneider *et al*., 1993). These two effectors are pivotal in order for BVDV to successfully infect the host (Meyers *et al*., 2007).

This thesis has provided evidence for the induction of both IFN-α and IFN-γ in response to ncpBVDV infection. In particular, there was a significant, transient increase in serum levels of both cytokines ($p < 0.05$) in response to BVDV infection. IFN responses to viral infection are often temporary and self-controlling in order to avoid pathological consequences that include disruption of both the normal immune response and blood cell development as a consequence of extended IFN stimulation (Abbas *et al*., 2005; Gånheim *et al*. 2003; Lin *et al*., 1998; Tråvén *et al*., 1991). Within group B, the highest producers of IFN-α tended to be the group B(D) animals; although, the group B(D) to B(S) comparison was not statistically significant. However, when comparing group B to group A, levels of serum IFN-γ were statistically different ($p < 0.01$). Additionally, group B(D) exhibited higher levels of serum IFN-γ ($p < 0.05$), which suggested that serum IFN-γ reflects the severity of ncpBVDV2-1373 infection.

4.1.2 Action of IFN

Following establishment of its presence within the host, evidence suggests that BVDV is unaffected by IFN-α if it's synthesized due to host cell infection by another pathogen. Past studies have demonstrated that cells treated with IFN-α prior to BVDV challenge are able to utilize this host defense to respond to BVDV while the same treatment, when provided post-infection, is ineffective (Peek *et al*., 2004; Schweizer *et al*., 2006). Notably, the ability of BVDV to avoid being cleared from the host during IFN-α response appears specific to this virus as BVDV-infected cells treated with IFN-α were guarded from infection by different viruses (Schweizer *et al*., 2006). Within *in vitro* analyses, BVDV is able to counter the action of IFN-α even after extended passages in the presence of elevated IFN-α levels (Schweizer *et al*., 2006). Consistent with the inability of BVDV-infected cells to induce IFN-mediated responses that clear the pathogen *in vitro*, treatment of BVDV-infected cattle with recombinant IFN-α failed to reduce viremia (Peek *et al*., 2004). Researchers have suggested that BVDV may impact the immune environment of the infected cells via regulating of NF-κB through the use of protein kinase R and/or IRFs (Taniguchi *et al*., 2002).

This study has provided evidence, which has shown that PBMCs from ncpBVDV infected animals remain responsive to both IFN-α and -γ. Notably, while immunocytochemical experiments demonstrated that BVDV was present within the WBCs, PBMCs represent a mixture of blood cell types with respect to their infection status. Therefore, both infected and uninfected cells might have differential capabilities in responding to IFN in that the infected cells do not respond to IFN. However, the ability of IFN to guide the clearance of unrelated viruses from BVDV-infected cells would also suggest that these cells still have functional IFN. Acknowledging the IFN responsiveness of the PBMCs questions whether BVDV is able to immunocompromise the host and suggests a complete downplay of IFN responsiveness. There was clear induction of gene expression of those genes known to be controlled by both IFN-α and –γ. Additionally, both the temporal characteristics and extents of induction of these genes trended similarly with the levels of the cytokines that regulated them.

PBMC responsiveness to IFN within BVDV-infected animals was further confirmed via kinome analysis. As a pivotal component to their infection, various pathogens, particularly those establishing chronic infections, manipulate host cellular signalling responses (Bowie *et al*., 2008;

Brodsky *et al*., 2009; Kim *et al*., 2008; Reddick *et al*., 2014). Therefore, studies examining host-pathogen interactions at the level of kinase-mediated signal transduction enable the understanding of cellular mechanisms connected with potent pathogen elimination; additionally, mechanisms used by the pathogen to avoid host defense. PBMC kinome analysis of ncpBVDV2-1373-infected cattle showed consistent and persistent activation of the JAK-STAT signalling pathway relative to the signalling activity of PBMCs before challenge was infected. Conversely, kinome analysis elucidated no evidence of JAK-STAT activation in the PBMCs of the group A animals. The combination of JAK-STAT activation with increased IFN-regulated gene expression is consistent with results of prior *in vitro* experiments (Baigent *et al*., 2004; Schweiser *et al*., 2006).

4.2 Clinical Outcomes

Characterising temperature and body weight changes is relevant as veterinarians and clinicians use these measures to track the health of an animal and the progression of illness. The temporal pattern of temperature change observed in this challenge follows a pattern that was previously observed under similar BVDV challenges (Ellis *et al*., 1998; Liang *et al*., 2006; Snider *et al*. 2014; van Drunen Littel-van den Hurk *et al*., 2013). Apart from clinically confirming the challenged animals were sick, the temperature change pattern added further evidence to classify the initiated BVDV infection as acute. Past evidence has shown that, in addition to leukopenia, increased body temperature is characteristic of acute BVDV infection (Liebler-Tenorio *et al*., 2003b), a result that contrasts with infection by low virulence strains (Bolin and Ridpath, 1992).

4.3 Predictive Markers for Mortality

While the mortality rate of the challenged cattle was unexpected, it enabled the testing of the clinical scores, the cytokines, and the regulated genes as predictive markers for mortality. While expectations would suggest that greater changes in clinical responses and higher spikes in immune system cytokines tend to favour increased likelihood of mortality; in this study, semi-quantitative and statistical assessments were performed to examine the usefulness of each marker as a mortality predictor. The desire to assess these markers was the reason for the subdivision of the group B animals. It was through subgroup B comparisons that these markers were examined.

Clinical signs are the most practical method to assess the course a disease may take as veterinarians are much more likely to examine changes in temperature and body weight over serum cytokine levels. These clinical changes are reliably used to assess an illness and were practical measures to assess BVDV infection in this challenge. During the course of the challenge, temperatures and body weights deviated; as expected, the group B(D) animals exhibited sharper variations in temperature change and body weight loss. However, a statistical comparison between both groups suggested the difference in clinical scores between both groups was insignificant. This was easy to see with temperature scores in Tables 3.2 and 3.15: four of the group B(D) animals ranked within the top five temperature responders, but the remaining two animals ranked very low. While five of the six group B(D) animals ranked among the top six animals in terms of body weight decrease, one animal within group B(S) exhibited the greatest weight drop. Additionally, the sixth animal in group B(D) exhibited the smallest body weight decrease among the fourteen. While decreased body weight change tended to favour increased likelihood of mortality, the outliers likely prevented statistical significance from being attributed.

The IFNs exhibited differential levels of reliability in predicting mortality. Firstly, while the highest IFN-α producers were among group B(D), the high and medium responders were equally divided between groups B(S) and B(D). Even before calculating a *p*-value to assess the difference between the groups, Tables 3.6 and 3.15 illustrated a lack of distinction between the two subgroups and strongly suggest IFN-α is an unreliable predictive marker. Conversely, IFN-γ was a more convincing predictive marker. Five of the six group B(D) animals ranked as high responders and the top two responders were both group B(D) animals. Additionally, the *p*-value calculated from cytokine levels summed from data on days 3, 7, and 10 showed the distinction between the two groups was significant. As such, IFN-γ arose as the only reliable marker for mortality within this challenge.

One difficulty in accepting the mortality results as valid is the sample size that was assessed. A fourteen-animal sample size is a small sample size under which to properly assess markers for mortality. This is particularly evidenced within the body weight data, which exhibited two outliers for each subgroup at the opposite ends of the animal ranks. It is well known that outliers in smaller sample sizes have greater abilities to skew trends within data. For example, body weight change is a well-known method of assessing severity of pathogen

infection; as such, it would be expected to be a more reliable predictive marker. The outliers in the ranks likely prevented statistical significance from being applied. As such, in order to properly assess predictive mortality markers, a larger sample size should be used. The results from this challenge suggest that IFN-γ warrants further investigation as a predictive marker.

4.4 Application of Peptide Arrays to Host-BVDV Interaction

While the only results presented in this study were related to the JAK-STAT pathway, the peptide arrays were utilized to examine global pathway changes between groups A and B relative to pre-challenge pathway profiles. Kinome analysis represents a novel tool through which further assessment of BVDV could be explored. The peptide array technology used to analyse kinase-mediated signal transduction is a novel and relatively new tool that has already been used to examine signalling alterations caused by antigen stimulation and pathogen infection (Arsenault *et al*., 2009; Arsenault *et al*., 2012; Arsenault *et al*., 2013).

As these arrays are assembled by synthetically creating and attaching multiple unique 15-mer peptides that represent phosphorylation sites of well-known protein kinases, data obtained from the arrays requires validation by additional experimental means, i.e. ELISAs, Western blots, or qRT-PCRs. Therefore, while the arrays provide a substantial amount of information, the information they elucidate must be confirmed. Interestingly, the peptide array data in this thesis is presented in reverse order in that JAK-STAT signalling is used to "validate" the cytokine and gene expression data. While these peptide arrays cannot validate the information from those experiments, they do provide another independent assessment of immune response. JAK-STAT signalling is often indicative of immune response as IFNs use this pathway to regulate various genes; as such, its activation provides additional evidence for the induction of IFN in this challenge.

InnateDB produces information on differential phosphorylation within both pathways and individual kinases (Lynn *et al*., 2008). These arrays have been used to examine the phosphorylation states of various JAKs and STATs, as well as well-characterised kinases within TLR pathways. As such, a more detailed examination of the phosphorylation states of select kinases could identify kinases as targets for BVDV infection; furthermore, as biomarkers on which to predict infection status or likelihood of mortality, or even against which to develop

novel therapeutics. Additionally, as these arrays are convenient tools to examine global signalling changes, they are very useful in guiding researchers to notable signalling events that could be further assessed. These events could include networks associated with growth, apoptosis, and the cell cycle. Overall, these arrays have been shown to detect changes in host-BVDV interaction (host signalling patterns caused by BVDV-infection) and represent novel tools on which to expand research into biomarker identification and characterisation of strain-specific BVDV infection.

4.5 Future Work

As the original goal of this study was to examine clinical and biochemical changes in animals persistently infected by BVDV, a future study could reattempt establishment of a persistent infection. This could be done by lowering the administered viral dose or by using a strain that is less virulent than the 1373 strain. The NY-1 strain is a potential substitute. The analyses conducted in this challenge could also be used in that future challenge. One additional component to that challenge could be the separation of PBMCs into their individual cell populations, or else isolating one of those populations to conduct clinical and biochemical tests.

This study has answered several questions; notably, addressed the contention regarding the ability of ncpBVDV to induce an IFN response. While this contention was mainly regarding the IFN-α, this study provided solid evidence that both IFN-α and -γ are induced by 1373. As mentioned, past studies have demonstrated that cpBVDV strains induce IFN response. Because 1373 exhibits both non-cytopathic and cytopathic characteristics, analysis of IFN induction should further be assessed by a less virulent, non-cytopathic strain; as such, NY-1 is a good candidate in both establishing a persistent infection and in further examining IFN induction. Results from that future challenge would further cement BVDV's status as an IFN-inducer.

Apart from demonstrating its induction, this study also identified IFN-γ as a potential predictive marker for mortality. As the animal sample size was relatively small, its status as a predictive marker should be further tested on a larger sample size. This could be done as a secondary objective in a future animal trial; albeit, in a challenge that would likely need to again utilize 1373 or another virulent BVDV strain. A challenge consisting of two groups – one

infected with NY-1 and the other infected with 1373 – would be useful in providing data on IFN induction and mortality assessment.

Finally, future experiments could expand on the use of peptide array technology to analyse animal kinotypes. It is clear from this thesis, in addition to previous studies, that kinome analysis was able to elucidate host cell signalling information caused by host-pathogen interaction. This technology could be further explored in future studies, particularly as a component of future BVDV challenges. Such uses of the arrays could include examining the changes of specific kinases between challenged animals; identifying potential biomarkers which could identify disease status, predict potential outcome, or act as targets against which new therapeutics could be developed.

5. CONCLUSIONS

In conclusion, this study identified ncpBVDV2-1373 as an inducer of both IFN-α and IFN-γ within bovine sera. These results help dispel past controversy regarding the status of non-cytopathic BVDV as an IFN-inducer. Additionally, this study provided solid evidence that the animals successfully responded to these cytokines. Genes known to be regulated by IFN-α – OAS-1, Mx-1, and STAT-1 – and IFN-γ – SOCS-1 and SOCS-3 – were shown to express in temporal patterns similar to their regulatory cytokines. The results of this challenge also suggested that IFN-γ shows promise as a potential predictor of mortality to BVDV infection as the cytokine's levels between groups B(S) and B(D) were statistically distinct on day 7 post-infection, as well as across the three post-infection days combined. Finally, peptide arrays assessing kinome profiles predicted increased phosphorylation within the JAK-STAT signalling network of PBMCs that were isolated from the group B cattle, a prediction which adds evidence that ISGs and GAS genes were signalled by IFNs. As such, these arrays show promise as novel tools through which to predict and further examine the specific mechanisms of host-BVDV interaction at the level of kinase-mediated phosphorylation.

6. REFERENCES

Abbas, A.K., Lithman, A.H. (2005). Cellular and Molecular Immunology, 5th edn. Philadelphia, PA.

Aderem, A., Underhill, D. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. *17*, 593–623.

Aderem, A., Ulevitch, R.J. (2000). Toll-like receptors in the induction of the innate immune response. Nature. *17*, 782–787.

Agapov, E.V., Murray, C.L., Frolov, I., Qu, L., Myers, T.M., Rice, C.M. (2004). Uncleaved NS2-3 is required for production of infectious bovine viral diarrhea virus. J. Virol. *78*, 2414– 2425.

Akhtar, L.N., Benveniste, E.N. (2011). Viral exploitation of host SOCS protein functions. J. Virol. *85*, 1912–1921.

Alcami, A., Koszinowski, U.H. (2000). Viral mechanisms of immune evasion. Immunol. Today. *21*, 447–455.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., Randall, R.E. (2004). The V proteins of paramyxoviruses bind to the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc. Natl. Acad. Sci. U.S.A. *101*, 17264–17629.

Arsenault, R.J., Jalal, S., Rabiuk L.A., Potter, A., Griebel, P.H., Napper, S. (2009). Kinome analysis of toll-like receptor signaling in bovine monocytes. J. Recept. Signal Transduct. Res. *29*, 299–311.

Arsenault, R.J., Li, Y., Bell, K., Doig, K., Potter, A., Griebel, P.J., Kusalik, A., Napper, S. (2012). Mycobacterium avium subsp. paratuberculosis inhibits gamma interferon-induced signalling in bovine monocytes: insights into the cellular mechanisms of johne's disease. Infect. Immun. *80*, 3039–3048.

Arsenault, R.J., Li, Y., Maattanen, P., Scruten, E., Doig, K., Potter, A., Griebel, P., Kusalik, A., Napper, S. (2013). Altered toll-like receptor 9 signaling in mycobacterium avium subsp. paratuberculosis infected bovine monocytes reveals potential therapeutic targets. Infect. Immun. *81*, 226–237.

Baca-Estrada, M.E. Godson, D.L. Hughes, H.P. Van Donkersgoed, J. Van Kessel, A. Harland, R. Shuster, D.E. Daley, M. Babiuk, L.A. (1995). Effect of recombinant bovine interleukin-1 beta on viral/bacterial pneumonia in cattle. J. Interferon Cytokine Res., *15,* 431–439.

Baccala, R., Gonzalez-Quintial, R., Lawson, B.R., Stern, M.E., Kono, D.H., Beutler, B., Theofilopoulos, A.N. (2009). Sensors of the innate immune system: their mode of action. Nat. Rev. Rheumatol. *5*, 448–456.

Baigent, S. J., Goodbourn, S. & McCauley, J. W. (2004). Differential activation of interferon regulatory factors -3 and -7 by non-cytopathogenic and cytopathogenic bovine viral diarrhoea virus. Vet. Immunol. Immunopathol. *100*, 135-144.

Baigent, S.J., Zhang, G., Fray, M.D., Flick-Smith, H., Goodbourn, S., McCauley, J.W. (2002). Inhibition of beta interferon transcription by noncytopathogenic bovine viral diarrhea virus is through an interferon regulatory factor 3-dependent mechanism. J. Virol. *76*, 8979–8988.

Bauer, S. (2006). Toll-earting self DNA. Nat. Immunol. *7*, 13–15.

Bauermann, F.V., Falkenberg, S.M., Vander Ley, B., Decaro, N., Brodersen, B.W., Harmon, A., Hessman, B., Flores, E.F., Ridpath, J.F. (2014). Generation of calves persistently infected with hobi-like pestivirus and comparison of methods for detection of these persistent infections. J. Clin. Microbiol. *52*, 3845–3852.

Bauermann, F.V. Ridpath, J.F., Weiblen, R., Flores, E.F. (2013). HoBi-like viruses: an emerging group of pestiviruses. J. Vet. Diagn. Invest. *25*, 6–15.

Baule, C., Kulcsar, G., Belak, K., Albert, M., Mittelholzer, C., Soos, T., Kucsera, L., Belak, S. (2001). Pathogenesis of primary respiratory disease induced by isolates from a new genetic cluster of bovine viral diarrhea virus type I. J. Clin. Microb. *39*, 146–153.

Bekeredjian-Ding, I.B., Wagner, M., Hornung, V., Giese, T., Schnurr, M., Endres, S., Hartmann, G. (2005). Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J. Immunol. *174,* 4043–4050.

Bolin, S.R. (2002). Bovine Viral Diarrhea Virus in Mixed Infection, In: Brogden, K.A., Guthmiller, J.M. (Ed.) Polymicrobial Diseases. ASM Press, Washington, D.C., pp. 41–68.

Bolin, S.R., Ridpath, J.F. (1992). Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am. J. Vet. Res. *53*, 2157–2163.

Bonjardim, C. A. (2005). Interferons (IFNs) are key cytokines in both innate and adaptive antiviral immune responses and viruses counteract IFN action. Microbes Infect. *7*, 569–578.

Bowie, A. G., Unterholzner, L. (2008). Viral evasion and subversion of pattern-recognition receptor signalling. Nat. Rev. Immunol. *8*, 911–922.

Brackenbury, L.S., Carr, B.V., Charleston, B. (2003). Aspects of the innate and adaptive immune responses to acute infections with BVDV. Vet. Microbiol. *96*, 337–344.

Brinkmann, V., Geiger, T., Alkan, S., Heusser, C.H. (1993). Interferon α increases the frequency of interferon γ-producing human $CD4^+$ T cells. J. Exp. Med. *178*, 1655–1663.

Brock, K.V., Deng, R., Riblet, S.M. (1992). Nucleotide sequencing of 5' and 3' termini of bovine viral diarrhea virus by RNA ligation and PCR. *38*, 39–46.

Brock, K.V., Grooms, D.L., Givens, M.D. (2005). Reproductive Disease and Persistent Infection, In: Goyal SM, R.J. (Ed.) Bovine Viral Diarrhea Virus. Diagnosis, Management and Control. Blackwell Publishing, Ames, Iowa, pp. 145–156.

Brock, K.V., Redman, D.R., Vickers, M.L., Irvine, N.E. (1991). Quantitation of bovine viral diarrhea virus in embryo transfer flush fluids collected from a persistently infected heifer. J. Vet. Diagn. Invest. *3,* 99–100.

Brock, K.V., Widel, P., Walz, P., Walz, H.L. (2007). Onset of protection from experimental infection with type 2 bovine viral diarrhea virus following vaccination with a modifiedlive vaccine. Vet Ther. *8*, 88–96.

Brodsky, I. E., Medzihtov, R. (2009). Targeting of immune signalling networks by bacterial pathogens. Nat. Cell. Biol. *11*, 521–526.

Bruschke, C.J., Haghparast, A., Hoek, A., Rutten, V.P., Wentink, G.H., van Rijn, P.A., van Oirschot, J.T. (1998). The immune response of cattle, persistently infected with noncytopathic BVDV, after superinfection with antigenically semi-homologous cytopathic BVDV. Vet. Immunol. Immunopathol. *62*, 37–50.

Chaplin, D.D. (2010). Overview of the immune response. J. Allergy Clin. Immunol. *125*, S3–S23.

Charleston, B., Brackenbury, L. S., Carr, B. V., Fray, M. D., Hope, J. C., Howard, C. J., Morrison, W. I. (2002). Alpha/beta and gamma interferons are induced by infection with noncytopathic bovine viral diarrhea virus in vivo. J. Virol. *76*, 923–927.

Charleston, B., Fray, M. D., Baigent, S., Carr, B. V., Morrison, W. I. (2001). Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type I interferon. J. Gen. Virol. *82*, 1893–1897.

Chen, Z., Rijnbrand, R., Jangra, R.K., Devaraj, S.G., Qu, L., Ma, Y., Lemon, S.M., Li, K. (2007). Ubiquitination and proteasomal degradation of interferon regulatory factor-3 induced by Npro from a cytopathic bovine viral diarrhea virus. Virology. *366*, 277–292.

Cook, J.R., Jung, V., Schwartz, B., Wang, P., Pestka, S. (1992). Structural analysis of the human interferon gamma receptor: a small segment of the intracellular domain is specifically required for class I major histocompatibility complex antigen induction and antiviral activity. Proc. Natl. Acad. Sci. U.S.A. *89*, 11317–11321.

Daigle, J., Van Wyk, B., Trost, B., Scruten, E., Arsenault, R., Kusalik, A., Griebel, P.J., Napper, S. (2014). Peptide arrays for kinome analysis of livestock species. Front. Vet. Sci. *14*, 1–15.

Decaro, N., Mari, V., Pinto, P., Lucente, M.S., Sciarretta, R., Cirone, F., Colaianni, M.L., Elia, G., Thiel, H.J., Buonavoglia, C. (2012). Hobi-like pestivirus: both biotypes isolated from a diseased animal. J. Gen. Virol. *93*, 1976–1983.

Deng, R., Brock, K.V. (1993). 5' and 3' untranslated regions of pestivirus genome: primary and secondary structure analyses. Nucleic Acids Res. *21*, 1949–1957.

Deregt, D., Bolin, S.R., van den Hurk, J., Ridpath, J.F., Gilbert, S.A. (1998). Mapping of a type 1-specific and a type-common epitope on the E2 (gp53) protein of bovine viral diarrhea virus with neutralization escape mutants. Virus Res. *53*, 81–90.

Desport, M., Collins, M.E., Brownlie, J. (1998). Genome instability in BVDV: an examination of the sequence and structural influences on RNA recombination. Virology. *246*, 352–361.

Diderholm, H., & Dinter, Z. (1966). Interference between strains of bovine virus diarrhea virus and their capacity to suppress interferon of a heterologous virus. Proc. Soc. Exp. Biol. Med. *121*, 976–980.

Dixit, E., Boulant, S., Zhang, Y.J., Lee, A.S.Y., Odendall, C., Shum, B., Hacohen, N., Chen, Z.J., Whelan, S.P., Fransen, M., Nibert, M.L., Superti-Furga, G., Kagan, J.C. (2010). Peroxisomes are signaling platforms for antiviral innate immunity. Cell. *141*, 668–681.

Doceul, V., Charleston, B., Crooke, H., Reid, E., Powell, P.P., Seago, J. (2008). The N^{pro} product of classical swine fever virus interacts with IκBα, the NF-κB inhibitor. J. Gen. Virol. *89*, 1881–1889.

Draghici, S. (2003). Elements of statistics. Data analysis tools for DNA microarrays, Etheridge, A.M., *et al.*, eds. (Boca Raton, Fla: Chapman & Hall/CRC), pp. 61–107.

Ellis, J. A., West, K. H., Cortese, V. S., Myers, S. L., Carman, S., Martin, K. M., Haines, D. M. (1998). Lesions and distribution of viral antigen following an experimental infection of young seronegative calves with virulent bovine viral diarrhea virus-type II. Can. J. Vet. Res. *62*, 161–169.

Everitt B. (1974). Cluster Analysis. (London: Heinemann Education).

Finlay, B.B., McFadden, G. (2006). Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell. *124*, 767–782.

Gånheim, C., Hultén, C., Carlsson, U., Kindahl, H., Niskanen, R., Waller, K. P. (2003). The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or Mannheimia haemolytica. J. Vet. Med. B Infect. Dis. Vet. Public Health *50*, 183–190.

García-Sastre, A., Biron, C.A. (2006). Type I interferons and the virus-host relationship: a lesson in détente. Science. *312*, 879–882.

Gil, L.H.V.G., Ansari, I.H., Vassilev V., Liang, D., Lai V.C.H., Zhong, W., Hong, Z., Dubovi, E.J., Donis, R. O. (2006). The amino-terminal domain of bovine viral diarrhea virus N^{pro} protein is necessary for alpha/beta interferon antagonism. J. Virol. *80*, 900–911.

Glew, E. J., Carr, B. V., Brakenbury, L. S., Hope, J. C., Charleston, B., Howard, C. J. (2003). Differential effects of bovine viral diarrhoea virus on monocytes and dendritic cells. J. Gen. Virol. *84*, 1771–1780.

Gong, Y., Shannon, A., Westaway, E.G., Gowans, E.J. (1998). The replicative intermediate molecule of bovine viral diarrhoea virus contains multiple nascent strands. Arch. Virol. *143*, 399–404.

Gong, Y., Trowbridge, R., Macnaughton, T.B., Westaway, E.G., Shannon, A.D., Gowans, E.J. (1996). Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. J. Gen. Virol. *77*, 2729– 2736.

Goodbourn, S., Didcock, L., Randall, R.E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. *81*, 2341–2364.

Griffin, S.D., Harvey, R., Clarke, D.S., Barclay, W.S., Harris, M., Rowlands, D.J. (2004). A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. J. Gen. Virol. *85*, 451–461.

Grummer, B., Beer, M., Liebler-Tenorio, E., Greiser-Wilke, I. (2001). Localization of viral proteins in cells infected with bovine viral diarrhoea virus. J. Gen Virol. *82*, 2597–2605.

Grummer, B., Grotha, S., Greiser-Wilke, I. (2004). Bovine viral diarrhoea virus is internalized by clathrin-dependent receptor-mediated endocytosis. J. Vet. Med. B. Infect. Dis. Vet. Public Health. *51*, 427–432.

Hahm, B., Trifilo, M.J., Zuniga, E.I., Oldstone, M.B. (2005). Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. Immunity. *22*, 247–257.

Haller, O., Kochs, G., Weber, F. (2006). The interferon response circuit: induction and suppression by pathogenic viruses. Virology *344*, 119–130.

Haloran, M.E. (1998). Concepts of infectious disease epidemiology, In: Rothman R, G.S. (Ed.) Modern Epidemiology. Lippincott-Raven, Philadelphia, PA, pp. 524–554.

Hansen, J.D., Vojtech, L.N., Laing, K.J. (2011). Sensing disease and danger: a survey of vertebrate PRRs and their origins. Dev. Comp. Immunol. *35*, 886–897.

Harada, T., Tautz, N., Thiel, H. (2000). E2-p7 region of the bovine viral diarrhea virus polyprotein: processing and functional studies. J. Virol. *74*, 9498–9506.

Hartigan J.A. (1975). Clustering algorithms. (New York: Wiley).

Hasbold, J., Hong, J.S., Kehry, M.R., Hodgkin, P.D. (1999). Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a. J. Immunol. *163*, 4175–4181.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y.H., Randall, R.E., McCauley, J.W., Goodbourn, S. (2006). The N^{Pro} product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. J. Virol. *80*, 11723e32.

Hilton, D.J., Richarson, R.T., Alexander, W.S., Viney, E.M., Willson, T.A., Sprigg, N.S. (1998). Twenty proteins containing a c-terminal SOCS box form five structural classes. Proc. Natl. Acad. Sci. USA, *95,* 114–119.

Hoffmann, J.A., Kafatos, F.C., Janewau, C.A., Ezekowitz, R.A. (1999). Phylogenetic perspectives in innate immunity. Science. *284*, 1313–1318.

Hornung, V., Barchet, W., Schlee, M., Hartmann, G. (2008). RNA recognition via TLR7 and TLR8. Handb. Exp. Pharmacol. *183*, 71–86.

Hou, F.J., Sun, L.J., Zheng, H., Skaug, B., Jiang, Q.X., Chen, Z.J.J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell. *146*, 448–461.

Houe, H. (1995). Epidemiology of bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. 11, 521-547.

Huber, W., von Heydebreck, A., Sueltmann, H., Poustka, A., Vingron, M. (2003). Parameter estimation for the calibration and variance stabilization of microarray data. Stat. Appl. Genet. Mol. Biol. *2*, Article 3.

Hughes, H.P. Rossow, S. Campos, M. Rossi-Campos, A. Janssen, S. Godson, D.L. Daflon, B. Voirol, M.J. Gerber, C. Babiuk, L.A. (1994). A slow release formulation for recombinant bovine interferon alpha I-1, Antiviral Res. *23*, 33–44.

Hussain, S., Zwilling, B.S., Lafuse, W.P. (1999). *Mycobacterium avium* infection of mouse macrophages inhibits IFN-gamma Janus kinase-STAT signaling and gene induction by down-regulation of the IFN-gamma receptor. J. Immunol. *163*, 2041–2048.

Ishii, K.J., Akira, S. (2005). TLR ignores methylated RNA? Immunity. *23*, 111–113.

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

Ito, T., Amakawa, R., Inaba, M., Ikehara, S., Inaba, K., Fukuhara, S. (2001). Differential regulation of human blood dendritic cell subsets by IFNs. J. Immunol. *166*, 2961–2969.

Iwasaki, A., Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nat. Immunol. *5*, 987–995.

Jalal, S., Arsenault, R., Potter, A.A., Babiuk, L.A., Griebel, P.J., Napper, S. (2009). Genome to kinome: species-specific peptide arrays for kinome analysis. Sci. Signal. *2,* 1–11.

Jensen, J., Schultz, R. D. (1991). Effect of infection by bovine viral diarrhea virus (BVDV) in vitro on interleukin-1 activity of bovine monocytes. Vet. Immunol. Immunopathol. *29*, 251–265.

Johns, H.L., Doceul, V., Everett, H., Crooke, H., Charleston, B., Seago, J. (2010). The classical swine fever virus N-terminal protease N^{pro} binds to cellular HAX-1. J. Gen. Virol. *91*, 2677–2686.

Kapil, S., Waltz, P.H., Wilkerson, M., Minocha, H.C. (2005). Immunity and immunosuppression. In Bovine Viral Diarrhea Virus e Diagnosis, Management and Control pp. 157-170. Edited by S. M. Goyal, J. F. Ridpath. Ames, IA: Blackwell Publishing.

Karikó, K., Buckstein, M., Ni, H.P., Weissman, D. (2005). Suppression of RNA recognition by toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity. *23*, 165–175.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawait, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T., Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. *205*, 1601–1610.

Kawai, T., Akira, S. (2009). The roles of TLRs, RLRs, and NLRs in pathogen recognition. Int. Immunol. *21*, 317–337.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. *6*, 981–988.

Kelling, C. L., Steffen, D. J., Topliff, C. L., Eskridge, K. M., Donis, R. O. & Higuchi, D. S. (2002). Comparative virulence of isolates of bovine viral diarrhea virus type II in experimentally inoculated six- to nine-month-old calves. Am. J. Vet. Res. *63*, 1379-1384.

Kierszenbaum, F., Mejia Lopez, H., Tanner, M.K., Sztein, M.B. (1995). Trypanosoma cruzi-induced decrease in the level of interferon-gamma receptor expression by resting and activated human blood lymphocytes. Parasite Immunol. *1*, 207–214.

Kim, M-J., Hwang, S-Y., Imaizumi, T., Yoo, J-Y. (2008). Negative feedback regulation of rig-I-mediated antiviral signaling by interferon-induced ISG15 conjugation. J. Virol. *82*, 1474–1483.

Kumagai, Y., Akira, S. (2010). Identification and functions of pattern-recognition receptors. J. Allergy Clin. Immunol. *125*, 985–992.

Kumar, H., Kawai, T., Akira, S. (2009). Pathogen recognition in the innate immune response. Biochem. J. *420*, 1–16.

Krey, T., Himmelreich, A., Heimann, M., Menge, C., Thiel, H.J., Maurer, K., Rumenapf, T. (2006). Function of bovine CD46 as a cellular receptor for bovine viral diarrhea virus is determined by complement control protein 1. J. Virol. *80*, 3912–3922.

Lackner, T., Muller, A., Pankraz, A., Becher, P., Thiel, H.J., Gorbalenya, A.E., Tautz, N. (2004). Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an RNA virus. J. Virol. *78*, 10765–10775.

Lamontagne, L., Lafortune, P., Fournel, M. (1989). Modulation of the cellular immune responses to T cell-dependent and T cell-independent antigens in lambs with induced bovine viral diarrhea virus infection. Am. J. Vet. Res. *50*, 1604–1608.

Larsen, L., Ropke, C. 2002. Suppressors of cytokine signalling: SOCS. APMIS. *110*, 833–844.

Le Bon, A., Thompson, C. Kamphuis, E., Durand, V., Rossmann, C., Kalinke, U., Tough, D.F. (2006). Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. J. Immunol. *176*, 2074–2078.

Lehtonen, A., Matikainen, S., Julkunen, I. (1997). Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophage. J. Immunol. *159,* 794–803.

Li, Y., Arsenault, R.J., Trost, B., Slind, J., Griebel, P.J., Napper, S., Kusalik, A. (2012). A systematic approach for analysis of peptide array kinome data. Sci. Signal. *5*, 1–16.

Liang, R., van den Hurk, J.V., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2006). Priming with DNA encoding E2 and boosting E2 protein formulated with CpG oligodeoxynucleotides induces strong immune responses and protection from bovine viral diarrhea virus in cattle. J. Gen. Virol. *87*, 2971–2982.

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

Liebler-Tenorio, E. M., Ridpath, J. F., Neill, J. D. (2003b). Lesions and tissue distribution of viral antigen in severe acute versus subclinical acute infection with BVDV2. Biologicals. *31*, 119–122.

Lin, Fan-Ching, Young, H.A. (2013). The talented interferon-gamma. Adv. Biosci. Biotechnol. *4*, 6–13.

Lin, Q., Dong, C., Cooper, M. D. (1998). Impairment of T and B cell development by treatment with a type I interferon. J. Exp. Med. *187*, 79–87.

Lin, S.C., Lo, Y.C., Wu, H. (2010). Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature. *465*, 885–890.

Liu, L., Lehmkuhl, H.D., Kaeberle, K.L. (1999). Synergistic effects of bovine respiratory syncytial virus and non-cytopathic bovine viral diarrhea virus infection of selected bovine alveolar macrophage functions. Can. J. Vet. Res. *63*, 41–48.

Lynn, D. J., Winsor, G. L., Chan, C., Richard, N., Laird, M. R., Barsky A., Gardy, J.L., Roche, F.M., Chan, T.H.W., Shah, N., Lo, R., Naseer, M., Que, J., Yau, M., Acad, M., Tulpan, D., Whiteside, M.D., Chikatamarla, A., Mah, B., Munzner, T., Hokamp, K., Hancock, R.E.W., and Brinkman, F.S.L. (2008) InnateDB: facilitating systems-level analysis of the mammalian innate immune response. Mol. Syst. Biol. *4*, 1–11.

Maattanen, P., Trost, B., Scuten, E., Potter, A., Kusalik, A., Griebel, P. & Napper, S. (2013). Divergent immune responses to *Mycobacterium avium* subsp. *paratuberculosis* correlate with kinome responses at the site of intestinal infection. Infect. Immun. *81*, 2861–2872.

Maurer, K., Krey, T., Moenning, V., Thiel, H.J., Rimenapf, T. (2004). CD46 is a cellular receptor for bovine viral diarrhea virus. J. Virol. *78*, 1792–1799.

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A. (2015). Type I interferons in infectious disease. Nat. Rev. Immunol. *15*, 87–103.

Medzhitov, R., Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. Immunol. Rev. *173*, 89–97.

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

Meylan, E., Tschopp, J., Karin, M. (2006). Intracellular pattern recognition receptors in the host response. Nature. *442*, 39–44.

Moes, L., Wirth, M. (2007). The internal initiation of translation in bovine viral diarrhea virus RNA depends on the presence of an RNA pseudoknot upstream of the initiation codon. J. Virol. *4*, 124.

Mogensen, T.H. (2009). Pathogen recognition and inflammatory signalling in innate immune defenses. Clin. Microbiol. Rev. *22*, 240–273.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., Tough, D.F. (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood. *99*, 3263–3271.

Moresco, E.M.Y., Beutler, B. (2010). LGP2: positive about viral sensing. Proc. Natl. Acad. Sci. U.S.A. *107*, 1261–1262.

Müller-Doblies, D., Ackermann, M., Metzler, A. (2002). In vitro and in vivo detection of Mx gene products in bovine cells following stimulation with alpha/beta interferon and viruses. Clin. Diagn. Lab. Immunol. 2002. 9, 1192–1199.

Neill, J.D. (2013). Molecular biology of bovine viral diarrhea virus. Biologicals. *41*, 2–7.

Neill, J.D., Ridpath, J.F. (2003). Gene expression changes in BVDV2-infected MDBK cells. Biologicals *31,* 97–102.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S. (2000). Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta. Vet. Scand. *41*, 93–99.

Nuttall, P.A. (1980). Growth characteristics of two strains of bovine virus diarrhoea virus. Arch. Virol. *66*, 365–369.

Palomares, R. A., Brock, K. V., Walz, P. H. (2014). Differential expression of pro-inflammatory and anti-inflammatory cytokines during experimental infection with low or high virulence bovine viral diarrhea virus in beef calves. Vet. Immunol. Immunopathol. *157*, 149–154.

Palomares, R., Walz, H., & Brock, K. (2013). Expression of type I interferon-induced antiviral state and pro-apoptosis markers during experimental infection with low or high virulence bovine viral diarrhea virus in beef calves. Virus Res. *173*, 260-269.

Parkin, J., Cohen, B. (2001). An overview of the immune system. Lancet. *357*, 1777–1789.

Peedel, D., Rinken, T. (2012). Effect of temperature on the sensitivity of cascaded lactose biosensors. Bioanalytical Chemistry. *61*, 306–313.

Peek, S. F., Bonds, M. D., Schaele, P., Weber, S., Friedrichs, K., Schultz, R. D. (2004). Evaluation of antiviral activity and toxicity of recombinant human interferon alfa-2a in calves persistently infected with type 1 bovine viral diarrhea virus. Am. J. Vet. Res. *65*, 865–870.

Pellerin, C., van den Hurk, J., Lecomte, J. Tussen, P. (1994). Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology. *203*, 260–268.

Persico, M., Capasso, M., Persico, E., Svelto, M., Russo, R., Spano, D., Crocè, L., La Mura, V., Moschella, F., Masutti, F., Torella, R., Tiribelli, C., Lolascon, A. Suppressor of cytokine signaling 3 (SOCS3) expression and hepatitis C virus-related chronic hepatitis: Insulin resistance and response to antiviral therapy. Hepatology. *46*, 1009–1015.

Peterhans, E., Jungi, T.W., Schweizer, M. (2003). BVDV and innate immunity. Biologicals. *31*, 107–111.

Peterhans, E., Schweizer, M. (2013). BVDV: a pestivirus inducing tolerance of the innate immune response. Biologicals. *41*, 39–51.

Potgieter, L.N. (1995). Immunology of bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. *11*, 501–520.

Randall, R.E., Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. *89*, 1–47.

Ray, M., Gam, A.A., Boykins, R.A., Kenney, R.T. (2000). Inhibition of interferon gamma signaling by Leishmania donovani. J. Infect. Dis. *181*, 1121–1128.

Reddick, L. E., Alto, N. M. (2014). Bacteria fighting back: how pathogens target and subvert the host innate immune system. Mol. Cell. *54*, 321–328.

Reikine, S., Nguyen, J.B., Modis, Y. (2014). Pattern recognition and signaling mechanisms of rig-1 and mda-5. Front Immunol. *5*, doi: 10.3389/fimmu.2014.00342.

Ridpath, J.F. (2003). BVDV genotypes and biotypes: practical implication for diagnosis and control. Biologicals. *31*, 127–131.

Ridpath, J. (2005). Classification and Molecular Biology, In: Goyal, S., and Ridpath JF. (Ed.) Bovine Viral Diarrhea Virus. Diagnosis Management and Control. Blackwell publishing, Ames, Iowa, pp. 65–80.

Ronecker, S., Zimmer, G., Herrler, G., Greiser-Wilke, I., Grummer, B. (2008). Fromation of bovine viral diarrhea virus E1-E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains. J. Gen. Virol. *89*, 2114–2121.

Rumenapf, T., Meyers, G., Stark, R., Thiel, H.J. (1991). Molecular characterization of hog cholera virus. Arch. Virol Suppl. *3*, 7–18.

Rumenapf, T., Unger, G., Strauss, J. H., Thiel, H. J. (1993). Processing of the envelope glycoproteins of pestiviruses. J. Virol. *67*, 3288–3294.

El-Saadany, S., Ziada, D.H., Bassat, H.E., Farrag, W., El-Serogy, H., Eid, M., Abdallah, M., Ghazy, M., Salem, H.A. (2013). The role of hepatic expression of STAT1, SOCS3 and PIAS1 in the response of chronic hepatitis C patients to therapy. Can. J. Gastroenterol. *27*, e13–e17.

Sadler, A.J., Williams, B.R.G. (2008). Interferon-inducible antiviral effectors. Nat. Rev. Immunol. *8*, 559–568.

Samuel, C. E. 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. *14*, 778–809.

Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., Tsujimura, T., Fujita, T., Akira, S., Takeuchi, O. (2010). LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc. Natl. Acad. Sci. U.S.A. *107*, 1512–1517.

Seth, R.B., Sun, L.J., Ea, C.K., Chen, Z.J.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF3. Cell. *122*, 669–682.

Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E. Thiel, H.J. (1993). Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science. *261*, 1169–1171.

Schneider, W.M., Chevillotte, M.D., Rice, C.M. (2014). Interferon-stimulated genes: a complex web of host defenses. Annu. Rev. Immunol. *32*, 513–545.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. J. Leukoc. Biol. *75*, 163–189.

Schweizer, M., Matzener, P., Pfaffen, G., Stalder, H., Peterhans, E. (2006). "Self" and "nonself" manipulation of interferon defense during persistent infection: bovine viral diarrhea virus resists alpha/beta interferon without blocking antiviral activity against unrelated viruses replicating in its host cell. J. Virol. *80*, 6926–6935.

Schweizer, M., Peterhans, E. (2001). Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J. Virol. *75*, 4692-4698.

Seago, J., Hilton, L., Reid, E., Doceul, V., Jeyatheesan, J., Moganeradj, K., McCauley, J., Charleston, B., Goodbourn, S. (2007). The Npro product of classical swine fever virus and bovine viral diarrhea virus uses a conserved mechanism to target interferon regulatory factor-3. J. Gen. Virol. 88, 3002–3006.

Shuai, K., Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. Nat. Rev. Immunol. *3*, 900–911.

Smirnova, N.P., Bielefeldt-Ohmann, H., Van Campen, H., Austin, K.J., Han, H., Montgomery, D.L., Shoemaker, M.L., van Olphen, A.L., Hansen T.R. (2008). Acute non-cytopathic bovine viral diarrhea virus infection induces pronounced type I interferon response in pregnant cows and fetuses. Virus Res. *132*, 49–58.

Smirnova, N.P., Ptitsyn, A.A., Austin, K.J., Bielefeldt-Ohmann, H., Van Campen, H., Han, H., van Olphen, A.L., Hansen T.R. (2009). Persistent fetal infection with bovine viral diarrhea virus differentially affects maternal blood cell signal transduction pathways. Physiol. Genomics. *36*, 129–139.

Smirnova, N.P., Webb, B.T., McGill, J.L., Schaut, R.G., Bielfeldt-Ohmann, H., Van Campen, H., Sacco, R.E., Hansen, T.R. (2014). Induction of interferon-gamma and downstream pathways during establishment of fetal persistent infection with bovine viral diarrhea virus. Virus Res. *183*, 95–106.

Snider, M., Garg, R., Brownlie, R., V, van den Hurk, J.V., van Drunen Littel-van den Hurk. (2014). The bovine viral diarrhea virus E2 protein formulated with a novel adjuvant induces strong, balanced immune responses and provides protection from viral challenges in cattle. Vaccine. *32*, 6758–6764.

Stokstad, M., Loken, T. (2002). Pestivirus in cattle: experimentally induced persistent infection in calves. J. Vet. Med. B. Infect. Dis. Vet. Public Health. *49*, 494–501.

Strober, W. (1997). Trypan Blue Exclusion Test of Cell Viability. Curr. Prot. Immun. A.3B.1–A.3B.2.

Szymanski, M.R., Fiebach, A.R., Tratschin, J.D., Gut, M., Ramanujam, V.M.S., Gottipati K, Patel, P., Ye, M., Ruggli, N., Choi, K.H. (2009). Zinc binding in pestivirus Npro is required for interferon regulatory factor 3 interaction and degradation. J. Mol. Biol. *391*, 438–449.

Takeuchi O., Akira, S. (2010). Pattern recognition receptors and inflammation. Cell. *140*, 805–820.

Taniguchi, T., Takaoka, A. (2002). The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr. Opin. Immunol. *14*, 111–116.

Tarry, D.W., Bernal, L., Edwards, S. (1991). Transmission of bovine virus diarrhoea virus by blood feeding flies. Vet Rec. *128*, 82–84.

Thurmond, M.C. (2005). Virus Transmission, In: JF, G.S.a.R. (Ed.) Bovine Viral Diarrhea Virus. Diagnosis, Management and Control. Blackwell Publishing, Ames, Iowa, pp. 91–104.

Tråvén, M., Alenius, S., Fossum, C., Larsson, B. (1991). Primary bovine viral diarrhoea virus infection in calves following direct contact with a persistently viraemic calf. Zentralbl. Veterinarmed. B. *38*, 453–462.

Trost, B., Kindrachuk, J., Maattanen, P., Napper, S., Kusalik, A. (2013). PIIKA2: an expanded, web-based platform for analysis of kinome microarray data. PLoS One. *8*, e80837. doi: 10.1371/journal.pone.0080837.

Vance, R.E., Isberg, R.R., Portnoy, D.A. (2009). Patterns of pathogenesis: discrimination of pathogenic and non-pathogenic microbes by the innate immune system. Cell Host Microbe. *6*, 10 – 21.

van Drunen Littel-van den Hurk, S., Lawman, Z., Snider, M., Wilson, D., van den Hurk, J.V., Ellefsen, B., Hannaman, D. (2013). Two doses of bovine viral diarrhea virus DNA vaccine delivered by electroporation induce long-term protective immune responses. Clin. Vaccine Immunol. *20*, 166–173.

Vercammen, E., Staal, J., Beyeart, R. (2008). Sensing of viral infection and activation of innate immunity by toll-like receptor 3. Clin. Microbiol. Rev. *21*, 13–25.

Versteeg, G.A., García-Sastre, A. (2010). Viral tricks to grid-lock the type I interferon system. Curr. Opin. Microbiol. *13*, 508–516.

Waldvogel, A.S., Hediger-Weithaler, B.M., Eicher, R., Zakher, A., Zarlenga, D.S., Gasbarre, L.C., Heussler, V.T. (2000). Interferon-gamma and interleukin-4 mRNA expression by peripheral blood mononuclear cells from pregnant and non-pregnant cattle seropositive for bovine viral diarrhea virus. Vet. Immunol. Immunopathol. *77*, 201–212.

Wasserman, S.A. (2010). Immunity takes a heavy toll. Nature. *465*, 882–883.

Weiskircher, E., Aligo, J., Ning, G., Konan, K.V. (2009). Bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes. Virol. J. *6*, 185.

Welsh, R.M., Bahl, K., Marshall, H.D., Urban, S.L. (2012). Type I interferons and antiviral CD8 T-cell responses. PLoS Pathog. *8*, e1002352.

Wilkins, C., Gale, M. (2010). Recognition of viruses by cytoplasmic sensors. Curr. Opin. Immunol. *22*, 41–47.

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

Xue, W., Zhang, S., Minocha, H. C. (1997). Characterization of a putative receptor protein for bovine viral diarrhea virus. Vet. Microbiol. *57*, 105–118.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K. Akira, S., Fujita, T. (2004). The RNA helicase RIG-1 has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. *5*, 730–737.

Yoshida, A., Koide, Y., Uchijima, M., Yoshida, T.O. (1994). IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. Biochem. Biophys. Res. Commun. *198*, 857–861.

Yu, H., Isken, O., Grassmann, C.W., Behrens, S.E. (2000). A stem-loop motif formed by immediate 5' terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. J. Virol. *74*, 5825–5835.

Zurcher, C., Sauter, K.S., Mathys, V., Wyss, F., Schweizer, M. (2014). Prolonged activity of the pestiviral RNase Erns as an interferon antagonist after uptake by clathrin-mediated endocytosis. J. Virol. *88*, 7235–7243.