Role of Intrauterine Bovine Viral Diarrhea Virus in Modulating Host Gene Expression at the Maternal-Fetal Interface

by

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Abstract

Bovine viral diarrhea virus (BVDV) is a member of the genus Pestivirus within the family Flaviviridae. Infections with BVDV have a substantial economic impact on beef and dairy producers in the United States and worldwide. This virus affects multiple organs including the respiratory, reproductive, and gastrointestinal systems, and immunosuppression is the primary underlying cause of clinical disease. Viral maintenance in cattle herds is due in large part to the capability of BVDV to cross the placenta and infect the fetus. Reproductive consequences of BVDV infection include stillbirths, abortions, calves with congenital malformations, or calves born persistently infected (PI) with the virus (Baker 1995). Once established on a farm, the virus can remain undetected in cattle herds because persistently infected animals continuously shed large amounts of virus and animals may show no clinical signs. These undetected persistently infected animals provide a continual source of the virus to the herd and maintain this cycle of BVDV exposure to serologically naïve heifers and cows. BVDV control programs have been established to increase protective serologic titers and to reduce the incidence of PI fetuses. Despite attempts to improve maternal immunity through vaccination programs, vaccines do not provide 100% fetal protection. To further understand why vaccination provides high levels of humoral immunity but does not adequately protect the fetus, we examined T cell associated responses during transplacental BVDV infections. We hypothesized efficient transplacental transmission of the virus is associated with a predominant Th-2 microenvironment of the

pregnant uterus. We identified evidence of immune recognition of the virus in placentomes with consequences of dysregulation of normal local immunoregulatory processes at the maternal-fetal interface. The primary objectives were to examine the gene expression profile of T cell-associated cytokines, including some T regulatory cell associated molecules in the normal bovine placenta, cytokeratin enriched primary placental cells, and peripheral blood mononuclear cells collected during normal, early to mid-pregnancy. The goal for this research was to determine if immune recognition by the local maternal cellular immune response occurs following BVDV infection and to characterize this response. We hypothesized the virus is more likely to avoid elimination by the host T cell response in the uterus than the systemic circulation due to the presence of negative regulators or inhibitory factors necessary for maintenance of pregnancy following implantation to gestational day 150.

The overall goals detailed in this dissertation were to establish normal immune regulatory parameters in bovine placentomes and the peripheral circulation during early to mid-gestation. Some of the experiments examined the role of bovine viral diarrhea virus on these immune parameters at the placental level. These investigations were performed with placental tissue and leukocytes obtained from peripheral blood samples collected from pregnant and non-pregnant heifers between 89-150 days of gestation, permitting three investigations. The first study was an *in vivo* experimental trial in which placental samples representing the maternal-fetal interface were analyzed to evaluate immune regulatory molecules potentially exerting an immunosuppressive function during mid-gestation. The literature provides minimal background regarding the anticipated cytokine and chemokine expression patterns during mid-gestation bovine pregnancy. This trial was carried out by experimental inoculation of pregnant heifers with

BVDV. This virus is capable of infecting fetuses by vertical transmission, and causing multiple adverse reproductive outcomes. Our hypothesis was that BVDV downregulates immune responses in the pregnant bovine uterus from gestational ages 75-150 to allow for persistence of the virus and escape from the maternal immune response. The second investigation centered on primary cell cultures propagated from maternal epithelial cells obtained from 75-day-gestation placentas from serologically negative heifers, and provided identification of immune regulatory genes in these primary cells. The hypothesis for the *in vitro* experimental study was that trophoblasts provide a substantial role in intercellular communication at the maternal-fetal interface, primarily functioning as an immunologic sensor involved with immune recognition and trafficking. Bovine trophoblasts were found to be susceptible to BVDV and appeared immunologically responsive to BVDV infection. For the third study, peripheral blood leukocytes from non-pregnant and pregnant heifers were treated as samples from control and treated groups, respectively, to evaluate expression patterns of potential immune regulatory molecules during normal bovine pregnancy during the 89 to 150-day period. The sampling time point was within the gestational period determined to be optimal for vertical transmission of BVDV and establishment of persistent fetal infection. Immunological signaling mechanisms in the bovine placenta during this phase of gestation are largely unknown. Our goal was to develop increased baseline knowledge about immune signaling in cattle at this specialized interface between the mother and fetus. This work provides a detailed comparison of immune regulatory networks between humans and cattle and expands on how infectious agents such as BVDV alter the normal mechanisms of immune homeostasis, disrupting the symbiotic relationship between the mother and fetus during pregnancy.

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List of Abbreviations

BVDV	Bovine viral diarrhea virus
$CCID_{50}$	Cell culture infectious dose 50%
GM	Geometric Mean
IHC	Immunohistochemistry
MDBK	Madin-Darby Bovine Kidney
QRT-PCR	Quantitative reverse transcription polymerase chain reaction
VI	Virus Isolation
VN	Virus Neutralization
Treg	T regulatory cell

Chapter I: Literature Review

History of Bovine Viral Diarrhea Virus

The first description of *Bovine viral diarrhea virus* (BVDV) in the United States was reported by Cornell University researchers Olafson, MacCallum and Fox (1946) during the spring and summer of 1946 following an outbreak in New York involving 6 dairy herds (Olafson, Mac et al. 1946). This contagious diarrheal disease of unknown origin was associated with acute clinical signs including pyrexia, diarrhea often containing blood, gastrointestinal and cutaneous erosions, ulcerations, and hemorrhages. Younger animals usually died between 7-10 days, while mortality was more rapid in adult animals, typically between 3-4 days after onset. Reduced milk production, abortions, leukopenia, and pneumonia were also described. Olafson, MacCallum, and Fox attempted to reproduce this new disease by inoculating a healthy cow with fecal samples from a clinically ill animal. The cow inoculated with fecal samples from severely affected animals did not become ill, but other cattle in the herd were ill 3 weeks later. A second attempt by Cornell University researchers to reproduce the disease was successful when healthy animals were inoculated subcutaneously with blood and a splenic emulsion, resulting in clinical signs in some animals. Mild to severe clinical signs were observed, but only the animals with clinical signs developed leukopenia. Clinical signs in leukopenic animals progressed to severe diarrhea, dehydration, and severe weight loss. Mucosal ulcerations were seen in these animals approximately 1 to 3 days after the onset of diarrhea. Peter Olafson described this condition as virus diarrhea (VD) of cattle (Olafson, Mac et al. 1946). The VD was a major concern because

lesions were similar to the foreign animal disease rinderpest; however, morbidity and mortality rates were different for these two viruses. VD had a morbidity rate of 33-88% and a 4-8% mortality rate and it was suspected rinderpest would have had a much higher mortality rate in the United States due to the susceptibility of U.S. cattle naive to the virus (Olafson, Mac et al. 1946). Rinderpest was discounted as the cause of the New York outbreak, partially based on the lack of serum neutralizing antibodies to rinderpest in animals recovering from diarrhea, and cattle that recovered from VD were susceptible to rinderpest (Walker and Olafson 1947). In 1996, Childs reported a similar diarrheal disease in cattle in Saskatchewan Canada associated with more severe clinical disease and high mortality characterized by severe diarrhea, depression, anorexia, and ulceration of oral mucosa. The clinical signs and postmortem findings for New York and Canadian diarrheal diseases were identical, but the cause was unknown and Childs could not experimentally reproduce or transmit what he called "X disease" (Childs 1946). However, the term "X-disease" lost favor and Olafson restricted use of "X disease" to toxic exposure of cattle to chlorinated naphthalenes (Goens 2002). In 1953, mucosal disease (MD) was named by Ramsey and Chivers (Ramsey and Chivers 1957). This highly fatal condition was believed to be distinct from VD because it affected fewer cattle, could not be transmitted between cattle, and inoculated cattle only showed clinical signs of fever. The importance of Child's discovery in Canada was not understood until 1963 when Pritchard reviewed early cases of BVDV and then summarized Child's description of "X-disease" as the first description of mucosal disease in cattle (Pritchard 1963). Pritchard classified BVDV into four categories: BVD-mucosal disease (BVDV-MD), chronic BVD (now recognized as a form of BVD-MD), mild acute BVD and severe acute BVD. The preliminary findings identifying viral characteristics in cell culture were

reported in 1957, where *in vitro* cytopathic effects were not observed in viral-infected cell cultures (Lee and Gillespie 1957), providing the first evidence of the noncytopathic (ncp) biotypes of BVDV (isolate NY-1), while cytopathic (cp) biotypes were identified in 1960 (Underdahl, Grace et al. 1957). Identification of a cp biotype opened a new realm of diagnostics based on virus neutralization and plaque assays and the ability to compare the antigenic relatedness of the viruses (Goyal and Ridpath 2005). Virus neutralization assays examining North American and European cases showed viral agents in these cases were the same and mucosal disease was a different manifestation of the virus. Bovine viral diarrhea-mucosal disease was named in 1968 (Casaro, Kendrick et al. 1971).

The virus disease of cattle, now known as BVDV has developed into one of the most economically impactful infectious diseases affecting cattle worldwide. A 2.5-billion-dollar economic impact has recently been reported. BVDV was added to the list of reportable diseases for cattle by the Office of International Epizootics in 2007 (Walz, Grooms et al. 2010). Bovine viral diarrhea virus causes infection, disease, or both in multiple organ systems in multiple animal species, including: cattle, sheep, goats, llamas, alpacas, pigs, white-tailed deer, and bison. While many species are susceptible to infection by BVDV, cattle appear to be the primary host. The two most economically important disease manifestations of BVDV are respiratory and reproductive tract infections. A role for BVDV in bovine respiratory disease (BRD) has been well documented. Respiratory tract infections can occur with BVDV alone or the virus can cause the animal to have increased susceptibility to bacterial infection. Bovine respiratory syncytial virus (BRSV), parainfluenza virus-3 (PI-3), and bovine infectious rhinotracheitis virus (IBR) are the viruses most frequently identified in mixed respiratory infections of cattle. One study that

evaluated cases of pneumonia identified BVDV more frequently than other respiratory viruses (Richer, Marois et al. 1988). BVDV can have a noteworthy comorbid association with *Mannheimia haemolytica Pasteurella multocida*, and *Histophilus somni* (Fulton, Ridpath et al. 2002). Speculation about synergism of BVDV and *Mycoplasma bovis* in bovine respiratory disease has been ongoing, but there is not conclusive evidence of enhancement of clinical disease in *Mycoplasma bovis* infections by BVDV (Prysliak, van der Merwe et al. 2011). Immunosuppressive effects of the virus can be synergistic with *Mannheimia haemolytica*, bovine herpesvirus type 1, and bovine respiratory syncytial virus resulting in more severe lesions, delayed serologic responses, greater dissemination of the virus, prolonged viral shedding, and more severe clinical disease (Bolin 2002). Calves naive to BVDV are at risk for secondary infections when exposed to PI calves and challenged with *Mannheimia haemolytica* by intratracheal inoculation 12 hours following exposure (Burciaga-Robles, Step et al. 2010). Reproductive consequences of BVDV are numerous, but the most critical method for maintenance and transmission of the virus to naïve cattle populations occurs with the generation of persistently infected (PI) offspring. The BVDV is associated with early testicular infections affecting bull semen quality and fertility (Coria and McClurkin 1978), (Barlow, Nettleton et al. 1986) (Meyling and Jensen 1988), (Revell, Chasey et al. 1988), (Whitmore and Archbald 1977), (Kirkland, Richards et al. 1991), chronic oophoritis and salpingitis (Grooms, Brock et al. 1998, Grooms, Brock et al. 1998), early embryonic death, delayed returns to estrus, abortion, persistent fetal infections, and congenital malformations including cerebellar hypoplasia (Kahrs, Scott et al. 1970, Kahrs, Scott et al. 1970), cataracts, retinal dysplasia, hydranencephaly (Badman, Mitchell et al. 1981), hydrocephalus, micrencephaly, porencephaly (Wohrmann, Hewicker-Trautwein et

al. 1992), hypomyelination (Binkhorst, Journee et al. 1983), hypotrichosis (Casaro, Kendrick et al. 1971), and a reduction in thymic size (Done, Terlecki et al. 1980). Calves congenitally infected with BVDV during the last trimester of gestation can have reduced neonatal performance and survivability (Munoz-Zanzi, Hietala et al. 2003).

Classification and Molecular Biology

Bovine virus diarrhea virus is a member of the genus Pestivirus within the family *Flaviviridae* (Collett, Anderson et al. 1988). The genus Pestivirus currently contains four recognized species of veterinary importance, BVDV genotypes 1 and 2 (BVDV-1 and BVDV-2), *Border disease virus*, and *Classical swine fever virus* (Darbyshire 1960) (Plant, Littlejohns et al. 1973). Data were used to group BVDV, border disease virus of sheep and goats, and classical swine fever virus (hog cholera virus) within the genus Pestivirus in 1978, and at that time pestiviruses became classified within the family *Togaviridae* (Porterfield, Casals et al. 1978). However, this genus would later be reclassified with the development of molecular biology techniques, and pestiviruses received official status as a member of the family *Flaviviridae*. This classification also was more suited to the transmission cycle for pestiviruses, which do not require an arthropod vector, unlike the alphaviruses (Eastern/Western/Venezuelan encephalitis) within the family *Togaviridae*. The family *Flaviviridae* comprises four genera, including Flavivirus, Pestivirus, Pegivirus, and Hepacivirus. The genus Flavivirus includes the prototypical flavivirus yellow fever virus, dengue virus, West Nile virus, Japanese encephalitis virus, louping ill virus, and others (Kuno, Chang et al. 1998). Finally, hepatitis C virus, discovered in 1989 (Choo, Kuo et al. 1989), is classified within the genus Hepacivirus. A few pestiviruses genetically dissimilar to BVDV have also been described and include: Giraffe virus, Pronghorn

virus, and HoBi-like virus. HoBi-like virus is classified as an atypical pestivirus, which has been occasionally referred to as BVDV-3 because there have been similar clinical findings with BVDV and HoBi-like viruses. However, this new classification has not been adopted because HoBi-like viruses are not closely related to BVDV-1 and BVDV-2 genetically. This dissimilar genetic background also causes some difficulty in identifying HoBi-like viruses with diagnostic techniques for BVDV-1 and BVDV-2 (Bauermann, Harmon et al. 2013). Contaminated fetal calf sera are believed to be the source for the recent discovery of the virus throughout several areas of the world (Stahl, Kampa et al. 2007). Natural infections with Hobi-like viruses have been reported in Italy (Decaro, Mari et al. 2012), Brazil (Weber, Mosena et al. 2016) and Thailand (Haider, Rahman et al. 2014), but not North America. Experimental inoculations overall have shown mild clinical disease in cattle, and mild increases in body temperature, nasal discharge, conjunctivitis, and leukopenia were primary findings (Decaro, Mari et al. 2012), (Ridpath, Falkenberg et al. 2013), (Schirrmeier, Strebelow et al. 2004), (Larska, Polak et al. 2012).

Pestivirus morphology and stability. There are few descriptions of the morphology of BVDV, which largely appear to be due to inherent characteristics of the virus making purification and visualization challenging. Physical separation is difficult because of the similar density of the virion to subcellular components and mature virions are viewed as fragile (Bielefeldt Ohmann and Bloch 1982). Early reports described variably sized enveloped particles measuring 35-100 nm. Later reports showed BVDV is an enveloped virus measuring 40-60 nm diameter (Chu and Zee 1984), and the underlying nucleocapsid with icosahedral symmetry measured approximately 27-30 nm diameter (Chasey and Roeder 1981), (Bielefeldt Ohmann and Bloch 1982). Pestiviruses are susceptible to inactivation by organic solvents, detergents, and

trypsin treatment (Moennig, Houe et al. 2005), electron beam irradiation (Preuss, Kamstrup et al. 1997), and gamma irradiation (Miekka, Busby et al. 1998). Virions are stable within a pH range of 5.7 to 9.3 (Hafez and Liess 1972).

Pestiviruses have a positive sense single stranded RNA genome. The genome is approximately 12.5 kilobases in length (Mendez, Ruggli et al. 1998), (Deng and Brock 1992), (De Moerlooze, Renard et al. 1991). Most of the viral genome is encompassed by one open reading frame of about 4000 codons flanked by a 5' untranslated region and a 3' untranslated region (Tautz, Elbers et al. 1997). The approximately 385 nucleotide long 5' untranslated region lacks a 5' methyl guanosine cap (Brock, Deng et al. 1992). In a separate publication, these authors suggested the primary and secondary structure of the 5 ' untranslated region of pestivirus more closely resembled the structure of picornavirus and HCV than the 5' untranslated region of flaviviruses (Deng and Brock 1993). In addition, the predicted cap independent translation mechanism of pestivirus was different from that of a number of the family Flaviviridae (Deng and Brock 1993). The most conserved nucleotide and amino acid sequences are located in the 5' untranslated region (5'UTR) and nonstructural protein p80 region, respectively (Deng and Brock 1992). The 5' untranslated region has a complex secondary structure, determined to be an internal ribosomal entry site for initiation of translation (Pellerin, van den Hurk et al. 1994). During translation, a single polyprotein is produced from the open reading frame. Viral and host proteases cleave this single polyprotein co- and post-translationally into individual viral proteins, including structural and nonstructural proteins. In BVDV infected cells, 13 polypeptides have been found, which are comprised of structural and nonstructural proteins (Akkina 1991), (Collett, Larson et al. 1988), (Donis and Dubovi 1987), (Donis and Dubovi 1987). Structural

proteins include capsid protein (core protein), E^{rns} (Rnase secreted envelope glycoprotein), E1 envelope glycoprotein, E2, and p7. The 5' end of the open reading frame encodes three structural proteins, while remaining nonstructural proteins are encoded by the highly conserved 3' end. E^{rns} is found on the surface of pestivirus-infected cells and is secreted into the medium (Rumenapf, Unger et al. 1993). Translocation across the cell membrane by its C-terminal domain and targeting of nucleoli has been described for E^{rns} (Langedijk, van Veelen et al. 2002). The largest target for neutralizing antibody production is the structural glycoprotein E2 (previously titled gp53). The host immune system recognizes E2 epitopes; however, the virus can escape antibody neutralization by viral modification of these epitopes. Genomic sequence analysis has determined hypervariable regions within the RNA encoding for E2, which is associated with antigenic diversity (Donis, Corapi et al. 1991), (Paton, Lowings et al. 1992). BVDV non-structural proteins are Npro, NS2-3, NS4A/B, NS5A, and NS5B. The N-terminal protein of BVDV codes for N^{pro}, a cysteine protease that cleaves the N-terminus from the core protein (Petric, Yolken et al. 1992), (Rumenapf, Unger et al. 1993). NS2-3 is a large 125 kD serine protease expressed in cytopathic BVDV isolates as NS2-3, but also expressed as two separate proteins (NS2 and NS3). In both cytopathic and non-cytopathic infections, formation of infectious viral particles appears to require expression of uncleaved NS2-3. These authors also documented that NS3 serves as a marker protein for cytopathic BVDV infections (Agapov, Murray et al. 2004). NS4A is a cofactor for serine protease NS23 (Tautz, Elbers et al. 1997), and NS4B appears to be important in cytopathic infections (Qu, McMullan et al. 2001). The replication complex requires NS5A. Finally, NS5B has RNA dependent RNA-polymerase activity.

Both cp and ncp biotypes are recognized for BVDV (Lee and Gillespie 1957), (Gillespie, Baker et al. 1960). These biotypes were identified based on tissue culture observations, in which distinct degenerative changes were found in cell monolayers with certain strains, designated as cp biotypes. Cytopathic BVDV infection results in cell vacuolation and death within 24 to 48 hours following inoculation. Noncytopathic BVDV induces no morphologic changes and does not affect survivability of cell cultures (Deregt and Loewen 1995). Noncytopathic and cp biotypes can be found with both BVDV-1 and BVDV-2. The predominant biotype in clinical cases is ncp, and the cp biotype is considered rare (Bolin 1990). Acute/transient infections may be caused by both cp and ncp BVDV, but only ncp isolates are capable of establishing persistent infections (Baker 1987), (Deregt and Loewen 1995). Both biotypes are involved in the pathogenesis of mucosal disease (Brownlie, Clarke et al. 1984) (Bolin, McClurkin et al. 1985) and both cp and ncp strains code for NS2-3. Only cp strains are capable of cleaving the nonstructural protein NS2-3 to NS2 and NS3 protein. Cytopathic variants produce high levels of NS3 as a free protein after the early phase of infection (Donis and Dubovi 1987), (Lackner, Muller et al. 2004), (Pocock, Howard et al. 1987). In contrast, during the early phase of infection, ncp BVDV variants largely produce NS2-3 and only small amounts of NS3 (Lackner, Muller et al. 2004).

Considerable antigenic diversity has been described for BVDV isolates, which is believed to arise from mutational events (Paton, Carlsson et al. 1995). Mutations involving the viral genome encoding for structural protein E2 and three hypervariable regions have been identified (Bolin, Littledike et al. 1991), (Donis, Corapi et al. 1991), (Paton, Lowings et al. 1992). E2 is the target for neutralizing antibody production by the humoral immune system. Consequently,

vaccine efficacy is impacted by antigenic diversity within E2. Vaccines containing a broad array of genotypes may provide improved protection. In addition to E2 variability, diversity of BVDV strains has been correlated with genotype differences based on nucleotide sequences and biotypes (Fulton, Ridpath et al. 2003).

Earlier classification of pestiviruses as BVDV, classical swine fever virus, and border disease virus was based on the host species of origin. However, some pestiviruses are not restricted to a single host, and BVDV can infect cattle, sheep, goats, and swine. Antigenic cross reactivity and antigenic variation were noted with attempts to separate pestiviruses by monoclonal antibody assays. Genomic sequencing and phylogenetic analysis of these sequences allowed for classification of BVDV into two genotypes, BVDV-1 and BVDV-2 (Pellerin, van den Hurk et al. 1994), (Ridpath, Bolin et al. 1994). The 5' untranslated region most frequently is used to differentiate pestivirus species (Ridpath, Bolin et al. 1994). The BVDV-1 was initially classified into two subgenotypes, BVDV-1a and BVDV-1b (Pellerin, van den Hurk et al. 1994), capable of being differentiated with monoclonal antibodies (Bolin and Ridpath 1998) and RT-PCR analysis (Ridpath and Bolin 1998). However, BVDV-1a–k and BVDV-2a and -2b have been described (Vilcek, Paton et al. 2001). The BVDV-1b strain is the most frequently identified type 1 subgenotype, and cases of pneumonia in which BVDV has been detected are also more commonly associated with the 1b subgenotype (Fulton, Ridpath et al. 2002). Late gestation fetal infections are reported to involve more 1a than 1b strains (Evermann and Ridpath 2002). Clinical findings in severe acute BVDV infections do not closely correlate with genotypes, in part due to substantial heterogeneity of strains within these two genotypes, and clinical presentation may be affected by stress, reproductive status, immune status and secondary

pathogens, which make it difficult to differentiate between BVDV1 and BVDV 2 infections (Ridpath 2005).

Transmission

There are two primary methods of BVDV transmission, horizontal transmission occurring transiently during postnatal acute infections, and vertical transmission to the fetus. Acute infections may arise from fomites, direct nose-to-nose contact, and vaccination with a modified live virus (MLV) (Duffell and Harkness 1985), (Brownlie 1990). Congenital transmission to the fetus may produce persistent fetal infection if acute/transient infection of the dam occurs between 40 and 125 days gestation. The outcome of congenital infection from gestational ages 120 to 150, the period of fetal immunocompetence, produces fetuses with serum neutralizing antibodies at birth and the potential for congenital malformations. Persistently infected calves continuously shed high levels of virus into the environment. Transiently infected animals shed for a duration of approximately one week. BVDV can be shed in saliva, urine, feces, nasal discharge, vaginal mucus, semen, tears, and milk (Houe, 1995). Fluids, gametes, and somatic cells can be infected by the virus; therefore, semen or embryos from infected animals can be other means of transmission (Gard, Givens et al. 2007).

The Clinical Manifestations of Bovine Viral Diarrhea Virus

Acute BVDV infection, severe acute BVDV infection, hemorrhagic BVDV infection, acute BVDV infection-bovine respiratory disease (BRD), and acute BVDV infection-immunosuppression are the five clinical forms of BVDV described in immunocompetent cattle (Grooms DL 2002). Clinical manifestations of BVDV have been reviewed and typical clinical responses were grouped based on host factors, such as: immune status (previous exposure to

BVDV or vaccinations), pregnancy status, level of environmental stress, and immunocompetence (Baker 1995). The ability of the host to respond immunologically to this virus is described as immunocompetence to BVDV, and these animals are not persistently infected with BVDV and will be able to mount an immune response to BVDV challenge. Animals without BVDV immunocompetence have altered immune systems resulting from previous *in utero* exposure, and these animals are persistently infected (PI) with a ncp strain of BVDV infecting the dam and crossing the placenta in early to mid-gestation. This important host factor causes an important difference in the clinical outcome to BVDV infection when compared to immunocompetent cattle. In 2005, Baker described immunocompetent, nonpregnant cattle as having several potential outcomes: subclinical BVDV infections, BVDV hemorrhagic syndrome, severe BVDV, BVDV infection with immunosuppression, BVDV and respiratory disease, and venereal infections. Infections in immunocompetent, pregnant cattle were described as having the following potential outcomes following BVDV fetal infection, including: embryonic death, abortions, stillbirths; congenital defects; normal calves born positive for BVDV; and immunotolerance to noncytopathic BVDV. Calves born immunotolerant to BVDV face several risks from persistent infection, most notably mucosal disease (MD). Forms of mucosal disease include acute MD, chronic MD, and MD with recovery (Baker 1995). In addition to host factors, viral factors are also important determinants of clinical disease. Variation in virus genotype and biotype, along with antigenic diversity, contribute to the complex spectrum of clinical manifestations observed with BVDV. Both genotypes, BVDV-1 and BVDV-2, can be attributed to both acute and persistent infections. Subclinical to fatal disease may be observed, but the majority of cases are subclinical, acute infections (Baker 1995).

Acute BVDV infections in immunocompetent calves and adults have historically been described as ‘acute’, ‘primary’, and ‘transient’. Currently, two types of infections are described based largely on the duration of viremia (Hanon, Van der Stede et al. 2014). This definition of the duration is largely associated with the clinical outcome, as transient cases are infected horizontally and develop 14-21 days of viremia, serum neutralizing antibody titers, and viral clearance. The second type of infection is persistent infection of the fetus following *in utero* vertical transmission of the virus from a transiently or persistently infected dam. Acute-transient infections may occur after an incubation period of 5-7 days in naïve cattle. The virus enters via the oral-nasal mucosa and initial replication occurs in the nasal mucosa and tonsils. Viremia typically begins around day 3 and peaks by day 7, but viremia has been documented to persist for up to 15 days. Viral replication is pronounced in the respiratory tract epithelium, spleen, thymus, lymph nodes, and gastrointestinal tract epithelium.

Acute-transient BVDV infections often are observed in 6 to 24-month-old seronegative cattle. The age group that appears to be most susceptible is 4-6-month-old calves, and this is thought to be due to the period of vulnerability caused by waning of maternally-derived BVDV specific antibodies. Maternally-derived antibodies are believed to be protective if there is not a great amount of antigenic diversity between the maternally-derived antibodies and the challenge strain of virus, and if there were adequate colostrum quality and consumption. Consequently, failure of passive transfer can be associated with acute disease in neonatal and preweaned calves, often causing enteric disease or pneumonia. A reduction in the level of maternal colostrum varies between dairy and beef calves; for dairy calves the estimated mean time to seronegativity was reported at 117.7+/- 37.7 days for BVDV-1 and 93.9 +-61.9 days for BVDV-2 (Munoz-Zanzi,

Hietala et al. 2003). In this study a longer interval until seronegative status was reached in beef calves and was 185.6+-59.8 (BVDV-1) and 157.8 +-56.1 (BVDV-2). A strong correlation of transient BVDV infection with bovine respiratory disease complex and diarrheal diseases has been documented. The risk of these conditions is often doubled by transient BVDV infection (Evermann 2005). This estimate took into consideration several field investigations of BVDV incidence in animals that received treatment for respiratory disease. In a group of animals entering a feedlot, 13/29 (45%) received treatment for respiratory disease, and 8/36 (22%) of untreated calves had seroconverted (Martin and Bohac 1986). Similar evaluations have been performed for the risk of bronchopneumonia in calves that received colostrum without BVDV antibody or calves receiving colostrum from seropositive dams. Among calves exposed to BVDV at birth, 68% (30/44) developed moderate or severe bronchopneumonia if they received seronegative colostrum, while 41% (35/86) of this group developed moderate to severe bronchopneumonia despite receiving colostrum with BVDV antibodies (Moerman, Straver et al. 1994). Other variables considered when evaluating the risk of other diseases following BVDV exposure, included incidence of respiratory or diarrheal disease requiring treatment in calves born into the herd when BVDV was present. Mortality rates of calves born during the period of BVDV introduction were also evaluated in this study. BVDV comorbidity with agents from the bovine respiratory disease (BRD) complex was associated with increased morbidity and mortality in calves. Antigen distribution of infectious bovine rhinotracheitis (IBR) was reported to be greater in calves previously exposed to BVDV (Potgieter, McCracken et al. 1984). Severity of *Mannheimia haemolytica* infections was more often associated with increased morbidity and mortality when BVDV is present (Potgieter, McCracken et al. 1984).

Bovine viral diarrhea virus is capable of infecting multiple organ systems, often concurrently. Clinical signs often observed include: oculonasal discharge, depression, anorexia, fever, oral erosions and ulcerations, diarrhea, decreased milk production in lactating cows, and increased respiratory rate. A consistent clinical finding during acute-transient BVDV infection is decreased total leukocyte counts, primarily due to a reduction in neutrophils and lymphocytes (Baker 1995). Virulence of the infecting BVDV strain is one of the most important factors influencing the outcome of infection, and higher virulence viruses are reported to cause increased viral loads in tissues and a faster rate of viral spread throughout the body, in contrast to viruses of lower virulence (Liebler-Tenorio, Ridpath et al. 2003). Strain virulence has been shown to be a determinant in dissemination of virus throughout the body, and subsequently animals with greater levels of viremia demonstrate more profound clinical signs (Walz, Bell et al. 2001). In low virulence infections, the intestinal mucosa was documented to be a specific location in which BVDV antigen could be readily found, but infections caused by high virulence strains demonstrated antigen in endocrine tissues, the nervous system, respiratory tract, and bone marrow (Liebler-Tenorio, Ridpath et al. 2003). A difference in tropism for a specific lymphoid tissue has not been observed between low and high virulence strains, and all strains of BVDV are reported to show viral replication and antigen localization within the lymph nodes, Peyer's patches, thymus, spleen, and tonsils. Several studies have examined potential reasons for differences in immune responses following low versus high virulence infections, and virulence and genotype have been noted to play a role in expression of innate immunity and pro-inflammatory and anti-inflammatory cytokines (Palomares, Brock et al. 2014).

Subclinical infections represent the majority of BVDV infections. Mild or subclinical

disease was estimated to encompass 70-90% of transient infections (Baker 1987). This estimate has been confirmed in large dairy herds in which virus was known to be present in the herd for approximately 2.5 years (Moerman, Straver et al. 1994). The primary impact of transient BVDV infection in cows is a decline in udder health. Reduced milk yield did not correlate with the level of BVDV antibody in bulk tank milk samples. However, there was an association of BVDV antibody with increased somatic cell counts in bulk milk tank samples (Grooms DL 2002). Retained placenta is also an issue for transiently infected cows delivering PI calves, as 5/12 (42%) dams with PI calves had retained placenta in comparison to 7/198 (4%) cows with retained placentas with non-PI calves.

Outbreaks of severe, peracute BVDV infections were reported in immunocompetent cattle in the 1990's (Pellerin, van den Hurk et al. 1994), (David, Crawshaw et al. 1994). These outbreaks were distinctive due to the peracute course, high morbidity, and mortality in all age groups. Mortality rates varied with 10-20% mortality rates reported for some herds (Pellerin, van den Hurk et al. 1994). Older cattle frequently demonstrated oral ulcers, diarrhea, fever, decreased milk production, and abortions. Based on samples collected during this period of atypical, peracute BVDV infections, diagnostic testing revealed noncytopathic BVDV to be implicated in these deaths. Nucleotide sequencing identified a novel genotype of BVDV, which was later designated as a separate species within the genus Pestivirus, BVDV-2 (Ridpath, Bolin et al. 1994).

A form of severe, acute BVDV infection in immunocompetent cattle is hemorrhagic syndrome. This cause of bloody diarrhea, epistaxis, hyphemia, ecchymotic hemorrhage, bleeding from injection sites or insect bites, and petechial hemorrhages is associated with

thrombocytopenia, in addition to severe lymphopenia and neutropenia. Bone marrow infection by BVDV, most notably megakaryocytes, may be a primary defect leading to thrombocytopenia (Ellis, West et al. 1998). Platelet defects, both quantitative and qualitative, are associated with platelet dysfunction and clinically noteworthy hemorrhage (Walz, Bell et al. 1999), (Walz, Bell et al. 2001). There is some strain association, as a majority of infections are associated with ncp, BVDV-2 ((Bolin and Ridpath 1992). One report described hemorrhagic syndrome in colostrum-deprived calves with a BVDV-1b strain (Blanchard, Ridpath et al. 2010).

The mechanism of BVDV-induced immunosuppression has not been clearly elucidated. However, one potential cause of immunosuppression relates to the ability of the virus to cause leukopenia, most notably neutropenia and lymphopenia. Severity of leukopenia is also reported to be greater with BVDV-2 than BVDV-1. Strain dependent decreases in helper (CD4+), cytotoxic (CD8+), and gamma/delta (γ/δ) T lymphocytes, B-lymphocytes and neutrophils have been described (Chase 2013). Speculation into the mechanism of leukopenia has involved the following theories: destruction of immune cells by BVDV, increased trafficking of immune cells into tissue sites of viral replication, and immune system removal of BVDV infected immune cells. Lymphoid depletion is a prominent feature during acute infection, primarily secondary to lymphocyte apoptosis and acute cytolysis of lymphocytes. Lymphoid tissues targeted during transient infection usually include thymus, spleen, Peyer's patches, and lymph nodes. Previous studies have shown an association between virulence and lymphocyte depletion. Immunohistochemical staining of lymphoid organs during infection with highly virulent BVDV strains correlated with antigen localization and lesions of lymphocyte apoptosis and depletion (Kelling, Steffen et al. 2002), (Liebler-Tenorio, Ridpath et al. 2003). A frequent clinical outcome

from BVDV immunosuppression is secondary bacterial pneumonia.

Viral Pathogenesis

Clinical findings during acute/transient infections point to immunosuppression as a consistent outcome. Transient leukopenia is the hallmark clinical finding, and one of the findings in an early report (Olafson, Mac et al. 1946). To date, precise mechanisms of immunosuppression have not been elucidated *in vivo*. Qualitative and non-qualitative leukocyte defects have been described as the basis for immunosuppression (Kapil S 2005). In vivo T cell responses to acute/transient BVDV infections showed depletion of CD4+ but not CD8+ or γδ-T cells, and this study associated this finding with prolonged shedding in transiently infected animals (Howard, Clarke et al. 1992). In ncp BVDV infections, a T helper 2 response is induced and cytotoxic T-lymphocytes (CTL) are compromised (Chase 2013). Humoral immunity provides some protection, but cross-protection between strains is unpredictable. There is also a reduction in antigen presentation, co-stimulatory molecules are down regulated, and T-lymphocyte numbers are reduced in a strain dependent manner (Chase 2013). *In vitro* studies have indicated that non-cytopathic (ncp) BVDV infection stimulates macrophage derived cytokines. Early studies examining the pathogenesis of BVDV immunosuppression identified BVDV infected cell cultures and monocytes expressing prostaglandin E2 (Welsh, Adair et al. 1995). Additionally, induction of an IL-1 inhibitor was believed to play a role in immunosuppression (Jensen and Schultz 1991). Reduced chemotactic response of monocytes also contributes to immunosuppressive potential of this virus (Ketelsen, Johnson et al. 1979). Other primary mechanisms of immunosuppression are believed to involve: *in vivo* induction of apoptosis of B and T cells in lymphoid tissue, a downregulation of MHC II and IL-2, or an

activation of a T-helper 2 response by ncp BVD (Chase, Elmowalid et al. 2004). *In vitro* suppression of TNF-α infection in bone-marrow derived macrophages has also been correlated with the immunosuppressive potential of BVDV (Adler, Jungi et al. 1996). The effect of BVDV on immune signaling was examined with monocyte derived dendritic cells (Mo-DC) infected with ncp and cp BVDV. Ncp BVDV down-regulated and cp BVDV up-regulated the expression of Mo-DC cell surface markers MHC I, MHC II, and CD86 (Rajput, Darweesh et al. 2014). This study also answered an important question regarding the involvement of dendritic cells in BVDV infections, or at least shed some light on the role of dendritic cell *in vitro* infections. The capacity of monocytes to produce infectious virus was reduced during the period of differentiation from a monocytic phenotype to monocytic-dendritic cell type. Other aspects of innate immunity were also evaluated, and superoxide anion was reduced in both cp and ncp infections in response to LPS (Adler, Frech et al. 1994). However, this study documented only ncp BVDV primed cells for enhanced production of nitric oxide.

Differing immune responses with noncytopathic and cytopathic BVDV infections. Adaptive and innate immune responses to cp and ncp BVDV have been previously evaluated in monocytes, macrophages, bone marrow derived macrophages, monocyte derived dendritic cells, and peripheral blood mononuclear cells. An *in vitro* model evaluating BVDV-reactive T-cell responses in transiently infected cattle found similar rates of seroconversion and viral clearance for cytopathic and non-cytopathic biotypes, but lymphoproliferation was observed earlier in animals infected with cytopathic virus. Additionally, seroconversion and virus specific lymphoproliferation were not detected until viremia resolved. Another study looking at the differences between cytopathic and noncytopathic viruses during immune responses evaluated

the relationship of cytopathic and noncytopathic viruses and apoptosis of peripheral blood mononuclear cells (PBMC). Cp virus, but not ncp virus, induced apoptosis in bovine CD4 (BoCD4), BoCD8 and BoWC1 T cells in whole PBMC cultures. However, cp virus induction of apoptosis was enhanced in the presence of monocytes and purified lymphocyte populations showed differing levels of apoptosis (Lambot, Hanon et al. 1998).

Reduced functional responses in neutrophils and lymphocytes has been documented in persistently infected cattle (Brown, Bolin et al. 1991). Persistent infection with BVDV bypasses B- and T- cell immune responses, essentially demonstrating immunotolerance to these arms of the immune system and bypassing the adaptive immune system (Peterhans and Schweizer 2013). Innate immune responses are also altered during BVDV infection, and one of these changes in innate immune function involves induction of type I interferon induction by cp biotypes, but ncp BVDV fails to induce interferon type I in cultured bovine macrophages (Peterhans, Jungi et al. 2003). Cultured cells infected with ncp BVDV also fail to induce interferon in the presence of double stranded RNA (Schweizer and Peterhans 2001). The BVDV envelope glycoprotein E^{rns} has been shown to play a key role in inhibiting IFN expression. The RNase activity of E^{rns} is required for this inhibition, and E^{rns} was shown to degrade ds- and ssRNA at neutral pH (Matzener, Magkouras et al. 2009). After cellular entry, E^{rns} is believed to degrade RNA within endosomes (Iqbal, Flick-Smith et al. 2000). However, once infection is established, the virus is resistant to the action of interferon (Peterhans and Schweizer 2013). Interestingly, when host cells are simultaneously infected with BVDV and other unrelated viruses, BVDV does not interfere with the action of IFN on other viruses replicating in these cells (Schweizer, Mätzener et al. 2006), (Chen, Rijnbrand et al. 2007). Later in life, PI cattle appear to maintain resistance to

the action of interferon, but the virus does not block the action of IFN against unrelated viruses in BVDV infected cells (Peterhans and Schweizer 2013). BVDV has other methods of antagonizing the IFN response, which include the ability of the N-terminal autoprotease, N^{pro} to resist dsRNA induced apoptosis and by inhibiting transcriptional activation of the IFN-βgene by preventing activation of the interferon promotor, interferon regulatory factor 3 (IRF-3) through promoting proteasome degradation of this protein (Hilton, Moganeradj et al. 2006). Another method by which the virus modulates the normal host defense response is by inhibiting an important host protein critical in restricting viral protein translation and replication in antiviral innate defense, protein kinase R (PKR). One pivotal study found PKR and NF-κB induction was inhibited in cells infected with BVDV following stimulation with dsRNA (poly I:C) (Gil, van Olphen et al. 2006). This study showed ncp prevented NF-κB induction in these experiments, but cp was a strong inducer of this downstream transcriptional activator.

BVDV is a major reproductive pathogen in cattle and consequences of reproductive infections include: infertility, embryonic death, fetal mummification, abortion, stillbirth, congenital disorders including a wide spectrum of fetal pathology, and the birth of viremic, persistently infected calves (Grooms 2004). While the incidence of PI calves is low and estimated to approach 1.0-2.0% of the cattle population, PI calves are a major threat to the cattle industry due to their continuous shedding of virus through nasal, oral, respiratory, fecal, and genitourinary routes (Grooms 2004). The frequency of BVDV infection primarily is due to the efficiency of this virus to cross the placenta of susceptible females (Fray, Paton et al. 2000, Bolin and Grooms 2004). Previous experimental studies have shown that BVDV crosses the placenta with almost 100% efficiency (Duffell and Harkness 1985). Viral transmission across the placenta

is believed to be achieved by hematogenous spread, and cell-to-cell transfer has not been documented. Experimental intranasal inoculation with BVDV in sheep has shown the virus crosses the placenta within 3 days (Swasdipan, Bielefeldt-Ohmann et al. 2001). The time of viral transmission across the placenta has been less clearly defined in cattle, and transplacental transfer appears to roughly correspond with the development of viremia (Scherer, Flores et al. 2001). One study indicated the earliest detection of viral antigen in the infected fetus was 14 days post infection (Fredriksen, Press et al. 1999). Viral antigen was observed in fetal liver and lung, but no antigen was detected in the placenta at 14 days postinfection, and this finding was used to support the theory that viral spread occurs from the vasculature, not local cell to cell spread. A spectrum of reproductive losses may occur, including infertility and early embryonic death causing reduced reproductive efficiency, abortion, persistent fetal infection, congenital defects, congenital infection in late pregnancy, and reduced bull fertility. Testicular infections by BVDV can be acute or persistent, and both can reduce semen quality (Coria and McClurkin 1978). There are three main phases of pregnancy that may be impacted by *in utero* BVDV infection: 1) infection before conception through the embryonic state (-9 to 45 days of gestation), 2) infection following the embryo stage (45-175 days gestation), and 3) infection in late gestation (185-285 days gestation) (Grooms 2004).

The primary reproductive consequence of BVDV is persistent fetal infection occurring in the latter part of early gestation to mid-gestation, in which BVDV is able to establish itself and persist as a life-long, chronic viral illness following *in utero* transmission of a ncp virus (Brownlie, Clarke et al. 1989). Some members of the *Flaviviridae* family, such as BVDV and hepatitis C virus, have a similar ability to evade the host immune response and establish

persistent infections in cattle and humans. One difference between these members of the *Flaviviridae* family is the efficiency of viral transmission to the fetus. It was reported efficiency of hepatitis C viral transmission to the fetus is dependent on the level of maternal viremia, and transmission is reported to occur only when serum HCV RNA is detectable (above 10^6 copies/ml) (Roberts and Yeung 2002). This author also stated, women with HCV viremia have a rate of maternal to infant transmission of 4-7% per pregnancy. Conversely, the ability of BVDV to establish persistence following *in utero* transmission across the maternal-fetal interface to the immunologically naïve fetus is dependent on gestational age of the fetus. The optimal time for fetal persistent infection is between 30 to 125 days gestation (Brownlie, Hooper et al. 1998), (Brownlie, Clarke et al. 1984), (Roeder, Jeffrey et al. 1986), (Radostits and Littlejohns 1988), (Moennig and Liess 1995). The chance for persistent fetal infection decreases near gestational day 100; however, persistent infections have been reported to occur up to 125 days gestation (Baker 1995). Circulating virus during the period of gestation when immunocompetence is developing (90-120 days) is a prerequisite for immunotolerance and persistent infection; however, the concentration of circulating virus necessary for persistent fetal infection has not been documented. This 90-120-day window of time is important because during this time period the immature fetal immune system does not recognize the virus (McClurkin, Littledike et al. 1984). By gestational day 163, it has been shown that the fetal antiviral immune system recognizes BVDV, but cannot eliminate the virus (Smirnova, Ptitsyn et al. 2009). The majority of dams carrying persistently infected fetuses are naïve to BVDV when first exposed to the virus, or they possess insufficient serum neutralizing antibodies to circumvent acute/transient infection. The other scenario for dams carrying PI fetuses is that these animals may also be persistently

infected, but this is a rare occurrence since less than 1% of the cattle population in the United States is believed to be persistently infected with BVDV. These PI dams are not naïve to the virus despite the absence of serum neutralizing antibodies to BVDV, and the chances of a PI animal producing a PI fetus are near 100% (Moerman, Straver et al. 1993), (Houe and Meyling 1991). Persistent fetal infection has a major epidemiological impact because persistently infected calves propagate large quantities of the virus into the environment and are a reservoir of infection for cattle naïve to BVDV (Grooms 2004). Up to 70% to 100% of susceptible, unvaccinated cattle have become infected by BVDV following exposure to a persistently infected animal (Fulton, Hessman et al. 2009). Following the development of fetal immune competence, approximately days 125 to 150 (McClurkin, Littledike et al. 1984), (Casaro, Kendrick et al. 1971), persistent fetal infection is less likely and the fetus becomes more susceptible to teratogenic effects of the virus during this period of rapid organogenesis (Duffell and Harkness 1985). Fetuses exposed to BVDV during organogenesis may show congenital anomalies at birth, including: hydranencephaly, microophthalmia, retinal dysgenesis, and cerebellar hypoplasia**.** Non-PI dams that later carry persistently infected fetuses are naïve to BVDV when initially exposed to the virus. The outcome in these serologically naïve dams is usually the same, if they carry a PI fetus or not, because the majority of these infections are subclinical. If the virus is not highly virulent and does not cause mortality, these dams will seroconvert and eventually eliminate the virus from their circulation through serum neutralizing antibodies. However, this protective response in the dam may occur after the virus has already crossed the placental barrier to infect the fetus. As mentioned previously, the efficiency of fetal infection is highly dependent on the gestational age of the fetus. The contribution of maternal

immunity may seem to play a role if there is clearance of the virus and no fetal infection; but when examined closely, it is clear factors such as fetal gestational age, dose of viral inoculum, and viral strain are consistent determinants regulating the efficiency of fetal infection. Clinical consequences of the virus on the dam appear to be minimal when infected with a strain that is not highly virulent; this is often evident through approximately 7-10 days of reduced feed intake, clear nasal discharge, mild febrile episodes, lethargy, and reduced leukocyte counts (Baker 1995). Infection of the dam with a moderate to highly virulent virus is likely to cause clinical illness and death on occasion, often impacting fetal survival. If dams carrying PI fetuses do not clear the virus from their circulation and they remain seronegative for BVDV, it is likely they will also be diagnosed as previously undetected PIs.

Maternal Innate and Adaptive Immune Responses to BVDV During Pregnancy.

Although there is a large amount of general knowledge about BVDV pathogenesis, direct conclusions about the mechanisms of BVDV transplacental infection have not been made based on an assumption the maternal immune response in the placenta is the same as the systemic circulation. Knowledge about the immunological barrier between the mother and fetus reminds us of this unique interface that is similar to other distinct anatomic sites with immune privilege, including the blood-testis barrier, blood brain barrier, eye, and hair follicle. Conclusions stating that immunological, physiological, and endocrinological impact of the virus in the placental microenvironment are the same as the maternal circulation are problematic due to the differences between systemic and mucosal immunity, and the large number of unknowns concerning normal immunoregulatory mechanisms in this complex organ. However, vaccine efficacy studies have shown there are challenges reconciling seemingly adequate systemic humoral serum neutralizing

antibody titers and cell-mediated immunity at the placental level. Studies evaluating BVDV at the maternal-fetal interface are limited, and these investigations have been made within the last five years. One notable experimental design throughout these more recent studies is a predominance of samples from maternal blood, amnionic fluid, and fetal tissues. Acute fetal infections have been documented to induce a pronounced type I IFN response in pregnant cows and fetuses with ncp BVDV with upregulation of ISG15 mRNA with qRT-PCR in blood of pregnant heifers and TI fetuses during acute non-cytopathic BVDV infection (Smirnova, Bielefeldt-Ohmann et al. 2008). Conversely, immunological responses, including T cell signaling and IFN induction, in heifers and cows maintaining persistently infected fetuses and transiently infected fetuses have been performed through evaluation of maternal and fetal blood samples (Shoemaker, Smirnova et al. 2009), (Smirnova, Ptitsyn et al. 2009), (Hansen, Smirnova et al. 2010). One of the first papers to investigate maternal gene expression in dams carrying persistently infected fetuses was performed by microarray analysis of RBC-depleted blood and qRT-PCR on whole blood for chemokine C-X-C motif receptor 4 (CXCR4), a ligand for stromal cell-derived factor-1 (SDF-1) (Hansen, Smirnova et al. 2010). SDF-1 is a potent chemotactic factor for multiple leukocyte and bone marrow cell types, but SDF-1 (CXCL12) is best known for its ability to exclusively bind CXCR4, the coreceptor for X4 HIV-1 *env* protein (Bleul, Farzan et al. 1996). Studies with *ex vivo* cytotrophoblast cultures in humans indicate SDF-1/CXCL4 plays an important role in pregnancy maintenance by promoting trophoblast cell survival by suppressing apoptosis in early, late, and term pregnancies (Jaleel, Tsai et al. 2004). In heifers carrying persistently infected fetuses, CXCR4 was downregulated at day 7 post-inoculation with BVDV and remained in downregulation for approximately 3 months post-

inoculation (gestational day 160) (Hansen, Smirnova et al. 2010). This experiment, primarily based on microarray and Western blot evaluations, was the first to document a fetal immune response to BVDV following inoculation with ncp BVDV at 75 days gestation. There was also down-regulation of CD8 T cells and several T-cell receptor (TCR) pathway members. Based on this study, it was proposed heifers carrying PI fetuses could be screened for persistent infection by evaluating maternal blood samples for a distinct chemokine profile. This study also showed transiently infected (TI) fetuses and PI fetuses are able to secrete ISG15 in response to the virus at gestational days 90, 182, and 190, but PI fetuses showed no difference in ISG15 expression, similar to controls, except for the 90-day gestation time point. TI fetuses were inoculated with BVDV at gestational day 178, and demonstrated differences in ISG15 expression at days 182 and 190. ISG15 is an important component of the type I interferon (IFN-I) response, which promotes cell survival during viral infection by anti-apoptotic effects.

Experiments evaluating type I interferon (IFN) responses to BVDV in the systemic circulation conclude ncp BVDV inhibits induction of type I IFN, providing foundational evidence for evasion of this arm of the host response as a central mechanism by which the virus maintains persistence (Magkouras, Matzener et al. 2008), (Glew, Carr et al. 2003), (Peterhans, Jungi et al. 2003). Evidence that ncp BVDV is capable of evading the type I interferon response *in vitro* has been provided by several studies (Charleston, Fray et al. 2001), (Schweizer and Peterhans 2001), (Schweizer, Mätzener et al. 2006). However, there appears to be a difference between *in vitro* and *in vivo* findings in other experiments, as ncp was shown to cause IFN alpha/beta and gamma induction *in vivo* (Charleston, Brackenbury et al. 2002), (Palomares, Marley et al. 2013). Earlier *in vitro* experiments, indicated type I interferon induction was

demonstrated in cp BVDV infected bone marrow-derived macrophages, which underwent activation-induced apoptosis after exposure to lipopolysaccharide (LPS) (Adler, Adler et al. 1997). In macrophages exposed to cp BVDV, a reduction in nitric oxide production and apoptosis was observed, but the opposite effect was detected when ncp was used to prime bone marrow-derived macrophages (Adler, Frech et al. 1994). More recently, attempts to provide additional detail for ncp *in vitro* BVDV infections have utilized novel cell type, lineage-negative (LIN^-) cells, a cell type described as being enriched for bovine IFN-alpha/beta production (Gibson, Miah et al. 2012). Other mechanisms of viral persistence described for BVDV, include: inhibition of viral cytolytic effect, infection of nonpermissive cells, and evolution of viral variants (Neill 2005).

Immunological Responses During Chronic and Persistent Viral Infections

Virus survival strategies encompass intrinsic properties of the virus and interactions of the virus with the host cells, host animals, and the host population (Peterhans and Schweizer 2013). Of all potential survival strategies, two appear to be most commonly employed; one aims to target the host population and the other is a survival strategy within the host. The population based method of ensuring spread of the virus in susceptible hosts is the "*hit and run strategy*" while sophisticated evasion methods are part of the "*infect and persist strategy*" (Ahmed 2002). Although the mechanisms that viruses utilize to establish and maintain persistence are well established in humans, the targets of persistence in veterinary species are not well established. Consequently, the specific mechanisms of viral persistence are largely described for human viruses.

Cytokines and chemokines are the most common host system exploited by viruses to maintain persistence in their host, essentially exploiting the "infect and persist" strategy. Disruption of cytokines and chemokines often target three common pathways, including: 1) interruption of cytokine production, 2) interference of receipt of cytokine signal, and 3) interference of cytokine effector function (Kane and Golovkina 2010). Viruses have adapted over time to disseminate throughout the mononuclear phagocyte system to infect various organ systems. A relatively simple way viruses achieve systemic spread is by manipulating the cytokine/chemokine axis in order to facilitate leukocyte chemotaxis to the primary site of infection, increasing likelihood of viral dissemination. More specific means by which chemokines and cytokines are manipulated by persistent viruses, include: 1) scavenger activity of viral proteins for host chemokines 2) disruption of cytokine binding to viral cellular receptors 3) inactivation of host cytokine receptors by downregulating cytokine receptor expression 4) neutralization of chemokines by novel molecules encoded by the virus 5) viral secretion of virokines that mimic host cytokines and block host cytokine receptors and 6) interference with target cell recognition of cytokines by exploiting a membrane-bound cytokine receptor (Zuniga, Macal et al. 2015). Persistence is also more likely in immune privileged sites where lymphocyte trafficking is reduced, lymphatic vessels are absent, and there is a lack of MHC class II antigen presenting cells. MHC I expression may be reduced in these more specialized cell types (Hamrah and Dana 2007). Other mechanisms of evasion used by viruses include: restricted expression of viral genes, antigenic variation, viral escape from T-cell recognition, suppression of cell-surface molecules required for T cell recognition, interference with antigen presentation (often defective interfering (DI) viral particles), and interference with cytokine function, and immunological

tolerance (Zuniga, Macal et al. 2015).

Over the years, BVDV researchers have been plagued by an inability to clearly elucidate how BVDV avoids immune detection in the fetus. Persistence in the bovine fetus is partially related to the immaturity of the fetal immune system, but this does not explain the lack of immune recognition. In 2013, Peterhans and Schweizer provided a detailed review describing BVDV's ability to induce tolerance of the innate immune response (Peterhans and Schweizer 2013). A basic feature of the virus permits persistence, which is the ability to infect host cells without cp effects (ncp biotypes). When cp BVDV is introduced into cell cultures, IFN type I and apoptosis are triggered (Adler, Adler et al. 1997), (Schweizer and Peterhans 1999), (Perler, Schweizer et al. 2000). It has been shown ncp BVDV uses several mechanisms to inhibit innate immunity, and a few of these studies have relied on evidence that IFN can be induced by synthetic double-stranded RNA to mimic virus infection, as infected cells can produce viral dsRNA as byproducts of viral genome replication (Jacobs and Langland 1996). Several studies have documented BVDV targets signaling through dsRNA. A synthetic analog of dsRNA, poly I:C (polyinosine-polycytidylic acid), was not able to induce interferon or apoptosis following BVDV treatment (Schweizer and Peterhans 2001). Poly I:C serves as a molecular pattern that is recognized by Toll-like receptor 3 (TLR-3), and TLR-3 ligation should activate NF-kB and inflammatory cytokines. However, it has also been shown the pathways activated by viruses and dsRNA are not identical and genes activated may not be identical (Sen and Sarkar 2007).

Evasion of type I IFN immune responses is achieved by two primary methods: 1) avoiding IFN induction 2) by failing to elicit a response to IFN when induced. Failing to respond to IFN is believed to be a purposeful response with a larger goal of "infect and persist."

Tampering host responses when there is a co-infecting virus increases the likelihood of BVDV persistence. Interferon induction is also inhibited by IFN antagonists, which most notably are key BVDV glycoproteins, N^{pro} and E^{rns}, that are unique to pestiviruses. Early BVDV infections cause relocalization of cellular interferon regulatory factor 3 (IRF-3) from the cytoplasm to the nucleus, and this step is blocked by the autoprotease Npro, which inhibits IRF-3 binding to DNA (Baigent, Zhang et al. 2002). Key outcomes of this block are inhibition of IFN-β activation, inhibition of dsRNA induced apoptosis, prevention of NF-kB activation, and triggering polyubiquitination and degradation of IRF-3 (Hilton, Moganeradj et al. 2006). The glycoprotein E^{rns} is a structural protein in the viral particle that possesses RNase activity (Schneider, Unger et al. 1993). Both ssRNase and dsRNase activity can be detected in BVDV infected cells and extracellularly, and preventing IFN synthesis in uninfected cells was suggested as a mechanism for preventing IFN synthesis by this group (Peterhans and Schweizer 2013).

By questioning why viral RNases are needed when host RNases are normally abundant, Peterhans and Schweizer have proposed a broader role for E^{rns} in evasion of the host immune response. They hypothesized E^{rns} acts as an enzymatically active decoy receptor, degrading extracellular viral RNA to avoid activation of the innate response. Furthermore, they concluded BVDV is unique in its ability to be established as "self", evident as immunotolerance, by bypassing the B- and T- cell arms of adaptive immunity. The virus also aims to fool the innate immune system by avoiding pattern recognition detection and TLR-3 ligation through E^{rns} activity, destroying extracellular virus not degraded by host RNases. This essentially avoids triggering the innate immune system and killing of the virus and host, facilitating the "infect and persist" strategy. Peterhans and Schweizer's review provided several specific mechanisms for

BVDV persistence: interference with IFN induction, expression of IFN antagonists, and degradation of viral BVDV glycoprotein E^{rns}.

Other factors favoring the success of BVDV exploit basic ruminant anatomy and physiology. Namely, the epitheliochorial type of placentation of ruminants ensures complete blockage of maternal to fetal transfer of antibody. Therefore, calves are born without immunoglobulins (agammaglobulinemic), and colostral uptake is required within the first 48 hours of life for transfer of maternal antibody. Colostrum provides a multitude of components that provide immediate, innate protection for the calf. Innate immunity is improved by colostrum, specifically enhancing NK cell activity, providing viable macrophages and neutrophils, cytokines, and antimicrobial proteins and peptides (lactoferrin, defensins, and cathelicidins) (Molenaar, Harris et al. 2009), (Stelwagen, Carpenter et al. 2009).

T regulatory cells are classically described as effector T lymphocytes that suppress potentially harmful immune responses. Early descriptions of T regulatory cells (Tregs) were discovered through studies involving systemic autoimmune diseases, which developed in normal rodents following depletion of a T cell subpopulation (Gery, Gershon et al. 1972). The phenotype classically described for T regs has primarily been CD4+ CD25+ Foxp3+, and they were initially named suppressor T cells because the presence of CD4+CD25+Foxp3+ cells in rodent autoimmune studies performed in the 1970s, reversed fatal disease. Today, detailed information describing the intricacies of T regs in humans and mice is abundant, but knowledge about Tregs in veterinary species is limited and resources for similar detailed evaluations are unavailable.

Bovine T regulatory cells are uncharacterized to date, but current evidence suggests there

may be more than one T lymphocyte subpopulation with regulatory activity. The earliest study describing bovine CD4+CD25+Foxp3+ T cells was performed by Seo, et al in 2007 when peripheral blood mononuclear cells (PBMC) were exposed to staphyloccal enterotoxin C1 (SEC1) (Seo, Lee et al. 2007). However, at this time the existence of bovine Tregs had not been confirmed. Stimulation of PBMC with SEC1 from 6 to 10 days postinoculation led to an increase in IL-10, TGF-B production, and Foxp3 expression. This experiment was performed to evaluate previous data suggesting Tregs can be induced by superantigen *in vivo* or *in vitro* upon exposure to superantigens (SAg). The first suggestions of non-CD4+CD25+Foxp3 expressing phenotypes with regulatory activity arose in 2009, when it was suggested gamma delta (γδ) T cells conferred regulatory activity (Hoek, Rutten et al. 2009). This study documented IL-10 activity, but not TGF-β or Foxp3. Another study documented foot and mouth disease virus (FMDV) antigen specific regulatory T cells were γδ lymphocytes (Guzman, Hope et al. 2014). Interestingly, this publication further concluded γδ T cells are the major regulatory T subset in peripheral blood of cattle, as subsets of γδ T cells secreted IL-10 *ex vivo* and proliferated in response to IL-10, IL-4, and TGF-β. Further data showed suppressive γδ T cells were not stained with Foxp3 antibody, but were present in both WC1+ and WC1- T cell TCR+ T cell populations. The third major antigen used to define a regulatory phenotype in cattle was *Mycobacterium avium paratuberculosis* (MAP), the causative agent of Johne's disease. This model appears to be more complicated for various reasons, but partly due to the tropism of MAP for macrophages, which secrete the Th2 immunomodulating cytokine, IL-10, similar to Tregs. Additionally, the phase of disease and interplay of variable IFN-γ production and antibody production have prevented definitive conclusions about the role of Tregs in MAP infections. Overall, there is strong

evidence MAP antigens induce a population of T cells that are CD4+CD25+, capable of producing IL-10 (de Almeida, Colvin et al. 2008). Futhermore, MAP antigens promoted reduced effector T cell activity in response to T regulatory cell signals (de Almeida, Colvin et al. 2008) (Coussens, Sipkovsky et al. 2012). Regulatory T cell phenotypes and associated cytokines have largely been described in these studies, but only one recent publication has proposed functional T cell regulatory activity in cattle (Guzman, Hope et al. 2014). In 2014, Roussey JA, et al indicated a shift in Th1 to Th2 during the subclinical phase of Johne's disease is due to nonresponsive T lymphocytes, not conversion to a Treg phenotype (Roussey, Steibel et al. 2014).

T regulatory responses have been proven to largely differ for acute versus chronic viral infections. The type of virus is an important determinant of the Treg cell response, but some of this variation in response is also dependent on the functional status of natural Treg (nTreg) populations in the host. Natural T regulatory cells (nTreg) often impact the magnitude, duration, and outcome of viral infections (Rouse, Sarangi et al. 2006). This review of regulatory cell activity during viral infection identified some of the cell types shown to be capable of expressing regulatory activity during viral infections in humans, including: natural regulatory T cells (CD4+CD25+Foxp3+), interleukin-10/antigen induced cells (T regulatory 1 cells: CD4+CD25+Foxp3-), CD8 regulatory cells (CD8+CD25-), and natural killer T regulatory cells (Vα24+Vβ11+). Kang et al. were successfully able to induce immunosuppressive function and FoxP3 expression in murine splenic-origin but not human blood-origin γδ T cells (Kang, Tang et al. 2009). Many acute viral infections are not associated with Treg-induced immune suppression or dysregulation of the immune response. Instead, Tregs are largely viewed to be beneficial in acute infections, as they have been shown to limit host tissue damage from pro-inflammatory

cytokines. Conversely, chronic viral infections and persistent viral infections often result in tissue damage from chronic inflammation. Additionally, chronic and persistent viral infections are often associated with immune dysfunction, which largely appears to be Treg-mediated (Rouse and Sehrawat 2010). Tregs are potent inhibitors of CD4+ and CD8+ T cells in humans, and inhibitory effects extend to some aspects involving CD4+CD8+ cell activation, proliferation, and effector function (Miyara, Yoshioka et al. 2009). One of the two major mechanisms described for Treg suppression of immune responses is CTLA-4 (cytotoxic T antigen-4/CD154) downregulation. There are several pathways involving CTLA-4, but the role of CTLA-4 in reducing CD80 and CD86's expression on dendritic cells is partially responsible. CTLA-4 deficiency will prevent a stable interaction between dendritic cells and T responder cells (Miyara, Yoshioka et al. 2009). Treg mediated downregulation of T cell responses reduces functional T cell responses by several mechanisms, but inhibition of IL-2 signaling is one of the major mechanisms of mediating suppression of CD4+ and CD8+ T cells. T cell proliferation and survival of natural Tregs are dependent on IL-2 (Fontenot, Rasmussen et al. 2005). The second major mechanism for Treg-mediated immune suppression is IL-2. IL-2 is believed to be necessary for sustenance of natural Tregs, required for induced Tregs upon stimulation of TGF-β, and Treg suppression (Sakaguchi, Wing et al. 2009). IL-2 upregulates Foxp3 expression via STAT5 (signal transducer and activator of transcription-5) (Burchill, Yang et al. 2007), (Villarino, Tato et al. 2007).

The effect of Treg cell-mediated immunosuppression on viral loads was examined to determine the importance of T regulatory cells (Tregs) during chronic viral infections. One of these studies showed T reg cell depletion had a profound effect on CD8+ T cell function.

Removal of Tregs enabled CD8+ cells to regain key antiviral functions including cytokine secretion and virus-specific cytolytic activity (Dietze, Zelinskyy et al. 2011). A similar role for Tregs in HIV-positive patients has been documented, in which there are discordant hematological parameters and virology data when comparing peripheral blood and lymph node samples. Examination of Treg cells within chronically HIV-infected lymphoid tissues helped resolve some of these questions when it was discovered the accumulation of Treg cells in lymphoid tissues correlates with local viral loads and dysfunction of virus specific CD8+T cells (Shankar, Russo et al. 2000). IL-2 levels have also been noted to contribute to dysfunctional HIV-specific CD8+ T cells, and cytotoxic function was restored following IL-2 exposure. This study evaluated HIV specific T cells in chronically infected HIV patients by flow cytometry. It was speculated these patients may have had functionally compromised HIV-specific CD8+T cells i*n vivo*. Levels of IL-2 also play a role in T cell exhaustion, effectively reducing CD8+ T cell functional responses. The duration of T cell exhaustion can vary depending on the type of virus and the viral load. During phases of high viral burden in persistent infections, severe CD8+ exhaustion and depletion are associated with upregulation of inhibitory receptors and possibly apoptosis of exhausted T cells; this progression toward severe exhaustion is associated with diminished effector function and the loss of possible T cell self-renewal (Kahan, Wherry et al. 2015).

A well described virus associated with persistent infections in humans is hepatitis C virus, a Hepacivirus within the family *Flaviviridae*. Historically, one of the most important prognostic indicators for patients with hepatitis C infections has been the effectiveness of therapeutically controlling the severity of liver inflammation with a goal of reducing hepatic

fibrosis and cirrhosis. The correlation between hepatitis, viral load, and the HCV-specific T effector response has not been consistent when comparing responses in the liver and peripheral blood. Recently, it has been shown Tregs infiltrating the liver inhibit the T effector response in a contact dependent manner during chronic HCV infection. In this study, the frequencies of intrahepatic Foxp3+ Tregs correlated directly with plasma HCV viral load and inversely with histological injury (Radziewicz, Dunham et al. 2009). Another evaluation of peripheral blood showed chronically infected HCV patients had increased CD4+CD25+Foxp3+ Tregs, and the number of Tregs in peripheral blood correlated with HCV-RNA load (Chang, Xue et al. 2014). Patients with chronic HCV infections typically do not mount a striking T effector cell response to the virus, largely due to dysfunctional HCV-specific CD8+ T lymphocytes, increased CD4+CD25+Tregs, and increased IL-10+ Tr1 cells (Chang 2007). CD8+ dysfunction during persistent HCV infection was highly correlated with increased expression of an inhibitory receptor associated with T-cell exhaustion and viral persistence, PD-1 (inhibitory receptor programmed death-1), on intrahepatic, HCV specific CD8+ cells (Xiao, Jiang et al. 2016). PD-1 is a member of the CD28 family of receptors, expressed on T cells, B cells, monocytes, and myeloid dendritic cells (Keir, Butte et al. 2008). Upregulation of PD-1 occurs with some chronic viral infections, causing dysfunctional virus specific T cells. PD-1 ligation with PD-L1 (programmed death ligand-1) and PD-L2 induces a negative signal to the responding cell. One model proposed the intrahepatic immune response to HCV was based on high PD-1 expression on liver infiltrating Tregs and T effector cells (Radziewicz, Dunham et al. 2009). In this model, PD-1 expressing T effector cells bind to PD-L1 on the surface of apoptotic hepatocytes, and PD-L1 ligation provides an inhibitory signal to T effector cells, causing Tregs to proliferate to

decrease T effector cell mediated liver injury. Upregulation of PD-1 follows, and PD-1 signaling leads to a defect in IL-2 signaling in Tregs, effectively reducing Treg proliferation and expansion(Franceschini, Paroli et al. 2009). Reducing intrahepatic Tregs worsens the clinical outcome in HCV infected patients by diminishing the normal homeostatic balance of Tregs and allowing unchecked, damaging responses to the liver by T cells.

There are several pathways controlling regulatory immune responses during viral infections, and some of these control mechanisms cause T cell dysregulation and alterations in the natural homeostatic balance following some viral infections. A marker of inducible Tregs is CTLA4 (CD152), which acts as a negative regulator of T cell responses by binding CD80 and CD86 to terminate or prevent further co-stimulation (Rudd 2009). Viral infections have been shown to cause loss of CTLA4 expression on Tregs, and the mechanism by which this occurs has been described by Rowe et al, in which the T regulatory cell set point is overridden or reaches signal zero (Rowe, Ertelt et al. 2012). Necessary factors for immune activation and mobilization of effector T cells following overriding the Treg set point have been documented. These include a) increased cell surface marker expression, including costimulatory molecule expression (CD80, CD86) or MHC expression b) secretion of mediators which promote maturation of dendritic cells including IL-6 c) activation of other pro-inflammatory cytokines which induce DC maturation through IL-6 independent means (such as PAMP stimulation). Additionally, adaptive responses may also be reduced following viral infections, explained by decreased expression of costimulatory factors or a decrease in antigen presenting cell costimulatory function (Rowe, Ertelt et al. 2012).

Asking these questions is not only important for an understanding of fundamental

immunologic pathways at the maternal fetal interface, but elucidating the significance of this unique immunological interface is necessary for vaccine development and treatment of microbial infections. One may also question how the precise mechanisms of local uterine immunosuppression will be elucidated due to the vast number of immunomodulatory molecules with constitutive and inducible functions acting in concert within complex cellular feedback loops. Mediators most often recognized to provide local immune suppression in the placenta involve: IL-10, TGF-β, and progesterone. One may also question if these molecules are capable of promoting a suppressor phenotype independently or complex immune interactions within the placenta provide a greater contribution to local immune suppression. Although IL-10 expression in the normal pregnant uterus is associated with suppressive function, IL-10 producing T cells are also generated *in vivo* during systemic infections. Pregnancy-induced alterations in normal uterine defense mechanisms may increase the likelihood of pathogen survival. Sequelae include an increased susceptibility of the fetus to primary and possibly secondary pathogen exposure, possibly promoting a pro-inflammatory cytokine profile directed towards defending against pathogens and skewing this response away from tolerogenic immune responses. The generation of IL-6 and IL-21 in the presence of TGF-β inhibit Tregs while driving Th17 differentiation, promoting a proinflammatory environment (Rowe 2013). One hypothesis is that the pathogen is indirectly responsible for inducing natural T regulatory cells to induced T regulatory cells (iTreg) by generating IL-10, one of the mediators which is known to promote conversion of natural T regulatory cells into inducible Tregs (Belkaid 2007). In the peripheral circulation, inducible T regs are known to express the high affinity IL-2 receptor CD25, Foxp3, CTLA4, glucocorticoid induced tumor necrosis family receptor (GITR), $\alpha_E\beta_7$-integrin (CD103), GITR (glucorticoid-

induced tumor necrosis factor family-related gene/protein) and neuropilin-1 (Wing, Fehervari et al. 2006). TGF-β is another molecule believed to have the potential to induce FOXP3+ Treg cells. TGF-β is expressed as an inactive complex in many immune and nonimmune cells. Activation into functional TGF-β is a tightly controlled process that provides regulation of TGF-β in numerous processes, but one of the most critical roles is signaling during adaptive immune processes. It is believed some pathogens target sites in which TGF-β is highly produced, possibly promoting pathogen persistence. Gastrointestinal tract, skin, and eye are proposed sites with high TGF-β expression and retention of T cells in peripheral tissues has been linked to TGF-β expression. (Belkaid 2007). One example of TGF-β's role in T cell retention has been explained in the gastrointestinal tract. TGF-β mediated induction of GPR15, the G protein-coupled receptor in Tregs, has been shown to be required for homing of Tregs to the large intestine (Travis and Sheppard 2014). Likewise, the placenta is a natural source of TGF-β (and IL-10 during certain stages of gestation), and this TGF-β-rich microenvironment is speculated to be responsible for the activation of natural T reg to induced T regs (Munoz-Suano, Hamilton et al. 2011). Studies of this cytokine and growth factor rich, endocrinologically-mediated microenvironment may provide insight into how normal immune function is regulated at this site. Addition of an infectious agent adds further complexity to this system.

Placentation

The placenta is often not given due credit in the literature, and there are often simplistic descriptors of the placenta as a tissue or series of membranes connecting the mother and fetus, not accounting for the complexity of this highly specialized organ. The placenta is a complex organ which has evolved to provide diverse roles in homeostatic regulatory functions for the

fetus. This organ provides numerous physiologic functions for the fetus during pregnancy, including: gas function which is later replaced by the fetal lung, excretory functions to include water balance and pH regulation, catabolic and resorptive functions later maintained by the gastrointestinal tract, synthetic and secretory functions of endocrine glands, metabolic and secretory functions of the liver, hematopoiesis of bone marrow during early pregnancy, heat transfer of skin, and immunological function (Benirschke K 2006). In contrast, there are a few roles not supported by the placenta including function of the locomotor apparatus and central nervous system of the fetus.

The clade Eutheria comprises the placental mammals (eutherians) and comprises approximately 4000 described species, and approximately 3000 genes are specifically expressed in placentas (Mess and Carter 2007). Although the placental mammals and marsupials are classified into separate intraclasses of eutheria and metatheria, metatherians such as marsupials also have placentae (Ferner and Mess 2011). The marsupial placenta is very short-lived, previously believed to be associated with the necessity for the short duration of pregnancy, in order to avoid immune detection by the maternal immune system. The trophoblast has little role in marsupial development, unlike eutherian mammals in which the trophoblast is important for maternal recognition of pregnancy and mediating immune responses at the maternal fetal interface.

Allantochorial placentas are the most common placental type in mammals. This type of placenta develops from the blastocyst wall and becomes vascularized by the allantois. Choriovitelline placentas develop from the blastocyst, but are vascularized by the yolk sac. Allantochorial placentas have the following features: 1) an outer shape and surface extension

around the chorionic sac 2) intricate interdigitation of maternal and fetal tissues 3) ordered structure forming tissue layers separating maternal and fetal blood 4) spatial arrangement of maternal and fetal vessels or of blood flow directions, respectively (Benirschke K 2006).

Placentas were categorized to describe the structure and composition by Strahl (Carter and Mess 2010) and Grosser (Kaufmann 1992), and this pioneering work was also reviewed by Enders (Enders 1965) and Ramsey (Ramsey 1985), providing an introduction to the concept of the maternofetal barrier. The placental barrier has also been described as the interhemal membrane, separating maternal and fetal blood. Descriptions of placental barrier types are based upon patterns of fetal trophoblast invasion, representing a continuum of degrees of invasion from minimal to only a superficial degree of maternal epithelial erosion observed with epitheliochorial-types and highly invasive patterns of erosion into the maternal endothelium for hemochorial-types of placentation. The number of placental layers serves as the basis for classification of three primary placental types: hemochorial, endotheliochorial, and epitheliochorial.

Hemochorial placentas are characterized by a marked degree of trophoblast invasion where trophoblasts migrate through the maternal endometrial epithelial layer, endometrial stroma, and invade maternal vascular endothelium. The trophoblasts of hemochorial placentas are the most invasive when compared to other placental types, enabling apposition of the trophoblast with maternal blood. Species with hemochorial placenta include rodents, lagomorphs, some insectivores, bats, aquatic herbivorous mammals such as manatees in the order Sirenia (Benirschke K 2006), and also primates and humans (Enders 1965). In 1965, Enders proposed further subdivision of hemochorial placentas based on the number of

trophoblastic epithelial layers into hemotrichorial (representing the rat, mouse, and hamster), hemodichorial (found in the beaver and rabbit placentas during the first trimester of human pregnancy), hemomonochorial (caviomorph rodents, guinea pigs, and the human placenta at term). Lastly, endothelioendothelial and hemoendothelial subtypes have been described in insectivores and bats (Wimsatt 1958).

The endotheliochorial type of placentation is described as an ancestral form of placentation for the eutheria (Carter and Mess 2007). Species with this placentation type include carnivores (Bjorkman 1973), insectivores (Wooding, Morgan et al. 1994), lower primates (Kaufmann 1985), tree shrews (Family Tupaiidae) (Luckhardt, Kaufmann et al. 1985), and bats (Wimsatt 1958). There is a direct apposition of trophoblast and maternal endothelium in endotheliochorial placentas, resulting from deeper invasive potential of the trophoblast and subsequent removal of endometrial connective tissue (Benirschke K 2006). The barrier between maternal and fetal blood is composed of 3 cellular and 2 modified tissue layers, forming endothelium, mesenchyme, and trophoblast on the fetal side, which are opposed by interstitium and endothelium on the maternal side (Mossman 1991), (Wooding, Morgan et al. 1994).

Domestic animal species with epitheliochorial types of placentation include horses, sheep, goats, and pigs (King, Atkinson et al. 1982). The epitheliochorial placenta is a non-invasive type of placentation representing a complete placental barrier, in which maternal and fetal blood are separated by 6 tissue layers. Maternal blood is lined on the maternal side by endothelium, endometrial connective tissue, and endometrial epithelium. The maternal endometrial endometrium lies directly adjacent to the layer of fetal trophoblast, followed by two more fetal layers, connective tissue and endothelium. A subdivision within epitheliochorial

placentation types includes the synepitheliochorial barrier, in which syncytia of maternal epithelial cells and fetal trophoblasts is demonstrated in ruminants (Wooding 1992). Further separation of placental types occurs between sheep/goats and cattle and largely relates to a difference in the timing for retention of fetomaternal syncytia. In sheep and goats, it has been determined syncytial formation is a prominent and continual process throughout gestation (Wooding, Morgan et al. 1994). These syncytia persist in sheep and goats, unlike cattle in which syncytial formation is a transient event. Syncytial formation in cattle is a short-lived process in contrast to sheep and goats, partially due to more rapid syncytial degradation and a rapid turnover of endometrial epithelial cells in cattle.

The shape of placentas has changed throughout evolutionary history to provide increased efficiency, allowing for increased surface area for maternal and fetal contact. Increased surface area is accomplished by anatomical modifications permitting maternal and fetal interdigitation, which can be observed in widespread areas or localized areas of the placenta. Increased surface area permits increased exchange of gases, nutrients, and waste. The chorion is divided into two types, chorion laeve and chorion frondosum. Chorion laeve is smooth and not capable of providing a function of material exchange with the mother, unlike the highly branching chorion frondosum. These anatomical modifications lead to variations in placental shape, resulting in four types of contact areas between the chorion and maternal endometrium: diffuse, cotyledonary, zonary, and discoid. The chorion frondosum represents the entire chorion in the mare and sow. This specialized chorionic surface is classified as a diffuse placenta, and this placental type provides a large surface area for maternal and fetal exchange. Ruminants possess a cotyledonary placenta, which is often referred to as a multiplex placental type. Cotyledons

represent discrete, nodular proliferations of chorion frondosum lined by vascularized villous trophoblasts. Chorion frondosum of cotyledonary areas infiltrate into maternal caruncles, which are localized areas of the non-glandular endometrium. The combined maternal-fetal unit of the cotyledon and caruncle is called the placentome. Fetal cotyledons begin to associate with maternal caruncles early in gestation, reported by day 35 in ewes and day 45 in cattle (Telugu 2008). The number of caruncles is species specific and the number is related to caruncular size, with three to eight caruncles described for deer which are larger than caruncles of domestic ruminants with approximately 20 to 150 in sheep, goats, and cattle (Telugu 2008). In domestic animal species, zonary placentation types are represented by carnivores. The placenta of the domestic cat provides the best representation of a typical endotheliochorial placenta (Enders 2009). Zonary placentas have a distinct band of chorion that surrounds the middle of the fetus to provide intimate contact between the fetus and uterus. Discoid placental types are represented by higher primates and rodents in which discrete, flattened, circular aggregates of chorion frondosum, or discs, are in intimate contact with maternal endothelium.

Although there are few anatomic differences regarding the number of intact cell layers between species with epitheliochorial placentas, differences in fetomaternal interdigitation, placental shape, and trophoblast patterns of invasion do exist among horses, pigs, goats, sheep, and cattle. Swine placentas are viewed as the simplest type, with a diffuse arrangement of chorionic villi over the entire surface of the luminal uterine epithelium (King, Atkinson et al. 1982). This large area of surface contact may seem desirable, but it has been reported that there is a correlation of the size of the litter in sows and gilts with the ability of histotrophic factors to communicate between the mother and fetus. Histotroph refers to the collection of uterine factors

primarily synthesized by the endometrial glands. These uterine factors include adhesion molecules, enzymes, cytokines, growth factors, ions glucose, transport proteins, and hormones (Leiser and Koob 1993). Sows require multiple fetuses, in the range of 5 to 6 per litter, for optimal placental development and growth. In this case, the large surface area does not increase efficiency of placental exchange. Horses also have a diffuse placenta but there are differences between horse and pig placentas based upon the invasive potential of the trophoblast in horses, which is absent in swine. Equid trophoblasts, or chorionic girdle cells, migrate into the uterine endometrium transiently and form endometrial cups. The equine placenta also has modified its diffuse architecture by formation of microcotyledons, which are localized regions of contact between the chorionic and uterine epithelium.

Although ruminants have very similar placentas, structural and functional differences exist between sheep/goats and cattle. One anatomic difference between placentas of sheep/goats and the cow is the shape of placentomes, which are convex in cattle, concave in sheep and goats, and flat in antelopes (Telugu 2008). Ruminant placentas have a cotyledonary/multiplex and villous pattern of epitheliochorial placentation, which describes 6 cellular and tissue layers separating maternal and fetal blood. This epitheliochorial type of placenta was first described by Grosser in 1909 and 1927 (Kaufmann 1992), and modified by Wooding, *et al*, to include the order Artiodactylae (ruminant ungulates, swine, horses, and camels) (Wooding 1982), (Wooding 1982). This type of placentation was associated with more recently evolved placental types in which the blastocyst did not invade the endometrium, but only remained attached to it superficially; a distinction from invasive endotheliochorial and hemochorial placentas. Microscopic and autoradiographic analysis of the ruminant placenta has allowed reclassification

of ruminant placentas into a distinct subtype. The synepitheliochorial subtype of epitheliochorial placentation is described as an adaptive modification of this maternal-fetal barrier in which the modifier "syn-" represents the fetomaternal syncytium and epitheliochorial signifies the persistence of a maternal epithelium at the fetal-maternal interface in ruminants (Wooding 1992). Uterine epithelium can be altered but remains intact for the most part in this system. The process of fetal trophoblast binucleate cell fusion (BNC) with the trophoblast epithelium uninucleate cell (UNC) differs between cattle/deer and sheep/goat placentas. The basic process results in BNC fusion with uterine epithelial cells to produce short-lived or transiently detected trinucleate cells in cattle. Trinucleate cells of cattle are resorbed following exocytosis of trinucleate cell secretory products into the maternal stroma, followed by a fairly rapid turnover of uterine epithelial cells. In sheep and goats, extensive migration and fusion of BNC with uterine epithelia produce a syncytial layer, derived from maternal and fetal cell types (Wooding, Morgan et al. 1994). The original description of this process was described by Wooding: In cattle and deer, “The BNC plasma membrane on the fetal side of the tight function is resorbed by the trophoblast UNC. The microvillar junction (MVJ) reforms and the trinucleate cell formed by the injection of BNC contents, releases the BNC granules to the maternal side by exocytosis, then dies and is resorbed by the trophoblast.” In the sheep and goat, “the continuous injection of BNC content forms a persistent fetomaternal syncytium (“uterine derivative”) replacing the original uterine epithelial cells” (Wooding, Morgan et al. 1994). In cattle, previous descriptions have concluded extensive syncytia are not present beyond day 40 of gestation (King, Atkinson et al. 1979). Gestational days following day 40 correspond with the generation of short-lived trinucleate cells associated with more rapid uterine epithelial cell turnover in cattle (Wooding and Wathes 1980).

There are four basic patterns of trophoblast interaction with the uterine epithelium. Trophoblast and uterine epithelial interactions initially require two basic steps for initiation of this process: loss of microvilli from these cells and subsequent cellular fusion events (Wooding, Morgan et al. 1994). The four basic patterns of trophoblast-uterine epithelial interactions described by Wooding, include: simple interdigitation of microvilli, displacement of, fusion with, or intrusion through the uterine epithelium (Wooding and Wathes 1980). Ruminants follow trophoblast-uterine fusion events during the process of implantation. The initial uterine basement membrane persists through the fusion process, but it has been described as being attenuated by processes emanating from syncytial plaques. During implantation, ruminants and other species with epitheliochorial types of placentation have minimal change within the stroma of the uterine mucosa, resulting in a non-deciduate endometrium. Non-deciduate placentas correspond with trophoblast function as minimally to non-invasive. Although minimal stromal changes occur in ruminants, local vascular changes do occur to increase blood flow and nutrient transport. Angiogenesis is responsible for expansion of the vascular bed in ruminants. In contrast, development of hemochorial placentas corresponds with differentiation of the endometrium into the decidua and the transformation of the uterine spiral arteries. This transformation is associated with extravillous trophoblast migration and replacement of the tunica intima and media of the spiral artery, resulting in expansion of the vessel diameter and lowered intervillous blood pressure (Benirschke K 2006). Caruncles in non-pregnant cattle (small, raised, non-glandular areas 0.5 to 1 cm diameter) become more prominent during the estrous cycle, and reach a diameter up to 10 cm during pregnancy. Bovine caruncles range from 80 to 140 and have a distinct stalk, unlike ewes with 80-100 caruncles and a concave shape with

broad attachments (Dantzer V 2007). Bovine chorion becomes opposed to endometrium around the 17th day of gestation. Adhesive contact occurs by day 18 with the proliferation of trophoblastic papillae (first near the uterine disc), which penetrate the openings of uterine glands. On day 36 in cattle, villous processes develop on the chorioallantoic membrane (cotyledons) in opposition to the caruncles (Dantzer V 2007). Primary villous processes extend from the chorion as finger-like projections towards the caruncles. As gestation proceeds, secondary and tertiary villi ramify from primary villi to form intricate interdigitations with endometrial epithelium, eventually reaching the deeper caruncular crypts. Chorioallantoic villi consist of vascular allantoic mesenchyme covered by a simple layer of epithelium composed of two cell types, columnar cells with rounded or irregularly shaped nuclei with large nucleoli and binucleate cells. Binucleate giant cells, characteristic of ruminant placentae, are formed from uninuclear trophoblasts. The mechanism by which this occurs has not been entirely elucidated, but several references imply the process of acytokinesis may be responsible, in which the nuclei undergo mitotic division without accompanying cytoplasmic division (Wooding 1992). Epithelial cells of the maternal crypts are cuboidal with spherical nuclei and distinct nucleoli. Among the cuboidal cryptal cells, binucleate cells of trophoblastic origin are present. Interdigitation of microvilli occurs between the trophoblastic and cryptal cells, increasing the maternofetal contact. Binucleate cells release granules by exocytosis into the maternal connective tissue, and their function may be transfer of complex molecules from foetal to maternal tissue. Interplacentomal areas, referred to as intercaruncular areas, separate the placentomes and occupy the majority of the placental surface area. Intercaruncular areas provide a surface for close apposition of maternal epithelium and chorion, but also have great importance because of the location of

uterine glands in these sites. The presence of uterine glands within the subjacent endometrial tissue is associated with a separation of maternal epithelium and chorion. The glandular endometrium is responsible for secreting large macromolecules into the uterine lumen by the 4th week of pregnancy. Furthermore, absorptive areas form in the chorion overlying the uterine glands suggesting that glandular regions are involved in transport of larger, less soluble nutrients. Following parturition, the chorionic villi are withdrawn from the crypts. Separation occurs at the interdigitation of the microvilli, and the foetal and maternal epithelia remain intact. When fetal villi separate from maternal crypts, expulsion of the foetal membranes occurs.

Products secreted by the bovine placenta include growth hormone (GH), prolactin, prolactin-related proteins, and placental lactogen. Placental hormone secretion in ruminants, similar to other mammals, include hormones related in structure to pituitary growth hormone (GH) and prolactin (Anthony, Liang et al. 1995), (Soares 2004), (Forsyth and Wallis 2002), (Goellner, Schubert et al. 2006). Placental growth hormone-like activity has also been described and growth hormone receptors (GHRs) are expressed in the bovine placenta (Scott, Kessler et al. 1992), (Kolle, Sinowatz et al. 1997). However, placental GH has not been detected in cattle (Gootwine 2004).

Microarray analysis of approximately 4000 mRNA clones isolated from bovine placental tissue identified approximately 2000 genes (Ishiwata, Katsuma et al. 2003). These findings strongly contrast extensive information collected previously concerning sheep GH, in which two primary GH transcripts encode two GH proteins (Lacroix, Jammes et al. 1996). Prolactin related protein genes have been described in the bovine placenta (Scott, Kessler et al. 1992). Prolactin is a type of somatomammotropin with structural and functional similarities to pituitary prolactin

(Soares 2004). Bovine prolactin-related proteins include nine proteins (I-IX) and placental lactogen (PL) also falls into this category (Ushizawa, Takahashi et al. 2005), (Ushizawa, Takahashi et al. 2007). Placental lactogens have various functions according to species, and placental and ovarian steroidogenesis, lactogenesis, and mammary gland development are primary roles (Talamantes, Ogren et al. 1980), (Colosi, Ogren et al. 1988). Placental lactogens were previously labeled as chorionic somatomammotropin. It has been shown there is a closer relationship of PL to prolactin/growth hormone family and pituitary prolactin than GH (Colosi, Ogren et al. 1988). Placental lactogens are believed to alter maternal metabolism to accommodate growth and development of the fetus. The stage of gestation has been estimated based on the level of peripheral plasma placental lactogen, but plasma levels cannot be used to estimate fetal number (Patel, Hirako et al. 1996). Bovine prolactin related protein (PRP) genes have several sources, but primary expression is found in bovine trophoblast cells from day 17 to term, especially in binucleate trophoblast cells (Hashizume, Ushizawa et al. 2007), and within the endometrium of the gravid horn (Yamada, Todoroki et al. 2002).

Pregnancy associated glycoproteins (PAG) of cattle, sheep, and goats have been grouped based on phylogenetic analysis (Garbayo, Green et al. 2000), (Hughes 2000). At least 21 distinct, full length cDNAs representing bovine PAG members had been cloned from cattle by 2006 (Hughes 2000), (Xie, Green et al. 1997). Uninucleate and binucleate trophoblasts secrete several PAG molecules throughout gestation. PAGs are expressed exclusively in the placenta of species in the Artiodactyla order, and at least 22 transcribed genes within the PAG gene family have been described (Telugu, Walker et al. 2009). Prior to day 28, BNG cells synthesize the pregnancy associated glycoproteins (PAG). Detection of PAG-1 from the dam's serum is

feasible due to the release of binucleate cell products into the maternal blood supply, and Pregnancy Specific Protein B (PSPB), is used as a confirmatory test for pregnancy (Sasser and Ruder 1987). PAG-1 is also referred to as pregnancy-specific protein B (PSPB) (Sasser and Ruder 1987). and pregnancy serum protein of MW 60kDA (PSP60) (Mialon, Camous et al. 1993). Additionally, PAG-1 also is a marker for trophoblast viability and proliferation in ruminants.

The Type I IFN, IFN-tau (IFNT), has been known as the major signal of pregnancy recognition or conceptus-maternal signaling in ruminants for many years (Roberts, Farin et al. 1990), (Hernandez-Ledezma, Sikes et al. 1992). This Type 1 interferon is exclusively found in ruminants, and is expressed within the bovine trophoectoderm from days 12 to 24 of pregnancy (Roberts, Farin et al. 1990). During embryogenesis, IFNT is exclusively produced by the mononuclear trophectoderm cells of the elongating conceptus during the pre-implantation period (Roberts, Farin et al. 1990). Properties of IFNT include: anti-luteolytic, spatial and temporal regulation of endometrial genes (Bazer, Spencer et al. 2009). Roberts described the primary function of IFNT in maintenance of pregnancy, by extending the functional lifespan of the corpus luteum (CL) to continue to produce progesterone, and promote endometrial homeostasis to support development of the embryo (Roberts 2007). He goes onto explain prevention of CL regression is accomplished by the ability of IFNT to prevent the release of prostaglandin F2α. INFT is most closely related to IFN-ω (IFN-omega/IFNW). The evolutionary relationship of IFNT and IFNW has been described as these IFN genes arose from interferon-α (IFNA) around 130 million years ago, approximately corresponding with the origins of mammals (Roberts, Ezashi et al. 2003). Furthermore, ruminant artiodactyls emerged approximately 36 million years

ago, which is believed to correspond with the approximate time for a duplication event leading to separation of IFNW and IFNT genes (Roberts, Ezashi et al. 2003). Gene reorganization from this duplication event was found by this group of experts to be the process leading directly or indirectly toward loss of viral responsiveness and expression of trophoblastic protein expression (Roberts, Liu et al. 1998). Primary cultures of bovine endometrial cells have been examined to identify candidate genes associated with IFNT gene regulation. IFNT was determined to regulate MSX1 (muscle segment homeobox gene family, a transcriptional repressor during embryogenesis) and CXCR7 (C-X-C motif receptor 7); and the following genes were determined to be IFNT gene targets: PTN (pleiotrophin), PLAC8 (placenta-specific 8), CXCL12 (C-X-C motif chemokine ligand 12) (Mansouri-Attia, Aubert et al. 2009).

Galectins are a family of endogenous lectins that play a role in diverse physiologic, developmental, cell signaling, and innate and adaptive immune responses. Placentomes and interplacentomal regions of the endometrium were examined for galectin expression from abattoir-collected placentas during bovine pregnancy from early to late gestation (Froehlich, Hambruch et al. 2012). In this study, galectins 1 and 3 were identified in interplacentomal regions while galectin 4 and 9 were found in placentomes. Furthermore, galectin-1 expression was observed in stromal cells and early gestation trophoblast giant cells, and galectin-3 expression was restricted to uterine epithelium. Galectin-4 expression within the endometrium of placentomes was localized to uterine epithelium and blood vessel walls. Uterine epithelial cells and late gestation trophoblast giant cells expressed galectin-9.

Placental specific gene transcription in cattle has been examined by oligonucleotide array examining gene expression profiles of the caruncular and intercaruncular areas of cattle in an

effort to compare differences in these regions by examining day 20 of the estrous cycle and implantation (Mansouri-Attia, Aubert et al. 2009). This study determined the immune response was more strongly impacted in caruncular regions than intercaruncular regions during pregnancy, and intercotyledonary areas showed greater alterations in metabolic function during pregnancy. Endometrial glands are crucial for the development of the conceptus and progesterone is a direct regulator of uterine receptivity to implantation (Gray, Bartol et al. 2001). Bovine primary endometrial cell cultures of glandular epithelial cells and fibroblast origin were used to identify gene expression of specific gene targets by RT-PCR, and *in situ* hybridization was used to confirm cellular localization. *In situ* hybridization indicated luminal epithelium during pregnancy was a prominent site for expression of C11ORF34 (chromosome 11 open reading frame) and MX1 (MX dynamin-like GTPase/type I interferon-induced myxovirus resistance protein) (Mansouri-Attia, Aubert et al. 2009). This study also related these gene expression patterns to IFNT target genes and IFNT-regulated genes, based on treatment with two concentrations of ovine recombinant IFNT. PTN (pleiotrophin), PLAC8 (placenta specific 8), and CXCL12 (C-X-C motif chemokine ligand 12) were identified as interferon- (IFNT) target genes and MSX1 and CXCR7 were noted IFNT-regulated genes, whereas C11ORF34 was not an IFNT-regulated gene.

Trophoblasts

Ruminants possess two morphologically distinct populations of fetal trophoblast cell types. Fetal uninucleate (mononucleate) and binucleate trophoblasts (Wooding 1992). The binuclear trophoblasts are referred to as fetal trophoblast cells which originate from the uninucleate trophoblast and migrate from the polarized layer of epithelial cells at the blastocyst

stage (trophoectoderm) to fuse with maternal uterine epithelial cells. The extraembryonic components of the placenta are formed by trophoectoderm (Rossant and Croy 1985). The secretory fetal binucleate cell contains intracytoplasmic granules which are released by exocytosis in the direction of the underlying maternal capillary beds (Wooding 1992). Mononucleate trophoblast and binucleate cells are both capable of expressing bovine pregnancy associated glycoprotein-2 (boPAG-2), while only BNC have been shown to secrete pregnancy associated glycoprotein-1 (PAG-1) (Hughes 2000) and placental lactogen (Duello, Byatt et al. 1986). Maternal trophoblasts or maternal caruncular epithelial cells are rarely described, except by Munson et al in 1988, who regarded maternal endometrial epithelial cells as difficult to cultivate with limited capacity for subculturing and cryopreservation (Munson L 1988). A bovine trophoblast cell culture system without using feeder cells has been isolated from *in vitro* fertilized blastocysts (Hashizume K 2006). This BT-1 cell line continually proliferated in Dulbecco's modified Eagle's/F12 culture medium supplemented with bovine endometrial fibroblast-conditioned medium. Cytokeratin expression, epithelial morphologic features, and dome-like structures (vesicles) were described. This BT-1 cell line was unique, in that cell culture media did not require FGF4 supplementation, and the cell population was capable of differentiated towar d binucleate cells when plated on a collagen substrate. Reverse-transcription polymerase chain reaction (RT-PCR) and microarray were used to confirm trophoblast-specific expression of the gene products: IFN-T, placental lactogen (PL), prolactin-related proteins (PRPs), and pregnancy associated glycoproteins (PAG). Other genes of interest expressed in BT-1 cells included: bovine IL-1α, human IL-18 binding protein c, bovine βA inhibin/activin, bovine U-serpin, human integrins B4 and α6, bovine Msx-1. Another reference described

bovine fetal trophoblasts (chorionic epithelial cells) in additional detail, including a polarized cytotrophoblast cell population and a second population of non-polarized, mostly binucleated trophoblast giant cells (TGCs), which are formed from unidentified stem cells through aytokinetic mitosis (Klisch, Pfarrer et al. 1999). Binucleated trophoblast giant cells migrate through the chorion to fuse with maternal epithelial cells, in a pattern of restricted trophoblast invasion, in which the maternal basement membrane is the deepest layer of binucleate trophoblast giant cell migration (Pfarrer, Hirsch et al. 2003). Adhesion of fetal and maternal epithelial cells to BM may be mediated by collagen receptor $\alpha 2\beta 1$, laminin receptor $\alpha 6\beta 1$, and laminin receptor $\alpha 6\beta 1$ due to the expression pattern of integrin subunits (Pfarrer, Hirsch et al. 2003). In 2007, a bovine caruncular epithelial cell line was described in a polarized cell culture model (Bridger, Menge et al. 2007). In this study, primary epithelial cell lines from caruncles of pregnant cows were subcultured 32 passages and these cells were characterized by demonstrating the intermediate filament, cytokeratin, tight junctional zonula occludens-1, and vimentin. Maternal origin of these caruncular epithelial cells, labeled BCEC-1, was confirmed by fluorescent in-situ hybridization (FISH).

Trophoblasts regulate several critical developmental processes necessary for establishment of the embryo. Gastrulation occurs in ungulates prior to implantation and leads to a flat disc with a long conceptus (Geisert, Brookbank et al. 1982). The bovine embryonic disc is covered by a trophoblast layer expressing both homeodomain-containing transcription factors, FGF4, GATA6, OCT-4, NANOG, ETS2, ASCL2, HAND1, and EOMES (Degrelle, Campion et al. 2005). Both OCT-4 and NANOG are markers of pluripotent embryonic cells, and there is also weak expression of the caudal-related homeobox transcription factor family member, CDX-2, in

trophoblasts covering the embryonic disc. Prior to implantation, the ungulate blastocyst remains detached in the uterus and is followed by a period of blastocyst elongation, which initiates and corresponds with a phase of rapid trophoblast development (Blomberg, Hashizume et al. 2008) and elongation (Chang 1952). Critial factors for early embryonic development in ruminants include TGF-β and EGF (Blomberg, Hashizume et al. 2008). Implantation is initiated when the pre-attachment blastocyst sheds the zona pellucida 8-10 days following fertilization, exposing the trophoectoderm. Subsequently, the embryo transitions from spherical to ovoid to elongate and filamentous (Betteridge, Eaglesome et al. 1980). Pre-contact and blastocyst orientation marks the second phase of implantation. In cows, around gestational day 12, trophoblast elongation is initiated and cellular elongation is evident at gestational day 14 and elongation is complete at 18 days (Guillomot 1995), (Guillomot, Reinaud et al. 1988). During the third phase of implantation, which is apposition of the conceptus, trophoectoderm and endometrial luminal epithelium are attached. The fourth phase of implantation follows, in which there is interdigitation of cytoplasmic projections of trophoectoderm cells and microvilli of the endometrial luminal epithelium (Spencer, Johnson et al. 2007). Implantation in cattle is complete by day 19, which also marks the time for initiation of trophoblast attachment in the region of the embryonic disk and binucleate cell migration begins (King, Atkinson et al. 1980), (Wathes and Wooding 1980). By gestational day 24, the embryonic membrane can extend the entire length of both uterine horns. Maternal and conceptus cross-talk and nutrient exchange are dependent on expansion of the trophoblast during this early phase (Stroband and Van der Lende 1990). The trophoectoderm cell layer in the blastocyst develops into the trophoblast cell lineage, which is destined to form the entire outer epithelial component of the placenta (Cross, Werb et

al. 1994, Rossant and Cross 2001). Trophoblast binucleate cells in ruminants express chorionic somatomammotrophin hormone 1 (CSH1/placental lactogen) of the growth hormone/prolactin gene family (Hashizume K 2006). GATA2 and/or GATA-3 are involved in the regulation of trophoblast-specific gene transcription in bovine trophoblast CT-1 cells (Bai, Sakurai et al. 2011). Bovine binucleate trophoblasts express MHC class I molecules (Bainbridge, Sargent et al. 2001), but following maternal-fetal epithelial cell fusion, trinucleate giant cells no longer express paternal antigens or major leukocyte antigens (Ellis, Sargent et al. 1998). At term, two trophoblast populations have been described, for which the majority express a low to intermediate level of class I genes and evidence class I protein expression (Ellis, Sargent et al. 1998). As this area of research has continued, the distinction between expression of classical versus non-classical MHC-I molecules has been noted, and most species do not express classical MHC-I molecules (Davies, Eldridge et al. 2006). This lack of classical MHC-I expression is attributed to preventing the maternal immune system from recognizing the fetus as foreign. Classical and non-classical MHC-I genes have been identified in the interplacentomal region of the bovine placenta, but expression patterns have varied between pregnancies. Classical to non-classical gene expression was theorized to be regulated by the MHC haplotypes carried by the fetus (Davies, Eldridge et al. 2006). Increased non-classical MHC-I gene expression may be protective and lessen the likelihood of fetal loss.

For humans, the trophoblast cell type that comes in closest contact to the fetus is the extravillous trophoblast, which is highly invasive and it invades deep into the maternal uterine wall (Moffett-King 2002). The diverse roles of the trophoblasts often go unrecognized, and only recently has the trophoblast been recognized as being capable of immune recognition and

immune signaling, providing an essential role in maternal-fetal communication. In 2004, Moffett and Loke wrote a summary describing unique trophoblast features, and this short paper was titled, “The Immunological Paradox of Pregnancy: A Reappraisal". They intended to persuade immunologists to discontinue their pattern of overlooking trophoblasts as immune cells (Moffett and Loke 2004). In this publication, “The Nature of the Trophoblast,” the trophoblast is described as a normal healthy, extraembryonic, not somatic cell that will form part of the embryo’s trophoectoderm. This paper clearly stated that conventional beliefs concerning the immunological relationship of the mother and fetus as a model for transplantation immunology are no longer valid. Furthermore, the authors explained how there is no similarity of the mother and fetus to the four models of immunological recognition. These models are: 1) the “self/non-self” model where B and T lymphocyte receptors are specific for a foreign entity and self-reactive lymphocytes are deleted by the fetus (Burnet 1969), (Medawar 1956); 2) “self/infectious non-self“ model discriminated by pattern-associated molecular patterns (PAMP) and germ-line encoded pattern recognition receptors (PRR) described by Janeway in 1992 (Janeway 1992); 3) the NK cell-MHC I inhibitory receptor recognizing ligands on normal cells describing the “missing self” model (Karre, Ljunggren et al. 1986); and 4) the “danger hypothesis” described by Matzinger in 1994 (Matzinger 1994), in which immune cells express receptors for products displayed on the surface of damaged cells. Other notable characteristics of trophoblasts mentioned by Loke and Moffett are more specific and distinctive characteristics, including: expression of endogenous retroviral products, expression of oncofetal proteins, expression of imprinted genes, unmethylated DNA, and unusual MHC (HLA-C, -E, and G), invasive properties, and finally frequent giant cells and syncytial formation. This list is worth mentioning

because several of these topics have been recently described for bovine placentas and/or trophoblasts (Moffett and Loke 2004).

Innate immunomodulatory functions are described for human trophoblasts, including inhibition of complement activity. One of these mechanisms includes DO/DAF (CD55) prevents complement attack and becomes incorporated into the trophoblast. Complement production is often associated with the capability of antigen presenting cells to provide protective functions of innate immunity in the face of antigenic challenge; however, other cell types may potentially have this ability. Production of complement components by human first trimester trophoblast cells (cytotrophoblast) and the trophoblast cell line HTR8/SVneo has been described (Bulla, Bossi et al. 2009). Complement components 3 (C3) and 4 (C4) were synthesized by both the trophoblast cell line and freshly collected cytotrophoblast cells. The addition of IFN-γ to cultures resulted in upregulation of C3 in cytotrophoblasts and C4 in HTR8/SVneo cells and cytotrophoblasts. Innate immunity in the female reproductive tract also involves antimicrobial proteins and peptides. These low molecular weight proteins with antiviral, antibacterial, and antifungal activity are prevalent in the female reproductive tract, and act to form an innate mucosal immune barrier. Some of these key molecules include: lysozyme, lactoferrin, and defensins. Human β-defensins 1-4 (HBD1-4), human α defensin-5, elafin, and SLPI (secreted leukocyte protease inhibitor) have been documented to be expressed by epithelial cells of the female reproductive tract in non-pregnancy (Wira, Fahey et al. 2010). SLPI continues to exert antimicrobial action in cervix, endometrium, and decidua during implantation and pregnancy (King, Kelly et al. 2007).

Trophoblasts inhibit NK cells by expressing NK cell ligands. One of the first mechanisms

describing tolerance included the altered HLA molecule expression in order to avoid cytolytic attack on the fetus. Other examples of trophoblastic expression of NK cell ligands, include: EVT expression of HLA class I molecules: HLA-C, HLA-E, HLA-G. HLA-E expressed on trophoblasts, interacts with CD94/NKG2 on NK cells (Loke and King 2000). MHC Classs Ia or Class II molecules are not expressed on the cell layer of the trophoblast in contact with maternal tissues. Following *in vitro* stimulation of co-cultures of PBMC and JEG-3 cells (trophoblast-like cells), it was shown that direct cell to cell contact of CD56+ NK cells and JEG-3 cells altered levels of intracellular TNF-α (Ntrivalas, Kwak-Kim et al. 2006). Down-regulation of intracellular CD56+/TNF-α (+) cell levels was induced by JEG-3 cells, a mechanism that may help trophoblasts escape from NK cell cytotoxicity (Ntrivalas, Kwak-Kim et al. 2006).

Trophoblasts avoid immune attack by cytotoxic T cells, and this is achieved by altering typical patterns of MHC expression, achieved by expressing non-classical MHC I molecules instead of classical MHC I forms. The missing self-hypothesis indicates trophoblasts should be susceptible to NK killing, and this contradicts the frequently used statement "all nucleated cells express MHC class I molecules." More specifically, "trophoblasts express the classical MHC class I molecule, human leukocyte antigen (HLA)-C and express non-classical MHC class I molecules HLA-C, G, E. This is a simplified description of different expression patterns of MHC, which are dependent on the phenotype and differentiation state of the trophoblast . Human placental HLA-G expression has been shown to be restricted to differentiated cytotrophoblasts (McMaster, Librach et al. 1995) and extravillous trophoblasts (Tilburgs, Crespo et al. 2015). This sophisticated system of trophoblast immune evasion from NK cell and cytotoxic T lymphocyte killing was clearly explained when HLA-E, expressed on the surface of

trophoblasts, was identified as the ligand for the NK cell receptor CD94/NKG2 (Loke and King 2000). This study indicated inhibition of decidual NK cell cytotoxicity was observed when ligation of HLA-E and CD94/NKG2 occurred.

Trophoblasts also suppress class Ia production, but express the class Ib molecule, HLA-G (McMaster, Librach et al. 1995). One function of HLA-G expression by trophoblasts is invasion, evidenced by this study which showed HLA-G is expressed only by cytotrophoblasts that invade the uterus. HLA-G expression levels were greatest in first and second trimester placentas, while virtually absent in term cytotrophoblasts. Later studies have expanded on the role of HLA-G in trophoblast invasion during implantation. Several immune cells are important for implantation, but none are more critical for this process than the uterine NK cell (uNK). Functional studies have shown membrane bound HLA-G stimulates proliferation and cytokine production by NK cells, but downregulation of uterine mononuclear cell responses (van der Meer, Lukassen et al. 2004).

Interstitial trophoblasts found within the decidua express high levels of FasL, which has been regarded as an appropriate arrangement due to the close proximity of this cell population to maternal leukocytes (Hammer, Blaschitz et al. 1999). Fas/FasL interactions are also involved in placental growth, primarily in the villous portion of the placenta (Hammer, Blaschitz et al. 1999). In this area, FasL is primarily located on cytotrophoblast cells, while low FasL expression is observed on syncytiotrophoblasts. Trophoblasts are susceptible to Fas/FasL-mediated apoptosis by Th1 cytokines, IFN-γ, and TNF-α, all of which promote Fas expression (Aschkenazi, Straszewski et al. 2002). Conversely, this group also showed Th2cytokines increase the resistance of trophoblasts to Fas-mediated apoptosis.

Trophoblasts are capable of suppressing T cell function by secretion of RANTES/CCL5 (Svinarich, Bitonti et al. 1996). Trophoblasts (Swan 71 trophoblast cell line) express the Th1-type chemokine, RANTES constitutively, and RANTES expression increases when trophoblasts are co-cultured with PBMC, conversely IFN-γ expression was low (Fraccaroli, Alfieri et al. 2009). Further evaluation of this system showed increased expression of IL-12p70, TNF-α, leukemia-inhibitory factor (LIF), nitrate production, along with increased RANTES/CCL5 expression to indicate communication between trophoblasts and leukocytes is consistent with a pro-inflammatory environment under the culture conditions established. Additional information provided from this study indicated RANTES secreted by trophoblast cells specifically suppresses maternal alloactivated T cell proliferation with mixed lymphocyte reaction (MLR) using maternal and paternal PBMC in the presence of serum free conditioned media (SFCM) obtained from the Swan 71 cell line. Suppression by RANTES was further investigated with anti-RANTES neutralizing antibody, which partially prevented suppression (Fraccaroli, Alfieri et al. 2009). Addition of RANTES following the addition of PBMC, increased expression levels of IL-12p70, TNF-α, leukemia-inhibitory factor (LIF), and nitrate production.

Placental Immunology

Immune receptors recognizing distinct pathogen signals such as pathogen associated molecular patterns (PAMPs) are necessary for innate immunity at the maternal-fetal interface, but few descriptions of cell sensors such as pattern recognition receptors (PRR) have been described in cattle. TLR-4 was identified in the bovine endometrium, but the role in local mucosal defense has been unknown (Herath, Fischer et al. 2006). Further understanding of the innate immune defense in the bovine endometrium in 2008 indicated TLRs 1-10 are expressed

by epithelial cells and stroma cells, while epithelial cells expressed TLRs 1-7 and 9 (Davies, Meade et al. 2008). In this study, stromal cells expressed all TLR receptors except TLR 5 and 8. In response to bacterial pattern associated molecular patterns (PAMPs), prostaglandin E2 was expressed by epithelial cells. Treatment with lipopolysaccharide (LPS), induced upregulation of the β-defensins, Tracheal and Lingual Antimicrobial Peptides (TAP and LAP) and the Membrane-associated Mucin, MUC-1. Muc-1 has been shown to regulate decidual antigen presenting cell chemokine and cytokine maturation and expression (Redzovic, Laskarin et al. 2013). Acute phase proteins (haptoglobulin or serum amyloid A) were not increased. The endometrium forms the first line of defense against pathogens and pattern recognition receptors, and other PRR likely play an important role in host defense and distinguishing self from nonself.

Cell types involved in bovine placental innate immunity include uterine NK cells, and neutrophils, along with cytokines/chemokines, and growth factors. Uterine NK cells have been described in sheep but not cattle. Uterine NK cells appear to share similar cytolytic function as granulated metrial gland cells described in other species, including rodents, guinea pigs, rats, but not primates (Peel 1989), (Peel and Stewart 1989). Macrophages have been documented in bovine placentas by Schlafer, et al, who found fetal macrophages (Hofbauer cells described in human placentas) in bovine cotyledonary villi from four months gestation through eight months of pregnancy. Fetal macrophages dramatically increased in frequency the last trimester of pregnancy, and post-partum following caesarian section (Schlafer, Fisher et al. 2000).

A quantitative and functional analysis of neutrophils during mid-gestation in the cow has not been documented. Peripartum and post-partum evaluation in dairy cows has provided some evidence of neutrophil function in the uterus of the cow. Post-partum total leukocyte numbers are

reduced, speculated to be associated with leukocyte infiltration into the uterus and mammary glands (Guidry, Paape et al. 1976). The mechanism for parturition associated decreases in leukocyte numbers is believed to be mediated by the effects of parturition on glucocorticoid receptor expression. A reduction in the level of glucocorticoid receptor expression in blood neutrophils was associated with neutrophilia, leukocytosis, and increased serum cortisol concentrations (Preisler, Weber et al. 2000). In addition to quantitative changes in leukocyte numbers, functional changes have been described for neutrophils peripartum and postpartum.

Cytokines/Chemokines: The microenvironment of the bovine placenta has not been characterized to provide detailed information concerning normal immunoregulatory processes in this synepitheliochorial type of placentation. Chemokines and cytokines in bovine placentas described to date, include: CCL2 (Chemokine (C-C motif) ligand 2 or monocyte chemotactic protein-1) expression in normal bovine placenta has been described (Oliveira, McClellan et al. 2010). In normal cattle at parturition, increased expression of CXCL8, CXCL6, and CXCL2 has been found (Streyl, Kenngott et al. 2012). Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine has been documented to be expressed in the caruncular and trophoblast epithelium of the placentomes, uterine glands, and vasculature of the caruncular area, often in a temporal pattern of reactivity suggesting this molecule may play a role in vascular development of the placenta in cattle (Paulesu, Pfarrer et al. 2012). MIF has also been shown to be an important component of T cell activation. Increased expression of IL-1β, IL-6 mRNA transcripts and the chemokine-IL-8 are present in the endometrium of clinically ill than normal animals (Turner, Cronin et al. 2014).

Growth factors such as TGF-β in the natural microenvironment of the bovine placenta is

anticipated due to the ubiquitous expression of this growth factor in a plethora of tissues. Although little is known about immune regulation in the normal bovine placenta, it is not surprising different isoforms (TGFβ1, TGβ2, and TGFβ3) are present and activated in bovine placentomes, and are secreted by bovine trophoblastic and endometrial epithelial cells in culture (Munson, Wilhite et al. 1996). GM-CSF has been identified immunohistochemically in the endometrium and oviduct and immunoreactive GM-CSF was identified in uterine flushings in pregnant and non-pregnant cattle (de Moraes, Paula-Lopes et al. 1999).

There is minimal information about the leukocyte composition in the pregnant bovine uterus. However, information that has been documented quantitated intraepithelial lymphocyte numbers in uterine epithelium in cyclic and pregnant heifers (Vander Wielen and King 1984). This study documented a decrease in lymphocyte numbers between cycling and pregnant uteri. This reduction in lymphocyte numbers was observed on day 19 and day 27 as the chorion and uterine epithelium developed, while there was an increase in degenerating cells. The concluding statement indicated a reduction in lymphocyte numbers and other potentially damaging cell types were means of altering the maternal immunological status to promote placental attachment and maturation. This hypothesis was further supported by evidence of continued reduction of lymphocyte numbers at the 4^{th} week of gestation, viewed as a time in which there is maternal exposure to chorion, which is potentially antigenic. At 4 weeks gestation, lymphocyte numbers were much lower than those counted at the period of placental attachment on days 19 and 21. Lymphocyte numbers counted during gestational days 19 and 21 were of interest because the numbers were comparable to lymphocyte numbers during the estrous cycle. Correlation with functional alterations during normal phases of placental development were associated with

gestational days 21-23 and days 25 and 27. On day 19, attachment begins in the middle segment of the ipsilateral horn and attachment extends into most of the uterine lumen during the next week (King, Atkinson et al. 1980). Finally, low lymphocyte numbers in the pregnant horn during pregnancy contrasted the high density of intraepithelial lymphocytes found in the contralateral/non-pregnant horn, which contained no chorion. This finding correlates lymphocyte density with placental development and function. In sheep, the phenotype of the normal placenta has been characterized to a greater extent than the immune populations described for cattle. There have also been noted differences between peripheral blood lymphocytes and endometrial lymphocytes (Hansen and Skopets 1992); subsequently, studies in sheep have provided tremendous insight into innate and adaptive immunologic responses and endocrine-mediated regulation of these pathways in ruminants (Hansen 2007), (Hansen, Henkes et al. 2010). Sheep have also been shown to have fluctuating numbers of a γδ-T cells during pregnancy, but the numbers increase later as an activated phenotype occurs during late pregnancy in sheep (Lee, Bazer et al. 1991), (Liu, Gottshall et al. 1997).

The role of gamma delta T cells in bovine placental immunology is likely important based on preliminary research. Cattle and other ruminants also have a unique T cell repertoire in which WC1.1+ and WC1.2+ T cells represent major populations in the blood of cattle in contrast to non-ruminant species (Hein, Dudler et al. 1991). It has been suggested that $WC1.1^{+}$, $WC1.2^{+}$, and $CD14^{+}$ monocytes act as regulatory cells in the peripheral circulation of cattle and $CD4^{+}CD25^{high}Foxp3^{+}$ and $CD4^{+}CD25^{low}$T cells do not function as Treg *ex vivo* (Hoek, Rutten et al. 2009). γδ-T lymphocytes at the placental level have been documented during the early immune response to *Neospora caninum* in the systemic circulation. This study identified a

substantial number of gamma delta T-cell receptors (TCR) immunohistochemically within the caruncles, intermixed with a more substantial population of CD4+ T lymphocytes (Maley, Buxton et al. 2006).

T regulatory cells in bovine placental immune responses were described following experimental inoculation with *Neospora caninum* (Almeria, Araujo et al. 2011), (Maley, Buxton et al. 2006). The study by Almeria et al, 2011 documented an infiltration of CD4+ and CD8+ T cells and a 46–49-fold increase in interferon-γ and interleukin-4 mRNA. Interleukin-12 p40, interleukin-10 and tumour necrosis factor-⟨ were also increased. One of these studies involving *Neospora caninum* showed an upregulation of Foxp3 following intrauterine infection at 110 days gestation (Almeria, Araujo et al. 2011). The significance and role of Foxp3 expression in modulating intra-uterine immune responses cattle has not been determined. T regulatory cells in the placenta of other veterinary species have been documented; the most extensive evaluation has been documented for feline immunodeficiency virus (FIV) infected cats (Coats 2005).

During bovine pregnancy, endometrial macrophages including a large number of CD14+ and CD18+ macrophages, have been localized to the bovine uterine endometrium (Oliveira, McClellan et al. 2010). Bovine whole genome array identified twelve genes characteristic of M2 activated macrophages in this study.

Humoral immune responses during pregnancy are not a topic of common discussion when the subject of local maternal immunity arises. When the secretory immune system (SIS) is evaluated at the maternal-fetal interface, this branch of the humoral immune system pertains to the broader function forming part of the placental barrier in humans. The barrier concept has largely been bypassed in the human literature regarding methods of cellular communication and

transfer between the mother and fetus. An immunohistochemical evaluation of the maternal and fetal portions of the human placenta showed each contained SIS protein elements including the secretory component (SC), J-chain, IgA, IgM, and IgG (Ben-Hur, Gurevich et al. 2001). This study showed the maternal and fetal parts of the placenta each contained two different SIS systems that showed alternative structure and orientation from one another.

The placenta is described as an immune barrier in which there is close apposition of two membranes forming an integrated physiological unit. In humans, there are several mechanisms that have been elucidated which permit the placenta to function as an immunologically privileged site, circumventing maternal rejection of the fetus. Historically, the maternal-fetal interface was described as an immune barrier, preventing bi-directional communication between the mother and fetus. Current evidence disputing this theory is the presence of maternal cells within fetal tissues along with fetal cells in the maternal circulation. Inflammation (Th1/Th2 balance) has also been considered part of this maternal-fetal barrier, which has been considered to be detrimental and a means of preventing maternal recognition of the fetus.

The focus of this investigation was local suppression of the immune system by immunological mediators regulating T cell activation. Immune suppression during pregnancy has classically revolved around endocrine mediators such as progesterone and estrogens, along with the trophoblast and decidua of the pregnant uterus forming a mechanical barrier which prevented activated T cells from the periphery from entering the implantation site (Mor and Cardenas 2010). Reduced immune reactivity during pregnancy has been a well-known requirement for successful pregnancy. However, in humans, this was found to be dependent on the stage of gestation. A Th1 or pro-inflammatory type response is associated with implantation

while mid-gestation a more quiescent immune environment favors continuation of pregnancy, and dominance of a Th2-type response. Current theory in humans states there is modulation of the maternal immune system during pregnancy, but there is no state of maternal immunosuppression (Mor and Cardenas 2010).

Over the years, attempts to explain the relationship between the maternal immune system and the fetus were made by several immunologists involved in the earliest transplantation studies (Brent and Medawar 1966). They asserted that the placenta contains lymphocytes with receptors for non-self, and the placenta also contains allogeneic cells. If these are encountered, there will be elimination of the infectious agent or rejection of the allograft (Billingham, Brent et al. 1953). The basis for this theory was built upon the classical self/non-self-model described by Burnet and Medawar, in which each lymphocyte expresses a single receptor for a foreign antigen and signaling through this triggers an immune response (Burnet 1959), (Burnet 1969). The correlation between the classical self/non-self-model and the maternal-fetal relationship as a model of transplantation has been categorized as Medawar's immunological paradox (Billingham, Brent et al. 1953), based upon his 1953 essay on this subject. Immunological models generated to explain the success of the maternal-fetal interaction have been summarized by Moffett and Loke, 2004, in what they describe as a reappraisal of the immunological paradox of pregnancy (Moffett and Loke 2004). They discuss four previously described models of immunological recognition in the context of the fetal maternal relationship and conclude none of the models correctly explain this relationship. The four models were previously mentioned in regard to immune recognition of trophoblasts, and the four components, include: a) Self/Non-self-described by Burnet in 1959 b) Self/infectious non-self (Janeway 1992) c) Missing Self

(Karre, Ljunggren et al. 1986) d) Danger Hypothesis (Matzinger 1994). Moffett and Loke also clearly state that "pregnancy is also not simply a case of acceptance or rejection like a transplant." In humans, current descriptions of tolerance mechanisms during pregnancy involve temporal regulation of numerous regulatory molecules throughout gestation. Gestational age dictates the overall expression pattern, including the anti-inflammatory Th2 cytokine profile that occurs post implantation (Wegmann, Lin et al. 1993). First and second trimesters are comprised of expanded populations of systemic and decidual CD4+CD25+ regulatory T cells (Aluvihare, Kallikourdis et al. 2004). The period of implantation is regarded as a stage of gestation in which a Th1 type cytokine response predominates over a Th2 type cytokine profile, but during formation of the placenta there is a reversal of this ratio with a dominant Th2 type response. Following implantation and subsequent placental development, this microenvironment is characterized as an immunologically quiescent, but complex period regulated by a plethora of mediators inducing a shift to a Th2 type cytokine response (Dealtry, O'Farrell et al. 2000). Shifting to a Th2 type response initiates activation and modulation of hormones, cytokines, biochemical processes, and cellular factors which promote anti-inflammatory processes to downregulate immune responsiveness in the pregnant uterus towards a state reminiscent of an anergic response (Arck, Hansen et al. 2007). It appears this microenvironment promotes downregulation of local immune responses via expression of a single or combination of expressed molecules or activated cell types. Gain and loss of function studies in mice have been extremely beneficial in determining the importance of individual suppressor molecules on this intricately interwoven signaling network of positive and negative regulatory factors whose function is to reestablish homeostasis to support pregnancy. Many of these experiments have

provided a foundation for elucidation of signaling pathways which have described galectin-1, CD4+CD25+ expressing Foxp3, M2 macrophages. IL-10, active forms of TGF-β, and metabolically regulated mediators such as indoleamine-2,3 dioxygenase (IDO) and arginase as key players in maintaining immune tolerance in the pregnant uterus (Munoz-Suano, Hamilton et al. 2011).

There are several innate and adaptive cell populations for promoting tolerance. Uterine macrophages are one of the more common leukocytes present in human and murine pregnancy, accounting for up to one-third of total leukocytes in the uteri of mice, and macrophages are present from early until late gestation in humans (Gomez-Lopez, Guilbert et al. 2010). The stimulus for macrophage activation and the presence of activating cytokines can polarize macrophages towards two primary pathways of differentiation. Macrophages destined to promote inflammation and cytotoxicity may be induced towards this M1 activation pathway in response to exposure to IFN-γ, TNF-α, and/or LPS. Stimuli for macrophages to follow the alternative, or M2 activation pathway are interleukin-4 (IL4) and -13 (IL13); activation of the M2 pathway is associated with immunosuppression and wound healing (Svensson-Arvelund, Mehta et al. 2015). Macrophages play an important role in modulating immune responses at the maternal-fetal interface (Svensson-Arvelund and Ernerudh 2015), and they are categorized as the major antigen presenting cell in the decidua (Bartmann, Segerer et al. 2014). Human and mice endometrial macrophages have demonstrated markers corresponding to the M2 activation pathway (Brown, von Chamier et al. 2014). If uterine macrophages do follow the M2 pathway during pregnancy, it is feasible for macrophages with this activation status to play a role in regulating immune responses during pregnancy since M2 subpathways, specifically M2c are

important for pregnancy maintenance in humans (Tang, Hu et al. 2015). The M2 activation pathway can be divided into three subpathways. The M2a pathway represents macrophages that are stimulated by IL-13 and IL-4; the M2b pathway involves macrophage differentiation under the influence of immune complexes via TLR and IL-1 receptor; and M2c macrophages differentiate under the influence of IL-10 and TGF-® or glucocorticoid hormoes (Brown, von Chamier et al. 2014). Hofbauer cells are human fetal macrophages found within the placenta, and are considered M2 macrophages (Kim, Romero et al. 2012). Hofbauer cells express IDO (Kudo, Boyd et al. 2004) and B7-2 (CD86) (Petroff and Perchellet 2010). B7-2 is the ligand for two receptors, the positive T cell regulator CD28, and the negative T cell regulator CTLA4. CD28 and T cell receptor complex interactions provide co-stimulatory signals for T cell activation, survival, and expansion. CD28 is the only B7 receptor expressed constitutively on naïve T cells (Petroff and Perchellet 2010). CTLA4 (cytotoxic T lymphocyte-associated molecule-4) is not found on resting lymphocytes. CTLA4 has been shown to have dramatic and rapid effects on downregulating T cell responses, and ligands are similar to CD28. CTLA4 binds both CD80 and CD86 on the surface of antigen presenting cells, and there is higher affinity of the CTLA4 and CD80/CD86 interaction than CD28 and CD80/86 binding (Petroff and Perchellet 2010). CTLA4 engagement with these ligands prevents production of IL-2-α, IL-4, and IFN-γ (Seder, Germain et al. 1994).

Trophoblasts play a large role in regulating CD8+ (CTL) T cell responses in the placenta to avoid cytotoxic responses that are deleterious to the fetus. CD8+T cells bind MHC I molecules on the surface of target cells, not classical paternal MHC antigens. Fetal expression of nonclassical MHC I molecules (HLA-A, HLA-E, and HLA-C) prevents fetal attack. HLA-G

expression by the trophoblast and placenta inhibits MHC-restricted CTL responses (Le Gal, Riteau et al. 1999).

Decidual gamma delta T cells provide a unique ability to promote homeostatic mechanisms controlling immune responses at the maternal fetal interface, partially due to their capability of recognizing unprocessed foreign antigen in the decidua, and the ability of γδ-T cells to divide in this location (Mincheva-Nilsson 2003). Stimuli for gamma delta T cell activation and expansion include recognition of fetal antigen, and exposure to environmental antigens in epithelial sites, and recognition of trophoblast-related antigens (Carding and Egan 2002). The ability of gamma delta T cells to exert cytotoxic functions is enabled by their capability to express five major cytolytic molecules, perforin, granzyme A, granzyme B, granulysin and Fas ligand (Mincheva-Nilsson, Nagaeva et al. 2000). Decidual gamma cells are gamma delta cells originating from the decidua, which are upregulated in early normal pregnancy in women. Gamma delta T cells exert a regulatory function by direct and indirect means, but tolerance promoting effects are gamma delta T cell recognition of non-classical HLA class I molecules by binding of KIRs (CD94) on the fetus ((Barakonyi, Kovacs et al. 2002). Imunosuppressive effects attributed to gamma delta T cells are direct inhibition of effector cells. through IL-10 and TGF-beta secretion. Conversely, IL-10 and TGF-beta secretion by gamma delta T cells may stimulate Th0 CD4+ Tcells to secrete IL-10, favoring generation of Tr1 cells. TGF-beta secretion by Th0 CD4+ T cells promotes Tr3 cell induction. The cytokine products of both Tr1 and Tr3 can indirectly suppress effector cell populations to limit immune responses (Mincheva-Nilsson 2003).

There is a B cell type that may play a role in Th2 immune responses in the placenta, the

regulatory B cell (Fettke, Schumacher et al. 2014). These regulatory B cells produce TGF-beta and IL-10 and are called B10 cells (Bregs in humans). IL-10 is believed to be important for modulating maternal-fetal immune responses, including preserving pregnancy following administration of lipopolysaccharide (LPS). IL-10 deficient mice have an increased rate of miscarriage (Robertson, Skinner et al. 2006) and proinflammatory effects of injected LPS were not observed when recombinant IL-10 was administered (Rivera, Olister et al. 1998). However, IL-10 is not essential for successful allogeneic pregnancies of IL10- null mutant ($Il10^{-/-}$) mice (Svensson, Arvola et al. 2001).

Uterine Natural Killer Cell-T lymphocytes (uNK) are distributed in much higher numbers in early versus late gestation, supporting previous evidence NK cells are important for placental development, implantation, and cellular communication with trophoblasts. At the time of implantation CD56 bright uNK are the predominate leukocyte population in the decidua. This population of uNK cells ubiquitously express natural-killer group 2 member D (NKG2D) and 2B4 (CD244) receptors (Apps, Gardner et al. 2008). NKG2D is a C-type lectin-like molecule, which functions as an activating receptor expressed by NK cells and T cell subsets (Raulet 2003). 2B4 (CD244) belongs to the family of signaling lymphocyte-activation molecules (SLAM) (Ma, Nichols et al. 2007). 2B4 is also expressed by some T cell subsets (Trinchieri, Kubin et al. 1993) and binds CD48 (Brown TI 2005). uNK also have killer cell immunoglobulin-like receptors (KIR) and leukocyte immunoglobulin-like receptors (LILR). Uterine NK cells in humans and mice have been shown to behave with hematopoietic stem cell-like properties, and the uterine microenvironment may represent a unique niche for progenitor cells (Manaster, Mizrahi et al. 2008). Other unique features of the decidual leukocyte population include

descriptions of a distinctive type of NK cell (comprising approximately 70%) described as a large granular lymphocyte/large granulated NK cell ($CD56^{bright}$/CD16-), while the remaining leukocyte populations were estimated to be composed of ~15% T cells and ~15% monocytes (Red-Horse, Drake et al. 2001). In contrast to peripheral NK cells, decidual NK cells have been previously characterized to possess limited cytolytic activity (Ferry, Starkey et al. 1990). More recent evidence suggests cytolytic activity may be correlated with the amount of intracytoplasmic granulolysin. Differential expression of natural cytotoxicity receptors by decidual NK cells (possess NKp46-, NKp30-, and NKp44-) is also observed in contrast to peripheral blood NK cells (which are devoid of NKp44-activating receptors) (El Costa, Tabiasco et al. 2009). The authors of this study concluded cytokine, chemokine, angiogenic factor secretion, and cytotoxic potential of human decidual NK cells is dependent on the specific engagement of natural cytotoxicity receptors. Differential expression of natural cytotoxicity receptors by decidual NK cells, possessing NKp46-, NKp30-, and NKp44-, has been shown in contrast to peripheral blood NK cells, which are devoid of NKp44-activating receptors (El Costa, Tabiasco et al. 2009).

When microbial disease is not present, most descriptions of the effect of T regulatory cells on various organ systems describe them as protective against inflammation induced immunopathology often observed with immune-mediated reactions and autoimmune diseases. Suppressor activity of Foxp3 has been debated in some species due to findings suggesting the molecule may be transiently expressed, possibly representing a state of activation. It has been shown that Foxp3 is not necessary for regulatory cell activity, mainly due to finding suppressor function in CD8+ T cells, gamma delta T cells, and other leukocytes. T regulatory cells (Tregs)

are necessary for successful pregnancy in humans and mice. Abortion could be induced in mice with allogenic pregnancy but not with syngeneic pregnancy with Treg depletion (Aluvihare, Kallikourdis et al. 2004). In 2005, it was shown adoptive transfer of Tregs prevented fetal rejection in abortion prone mice (Zenclussen 2005), (Leber, Teles et al. 2010). Using anti-CD25 monoclonal antibody treatment, a series of experiments provided evidence Tregs are necessary during implantation and early allogeneic pregnancy, but this study did not provide evidence for the necessity of Tregs in late allogeneic pregnancy (Shima, Sasaki et al. 2010). In humans, decreased decidual T reg numbers have been associated with higher miscarriage rates (Sasaki, Sakai et al. 2004).

Generation and expansion of CD4+CD25+ T regulatory cells during pregnancy: A single mechanism to account for generation and expansion of T regulatory cells during pregnancy has not been found to date. It appears some of the factors associated with pregnancy associated increases in CD4+CD25+ cell populations include hormonal regulated expansion by estrogen (Tai, Wang et al. 2008), while data collected from other studies has shown no correlation between estrogen and/or progesterone on the frequencies of Treg numbers in ovariectomized mice (Tai, Wang et al. 2008). Researchers have shown maternal exposure to seminal fluid induces expansion of Tregs, (Zenclussen, Thuere et al. 2010), due to exposure to paternal antigens and also cytokines and other potential immune-modulating components present in seminal fluid (Robertson, Guerin et al. 2009). Paternal antigens have been shown to induce Treg expansion by antigen-dependent and antigen-independent mechanisms. Antigen specificity for T reg expansion following exposure to seminal fluid has been documented. A target of cytotoxic T lymphocytes (CTL) is fetal inherited paternal alloantigen, producing, HLA (van Kampen,

Versteeg-van der Voort Maarschalk et al. 2001) and minor HLA antigen specific CTL (Verdijk, Kloosterman et al. 2004). A difference in Treg expansion has been observed between allogeneic and syngeneic pregnancies, in which allogenic combinations of paternal antigens promote a greater increase in Treg cell expansion (Aluvihare, Kallikourdis et al. 2005), (Robertson, Guerin et al. 2009, Zenclussen, Thuere et al. 2010). Finally, the fetus itself has been shown to be an inducer of CD4+CD25 Tregs, as fetal antigen specific T regulatory cells (Kahn and Baltimore 2010).

Uterine Dendritic cells (uDC) populations represent approximately 1-2% of the decidua basalis and decidua parietalis are populated by uDC (Kammerer, Schoppet et al. 2000). Although dendritic cells represent a small population of cells within the decidua, their numbers do not reflect their importance. Dendritic cells are critical for pregnancy, evidenced by uterine dendritic cell depletion studies which resulted in a severely impaired implantation process (Pollard 2008). Conditional ablation of uterine dendritic cells (uDC) in a transgenic mouse model was also performed, and investigators observed an impaired proliferation and differentiation of the decidua, even in the absence of embryos (Plaks, Birnberg et al. 2008). This study indicated dendritic cells were necessary for critical pathways involving TGF-β and FMS-like tyrosine kinase I signaling, a receptor for vascular endothelial growth factor (VEGF). Outside of the period of implantation, the immunoregulatory properties of uDC were investigated due to their important role in the systemic immune system to act as "sentinels" in innate immunity, in addition to positive and negative immune regulators of the adaptive immune system of humans, with the hope of understanding the role of dendritic cells in mediating maternal tolerance to fetal antigens. The functional activity of dendritic cells has been correlated with their state of

differentiation within their "life cycle", from an immature state into a maturation program until senescence, primarily initiated through stimulation by exogenous antigens. DC are resistant to maturation induced by TLR ligands or by signaling through CD40 (Morelli and Thomson 2007). The outcome of immune regulation, manifested either as immune activation, downregulation, dysregulation, or a lack of immune responsiveness may be attributed to the diversity of responses dendritic cells are capable of eliciting. This broad functional capability and the diversity of dendritic cell subsets are a function of the state of maturation (Boonstra, Asselin-Paturel et al. 2003). Regarding decidual dendritic cells, it has been questioned whether a special subpopulation of "tolerance inducing" antigen presenting cells may be responsible for skewing immune responses towards a state of tolerance. Immature or "semi-mature" states could promote tolerance inducing DC in the mucosa. Dendritic cells have been described as pivotal for tolerance in gastrointestinal models of oral tolerance (Weiner 2001); (Alpan, Rudomen et al. 2001). *In vitro* cytokines such as IL-10 (Buelens, Willems et al. 1995) and anti-inflammatory mediators such as glucocorticoids promote a conversion of dendritic cells into promoters of Th2-type responses (Piemonti, Monti et al. 1999). Tolerogenic DC are associated with production of large amounts of IL-10 and IDO, but low amounts of IL-12p70 and TNF-α (Grohmann, Fallarino et al. 2003), (Mellor and Munn 2004). This subpopulation also expresses low levels of HLA-DR, CD80, and CD86 (Morelli and Thomson 2007). With regard to Tregs, tolerogenic DC are capable of iliciting Treg expansion but show an impaired ability to expand CD4+ effector cells (Morelli and Thomson 2007).

Immunological responses in the pregnant uterus are most commonly performed following isolation from tissue, such as the decidua of humans. However, humans have tremendous

capabilities for making prenatal diagnosis useful for both diagnostic and therapeutic purposes. Amniocentesis and chorionic villus sampling enables practitioners with a multitude of testing possibilities, including the ability to diagnose microbial infections and to perform genetic and immunologic analyses. Since the immunologic homeostatic balance is crucial for tolerance during pregnancy, alterations in this complex immune regulatory network may produce consequences for the fetus. Cytokine alterations during pregnancy are implicated in several disorders including eclampsia. The ability to sample amniotic fluid has provided evidence of pro-inflammatory cytokine alterations. Additionally, potential suppressors of cytokines associated with a pro-inflammatory profile have also been detected in amniotic fluid, including an IL-23 binding protein (Herway, Bongiovanni et al. 2009). This protein was found in amniotic fluid from mid-trimester when the Th2-type responses predominate over Th1-type mediators in the decidua, corresponding with the proposed potential role described by these authors of this IL-23 binding protein as a suppressor of intra-amniotic neutrophil-derived inflammation.

Six of the seven B7 family members are expressed in the placenta: the ligands, B7-2 (CD86), B7-H2, B7-H1 (PD-L1), B7-DC (PD-L2) are expressed on both RNA and protein levels, while expression of B7-H3 and B7-H4 RNA has been documented in the placenta (Petroff, Chen et al. 2002). B7-H2 only exerts positive regulatory effects on T cells, B7-H4's only influence T cells in a negative regulatory role. The remainder of B7 family members listed above may have either a positive or a negative effect on T cells. B7-H1 is expressed throughout gestation in humans, particularly by decidual macrophages and all trophoblast populations. Interestingly, syncytiotrophoblast expression of B7-H1 is higher than the underlying cytotrophoblast expression pattern of B7-H1, possibly due to the proximity of the

syncytiotrophoblast to maternal blood (Petroff, Chen et al. 2002). The author speculates the degree and temporal expression of B7-H1 in trophoblast subpopulations throughout gestation are believed to be associated with trophoblast functional requirements and differentiation states. The ligand for the inhibitory receptor of the CD28 family PD-1 is B7-DC. B7-DC is associated with endothelial expression within the villus mesenchymal core, and has implications in tolerance of lymphocytes to self-antigens through B7-DC/B7-H1/PD-1 signaling. B7-H1 and B7-H4 expressed by cytotrophoblasts and syncytiotrophoblasts also promote tolerance during pregnancy. Besides B7-H1 signaling through PD-1, ligation of B7-H1 expressed by trophoblasts with Fas/FasL and IL-10 signaling pathways has been suggested (Dong, Zhu et al. 1999). B7-H4 ligation with B and T Lymphocyte Attenuator (BTLA) activates and induces BTLA4 expression on Th1-type lymphocytes to control Th1 responses (Petroff 2006). B7-H2 is expressed on both syncytiotrophoblast and cytotrophoblast and upon ligation with its stimulatory receptor, ICOS (Inducible Costimulator of T cells), Th2-type effector cell functions are directed by signaling through the B7-H2/ICOS pathway (McAdam, Chang et al. 2000).

In allogeneic murine pregnancy models, indoleamine 2,3-dioxygenase (IDO) has been shown to be necessary for fetal survival (Munn, Zhou et al. 1998). Induction of this tryptophan catabolizing enzyme has been reported to starve T cells of this essential amino acid, preventing T cell proliferation (Moffett and Namboodiri 2003); however, several opinions indicate complete depletion of tryptophan *in vivo* and *in vitro* is unfeasible (Terness, Kallikourdis et al. 2007). It has been shown that tryptophan metabolites, 3-OH-kynurenine and 3-OH-anthranilic acid are primarily involved, while kynurenine and picolinic acid are less important in inhibiting T cell proliferation (Frumento, Rotondo et al. 2002).

Chemokines such as RANTES/CCL5 are expressed by the trophoblast cell line, Swan 71, and RANTES has been described in normal human endometrial tissues and endometriosis affected tissues and cells (Hornung, Ryan et al. 1997). Placental explants from first trimester pregnancies expressed IL-8, IL-6, CCL4, GM-CSF, and CCL2, but when TNF-α was added to culture media there was evidence of increased secretion of CCL5 (2.2 fold), GM-CSF (1.7 fold), and IL-10 (1.8 fold) (Siwetz, Blaschitz et al. 2016). CCR2 is the receptor for the CC chemokine, CCL2. Regulation of Th1 and Th2 type immunity is believed to involve CCL2 and CCR2 interactions (He, He et al. 2012). Furthermore, these authors concluded CCL2 is produced by dendritic stromal cells (DSC), and expression of CCL2 is stimulated by Th2 cytokines in the placenta during early pregnancy. CCL2 is believed to be a strong mediator of Th-2 polarization at the maternal-fetal interface, partially by inducing IL-4 production and reducing IFN-γ secretion (He, He et al. 2012). CCL2 deficient mice maintain their level of IFN-γ and IL-2 expression, but Th2 responses are reduced.

Chemokines and chemokine receptors, including: CCL3, CCL4, and CCL5 are all ligands for CCR5, which is believed to be expressed on effector T cells previously sensitized to antigen, and this CCR5+ T cell population is targeted to inflammatory sites peripheral to secondary lymphoid organs (Tanel, Fonseca et al. 2009). CCL4 is expressed on professional antigen presenting cells following activation, and migration of Th1 cells towards CCL4 has been linked to CTLA4 (Knieke, Hoff et al. 2009). Murine and human Tregs have been shown to produce CCL3 and CCL4 (Patterson, Pesenacker et al. 2016). Production of these chemokines was proposed to be a mechanism of attracting CD4+ and CD8+ T cells to Tregs. Proinflammatory and anti-inflammatory roles have been described for CCR5+ T cell populations, and a

suppressive phenotype is associated with CCR5+ Tregs that accumulate in the uterus during pregnancy (Kallikourdis, Andersen et al. 2007). Foxp3 expression was found on both CCR5+ and CCR5- populations of CD4+CD25+ T cells, but CCR5+ T cells expressed Foxp3 at a slightly lower level. CCR5 negative Treg cells were proposed to impair maternal-fetal tolerance. Kallikourdis, et al summarized CCR5 + CD4+ CD25+ Tregs are highly suppressive, and this population forms the effector arm of regulatory T cells. Activation by paternal antigens was believed to increase the likelihood of CCR5+ Tcell accumulation in the pregnant uterus.

Negative regulators such as costimulatory molecules and their associated ligands PD-1/PD-L1 can play a role in pregnancy loss. One allogenic murine pregnancy model demonstrated PDL1 blockade resulted in increased rejection rates of allogenic concepti but not syngeneic concepti (Guleria, Khosroshahi et al. 2005). Decreased fetal survival and expansion of Th-type 1 immune responses were observed in PDL1 deficient females. An association was described between the PD-1/PD-L1 pathway, decidual stromal cells, and T cells through constitutive expression of PD-L1 by decidual stromal cells, and PD-L1 expression is increased on decidual stromal cells by exogenous TNF-α and IFN-γ (Zhang, Tian et al. 2015). PD-1 expression levels on CD3+ T cells increased in the decidua of first-trimester pregnancy and remained elevated throughout pregnancy (Taglauer, Trikhacheva et al. 2008). There is evidence modulation of T cell responses and maternal-fetal tolerance at the maternal-fetal interface involves the PD-1/PD-L1 pathway (Zhang, Tian et al. 2015). Both Th1 cell expansion *in vivo* and increased allogenic fetal abortions following PD-L1 blockade (Guleria, Khosroshahi et al. 2005).

Endocrine mediated downregulation of immune responses during pregnancy may provide systemic and local immunosuppression. Hormones involved in the stress response cascade,

primarily initiated in the hypothalamic-pituitary axis, exert an adverse influence on T cell function during pregnancy. Corticotrophin releasing hormone (CRH), adrenocorticotrophic releasing hormone (ACTH), cortisol, catecholeamines, neurotrophin nerve growth factor (NGF), and neuropeptides have all been implicated in stress associated neuroendocrine-feedback mechanisms during pregnancy. Neuropeptides such as substance P and vasoactive intestinal peptide (VIP) also play roles in modulating immune responses in the placenta. The immunomodulatory properties of VIP have been largely described as negative regulatory properties to include: inducing a Th2-cell type response and inhibiting cytokine and chemokine expression by macrophages and T cells (Delgado and Ganea 2008). Additional studies have elucidated the potential role of VIP at the maternal fetal interface during pregnancy. It has been suggested VIP promotes an anti- inflammatory environment, supporting pregnancy with a Th2-dominant and tolerance-promoting response. In the associated study, co-cultures of maternal and paternal peripheral blood mononuclear cells (PBMC) with Swan 71 cells and VIP increased T regulatory cell expression, as increased TGF-β and Foxp3 expression was found, along with an elevated proportion of CD4+CD25+Foxp3+ cells within maternal PBMC (Fraccaroli, Alfieri et al. 2009). T cells express receptors for substance P, calcitonin gene-related peptide, somatostatin, and vasoactive intestinal peptide (Lambrecht 2001). Of particular interest is substance P (SP) because several studies have shown SP has immunostimulatory properties. Increased IL-2 production and IL-2 receptor expression in T lymphocytes stimulated by antigen and mitogen stimuli has been observed *in vitro* and *in vivo* following exogenous physiologic doses of SP (Lambrecht 2001). This review paper also indicated that CGRP, somatostatin, and VIP primarily have a suppressive effect on T cell function. Neuropeptides can also promote Th17 activation by

triggering monocytes to secrete IL-23. Substance P mediated abortion has been described (Joachim, Hildebrandt et al. 2001), (Arck, Hansen et al. 2007).

Steroid mediated suppression by estradiol regulates systemic and local immune responses, but has especially important implications for innate immune responses. Estradiol is capable of suppressing pro-inflammatory chemokines and cytokines expressed by epithelial cells of the female reproductive tract, altering innate defenses in the face of pathogen challenge. Evidence this suppressive effect on innate immune function is receptor mediated was explained by experiments with the cervical epithelial cell line (ECC-1) and stimulation with IL-1β. Products of ECC-1 stimulation with IL-1β were IL-8 and human beta defensin-2 (HBD2), and both were blocked upon treatment with estradiol. Substitution of estradiol with an inhibitor of the estradiol receptor, ICI 182780, blocked downregulation of IL-8 and HBD2 following IL-1beta stimulation (Fahey, Wright et al. 2008).(Rukavina and Vince 2000)

Progesterone has a number of interactions with the immune system. A few of the better known immune regulatory functions of progesterone, include: modifying decidual lymphocyte function by downregulating innate immune factors and also upregulating Th1 type immune responses. Downregulation of perforin expression in decidual lymphocytes occurs directly or via progesterone-induced blocking factor (Rukavina and Podack 2000, Rukavina and Vince 2000), (Laskarin, Tokmadzic et al. 2002). Progesterone can upregulate Th1 type chemokines such as CCL5/RANTES production in endometrial CD4 and CD8 lymphocytes (Fraccaroli, Alfieri et al. 2009). At physiological concentrations of progesterone, this group saw an additive effect on the suppression of maternal response of SFCM and progesterone. Lymphocyte proliferation was also inhibited when anti-RANTES antibody specifically prevented the reduction in lymphocyte-

thymidine uptake. The authors speculated RANTES may be a protective response to reduce inflammation induced by exposure to paternal antigens in the presence of the suppressive effects of progesterone (Fraccaroli, Alfieri et al. 2009). Alpha2-macroglobulin, alpha-fetoprotein, interferons, phospholipids and human chorionic gonadotropin have all been implicated in regulating immune responses during pregnancy. A chemotaxis function has been described for hCG at the maternal-fetal interface during very early pregnancy, potentially to attract Tregs (Schumacher, Brachwitz et al. 2009).

Local steroid synthesis in animals is tightly controlled by progesterone. The endometrium and trophoblast directly and indirectly promote placental development and growth during pregnancy by secreting factors necessary for corpora lutea maintence, essentially providing a luteotrophic effect in most animals except dogs and cats. Corpora lutea of dogs, goats, mice, pigs, and rabbits secrete sufficient quantities of progesterone to maintain pregnancy throughout gestation. In contrast, cows, cats, guinea pigs, horses, and sheep can supplement or replace progesterone production of the corpus luteum with placental-derived progesterone. In cows, maternal plasma progesterone levels are maintained at 10 ng/ml through approximately 50 days of gestation when levels slightly reduce until 150 days gestation when a steady progesterone concentration is maintained until parturition approaches. The placenta of cows is able to convert progesterone into estrogens, unlike placentas of ewes, sows, and mares. In most species, placental-derived estrogen synthesis usually begins only after the first fifth or quarter of gestation. Plasma estrogens in cows and ewes remain stable throughout gestation and peak only just before parturition. Goats have levels and patterns of estrogen secretion that vary from other ruminants, in which estrogens increase constantly, reaching levels comparable with those in sows

and mares a few days before full term is reached. Although there is great similarity in placental structure anatomically between ruminants, functional differences may have implications for immunological signaling patterns. Molecules known to have direct and/or indirect roles in inhibiting lymphocyte activation in the sheep placenta, include: IFN-τ, progesterone, prostaglandin E2 (PGE2), and uterine milk protein (uTMP) (Moffatt, Bazer et al. 1987). Steroid hormone regulation of lymphocyte number and distribution and the effect on lymphocyte function during various stages of gestation has not been examined in cattle. Immunological effects of progesterone in cattle, goats, and pigs are unknown, while the uterine serpin remains the major progesterone induced immunoregulatory molecule in sheep (Arck, Hansen et al. 2007). In sheep, uterine serpin blocks lymphocyte proliferation *in vitro*.

Local progesterone-mediated immune suppression is mediated by indirect and direct effects on lymphocyte proliferation and differentiation in humans. Functional suppression of lymphocytes is achieved by: binding of progesterone induced blocking factor (PIBF) to suppress T cell activation (Chien, Chang et al. 2006), increasing IL-4 mRNA expression and IL-4 production in Th1-type lymphocytes (Piccinni, Giudizi et al. 1995), inhibiting mature dendritic cells, or inhibiting dendritic cell-induced T cell proliferation in a receptor mediated fashion (Butts, Shukair et al. 2007). Specific T lymphocytes, notably gamma delta T cells expressing the nature progesterone receptor 31 (nPR31), mediate this action by binding PIBF to suppress T cell activity (Hansen and Liu 1997), (Meeusen, Fox et al. 1993). PIBF induces a Th2-type dominant cytokine response. Additionally, uNK activation and cytotoxicity may be altered by progesterone due to the ability of progesterone to act as a ligand for various NK inhibitory receptors, including progesterone mediated upregulation of HLA-G, one ligand for both NK inhibitory and activating

receptors (Szekeres-Bartho, Halasz et al. 2009) and its ability to alter homing of uNK cells Additionally, progesterone promotes the production of leukemia inhibitory factor (LIF) and macrophage-colony stimulating factor (M-CSF) by T cells (Szekeres-Bartho, Halasz et al. 2009), (Szekeres-Bartho 2009). LIF receptors are produced by several trophoblast types, especially villous syncytiotrophoblast and cytotrophoblast cells (Sharkey, King et al. 1999).

Signals indicating maternal recognition of pregnancy not only provide histotrophic factors to the trophoectoderm to support and maintain pregnancy, some of these factors also provide signals to downregulate immune responses to promote fetal tolerance. Asking these questions is not only important for an understanding of fundamental immunologic pathways at the maternal fetal interface, but elucidating the significance of this unique immunological interface is necessary for vaccine development and treatment of microbial infections. One may also question how the precise mechanisms of local uterine immunosuppression will be elucidated due to the vast number of immunomodulatory molecules with constitutive and inducible functions acting in concert within complex cellular feedback loops. To date a few of the more important cell types for mediating local immune responses in the human placenta are believe to involve: CD4+ CD25+ Foxp3 T regulatory cells, uterine NK cells, decidual antigen presenting cells such as macrophages and dendritic cells, maternal and fetal trophoblasts.

Chapter II: Gene Expression in Placentomes of Normal Heifers and Heifers Carrying BVDV Persistently Infected Fetuses

Abstract

Bovine viral diarrhea virus (BVDV) is a single stranded, positive sense RNA virus within the family *Flaviriridae*. Other members of the *Flaviviridae f*amily include hog cholera virus, border disease virus, and hepatitis C virus (HCV). Some members of the *Flaviviridae* family, such as BVDV and hepatitis C virus, have the ability to evade the host immune response and establish persistent infections. BVDV establishes persistence following *in utero* transmission of a non-cytopathic biotype of the virus across the maternal-fetal interface to the immunologically naïve fetus from gestational days 30 to 125. The hypothesis of this study was that BVDV exploits natural immunosuppressive mechanisms at the maternal-fetal interface to promote virus survival and subsequent persistent fetal infection. Altering the balance of pro-inflammatory and anti-inflammatory mediators decreases the likelihood of a predominant Th1 response, which is associated with decreased fetal survival in humans. *In utero* BVDV infections prior to day 75 typically result in fetal death, and we speculated the virus is able to persist following day 125 due to a natural influx of Tregs into the bovine placenta. To evaluate the effect of BVDV on host gene expression at the maternal-fetal interface following transplacental transmission, pregnant heifers were inoculated with ncp BVDV2 (strain PA131) between 67 and 89 days gestation. Placentomes were collected 70 days post-inoculation with BVDV at gestational day 150 from 11 control and 11 BVDV infected heifers for quantitative real-time PCR (qRT-PCR) using primers for FOXP3, CTLA4, TGF-β, CD25, IL-10, CCL5, CXCL10, and indoleamine-2,3 dioxygenase

(IDO). *In utero* infection with BVDV between 2 to 3-months gestation altered maternal immune surveillance mechanisms by dysregulating T cell-associated responses including T regulatory associated markers FOXP3, CTLA4, and TGF-β ($p<0.05$). This study provides new evidence that 150-day bovine placentomes express immunoregulatory molecules which are involved in T-cell recruitment (CXCL10/IP-10 and CCL5), T cell activation (CD25/IL-2α receptor, IDO), T cell signaling (CTLA4), and T cell regulation (FoxP3, CD25, CTLA4, TGF-β, IL-10). CTLA4 is expressed on activated T cells and a large number of FOXP3 expressing T regulatory cells in humans (Sakaguchi, Miyara et al. 2010). CCL5 (RANTES), IDO, and CXCL10, are pivotal mediators of the immune trafficking and maternal-fetal tolerance in humans and mice.

Introduction

Persistent fetal infection with BVDV has a major epidemiologic impact on the cattle industry because persistently infected calves shed large quantities of the virus into the environment and are a reservoir of infection for cattle naïve to BVDV. The susceptibility of the fetus to persistent fetal infection is greatest during the window of days 30-125, while susceptibility steadily decreases near gestational day 100. However, persistent infections have been reported to occur following infection up to 125-days gestation. Circulating virus during the period of gestation when immunocompetence is developing (90-120 days) is a prerequisite for immunotolerance and persistent infection (Radostits and Littlejohns 1988), due to the immaturity of the fetal immune system, producing a state of immunotolerance. Dams carrying persistently infected fetuses are naïve to BVDV or have non-protective serum antibody titers when exposed to the virus. Conversely, immunocompetent dams clear the virus by mounting a vigorous serum neutralizing antibody response. *In vivo* T cell responses to acute BVDV infections have been

previously evaluated, and depletion of CD4+ but not CD8+ or γδ-T cells was associated with prolonged shedding in transiently infected animals (Howard, Clarke et al. 1992). An *in vitro* model evaluating BVDV-reactive T-cell responses in transiently infected cattle demonstrated seroconversion and virus specific lymphoproliferation were not detected until viremia resolved (Lambot, Douart et al. 1997). This group also found similar rates of seroconversion and viral clearance with both biotypes, but there were differences in the onset of lymphoproliferation which was earlier with cytopathic biotypes than noncytopathic biotypes.

Experiments evaluating type I interferon (IFN) responses to BVDV in the systemic circulation of acutely infected non-pregnant heifers and steers, in addition to *in vitro* studies, conclude ncp BVDV inhibits induction of type I IFN, a central mechanism by which the virus maintains persistence. Acute fetal infections have been documented to induce a pronounced type I IFN response in pregnant cows and fetuses. During acute ncp BVDV infection 3 to 15 days after viral inoculation, there was upregulation of ISG15 mRNA with qRT-PCR in blood of pregnant heifers and TI fetuses (Radostits and Littlejohns 1988). Similarly, in 2012, Smirnova *et al* confirmed there is an early and prolonged upregulation of type I interferon stimulated genes, as cotyledonary tissue showed changes in ISG expression, while fetal blood and caruncular tissue did not show a response (Smirnova, Webb et al. 2012). Immunologic responses, including T cell signaling and IFN induction in heifers and cows maintaining persistently infected fetuses and transiently infected fetuses have been measured through evaluation of maternal and fetal blood samples. The role of BVDV on maternal gene expression in dams carrying persistently infected fetuses has also been evaluated. Microarray analysis of RBC-depleted blood and qRT-PCR on whole blood for chemokine C-X-C motif receptor 4 (CXCR4), a ligand for stromal cell-derived

factor-1 (SDF-1) found that maternal immune responses were altered in dams carrying a persistently infected fetus (Weiner, Smirnova et al. 2012). CXCR4 was downregulated at day 7 post-inoculation with BVDV and remained in downregulation for approximately 3 months postinoculation (gestational day 160). Speculation about an adaptive immune response during persistent BVDV infections was discussed in 2014, when Smirnova *et al* indicated fetal IFN-γ was induced by BVDV in acute infection of transiently infected and persistently infected fetuses (Smirnova, Webb et al. 2014).

The pregnant uterus represents a unique microenvironment maintained by a complex regulatory network of cytokines, chemokines, growth factors, and hormones promoting a presumed state of active immunosuppression. Recent studies in humans indicate a clear distinction between immunoregulatory functions of the uterine microenvironment and the maternal blood during pregnancy. Immunoregulatory functions of some subsets of lymphocytes, NK cells, and macrophages show quantitative and functional differences between the systemic circulation, the pregnant uterus, and even the non-gravid uterus. These quantitative differences in cell phenotypes are no longer solely associated with the immune barrier described at this immune privileged site. Since immune privilege has been adapted to include both intrinsic (placenta, eyes, testes, central nervous system) and acquired classifications, it has become apparent the immune barrier only partially explains mechanisms regulating local responses to foreign antigens, placental inflammatory responses and immunosuppression. Since the 1990's, Th1/Th2 polarization in the maternal blood and decidua of humans and mice has been viewed as the primary cause of pregnancy-associated immunosuppression. This local microenvironment is believed to be maintained by a complex regulatory network that maintains a state of active

immunosuppression, and many of these local controls are believed to be mediated by T regulatory cells (Tregs). Multiple studies in humans and mice have linked cytokine mediated immunosuppression during pregnancy with the immunomodulatory role of Tregs. Although Th1/Th2 bias and T regulatory cells have been extensively researched in the systemic circulation, quantitative and functional analysis of this network and its role in regulating immune responses at the placental level are far less extensive, especially in veterinary medicine. In 2013, Oliveira, *et al* found there was no T cell response to the presence of an embryo in the bovine endometrium (Oliveira, Mansouri-Attia et al. 2013). The NK cell populations examined, NKp46 (CD335+) did not increase, and the number of cells expressing NKp46 was lower in pregnant animals than in cyclic endometrium. There was no evidence of Treg local expansion during implantation, but increased Tregs (CD4+CD25+ T cells) at Days 33-34 of pregnancy in beef heifers was found (Oliveira, Mansouri-Attia et al. 2013). Additionally, Foxp3 expression was not changed during days 5, 7, 13, and 16 of the estrous cycle.

Our experimental trial provides a novel perspective for quantitating phenotypic markers associated with T regulatory cells at the placental level to evaluate the maternal and fetal response to BVDV. We studied several genes previously described as negative regulators of T cell responses (FOXP3, CTLA4, TGF-β, IL-10), Th1-type molecules CCL5 (RANTES), CXCL10 (C-C chemokine, IP-10), CD25 (IL-2-α chain receptor), CXCL10 (IP-10, IFN γ inducible protein), and IDO to more fully evaluate their expression in cattle.

Materials and Methods

Synchronization and Breeding. This work was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University. Twenty-two, approximately

18-month-old heifers were selected and synchronized for pregnancy. Heifers were synchronized for estrus using a controlled internal drug release (CIDR)® protocol designed for beef cattle. All cattle were given 2.0 ml (100 mcg) of gonadorelin diacetate tetrahydrate (Cystorelin®**,** Duluth, GA) intramuscularly to induce follicular ovulation. Additionally, a progesterone-containing insert (EAZI-BREED™ CIDR®**,** Zoetis Animal Health: controlled internal drug releasing device) was placed intravaginally. Seven days later, the CIDR® was removed and 5 ml of prostaglandin $F_2\alpha$ was administered IM (Dynoprost tromethamine; Lutalyse®, Zoetis Animal Health, Kalamazoo, MI). Two days later, day 0, heifers were artificially inseminated with semen from a BVDV seronegative and virus isolation negative bull. Two days after administration of GnRH, two healthy bulls, seronegative and virus isolation negative for BVDV, were placed with the heifers for breeding. Bulls remained with the heifers for 30 days. Pregnancy diagnosis was performed by transrectal palpation 85 days after artificial insemination and 63 days following administration for heifers carrying fetuses obtained by natural breeding. All heifers became pregnant, and fifteen heifers were estimated to be pregnant by artificial insemination, while 7 heifers were classified as bull-serviced pregnancies based on ultrasound measurements of fetal trunk diameters (Curran, Pierson et al. 1986). A fetal trunk diameter of less than 2.69 cm was estimated to be 76 days gestation and derived from natural breeding. Pregnancies produced by artificial insemination were based upon fetal trunk diameters greater than 3.1 cm, and this measurement corresponded to a gestational age of 82 to 86 days. Upon ultrasound confirmation of approximate gestational age, heifers were randomized (and divided into principal (n=11) and control groups (n=11) (Figure 2.1).

Virus propagation and animal inoculation. The target intranasal inoculation for the

principal group was $1.0X10^5$ $CCID_{50}$/ml of the BVDV-2 strain PA131 between 67 and 89 days gestation. The stock BVDV strains used in this study were biologically cloned via successive passages in Madin-Darby bovine kidney (MDBK) cells with subsequent minimal propagation by incubation of the MDBK monolayer for 48 hours at 37 °C and 5% CO_2 to produce an adequate amount of stock virus with the desired $CCID_{50}$/mL for characterization and animal challenge exposure studies. The inocula used consisted of cell culture supernatants containing $1.3 X 10^5$ $CCID_{50}$/mL of BVDV-2 strain PA131 in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% equine serum and 1% L-glutamine. The inocula were obtained following freeze-thaw cycles to disrupt the cells and release the viral particles. Inoculum cell culture supernatant was stored at -80 °C until the day of inoculation. After freezing, an aliquot of the BVDV inoculum was titrated by using the Reed-Muench method to determine the final $CCID_{50}$/mL for inoculation. The inocula were aliquoted (5 mL) in individual tubes within 1 hour of inoculation and stored on ice until use. Inoculation was performed by intranasal aerosolization of 5 mL of inoculum by use of an aerosolizer and a vacuum pump. One sample of each inoculum was transported on ice and returned to the lab to determine the $CCID_{50}$/mL after inoculation.

Sample collection. Maternal blood was collected by jugular venipuncture at days 0, 8, 14, 28, 42, 56, and 70. The uterus, placenta, and fetal spleen, liver, kidney, heart blood, and lung were collected at 150-days gestation immediately after humane slaughter of each heifer (Figure 2.2). Between 20-25 placentomes from each animal were randomly selected and each tissue section collected contained representative samples of the maternal-fetal interface comprising areas where fetal villi and maternal septa would interdigitate microscopically (Figure 2.3). Samples were cut into 5-8 mm^3 pieces or an equivalent of 30mg of tissue and placed in cryotubes

containing RNAlater®(Invitrogen) and stored at 4°C for 24 hours prior to removal of RNAlater® and long term storage at -80°C. Fetal heart blood (2 ml), spleen, and amniotic fluid (2 ml) were obtained for BVDV virus isolation and PCR testing.

Sample preparation. Placentomes were placed in RLT lysis Buffer®(Qiagen) homogenized with 0.5mm zirconium oxide beads in the BBX24 Bullet Blender homogenizer® (NextAdvance,Inc.Averill Park, NY 12018). The supernatant was collected and processed for total RNA with RNeasy mini® (Qiagen). RNA was quantified with the NanoDrop1000® spectrophotometer (ThermoFischer,Wilmington, DE19810) and 1 microgram of RNA was used per reaction for subsequent reverse transcription cDNA preparation with the Transcriptor First Strand cDNA Synthesis kit®(Roche). cDNA was quantified with the NanoDrop1000® (ThermoFischer, Wilmington, DE 19810), then diluted to 200 ng/µl and used for quantitative PCR at 20ng/lµl per reaction (Roche, Light Cycler 2.0®) to quantify gene expression. Primer sequences were identified in current literature (Table 2.1) and purchased from TIB MOLBIOL LLC (PO Box 190 Adelphia, NJ 07710). PCR reactions were performed with the LightCycler® FastStart DNA Master SYBR Green I kit (Roche) and the supplied 25mM $MgCl_2$ was titrated for each primer with the appropriate annealing conditions (Table 2.2).

Virus isolation. For virus isolation, 250 µl of each sample was added to individual 25 cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% equine serum, and L-glutamine was used. After three-days incubation at 37° C and 5% CO_2, flasks were frozen and upon thawing, 50 µl of the cell suspension from each flask was transferred to a 96 well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated 3 days and the

culture medium from each well was transferred to the corresponding well of a new 96-well plate previously seeded with MDBK cells (second plate). Both 96-well plates (first and second) were tested for BVDV antigen by using immunoperoxidase staining, using a bovine BVDV specific polyclonal antibody (B-224).

BVDV serum neutralizing antibody titers. Serum virus neutralization was performed at serial two-fold dilutions of heat-inactivated serum starting at 1:5 through 1:640 using 100 CCID50 of noncytopathic BVDV-1a (SD-1) and BVDV-2 (PA131) and incubated for 1 hour at room temperature before the addition of MDBK cells (1.5 x 105 cells / ml). Cultures were incubated for 72 hours at 37° C in 5% CO2. The VN antibody titer of each sample was determined using the Reed-Muench method (Reed and Muench 1938). The endpoint dilutions reflected the highest dilution of serum that inhibited the replication of virus in cell culture. This was determined by immunoperoxidase (IP) staining using a bovine BVDV specific polyclonal antibody (B-224).

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of placentomes. Following fixation, tissues were processed routinely and embedded in paraffin wax for routine histopathology and immunohistochemistry (IHC). Sections (4 μm) were cut on silane-coated slides and dried. Sections were then dewaxed and rehydrated by sequential immersion in xylene followed by graded concentrations of ethanol, then tap water. IHC was performed with a commercial autostainer (DakoNorth America Inc., Carpinteria, California,USA). Blocking of endogenous peroxidase activity was performed with 3% H_20_2 and sections were pretreated with proteinase K prior to application of primary antibody. The monoclonal antibody (MAb) 15C5 (Syracuse Bioanalytical, East Syracuse, New York,

USA) was utilized, for detection of BVDV antigen. Following incubation with the primary antibody, BVDV antigen was detected using a biotinylated link antibody followed by peroxidase labeled streptavidin (Dako). The substrate was NovaRED (Vector Laboratories, Burlingame, California, USA). The sections were counterstained with haematoxylin and coverslipped under non-aqueous mounting medium. Each BVDV-labelled tissue section was accompanied by a negative control slide in which BVDV antibody was replaced with primary antibody diluent.

Quantification of target genes. The cDNA from each animal (n=22) was amplified simultaneously in one qRT-PCR run (number of capillaries available per run =32). Three technical replicates (separate PCR runs) for each gene were performed, and the crossing points (Cp) for each gene target and housekeeping gene were compiled and averaged for each group. Evaluation of the difference between treatment and control groups was based on using housekeeping genes as an internal normalization method to account for any differences in starting cDNA levels. Quantitative reverse transcriptase PCR (qRT-PCR) data generated with the Light Cycle 2.0 software (Roche®**,** Indianapolis, IN) was expressed in crossing points (Cp) or the threshold cycle (CT), corresponding to the PCR cycle wherein the amount of DNA of the amplified gene generates a fluorescent signal higher than baseline. The CT value therefore correlates negatively to the amount of target mRNA, i.e. the higher the amount of mRNA, the sooner the threshold is reached and the lower the CT value obtained. Results for gene expression were analyzed with the comparative ΔΔCT method for relative gene quantification using the Delta Delta CT difference (ΔΔCT**)** (Livak 2001). ΔΔCT= (CT (Target, Untreated)- CT (Reference, Untreated)) – (CT (Target, Treated) – (CT (Reference, Treated)). Gene expression analysis was performed through the relative quantification of the mRNA level or fold regulation

of target genes normalized to a housekeeping gene (unaffected by the studied conditions) and compared with the uninfected control group. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase subunit A (SDHA) were used as the endogenous reference genes. The efficiency of the qRT-PCR (necessary for the comparative ΔΔCt method), was determined for each target gene by creating a standard amplification curve using 1μg, 100 ng, 10 ng, 1 ng and 0.1 ng per reaction of a cDNA template mix. The cDNA template mix was created by mixing equal amounts of cDNA from placentomes collected from treatment and control groups.

Statistical Analysis. Data were analyzed using JMP statistical software (SAS Campus Drive Building T Cary, NC). Placental data were compared by the student's t-test and differences between groups were considered significant when $P<0.05$. For serum neutralizing titer determination, a log base 2 transformation was applied to virus neutralizing titers before calculation and comparison of the means with repeated measures analysis of variance (ANOVA).

Results

Placental gene expression in BVDV inoculated heifers (treatment group) and uninfected heifers (control group) was performed with real time PCR analysis, and of the genes analyzed the most consistent finding was expression of FOXP3, CCL5 (RANTES), CXCL10 (IP-10), CTLA4, and TGF-β with raw data crossing points less than 30 in placentomes of both principal and control groups (Figure 2.4). CD25 and IL-10 were expressed at lower levels with crossing points of 31-33, respectively, in placentomes of both principal and control groups. Crossing points 32 or lower are considered equivalent to levels of expression of biological relevance. The mean difference reported reflected changes between treatment and controls when the difference

between target genes and the average of the housekeeping genes SHDA and GAPDH were examined (Table 2.3). A significant ($p<.05$) upregulation of Foxp3, CTLA4, and TGF-β was observed following comparison of target genes and the average of housekeeping gene SDHA and GAPDH for the mean Cp values for infected and control animals (Table 2.4 and Figure 2.5).

Fetal spleen and fetal heart blood (serum) virus isolation and PCR on fetal spleen showed corresponding results for three fetuses (Table 2.5). These fetuses, numbers 8043, 8086, and 8119 were virus isolation and PCR positive. These results confirmed that these fetuses were persistently infected with BVDV. Eight fetuses were negative for BVDV by PCR and virus isolation on fetal spleen and fetal heart blood (Table 2.5). Amnionic fluid was also tested for BVDV by standard PCR, and amnionic fluid from the persistently infected fetuses and one additional fetus were positive. There were also additional fetal spleens and amnionic fluid samples with faint PCR positive results (Table 2.5).

Geometric mean titers for the heifer treatment group showed low levels of antibody following challenge (Figure 2.6). Seroconversion was noted in the heifers that would eventually carry a persistently infected fetus (Table 2.6). Titration of the viral inoculum on day 89 (day 0) was $1.0X\ 10^3$.

Discussion

Humans, mice, and cattle share 93 genes in their placentae, mostly related to immune function (based on analysis of gene ontology) (Barreto, Bressan et al. 2011). TGF-β (Munson, Wilhite et al. 1996), MIF (macrophage migration inhibitory factor (MIF) (Paulesu, Pfarrer et al. 2012); and galectin expression (Froehlich, Hambruch et al. 2012) have been previously characterized in the normal bovine placenta. Experimental *Neospora caninum* infections have been evaluated at the

bovine fetal-maternal interface. Almeria *et al* documented maternal and fetal portions of the placenta showed upregulation of Th1, Th2, and Treg cytokines three weeks following intravenous inoculation of *N. caninum* tachyzoites. There was upregulation of interferon-γ, IL-12p40, IL-6, and IL-10 in caruncles of the infected group. Upregulation of IFN-γ and downregulation of TGF-β was identified in fetal cotyledonary tissue (Almeria, Araujo et al. 2011). In a separate study of transplacental neosporosis, a pronounced Th1 type response involving IL-12, IFN-γ, and TNF-α was observed in the placentas during early gestation, but Th1 cytokines were mildly increased in mid-gestation and minimal in late gestation (Canton, Katzer et al. 2014). Information concerning T cell responses following viral infection at the bovine maternal-fetal interface during mid-gestation is sparse. In 2015, Hansen et al expanded on their previous descriptions of innate responses during *in utero* BVDV infections, and included their work on adaptive responses in fetal PI liver, indicating increased expression of MHCI and MHCII in fetal Kupffer cells (Hansen, Smirnova et al. 2015). IFN-γ regulation was proposed to be the likely mechanism behind this increase, which was believed to be a consequence of the MHC I to MHC II ratio. Phagocytic activity was also increased *in vitro* when compared with non-PI Kupffer cells and IFN-γ production increased in PI fetal Kupffer cells in contrast to control Kupffer cells (Hansen, Smirnova et al. 2015). Similarly, fetal liver, spleen, and thymus cells showed a dramatic increase in IFN-γ by day 97 of gestation. Fetal blood contained an increase in IFN-γ mRNA. Adaptive responses described in PI fetal spleen microarray analysis, included: upregulation of T cell receptor gamma 6 (TCRG6), lymphocyte-specific protein tyrosine kinase (LCK), zeta chain TCR associated protein kinase 70 (ZAP70), and natural killer cell receptor 2B4, CD244, delta CD3-TCR complex (CD3D). There was a 2-4.8-fold

upregulation of IFN-γ induced genes in this fetal spleen microarray study, and signal transducer and activator of transcription 1 (STAT1) and the ATP-binding cassette (ABC) transporter; transporter 1, ATP-Binding cassette, (TAP1), which are activated downstream within the IFN-γ signaling pathway. Chemokines upregulated were CXCL10, CXCL16, and CXCR6. Furthermore, IFI16 (a viral restriction factor) was induced. Quantitative RT-PCR on day 97 fetal spleen and blood confirmed upregulation of STAT1, IFI16, CXCL10, CLCL16, and CXCR6 (Hansen, Smirnova et al. 2015).

The data from this study demonstrated that placentomes from 143-165 days gestation heifers constitutively express gene targets with a T regulatory phenotype, based on analysis of Foxp3, TGF-β, CTLA-4, and IL-10 gene expression. At one time, T regulatory cells were classically described as CD4+CD25+, but today a diverse phenotypic profile of T cells fits this classification according to the human literature, including: CD4+CD25+, T regulatory 1 (Tr1), T helper 3 (Th3), CD4+CD25− lymphocyte-activation gene 3+ (LAG3+), CD8+, CD3+CD4−CD8−, γδ TCR+, and natural killer T (NKT) cells (Sakaguchi, Miyara et al. 2010). There is a great deal of literature describing the suppressive role of Foxp3 and the cell types which express this transcription factor in humans and mice. Foxp3 expression in humans, unlike mice, may not be specific for cells with a regulatory phenotype and may only be a consequence of activation status, activation by paternal alloantigen expressed by the fetus, or an anti-fetal response by the T regulatory cell population (Sakaguchi, Miyara et al. 2010). These findings in humans could suggest there are cell types distinct from the CD25 lineage that may be responsible for the expression of Foxp3 in cattle due to the low expression of CD25 (average Cp=31) in placentomes in contrast to Foxp3 expression (average Cp=25). In cattle, Foxp3 expression has

been documented in WC1.1+, WC1.2+ T lymphocytes (workshop clusters 1 and 2) and CD14+ monocytes in *ex vivo* studies with co-culture suppressor assays (Hoek, Rutten et al. 2009). This group also examined Foxp3 expression in CD4+CD25+low T cells, NK cells, and CD8+ T cells, but these cell types did not express Foxp3. Regulatory activity found only the WC1.1+ (T19+/CD163+) and WC1.2+ T cells (gamma delta TCR) and CD14+ monocytes were supported by IL-10 transcription/expression. WC1.1+ and WC1.2+ T cells represent major populations in the blood of cattle in contrast to non-ruminant species. In 2014, Guzman *et al* identified WC1+ and WC1-γδ-T cells expressing IL-10, found in approximately 15% of the proliferating cells in culture (Guzman, Hope et al. 2014). Approximately 50% of this cell population expressed IL-7R and the majority were CD45RO+ (both are proposed markers to identify T cells with regulatory potential). Foxp3 was not detected in expanding IL-10+ γδ T cells. These authors suggested Foxp3 and other markers used to identify T reg populations in humans and mice do not apply to cattle. This is further evidence that the role of Foxp3 expression in modulating peripheral and intra-uterine immune responses in cattle has not been determined. Immunoregulatory cells expressing phenotypic markers for Treg cells have only been recently described at the bovine maternal fetal interface. *Neoospora caninum* infection at 110 days of pregnancy induced upregulation of Th1, Th2 and T-regulatory (Treg) cytokine gene expression in both the maternal and the fetal placenta of the infected group (Almeria, Araujo et al. 2011). IFN-γ, IL-12p40, IL-6 and IL-10 were upregulated in the caruncle, and IFN-γ was reported to be upregulated in the cotyledon. Downregulation of TGF-β, was observed in cotyledons of the infected group. Late gestational cows have increased γδ-T cells in the periphery close to parturition (Oliveira, Mansouri-Attia et al. 2013). It has been speculated that γδ-T cells possibly recirculate from the

uterus to the systemic circulation close to parturition. We have provided data to support the theory of an immunoprotective function of the bovine uterus during pregnancy by describing gene expression profiles in placentomes analyzing regulatory cell phenotypes including IL-10 (produced by Th2 and by activated Th1 cells), TGF-β, CD25 (IL-2αR chain), Foxp3 (primarily expressed by CD4+CD25 T cells), and CTLA-4 (CD152, ligand for CD80 and CD86). For the targets examined, basal expression of FOXP3, CTLA4, and TGF-β had crossing points below 32 in control animals at 150-days gestation with quantitative PCR. Interestingly, despite relevant levels of gene expression, which should correlate with visible evidence of T cell infiltration into placentomes, histopathologic examination did not identify severe inflammation. Theories for this finding may include other cell types (non-leukocytes) that may be contributing to signaling and immune regulation in the placenta, such as dendritic cells, trophoblasts, stromal cells or leukocytes, but may not be clearly evident with immunohistochemical staining. The data from this study suggests transplacental BVDV infection alters normal immunologic parameters in the pregnant uterus at 150-days gestation. Viral induced alterations of normal immune signaling during this gestational window could be a mechanism to promote a state of immunologic non-responsiveness at the maternal fetal interface increasing the possibility of persistent fetal infection.

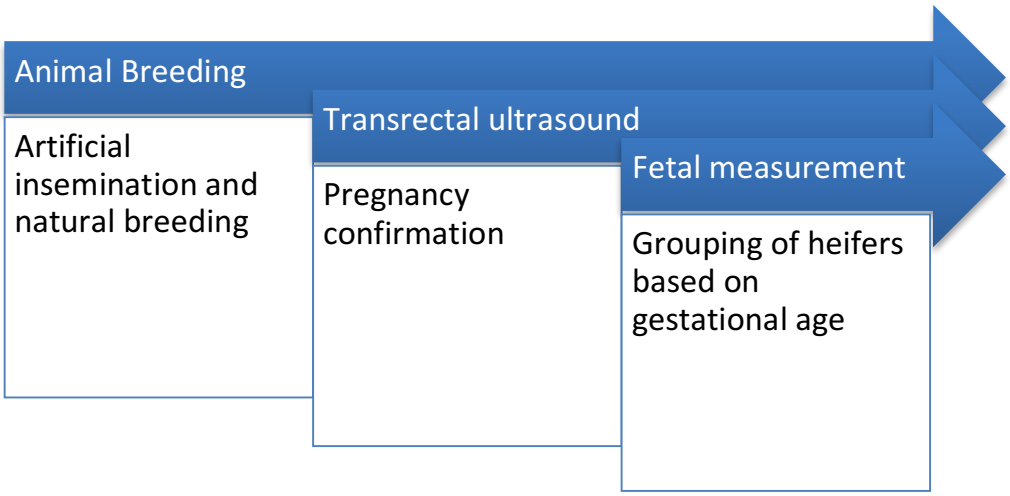

Figure 2.1. Experimental design for animal breeding and pregnancy determination

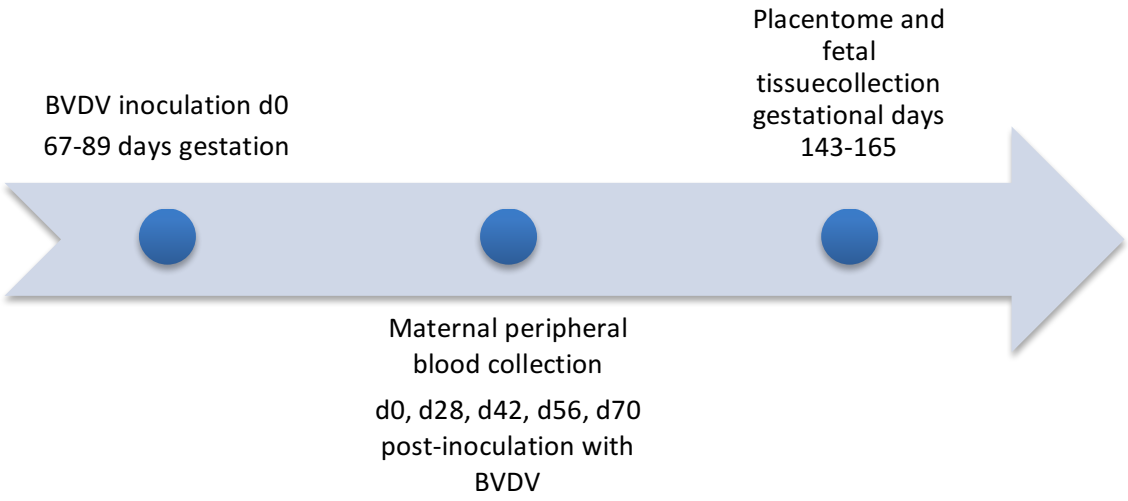

Figure 2.2. Experimental design for virus inoculation and sample collection.

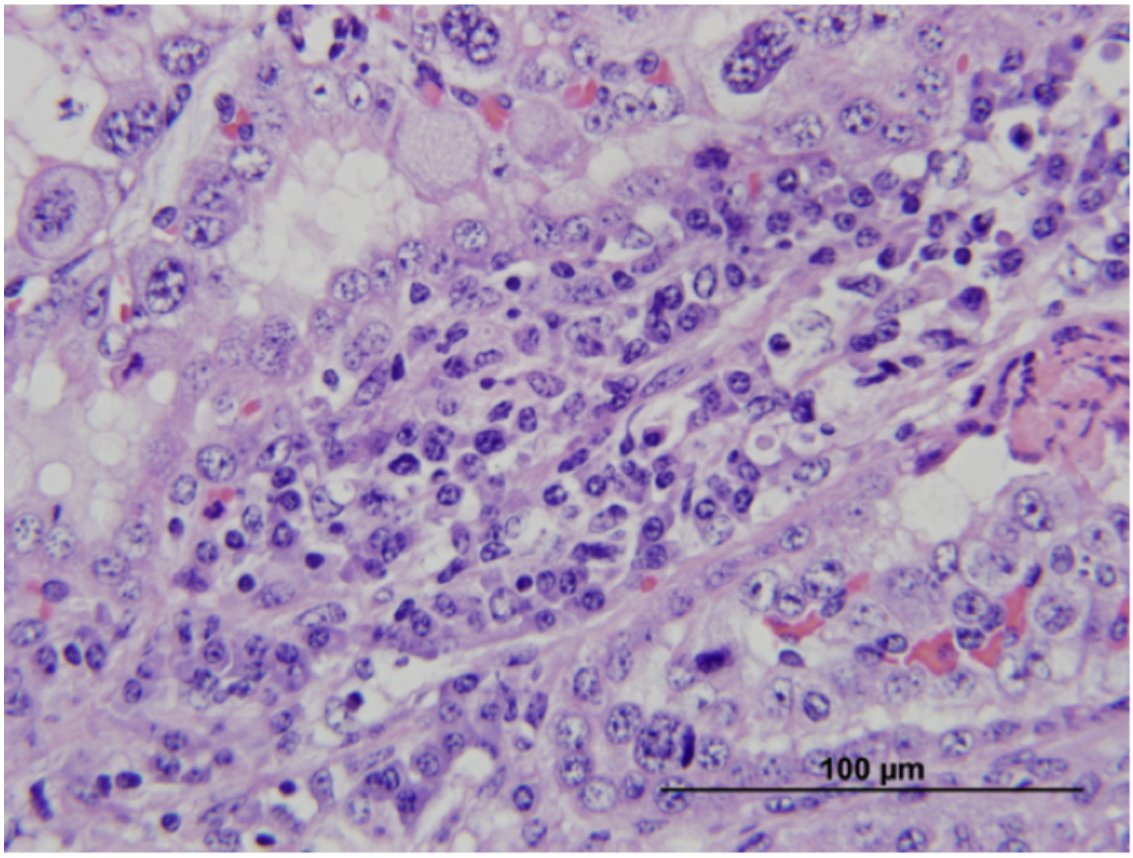

Figure 2.3. Hematoxylin and eosin (H&E) section of a persistently infected fetus (159 days gestation). Rare areas of the placentome contained mild infiltrates of lymphocytes and plasma cells within the stroma lining the single row of maternal caruncular epithelial cells. Fetal trophoblasts are visualized at the top of the photomicrograph with swollen, pale foamy to granular cytoplasm and single nuclei. 40X magnification

Table 2.1

Primers used for quantitative RT-PCR during the mRNA expression analysis of chemokines and T regulatory markers in placentomes from 150-day gestation heifers inoculated with BVDV at gestational day 89

Primers	**Primer Source References**	**Primer Sequence**
CCL5 (RANTES)	Veterinary Immune Reagent Network	F: 5'-CCATGAAGGTCTCTGCCACT-3' R: 3'-CCACCCTAGCTCAACTCCAA-5'
CXCL10 (IP-10)	Veterinary Immune Reagent Network	F: 5'-AGTTGCAGCACCATGAACAA-3' R: 3'-GCTTCTCTCTGGTCCATCCTT-5'
CD25	NM174358 Seo, et al	F: 5'-GCAGGGACCACAAATTTCCA-3' R: 3'-GGTACTCAGTGGTAAATATGAACGTATCC-5'
CTLA4	X93305 Seo, et al	F: 5'-GCAGCCAGGTGACCGAAGT-3' R: 3'-TCATCCAGGAAGGTTAGCTCATC-5'
FOXP3	NM DQ322170 Seo, et al	F: 5'-AAGAGCCCAGGGACAACTTTC-3' R: 3'-GGGTTCAAGGAGGAAGAGGAA-5'
IL-10	U00799 Seo, et al	F: 5'-TTCTGCCCTGCGAAAACAA-3' R: 3'-TCTCTTGGAGCTCACTGAAGACTCT-5'
TGF-β1	M36271 Seo, et al	F: 5'-CATCTGGAGCCTGGATACACAGT-3' R: 3'-GAAGCGCCCGGGTTGT-5'
SDHA	NM 174178 Gossens, et al	F: 5'-GCAGAACCTGATGCTTTGTG-3' R: 3'-CGTAGGAGAGCGTGTGCTT-5'
GAPDH	XM_618013 Gossens, et al	F: 5'-TTCAACGGCACAGTCAAGG-3' R: 3'-ACATACTCAGCACCAGCATCAC-5'
IDO	NM_001101866 XM_001490681.1 Plain, KL, et al	F: 5'-CGAATATACTTGTCTGGTTGG R: 5'-GGAGAACATCAAAGCACTG

Table 2.2

Optimization of primers for quantitative PCR using LightCycler® FastStart DNA Master SYBR Green I on placentomes obtained from 150-day gestation heifers inoculated with BVDV at gestational days 67-89

Primer	**Temperature**	**Microliters of $MgCl_2$**
TGF-β1	73°	1.6
CXCL10	71°	1.6
IL-10	70°	0.8
CD25	67°	0.8
CCL5 (RANTES)	71°	1.6
SDHA	71°	1.6
GAPDH	71°	0.8
FOXP3	71°	0.8
CTLA4 (CD152)	69°	2.4
IDO	61°	0.8

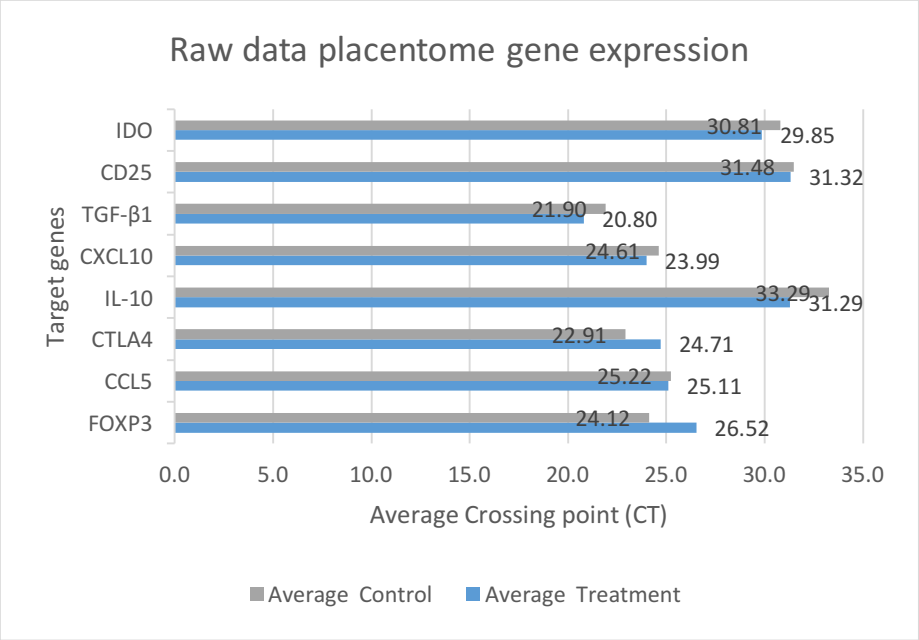

Figure 2.4. Raw Crossing point (CT) values averaged for 11 placentomes in each treatment group.

Table 2.3

Mean change in placentome gene expression for treatment and control groups inoculated between gestational days 67-89. Data are shown as the difference between the target gene and the average of housekeeping genes SDHA and GAPDH

Target	Treatment	Control
FOXP3	5.46	1.34
CCL5	4.05	2.44
CTLA4	3.65	0.14
IL-10	10.22	10.51
CXCL10	2.93	1.83
TGF-β	-0.26	-0.88
CD25	10.26	8.7
IDO	8.78	8.03

Table 2.4

Descriptive statistics for mean change in placentome gene expression for treatment and control groups which were inoculated with BVDV between gestational days 67-89

		Foxp3	**CCL5**	**CTLA4**	**IL-10**	**CXCL10**	**TGF-β**	**CD25**	**IDO**
Mean	**Treatment**	5.46	4.05	3.65	10.22	2.93	-0.262	10.26	8.78
	Control	1.34	2.44	0.138	10.51	1.83	-0.876	8.70	8.03
Std. error	**Treatment**	0.680	0.557	0.631	0.873	0.517	0.197	0.686	0.885
	Control	0.744	0.536	0.744	0.412	0.600	0.343	0.664	0.553
Std. deviation	**Treatment**	2.25	1.85	2.09	2.90	1.72	0.654	2.27	2.93
	Control	2.47	1.78	2.47	1.37	1.99	1.14	2.20	1.83
p-value		0.002	0.082	0.005	0.577	0.358	0.015	0.250	0.470

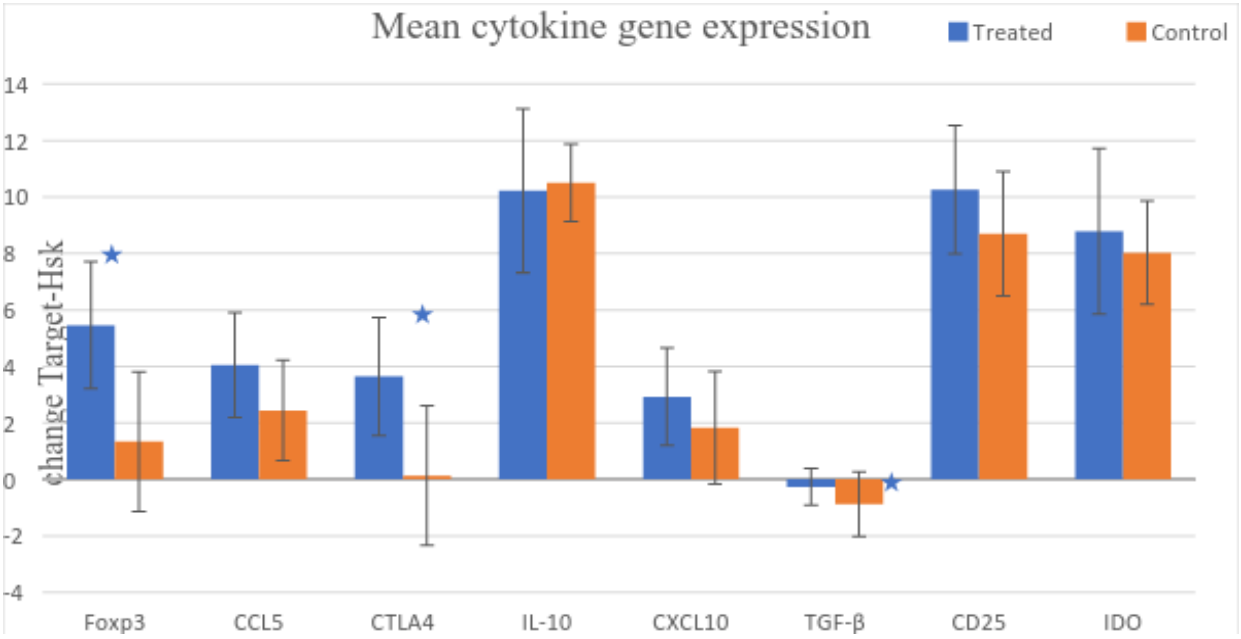

Figure 2.5. Difference in means of treatment and control groups based on the difference between the target gene and the average of housekeeping genes SDHA and GAPDH. Each star (★) indicates significance ($p<0.05$). Error bars are reflective of the standard deviation for each group.

Table 2.5

Summary of fetal virology results including testing of amniotic fluid from fetuses in heifers inoculated with BVDV at 67-89 days gestation and fetal collection occurred on gestational day 143-165

Fetus	VI Spleen	PCR Amniotic Fluid	VI Fetal Heart Serum
8029	NEGATIVE	NEGATIVE	NEGATIVE
8033	NEGATIVE	POSITIVE	POSITIVE
8034	NEGATIVE	POSITIVE	NEGATIVE
8036	NEGATIVE	FAINT	POSITIVE
8043	POSITIVE	POSITIVE	POSITIVE
8044	NEGATIVE	NEGATIVE	POSITIVE
8060	NEGATIVE	NEGATIVE	NEGATIVE
8086	POSITIVE	NEGATIVE	POSITIVE
8109	NEGATIVE	NEGATIVE	POSITIVE
8119	POSITIVE	POSITIVE	POSITIVE
8141	NEGATIVE	FAINT	NEGATIVE

Table 2.6

Geometric mean antibody titers of cows in each treatment group to BVDV noncytopathic strain PA131 which was used as the challenge strain

Treatment Group		Day 0 (challenge)	Day 28	Day 42	Day 56	Day 70
Control	Geometric mean antibody titer	1.0	1.0	1.0	1.0	1.0
	proportion of cows that seroconverted	0%	0%	0%	0%	0%
BVDV Challenged	Geometric mean antibody titer	1.0	1.8	5.5	9.7	9.7
	proportion of cows that seroconverted	0%	18%	27%	45%	45%

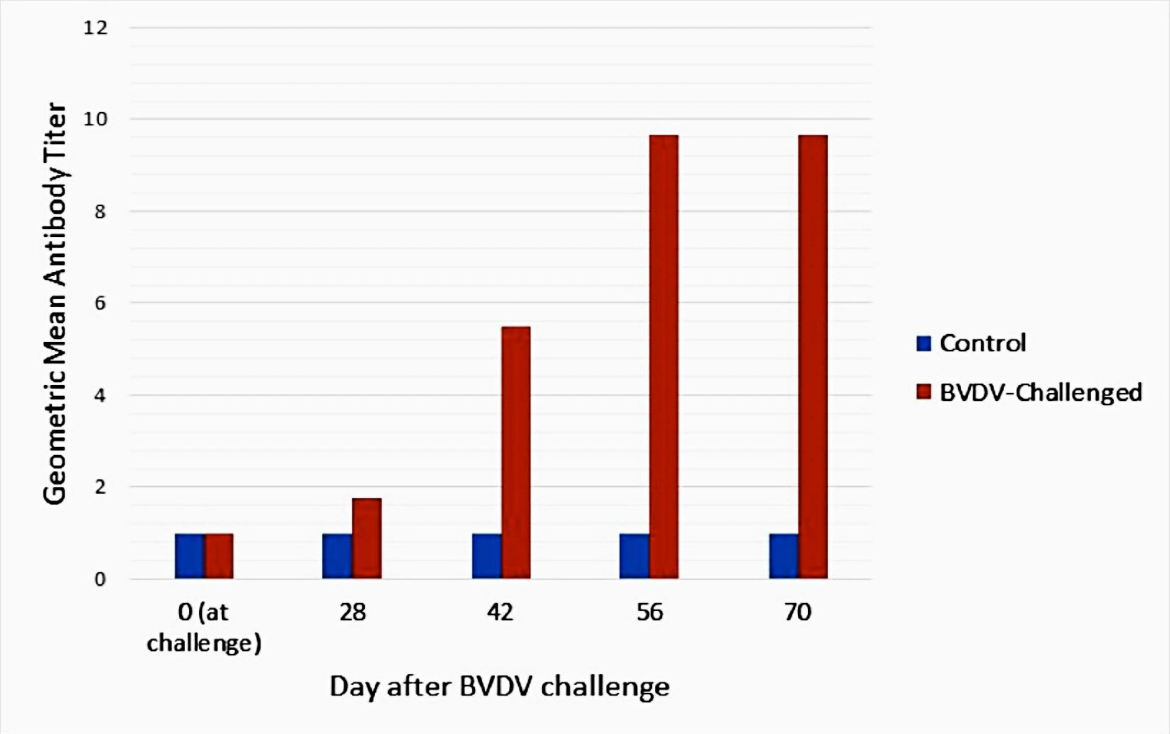

Figure 2.6. Geometric mean antibody titers of control heifers (n=11) and heifers intranasally inoculated with BVDV 2 strain PA131 (n=11). All heifers were challenge-exposed at approximately 67-89 days of gestation. No significant differences were noted between groups (*P*=0.1892).

Chapter III: Constitutive Gene Expression Profiles of Normal Cytokeratin Enriched Bovine Placental Cells and Changes in Gene Expression following *in vitro* infection with Non-Cytopathic strains of BVDV

Abstract

Bovine viral diarrhea virus (BVDV) is a positive sense single stranded RNA virus within the family Flaviviridae. BVDV is associated with a variety of disease manifestations in the respiratory, gastrointestinal, hemo-lymphatic, genital, and central nervous systems of cattle. The reproductive consequences of infection following viral transmission to BVDV naïve heifers include delayed returns to estrus, infertility, abortion, stillbirth, and persistent fetal infections. The mechanism by which BVDV establishes persistent fetal infections is unknown, but previous authors have suggested the virus is transmitted across the maternal-fetal interface by maternal viremia and subsequent hematogenous viral transmission to the fetus. Unlike species with hemochorial placental architecture, antibody (primarily IgG) is restricted from crossing the maternal-fetal barrier in cattle. Consequently, circulating maternal serum neutralizing antibodies to BVDV cannot eliminate infection within the fetus. The best chance for eliminating the virus is developing fetal-origin serum neutralizing antibodies, which is only possible following immune competence. Immune competence is associated with approximate gestational ages of days 100 to 150 in cattle. Infection prior to gestational day 120 has been shown to be the optimal period for persistent fetal infection with BVDV.

Interactions between the fetal placenta and the local maternal immune system are of interest due to the potential for vaccine development and possible therapeutic interventions. Human and mouse studies have demonstrated the complexities of this immunologic interface

between the mother and fetus, but this relationship has not been detailed in domestic animal species. Sheep have provided the majority of information in veterinary reproductive immunology since the 1970-1980's and they continue to provide tremendous contributions to this field. A strong interest in the unique immunologic relationship between the mother and fetus has also been documented in cats, horses, swine, and cattle recently. Trophoblasts from these species have been examined because of the role of the trophoblast in developmental, hormonal, and immunologic signaling pathways. In this study we describe a detailed analysis of constitutive and inducible gene expression patterns in primary bovine maternal caruncular cell cultures and examine the potential for maternal placental epithelial cells derived from 75-day gestation placentas to respond immunologically to *in vitro* challenge with BVDV.

Introduction

Trophoblasts are derived from the trophectoderm layer in the blastocyst, destined to form the epithelial cell compartment of the placenta (Cross, Werb et al. 1994), (Rossant and Cross 2001). The number of maternal and fetal epithelial cell layers supported by mesenchymal tissues such as endothelium and stromal constituents, varies among humans and animal species, reflecting diverse placentation types. Consequently, this dissimilar microanatomic architecture is believed to result in immunologic variation between species due to the extent of contact between the maternal tissue and trophoectoderm. Therefore, the highly invasive hemochorial placenta of humans, mice, and non-human primates is expected to show divergent immunological responses from other species, especially animals with noninvasive epitheliochorial placentas. The placental barrier in cattle has been classically described as non-invasive and epitheliochorial due to the

lack of trophoblast invasion, which maintains the presence of 6 cell layers separating the vasculature of the mother and fetus. However, improved microscopic techniques have shown that the bovine placenta is "minimally invasive" and can be classified as synepitheliochorial (Wooding 1992). Reclassification became necessary in cattle, sheep, and goats because the 6-cell layer epitheliochorial placenta is a transient interface between the dam and fetus due to the ability of specialized fetal binucleated trophoblasts to migrate and fuse with single maternal epithelial cells, producing a trinucleate morphology (Wooding and Wathes 1980). This process of migration and fusion continues to produce syncytial plaques, which are aggregates of trinucleate trophoblasts intermixed with adjacent apoptotic endometrial epithelial cells. Syncytial plaques replace the uterine epithelium transiently in cattle, unlike sheep and goats. Syncytia are replaced by residual endometrial epithelial cells with proliferative capacity, which continue to fuse with migrating binucleate fetal trophoblasts to produce trinucleate trophoblasts (Wooding 1992). Additionally, the source and concentration of placental progesterone varies between cattle and sheep, and there may be several endocrinologic mediators responsible for potential differences in female reproductive signaling mechanisms for sheep and cattle. Despite the similarity of placental architecture in cattle, sheep, and goats, these microanatomic and endocrinologic differences cause us to examine the immunologic signaling mechanisms by individual species rather than as a group.

In humans, the maternal fetal interface has classically been described as a site of immune privilege. One of several proposed mechanisms supporting the concept of immune privilege in the placenta includes the hypothesis of the trophoblast and decidua serving a barrier function, preventing immune cell trafficking between the placenta and fetus. The maternal-fetal

relationship has evolved from the idea of fetal survival due to escape from potentially destructive maternal immunosurveillance mechanisms. Fetal immunoevasion and/or a failure of the maternal immune system to recognize the fetus as a semiallograft was believed to be necessary for a successful pregnancy (Smirnova, Webb et al. 2014). The maternal immune system is no longer thought to be a quiescent system due to failure to attack the fetal allograft. Instead the maternal immune system represents a robust network of mediators that promotes homeostasis and maintenance of pregnancy.

In contrast to other tissues, unique molecules are expressed in the placenta, increasing the necessity for tolerance. Several of these unique molecules are expressed by trophoblasts. Trophoblasts have been described as immunologic sensors, integrating innate and adaptive immune responses (Mor and Cardenas 2010). Extravillous trophoblasts express B7-H2 and B7-H3 molecules, and B7-H1 is expressed on all trophoblast populations throughout pregnancy, while B7-DC is expressed by syncytiotrophoblasts in early pregnancy (Garden, Pinheiro et al. 2011). Trophoblasts and glandular epithelial cells in humans and mice express indoleamine-2,3-dioxygenase (IDO) (Kudo, Boyd et al. 2004). Inhibition of both B7-H1 and IDO have been shown to cause T cell-dependent rejection of allogenic fetuses in mice (Murphy and Stockinger 2010). Extravillous trophoblasts express the non-classical major histocompatibility (MHC) antigens: human leukocyte antigen (HLA)-C, HLA-E, HLA-F, and HLA-G (Holets 2006). Galectin expression by the syncytiotrophoblast is associated with T cell apoptosis (Kopcow, Rosetti et al. 2008). Galectin-1 is a member of beta-galactoside binding proteins, a lectin capable of inducing apoptosis on activated Th1, Th17+ CD4+ T cells, and CD8+T cells (Perillo, Pace et al. 1995), (Kopcow, Rosetti et al. 2008).

Depending on the stage of pregnancy and individualized factors (circumstances), trophoblasts likely interact with each of the different pathways of the immune system. The close microanatomical proximity of trophoblasts to innate immune cells facilitates cross talk between these populations, which is facilitated by cell to cell contact and paracrine mediated signaling of cytokines and chemokines. Trophoblasts have been documented to provide immunoregulatory signals to multiple cell types including: dendritic cells (Salamone, Fraccaroli et al. 2012), macrophages (Fest, Aldo et al. 2007), CD4+CD25+ T regulatory cells (Ramhorst, Fraccaroli et al. 2012), CD3+ T lymphocytes (Chen, Liu et al. 2012), NK cells (Parham 2004), CD8+ (Tilburgs and Strominger 2013), and γδT cells (Heyborne, Fu et al. 1994). Decidual stromal cells (Sharma, Godbole et al. 2016) provide signals to trophoblasts, including the release of mediators to initiate trophoblast invasion. The role of trophoblasts as immune regulators is strongly supported by the constitutive expression of IL-8, macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), and growth related oncogene α (GRO-α) (Aluvihare, Kallikourdis et al. 2004). Inducible mediators, include: indoleamine-2,3-dioxygenase (IDO) (Mellor and Munn 2004), CCL5 (Fraccaroli, Alfieri et al. 2009), type I interferons (IFN) (Aboagye-Mathiesen, Toth et al. 1994), the type III interferon, IFN lambda 1 (IFNλ1) (Bayer, Lennemann et al. 2016), MIC-1 (Segerer, Rieger et al. 2012), and chemokines (Du, Wang et al. 2014). Trophoblasts also have been shown to express the following immunologic mediators constitutively: indoleamine 2,3-dioxygenase (IDO), interferon-γ (IFN-γ) (Platt and Hunt 1998), CCL5 (RANTES) (Sharma, Godbole et al. 2016), and type I interferons. This expression profile demonstrates this cell type is not a typical epithelial cell. A few of the mediators that are not constitutively expressed by trophoblasts include: IL-2, IL-12, and IL-15.

Descriptions of trophoblasts as immune sensors are based on evidence of pattern receptor expression in humans. Toll like receptors (TLR) 1-9 are expressed by trophoblasts in humans, directly linking trophoblasts to placental innate immunity (Abrahams and Mor 2005). Functional studies with TLR have shown the ability to induce antimicrobial signaling cascades following exposure to TLR ligands such as peptidoglycan (Abrahams, Aldo et al. 2008), LPS (Abrahams, Bole-Aldo et al. 2004), and viral single stranded RNA (ssRNA) (Aldo, Mulla et al. 2010), (Potter, Garg et al. 2015). The dsRNA analogue, poly I:C, induces ligation of TLR3 in human trophoblasts (Abrahams, Schaefer et al. 2006). Viral infection has been shown to induce trophoblast derived antimicrobial peptides, including human beta defensins 1 and 3, and secretory leukocyte protease inhibitor (SLPI). SLPI is referred to as a homeostatic protein expressed by epithelial cells and this protein has been shown to interfere with class-switching in B cells. Trophoblasts have been shown to be permissive to cytomegalovirus (Hemmings, Kilani et al. 1998). CD4 expression by trophoblasts renders this cell population susceptible to human immunodeficiency virus-1 (HIV-1) (David, Autran et al. 1992). In veterinary medicine, the relationship between trophoblastic gene expression and viral infection has been documented in the feline immunodeficiency virus (FIV) infected cat. In normal tissues an array of pro- and anti-inflammatory immunomodulators was expressed from early to late pregnancy, but detection of FIV RNA in trophoblasts was rare (Scott, Shack et al. 2011). Trophoblasts may have the potential to express a wide variety of mediators based on the constitutive expression patterns described above. The potential of trophoblasts to interact with these circulatory networks permits the trophoblast to serve an immunosurveillance role, providing the necessary signals to promote protection of the fetus by downregulating immune responses necessary for tolerance or to

upregulate immune response in the face of microbial challenge. Bovine traophoblasts could provide immunologic regulatory functions at the maternal-fetal interface, and BVDV could also exploit the signaling mechanisms of this cell type to promote persistent fetal infection.

Materials and Methods

Collection of bovine primary trophoblasts. Experimental protocols for the use of two, 75-day gestation, Angus cross cattle in this study were approved by the Auburn University Institutional Animal Care and Use Committee. Estrous synchronization and artificial insemination were performed as described previously (Macmillan, Taufa et al. 1988). At least 25 placentomes were collected at 75 days gestation and processed for primary placental cultures. Cells were grown in RPMI media supplemented with certified BVDV free 10% fetal bovine serum (HyClone® FetalClone III, Thermo Scientific, Waltham, MA), 1.0 ng/ml epidermal growth factor (EGF), 1% sodium bicarbonate, 1% sodium pyruvate, and 1% penicillin/streptomycin/fungizone (Gibco) (adapted from Stringfellow DA, et al, 1987). Cell culture flasks were coated with 5 to 10 micrograms/cm2 (µg/cm2) of type I bovine dermal collagen and stored at 4° until use (Becton-Dixton #354231). Mixed placental cell cultures were expanded until cell numbers reached approximately 10^6, per manufacturer recommendations for magnetic-bead separation of cytokeratin-positive cells. Epithelial cells were obtained by the use of the cytokeratin monoclonal antibody (AEC/AEC3, Santa Cruz Biotechnologies, Santa Cruz, CA). AEC1 and AEC3 clones are combined in the AEC1-AEC3 product. This antibody served as a marker of epithelial cells. Clone AEC1 detects high molecular weight keratins 10, 14, 15, and 16 as well as low molecular weight cytokeratin-19. Clone AEC3 detects the high molecular weight cytokeratins 1, 2, 3, 4, 5, and 6, and the low molecular weight cytokeratins 7 and 8. This

monoclonal cytokeratin antibody was labeled with the DSB-X™ Biotin Protein Labeling Kit (Molecular Probes®, Life Technologies™, Grand Island, NY) according to manufacturer instructions. Biotin labeled cytokeratin antibody was subsequently added to mixed placental cell cultures, then processed for isolation of cytokeratin-positive cells with strepavidin-linked magnetic beads provided in the Dynabeads® FlowComp™ kit (Invitrogen). The number of cytokeratin separated cells was low, and changing the media for a minimum of one week was necessary until confluency was achieved in one T25 cell culture flask. Typically, from this starting point with low cell density, three passages were required to obtain sufficient cell numbers for inoculation with virus. The mesenchymal marker vimentin (Santa Cruz Biotechnologies, RV203: sc-58899) was used to confirm the cells were not a spindle-cell population such as fibroblasts or endothelial cells. Cell cultures were stained with AE1-AE3 following each separation to confirm an epithelial phenotype (Figures 3.1 and 3.2). Separated cells were expanded for several passages until confluency in T25 cell culture flasks and infected with 1.0 X 10^5 $CCID_{50}$ of the type I BVDV strain SD-1 and 1.0 X 10^5 $CCID_{50}$ of the type 2 strain PA131 for 72 hours at 37°C (Figure 3.2). An uninfected control flask was processed separately but maintained in conditions identical to infected cell cultures. BVDV positive primary cell cultures were stained with the BVDV polyclonal antibody B224 with the immunoperoxidase monolayer assay (Figure 3.3). Upon culture termination, buffer RLT^{plus} (Qiagen™, Valencia, CA) was added directly to the flask for direct lysis of cells grown in monolayers. The lysate was collected with the use of 1.8 cm cell scrapers (Fisherbrand™ Cell Scrapers, ThermoFisher Scientific, Waltham, MA). The lysate was collected and homogenized by vortexing for one minute, then the protocol for total RNA extraction with RNeasy® (Qiagen™) was followed for

total RNA collection from each infected cytokeratin-enriched cell culture (Figure 3.2). This RNA extraction procedure removes genomic DNA with gDNA Eliminator spin columns. RNA was quantified with the NanoDrop1000® spectrophotometer (ThermoFischer,Wilmington, DE19810) and 1 microgram of RNA was used per reaction for subsequent reverse transcription cDNA preparation with the Transcriptor First Strand cDNA Synthesis kit® (Roche). cDNA was quantified with the NanoDrop1000® (ThermoFischer, Wilmington, DE19810), then diluted to 200 ng/ul and used in quantitative PCR experiments at 20ug per reaction (Roche, Light Cycler 2.0®) to quantify gene expression. Primer reference information was obtained from current literature (Table 3.1) and purchased (TIB MOLBIOL PO Box 190, Adelphia, NJ). PCR reactions were performed with the LightCycler® FastStart DNA Master SYBR Green I kit (Roche) and the supplied 25mM $MgCl_2$ was titrated for each primer with the appropriate annealing conditions (see Table 3.2).

Normalization and relative quantification of target genes. Quantitative reverse transcriptase PCR (qRT-PCR) data generated with the Light Cycle 2.0 software (Roche®, Indianapolis, IN) was expressed in crossing points (Cp) or the threshold cycle (CT), at a continuous level of fluorescence. The qRT-PCR procedure yielded crossing points or threshold cycle (CT) as the fundamental quantitative units, corresponding to the PCR cycle wherein the amount of DNA of the amplified gene generates a fluorescent signal higher than the baseline. The CT value therefore correlates negatively to the amount of target mRNA, i.e. the higher the amount of mRNA, the sooner the threshold is reached and the lower the CT value obtained. Results for gene expression were analyzed with the comparative ΔΔCT method (Livak 2001). Gene expression analysis was performed through the relative quantification of the mRNA level

or fold regulation of target genes normalized to a housekeeping gene (unaffected by the studied conditions) and compared with the uninfected control group (Livak 2001).. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase subunit A (SDHA) were used as the endogenous reference genes. The efficiency of the qRT-PCR (necessary for the comparative ΔΔCt method), was determined for each target gene by creating a standard amplification curve using 1μg, 100 ng, 10 ng, 1 ng and 0.1 ng per reaction of a cDNA template mix. The cDNA template mix was created by mixing equal amounts of cDNA from cytokeratin enriched primary cells obtained from treatment and control groups. Evaluation of the difference between treatment and control groups was based on using housekeeping genes as an internal normalization method to account for any differences in starting cDNA levels. The change in crossing threshold for relative gene quantification compares the CT values of the target gene in the treated sample relative to the untreated sample with the equation: Change in crossing threshold = (CT (Target, Untreated)- CT (Reference, Untreated)) – (CT (Target, Treated) – (CT (Reference, Treated)) (Livak 2001). With this method, the amplification efficiencies (E) of the housekeeping and target genes are assumed to be approximately equal (E=1) and amplification efficiencies for each PCR cycle will double the amount of material in the sample being analyzed (E=100%).

Results

Nineteen genes were expressed in cytokeratin enriched primary placental cells analyzed by quantitative PCR. The nineteen genes expressed included: FOXP3, CTLA4, IL-10, IL-4, TGF-β, CCL5, IFN-γ, IFN-β, IFN-α, CXCL9, CXCL10, CXCL11, IDO, CCL2, CCR7, ISG15,

Mx1, OAS, and PKR. Thirteen genes with CT values between 23 to 34 were expressed, including CTLA4, IL-4, TGF- β, CCL5, IFN-β, CXCL9, CXCL10, CCL2, ISG15, Mx1, OAS, and PKR. TGF-β, CCL2, IFN-γ**,** OAS, and CTLA4 expression values were higher than the other genes tested with CT values ranging from 22-27. Lower concentrations of FOXP3, IFN-β, CXCL9, CXCL11 expression levels were found, but were still detected in the range of expression values associated with biological importance (crossing points of 32 or less). Most notable is the number and types of genes expressed in a cell type for which there is no baseline immunologic data published at this time (Figure 3.4). T regulatory cell associated molecules such as FOXP3, CTLA4, TGF-β, and IL-4 were expressed with crossing points of 32 or less, except IL-10 had a crossing point of 33 (denoted by a * in Figure 3.5). Epithelial cells have been shown to express the majority of these molecules; however, this is a novel finding for epithelial cells originating from the bovine placenta. Individual PCR runs showed graphical changes in raw CT values between uninfected control and virus-inoculated cultures with occasional genes (Figures 3.6 and 3.8). However, analysis of the mean CT values compiled from all passages (averaged) did not show notable differences between groups when the individual housekeeping genes (Figures 3.9 and 3.10) and an average of the housekeeping genes were included (Figure 3.11 and Table 3.3). These changes suggest if sufficient replicates were obtained for one passage, there is the potential for strong alterations in gene expression of cytokeratin-enriched placental primary cells following BVDV infection for 72 hours. Averaging Ct values across passages was required, but likely reduced the response of individual cell cultures, as depicted in figure 3.8. There was limited cell expansion capability, and 4-5 days were required for a monolayer to form, despite a fairly heavy seeding density. Additionally, there were issues obtaining necessary total

RNA concentrations unless at least a surface area equivalent to a T25 cell culture flask was used. This procedure was more challenging than collecting MDBK cells in a monolayer, as after trying both trypsinization and direct in-well lysis buffer, the cell layers could not be visualized in trypsin or lysis buffer. Confirmation of cellular removal was achieved by using cell scrapers, followed by microscopic examination of the flasks to ensure low RNA concentrations were not due to this aspect of the procedure. Later in these experiments, a newer RNA preparation kit (RNeasy Micro Kit, Qiagen) was used to generate total RNA from very small amounts of starting material. This approach enabled additional treatment flasks (replicates), while maintaining an adequate RNA concentration for cDNA synthesis and PCR runs. Overall results suggest cytokeratin enriched primary bovine placental cells are immunologically responsive to BVDV infection and this cell population expresses T regulatory associated cytokines.

Discussion

Our assumptions and conclusions from these studies with bovine trophoblast cell cultures are not intended to draw comparisons with other species. It was necessary to gather background information from other species since primary bovine trophoblast cultures have been uncharacterized for their immunologic function. Steroid synthesis, bovine placental lactogen, and genes associated with developmental regulatory pathways have been characterized, primarily in early gestation. However immunologic factors are only beginning to be described in early gestation and early to mid-gestation. Various gestational ages have been analyzed for innate immune responses; specifically, induction of interferon type I and downstream IFN-γ induced pathways in fetal blood, amnionic fluid, and fetal lymphoid tissues during persistent fetal infection with BVDV (Hansen, Smirnova et al. 2015).

Previous descriptions of primary bovine placental epithelial cells have indicated cultivation of these populations is challenging and not ideal for long-term studies (Munson L 1988). Other research in this area involved immortalizing bovine endometrial epithelial cells due to their short life span and rapid loss of responsiveness to hormone stimulation. Epithelial cells characterized as fetal trophoblasts have been cultivated with non-feeder cell lines, including: bovine trophoblast stem cell line, BT-1 and a bovine cotyledonary trophoblast cell line (F3) (Hashizume K 2006). Immortalized bovine endometrial epithelial cells (CT-1 cells) were characterized by Bai, *et al* in 2014 (Bai, Sakurai et al. 2014) The morphologic appearance of the epithelial cells analyzed in this study corresponds with typical cytoplasmic features of maternal epithelial cells described in the literature as both maternal trophoblasts and maternal caruncular epithelial cells. However, we could not identify a specific marker to conclusively identify these cells as being derived from either maternal or fetal epithelium. Some immunologic markers analyzed have been identified in epithelial cells in other locations and also tumor cells. Indoleamine 2.3-dioxygenase can be induced in both dendritic cells and epithelial cells. An *in vivo* experimental trial performed in our lab showed a moderate level of IDO expression in placentomes of approximately 150-day gestation heifers. Our magnetic bead labeling experiments to separate cytokeratin positive cells from mixed cell cultures indicates cytokeratin enriched placental cells, presumed to be trophoblasts or maternal caruncular epithelial cells, are at least partial contributors to IDO expression. However, expression of IDO metabolites and protein expression would be necessary to place more significance on IDO gene expression in our cell cultures. Foxp3 is also expressed in epithelial cells, despite the nearly universal association of Foxp3 expression in T regulatory lymphocytes. Foxp3 has also been shown to play a role in

tumorigenesis, similar to IDO. Additional molecules expressed by our epithelial cells could potentially be related to a common signaling pathway. Since T regulatory cells have been shown to express Foxp3, IL-10, TGF-β, and CTLA4; this seemed to be a notable finding in this case. Additionally, recognized role of IDO is induction of Foxp3 T regulatory cells in response to pro-inflammatory stimuli, and directing the immune response away from a Th-17 effector response. Finally, IL-4 is a Th2 cytokine with immunosuppressive properties, and similarly has a role in T regulatory cell activation (Petroff, Chen et al. 2002).

This research provided evidence of constitutive and inducible gene expression in primary bovine placental cell cultures, and suggested there is potential for primary placental epithelial cells derived from 75-day gestation placentas to respond immunologically to *in vitro* challenge with BVDV.

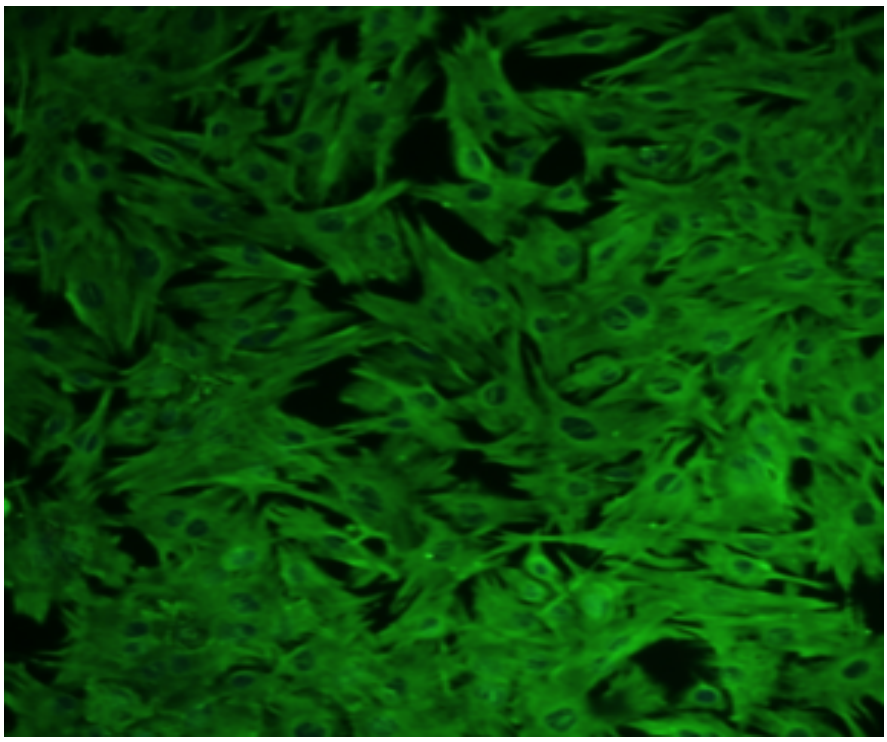

Figure 3.1. Monomorphic, occasionally binucleate primary placental cells in a monolayer labeled with AE1-AE3 monoclonal pancytokeratin antibody. 400X magnification.

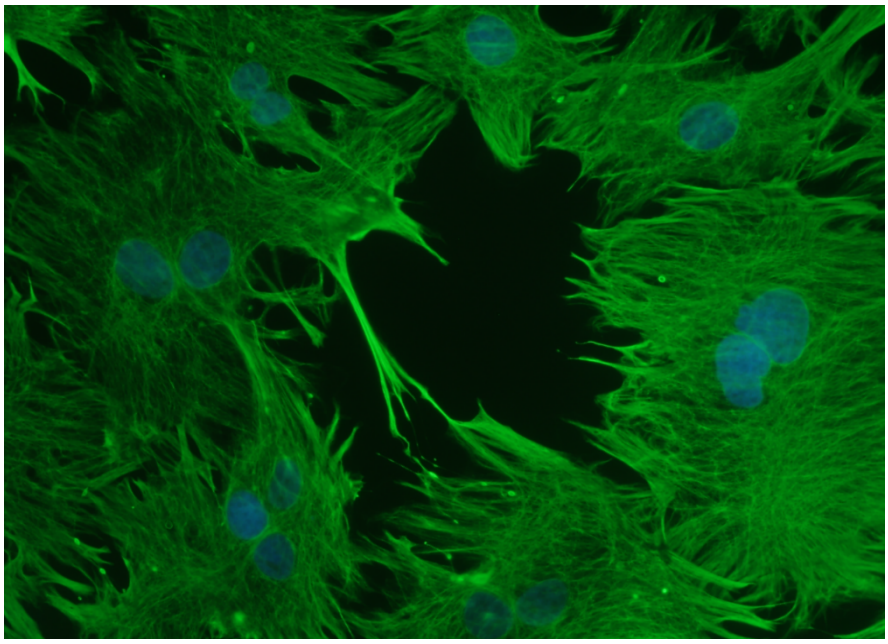

Figure 3.2. Cytokeratin positive (clone AEC/AEC3) placental epithelial cells with DAPI nuclear staining in indirect immunofluorescence. Epithelial cells are large, polygonal, and often binucleate with irregular, tapered to fibrillar cytoplasmic borders, 1000X magnification.

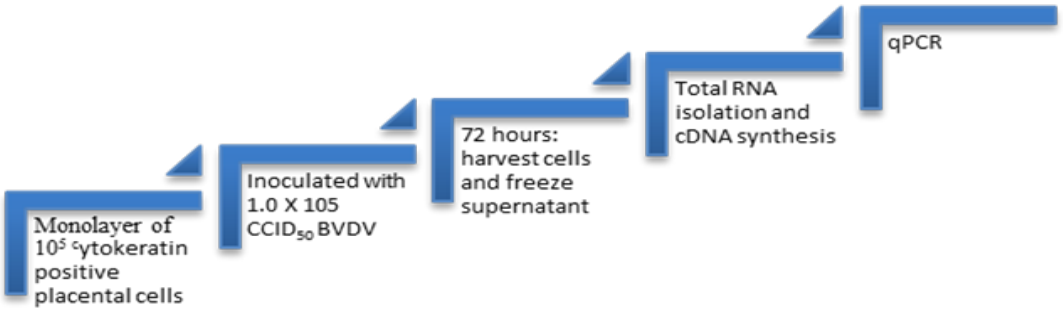

Figure 3.3. Experimental design for propagation and cultivation of cytokeratin enriched primary cells

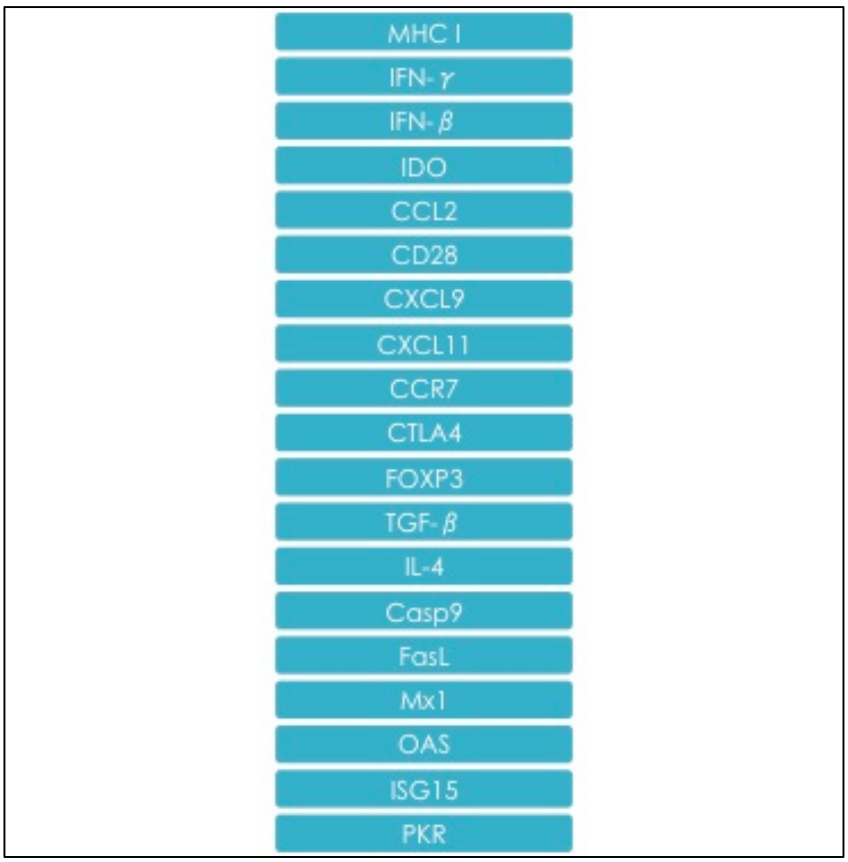

Figure 3.4. Constitutive gene expression profiles of cytokeratin enriched placental epithelial cells obtained from 75-day gestation heifers.

Table 3.1.

Primer sequences and references. *Primers used for quantitative RT-PCR during the mRNA expression analysis of chemokines and T regulatory markers in cytokeratin enriched primary placental cells.*

Primers	**Primer Source References**	**Primer Sequence**
CCL5 (RANTES)	Veterinary Immune Reagent Network	5’-CCATGAAGGTCTCTGCCACT-3’ 3’-CCACCCTAGCTCAACTCCAA-5’
CXCL10 (IP-10)	Veterinary Immune Reagent Network	5’-AGTTGCAGCACCATGAACAA-3’ 3’-GCTTCTCTCTGGTCCATCCTT-5’
CD25	NM174358 Seo, et al	5’-GCAGGGACCACAAATTTCCA-3’ 3’-GGTACTCAGTGGTAAATATGAACGTATCC-5’
CTLA4	X93305 Seo, et al	5’-GCAGCCAGGTGACCGAAGT-3’ 3’-TCATCCAGGAAGGTTAGCTCATC-5’
FOXP3	NM DQ322170 Seo, et al	5’-AAGAGCCCAGGGACAACTTTC-3’ 3’-GGGTTCAAGGAGGAAGAGGAA-5’
IL-10	U00799 Seo, et al	5’-TTCTGCCCTGCGAAAACAA-3’ 3’-TCTCTTGGAGCTCACTGAAGACTCT-5’
TGF-β1	M36271 Seo, et al	5’-CATCTGGAGCCTGGATACACAGT-3’ 3’-GAAGCGCCCGGGTTGT-5’
SDHA	NM 174178 Gossens, et al	5'-GCAGAACCTGATGCTTTGTG-3’ 3’-CGTAGGAGAGCGTGTGCTT-5'
GAPDH	XM_618013 Gossens, et al	5'-TTCAACGGCACAGTCAAGG-3' 3’-ACATACTCAGCACCAGCATCAC-5'
IDO	NM_001101866 XM_001490681.1 Plain, KL, et al	5’-CGAATATACTTGTCTGGTTGG 5’-GGAGAACATCAAAGCACTG

Table 3.2

Optimization of primers for quantitative PCR using LightCycler® FastStart DNA Master SYBR Green

TGF-β1	73°	1.6
CXCL10	71°	1.6
IL-10	70°	0.8
CD25	67°	0.8
CCL5 (RANTES)	71°	1.6
SDHA	71°	1.6
GAPDH	71°	0.8
FOXP3	71°	0.8
CTLA4 (CD152)	69°	2.4
IDO	61°	0.8

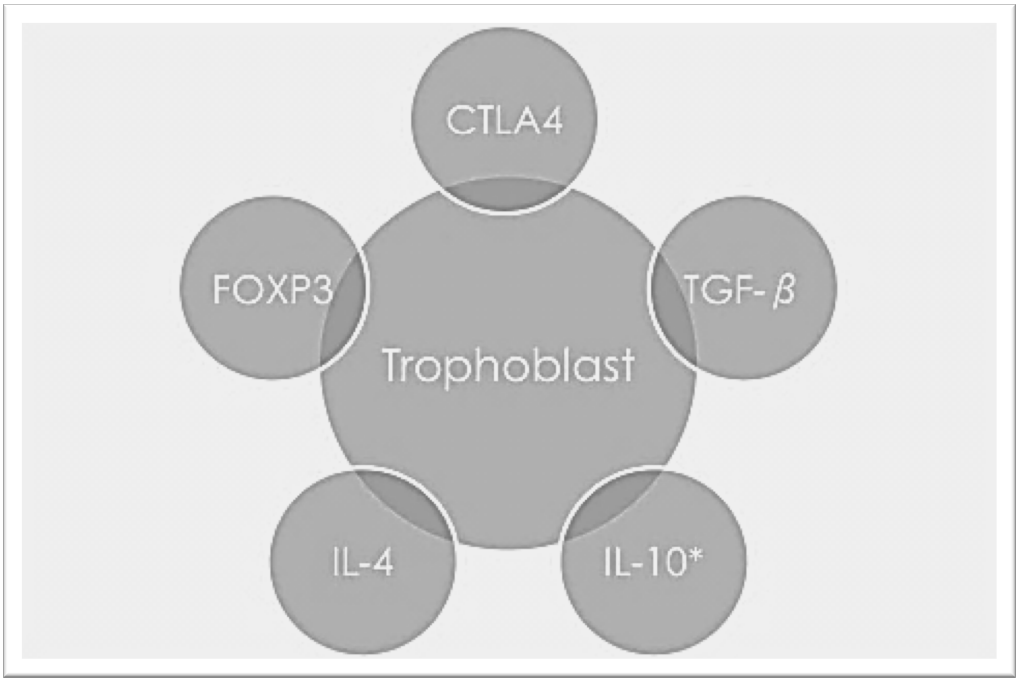

Figure 3.5. Cytokeratin-enriched epithelial cells expressed molecular markers of T regulatory cells. *Denotes IL-10 expression was low with a low CT value of approximately 33.

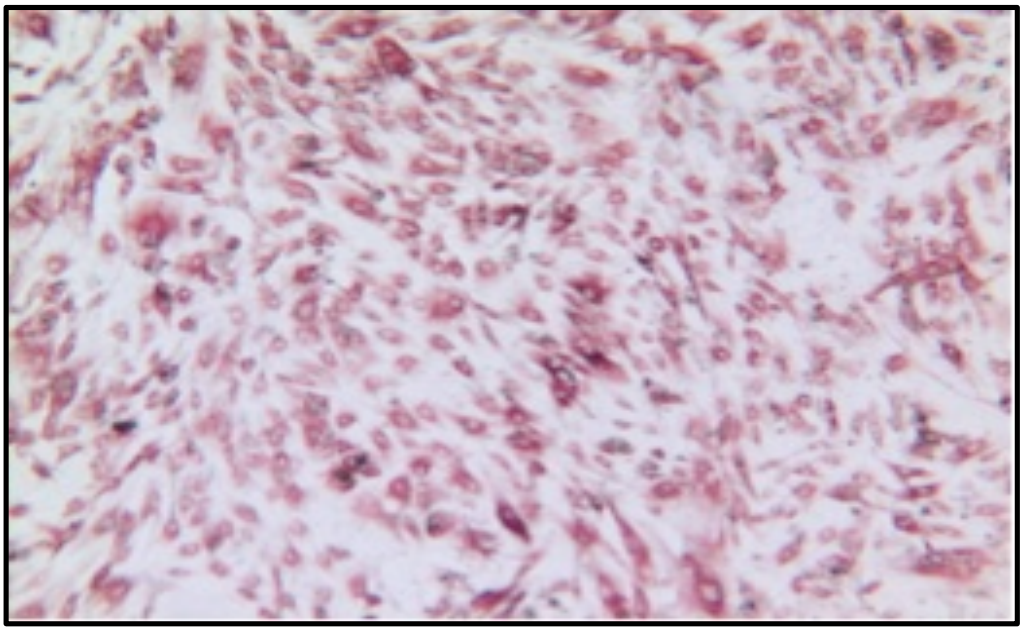

Figure 3.6. Cytokeratin enriched placental epithelial cells infected with BVDV type 2 strain PA131 and stained with the BVDV polyclonal antibody B224 at 72 hours postinoculation. Immunoperoxidase monolayer assay (IPMA).

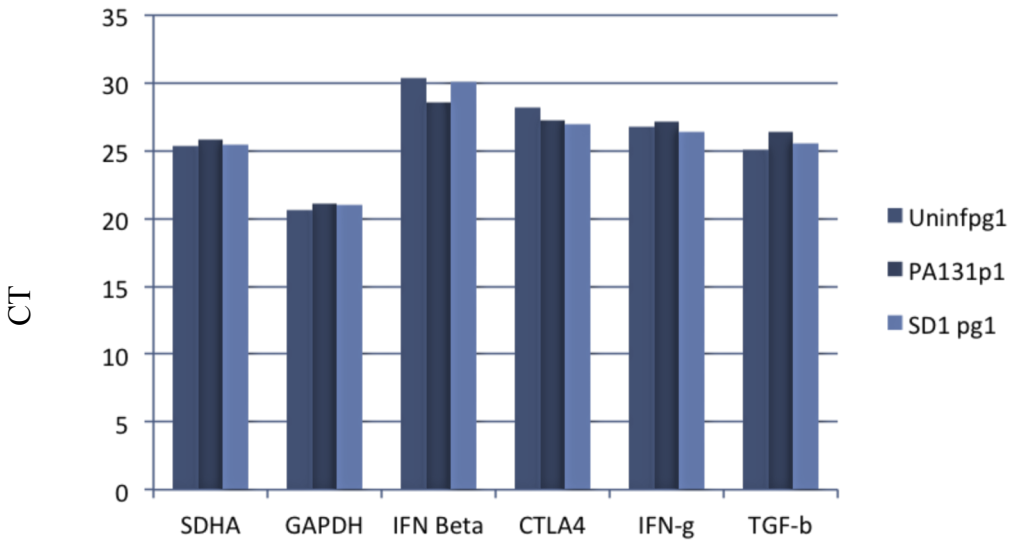

Figure 3.7. Raw mean CT values for cytokeratin enriched placental epithelial cells.

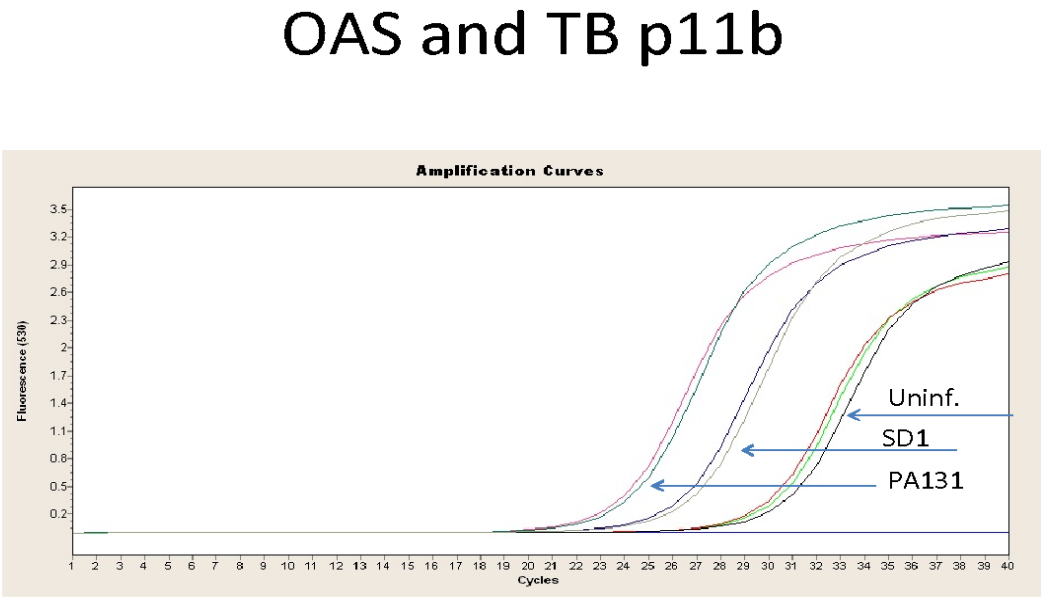

Figure 3.8. Amplification curves for 2′,5′-oligoadenylate synthetase (OAS) in cytokeratin enriched placental epithelial cells. Without consideration of housekeeping genes, the crossing threshold values (CT values) show differences between BVDV uninfected cultures and BVDV cultures infected with the BVDV type 1 strain SD-1 and the type 2 strain PA131.

Table 3.3

Descriptive Statistics and 1-way ANOVA results for quantitative PCR targets expressed in BVDV infected cytokeratin enriched primary cells using the average of housekeeping genes SDHA and GAPDH for normalization and the difference between treatment and control groups.

One Way ANOVA Summary					
		p-value	Mean	Standard Error	**Standard Deviation**
	Control		8.23	0.594	1.68
Foxp3	**PA131**	0.4002	7.01	1.23	3.01
	SD1		6.19	1.49	3.33
	Control		4.77	0.673	1.9
CTLA4	**PA131**	0.1228	4.02	1.01	2.66
	SD1		2.08	0.953	2.45
	Control		1.91	0.899	2.64
TGF-β	**PA131**	0.2388	3.93	0.862	2.28
	SD1		1.70	1.22	2.73
	Control		3.79	0.973	2.75
IFN-γ	**PA131**	0.7463	3.57	1.44	3.53
	SD1		2.47	1.37	3.07
	Control		7.59	1.54	4.36
IFN-β	**PA131**	0.8740	6.46	1.59	3.88
	SD1		6.52	2.27	5.55
	Control		5.63	1.74	4.92
CCL5	**PA131**	0.8169	7.11	1.78	4.36
	SD1		6.36	1.27	2.83
	Control		0.720	1.45	3.83
CCL2	**PA131**	0.9466	0.403	1.39	3.67
	SD1		0.008	1.53	3.42

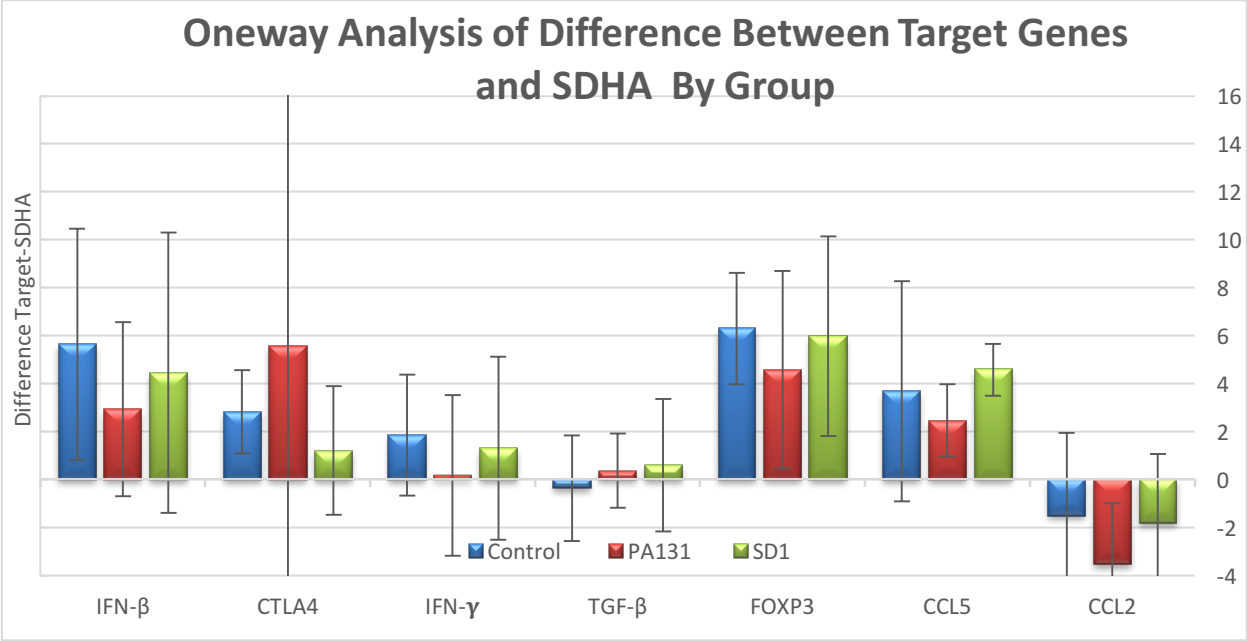

Figure 3.9. The mean crossing threshold difference between the target genes and the housekeeping gene, SDHA, were compared among groups with Oneway Analysis of Variance (ANOVA). There were no significant differences among the groups (P>.05). Error bars reflect standard deviation for each group.

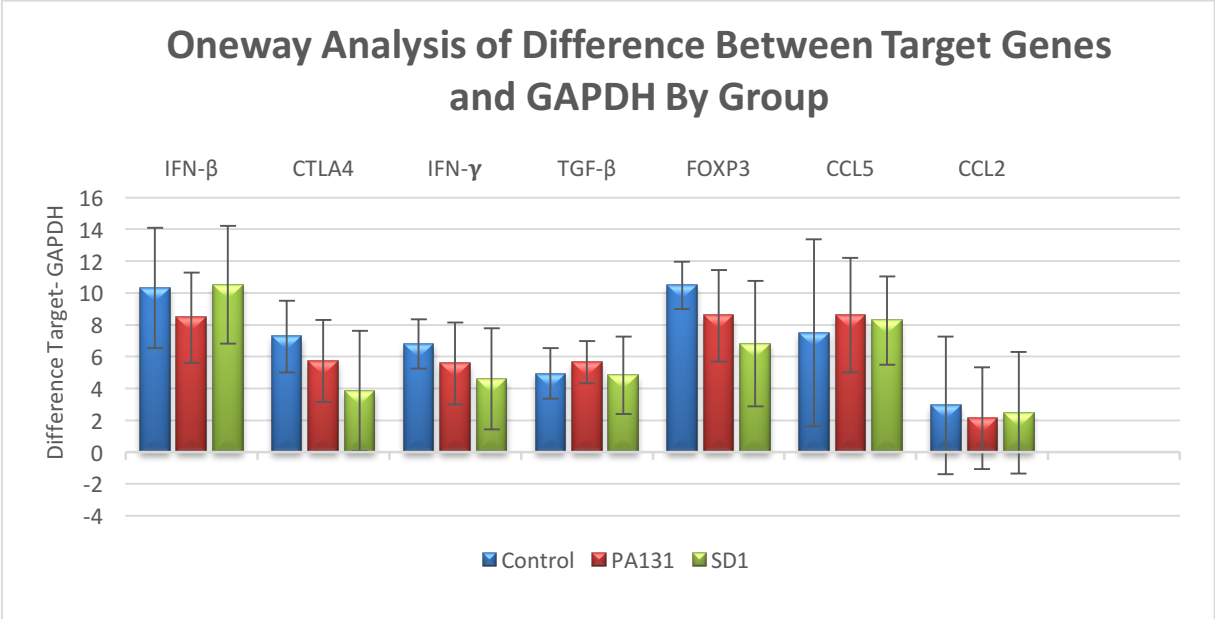

Figure 3.10. The mean crossing threshold difference between the target genes and the housekeeping gene, GAPDH, were compared by Oneway Analysis of Variance (ANOVA). No significant differences were found among groups. (P>0.05). Error bars reflect standard deviation for each group.

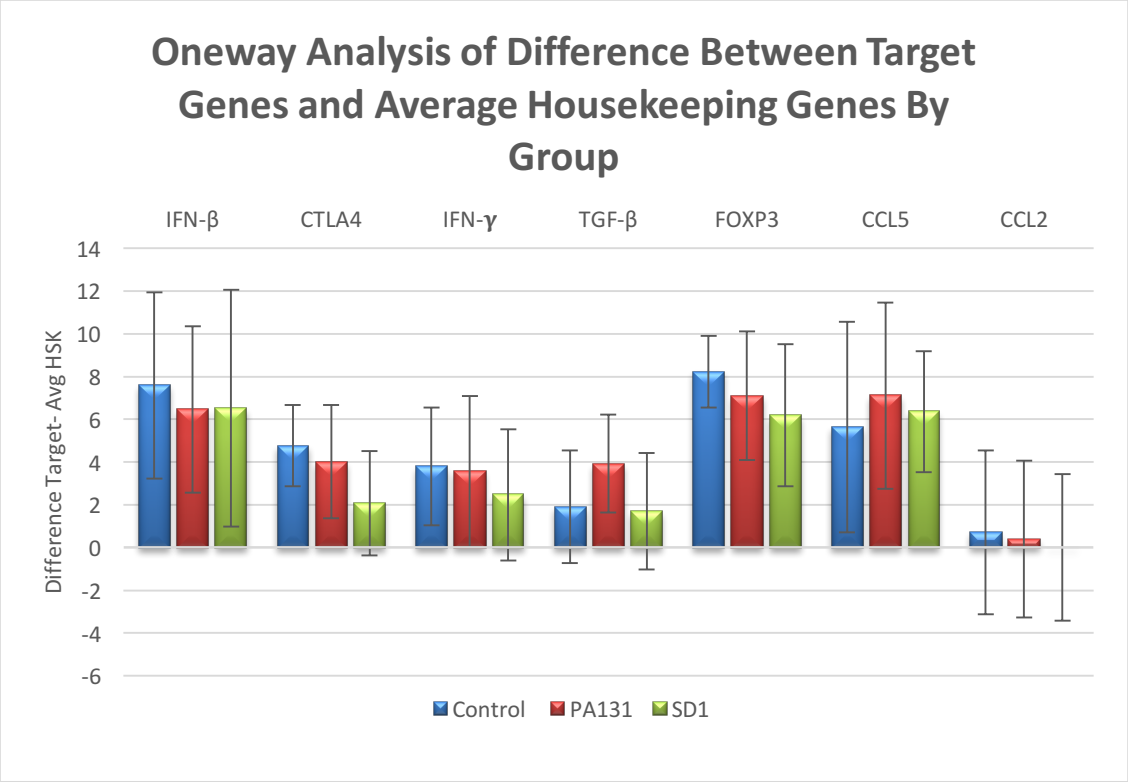

Figure 3.11. The mean crossing threshold difference between the target genes and an average of the two housekeeping genes (HSK), SDHA and GAPDH, are shown. No significant differences were found among groups. (P>0.05, One way ANOVA). Error bars reflect standard deviation for each data set.

Chapter IV: Quantitative Analysis of T-regulatory cell Associated Genes in Bovine Peripheral Blood Mononuclear Cells During Pregnancy

Abstract

The maternal immune system during pregnancy is classically described as being in a state of immunosuppression. Pregnancy-associated increases in estrogens, progesterone, and glucocorticoids historically are believed to be the primary endocrinologic factors causing a cooperative down-modulation of systemic maternal immune responses in humans and animals. However, there has been an evolution in theory from a fetus being analogous to an allograft to a current more complex understanding of the immunologic interface with immune cells as supporting cells, not an immunologic barrier formed in response to the fetus. Systemic and placental Th2 regulation during normal bovine pregnancy are sparsely documented, and there is also no baseline immunologic data published about temporal alterations in the bovine immune system during pregnancy. Using quantitative reverse transcriptase-PCR (qRT-PCR) on peripheral blood mononuclear cells collected from healthy pregnant heifers at days 89 or 141 gestation, we examined baseline T-regulatory and T cell associated markers Foxp3, CTLA4, CCL5, IDO, and TGF-β, and compared expression differences during these gestational periods. Significant differences in Foxp3 and CTLA4 expression were found between gestational days 89 and 141 ($p<0.05$), while the change in gene expression between these time points was not significant for CCL5, IDO, and TGF-β ($p>0.05$). The data showed increased expression of T-regulatory cell markers, Foxp3 and CTLA4, as pregnancy progressed from early to mid-gestation. These results indicate more baseline immunological data is needed from early to mid- and late gestation to further elucidate the normal immunologic signaling mechanisms during

bovine pregnancy.

Introduction

Current theory regarding immune recognition during pregnancy is that the maternal immune system is a natural homeostatic state, not an anti-inflammatory state necessary to protect the fetus from maternal immune attack (Mor and Cardenas 2010). Several leaders in this field have proposed the maternal immune system is modulated and directed by the fetal-placental unit. This is supported by current research showing there is recruitment of fetus-specific regulatory cells from the maternal blood to the placenta (Tilburgs, Roelen et al. 2008). A clear distinction between immunoregulatory functions of the uterine microenvironment and the role of the maternal immune system during pregnancy is becoming less clear, as there is less emphasis on the uterine environment being an immunologically privileged site and immune barrier. Current theory maintains it is likely not only the placenta that exerts a protective role in maintaining immune homeostasis during pregnancy. Peripherally generated, pregnancy-protective mechanisms also play a major role in fetal protection. Fetal-antigen specific CD4 Tregs have been well characterized in humans and mice (Guleria, Khosroshahi et al. 2005, {Kallikourdis, 2007 #3356). (Kallikourdis, Andersen et al. 2007) Previous studies in humans have shown immunoregulatory functions of some subsets of lymphocytes, NK cells, and macrophages differ between the systemic circulation and the uterus, and the pregnant uterus represents a unique immunologic niche. A more complete understanding of changes in systemic and uteroplacental immune cell populations, cytokines, and other immune mediators throughout pregnancy progression would be beneficial to understand implantation, adverse pregnancy outcomes, and transplacental viral infections. These fluctuations in lymphocyte dynamics locally and within the

systemic circulation are necessary to further our knowledge of bovine maternal immunology, specifically as this information may aid our understanding of the degree of immunosuppression or immunocompetence and risk of infectious disease transmission.

Material and Methods

Ten approximately 1-year-old, mixed bred heifers were artificially inseminated and bull bred then comingled with 10 non-pregnant, approximately 1-year-old mixed bred heifers for the duration of this trial. At days 89 and 144 of pregnancy, peripheral blood was collected by jugular venipuncture and placed in EDTA-collection tubes. Blood collection tubes were quickly transported on ice to the laboratory and buffy coats were isolated by centrifugation (1500 g, 20 min). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as previously described with minor modifications (Collins, Howard et al. 1999). The buffy coat was washed with phosphate buffered saline (PBS) (Sigma Aldrich, UK) (300 g for 10 min at 4 °C). Red blood cells were lysed by incubation in ammonium-chloride lysis buffer for 10 min and white blood cells washed three times with PBS (600 g for 10 min at 4 °C). Washed white blood cells were preserved in RNAlater (Qiagen™) and frozen at -80°C. Following rapid thawing of the buffy coat, total RNA extraction with RNeasy Mini Kit® (Qiagen™) was followed for total RNA collection. This RNA extraction procedure removes genomic DNA with gDNA Eliminator spin columns. RNA was quantified with the NanoDrop1000® spectrophotometer (ThermoFischer,Wilmington, DE19810) and 1 microgram of RNA was used per reaction for subsequent reverse transcription cDNA preparation with the Transcriptor First Strand cDNA Synthesis kit®(Roche). cDNA was quantified with the NanoDrop1000® (ThermoFischer, Wilmington, DE 19810), then diluted to 200 ng/ul and used in quantitative PCR experiments at

20ug per reaction (Roche, Light Cycler 2.0®) to quantify gene expression. Primer sequences and optimal housekeeping genes (endogenous reference genes) for this study were obtained from the literature (Table 4.2) and purchased (TIB MOLBIOL LLC PO Box 190 Adelphia, NJ 07710). PCR reactions were performed with the LightCycler® FastStart DNA Master SYBR Green I kit (Roche) and the supplied 25mM $MgCl_2$ was titrated for each primer with the appropriate annealing conditions (Table 4.1). The cDNA from each animal (n=20) was amplified simultaneously in one qRT-PCR run (number of capillaries available per run =32). Three technical replicates (separate PCR runs) for each gene were performed, and the crossing points (Cp) for each gene target and housekeeping gene were compiled and averaged for each group. Evaluation of the difference between treatment and control groups was based on using housekeeping genes as an internal normalization method to account for any differences in starting cDNA levels. Quantitative reverse transcriptase PCR (qRT-PCR) data generated was expressed in crossing points (CP) or the threshold cycle (CT), corresponding to the PCR cycle wherein the amount of DNA of the amplified gene generates a fluorescent signal higher than the baseline. The CT value therefore correlates negatively to the amount of target mRNA, i.e. the higher the amount of mRNA, the sooner the threshold is reached and the lower the CT value obtained. Results for gene expression were analyzed with the comparative ΔΔCt method for relative gene quantification using the Delta Delta CT" difference (ΔΔCT**)** (Livak and Schmittgen 2001). ΔΔCT= (CT (Target, Untreated)- CT (Reference, Untreated)) – (CT (Target, Treated) – (CT (Reference, Treated)). Gene expression analysis was performed through the relative quantification of the mRNA level or fold regulation of target genes normalized to a housekeeping gene (unaffected by the studied conditions) and compared with the uninfected

control group (Livak 2001). The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase subunit A (SDHA) were used as the endogenous reference genes. The efficiency of the qRT-PCR (necessary for the comparative ΔΔCT method), was determined for each target gene by creating a standard amplification curve using 1µg, 100 ng, 10 ng, 1 ng and 0.1 ng per reaction of a cDNA template mix. The cDNA template mix was created by mixing equal amounts of buffy coat cDNA collected from pregnant (treatment) and non-pregnant (control) heifers.

JMP statistical software was used for statistical analysis (SAS Campus Drive, Cary NC). Data from pregnant and non-pregnant cows at 89 and 144 days gestation was examined using the one-way analysis of variance followed by the Student's t-test. For all analyses, $P<0.05$ was considered significant.

Results

The data showed increased expression of T-regulatory cell markers, Foxp3 and CTLA4, as pregnancy progressed from mean gestational day 89 to mid-gestational day 144 (Table 4.3, Figures 4.1and 4.2). One way analysis of variance (ANOVA) of the difference between the target genes and average housekeeping genes for pregnant and non-pregnant cattle showed significant results for Foxp3 and CTLA4 when comparing mean gestational days 89 and 144 (Figure 4.9). One way Analysis of Variance for Foxp3 indicated the p-value was <0.0001. One way Analysis for CTLA4 also showed significance with a p-value of 0.0039. Other genes analyzed, IDO, TGF-β, and CCL5 did not show significance (p-values of 0.28, 0.21, and 0.96 respectively) (Table 13). The fold change in gene expression ($2^{-\Delta\Delta Ct}$) between pregnant and open heifers at 89 days gestation for Foxp3 was increased 15.7-fold, CTLA was elevated 1.72-fold,

TGF-β 1.92 times, a 1.28 fold increase in IDO was observed, and CCL5 was increased 0.71 fold (Figure 4.10) (Table 4.5). Data from 144 days gestation showed there was a 61 times fold change in Foxp3 expression when pregnant and open heifers were compared at this time point (Table 4.6 and Figure 4.11). CLTA4 had a 6-fold increase, while TGF-β was increased 1.5 times, IDO was increased 1.49-fold in pregnant heifers, and CCL5 showed a very similar 1.36-fold increase (Table 4.6 and Figure 4.11). Overall findings were increased Foxp3 and CTLA4 expression occurred as pregnancy progressed from mean gestational days 89 to the final sampling point at mean gestational day 144. Expression of TGF-β, IDO, and CCL5 expression was not increased as pregnancy progressed from approximately 3 to 5 months gestation.

Discussion

We found that T cell associated molecules such as Foxp3, CTLA4, TGF-β, IDO, and CCL5 are expressed from approximately 3 to 5 months gestation in approximately 1 year old mixed breed heifers within a range of biological relevance with quantitative RT-PCR analysis. The regulatory function of Foxp3, CTLA4, TGF-β, and IDO in cattle is unknown. However, this information provides valuable insight for bovine immunology, especially bovine reproductive immunology involving transplacental viral infections such as bovine viral diarrhea virus (BVDV). In humans immune cells within the local placental bed likely control the systemic immune response in women during pregnancy, but studies in cattle are only beginning to associate endocrine hormone levels with fluxes in cytokine levels during pregnancy. In humans and mice, it has been shown that pregnancy induces an accumulation of regulatory T cells in the gravid uterus (Kallikourdis, Andersen et al. 2007). Similarly, the maternal pool of CD4+CD 25+ T cells is also expanded in the periphery during pregnancy (Aluvihare, Kallikourdis et al. 2004).

T regulatory cell expansion in maternal peripheral blood during bovine pregnancy has been incompletely characterized. However, preliminary information was documented by Oliveiria and Hansen in 2008, describing an increase in CD4+CD25+ expression in bovine peripheral blood mononuclear cells in early pregnancy (33-34 days gestation) and preparturient cows with flow cytometry (Oliveira and Hansen 2008). In this study, there was also an increase in the proportion of γδ-T cells in peripheral blood in preparturient cows. A previous report indicated elevations in γδ-T cells 3 weeks prior to parturition in cattle and a return to baseline at parturition (Karcher, Beitz et al. 2008). In the Oliveira study, monocytes (CD68 positive population) were also decreased in PBMC during late pregnancy (preparturient), and this corresponded with a large influx of CD68+ and CD14+ cells (macrophages) within the endometrial stroma during this time period, suggesting this was a response to recruitment and uterine demand. In contrast, CD4+ and CD8+, CD68+ monocytes, and γδ-T cells were not changed 33-34 days after insemination, compared to non-pregnant controls (Oliveira and Hansen 2008). A separate study involving PBMC signaling was described in 2013, in which the effect of progesterone on Th1/Th2/Th17 and regulatory T cell-related genes in peripheral blood mononuclear cells (PBMC) of pregnant cows was reported when *ex vivo* PBMC were stimulated with progesterone (Maeda Y 2013). In this study, progesterone exerted a dose-dependent inhibition of IFN-gamma and IL-17 in both pregnant and non-pregnant cows. However, PBMC from both pregnant and non-pregnant cows also showed Foxp3 and TGF-beta were not altered by progesterone. A few studies performed the past few years have provided some insight into immune regulation during bovine pregnancy. Pregnancy loss and cytokine activation has been discussed as an outcome of Th1 cytokine production. However, pregnancy in ruminants has not been characterized as a Th1/Th2 cytokine

balance, as described in mice and humans (Wilczynski 2005), (Szekeres-Bartho 2002). In 2013, Maeda et al showed *ex vivo* stimulation of PBMC with progesterone (P4) at 0, 0.1, 1, or 10 μg/ml from 8 pregnant and 8 non-pregnant luteal phase cows at 163.1 +/- 16.9 days gestation resulted in a higher level of Th2 immunity in pregnant cows (Maeda Y 2013). The overall findings indicated P4 is an essential regulator of Th1/Th2/Th17 and T reg immunity. Expression patterns of T reg cytokines (IFN-γ, IL-4. IL-17, and TGF-β) and transcription factors (Tbet, GATA-3, RORC and Foxp3) and P4 receptors (PGR, PGRMC1, and PGRMC2) were evaluated and IFN- γ, and IL-17 were decreased by P4 in a dose dependent fashion. TGF- β and FoxP3 were not changed in the presence of P4. Finally, Tbet and RORC expression decreased, but there was enhanced IL-4 expression. PGRMC1 was increased following progesterone treatment in pregnant, but not non-pregnant cows. These described studies have provided preliminary information about the maternal immune response during pregnancy, suggesting there are temporal changes in leukocyte populations peripherally and at the uterine level. Mating in mice also increases T reg populations, as Tregs have been found to be increased in the vaginal lumen and vagina (Kallikourdis and Betz 2007). In cattle, it would also be beneficial to further evaluate temporal fluxes in leukocyte populations and associated signals throughout breeding and all phases of gestation. This trend in leukocyte numbers and T regulatory cell populations would be especially important to understand during mid-gestational bovine pregnancies when bovine viral diarrhea virus (BVDV) can cross the placenta and produce persistent fetal infections.

Regarding the role of T regulatory cells during pregnancy, comparisons between uterine and peripheral blood immune function are problematic due to alterations in the proportions of T regulatory cells expressed in the decidua versus the peripheral blood of pregnant women in

studies with paired samples. One comparison of uterine and peripheral blood T regulatory cell populations showed a similar pattern with a decidual population of CD4+ CD25- Foxp3+ cells with a Th3-like cytokine expression profile, which was enriched 10-fold in decidual samples compared with peripheral blood. Additionally, pregnant and non-pregnant women did not show any difference in numbers of circulating Treg cells. This reference provided evidence there is local enrichment of T reg cells in decidua with a CD4+ CD25- Foxp3+ phenotype, and the authors speculated a population of T cells may potentially be converted from "naïve to classical" T reg cells in the uterus (Dimova, Nagaeva et al. 2011). Therefore, Tregs infiltrate the pregnant uterus during pregnancy and systemic evaluation of T regulatory cells from peripheral blood during pregnancy may not explain the functional immune status of the local placental bed.

Documentation of human natural T regulatory cells as a normal component of the human decidua, partially initiated this study examining the normal immunoregulatory profile at the bovine fetal-maternal interface in the context of the induction of natural T regulatory cells to inducible T regulatory cells by TGF-β in humans and mice (Mellor and Munn 2008). However, there is a great deal of debate concerning the association of Foxp3 with a suppressor phenotype in humans due to findings suggesting the molecule may be transiently expressed, possibly representing a state of activation. Furthermore, the function of Foxp3 and other molecules associated with a T-regulatory phenotype in mice and humans is unknown in cattle and many veterinary species. The data from this study indicates T regulatory-associated molecules Foxp3 and CTLA4 may play a role in regulating maternal systemic immune responses at specific gestational phases, and further *in vivo* studies are needed to determine what factors control expression of T regulatory associated molecules between the systemic circulation and maternal-

fetal interface.

Table 4.1.

Primer references and sequences used to evaluate gene expression profiles in bovine buffy coats in pregnant and non-pregnant heifers

Primers	**Primer Source References**	**Primer Sequence**
CCL5 (RANTES)	Veterinary Immune Reagent Network	F: 5'-CCATGAAGGTCTCTGCCACT-3' R: 3'-CCACCCTAGCTCAACTCCAA-5'
CTLA4	X93305 Seo, *et al*	F: 5'-GCAGCCAGGTGACCGAAGT-3' R: 3'-TCATCCAGGAAGGTTAGCTCATC-5'
FOXP3	NM DQ322170 Seo, *et al*	F: 5'-AAGAGCCCAGGGACAACTTTC-3' R: 3'-GGGTTCAAGGAGGAAGAGGAA-5'
IDO	NM_001101866 XM_001490681.1 Plain, KL, *et al*	F: 5'-CGAATATACTTGTCTGGTTGG R: 5'-GGAGAACATCAAAGCACTG
TGF-β1	M36271 Seo, *et al*	F: 5'-CATCTGGAGCCTGGATACACAGT-3' R: 3'-GAAGCGCCCGGGTTGT-5'
SDHA	NM 174178 Gossens, *et al*	F: 5'-GCAGAACCTGATGCTTTGTG-3' R: 3'-CGTAGGAGAGCGTGTGCTT-5'
GAPDH	XM_618013 Gossens, *et al*	F: 5'-TTCAACGGCACAGTCAAGG-3' R: 3'-ACATACTCAGCACCAGCATCAC-5'

Table 4.2.

Optimization of primers for quantitative PCR using LightCycler® FastStart DNA Master SYBR Green I for quantitative RT-PCR analysis of buffy coats from pregnant and open heifers.

Target	**Temperature**	**Microliters $MgCl_2$**
TGF-β1	73°C	1.6
CCL5 (RANTES)	71°C	1.6
SDHA	71°C	1.6
GAPDH	71°C	0.8
FOXP3	71°C	0.8
CTLA4 (CD152)	69°C	2.4
IDO	61°C	0.8

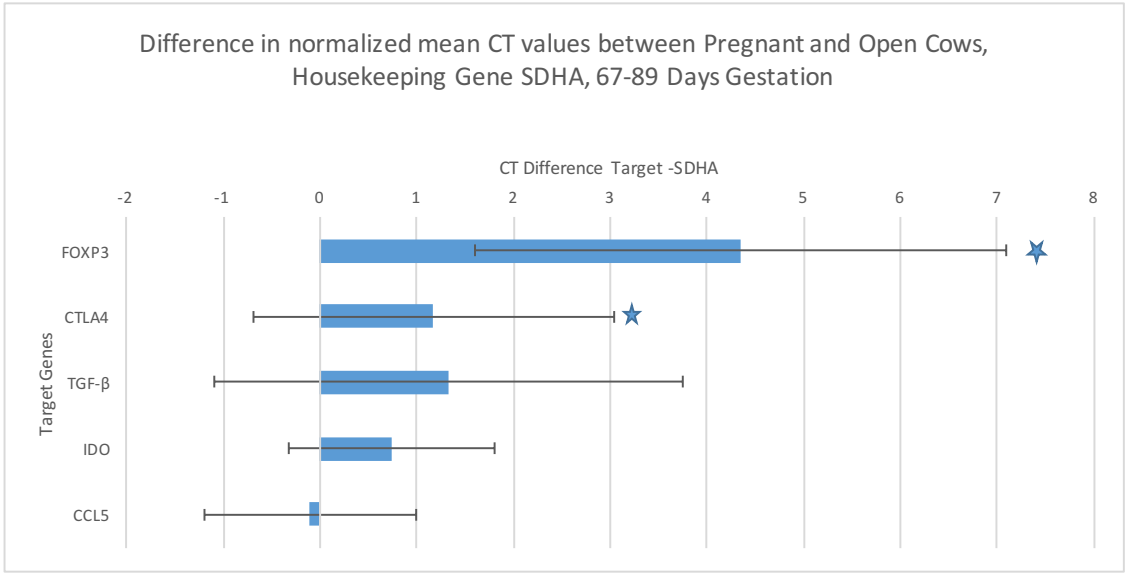

Figure 4.1. Difference in mean CT values between Pregnant and Open Cows at 89 days. Buffy coats, housekeeping gene=SDHA; Mean gestation length = 89 days; range=67-89 days. Error bars represent standard deviation. ★ Indicates $p<0.05$

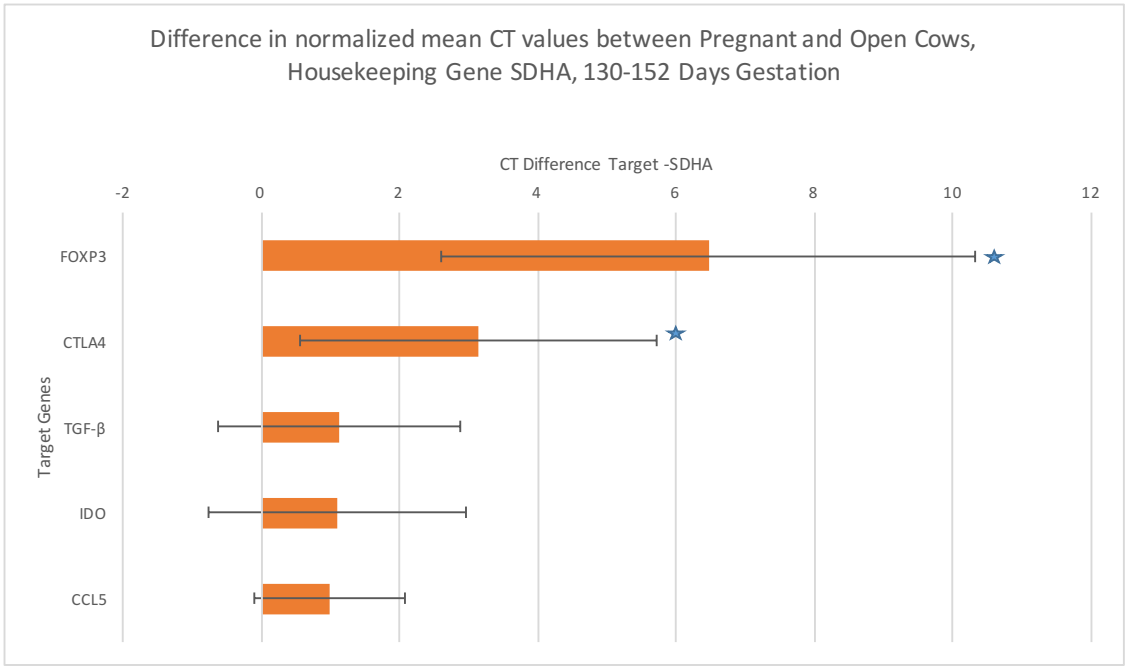

Figure 4.2. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping gene=SDHA; Mean gestation length = 144 days; range=130-152 days. Error bars represent standard deviation. ★ Indicates $p<0.05$

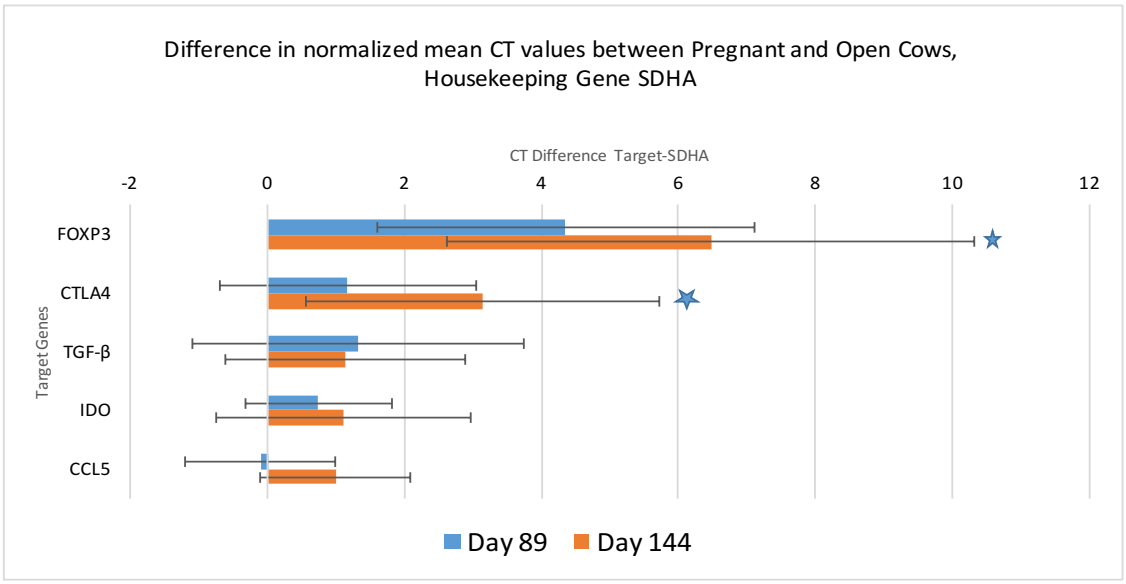

Figure 4.3. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping gene=SDHA. Error bars reflect standard deviation. ★ Indicates $p<0.05$

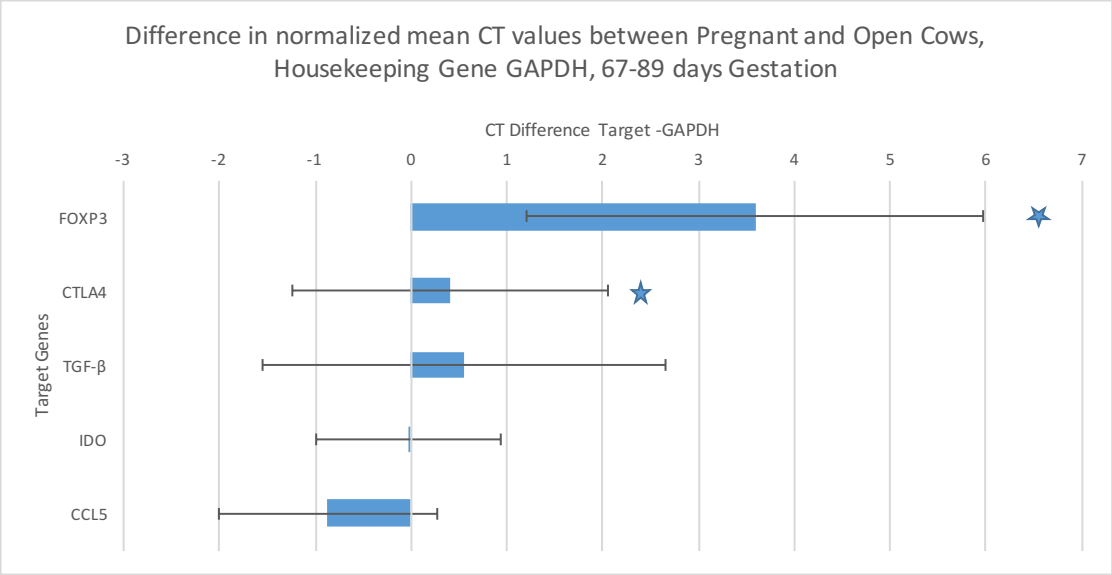

Figure 4.4. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping gene=GAPDH; Mean gestation length = 89 days; range=67-89 days. Error bars represent standard deviation. ★ Indicates $p<0.05$

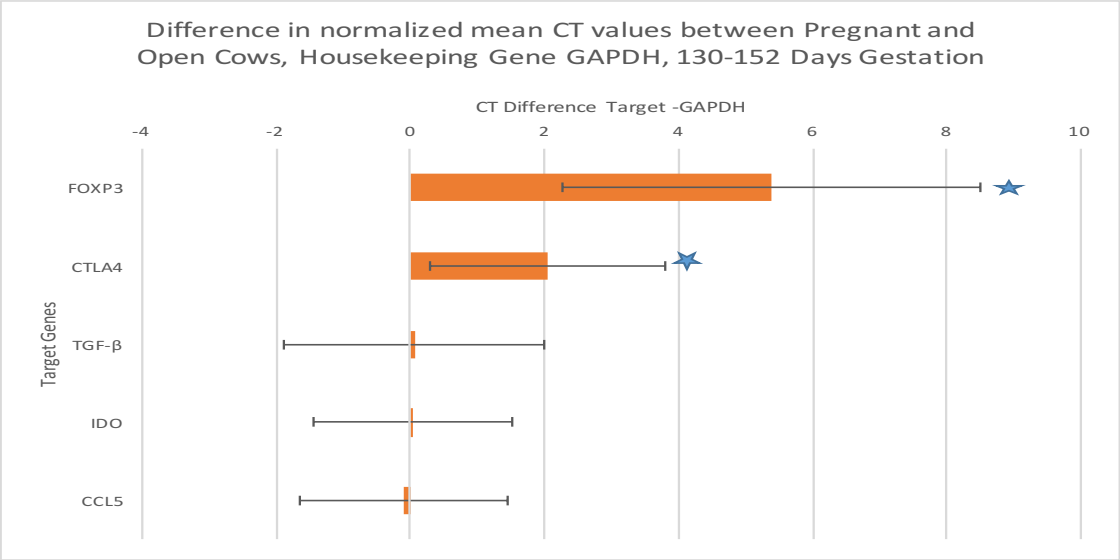

Figure 4.5. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping gene=GAPDH; Mean gestation length = 144 days; range=130-152 days. Error bars represent standard deviation. ★ Indicates $p<0.05$

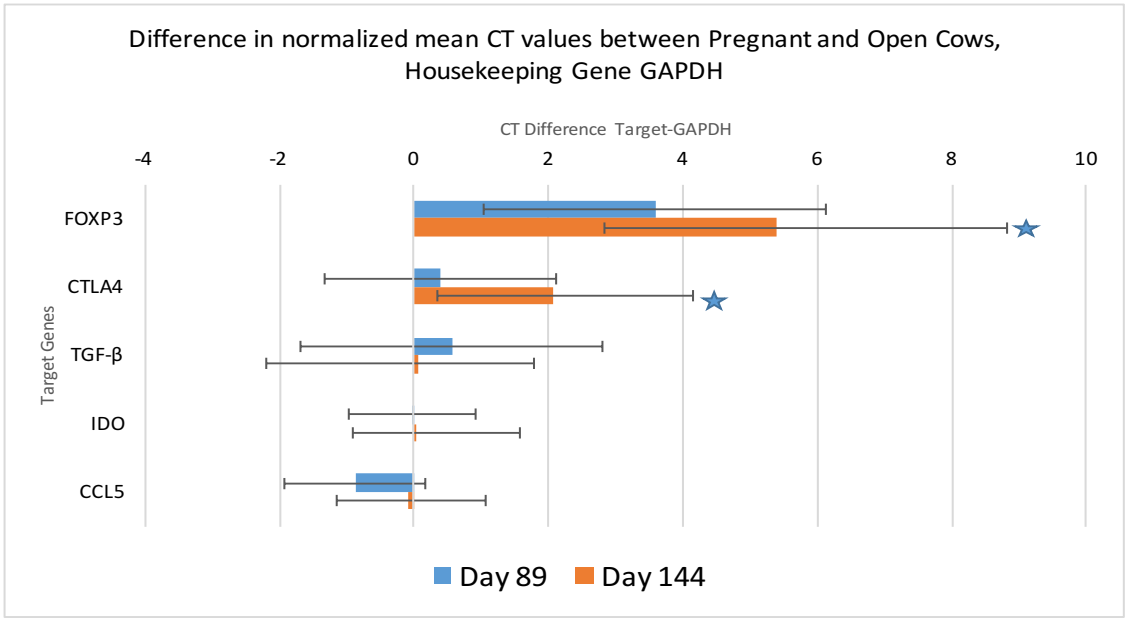

Figure 4.6. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping gene=GAPDH; Error bars represent standard deviation. ★ Indicates $p<0.05$

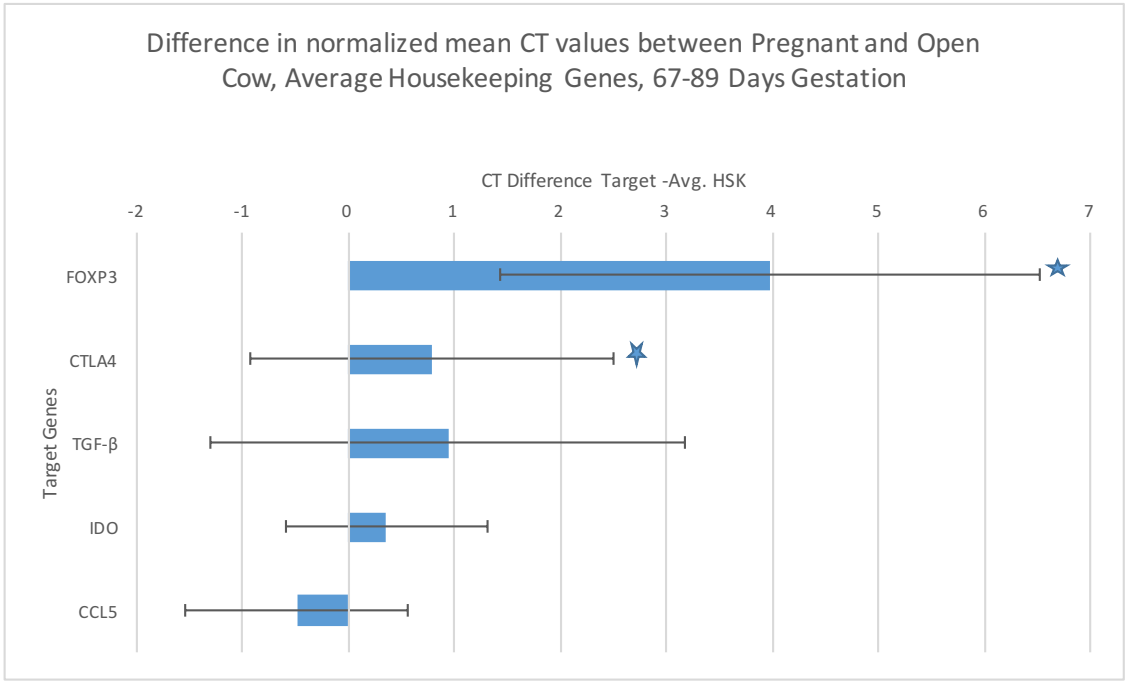

Figure 4.7. Difference in mean CT values between Pregnant and Open Cows at 89 days. Buffy coats, housekeeping genes = average of SDHA and GAPDH; Mean gestation length = 89 days; range=67-89 days

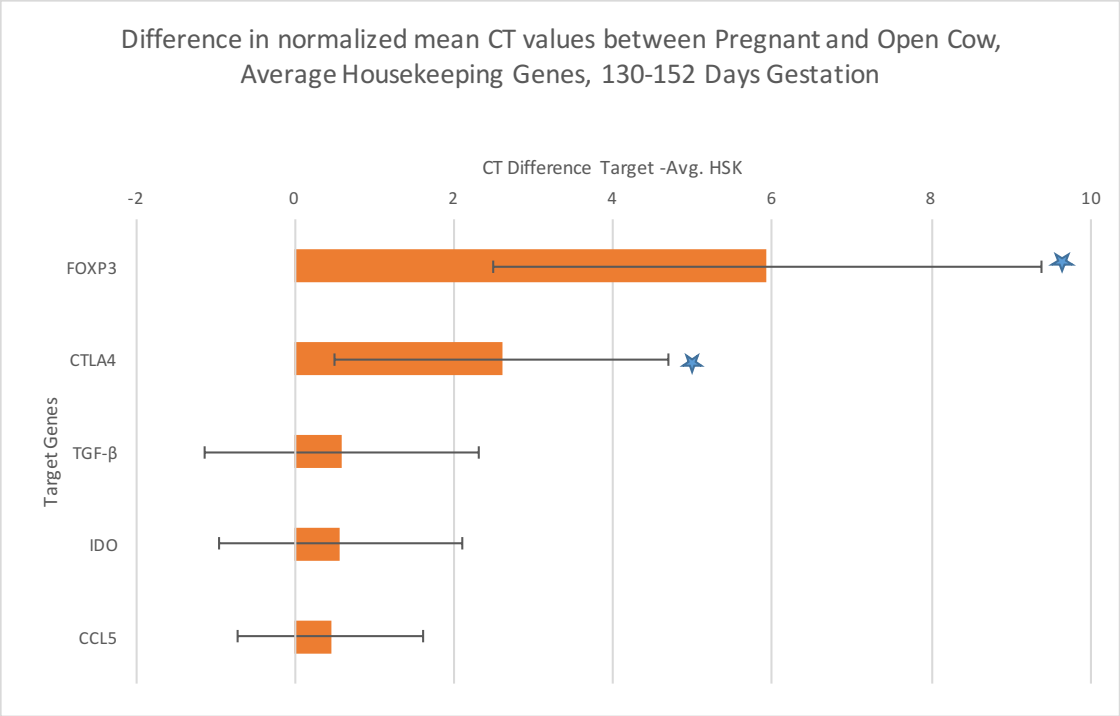

Figure 4.8. Difference in mean CT values between Pregnant and Open cows. Buffy coats, housekeeping genes = average of SDHA and GAPDH; Mean gestation length = 144 days; range=130-152 days. ⋆ Indicates $p<0.05$

Table 4.3

Summary of changes in mean crossing point values between pregnant and open heifers at gestational days 89 and 144, as reflected in Figures 4.1 and 4.2.

Difference in means of target genes and average of two housekeeping genes	FOX	CTLA4	TGF-β	IDO	CCL5
Day 89	3.97	0.79	0.94	0.36	-0.49
Day 144	5.93	2.60	0.59	0.58	0.45

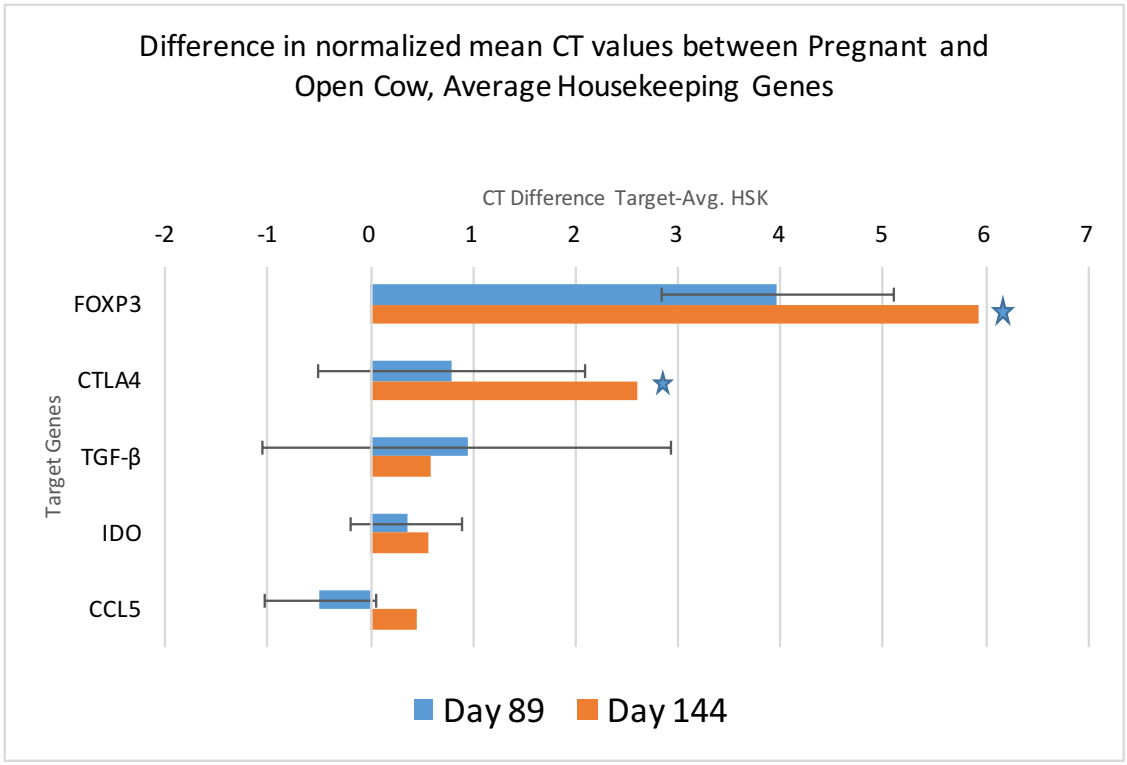

Figure 4.9. Difference in mean CT values between Pregnant and Open Cows Sampled at Average Gestational Days 89 and 144. Buffy coats, housekeeping genes = average of SDHA and GAPDH. ★ Indicates $p<0.05$

Table 4.4.

Descriptive statistical data for the difference in means for Pregnant and Open heifers at two gestational days.

			FOX	CTLA4	TGF-B	IDO	CCL5
Open	Day 89	MEAN	4.05	0.48	-1.65	3.84	0.34
		SD	1.14	1.30	1.99	0.54	0.95
Preg	Day 89	MEAN	0.08	-0.31	-2.59	3.48	0.83
		SD	1.88	2.02	2.45	1.21	1.13
Open	Day 144	MEAN	4.17	0.43	-1.69	5.10	0.24
		SD	1.37	1.41	2.26	1.39	1.12
Preg	Day 144	MEAN	1.87	1.88	1.09	1.67	1.20
		SD	5.93	2.60	0.59	0.58	0.45

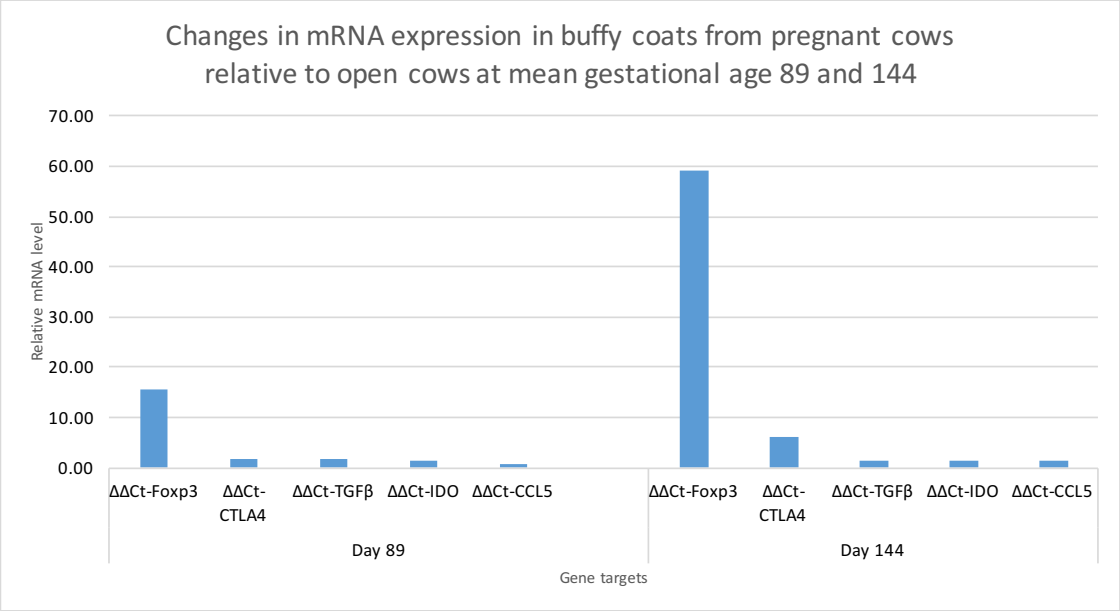

Figure 4.10. Changes in mRNA expression of Foxp3, CTLA4, TGF-B, IDO, and CCL5 in buffy coat samples of open and pregnant heifers with a mean gestational age of 89 and 144 days. Data are expressed as fold difference of mRNA expression normalized to an average of two housekeeping genes (SDHA and GAPDH), relative to the values obtained for open heifers.

Table 4.5.

Changes in mRNA expression of Foxp3, CTLA4, TGF-B, IDO, and CCL5 in buffy coat samples of open and pregnant heifers with a mean gestational age of 89 and 144 days. Data are expressed as fold difference of mRNA expression normalized to an average of two housekeeping genes (SDHA and GAPDH), relative to the values obtained for open heifers.

	Fold change in gene expression: $2^{-\Delta\Delta Cp}$				
Day 89	FOX	CTLA4	TGF-β	IDO	CCL5
	15.7	1.72	1.92	1.28	0.71
Day 144	FOXP3	CTLA4	TGF-β	IDO	CCL5
	61	6.06	1.5	1.49	1.36

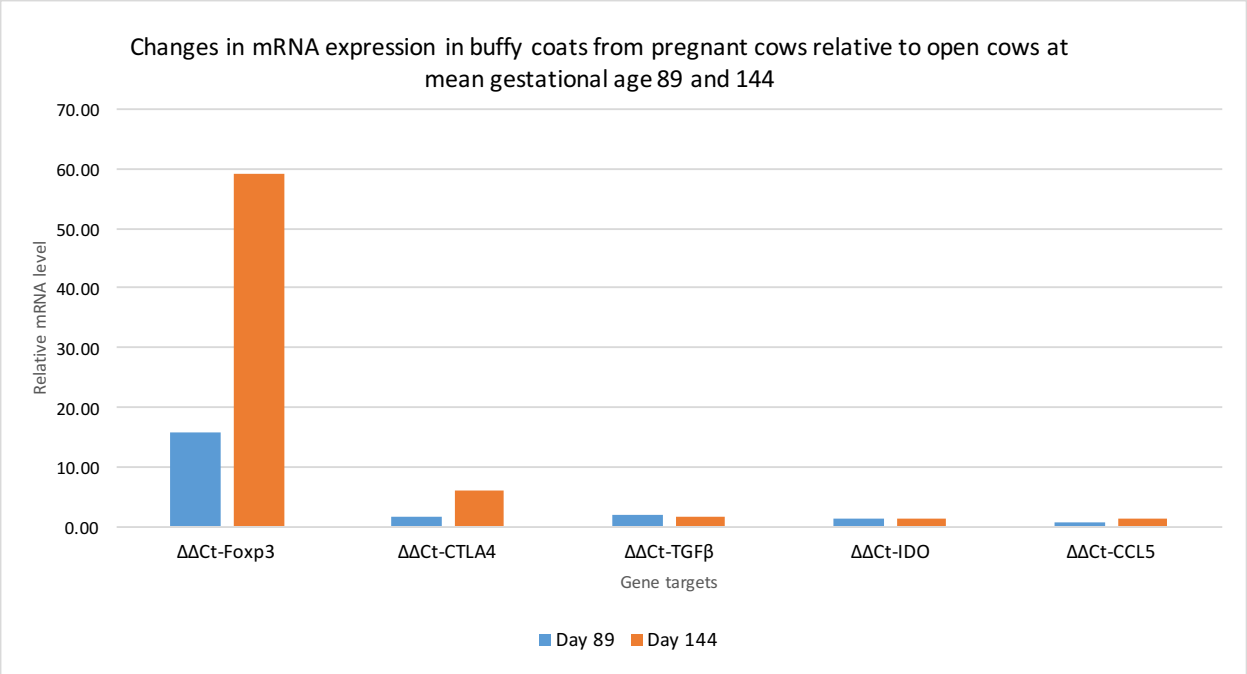

Figure 4.11. Changes in mRNA expression of Foxp3, CTLA4, TGF-B, IDO, and CCL5 in buffy coat samples of open and pregnant heifers from mean gestational days 89 and 144. Data are expressed as fold difference of mRNA expression normalized to an average of two housekeeping genes (SDHA and GAPDH), relative to the values obtained for open heifers.

General Conclusions

This trial was carried out by experimental inoculation of pregnant heifers with bovine viral diarrhea virus (BVDV), a Pestivirus within the family *Flaviviridae*, capable of infecting fetuses by vertical transmission, and causing multiple adverse reproductive outcomes. Fetuses of pregnant cattle infected with BVDV between 45 to 125-days gestation are highly susceptible to infection with noncytopathic biotypes of this virus, which are highly efficient at crossing the placenta. Fetuses infected with BVDV during these gestational days have the potential to become immunotolerant and persistently infected with the virus. Once these persistently infected fetuses are born, these animals serve as a continual source of BVDV in the environment. In cattle, immunologic regulatory processes in the pregnant uterus have not been elucidated, complicating our understanding of transplacental BVDV infections. The overall focus of these studies was to characterize normal immune parameters in the bovine placenta at mid-gestation, and investigate if BVDV alters immune signaling related to T cell activation.

The objectives of the studies described in this dissertation were focused on establishing normal immune regulatory parameters in bovine placentomes and in the peripheral circulation during mid-gestation. These investigations were performed with placental tissue and leukocytes obtained from peripheral blood samples collected from pregnant and non-pregnant heifers between 89-150 days of gestation, permitting three investigations. The first study was an *in vivo* experimental trial in which placental samples representing the maternal fetal interface were analyzed to evaluate immune regulatory molecules potentially exerting an immunosuppressive function during mid-gestation. In cattle, immunologic regulatory processes in the mid-gestational pregnant uterus have not been elucidated, complicating our understanding of transplacental

BVDV infections. The overall focus of these studies was to characterize normal immune parameters in the bovine placenta at mid-gestation, and investigate if BVDV alters immune signaling related to T cell activation. Our hypothesis was BVDV downregulates immune responses in the pregnant bovine uterus from gestational ages 75-150 to allow for persistence of the virus and escape from the maternal immune response. The second investigation centered on primary cell cultures propagated from maternal epithelial cells obtained from normal 75-day-gestation placentas, and these cells were also evaluated for genes involved in immune regulation. This *in vitro* experimental study was based on the hypothesis of the trophoblast providing a substantial role in intercellular communication at the maternal fetal interface, essentially functioning as an immunologic sensor involved with immune recognition and trafficking. Bovine trophoblasts were found to be susceptible to BVDV, and appeared immunologically responsive to BVDV infection. For the third study, peripheral blood leukocytes from non-pregnant and pregnant heifers were treated as samples from control and treated groups, respectively, in order to evaluate expression patterns of potential immune regulatory molecules during normal bovine pregnancy in the 89 to 150 day time period. The sampling period was similar to the gestational period optimal for vertical transmission of BVDV to cause persistent fetal infections. Immunologic signaling mechanisms in the bovine placenta during this phase of gestation are largely unknown. Our goal was to develop increased baseline knowledge about immunologic signaling in cattle at this specialized interface between the mother and fetus. This work provides a comparison of immune regulatory networks between humans and cattle and expands on how infectious agents such as BVDV alter the normal mechanisms of immune homeostasis, disrupting the symbiotic relationship between the mother and fetus during pregnancy.

We also performed a preliminary trial with BVDV seronegative heifers inoculated with BVDV at 75 days gestation. There was no inflammatory response at 5 and 7 days post-inoculation with the type I BVDV strain SD-1; however, viral antigen was strongly expressed in uterine glands of the 5 day post-inoculation heifer and expressed in the placentomes of the heifer infected with BVDV for 7 days. We speculated this finding could be due the ability of BVDV to evade immune detection or possibly there is the lack of an immune response due to a local presence of naturally occurring T regulatory cells in the bovine placenta. Speculation about the local numbers of naturally occurring T regulatory cells remained a theory because the proportions of different lymphocyte classes and baseline expression patterns of key T cell mediators expressed during various stages of gestation are still largely unknown in cattle, unlike humans and mice.

The maternal immune system in the pregnant uterus must be highly specialized in its ability to downregulate maternal immune responses toward the fetus and at the same time be able to detect and destroy invading viruses. The interaction of cytokines, chemokines, and immune cells is necessary to coordinate an effective antiviral immune response. The ability of the virus to be cleared from the maternal circulation but not the uterine mucosal defense system raises questions as to how the uterine immune system recognizes and responds to the virus. Additionally, mechanisms of recruitment and sequestration of responder immune cells within the bovine placenta is unknown. The opinions concerning the potential for classifying the placenta as an immune privileged site are mixed, but overall this theory has largely been discounted due to the bidirectional traffic of the hemochorial placenta of humans and mice, but immune privilege should still be a consideration for the bovine placenta due to the unique epitheliochorial placental

microanatomy. The scope of this study was designed to provide answers to several ongoing questions about basic normal bovine immunology at the maternal-fetal interface, and relate these findings to potential immunologic mechanisms by which BVDV crosses the maternal-fetal interface. Based on BVDV immunohistochemical staining patterns in persistently infected fetuses, several cell types frequently demonstrate strong and intense immunoreactivity, including: macrophages, epithelial cells, lymphocytes, and stromal cells (fibroblasts, endothelial cells, and also dendritic cells which appear as reticular-like cells within lymph nodes infected with BVDV). Consistent staining of these cell types has led to the conclusion BVDV has a tropism for these cell types, especially lymphocytes and epithelial cells. Fetal immune function would be well established in later gestation, and it seems reasonable to conclude the virus could at one point be eliminated by local intra-uterine immune responses, but maintained within a persistently infected cell type which is not recognized as infected by the immune system. This theory is not compatible with the pathogenesis of transient BVDV infections, in which the dam is viremic for only approximately 21 days. continual source of virus in a persistently infected cell type may deserve further consideration for conditions in which dams carrying persistently infected fetuses may have a fluctuating antibody titer, believed to be secondary to shifts of viral antigen recirculating from the persistently infected fetus back to the dam as pregnancy progresses. Viral pathogenesis studies in humans have broadened our perspective of mechanisms involving transplacental viral infections, and one example involves fetal transmission of hepatitis B. Hepatitis B first infects the placental barrier, then infects the uterus, and evidence for this conclusion was early infection of permissive trophoblasts (Bai, Zhang et al. 2007). A more precise description of these events has not been described in cattle. BVDV is transferred

hematogenously, but also cellular transmission of the virus likely occurs at the interface where maternal and fetal villi interdigitate. It does not seem possible there is sufficient cellularity in early placental development; including stromal, leukocytic, or epithelial, in perivascular areas of this interface based on the cellularity visualized in microscopic evaluations performed. Therefore, cell to cell transfer of the virus in early gestation does not seem feasible in placentomes when the placenta is developmentally immature, but increased leukocyte infiltration during later gestational phases supports this possibility. A Th1 type immune response predominates in early gestation during implantation in humans, and it seems logical an intrauterine viral infection during this time period in cattle may cause similar embryo and early fetal loss due to pro-inflammatory cytokine cascades already present locally in preparation for implantation. Furthermore, 75-125 day placentas may be composed of a somewhat different cellular phenotype which accumulate for immunosurveillance, favoring modulation of local immune responses to protect the fetus.

Within the past few years, there have been recent advances to the field of innate and adaptive immune responses during bovine pregnancy. These contributions have shown the virus is detected by the fetal immune system based on recent work by Hansen, et al (Smirnova, Webb et al. 2014, {Smirnova, 2012 #967), (Hansen, Smirnova et al. 2015). This group of researchers indicated a failure of IFN-γ signaling is the primary hypothesis for persistent BVDV infections. How and why BVDV exploits the 75-125-day gestation window to establish persistent fetal infections is intriguing. Microscopic examination of 75-day gestation placentas during this research identified few to no leukocytes with hematoxylin and eosin (H&E), within placentomes. Conversely, 150-day gestation placentas demonstrated a fairly high number of leukocytes in a

pilot study. Serial sampling of bovine placentas throughout early, mid, and late gestation would further our understanding of the immunologic dynamics present in normal bovine pregnancy. To conclude, we feel this research supported T regulatory cell-associated molecules are strongly expressed in mid-gestational peripheral blood, placentomes, and primary cytokeratin enriched placental cells. Furthermore, there was strong evidence the bovine immune response during pregnancy has the potential for a similar complexity as humans and mice. We feel potential mechanisms that may play a role in maintence of a Treg phenotype include normal cyclical changes which are primarily dictated by endocrinologic factors normally fluctuating during various phases of gestation, and local placental induction of a Treg phenotype is responsible for the expression of FoxP3, CTLA4, and TGF-β. If an inducible Treg population is associated with temporal Treg fluxuations, this is likely mediated physiologically or controlled by another factor besides BVDV infection, as there was evidence of T regulatory cell type patterns of gene expression in normal placenta. This research showed complex patterns of immunologic signaling during mid-gestation bovine pregnancy, indicating there is potential for modulation of local and systemic immunity in response to transplacental microbial infections such as bovine viral diarrhea virus.

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Appendix A

Table 1. Cytokeratin enriched primary cells inoculated with BVDV strain PA131 and passaged 3 consecutive times. Cultures terminated at 72 hours, frozen and stored at -80°C. Upon thawing, the $CCID_{50.}$ was determined by the Reed and Muench method.

Virus dilution	Infected	Uninfected	Infected (A)	Uninfected (B)	Total (A+B)	Ratio A/(A+B)	% infected
-1	3	0	15	0	15	15/15	100%
-2	3	0	12	0	12	12/12	100%
-3	3	0	9	0	9	9/9	100%
-4	3	0	6	0	6	6/6	100%
-5	3	0	3	0	3	3/3	100%
-6	0	0	0	3	3	0/3	0%

Appendix B

Table 2. Cytokeratin enriched primary cells inoculated with BVDV strain SD1 and passaged 3 consecutive times. Cultures terminated at 72 hours, frozen and stored at -80°C. Upon thawing, the $CCID_{50}$ was determined by the Reed and Muench method.

Virus dilution	Infected	Uninfected	Infected (A)	Uninfected (B)	Total (A+B)	Ratio A/(A+B)	% infected
-1	3	0	14	0	15	15/15	100%
-2	3	0	11	0	12	12/12	100%
-3	3	0	8	0	9	9/9	100%
-4	3	0	5	0	5	6/6	100%
-5	2	1	2	1	3	2/3	66%
-6	0	0	0	3	3	0/3	0%

Appendix C

Table 3. Cytokeratin enriched primary cells inoculated with BVDV strain BJ and passaged 3 consecutive times. Cultures terminated at 72 hours, frozen and stored at -80°C. Upon thawing, the $CCID_{50}$ was determined by the Reed and Muench method.

Virus dilution	Infected	Uninfected	Infected (A)	Uninfected (B)	Total (A+B)	Ratio A/(A+B)	% infected
-1	3	0	15	0	15	15/15	100%
-2	3	0	12	0	12	12/12	100%
-3	3	0	9	0	9	9/9	100%
-4	3	0	6	0	6	6/6	100%
-5	3	0	3	0	3	3/3	100%
-6	0	0	0	3	3	0/3	0%