THESIS

INNATE AND ADAPTIVE IMMUNE RESPONSES AFTER BOVINE VIRAL DIARRHEA VIRUS INFECTION IN THE FETAL THYMUS

Submitted by

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ABSTACT

INNATE AND ADAPTIVE IMMUNE RESPONSES AFTER BOVINE VIRAL DIARRHEA VIRUS INFECTION IN THE FETAL THYMUS

Bovine viral diarrhea virus (BVDV) infection of bovine fetuses in the first 125 days of pregnancy results in persistently infected (PI) cattle and transiently infected (TI) cattle become infected, but have a developed immune system enabling them to clear the virus. PI cattle are the main source of BVDV infection in populations causing significant economic losses to the industry worldwide. The mechanisms that lead to this "immunotolerant" state are not well defined. Following maternal inoculation with BVDV on day 75 of gestation, fetal infection was determined by presence of viral mRNA. Fetal infection occurred by day 89 of gestation with a peak at day 97 and a significant 10-fold decrease in viral titer at days 192 and 245 of gestation. BVDV was never completely eliminated in the fetus and persisted at lower, but significant levels. We hypothesized that there is dysgenesis of the fetal thymus, inhibiting functional innate and adaptive immune responses following maternal infection with BVDV. Total RNA was extracted from the thymus of uninfected control and BVDV-infected fetuses at days 89, 97, 190, and 245 of gestation. Genes representing the innate immune system, T and B cell receptor signaling, and the antigen processing and presentation in both the MHC I and MHC II pathways were explored using RT-qPCR. There were significant increases ($P \leq 0.05$) observed in all innate genes analyzed (*RIG-I*, *IRF7*, *NF-kB*, *IFN*β, and *ISG15*) in TI fetuses, with minimal responses in PI fetuses. There were significant decreases ($P \leq 0.05$) of antigen presentation genes and adaptive immune system genes *LMP2*, *TAP1*, β*2M, CD8a, CD8b, CIITA, CD4, IFI16, CXCL16, CXCR6,* and *CD79b* in day 190 PI fetuses. Persistent BVDV infection may initiate fetal adaptive immune responses, which are not fully activated over time because

of “immunotolerance” caused by impaired antigen processing. Longer-term consequences of fetal PI remain to be determined in context of postnatal impaired immune responses to secondary infections.

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LIST OF KEYWORDS

Bovine viral diarrhea virus, Cattle, Fetus, Immune response, Persistent infection, Thymus

CHAPTER I.

LITERATURE REVIEW

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a small (12.5 kb genome) enveloped RNA virus included in the genus *Pestivirus* and Family *Flaviviridae.* It was first discovered in the 1940s and is an infectious agent that affects herd productivity and reproduction of cattle worldwide (Childs 1946). BVDV causes significant economic losses through increased morbidity and mortality, due to immunosuppression decreasing performance in cattle herds and costing the industry millions of dollars each year. This industry loss is inflated when proper control methods are not maintained, allowing for infected calves to continue spread of the virus (Pinior et al., 2017, Richter et al., 2017). In the United States, control standards include the identification and elimination of persistently infected animals, preventing entry of persistently infected animals to the herd using diagnostic testing, and vaccination of animals (Fulton et al., 2006, Van Campen 2010). The more recent vaccines that have been developed reduce the risk of fetal infection, but their efficacy is increased when the vaccine strain matches the strain to which the herd may have contact (Newcomer et al., 2015). The type of vaccine and time that it was given can also have an effect. If a modified-live vaccine is given to a pregnant dam, the fetus would develop a persistent infection (Palomares et al., 2013). Vaccination also interferes with BVDV diagnostic testing in which cattle vaccinated against BVDV can have a false positive result when using a diagnostic technique that tests for antibodies. This false positive may prevent the selling and moving of animals to other locations. Financial losses due to BVDV ranged from 50 – 687.80 USD per animal worldwide, but regardless of the size of the herd, losses peak one year after the virus is introduced in the herd, when reproductive failures and decreased productivity are observed (Damman et al., 2015, Richter et al., 2017). A better understanding of mechanisms that lead to the establishment of persistent BVDV infection may aid in disruption of the transmission cycle.

HISTORY

BVDV was first described in 1946 after a disease of unknown origin, "X", spread through a Canadian dairy herd as both acute and subacute forms (Childs 1946). Within this herd, young cattle that became infected had a sudden onset of illness with death following 7 to 10 days later. The more severe type of the disease led to death in mature naïve cattle within 3 to 4 days with clinical signs including pyrexia, tachypnea, tachycardia, anorexia, dehydration, and diarrhea, with some cattle exhibiting ulcerative lesions on mucosal tissues (Childs 1946). Post-mortem findings unveiled extensive loss of gastrointestinal epithelium, with erosive lesions observed from the nares to esophagus and throughout the upper and lower alimentary system. Hemorrhage in the kidneys was also reported as well as with petechiation in the ureters and bladder and possible thrombosis within the spleen (Childs 1946). In the same year, Cornell University, also reported an outbreak of gastroenteritis and severe diarrhea, along with pyrexia, tachypnea, and ulcers present in the mouth, nares, and muzzle in cattle (Olafson et al., 1946). The post-mortem findings from this herd were consistent with those described for "X" disease and the disease became known as virus diarrhea (VD) of cattle (Childs 1946, Olafson P et al., 1946).

In the 1950s, Iowa farmers reported a disease in cattle with clinical signs consisting of mucopurulent nasal discharge with gross erosions and hemorrhages of the intestinal tract which they termed mucosal disease (MD) (Ramsey et al., 1951). BVDV consisting of two genotypes, 1 and 2, and two biotypes, cytopathic (cp) and noncytopathic (ncp) (Goens 2002). The cp biotype was named in reference to its lytic behaviors in *in vitro* cell cultures (Gillespie et al., 1961). The majority of worldwide economic losses that occur are connected to the ncpBVDV type and its ability to cause persistent infection (PI), which is how the virus continues to spread throughout herds (Bissey et al., 1991, Moerman et al., 1994, Peterhans et al., 2010, Richter et al., 2017).

TRANSMISSION AND PATHOGENESIS OF TRANSPLACENTAL INFECTION

BVDV is transmitted either directly from infected animals through excretions or secretions including nasal discharge, tears, saliva, urine, feces, milk, and semen, or indirectly through fomites such as equipment (i.e. halters or nose tongs). It can survive up to 3 weeks in the environment (Khodakaram-Tafti et al., 2017). BVDV is also able to transmit vertically from dam to fetus at any age of gestation. Cows have an epitheliochorial (cotyledonary) placenta which consists of three fetal layers (fetal endothelial cells, fetal connective tissue, and chorionic epithelial cells) and three maternal layers (endometrial epithelial cells, maternal connective tissue, and maternal endothelial cells). This type of placenta inhibits large maternal immunoglobulins (IgG, IgM) from crossing the placenta to protect the fetus. BVDV has a high predilection for the walls of blood vessels, in particular the smooth muscle cells in the tunica media as well as epithelial tissues including the reproductive, central nervous system, and lymphoid tissues, therefore, BVDV can be detected in intercotyledonary fetal membranes and placentomes of the placenta (Ohmann 1983, Booth et al., 1995, Fredriksen et al., 1999a, Fredriksen et al., 1999b). Additionally, transplacental-BVDV infection affects multiple organs and causes lesions that may appear in the fetal lymphatic, digestive, pulmonary, urogenital, nervous, and reproductive systems (Shin et al., 2001, Bolin 2002). The Peyer's patches, of the fetal digestive system, have been reported to have the highest amount of virus (Yasuda et al., 2006). The main route of infection is caused by the presence of persistently infected cattle in the herd that infect naïve cattle (Grooms 2004).

Naïve heifers infected prior to conception through day 45 of gestation, have a decreased conception rate and there is an increase in early embryonic deaths (Houe et al., 1993). Infection of a naïve pregnant cow between days 45 to 175 of gestation can cause congenital defects, abortions, or more commonly, if the fetus survives, it may become persistently infected (PI) and continuously shed virus throughout its life. Congenital defects range from hydrocephalus, hydranencephaly, porencephaly, microencephaly, or cerebellar hypertrophy with cerebellar hypertrophy being the most common

(Agerholm et al., 2015). These central nervous system defects mainly occur through direct damage to the cells or the inflammatory responses that occur due to the fetus's response to the virus (Agerholm et al., 2015). While the mechanisms of fetal infection are unclear, there is evidence that a vasculitis on the maternal side of placentation may be the main source of virus to be passed to the fetus via circulation (McClurkin et al., 1984, Murray 1991, Fredriksen et al., 1999a). BVDV has also been identified in binuclear trophoblast cells of the fetus (Fredriksen et al., 1999a). Normally, fetuses that are infected between days 18 and 125 of gestation with the ncpBVDV biotype can become a PI and therefore immunotolerant to the virus (McClurkin et al., 1984, Virakul et al., 1988). Calves born apparently normal yet persistently infected, when bred, are able to produce persistently infected calves (McClurkin et al., 1984).

A persistent infection occurs prior to 120 days of gestation in which a competent immune system has not developed and the fetus is able to shed virus throughout its lifetime (Brownlie et al., 1987, Lanyon et al., 2014). When infection occurs later in gestation, greater than 135 days of gestation, the fetus may also end up with congenital defects or they can become persistently or transiently infected. A transient, or acute, infection occurs after the immune system is more established after day 120 of gestation, in which the fetus can mount an effective immune response and successfully clear the virus with no lingering side-effects (Casaro et al., 1971, Chase et al., 2004).

BVDV persistently infected animals can also act as a synergistic agent for various infections due to the immunosuppression caused by the virus (Bolin 2002, Fulton 2013). These synergistic infections can be of respiratory, enteric, or reproductive infections. Bovine herpesvirus 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza virus 3 (PI-3V), and bovine coronavirus are viral infections that have been isolated in BVDV infected animals (Bolin 2002, Fulton 2013). Less is known about mixed infections with BVDV causing enteric disease, but BVDV as well as BRSV has led to more pronounced rotavirus, *Salmonella spp.*, *Escherichia coli*, or *cryptosporidiosis* infections (Bolin 2002,

Fulton 2013). Reproductive disease is more common than enteric disease especially because abortions make it relatively easy to identify. BVDV has been linked with numerous mixed infections and a variety of agents which include *Leptospira borgpetersenii* serovar hardjo, *Coxiella burnetti*, *Campylobacter fetus*, *Arcanobacterium pyogenes*, *Bacillus spp*., or *Neospora caninumm (Bolin 2002, Fulton 2013)*. For all diseases related to the different organ systems, the severity of a secondary infection typically has more severe clinical signs in BVDV PI calves, than healthy calves.

BVDV AND IMMUNITY

Persistently infected cattle are immunotolerant to BVDV and are unable to clear the virus. The inability of the host to clear the virus may be because of either a 'hit-and-run' method, which generates transiently infected animals or an 'infect-and-persist' method which generates PI animals (Rhodes et al., 1999, Endsley et al., 2003, Peterhans et al., 2003b, Grooms 2004). The 'hit-and-run' method is the explanation for how acute infection moves through a herd, in which animals become infected and transfers the disease to a second animal before the initially infected animal clears it, whereas the 'infect-and-persist' is how the disease achieves persistence within a herd. This is the same or similar method that HIV and herpes infection are associated within their hosts (Peterhans et al., 2003a, Schweizer et al., 2006). It is currently thought that fetuses become PI because of an impaired innate immune response coupled with evasion of the adaptive immune system, thus the 'infect and persist' (Peterhans et al., 2013).

In a previous study, at Colorado State University, naïve heifers were inoculated with BVDV and viral blood titers were measured in the dam and fetus at various time points throughout gestation (Smirnova et al., 2008, Smirnova et al., 2012). Naïve pregnant heifers were inoculated with BVDV at day 75 of gestation (Figure 1). Viral maternal titers were first detected at day 82 with a negligible titer by

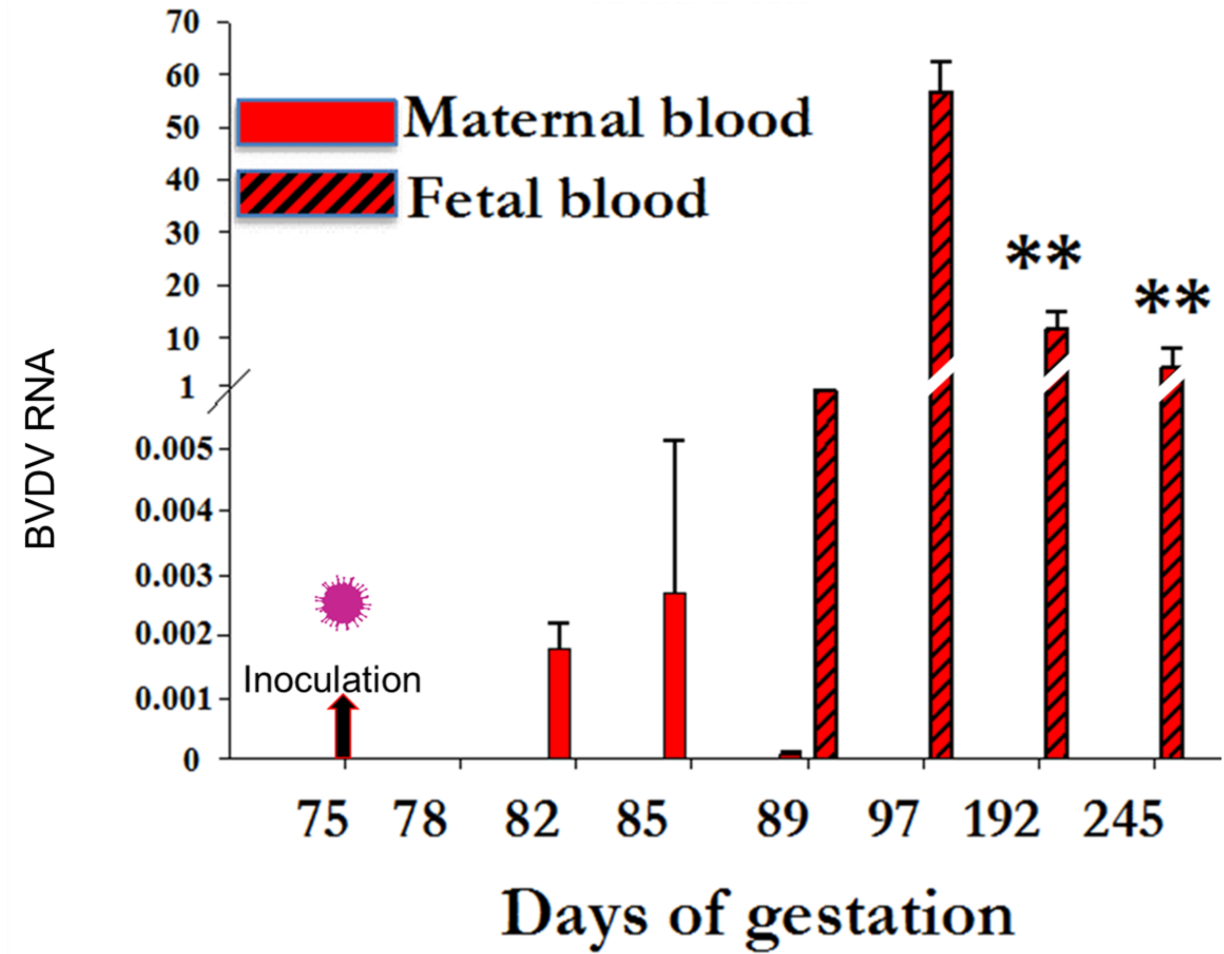


Figure 1. Viremia (BVDV RNA expression; $2^{-\Delta Ct}$) following infection with ncpBVDV on day 75 in heifers and their fetuses. ** represents $P < 0.01$ compared to day 97. Figure modified from (Smirnova et al., 2012).

day 89 indicating a rapid immune response resulting in clearance of the virus within 2 weeks of infection (Smirnova et al., 2012). Fourteen days after inoculation of the heifer, BVDV was present in the fetus as seen in the viral titers. At this 2 week presence of BVDV in the fetus (day 97 of gestation), the viral load peaks and a significant decrease, but not clearance, of BVDV RNA is observed on days 192 and 245. It is important to note that even with the significant decrease of viral titer at days 192 and 245, there is still significant titer at these days. This decrease may be indicative of partial clearance of the virus, which supports the idea that during the early stages of the infection, a slight fetal immune response occurs, however, there is a lack of complete clearance of the virus (Smirnova et al., 2012). The inability of the fetus to completely clear the virus may indicate a dysfunctional immune system, given the fact that no antibodies are present to clear or seroconvert these underdeveloped immune systems.

INNATE IMMUNITY

The innate immune system is the first line of natural defense against foreign pathogens and is mediated by pattern recognition receptors (PRR). Cellular toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) are on the endosome membrane of a variety of cells including phagocytes and epithelial cells (Saito et al., 2007, Blasius et al., 2010, Loo et al., 2011). RLRs include RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology). These receptors, as well as others, recognize viral nucleic acids, such as pathogen-associated molecular patterns (PAMPs), in order to initiate an innate immune response (Figure 2). Once the receptors recognize a viral infection, downstream signal transduction is activated with the phosphorylation of transcription factors and upregulation of interferon (IFN) type 1 transcription, which then induces intracellular immune responses and elicit antiviral processes (Alexopoulou et al., 2001, Samuel 2001, Loo and Gale 2011). These receptors are able to trigger the innate defenses of myeloid cells, epithelial cells, and cells of the central nervous system which initiate production of IFNs (Loo and Gale 2011).

Another division of innate immunity is the complement system that opsonizes and destroys pathogens based on the PAMP recognition mechanism, where the PAMPs interact with the PRR domain of the TLR (Liszewski et al., 2013). In addition to extracellular recognition, PAMPs can also be detected intracellularly, which is critical for recognizing intracellular viruses such as BVDV (Mogensen 2009, Blasius and Beutler 2010, Takeuchi et al., 2010). *In vivo* studies of BVDV in infected fetuses showed an upregulation of viral recognition receptors, *RIG-I* and *MDA5* helicases. Additionally, interferon stimulated genes (*ISG*s) were also found to be upregulated in fetal blood, fetal splenic tissue as well as in PI post-natal steer blood. The *in vivo* increase in ISGs are indicative of a type I IFN response. ISGs can also have positive feedback effects, increasing type I FIN transcription until the infection is cleared (Charleston et al., 2002, Smirnova et al., 2008, Shoemaker et al., 2009). However, *in vitro* studies showed an opposite effect in which ISGs and type I IFN production were inhibited (Baigent et al., 2002). Viral recognition receptors signal IFN production through *IRF3* or *IRF7*. IRF3 and IRF7 are phosphorylated in the cytoplasm by TBK1 or IKKε, then translocate into the nucleus where they induce transcription of IFNs (Shen et al., 2014). One of the reasons for the persistence of BVDV was a failed activation of IRF3 and IRF7, thus a lack of ISG production interferon response (Baigent et al., 2002). In addition to IRF3 and IRF7, production of IFNs can also be induced and regulated through the nuclear factor kappa B (NF-kB) transcription factor (Pfeffer 2011). There are five members in the NF-kB family and like IRF3 and -7, the NF-kB pathway is activated by TLRs, RLRs, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Dev et al., 2010). However, upon repeated exposure to an insult, a reduced response is observed. This is directly caused by NF-kB in which excess dimerization of NF-kB occurs which bind to transcriptional targets causing the attenuation of signaling cascades or the receptors, and are not as sensitive to this repeated challenge (Dev et al., 2010). This attenuation or decreased receptor sensitivity may be important for the tolerance, or "immunotolerance" that is seen with BVDV.

Interferons serve as the first line of defense against pathogens through their antiviral, anti-proliferative, and immunomodulatory functions. IFN-α/β are integral for the innate immunity by inhibiting viral replication in infected cells, whereas IFN-γ is the linking cytokine of the innate and adaptive immune system (Kadowaki et al., 2000). The innate immune response ends in the production of type I interferons (α, β, and ω) which then activates the type II interferon (IFN-γ), where IFN-γ signals and initiates the beginning of the adaptive immune response. The type I IFNs initiate the activation of ISGs through the JAK/STAT pathway (Malakhova et al., 2003). Type I IFNs bind to its receptors on the cell surface of neighboring cells, leading to phosphorylation of *STAT* protein. Once *STAT* enters the nucleus it then initiates transcription of IFN-inducible genes, such as gamma-interferon-inducible gene 16 (*IFI16*), leading to the transcription of other interferon stimulated genes including *ISG15* (Veeranki et al., 2011, Thompson et al., 2014). ISG15 is a 15 to 17 kDa ubiquitin-like protein that exists either free or conjugated. The conjugated form can target and conjugate with proteins for post-translation modification (ISGylation). This ISGylation results in modulation of activity of various proteins and various innate anti-viral functions against both DNA and RNA viruses including destabilization of proteins, inhibition of viral replication, disruption of viral ubiquitination within host cells, or with certain viruses disruption of viral budding (Campbell et al., 2013, Cruz et al., 2017). Thus, the innate immune system attempts to inhibit viral replication, but when it fails to do so, the innate system signals the adaptive immune system.

IFN-γ is a type II interferon, produced by natural killer cells, as part of the innate immune system. It is also produced by T helper and cytotoxic T lymphocytes as part of the adaptive immune response (Schoenborn et al., 2007). After infection with ncpBVDV, IFN-γ measured in fetal blood spiked significantly twenty-two days after maternal infection, but for reasons that have yet to be identified, the IFN-γ was no different than controls at day 245 (Hansen et al., 2015). *In vitro* infection of mononuclear cells with ncpBVDV also showed this same increase of IFN-γ after 48 hour exposure to the virus (Choi

2017). Since IFN-γ is one of the many interferons produced during the innate immune response its production may indicate a functional innate immune response. Additionally, IFN-γ is an important activator of macrophages and induces MHC molecule expression in the adaptive immune system.

ADAPTIVE IMMUNITY

The adaptive or acquired immune system is geared toward reacting against specific antigens. Immunological memory is created after the first exposure of the system to foreign antigens. There are two divisions of the adaptive immune system: cell-mediated immune responses, directed by T cells, and humoral (antibody-mediated) immune responses, which is executed by B cells. Both cells types originate in the bone marrow, but T cell progenitors migrate to the thymus to mature into T cells through a positive and negative selection process, which is described in greater depth in the *Thymus Development* section.

There are several types of T cells, but the two major subsets are helper (Th) and cytotoxic (Tc) T cells which are of particular interest. The Th cells recognize peptides presented on MHC class II molecules through the exogenous pathway, whereas Tc recognize peptides presented on MHC class I molecules through the endogenous pathway. The binding of Tc then causes a release of cytokines that recruits and directs phagocytic cells to the foreign entity (Goddeeris et al., 1994, Blum et al., 2013).

B cells are involved in the activation of other B cells through the interaction of CD4+ T cells and the MHC complex on the cell surface of B cells. The B cell receptor binds to an antigen which is then internalized and processed and presented via MHC II complexes on the B cell surface to CD4+ T cells. These T cells then release cytokines that signals B cells to activate, proliferate, and differentiate into memory B cells, allowing for a quicker immune response the next time they come into contact with a foreign protein, or plasma cells which produce large amounts of antibodies directed at a specific foreign protein (Coutinho et al., 1984). On the surface of B cells are the glycoproteins CD79a and -b that are

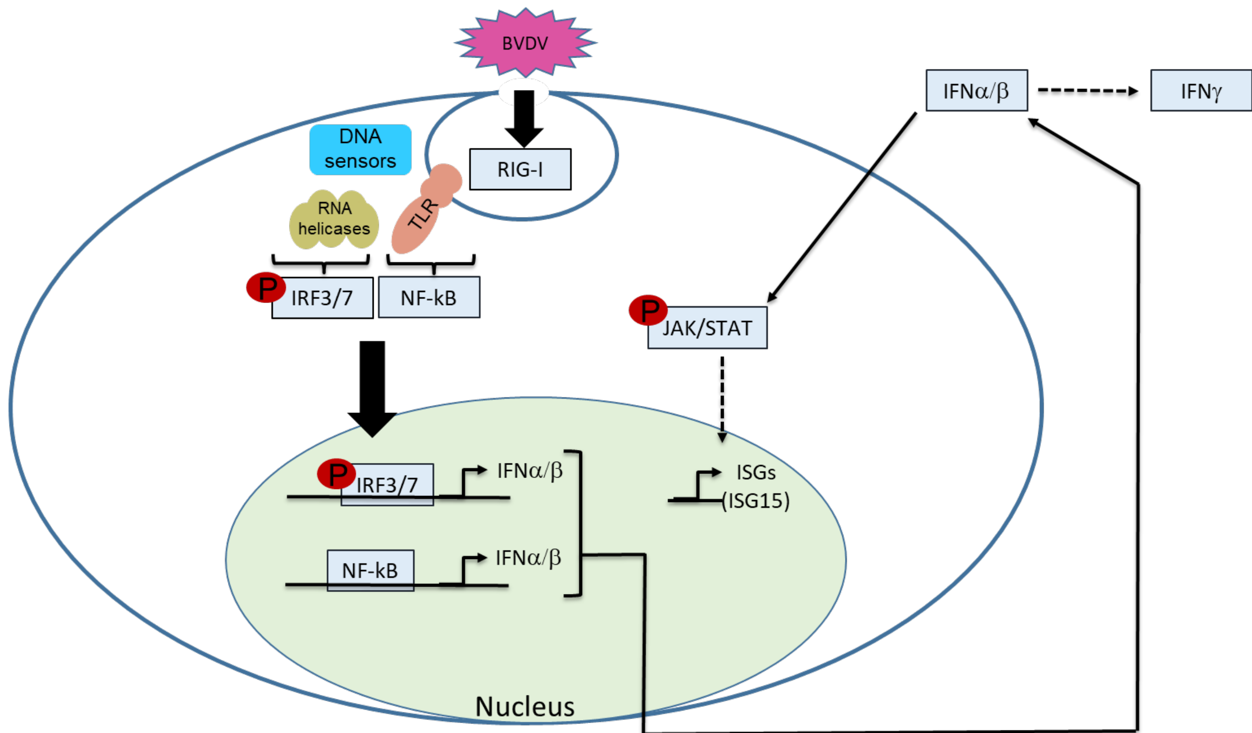


Figure 2. Pathways of activation and regulation of the innate immune response. Modified from (Takeuchi et al., 2009, Cui et al., 2014).

linked together as a heterodimer through a disulfide bond and together along with membrane bound IgM, they make up B cell receptor signaling complex (BCR) which is required for cell surface expression and signal transduction. CD79a and -b are important for B cell maturation and survival and remain on the cell surface until differentiation of B cells into plasma cells (Fuentes-Pananá et al., 2006, Huang et al., 2011).

ANTIGEN PROCESSING AND PRESENTATION

For proper activation of T and B cells, antigen presentation is critical. Presentation is established through the function of antigen presenting cells (APCs) like macrophages and dendritic cells. These APCs internalize, process, then present pathogen-derived peptides on the cell surface to allow CD4+ or CD8+ glycoproteins on the surface of T cells to bind. Antigen processing and presentation is done through two pathways: major histocompatibility complex (MHC) I and II. The genes of the MHC are highly polymorphic, which allows for recognition and binding of a variety of pathogens (Sette et al., 1999).

Systematically, MHC I and II glycoproteins bind to antigenic peptides and present them to T lymphocytes, referred to as cell-mediated immunity. The MHC complex in bovine is referred to as the bovine major histocompatibility complex or the bovine leukocyte antigen (BoLa) gene locus (Spooner et al., 1979, Nascimento et al., 2006). To avoid complexity, in this paper BoLa and MHC will be used interchangeably since their processes are identical.

The MHC class I molecules follow the endogenous pathway (Figure 3), meaning that proteins present in the cytosol of antigen presenting cells are degraded into short peptides by the immunoproteasome via low-molecular-mass protein (LMP) 2, 7, or multicatalytic endopeptidase complex subunit 1 (MECL-1). The immunoproteasome unit is upregulated by interferon gamma (IFN-γ). The LMPs and MECL-1 are structurally specific subunits to organs related to the immune system, such as

the spleen and thymus (Groettrup et al., 1997, Eleuteri et al., 2000, Ferrington et al., 2012). When the subunits of the proteasome are upregulated, they play a role in stimulating the immunomodulator IFN-γ, signifying their role in the antigen processing pathway (Eleuteri et al., 2000). The degraded antigenic peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) 1 and 2 proteins. The MHC class II transactivator (CIITA) is mainly associated with MHC class II function, but it also plays a role in the MHC class I promoter function, surface antigen expression, and gene induction (Martin et al., 1997, Nikcevich et al., 1999). In the ER, the α chain and beta 2-microglobulin (β2-microglobulin) proteins will be assembled into a functional stable MHC I complex through various chaperone molecules. The complex then allows for binding of the antigenic peptide within the ER. This complex is then trafficked from the ER to the Golgi apparatus where post-translational processes occur, and then finally to the surface membrane where it interacts with T cell receptors (TCRs) that are expressed on the surface of CD8+ T cells (Alberts et al., 2002b).

The MHC I complex is especially important in cows during pregnancy. It is imperative that maternal immunity not react to fetal antigens while still able to fight off any other infection that it may become exposed (De Mestre et al. 2010). There are two classes of MHC I: the classical, highly polymorphic, and the nonclassical, nonpolymorphic. The classical is able to present many different types of antigen, while the non-classical presents a “zero” antigen, an antigen that fills the MHC groove in order to keep natural killer cells from attacking it, which is how leukocytes recognize these aspects as being self (Rapacz-Leonard, Dąbrowska and Janowski 2014). Antigen presenting cells can become infected with the BVDV virus in which more virus is produced. These cells along with a multitude of different proteins can be altered after infection with BVDV which include: a variety of TLRs, both MHCs, and a number of other cytokines (Lee et al., 2006, Rajput et al., 2014). While these changes were shown in adult cattle, their fetuses were not analyzed and thus can only speculate the changes in the fetus are due to the changes in the adult.

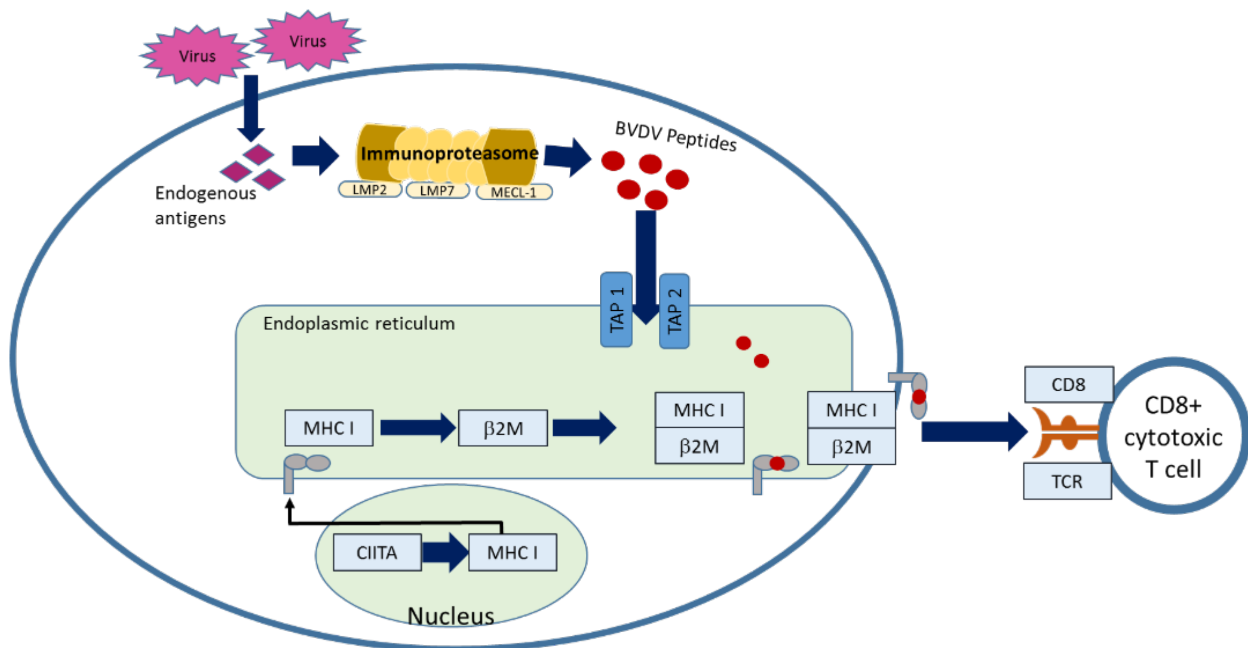


Figure 3. Antigen processing and presentation by the MHC class I pathway inside of an antigen presenting cell. Modified from (Inaba et al., 2005, Trombetta et al., 2005).

The MHC class II molecules follow the exogenous pathway (Figure 4), in which antigen-presenting cells (APCs), namely macrophages or dendritic cells, can take up antigen either through phagocytosis, endocytosis, or both. In addition to being a signaling cytokine to activate the adaptive immune system, IFN-γ, produced by T lymphocytes and natural killer (NK) cells, has macrophage activation, anti-viral, and anti-proliferative properties. IFN-γ has the ability to cause upregulation of both MHC class I and II molecules on cells (Kindt et al., 2007). IFN-γ can then induce *CIITA* to transcriptionally induce most genes in the MHC class II pathway (Steimle et al., 1994, Muhlethaler-Mottet et al., 1998).

Gamma-interferon-inducible lysosomal thiol reductase (GILT), also known as IFI30, is localized within endosomes where it plays a role in the unfolding and degradation of proteins (West et al., 2013). It is also constitutively expressed by APCs however, it is not regulated by CIITA, instead it is regulated by STAT1 and induced by IFN-γ (Hastings 2013). In the ER a chaperone protein, invariant chain (Ii) binds to the MHC II molecule (Busch et al., 1996, Martin et al., 1997). This complex is then moved through the Golgi to endocytic or phagocytic compartments where the Ii is degraded leaving an Ii-derived class II invariant chain peptide (CLIP) fragment. The CLIP is then exchanged for antigenic peptide by HLA-DM, which is then transported to the surface membrane to allow for interaction with TCRs that are expressed on the surface of CD4+ T cells (Robinson et al., 2002, Kindt et al., 2007).

It is important to mention that there are conflicting results shown previously between *in vivo* and *in vitro* studies. For instance, one study measured *in vitro* IFN-γ production and MHC II complexes in bovine monocyte cells and Madin-Darby bovine kidney (MDBK) cells after infection with both noncytopathic biotype strains (ncpBVDV1 and ncpBVDV2). In these cells, both the IFN production and MHC II complex protein were diminished (Alkheraif et al., 2017, Choi 2017).

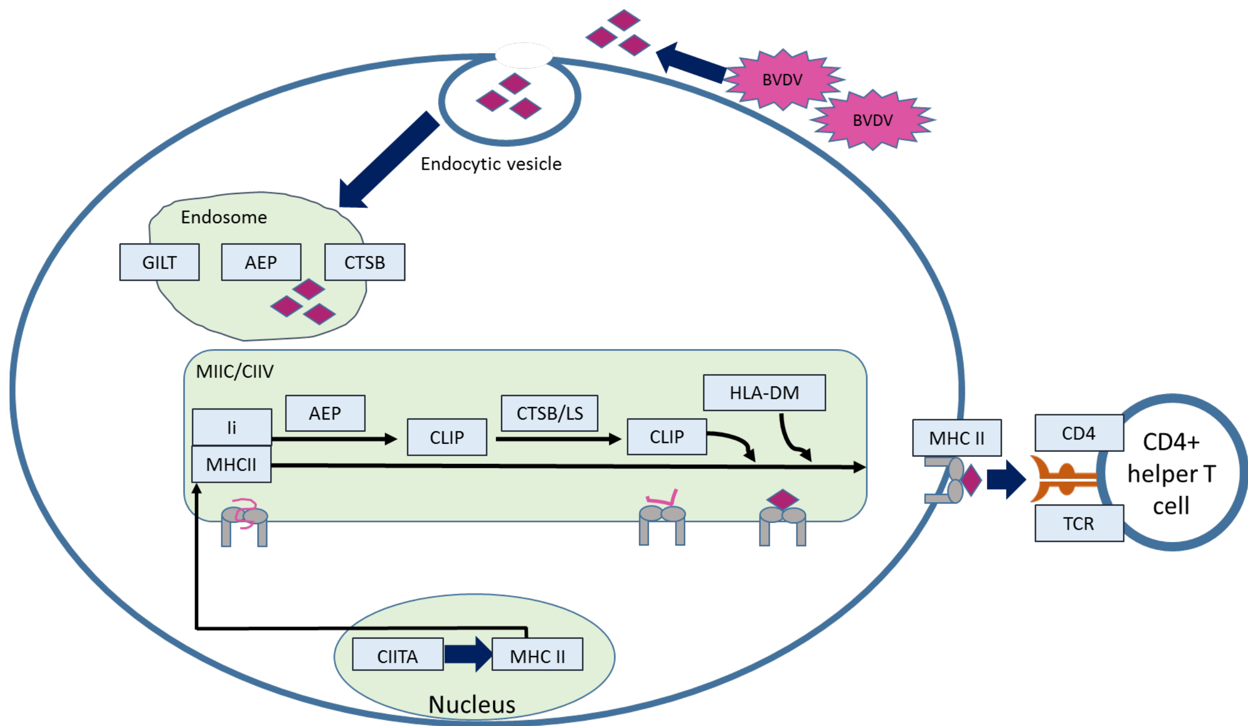


Figure 4. Antigen processing and presentation by the MHC class II pathway. Modified from (Inaba and Inaba 2005, Trombetta and Mellman 2005).

However, *in vivo* studies in young calves had the opposite results in which IFN production and MHC II complexes were shown to be increased (Smirnova et al., 2012, Smirnova et al., 2014, Choi 2017). The differences in these studies could be due to a difference between the models. In the latter studies a different strain was used to infect cattle, but also the animals challenged were old enough to have a developed immune system, which could account for the increases measured.

THYMUS AND BVDV

The thymus is a target of BVDV and in order to gain an understanding of possible immunosuppressive or immunotolerant pathways, it is imperative to understand the development of the thymus as well as known effects of BVDV on various lymphoid tissues (Romero-Palomo et al. 2015). In ruminants, the thymus can first be visualized in one month old fetuses and lymphoid development occurs by day 42 of gestation (Goddeeris and Morrison 1994). Colonization of T cells in the thymus occurs in a wave-like pattern where precursors enter the tissue, mature, and leave (Goddeeris and Morrison 1994).

The thymus is a primary lymphoid organ where progenitor T cells migrate to mature. Over 99% of the cells in the thymus are T cells, with the remaining being B cells, null cells, and other cells such as granulocytes, macrophages, and other hematopoietic cells (Shewen 1988). In the thymus, T cells undergo positive and negative selection process before being expelled into the peripheral blood (Figure 5). Positively selected T cells are able to recognize complex comprised of antigenic peptides and the MHC molecule, whereas cells that recognize self-antigen on MHC molecules are eliminated via apoptosis (Takaba et al., 2017). There are three populations of T cells, which are defined by the expression of three surface molecules. The CD4+ and CD8+ antigen identifies the MHC class II- and MHC Class I-restricted subpopulations of $\alpha\beta$ $TCR^{+}T$ cells, respectively. The third population is $CD4^{-}$ and $CD8^{-}$ that is identified by the WC1 surface molecule and expresses the $\gamma\delta$ T-cell receptor (TCR) (Howard et al., 1992).

These T cells can account for up to 60% of all lymphocytes in bovine peripheral blood, and they may serve a regulatory function (Guzman et al., 2014). What separates these T cells from other lineages is their ability to become activated by directly binding to antigens, without interaction with MHC complexes.

During BVDV infection, the virus is found in lymphocytes, lymphoid follicles, and the cortex of the thymus (Liebler-Tenorio et al., 2004). Following infection, thymic lymphoid depletion has been commonly reported, but the mechanism behind this has not been fully investigated (Ohmann 1982, Raya et al., 2012, Falkenberg et al., 2014, Romero-Palomo et al., 2015, Falkenberg et al., 2017). Histological cellular depletion of the thymus appears to correlate with clinical presentations in which infected animals with clinical disease had the most pronounced cortical atrophy, followed by acutely infected calves. However, clinical PI calves had similar thymic patterns to control calves (Falkenberg et al., 2017). One theory behind this lymphoid depletion is related to thymic epithelial cells (TECs), which are in the cortex of the thymus and play a role in positive selection of maturing lymphocytes. Without the signal from TECs to allow for continued maturation of lymphocytes, the lymphocytes undergo apoptosis (Raya et al., 2015). TECs are target cells of BVDV and can produce cytokines that stimulate the proliferation of lymphocytes (Raya et al., 2015). Since BVDV tends to translocate to epithelial cells early in the course of the disease, maturation of lymphocytes may be restricted and increased thymic atrophy may occur. Even with the depletion of cells in the thymus, peripheral blood mononuclear cells (PBMCs) were still found to elicit an MHC class I- and II-restricted T cell response indicating that BVDV may not cause a complete suppressive effect of the antigen processing and presentation pathway (Glew et al., 2001).

Previous research has indicated that there is a function innate immune response in both blood and spleen, however the thymus tells a different story. Atrophy is commonly seen in the thymus and

thus dysgenesis of the thymus during gestation leads to suppression of the innate and adaptive immune responses in the fetus against maternal BVDV infection.

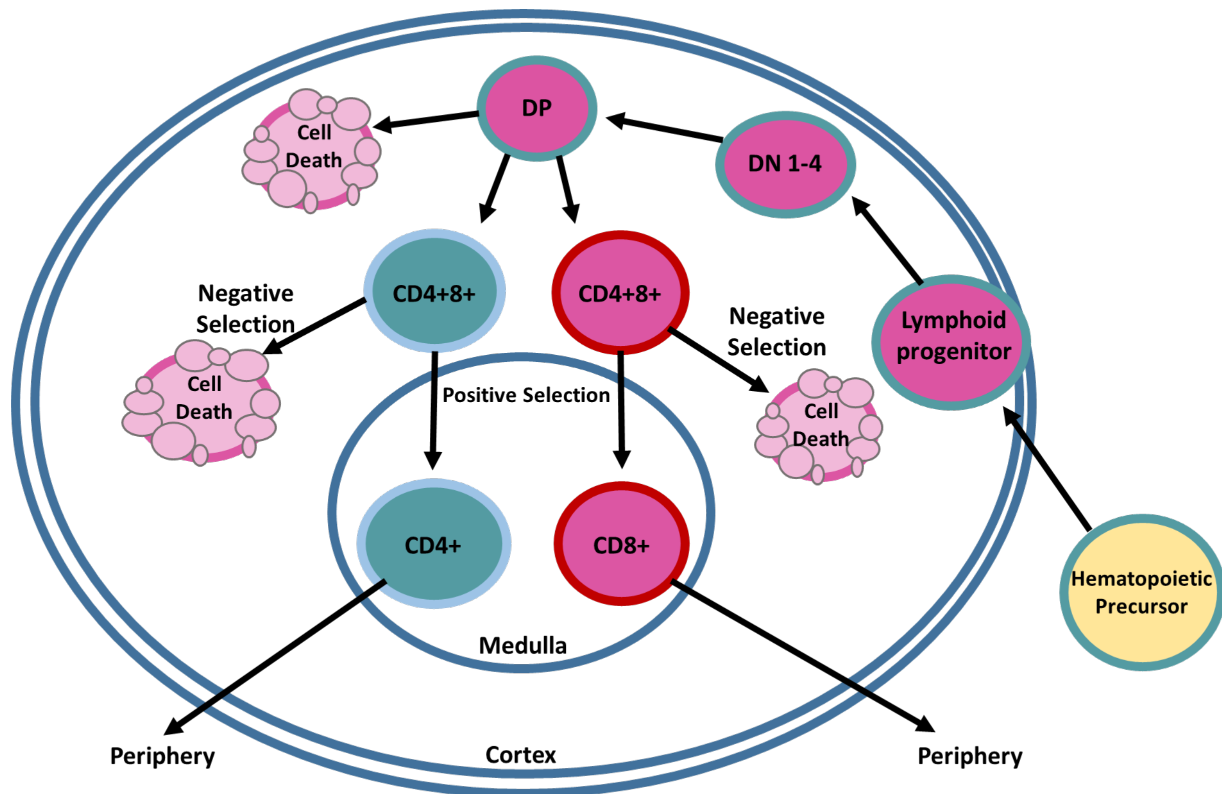


Figure 5. Illustration of T cell maturation in the thymus. This illustration follows a hematopoietic precursor in the cortex and medulla to lymphoid progenitor and the stages of double negative (DN) for CD4-CD8- then maturing to double positive (DP) for CD4+CD8+ before becoming committed to the end stage of CD8+ and CD4+ expressed T-cells prior to emigration to the peripheral blood. Modified from (Germain 2002).

CHAPTER II

INNATE AND ADAPTIVE IMMUNE RESPONSES AFTER BOVINE VIRAL DIARRHEA VIRUS INFECTION IN THE FETAL THYMUS

INTRODUCTION

Infection with BVDV, a *Pestivirus* in the family *Flaviviridae*, leads to significant economic losses for cattle producers in all bovine sectors worldwide (Larson et al., 2002, Moennig et al., 2005, Pinior et al., 2017, Richter et al., 2017). BVDV is a small, enveloped, RNA virus that is classified into two genotypes. Each genotype has two biotypes which is based on their lytic activity in cell culture - cytopathic (cp) and noncytopathic (ncp) (Gillespie et al., 1961). Both BVDV biotypes can cause acute infection in immunocompetent animals with diverse clinical presentations, ranging from subclinical disease, to fever, nasal or ocular discharge, to more sever systemic disease involving reproductive loss, including early embryonic death, abortion, and stillbirth (Childs 1946, Brownlie et al., 1987, Lanyon et al., 2014). BVDV has a multiple organ tropism and previous data has illustrated that infection affects fetal growth and normal development of multiple organs. The immune function in blood, spleen, liver and other organs is also altered due to persitent infection with BVDV (Smirnova et al., 2008, Hansen et al., 2015). When infection occurs after development of the immune system, which is around day 160, the animal develops a transient infection (TI) that can eventually be cleared through seroconversion (Done et al., 1980, Hanon et al., 2014).

Cows have an epitheliochorial placenta in which placentomes are mutlifocal and allow for nutrient exchange between dam and fetus (Brownlie et al., 1987). The BVDV virus can be vertically transferred from dam to fetus at any stage of gestation via the placenta, but only the noncytopathic biotype has been reported to establish a persistent infection (PI) in the fetus (McClurkin et al., 1984, Virakul et al., 1988). Once in the fetus, the virus can replicate in a variety of cell types including

epithelial cells, monocytes and macrophages, thymocytes and lymphocytes, and dendritic cells; albeit the virus tends to replicate in epithelial cells and macrophages earlier than lymphocytes (Bolin 2002, Bachofen et al., 2013). The replication of BVDV in the cells that it has a predilection for, may cause the changes in the immune responses that have been previously reported.

The innate immune response is initiated by retinoic acid inducible gene I (RIG-I), which is an RNA helicase that recognizes viral RNA in the cytosol (Loo and Gale 2011). Viral recognition receptor, RIG-I, then leads to further activation of interferon regulatory factors 3 and 7 (IRF), as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways, which eventually lead to transcription of type I and II interferons (Figure 2). The type I interferons lead to activation and of interferon stimulated genes (ISGs). ISG15, amongst other ISGs, the type II interferons, as well as tumor necrosis factor-alpha (TNF-α) lead to the initiation of the adaptive immune response (Loh et al., 1992, Sentsui et al., 1998, Kadowaki et al., 2000, Samuel 2001, Taniguchi et al., 2001, Charleston et al., 2002). Following the type I IFN response, IFN-γ and TNF-α initate the adaptive immune response for a more robust and specific response to the viral infection.

IFN-γ, an initiator of the adaptive immune response pertaining to the MHC class I pathway, is able to increase the expression of the immunoproteasome which is made up of LMP2, -7, and MECL-2 (Niewerth et al., 2014). The immunoproteasome processes antigens to make peptides which then translocate into the endoplasmic reticulum via TAP proteins. These peptides then bind to MHC complexes where they are presented on antigen presenting cells (APCs) to CD8+ T cells (Groettrup et al., 1997, Eleuteri et al., 2000, Ferrington and Gregerson 2012).

The following experiments were designed to determine the timing of persistent infection in thymic tissue as well as the periods when the innate and adaptive immune pathways are affected by viral infection. As seen in the previous viral titer data (Figure 1), there is a viral titer peak in fetal blood

on day 97 of gestation, which then incurs a robust drop in viral mRNA at days 192 and 245 of gestation. This decrease between days 97 and 192 of gestation may indicate a partially active immune system. We hypothesize that BVDV fetal infection during early gestation results in dysgenesis of the thymus, leading to a suppressed innate and adaptive immune responses in the fetus against BVDV infection. The aim was to identify at what point the fetal immune response is no longer adequately responding to the virus as well as to identify which genes and proteins, in both the innate and adaptive immune systems, are malfunctioning.

MATERIALS AND METHODS

Animals

Animal experiments were performed following the experimental design previously described and below (Smirnova et al., 2008, Smirnova et al., 2012). All animal experiments were approved by the Institutional Animal Care and Use Committee at Colorado State University and the University of Wyoming. Naïve Hereford heifers were purchased from a source that did not vaccinate against BVDV and were serologically negative for BVDV. Heifers were confirmed negative for BVDV using an ear notch sample for ELISA capture sent to IDEXX Laboratories (Westbrook, ME, USA) as well as a standard serum neutralization assay. Heifers were re-tested prior to BVDV inoculations to confirm negativity. Estrous cycles were synchronized and heifers were artificially inseminated with BVDV-free semen around 12 months of age. Ultrasound examination confirmed pregnancy on days 32 and 70 of gestation.

Experimental design: BVDV infection and fetal collections

Two experiments were performed, as previously described, experimental design for both is depicted in Figure 6 (Smirnova et al., 2008, Smirnova et al., 2012). Briefly, experiment 1 used 18 pregnant heifers, naïve for BVDV, and they were inoculated with a 2 ml aliquot of 4.4 $\log_{10}$ TCID50/ml of ncp BVDV2 strain 96B222 intranasally to create infected fetuses or were sham inoculated with culture

media as controls. Animals were inoculated with virus or media on day 75 of gestation to generate PI or control fetuses or they were inoculated on day 175 to generate TI or control fetuses (n=6 control/6 PI/ 6TI). Fetuses were collected by Cesarean section on day 190 of gestation for experiment 1 and day 192 of gestation for experiment 2.

The second experiment used the same dose and route of ncp BVDV for inoculation. Animals were inoculated on day 75 of gestation and fetuses were collected by Cesarean section on days 82, 89, 97, 192, and 245 of gestation (n = 4 control/4 PI fetuses for days 82, 89, 97, 192, and 245; n = 4 control/3 PI fetuses for day 245: one PI fetus in the 245 day group was lost due to abortion prior to collection for reasons that were not related to the experimental infection). Fetal tissues for RNA and protein analysis were snap frozen in liquid nitrogen and stored at -80°C.

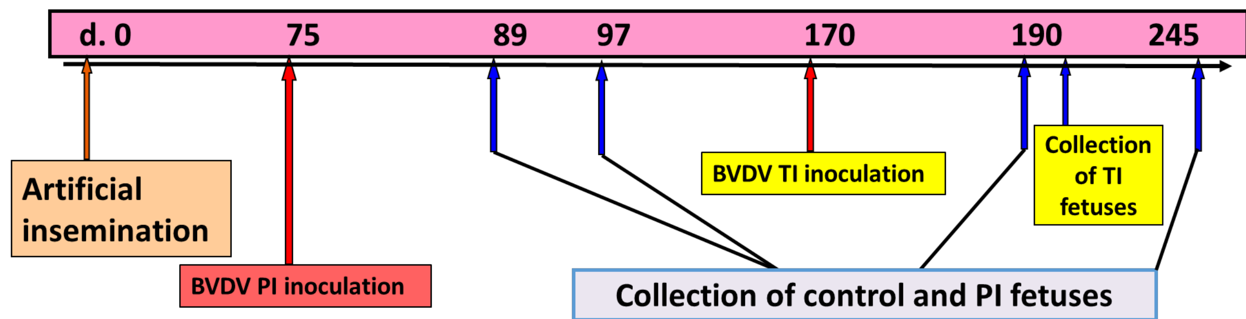


Figure 6. Experimental design to examine fetal response to persistent infection with ncpBVDV. Heifers were artificially inseminated (AI) on day 0. Confirmed pregnant heifers were infected with ncpBVDV type 2 viral strain intranasally on days 75 and 170 of gestation to generate PI and TI fetuses, respectively, or they were kept BVDV-naïve to generate control, uninfected fetuses. Cesarean sections were performed on day 89, 97, 190, and 245 of gestation to collect fetuses. Modified from (Hansen et al., 2015).

RNA Extraction and RT-qPCR

Total RNA from 70 mg of frozen thymus tissues was isolated with TRIzol reagent according to manufacturer's instructions (Invitrogen, USA). The isolated RNA was treated with DNAase I (Qiagen, Germantown, MD) and was purified using RNeasy MiniElute Cleanup Kit (Qiagen, USA). RNA concentration, 260/280 and 260/230 ratios were measured using NanoDrop 1000 Spectrophotometer (ThermoScientific, Rockford, IL). All amplicons were sequenced (GENEWIZ, LLC) to confirm identity of each target gene. Primers were designed to generate 90 to 200 bp amplicons and were optimized for melting point as well as lack of self-annealing or folding at high temperatures. Analysis of efficiency of amplification for each primer pair was completed. Primer efficiencies were performed in fetal thymic tissues and considered acceptable if between 90 to 120 percent. Primer sequences and their accession numbers are listed in Table 1. One microgram of RNA was then reverse transcribed to synthesize cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). Reverse transcription (RT) was performed according to manufacturer protocol. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed with iQ ™ SYBR® Green Supermix according to manufacturer's instructions (Bio-Rad, Hercules, CA,). Briefly, upon generation of dsDNA, iQ™ SYBR® Green Supermix intercalates between strands of DNA as they are formed. With increasing copies of DNA, the signal is increased. Synthesized cDNA was then diluted 5-fold with RNAase free water. Final primer concentrations were 5 μM. Technical duplicates of each reaction was placed on the same plate. Three biological replicates were also performed.

The RT-qPCR reaction was performed in a 384-plate format and conducted at one cycle of 95°C for 3 minutes, 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 15 s with a final 5 minute elongation using a LightCycler-480 Instrument (Roche, Basel, Switzerland). Gene expression was calculated after normalization with 18S ribosomal RNA (18S rRNA), an endogenous control, using the comparative ΔCt

method and was presented as relative expression ($2^{-\Delta Ct}$). Upon completion of RT-qPCR, amplification melting curve analysis was performed to assess the quality of amplification. RT-qPCR was evaluated based on MIQE guidelines (Bustin et al., 2009).

Table 1. RT-qPCR primer sequences. F – forward, R – reverse

Target gene	Accession number	Primer sequences
	Housekeeping Genes	
GAPDH	DQ403066	F: TGACCCCTTCATTGACCTTC R: CGTTCTCTGCCTTGACTGTG
18S	NR_036642.1	F: GAACGAGACTCTGGGCATGC R: CTGAACGCCACTTGTCCCTC
	Innate Immune Genes	
RIG-I	XM_580928.8	F: CAGGAAGATCCTGGACCCTA R: TTTTTCTTGGCCTGAATGTG
IRF7	XM_015461322.1	F: GCCTCCTGGAAAACCAACTT R: CCTTATGAGGGTCGGTAGGGG
NF-KB	XM_010819902.2	F: CGAGGTTCGGTTCTACGAGG R: TGCAGGAACACGGGTTACAGG
IFNβ	XM_015464447.1	F: TCCAGCACATCTTCGGCATT R: TTCCCTAGGTGGGGAACGAT
STAT1	CK849770	F: CCTGTATCTGGGCAGAGGAT R: CCAAAAGCTAATTCTCCCAAGA
IFI16	CK952947	F: GGAGTGATGGAAGTCGTGGT R: CCCACTTTCTCCCAATTCAA
ISG15	NM_001009735.1	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT
	Adaptive Immune Genes – MHC Class I	
LMP2	NM_001034388.2	F: ATCTACCTGGCCACCATCAC R: AGGAGAGTCCGAGGAAGGAG
TAP2	NM_174222.2	F: TGGAGCTGGATTTAGACCGTG R: GGCAAAGCTCTGTTCGTCAA
β*2M*	AC_000167.1	F: AFTAAGCCGCAGTGGAGGT R: CGCAAAACACCCTGAAGACT
CD8a	NM_174015.1	F: TACATCTGGGCTCCCTTGGT R: CCACAGGCCTGGGACATTTG
CD8b	NM_001105344.2	F: AGCTGAGTGTGGTTGATGTTCT R: TTCTGAGTCACCTGGGTTGG
	Adaptive Immune Genes – MHC Class II	
CIITA	XM_005196914.3	F: AGTCTGGAAGTTGCTGCCGTC R: CACTGTCCACTGCCTTGGG
GILT (IFI30)	AY581199.1	F: GCATGCAGCTCTTGCACATC R: GGCCCCAAGAGTTCTTACCC
CD4	NM_001103225.1	F: GGGCAGAACGGATGTCTCAA R: ATAGGTCTTCTGGAGCCGGT
	B Cell Gene	
CD79b	M_003583611.4	F: TGATTCCCGGGCTCAACAAC

		R: CTGCCAGATCCGGGAACAAG
IFN Inducible Genes		
CXCR6	CB170970	F: CAGGTGCCTGACAGAAATGA R: CTTCCAGGGTCAAGCAAAGA
CXCL10	CB533091	F: ACACCGAGGCACTACGTTCT R: TAAGCCCAGAGCTGGAAAGA
CXCL16	CK770974	F: CTTGTGAGGGCAGATTGTGA R: GGTCAATAGCTGGTTAGTTGTGAA
BVDV Cell Receptor Gene		
CD46	XM_005217324.3	F: GCTGGACTCAGCAAGGTCTC R: AAGAAGCCAGTCTTTCGGGG

Western blotting

Protein was generated from thymic tissues homogenized in 1X RIPA buffer and Halt™ protease and phosphatase single-use inhibitor cocktail according to manufacturer instructions (ThermoScientific, Rockford, IL). Concentrations were measured using the Pierce™ BCA Protein Assay Kit (ThermoScientific, Rockford, IL) and analyzed in a BioTek Synergy 2 reader (Winooski, VT). Proteins were subjected to 12% SDS-PAGE made from TGX™ FastCast™ Acrylamide Solutions Kit (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes, and blocked with Bio-Rad Blocking Buffer (Bio-Rad, Hercules, CA)/Tris-buffered saline-Tween 20 (TBS-T) at room temperature for one hour. Western blotting was carried out using the following dilutions of primary antibodies in blocking buffer: 1:1,000 anti-IRF7 (rabbit IgG; abcam ab62505), 1:1,000 anti-CD4 (mouse IgG, abcam ab25804), 1:25,000 5F10 mouse monoclonal anti-ISG15 antibody (Austin et al., 2004) and 1:2,000 β-actin (mouse monoclonal antibody conjugated to HRP, Santa Cruz BioTechnology sc-47778). Washed blots were then incubated in 1:2,000 dilutions in 1% Bio-Rad Blocking BufferTBS-T of the appropriate secondary antibody-horseradish peroxidase (HRP) conjugate (Santa Cruz BioTechnology). Clarity™ Western ECL Blotting Substrate (Bio-Rad, Hercules, CA) was used to activate the HRP and imaging was performed using Molecular Imager ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad, Hercules, CA).

Morphogenesis of Thymus during Bovine Fetal Development and Effect of in utero BVDV Infection

Thymus samples, collected previously (Smirnova et al. 2008, Smirnova et al. 2012) and fixed for 48 hours in 10% neutral-buffered formalin, followed by routine processing for paraffin-embedding, were sectioned (4-5 μm) and stained with eosin and hematoxylin (H&E). Microscopic assessment was performed with special attention to the components of the reticulo-endothelial network, myeloid, and lymphoid cells, including when and where the latter two cell types appear in the organ primordium. For the thymus, the relative cortical-to-medulla ratio was assessed, including full encirclement of the medulla by cortical thymocytes. Additionally, attention was paid to myoid cells and Hassall's corpuscles in terms of appearance, frequency, and size. Histopathology was performed in collaboration with Helle Bielefeldt-Ohmann at the University of Queensland.

Statistical analysis

Statistical analysis of data obtained by RT-qPCR was performed in GraphPad Prism 7. For BVDV, the control group was expected to have very low (or no) expression. This is seen in summary statistics and graphs, but it creates an "artifact" in the analysis due to essentially zero variability and sample size (especially at day 245 of gestation), thus a Wilcoxon test was used to compare PI versus control at each day separately. For day 190 of gestation and analyzing BVDV, a Kruskal-Wallis analysis was used to compare the three different groups (Control, PI, and TI). For all other genes, a two way ANOVA was used to compare control and PI for all days. For genes at day 190, a one way ANOVA was used to compare control, PI, and TI. Differences between groups (infected, day, and infected/day) were considered significant when p-value was less than 0.05. Data are presented as mean ± standard error. Statistical analysis was done in collaboration with Dr. Ann Hess, a statistician from Colorado State University.

RESULTS

Infection with BVDV induces innate gene immune responses in the fetal thymus of TI, but not PI fetuses

Thymus *RIG-I* mRNA was significantly increased in the TI fetuses compared to control ($P \leq 0.001$) and PI ($P \leq 0.0001$) at day 190 of gestation (Figure 7A). The TI fetuses were only collected at day 190 of gestation. *RIG-I* was not significantly different in control versus PI fetal thymic tissue at day 89, 97, or 245 of gestation (Figure 7A).

IRF7 mRNA was significantly increased in the TI fetuses compared to control ($P \leq 0.001$) and PI ($P \leq 0.0001$) fetuses at day 190 of gestation (Figure 7B). There were no significant difference between control and PI at day 89, 97, or 245 of gestation (Figure 7B). *NF-kB* mRNA was significantly increased in the PI fetuses compared to the control fetuses ($P \leq 0.01$) at day 97 of gestation, whereas the PI fetuses were significantly decreased compared to the control ($P \leq 0.01$) and TI ($P \leq 0.0001$) fetuses at day 190 of gestation (Figure 7C). The TI fetuses were also significantly increased compared to the control fetuses ($P \leq 0.01$) also at day 190 of gestation (Figure 7C).

IFNβ mRNA was significantly decreased in the PI fetuses compared to control ($P \leq 0.05$) and TI ($P \leq 0.0001$) fetuses at day 190 of gestation (Figure 7D). There were no *IFNβ* differences between control and PI fetuses at day 89, 97, or 245 of gestation (Figure 7D). When analyzed in BVDV fetal thymuses there was no change of *ISG15* gene expression at any day of gestation, except in the TI fetus where the TI is significantly higher than control ($P \leq 0.01$) and PI ($P \leq 0.01$) fetuses at day 190 of gestation (Figure 7E).

Western blot analysis of innate immune responses after BVDV infection in fetal thymus

IRF7 from fetal thymus was measured at day 89, 97, 190, and 245 gestation. There was no difference in IRF7 after BVDV infection at day 89, 97, 190, or 245 of gestation (Figure 8). Protein was

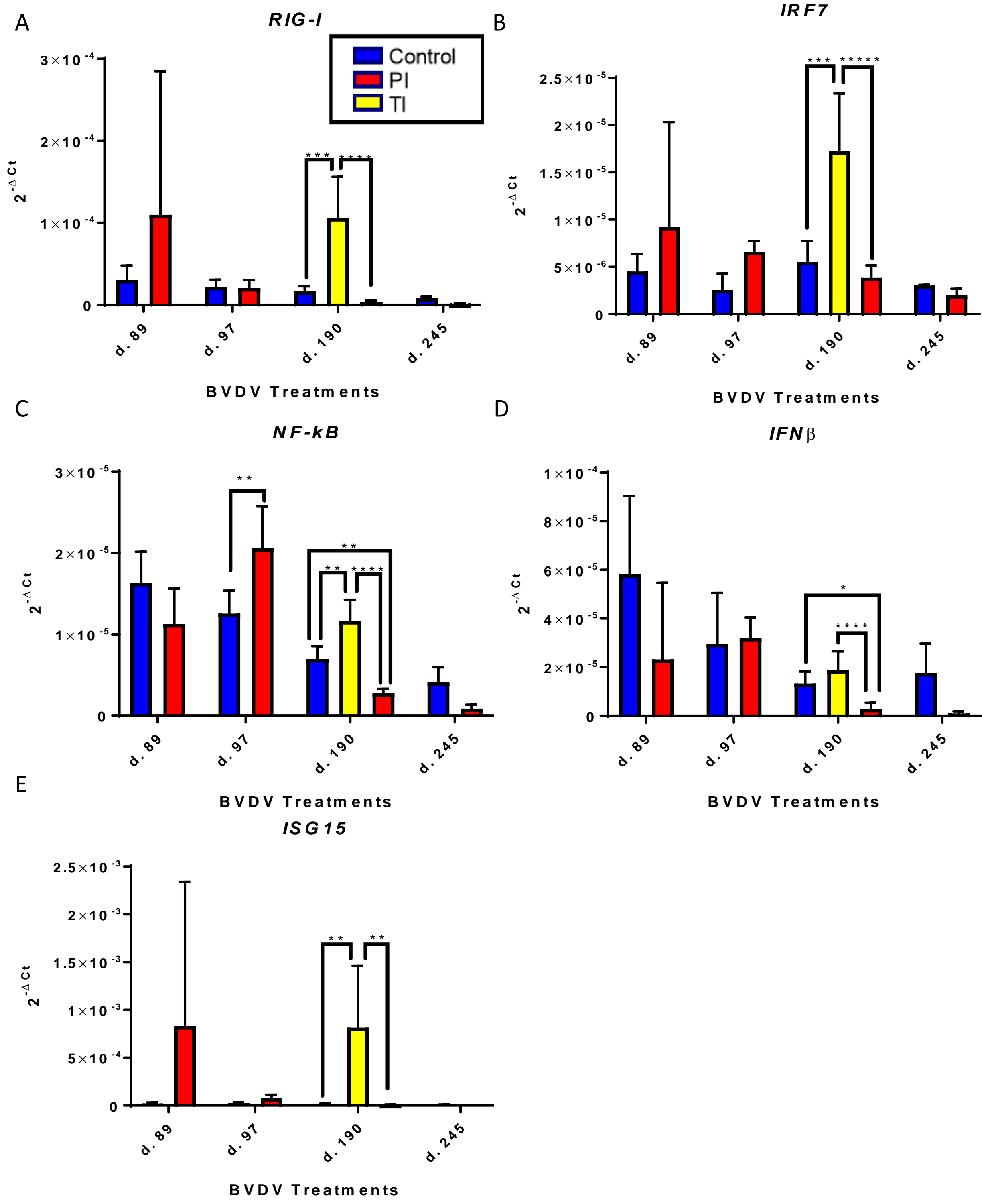


Figure 7. Responses of innate immune response genes in the fetal thymus after maternal inoculation with ncpBVDV at day 75. (A) *RIG-I* mRNA; (B) *IRF7* mRNA; (C) *NF-kB* mRNA; (D) *IFNβ* mRNA; and (E) *ISG15* mRNA. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, *** $P \leq 0.0001$.

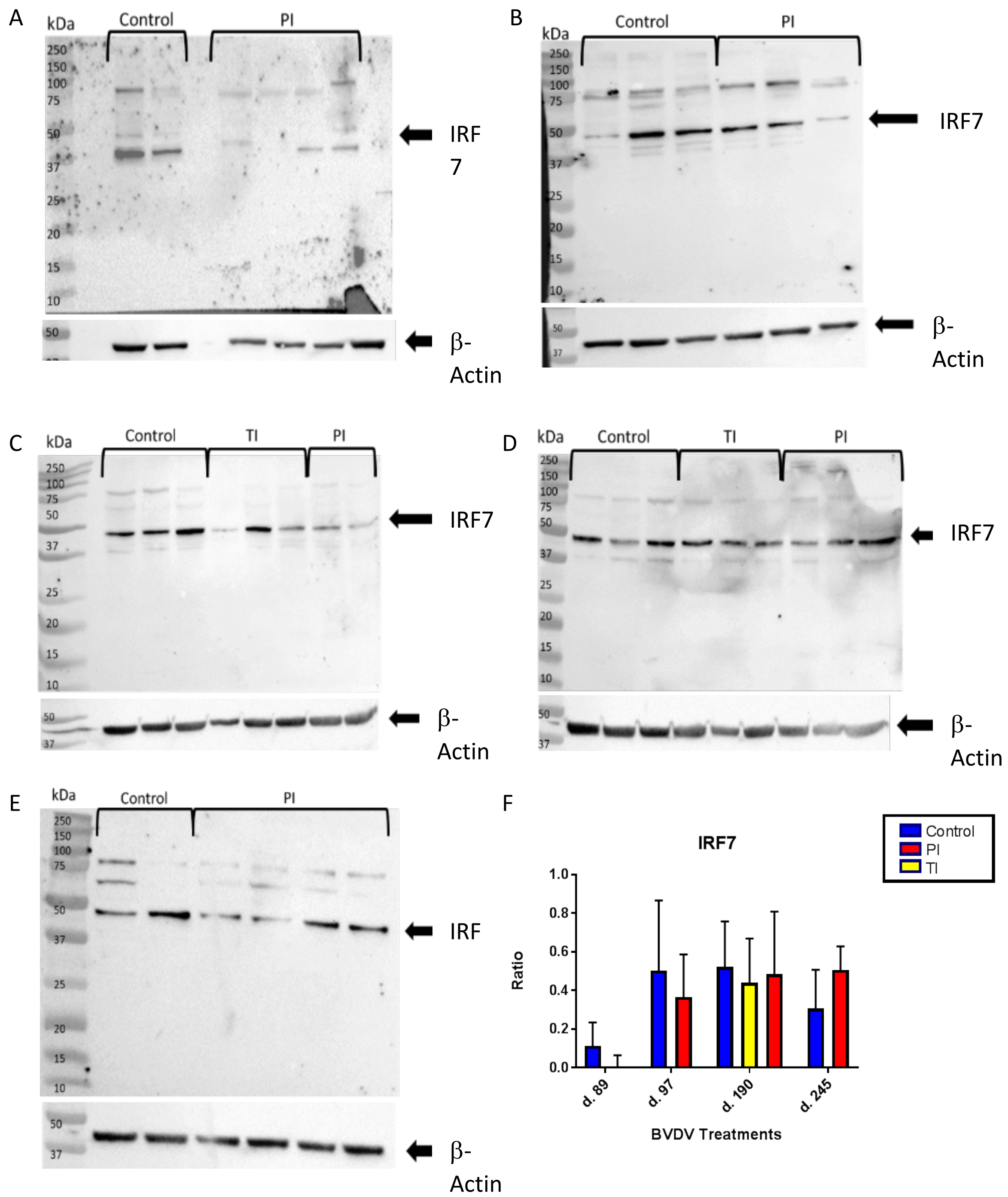


Figure 8. Western blots of IRF7, normalized to β-actin from day (A) 89, (B) 97, (C and D) 190, (E) 245 and (F) ratio of IRF7 and β-actin. Data are presented as mean ± SE.

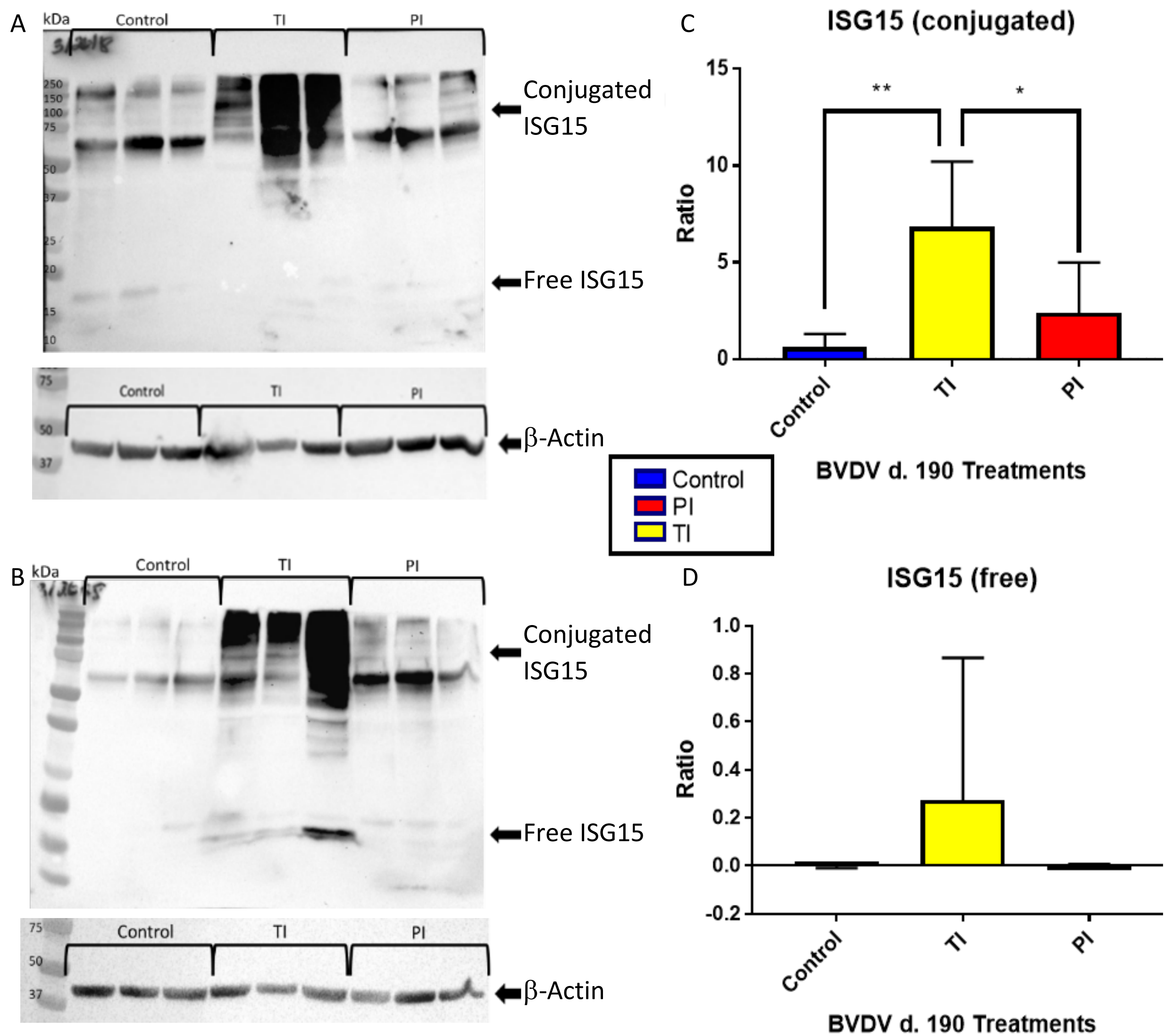


Figure 9. Increased protein expression in TI fetuses at day 190 of gestation after maternal inoculation with ncpBVDV. Western blots of ISG15 at day 190 (A and B) followed by (C) the ratio of the conjugated ISG15 and β-actin and (D) the ratio of the free ISG15 and β-actin. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$.

normalized to β-actin (Figure 8F). The IRF7 protein data does not correlate with day 190 of gestation mRNA. ISG15 exists in both a conjugated and free form. Protein measured from 37 kDa and above is considered conjugated and the free form is at 17 kDa (Figure 9A). The TI fetuses conjugated form of ISG15 was significantly higher than both the control ($P \leq 0.01$) and PI ($P \leq 0.05$) fetuses at day 190 of gestation (Figure 9B). However, the free form of ISG15 was not significantly different from control ($P \leq 0.36$) or PI ($P \leq 0.37$) thymuses, but it does follow the same trend that the TI fetuses have an increased amount of protein compared to control and PI fetuses at day 190 of gestation (Figure 9C).

Expression of IFN induced genes in fetal tissues

Type I and II IFNs are able to initiate activation of various interferon inducible genes which serve various roles in both immune pathways. The genes that we studied that are associated with these pathways include *STAT1*, *IFI16*, *CXCL10*, *CXCL16*, and *CXCL6*. RT-qPCR was performed on these listed genes at days 89, 97, 190, and 245 of gestation.

STAT1 mRNA concentrations did not change on days 89, 97, 190, or 245 of gestation between any treatment group (Figure 10A). Whereas RT-qPCR showed a significant difference between PI and control ($P \leq 0.001$) and PI and TI ($P \leq 0.05$) fetuses at day 190 of gestation for *IFI16* (Figure 10B). There were no other significant differences between treatment groups at day 89, 97, or 245 of gestation for IFI16.

Chemokines *CXCL10*, *CXCL16*, and *CXCR6* are generally induced by IFN-γ. There was no significant difference between fetal thymuses after maternal BVDV infection in any treatment group at day 89, 97, 190, or 245 of gestation for *CXCL10* (Figure 10C). However, both chemokines *CXCL16* and *CXCL6* showed significant differences at day 190 of gestation (Figure 10D and E). For *CXCL16* the PIs were significantly lower than both the control ($P \leq 0.01$) and TI ($P \leq 0.05$) fetuses at day 190 and for

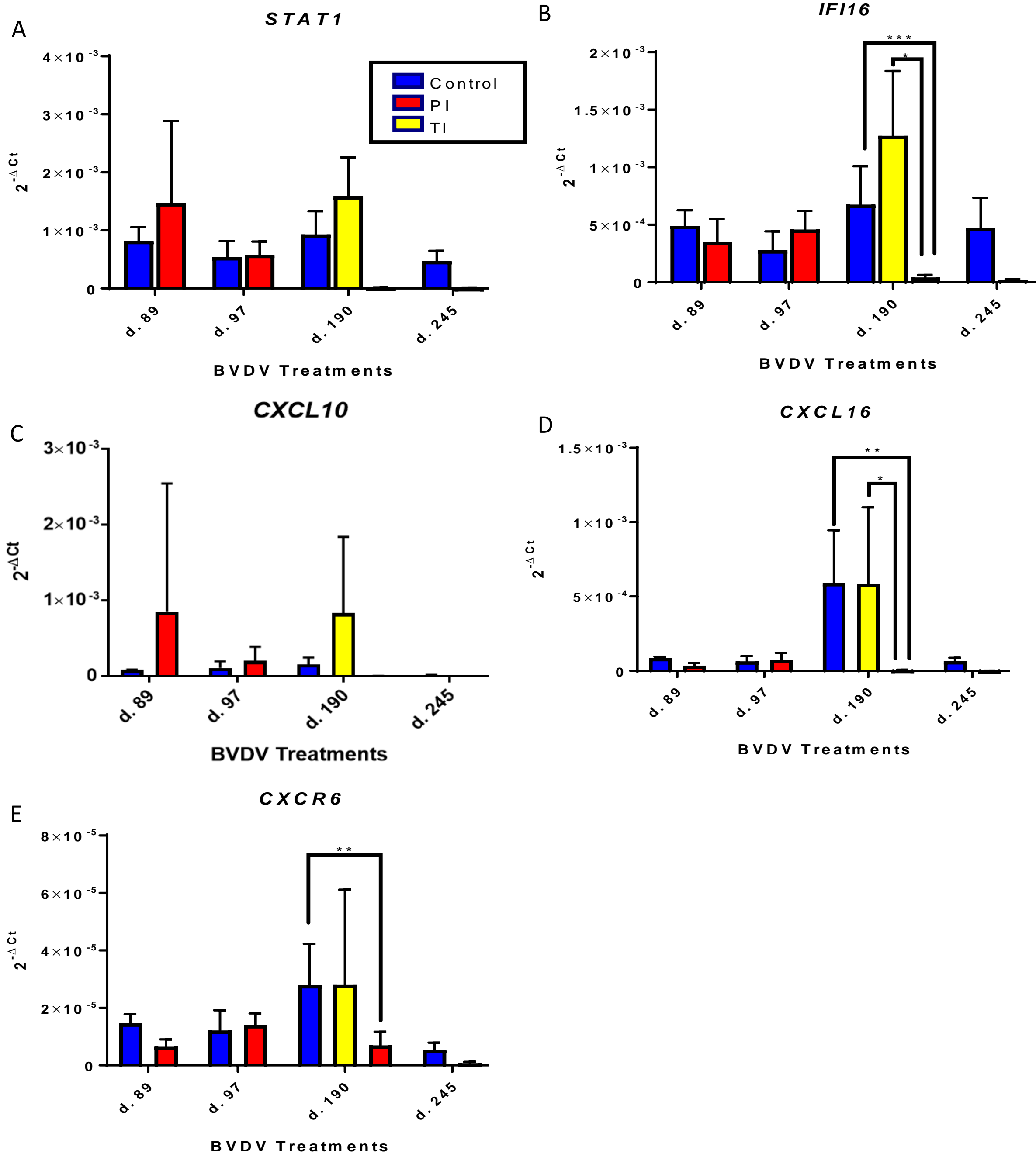


Figure 10. Concentrations of mRNA for *IFN-γ* induced genes in fetal thymus after maternal inoculation with ncpBVDV. Gene expression of *IFI16*, *CXCL16*, and *CXCR6* mRNA, significantly decreased in PI fetal thymus on day 190 when compared to control and TI fetuses. (A) *STAT1* mRNA, (B) *IFI16* mRNA, (C) *CXCL10* mRNA, (D) *CXCL16* mRNA, and (E) *CXCR6* mRNA. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

*CXCR*6 the PI was significantly lower than control (P ≤ 0.01) fetuses however, there was too much sample to sample variablity in the TI fetuses to show a significant difference.

Downregulation of genes along the MHC class I (BoLa) Pathway in response to fetal persistent infection in the thymus

No differences were found for *IFN-*γ for mRNA measurement between control and PI groups on any of the days of gestation analyzed (Figure 11A). The immunoproteasome is a beginning step of antigenic peptide breakdown. RT-qPCR was performed on thymus tissue for various genes along the MHC class I Pathway. After maternal BVDV infection, there is an increase of fetal thymic *LMP2* mRNA of persistently infected fetuses compared to the control group at day 97 of gestation (P ≤ 0.001), but is then downregulated at day 190 and 245 of gestation (P ≤ 0.01). The TI fetuses were also significantly increased compared to the PI (P ≤ 0.05), but not different than the sham infected controls (Figure 11B).

The *TAP 1* mRNA was significantly increased in PI fetuses at day 97 (P ≤ 0.05), but the day 190 samples showed a decrease of PI fetuses compared to the control group (P ≤ 0.0001). The day 245 PI fetuses were trending toward a decrease, but they were not significantly different (P = 0.09) (Figure 11C). β*2M* mRNA was significantly decreased at day 190 and 245 in the PI fetuses compared to the control group (P ≤ 0.01) and TI fetuses compared to PI fetuses at day 190 (P ≤ 0.0001) (Figure 11D). The *CD8a* and *CD8b* mRNA from thymus tissue at all days of gestation tested had the same pattern of change. The *CD8a* gene was significantly decreased in PI compared to the control (P ≤ 0.01) fetuses at day 190 gestation of PI animals as well as in the PI compared to TI (P ≤ 0.001) fetuses (Figure 12A). On day 245, *CD8a* was significantly decreased in the PI compared to control (P ≤ 0.001) fetuses. The *CD8b* gene was also significantly decreased in PI animals compared to control (P ≤ 0.05) and TI (P ≤ 0.01) fetuses at day 190 of gestation (Figure 12B). Day 245 PI fetuses were also significantly decreased compared to control (P ≤ 0.05) fetuses (Figure 12B).

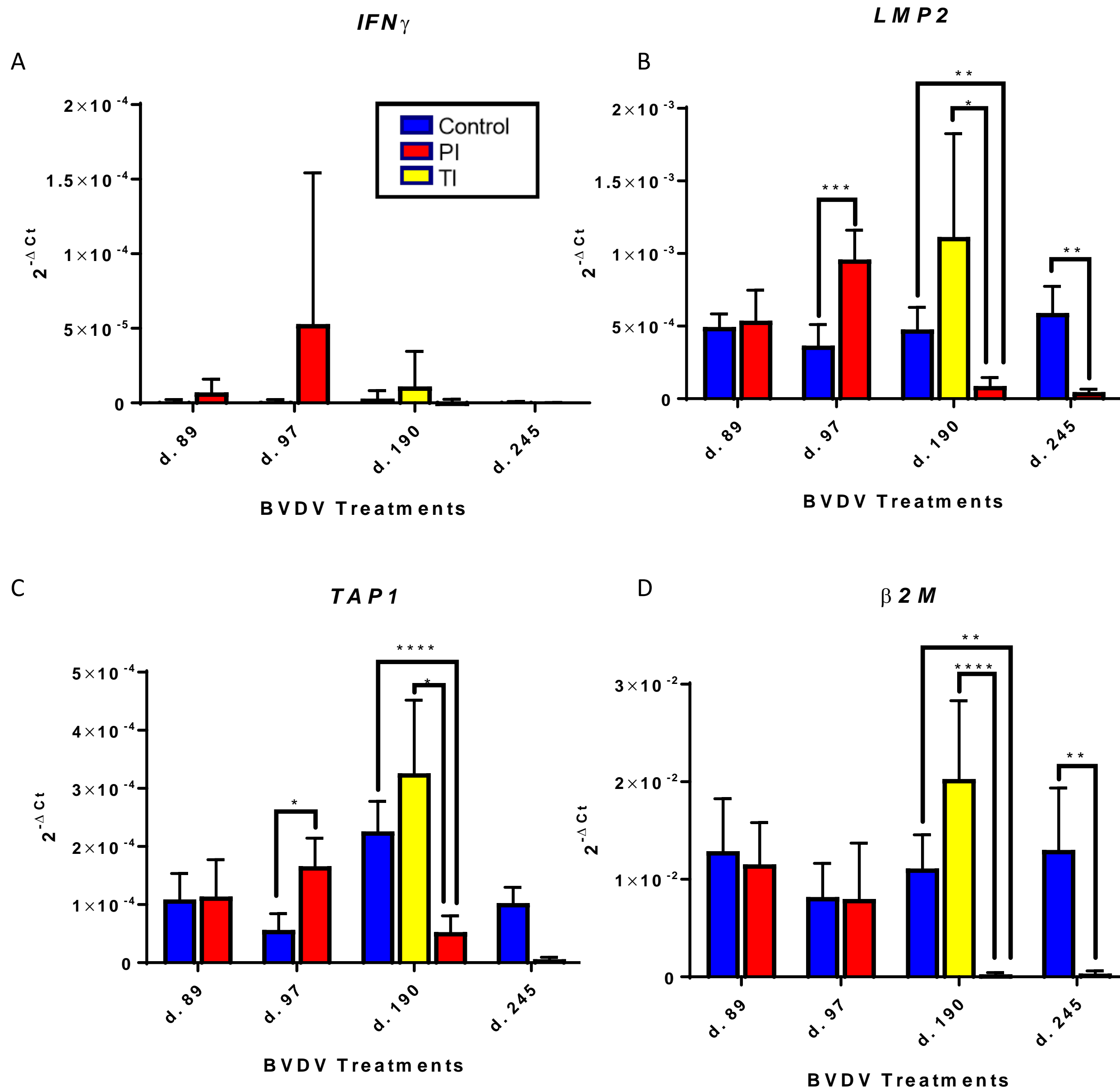
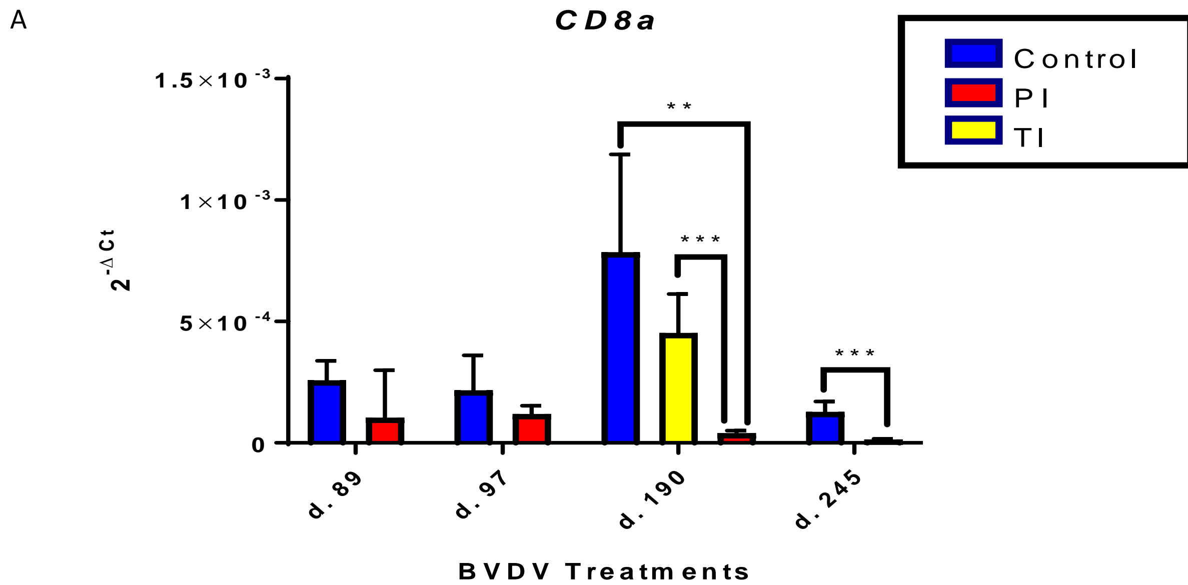


Figure 11. Responses of adaptive immune response genes in the fetal thymus after maternal inoculation with ncpBVDV. (A) *IFN-γ* mRNA, (B) *LMP2* mRNA, (C) *TAP1* mRNA, and (D) *β2M* mRNA. Data are presented as mean ± SE. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

A



B

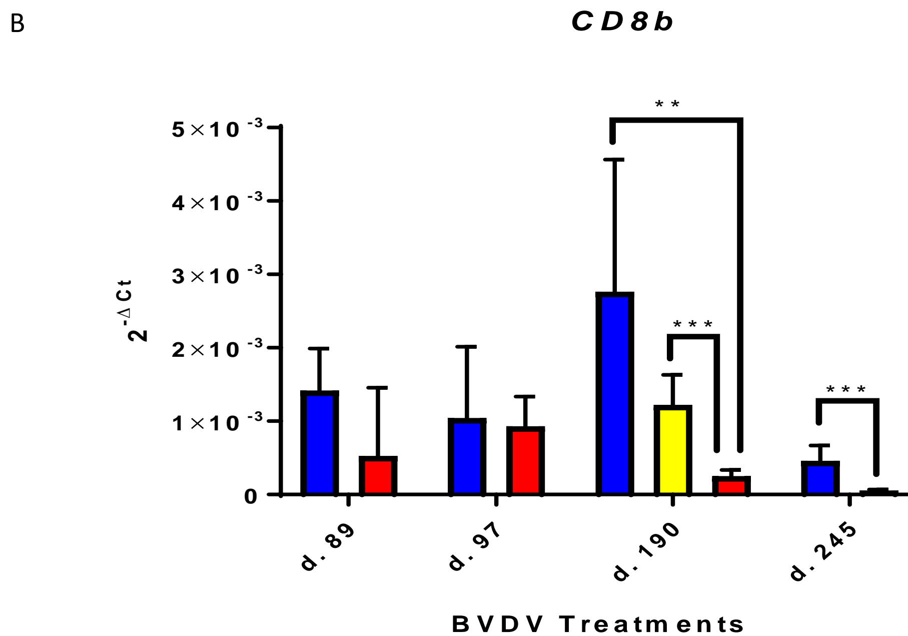


Figure 12. Responses of *CD8a* and *CD8b* in the fetal thymus after maternal inoculation with ncpBVDV. (A) *CD8a* mRNA and (B) *CD8b mRNA*. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Downregulation of genes along the MHC class II Pathway in response to fetal persistent infection in the thymus

RT-qPCR was performed on thymus tissue for *CIITA*, *GILT*, and *CD4+* mRNA on four selected time points representing the course of the establishment of fetal persistent infection at days 89, 97, 190, and 245. There was a significant increase in *CIITA* mRNA concentration at day 97 in the PI fetuses compared to control groups, but *CIITA* mRNA at significantly decreased in both day 190 and 245 compared to control groups (Figure 13A).

There was a significant increase in *GILT* mRNA concentration in PI versus the control group at day 97, with no differences at day 89 ($P = 0.66$), 190 ($P = 0.98$), or 245 ($P = 0.80$) (Figure 13B). CD4 had decreased gene expression at day 190 and 245 in PI fetal thymus. CD4 was significantly lower in fetal PI thymuses at day 190 compared to both the control ($P \leq 0.0001$) and TI ($P \leq 0.001$) samples (Figure 13C).

Following the RT-qPCR data, CD4 protein concentration was evaluated using Western blot. CD4 from days 89 ($P = 0.99$), 97 ($P = 0.99$), 190 ($P = 0.43$), and 245 ($P = 0.99$) of gestation did not show any significant difference between the treatment groups (Figure 14). It is important to note that this did not correspond with the mRNA data.

Additional genes measured in the thymus after BVDV maternal infection using RT-qPCR

RT-qPCR of *CD79b* in the fetal thymus after maternal BVDV infection showed significant difference between PIs and control ($P \leq 0.05$) and PI and TI ($P \leq 0.01$) fetuses at day 190 of gestation (Figure 15). There were no other differences between treatments at day 89, 97, or 245. The *BVDV* mRNA viral titer using RT-qPCR was also measured at days 89, 97, 190, and 245 of gestation. *BVDV* mRNA levels were not expressed in any of the control fetal thymus tissue. The PI BVDV mRNA relative expression ranges from 0.001 to 0.004 ($2^{-\Delta Ct}$)over the four various time points at +/- the standard error

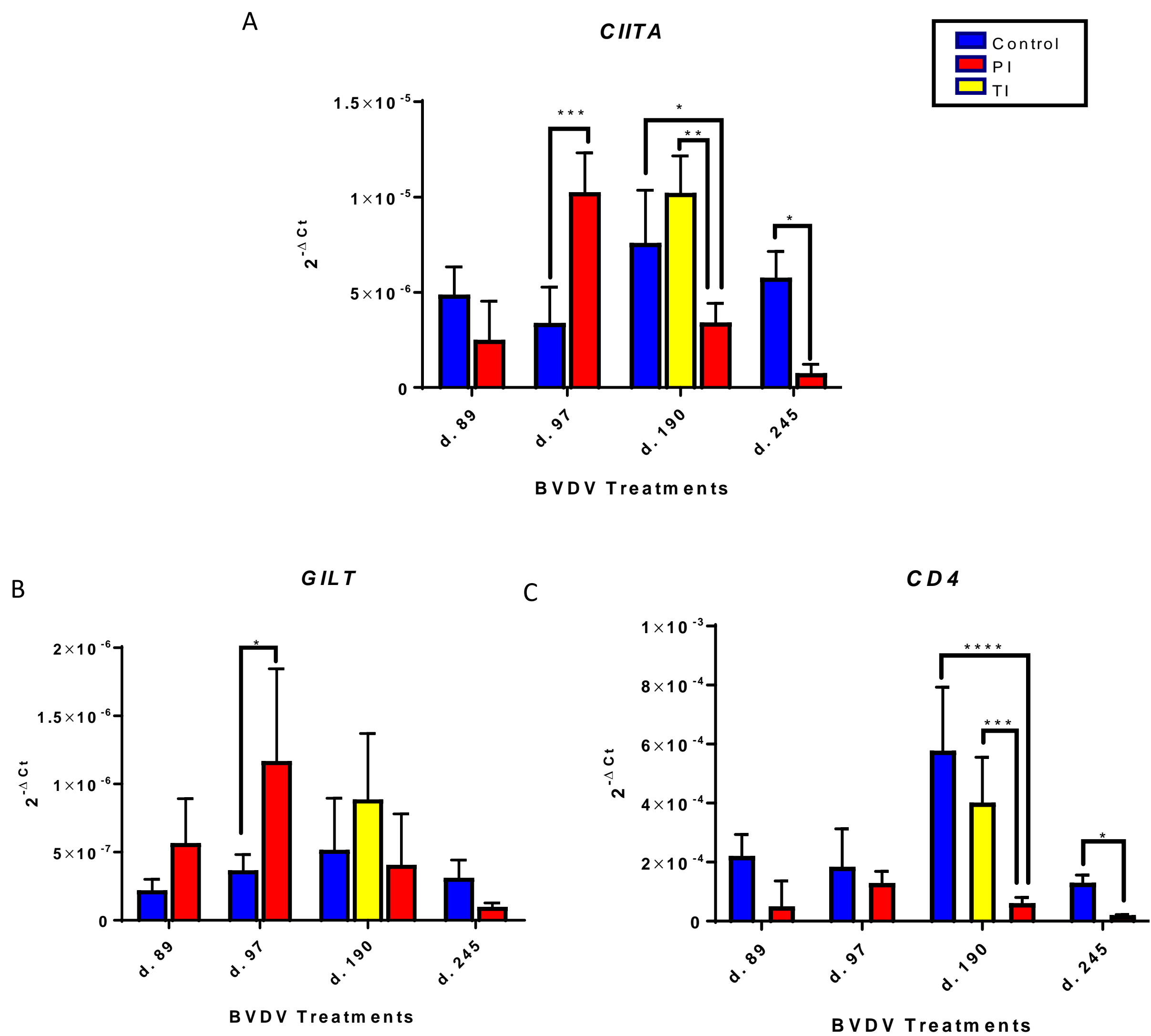


Figure 13. Responses of adaptive immune response genes in fetal thymus after maternal inoculation with ncpBVDV. (A) *CIITA* mRNA, (B) *GILT* mRNA, and (C) *CD4* mRNA. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

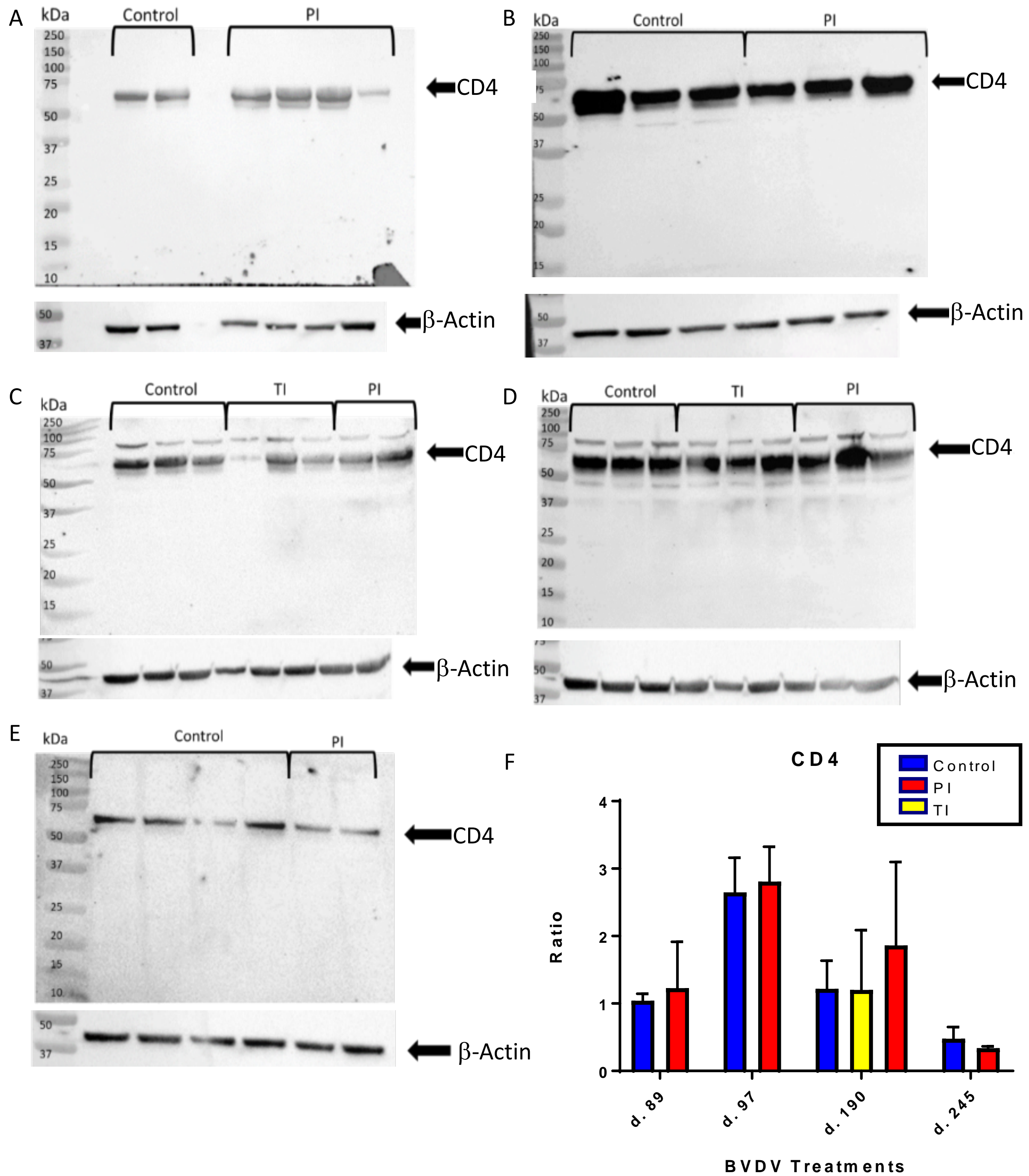


Figure 14. Protein expression of CD4 in fetal thymus after maternal inoculation with ncpBVDV. Day (A) 89, (B) 97, (C and D) 190, and (E) 245 of gestation. Figure (F) is the ratio of CD4 to β-actin at each day. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$.

of the mean. There was no significant difference in the BVDV receptor, *CD46,* expression from fetal thymus after maternal BVDV infection at day 89, 97, 190, or 245 (Figure 16).

Morphogenesis and Histology of thymus during bovine fetal development and the effect of in utero BVDV infection

Fetal thymuses from control, TI, and PI fetuses were evaluated at days 82, 89, 97, 190, and 245 of gestation for morphological differences after maternal infection with BVDV. One day 82 infected, two day 89 control, and one day 89 infected thymus were examined (Figure 17 A & B). These four thymuses have clearly delineated cortex and medulla. The cortex is relatively narrow compared to later fetal stages, and has a “leaflet”-like outline, whereby the medulla in some places is directly adjacent to the interstitial parenchyma. However, the cortical cell density is comparable to that of later fetal stages. The medulla contains well developed Hassall’s corpuscles and rare myoid cells. Extramedullary hematopoietic activity is present in the septae between the cortical “leaflets”.

Four control and four PI fetal thymuses from day 97 are similar in morphology to the day 89 tissue, except for a slightly greater variation in the cortex-to-medulla ratio (Figure 17 C &D). However, there are no discernible differences between thymuses from the control or PI fetuses at day 97 of gestation.

At day 190, the morphology of the tissue has attained almost a neonatal appearance, with the cortex having expanded to completely surround the medulla and the cortico-medullary ratio being ≥ 2 (Figure 17 E & F). Six-fetal thymi were examined from control, TI, and PI maternally infected animals. The medulla contains large, often complex Hassall’s corpuscles, large numbers of myoid cells and eosinophilic granulocytes. The latter can be seen throughout the medulla, but appears in highest frequency in the cortico-medullary border zone. The myoid cells are large, round cells with a deeply

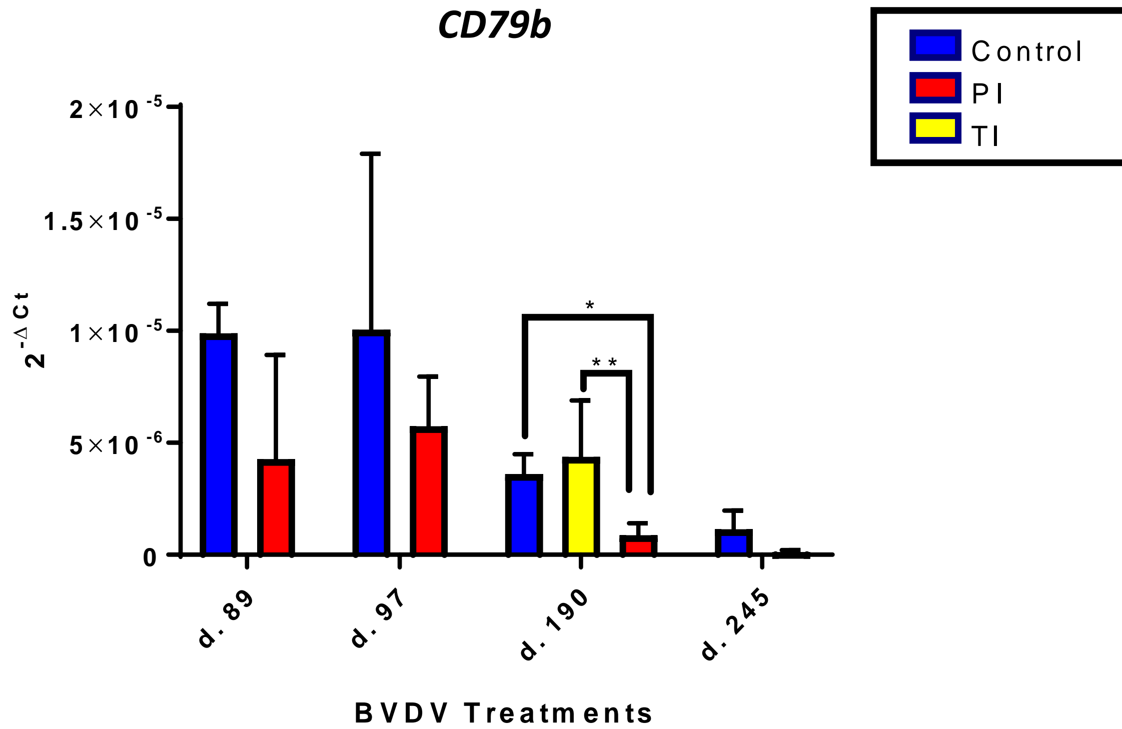


Figure 15. Expression of *CD79b* mRNA in response to BVDV. *CD79b* was measured in fetal thymus at day 89, 97, 190, and 245 of gestation. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

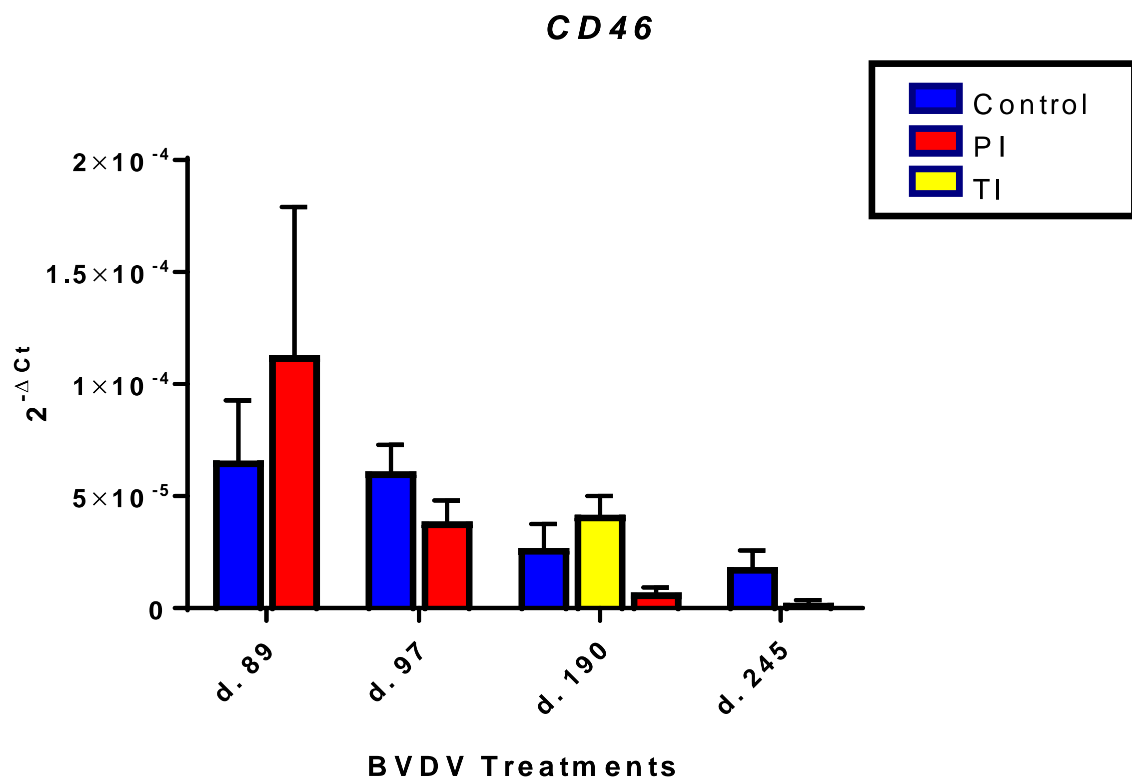


Figure 16. There are no *CD46* gene expression changes in fetal thymus collected at day 89, 97, 190, or 245 of gestation. Data are presented as mean ± SE. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

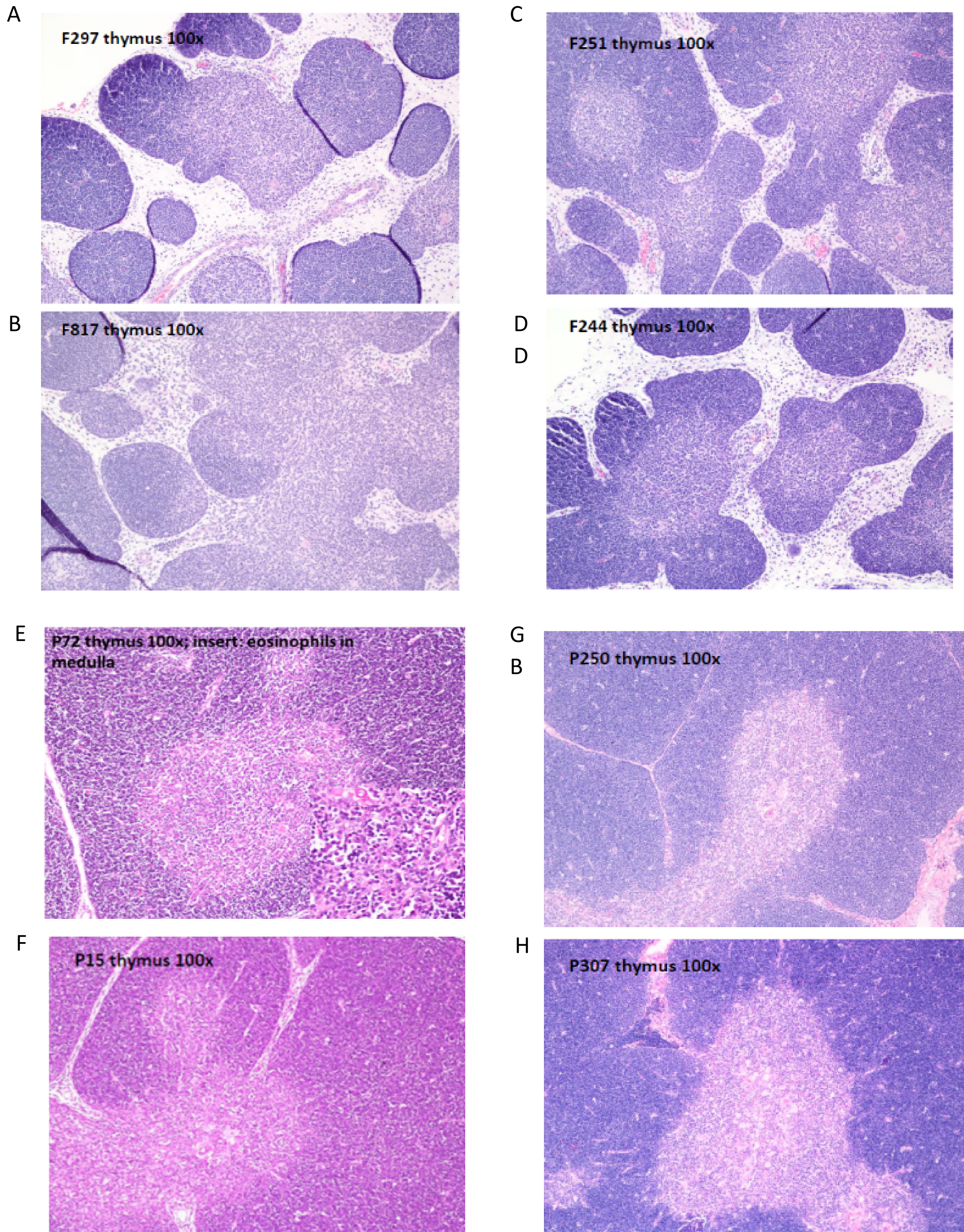


Figure 17. H&E Staining of fetal thymus comparing control and PI at various days of gestation. (A) Control and (B) PI day 89; (C) Control and (D) PI day 97; (E) Control and (F) PI day 190 (G) Control and (H) PI day 245.

eosinophilic cytoplasm, either homogenous or with finely fibrillar patterning. The almost euchromatic nucleus is round and centrally located or more oval and acentric. There may be a small amount of chromatin clumping along the nuclear membrane and occasionally a small nucleolus is present. These cells may in some cases be considered “single cell” Hassall’s corpuscles. There are no discernible differences between the control, TI, or PI fetuses with regard to the thymus morphology at day 190. Four control and 3 infected fetal thymi were evaluated from day 245 of gestation. The thymus appears as is normal for neonates. There are no discernible differences between the control and PI fetuses, except that one infected thymus may have more myoid cells, but morphometry would be required to ascertain or refute this impression.

DISCUSSION

BVDV is a remarkable virus because of its ability to evade the immune system and continuously spread through herds by only a single persistently infected animal. In this study, we have shown that BVDV affects the innate and adaptive immune systems in the fetal thymus after transplacental infection. The thymus is a target organ for BVDV and it is also a primary lymphoid organ which serves as the primary site of T cell maturation. It is mostly associated with the adaptive immune response, but it also has some functionality related to innate immunity. Innate immunity is a non-specific defense mechanism and is initiated soon after antigens enter the body. The innate immune response is the first line of defense to fend off foreign antigens, while the adaptive immune response focuses on the antigen processing and presentation, mediation of viral replication, production of antibodies, and co-stimulatory signals. It has been shown that after infection of BVDV, maternal blood viremia is first detected at day 82 and cleared by day 89, which is 2 weeks after initial inoculation however, viremia in PI fetal blood is first detected at day 89, peaks at 97, and then significantly decreases by day 192 and 245 of gestation, but is not cleared (Figure 1). This observation is of interest because this pattern of viremia indicates a functional adult immune response, but a compromised fetal immune response.

Infection with BVDV induces innate immune responses in the fetal thymus of TI, but not PI fetuses

A functional innate immune response in blood and spleen has previously been described, but as to why it is not able to resolve persistent BVDV infections is unclear (Hansen et al., 2010, Hansen et al., 2015). However, this does not explain the attenuation in viral titer. For this reason, we suspected that the innate immune response might be mostly intact. To test this idea, five early response genes to viral infection (*IFNβ*, *NF-kB, RIG-I*, *IRF7*, and *ISG15*) were analyzed in fetal thymus using real-time PCR within the innate immune system at days 89, 97, 190, and 245 of gestation. All genes evaluated had an increased gene expression (mRNA) in TI fetal thymuses at day 190 of gestation. The IRF7 protein concentration was also increased in the TI fetuses at day 190. TI fetuses have a competent immune system and a normal response to foreign antigens as expected. However, it is not yet known whether this can have long lasting detrimental effects postnatally. There was only one gene with increased gene expression in PI fetal thymuses, NF-kB, this gene only had increased mRNA at day 97 of gestation, which coincides with an *in vitro* study in MDBK cells in which BVDV increases translocation of NF-kB to the nucleus which leads to pro-inflammatory responses that regulate the host-virus interaction (Zahoor et al., 2010, Fredericksen et al., 2015). However, we saw decreased expression of NF-kB in the PI fetal thymus at day 190 which significantly alters the normal host-virus interaction. The initial increase of NF-kB at day 97 of gestation may lead to decreased receptor sensitivity and attenuation of signaling cascades through the accumulation of dimers which could explain the subsequent decrease in gene expression at day 190 and 245 of gestation.

IFNβ is a type I IFN that activates other molecules to prevent the virus from replicating, creating an "anti-viral" state within the cell. *IFNβ* gene expression was diminished in the PI thymus at day 190. In a previous *in vivo* study, naïve heifers were inoculated with ncpBVDV type 2. *IFNβ* mRNA was measured at various days of gestation in fetal blood, caruncles, and cotyledons. The only difference

seen with this gene was an increase in PIs in the cotyledon at day 192 of gestation (Smirnova et al., 2012). However, another study exposed adult PBMCs to BVDV type 1 and *IFNβ* was found to be diminished compared to control samples (Weng et al., 2015). These two studies may show conflicting results because they were exposed to different types of BVDV and studies were done either *in vivo* or *in vitro*. However, IFNα/β secretion in the thymus is induced by dendritic cells and the decreased *IFNβ* in the thymus may be because of a shift of the various cell types within the thymus, or a decrease in transcription within cell types, but immunohistochemistry will be needed to test this theory (Colantonio et al., 2011). NF-kB may be up at the initial exposure to the virus, but since there is no increase in IFNβ, the ability for NF-kB to induce IFNβ may be inhibited.

RIG-I, *IRF7*, and *ISG15* were not statistically different in control compared to PI fetal thymuses at any day of gestation. RIG-I is a cytosolic pattern recognition receptor that is essential for antiviral immunity in the innate immune response. Various viruses (dengue virus, procaine reproductive and respiratory syndrome virus, the hepatitis C virus, and porcine epidemic diarrhea virus) are able to mask their PAMP signatures preventing RLR activation (Zinzula et al., 2013, Schulz et al., 2016). *IRF7* is expressed in mature thymocytes and in a tissue that is still developing, its expression may be low due to the immaturity of the thymus and thymocytes which may explain why there is no change in gene expression or protein concentration (Colantonio et al., 2011). *ISG15* is known to be secreted from various immune and nonimmune cells and is induced by IFNs, viral infection, and bacterial endotoxins thus with no increase in IFNs, *ISG15* is not expected to change (Osiak et al., 2005). These data analyses are summarized in Figure 18.

IFN induced genes in fetal thymus

Five IFN-γ inducible genes (*STAT1*, *IFI16*, *CXCL10*, *CXCL16*, and *CXCR6*) were analyzed using RT-qPCR in the fetal thymus at days 89, 97, 190, and 245 of gestation. Type I IFNs are transcribed during

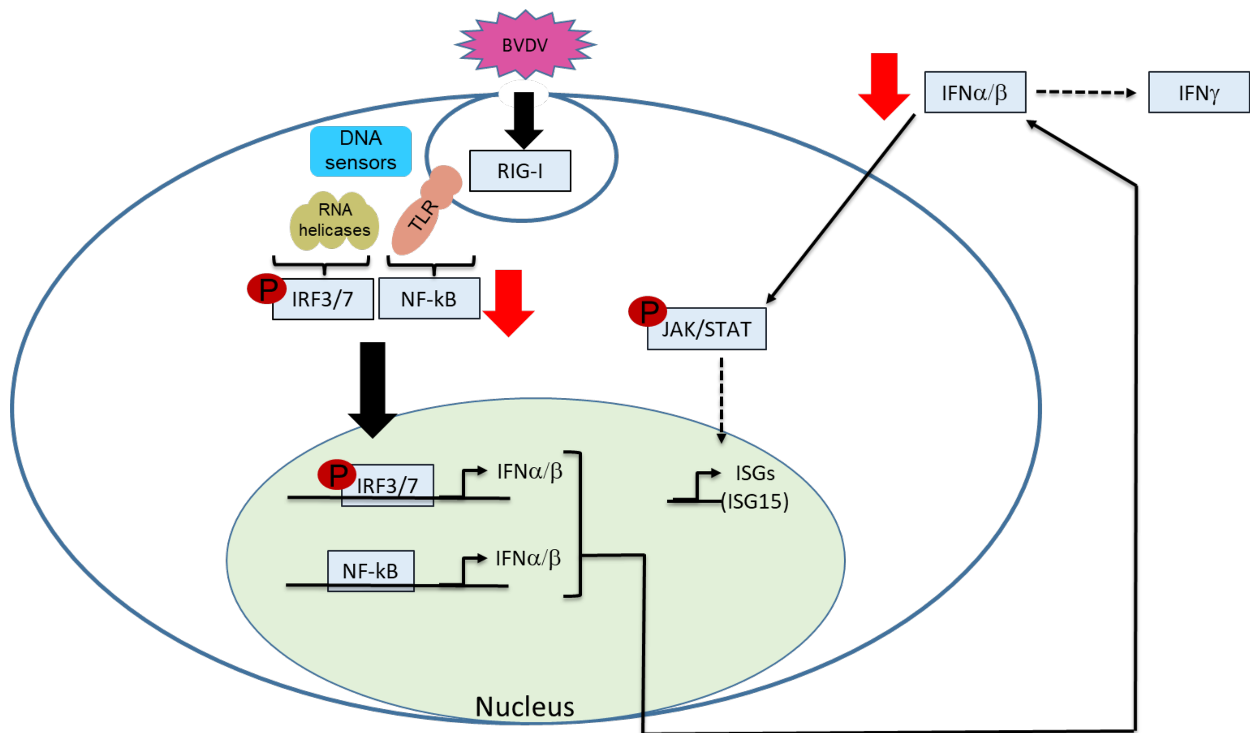


Figure 18. Attenuated innate response in PI fetal thymus at day 190 of gestation after maternal infection. The red arrows indicate genes that were decreased at day 190 of gestation in the PI fetal thymus.

the innate immune response and production of *IFN-γ* is the link between the innate and adaptive immune systems. The innate immune response eventually leads to activation and production of *IFN-γ*, this in turn then leads to activation of the adaptive immune response. *STAT1* is phosphorylated and translocates to the nucleus where it binds to gamma-activated sequence (GAS) to stimulate transcription and induce IFN signal transduction pathways (Decker et al., 1997). In the fetal thymus, there was no change in *STAT1* gene expression in any treatment group at any day. However, *STAT1* in BVDV fetal spleen and blood was previously shown to be increased during gestation at day 97 in PIs, followed with a decrease at day 192 (Smirnova et al., 2014). These data and another experiment have shown a pronounced IFN response to the virus in blood and the spleen in acutely infected animals and to a lesser degree PI animals compared to controls (Shoemaker et al., 2009). Fetal liver and spleen have also shown increases in *IFN-γ*, which may serve as possible sources for secreted *IFN-γ* (Smirnova et al., 2014). Other Flaviviruses such as dengue, yellow fever, and West Nile virus have been reported to inhibit the JAK/STAT signaling pathway by decreasing STAT1 phosphorylation using IFN antagonists (Randall et al., 2008, Cumberworth et al., 2017). It has previously been shown that IFN antagonistic proteins have also contributed to "immunotolerance" of BVDV (Peterhans et al., 2010). *IFI16* is an IFN-γ inducible gene that is able to detect foreign DNA and RNA virus in the cytoplasm and nucleus triggering antiviral cytokines to DNA and RNA viruses (Thompson et al., 2014, Diner et al., 2015). IFI16 along with apoptosis-associated speck-like protein (ASC) and caspase-1 form a protein complex called an inflammasome that is able to detect pathogenic microorganisms and activate various proinflammatory cytokines (Latz et al., 2013, Diner et al., 2015).

Three different IFN-γ inducible chemokines were analyzed. *CXCL10* is able to stimulate monocyte, NK, and T cell migration as well as having a role in the antiviral response to viruses which includes *Flavivirus*es (Brownell et al., 2013, Chen et al., 2013). CXCL16 is expressed on the surface of

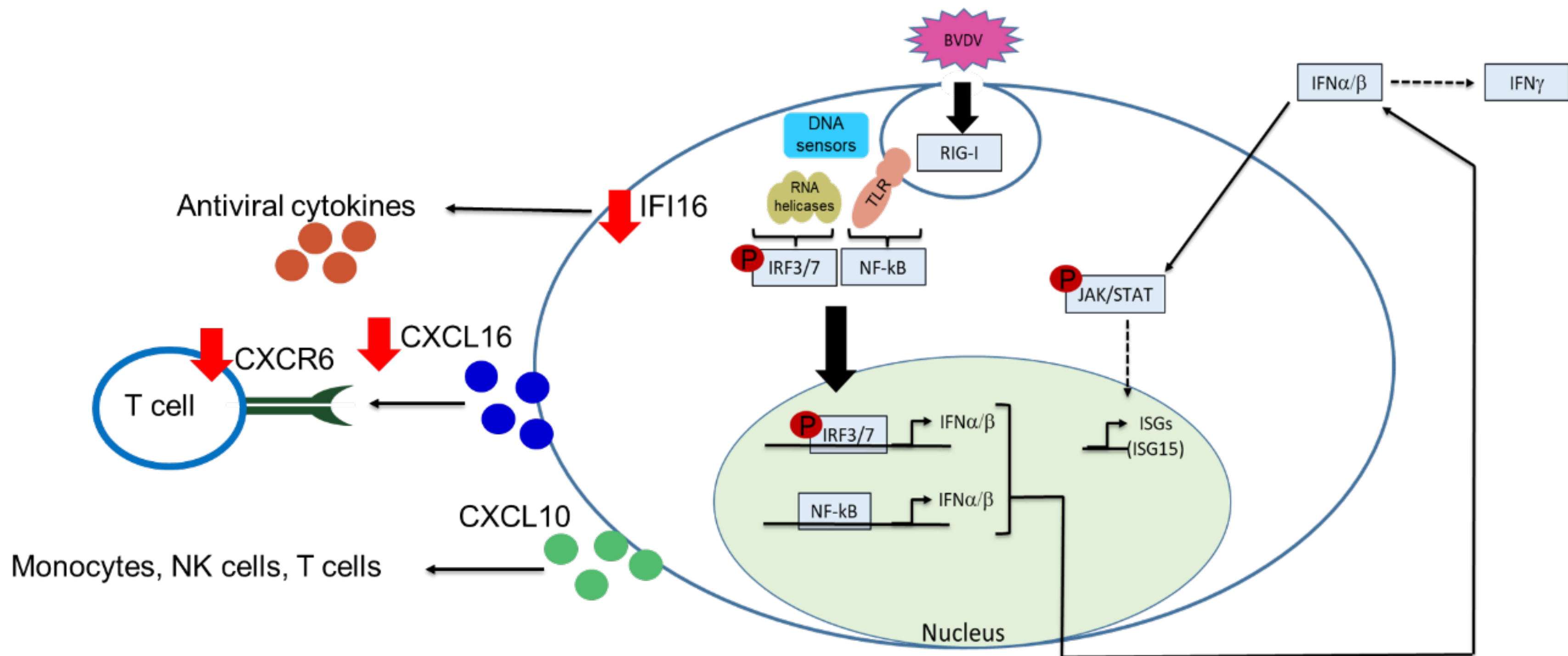


Figure 19. IFN induced chemokines suggests a decrease in T cell migration to the thymus in fetal thymuses after maternal infection at day 190 of gestation. The red arrows indicates genes that were decreased in day 190 of gestation in the PI fetal thymus.

APCS and functions as a chemoattractant for T cells in addition to being an adhesion molecule with CXCR6 being its specific receptor (Abel et al., 2004, Hara et al., 2006).

IFN-γ inducible genes *IFI16*, *CXCL16*, and *CXCR6* were significantly decreased in thymuses of PI fetuses at day 190 of gestation (Figure 19). These data suggest that the adaptive immune response of the thymus is not functional, which contributes to the unleashed replication of the virus in host cells. IFN-γ inducible genes are suppressed in day 190 which may contribute to the virus's ability to evade the host antiviral mechanisms and result in "immunotolerance" to the persisting virus. These data coincide with previous studies showing that these genes were decreased in the fetal spleen at day 190 of gestation. This may indicate a persistence of the *BVDV* mRNA in the blood (Figure 1).

Decreased expression of genes along the MHC class I Pathway in response to fetal persistent infection in the thymus

The MHC class I pathway is an important aspect of the adaptive immune response, especially during pregnancy. IFN-γ and TNF-α play roles in upregulating MHC class I antigen processing and presentation along with TNFα. IFN-γ is important for defense against intracellular pathogens. IFN-γ is produced through IRF or NF-kB pathways in the innate immune system, with the majority of its production being through CD4+ and CD8+ T cell subsets (Ye et al., 1995). Six genes (*IFN-γ*, *LMP2*, *TAP1*, *β2M*, *CD8a*, and *CD8b*) associated with the adaptive immune response were analyzed using real-time PCR within the MHC class II pathway at days 89, 97, 190, and 245 of gestation. There was no change in expression of these genes between any of the treatment groups at any day of gestation. However, this contradicts another study in which steers that had the mucosal disease, a mutation of ncpBVDV and concurrent infection with cpBVDV, of BVDV reported increased IFN-γ in the blood (Charleston et al., 2002). This difference may be due to the differences between blood and thymus or the increased severity of mucosal disease, which is caused by a mutation of ncpBVDV. When facing mucosal disease

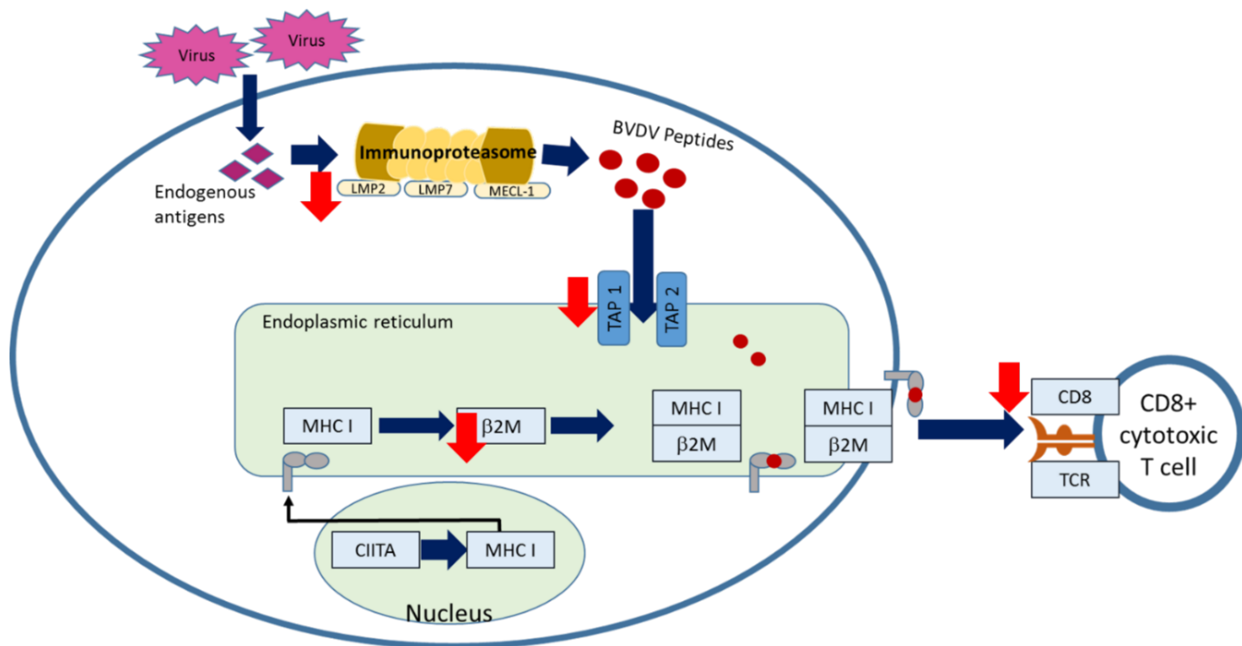


Figure 20. Decreased antigen presentation and cell mediated response at day 190 of gestation in PI fetal thymuses in the MHC I pathway. The red arrows indicates genes that were decreased in day 190 of gestation in the PI fetal thymus.

the immune system may be pushed into survival mode. *LMP2*, *TAP1*, and *β2M* gene expression was increased in TI fetal thymuses at day 190 of gestation compared to control and PI thymuses. The *CD8* and *CD8b* gene expression in TI fetuses were significantly higher in PI, but not compared to the controls at day 190 of gestation.

The immunoproteasome is made up of LMP2, 7, and *10* (*MECL-1*) and as a whole it allows for BVDV endogenous antigens to be broken down into smaller peptides through proteolysis. In this study *LMP2* mRNA gene expression in PI thymuses was increased at day 97, but then decreased at both day 190 and 245 of gestation. This suggests that a normal immune response occurs early in the progression and detection of viral infection, but later, normal function is inhibited by the virus's ability to manipulate host gene regulation pathways (Harwig et al., 2017). This same increase of *LMP2* mRNA at day 97 of gestation has also been reported in splenic tissue (Hansen et al., 2015). Since *IFN-γ* is not increased, the initial *LMP2* mRNA increase may be due to *TNFα*, but *TNFα* was not analyzed here.

After antigenic peptide is broken down by the immunoproteasome, TAP (1 or 2) transports these peptides into the endoplasmic reticulum (ER) where they can bind to MHC class I molecules prior to being presented on the cell membrane. *TAP1* mRNA gene expression was increased in PI thymuses at day 97 and then dropped at day 190 and had a decreasing mRNA trend at day 245 of gestation. This same increase of *TAP1* mRNA at day 97 of gestation has also been reported in splenic tissue (Hansen et al., 2015). These changes may indicate that while BVDV antigen can be transported normally, at first, it like the other aspects of the MHC class I pathway change in response to the virus intracellularly.

Further downstream in the MHC class I pathway β2M protein assembles with the MHC I molecule in the ER to form a stable MHC I complex. *β2M* mRNA in PI thymuses was decreased at day 190 and 245 of gestation. In a previous study, *β2M* in PI fetal spleens at day 97 was found to be increased (Hansen et al., 2015). When β2M is not present, MHC remains in the ER and the MHC I

complex is not expressed on the cell surface (Benedictus et al., 2015). The thymus plays a major role in the T cell development, thus the adaptive immune response may be lagging behind accounting for the discrepancies between the spleen and thymus data. These data in this section are summarized in Figure 20.

After the MHC I complex is presented on the surface of antigen presenting cells, the T cell co-receptor, CD8, on cytotoxic T cells binds and the cytotoxic cell becomes an effector cell that can target cells infected with the same pathogen (Alberts et al., 2002a). Cytotoxic T cells can release perforin, a protein that creates channels in the plasma membrane which causes infected cells to undergo apoptosis. *CD8a* and *CD8b* mRNA in PI thymuses were decreased at day 190 and 245 of gestation. However, BVDV can induce the CD8+ T cell subpopulation in BVDV infected cattle, four to twenty-four months old, using flow cytometry (Rhodes et al., 1999). CD8+ T cell induction was seen in naïve calves in which the adaptive immune system is fully developed and not in the development stages as was evaluated in this study.

Downregulation of genes along the MHC class II pathway in response to fetal persistent infection in the thymus

The MHC class II pathway is another important aspect of the adaptive immune response. CD4+ T cells recognize processed antigenic peptides that are on the surface of antigen presenting cells (APCs) using this pathway; it uses endosomes or lysosomes to process the antigenic proteins and make them into small peptides. In the case of BVDV, when CD4+ T cells decreased, there was an increased period of virus shedding (Chase et al., 2004).

Three genes (*CIITA*, *GILT*, and *CD4*) were analyzed using RT-qPCR within the MHC class II pathway at days 89, 97, 190, and 245 of gestation. *CIITA* is an IFN-γ stimulated gene, but it also serves a role as the major histocompatibility class II transactivator that allows for activation of MHC class II

expression (Xu et al., 2004, Shi et al., 2018). *CIITA* and *CD4* mRNA in the TI thymuses were not different compared to control thymuses. However, these genes were decreased in the PI thymuses compared to control and TI fetal thymuses. In addition to this, there was an increase of *CIITA* mRNA in PI fetal thymuses at day 97 followed by a decrease at days 190 and 245 of gestation. Human cytomegalovirus (HCMV) and Epstein-Barr viruses can cause life-long infection in normal hosts and are able to block expression of MHC class II by inhibiting transcription of *CIITA* (Lee et al., 2011). With HCMV infection, like shown with BVDV, after infection a transient increase of CIITA is initially observed, followed by reduction in gene expression at a later time point (Lee et al., 2011). These changes that occur keep MHC class II molecules within cells, decreasing their normal presence on the surface membrane, subsequently reducing APCs ability to present antigens to CD4+ T cells which may account for the changes in the various gene transcripts measured in this study.

GILT (IFI30) is also induced by interferon gamma and is expressed constitutively in antigen presenting cells. One of its main functions is proteolysis in endosomes to make peptides available for antigen processing (Phan et al., 2000). The only difference with gene expression of *GILT* was an increased in the PI thymuses at day 97 of gestation. *GILT* expression has been previously shown to be increased after bacterial infection or under inflammatory conditions (Maric et al., 2001, Lackman et al., 2006). Along with IFN-γ, *GILT* is also induced by *NF-kB, IL-1b,* and *TNF* (Lackman and Cresswell 2006, Hastings et al., 2011). In these studies, *GILT* mRNA only increased when *NF-kB* increased, but there was no decreased of *GILT* when *NF-kB* was decreased, likely because *GILT* is a constitutively expressed by APCs and can be induced by other signals.

CD4 is a co-receptor expressed on helper T cells and is needed to bind to class II MHC proteins. Helper T cells stimulate macrophages to destroy intracellular microorganisms and help B cells and cytotoxic T cells respond to antigens (Alberts et al., 2002a). *CD4* mRNA was decreased in PI fetal

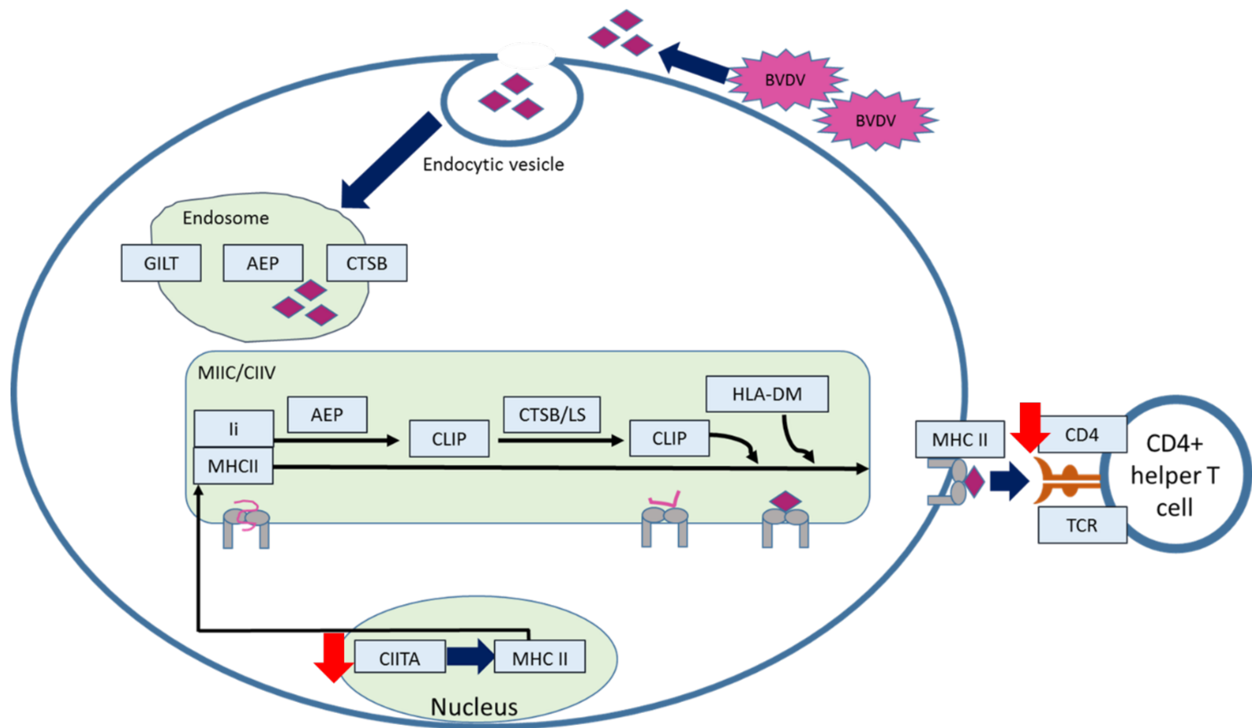


Figure 21. Decreased antigen presentation and cell mediated response at day 190 of gestation in PI fetal thymuses in the MHC II pathway. The red arrows indicates genes that were decreased in day 190 of gestation in the PI fetal thymus.

thymuses at day 190 and 245 of gestation. There was no change with CD4 protein, which may indicate translational disruption. Human immunodeficiency virus type 1 (HIV-1) is an RNA virus that is able to inhibit translation and this same mechanism may also be true for BVDV however, more studies would need to be done to demonstrate it (Bour et al., 1997). CD4+ T cells were also decreased in HCMV and Epstein-Barr infections which may be due to their inability of APCs to present antigen on their cell surface, which has also been seen with BVDV. These data can all be summarized in Figure 21.

CD79b and CD46 in the thymus after BVDV maternal infection using RT-qPCR

Two additional genes (*CD79b* and *CD46*) were analyzed using real-time PCR in fetal thymus at days 89, 97, 190, and 245 of gestation. C*D79b*, a B cell receptor gene, in cooperation with *CD79a,* is an important activator of B cells through the interaction with CD4+ T cells. After this interaction, B cells then have the capability to proliferate, secrete antibodies, cytokines, and chemokines, and initiate memory cell formation (Bishop et al., 2003). Differences with CD79b gene expression are only seen at day 190 of gestation with PI thymuses being considerably lower than both the control and TI data. B cells are dependent on T cell activation and CD4 expression was shown to be decreased on the same day, these results are expected (Parker 1993).

CD46 is the receptor that BVDV uses to enter the cell (Maurer et al., 2004, Krey et al., 2006). There are no differences in *CD46* in any treatment group at any day of gestation. While CD46 serves as the receptor for BVDV, its numbers are not changed by its presence and thus does not affect entry of BVDV into cells (Krey et al., 2006).

Histology of control, TI, and PI fetal thymuses at day 89, 97, 190, and 245 of gestation

The rudimentary thymus is first detected around day 27 of gestation in ruminants. Colonization and lymphoid development occurs around day 42 of gestation. The day 42 progenitor cells comes from the fetal liver, until the bone marrow develops and matures enough and around day 70 and is able to

take over as the primary source of thymocytes (Goddeeris and Morrison 1994). During thymocyte development cells can express CD4 or CD8 molecules on their surface. The CD8 molecules expressed consist of CD8a and CD8b heterodimers. However, extrathymic lymphocytes only express CD8a homodimers (Kioussis et al., 2002). The CD8+ T cells are able to recognize cytosolic processed antigenic peptides from the MHC class I pathway.

There were no gross differences between control, TI, and PI fetal thymuses during *in utero* development at any day of gestation with H&E staining, but since the ncpBVDV was used in these studies, no lysis was expected. However, differences in T cells are expected, but H&E staining does not differentiate T cells. IRF7 and STAT1 are essential for the development of medullary thymic epithelial cells (mTECs) and maintenance of thymic architecture and because we see no decrease in any of these genes, it ties in with the fact that there is no gross histology changes seen with H&E (Otero et al., 2013). Extensive immunohistochemistry testing will need to be performed in order to evaluate changes in specific cell populations after maternal infection with BVDV. In a previous study that analysed the spleen, there were no histopathological changes in the PI infected fetuses except for an increase in the number of megakaryocytes an early development of some periarteriolar lymphoid sheaths and as we saw in the thymus, the spleen also had normal development in TI infected fetuses (Shoemaker et al., 2009). BVDV antigen and ISG15 were both detected in macrophage-like cells in the spleen thus would be a proper follow-up experiment in the fetal thymus tissues (Shoemaker et al., 2009). These same experiments also detected BVDV in Kupffer cells of the fetal liver as well as an increase in antigen presenting cells (Morarie-Kane et al., 2018).

Thus far, experiments measuring BVDV have been performed in the spleen, liver and now the thymus. The spleen and liver have indicated that an activated immune response occurs in these lymphoid tissues, however, in the thymus the innate and adaptive immune response appears to be

attenuated. This is interesting because it may suggest that BVDV affects primary lymphoid organs first, which gives additional information to the mechanism of BVDV tolerance in PI fetuses.

CHAPTER 3

OVERALL SUMMARY AND FUTURE DIRECTIONS

These studies investigated numerous genes in the innate and adaptive immune systems. The experimental model used for the scope of this work allowed for analysis of acute and chronic stages of PI. The major finding that emerged from the examination of the innate immune system of the fetal thymus in PIs was that there was no increase in any gene tested of the innate immune response, with the exception of NF-kB in PI fetal thymus. This may be an indication that "immunotolerance" reflects a lack of development of the immune system or the virus evading the immune system. When the immune system is unable to immunologically recognize BVDV, this is referred to as "immunotolerance".

Historically it was always portrayed that BVDV creates "immunotolerance" however, genes of adaptive immune system were actually downregulated, which indicates that the virus may be causing dysfunction of the adaptive immune response in the fetal thymus. The decrease of genes in the adaptive immune system was an unexpected finding that could be directly caused by BVDV altering host cells. "Immunotolerance" may be exacerbated because the day 190-245 fetal thymus is still in its development stages.

Innate genes from the TI fetal thymus had a global increase in expression. At 190 days of gestation, the TI fetal thymuses had a normal functioning and developed immune system and thus the increase in gene expression was expected. This was also true for the IFN induced genes *IFI16* and *CXCL16*, as well as genes in the adaptive pathway including *LMP2*, *TAP1*, *β2M*, and *CIITA*. However, it is unclear if TI with BVDV during fetal development has detrimental postnatal epigenetics effects. A postnatal study would clarify if there are any long lasting effects.

This study compared protein and RNA of IRF7, ISG15, and CD4. The protein data of IRF7, CD4, and the free form of ISG15 did not reflect the mRNA data. It was very likely that any free ISG15 protein

was being consumed through formation of conjugated ISG15. The ISG15 conjugated form protein mirrored that of RNA, in which conjugated ISG15 was significantly upregulated with BVDV TI. Up to this point, there is not enough data to explain why IRF7 and CD4 western blot analyses were different from RT-PCR data. There are many viruses that inhibit translation of the host cell, which is known as "shutoff" (Toribio et al., 2010, Li et al., 2015). In response to viral infection, ISGs are supposed to block viral replication, but as shown, ISG15 protein is not increased in PIs, like it is in TIs, which may be contributing to host translational shutoff, but further studies are necessary to determine if this strategy applies to BVDV. Also, there may be discrete cell-specific changes in protein that can be discernable through sensitive immunohistochemistry approaches and that won't be detected by using western blot. For example, in a previous study it was demonstrated that ISG15 protein did not change in fetal PI spleens based on western blot, but when tissue sections were examined by immunohistochemistry, there was very specific upregulation of ISG15 protein in macrophage-like cells from PI compared to control spleens (Shoemaker et al., 2009). Western blot analyses of CD8, CD79b, and LMP2 protein was considered for the present studies however, either the antibodies of interested were not available in bovine or the antibody of interest in a different species had a low protein sequence homology to bovine. For these reasons, analyses of the protein to the RNA tested was limited. Thymus protein and RNA analyses was then followed up with histological interpretation of the tissue.

BVDV has a tropism for thymus tissue and has been reported to cause thymic atrophy which include a reduction of the cortex:medulla ratio. The studies that showed atrophy in the thymus used 3 to 5 week old calves to evaluate the thymus after the acute or persistent infection (Romero-Palomo et al., 2015, Falkenberg et al., 2017). However, thymus tissues collected in this study were all done *in utero* which may help pinpoint the time of thymic change. As discussed chapter 2, histopathology was evaluated at day 89, 97, 190, and 245 of gestation and at each day there were no developmental differences seen between control, TI, or PI thymuses. Normal development of the lymphoid follicles and

the cortex:medulla ratio was as it should be for each day of gestation. The normal development of the thymus, may indicate that the thymic atrophy does not begin until after birth. In addition to the infection, environmental stressors such as prolonged physical or emotional stress, can lead to thymic atrophy which is another reason for postnatal studies of BVDV to be performed (Gruver et al., 2008). Various stressors cause an increase production of cortisol, a stress hormone, which then leads to thymus involution as well as decreased thymopoiesis.

There has been some question as to whether epigenetics mechanisms are altered by various pathogens, including BVDV. DNA methylation is an important epigenetic mechanism that has roles in silencing gene transcription, protein expression, and regulating miRNAs. A previous *in vitro* study showed decreased methylation levels of various promoters in MDBK cells after they were infected with BVDV (Fu et al., 2017). One of these promoters was DNMT1, a DNA methyltransferase that helps maintain methylation, and by silencing it the BVDV NADL strain was able to be suppressed (Fu et al., 2017). Additional *in vivo* experiments with BVDV would need to be performed in the fetal thymus in order to ascertain if epigenetic changes seen *in vitro* occur *in vivo*. These epigenetic changes may be the culprit behind the long term immunosuppression that is associated with this disease.

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