EVALUATION OF IMMUNE SYSTEM TRAITS IN RECIPROCAL ANGUS-BRAHMAN F_1 CROSSES

A Thesis

by

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ABSTRACT

The influence of sex and reciprocal cross type on cell-mediated immune responses following *in vitro* exposure of Bovine Viral Diarrhea Virus (BVDV) were evaluated in Brahman-Angus F_1 cattle for across two trials. Lymphocyte populations were stimulated with BVDV Type 1 (BVDV1) and Type 2 (BVDV2) genotypes. In Trial 1 (n = 48) T cells from Brahman-sired animals consistently ranked approximately 20% higher for BVDV positivity (BVDV+) than those from Angus-sired animals pre-MLV vaccination. Post-MLV vaccination, T cells from Angus-sired animals were ranked 20% higher ($P < 0.05$) for CD2+, CD4+, and CD8+ cells. Post-MLV steers consistently had 30-35% higher ($P < 0.05$) frequencies of T cells BVDV+ following viral exposure; steers had higher frequencies ($P < 0.05$) of CD4+ and CD335+ cells expressing IFN-γ, and ranked higher for CD2+ and CD8+ cells when compared to heifers in both crosses. There were no significant correlations involving BVDV antibody titers and T cell responses. When stimulated with BVDV2, positive correlations of 0.33 to 0.39 ($P < 0.05$) were observed between cell frequencies for BVDV presence and IFN-γ expression within cell type, but these correlations were not significant when stimulated with BVDV1, except for CD335+ ($r = 0.41$, $P = 0.003$). In Trial 2 (n = 19), Angus-sired animals had an 85% higher ($P = 0.032$) proportion of CD335+ cells, while having 38% lower ($P < 0.001$) proportion of CD4+ cells regarding total lymphocyte numbers. Angus-sired cattle were 26% higher for cell frequency of Indoleamine 2,3-dioxygenase-1 (IDO) expression in CD335+ cells ($P = 0.001$) and 58% higher ($P = 0.018$) for total lymphocytes. Bulls (n = 4) consistently ranked higher than steers (n = 15) for cell frequency of IDO expression for all cells but were 73% higher ($P = 0.012$) for total lymphocytes. Bulls also ranked higher for cell frequency of programmed cell death-1 (PD-1) expression by 58.2% in CD4+ ($P = 0.025$). Bulls had a tendency to rank higher in frequency of PD-1 and IDO expression

across all cell types. Differences in cell mediated responses involving reciprocal cross types and sex observed in this project may relate to widely reported reciprocal cross differences in birth weight in *Bos indicus-Bos taurus* crossbred cattle and warrant additional study.

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CHAPTER I

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most prevalent viruses throughout the cattle industry globally and is an important pathogen for bovine respiratory disease (BRD). Infection with BVDV is commonly a precursor to morbidity as it leads to depression in various immune system functions, and many times is found with co-infection involving other BRD pathogens. Both the innate and adaptive immune system components have shown immunosuppression from BVDV infection through apoptosis of Natural Killer cells (innate system), and decreases in B and T lymphocytes (adaptive system). Due to the different genotypes (Type 1 and Type 2), vastly numerous strains, and its multiple influences on immune suppression, detailed study of different immune system traits is crucial to better understand BVDV and limit its susceptibility on beef cattle production. Some studies have shown genetic composition of cattle may account for variations in immune response to BRD, and more research is needed about animal-pathogen interactions.

Cell-mediated immunity is largely operated by various classifications of B and T lymphocytes. The frequency of BVDV-positive T cells was studied to evaluate T cell immune system traits.

Interferon-γ (IFN-γ) is a part of the interferon cytokine family, which is produced in response to virus infection or immune stimulation. Interferon-γ is related to cell-mediated immunity, and possesses antiviral properties by interrupting viral RNA protein synthesis. Because of these properties, IFN-γ has been considered as a vaccine adjuvant for vaccines against viruses.

Programmed cell death-1 (PD-1) acts as an inhibitory, immunosuppression protein that is typically expressed in response to immune challenges, and downregulated in acute challenges to allow for immune reaction to occur. However, prolonged upregulation of PD-1 leads to immune exhaustion, at which time immune system efficiency decreases. Prolonged exposure to viruses has been connected to CD8+ T cell exhaustion.

Indoleamine 2,3-dioxygenase-1 (IDO) is another protein in immunoregulation and is typically expressed on antigen presenting cells, which are necessary in the onset of immune reaction. The consistent upregulation of IDO leads to T cell apoptosis, bias against cytotoxic T cells, and impairs the efficiency of Natural Killer cells. High expression of IDO encourages the immune system toward a tolerogenic capacity from an immunogenic role.

Among *Bos indicus*-*Bos taurus* F_1 crosses, an unusual pattern for calf birth weight occurs where *Bos indicus*-sired calves from *Bos taurus* dams have much heavier birth weight, and much heavier male vs. female calves as compared to the reciprocal cross (from *Bos taurus* sires x *Bos indicus* dams). It has been speculated that genomic imprinting may be involved with these birth weight differences. Investigation of potential reciprocal effects on immune responses has not been conducted. This thesis investigated potential reciprocal F_1 effects for T cell responses for cell frequency of BVDV presence and IFN-γ in Trial 1 and PD-1 and IDO in Trial 2, with the overall goal of improving understanding about cattle immune system profiles and responses under typical production conditions.

Objectives

The objectives of this thesis were to investigate potential Angus-Brahman reciprocal F_1 cross effects following MLV vaccination for T cell responses when stimulated *in vitro* to Bovine Viral Diarrhea Virus and frequency of cell expression of Interferon-γ. In Trial 2 of this study, the

objectives were to understand the impact of reciprocal F_1 cross effect and sex on the frequency of cell expression of PD-1 and IDO to further understand immune system response in typical production settings.

CHAPTER II

LITERATURE REVIEW

The immune system is exceptionally complex and interconnected. One traditional method to study the immune system has been through vaccine response. Vaccine response has often previously been studied through changes in virus-neutralizing antibody titers, however this method has been found to offer little insight to the level of cell-mediated immunity. This chapter will review various properties of cell-mediated immunity, with particular interest in lymphocyte function and the cytokine interferon-γ This literature review will explore properties of immune function, as well as its relation to vaccination and the bovine viral diarrhea virus (BVDV) specifically. Of particular interest to this literature review are the influence of genetics on immune response, as identifying ways to breed for disease resistance or favorable vaccine response that could benefit the beef industry.

Overview of Basic Anatomy

The immune system can largely be separated into two parts, the innate and adaptive immune system. This distinction comes largely from the specificity of reactions and time of reactions, however the two parts are largely integrated (Daha, 2011; Tizard, 2018). The innate system is tasked with forming an immediate, non-specific immune response to challenge. Innate responses are activated immediately when a pathogen enters the organism, and is very effective as approximately 95% of all antigen challenges are thought to be neutralized by cells of the innate immune system, before the adaptive system is initiated. In the scope of this review, the adaptive immune system functions will be emphasized.

The adaptive immune system

The adaptive immune system, mediated by various lymphocytes, is delayed by 4 to 7 days after initial immune challenge in order to form a specific response to the challenge (Janeway et al., 2001). Within the adaptive immune system there are cell surface receptors specific to foreign invaders which aide in their destruction and the subsequent memory response after each encounter. If the animal experiences the same foreign invader, the adaptive immune system is able to respond faster and more efficiently through the creation of memory B and T cells (Tizard, 2018). The adaptive immune system is exceptionally diverse and complex, which allows for defense against many different foreign invaders.

The adaptive immune system is composed of two branches, the humoral and cell-mediated immune response. The humoral immune response is largely mediated by specialized white blood cells classified as B cells. The B cells of the humoral immune response are responsible for secreting antigen-specific antibodies directed against foreign invaders (Tizard, 2018). Antibody-mediated immune responses are commonly referred to the humoral immune response due to antibodies being found in body fluids, historically referred to as humors (Janeway et al., 2001; Tizard, 2018). The cell-mediated immune response is largely regulated The T lymphocytes are developed in the thymus and are known as naïve T cells until an encounter with antigen (Janeway et al., 2001). These cells function by destroying infected cells, therefore referred to as the cell-mediated response (Tizard, 2018). Extracellular invasions are typically combated by the B cells of the humoral branch of adaptive immunity, whereas T cells of the cell-mediated branch of adaptive immunity fight against intracellular invasion. Extracellular invasions are typically a consequence of bacteria or fungi, whereas intracellular infections are commonly the consequence of virus (Tizard, 2018). Antibody-mediated responses

can offer protection with intracellular infection by blocking the uncoating and fusion mechanisms of viruses within cells, which in turn stops progression of virus (Bottermann and Caddy, 2022).

The humoral and cell-mediated immune responses work independently and in conjunction with one another to allow the adaptive immune system to function (Janeway et al., 2001). Antigens are trapped when entering the body and recognized as foreign. Dendritic cells, or other antigen-processing cells (APCs), encounter and capture antigen, and receive signals from pathogens that influence the immune response (Liu, 2015). When the APC binds to antigen, a sample of the foreign material is presented on major histocompatibility complex molecules for B and T cells to recognize antigen (Tizard, 2018). Based on signals from APCs, T cell differentiation is able to be antigen specific (Liu, 2015). These antigen-specific cytotoxic T lymphocytes then destroy cells infected with antigen. When the B cells are presented antigen they then produce antigen specific antibodies in order to neutralize antigen (Tizard, 2018). The adaptive immune system relies heavily on the creation of memory B and T cells that remember this antigen event, and will react faster and more efficiently the next time the antigen is encountered (Tizard, 2018).

Virus-neutralizing antibodies have been used in research as an indication of protection against infection of BVDV (Alpay and Yeşilbağ, 2015), as well as a way to find similarities and differences between strains (Mosena et al., 2020; Falkenberg et al., 2021). Immune protection can occur without serum antibodies present (Ridpath et al., 2003; Downey-Slinker et al., 2016; Falkenberg et al., 2020). This concept is not species-specific or pathogen-specific. Neal and Splitter (1998) found protection accredited to the CD8+ cells of the cell-mediated immune system in mice challenged with encephalomyocarditis in the absence of serum antibodies. Others

have also observed that protection that occurs without the presence of serum virus-neutralizing antibodies may be accredited to mucosal immunity or cell-mediated immunity functions (Neal and Splitter, 1998; Platt et al., 2006; Stevens et al., 2009; Van Anne et al., 2018).

T lymphocytes of the cell-mediated immune system

The T cells account for 60-80% of lymphocytes in circulation in the blood at all times in healthy animals not experiencing immune system depression (Tizard, 2018). The cell-mediated immune response is driven by three major types of T cells. Those responsible for the destruction of infected or abnormal cells are the cytotoxic T cells, also referred to as CD8+ T cells (Tizard, 2018). The T helper cells (CD4+ T cells) provide signals, usually through the action of cytokines, that activate adaptive immune responses. Within the T helper cell designation, there are 3 groups. T helper 1 (Th1), T helper 2 (Th2), and regulatory T cells (T reg) (Tizard, 2018). The Th1 cells are important in destroying intracellular bacterial infections (Janeway et al., 2001). Th1 cells activate macrophages and release cytokine and chemokines which attract additional macrophages to the area (Janeway et al., 2001). The T cells also act to destroy extracellular pathogens by activating the B cells of the humoral immune system, which is the main role of the Th2 cells (Janeway et al., 2001). The Th1 cells promote cell-mediated immune responses, and thus down-regulate humoral immune responses that are driven by antibodies. Complete maturity of Th1 cells is achieved by additional stimulation from IFN-γ. Once activated, Th1 cells produce cytokines IL-2 and IFN-γ (Tizard, 2018). Regulation of immune responses is the responsibility of regulatory T cells (Tizard, 2018).

The T helper cell (CD4+) responses occur simultaneously with humoral immune responses, assumed due to the requirement for T cell help eliciting a humoral immune response (Tizard, 2018). The classes of the adaptive immune system work simultaneously and in

conjunction, it has become clear that measuring serum antibody titers alone does not explain an animal's complete immune response (Platt et al., 2006; Van Anne et al., 2018). Potential protection through vaccination is commonly estimated through antibody titers (Alpay and Yeşilbağ, 2015; Neill et al., 2019), so many vaccines have a Th2, or humoral immune system, bias. This may be due to a bias of research focusing on the humoral immune response onset by vaccination. Additionally, Th2 cells favor the humoral immune functions, and secrete anti-inflammatory cytokines such as IL-4, IL-5, and IL-6 (Ott and Gifford, 2010). The Th1 cells favor cell-mediated immune functions, such as cytotoxicity and inflammatory responses, mediated by the cytokines IL-2 and interferon-γ (Janeway et al., 2001).

Bovine natural killer cells are distinguished by the activation receptor CD335+ (Osman and Griebel, 2017). Natural killer cells (CD335+) are non-specific innate lymphocytes that carry out the cytotoxic activity of the innate immune response (Osman and Griebel, 2017). The activation of natural killer cells release cytotoxic granules which kill target cells through perforin (Bryceson et al., 2006). The CD2+ receptor is one of the earliest receptors that identified T cells (Haynes et al., 1989) and is a crucial receptor in the activation of T cells. The CD2+ receptor enhances the connection of the T cell receptor to antigen presenting cells and aides in recruitment of other T cells (Krensky et al., 1983). The CD2+ protein is crucial in cell adhesion and interacts with antigen (van der Merwe et al., 1994). Table 2.1 summarizes relevant cell types central to this thesis along with their general functions.

Table 2.1 Classifications and functions of relevant lymphocytes.

Type of cell	Immune response	Action/ Function
CD2+ T cell	Cell-mediated	Enhance TCR mediated activation of T cells. Stabilize cellular adhesion between T cells and APCs.
CD4+ T cell	Cell-mediated/ humoral - T helper cells - Th1and Th2	Receptor for MHC II molecules, which aides in antigen recognition
CD8+ T cell	Cell-mediated- Cytotoxic T cells	Cytotoxic activity, attack and kill abnormal cells.
CD335+ cell	Innate	Commonly found on natural killer cells, kill abnormal cells.
B Cell	Humoral	Secretion of antibodies.

Cytokines

Cytokines are signaling proteins that control the immune system in the body (Tizard, 2018). These signaling proteins connect various immune functions, and coordinate immune responses by impacting virtually every biological process, as signaling, inhibitory, or stimulatory proteins (Nathan and Sporn, 1991). Cytokines have been shown to be dual purpose, as well as contradictory. Cytokines can impact many cell types, and immune cells rarely produce one cytokine at time. Cytokines are also redundant as many may elicit the same effect (Tizard, 2018). Cytokines are produced in response to stimuli, such as the binding of antigen to B or T cell receptors (Tizard, 2018). Cytokines act as the communicator between cell-mediated and humoral immune systems by acting as the mediator between Th2 and Th1 designated T cells (Nathan and Sporn, 1991; Tizard, 2018). When cytokines bind to receptors they dictate cell behavior by initiating or inhibiting cell division, differentiation, or protein synthesis (Tizard, 2018). Cytokines can be classified by their role in the adaptive immune system. Type I cytokines act in cell-mediated immune responses, while type II cytokines mediate antibody driven immune

responses (Tizard, 2018). Defense against infections require type I cytokines to act quickly to initiate a cell-mediated response. The most important cytokines in the type I response are interferon-g (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor a (TNFα, (Tizard, 2018). The Th1 T cells secrete the type I cytokines such as IL-2 and IFN- γ, which involves an upregulation in phagocytic activity (Spellberg and Edwards, 2001) . Cytokines from type I responses have the tendency to suppress type II immunity and vice versa, which encourages bias towards a cell-mediated or humoral immune response (Spellberg and Edwards, 2001; Platt et al., 2009; Tizard, 2018).

The cytokine interferon-γ (IFN- γ) is a part of the interferon cytokine family. This family of cytokines is produced in response to virus infection or immune stimulation (Tizard, 2018). The interferon cytokines interrupt viral RNA and protein synthesis, therefore have antiviral functions (Tizard, 2018). The cytokine IFN- γ is mainly produced by activated T cells (CD4+ and CD8+ cells) and natural killer (NK) cells (Tau and Rothman, 1999). This promotes activation of macrophages, and encourages antivirial and antibacterial immunity (Tau and Rothman, 1999), and enables NK cell activity (Platt et al., 2006). Interferon-γ is produced only by memory CD4+ and CD8+ cells, those that have been exposed to antigen before (Van Anne et al., 2018), which means it is a strong indicator of acquired immunity when looking at vaccine efficacy or immunity from previous exposure. When IFN- γ is produced by CD4+ and CD8+ cells, it is initiated by foreign antigen binding to the T cell receptor on a CD4+ Th1 cell (Tizard, 2018).

The use of cytokines as adjuvants has gained interest in animal vaccines, as they offer general enhancement for the preexisting immune response performed when a vaccine is given, as well as modification of the classification of immune response (Lowenthal et al., 1998; Wedlock

et al., 2008; Burakova et al., 2018). Utilizing cytokines as adjuvants in some studies, has shown to enhance the immune response while utilizing lower levels of antigen, as well as reducing the number of vaccinations required to reach a satisfactory immune response (Lowenthal et al., 1998; Foss and Murtaugh, 2000). Interferon-γ has been of interest for its adjuvant capabilities because it promotes Th1 cell activity while also stimulating the uptake and presentation of antigen (Burakova et al., 2018). This process is performed by stimulating the MHC II on antigen presenting cells (APCs) (Burakova et al., 2018). This cytokine activates macrophages, activates CD8+ cells, and increases the cytotoxic activity of NK cells, all of which aide in virus disposal and neutralization (Burakova et al., 2018). Fan et al. (2016) found pigs receiving a vaccine for classical swine fever virus (CSFV) with species-specific IFN-γ as an adjuvant demonstrated normal behavior and temperatures after virus challenge, whereas pigs that received the vaccine alone experienced mild, transient pyrexia. There was a greater viral load in the spleens of pigs vaccinated without the IFN-γ adjuvant, and lower expression of MHC I and II, which showed evidence of lower rates of antigen presentation (Fan et al., 2016). Anti-CSFV IgG2 antibodies were significantly elevated in pigs that had received IFN-γ as an adjuvant (Fan et al., 2016), which indicated an enhanced Th1, cell-mediated, immune response.

Major Histocompatibility Complex Class II

Major histocompatibility complex class II (MHC II) molecules play a crucial role in initiating immune responses and are typically found on cortical epithelial cells, dendritic cells, and antigen presenting cells (APCs) (Tizard, 2018). The main function of MHC II molecules is to bind to phagocytized peptides from antigen molecules. The MHC II molecule displays antigen on the plasma membrane of APCs which then allows CD4+ cells (T helper cells) to recognize antigen (Villadangos, 2001). Within antigens, there are immunodominant determinants, which

refers to specific epitopes within antigens, and MHC II molecules only associate with the immunodominant determinants of each antigen (Villadangos, 2001; Tizard, 2018). The T cells are antigen restricted and will only respond to antigen when bound to a particular MHC molecule. The CD4+ cells are restricted by the MHC II molecules, and CD8+ cells are restricted by MHC I molecules (Villadangos, 2001; Tizard, 2018).

Bovine viral diarrhea virus has been found to decrease the percentage of T cells and B cells in circulation (Marshall et al., 1994) This in turn decreases the proportion of cells that express MHC II on their surface membranes. By decreasing MHC II expression, antigen presentation function decreases and so does efficiency of immune function (Marshall et al., 1994). Brodersen and Kelling (2009) found that concurrent infection of bovine respiratory syncytial virus (BRSV) and BVDV reduced the percentage of cells expressing MHC II within the spleen and Peyer's Patches, which further indicated the negative effect of BVDV on antigen presentation and cell-mediated immune system. Brodersen and Kelling (2009) found that concurrent infection of BRSV and BVDV reduced the percentage of cells expressing MHC II within the spleen and Peyer's Patches, which further indicated the negative effect of BVDV on antigen presentation and cell-mediated immune system (Brodersen and Kelling, 1999).

Major histocompatibility II (MHC II) molecules provide the first signal of antigen recognition and therefore begins actions of the adaptive immune system. Bovine viral diarrhea virus infected antigen presenting cells appear to have a reduction in Fc and C3 receptor expression (Chase, 2013) that activate phagocytic processes. Bovine viral diarrhea virus impacts antigen presentation in several ways which seem to be dependent on the BVDV type and type of the antigen presenting cell itself (Chase, 2013). Glew et al. (2003) found *in vitro* infection of

BVDV impacted monocytes to a larger degree than dendritic cells. Monocytes are necessary to present antigen to T helper cells.

The BVD virus exists in two biotypic forms, cytopathic (cp) and non-cytopathic (ncp) (Glew et al., 2003). The two biotypes are differentiated by which causes death in cultured cells. Cytopathic types cause death in cultured cells, where non-cytopathic types fail to induce death in cultured cells (Peterhans et al., 2010). The infection with cp BVDV kills cells by initiating the process of apoptosis. When stimulated with cp BVDV, viable monocyte count has decreased from 76% one hour after infection to 26% 96 hours after infection. Conversely, viable dendritic cell count did not fall below 75% at any point (Glew et al., 2003). When stimulated with a ncp BVDV type, Glew et al. found no difference in cell viability 24 hours post infection, however, 72 and 96 hours post infection, difference in cell viability became apparent, with monocytes being impacted more severely. Lee et al. (2009) found that using both cp BVDV1a and ncp BVDV1b subgenotypes decreased MHC II expression; however, the largest decrease was seen utilizing the ncp BVDV1b strain (Lee et al., 2009). Archambault et al. (2000) found that a ncp BVDV type 2 strain caused an increase in morbidity and mortality in PMBCs, which in turn decreased MHC II surface expression.

Programmed cell-death-1

Programmed cell-death-1 (PD-1) is an inhibitory immune receptor that is encoded in the *Pcd1* gene (Wang et al., 2005; Francisco et al., 2010; Bally et al., 2016). The PD-1 protein and related ligands deliver inhibitory signals to regulate the reaction of T cells to challenge, T cell activation, and immune tolerance (Francisco et al., 2010). Response to foreign and self-antigen requires cytotoxic activity to clear antigen, but also maintain tolerance to not create tissue damage (Allie et al., 2011). The PD-1 protein can be expressed on various effector cells, such as

T cells, B cells, natural killer (NK) cells, macrophages, and dendritic cells (Francisco et al., 2010). A few hours following T cell activation, the functional effects of PD-1 expression have been observed (Allie et al., 2011). The PD-1 protein has a large impact on cytokine production, notably IFN- γ. Th1 cytokine production (IFN- γ), is increased in the absence of PD-1 (Wang et al., 2005). Inhibitory effects regulated by PD-1 rely largely on the strength of the T cell receptor signal, with larger effects seen at lower signal (Wang et al., 2005; Sharpe and Pauken, 2017).

The inhibitory immune receptor PD-1 is expressed on T cells in response to most immune challenges, however the nature of the challenge impacts expression of PD-1. In acute antigen challenge settings, PD-1 is downregulated for typical immune responses to occur. During prolonged chronic challenge, PD-1 expression remains high, which results in an ineffective response to antigen. The immune regulator PD-1 is closely related to immune system exhaustion (Zhang et al., 2007; Bally et al., 2016). When T cells are in a resting state, PD-1 is expressed at very low levels, which is heavily linked to its immune-tolerance function (Allie et al., 2011). The PD-1protein is also downregulated in instances of acute antigen stimulation, until antigen clearance, at which time, PD-1 expression returns to intermediate expression (Allie et al., 2011; Bally et al., 2016). After acute antigen stimulation, many effector T cells die due to various inhibitory factors, such as PD-1, to ensure that uncontrolled activation and tissue damage does not occur due to an unregulated CD8+ T cell response (Allie et al., 2011).

Immune exhaustion due to high expression of PD-1 is seen during chronic infection. Increased expression of PD-1 has been connected to CD8+ T cell exhaustion during chronic exposure to viruses (Bally et al., 2016). Exhaustion of CD8+ T cells leads to decreased immune function as exhausted CD8+ T cells are hindered in their ability to proliferate and produce cytokines, and have decreased cytotoxic activity (Bally et al., 2016; Sharpe and Pauken, 2017).

High PD-1 expression is not solely responsible for immune exhaustion, and is necessary for the development of regulatory T cells, which pushes the immune system towards a regulatory role, indicting that PD-1 is necessary to bring the immune system back to homeostasis through regulatory T cells (Allie et al., 2011). High expression of PD-1 can be combatted with antibody-based immune checkpoint blockades (Bally et al., 2016), in which antibodies block PD-1/ PD ligand-1 interactions (Sharpe and Pauken, 2017). Antibody blockade therapies have been useful in advanced cancer treatments by stimulating the antitumor immune response (Allie et al., 2011; Bally et al., 2016).

Vaccination response is also largely impacted by PD-1 expression. Allie et al. (2011) demonstrated increased memory cell activation in the absence of PD-1 expression, as well as increased CD8+ antigen-specific activity. The absence of PD-1 expression was found to generate more memory cells. This indicates that vaccine response, notably the ability to create memory cells, may be improved in the absence of PD-1 expression, either through antibody blockage or other function (Allie et al., 2011).

Studies have been conducted on mice deficient in PD-1 expression. Wang et al. (2005) found mice deficient in PD-1 experienced an acceleration of Type I diabetes, with 100% of mice deficient in PD-1 developing Type I diabetes by week 10, when compared to “wild-type” mice, which did not develop Type I diabetes until 17 weeks, with only 30% developing the condition (Wang et al., 2005). This may indicate that excessive PD-1 suppression could possibly be linked to various diseases

Epigenetic regulation of the *Pcd1* gene has been evaluated. Epigenetics refers to the heritable mechanism by which cells maintain transcriptional profiles across cell generations, but without modifying the core genetic makeup (Jablonka and Lamb, 2006). Utzschneider et al.

(2013) found that exhausted T cells, which experienced an upregulation of PD-1 expression, maintained their exhausted phenotype in following cell divisions, even following the removal of antigen (Utzschneider et al., 2013). Utzschneider et al. (2013) discovered that exhausted CD8+ T cells transferred from chronically infected mice into naïve mice maintained the exhausted phenotype and continued expressing PD-1 at a high level.

Indoleamine 2,3-dioxygenase (IDO)

Indoleamine 2,3-dioxygenase (IDO) is an immunsuppressive regulator protein. The main function of IDO is to catabolize tryptophan to kynurenine for the kynurenine pathway (Mellor, 2005). The kynurenine pathway is the primary route for tryptophan catabolism (Davis and Liu, 2015), and tryptophan is an essential amino acid largely tied to immune system through T cell activation. Tryptophan is an essential amino acid critical for cell survival, and the enzymatic activity of IDO is the rate-limiting factor in the kynurenine pathway (Mellor, 2005). The protein IDO is expressed on antigen-presenting cells (dendritic cells, macrophages, and tumor cells) (Mellor, 2005; Savas et al., 2015). In normal settings, IDO is expressed in areas that experience chronic inflammation as well as tissues that have mucosal surface areas (Mellor, 2005), such as the placenta where its expression is crucial in preventing rejection of allogenic fetuses (Munn et al., 1998).

The immunosuppressive properties of IDO operate by decreasing the amount of tryptophan in circulation, and an increase in IDO expression would increase its enzymatic activity on tryptophan (Munn and Mellor, 2016) . Cell survival is tryptophan reliant, so T cells are highly sensitive to a decrease in tryptophan concentration. Inadequate levels of tryptophan ultimate leads to a hindrance in T-cell ability to proliferate and function properly (Munn and Mellor, 2016). Tryptophan shortage leads to T cell dysfunction and initiates apoptosis (Théate et

al., 2015). In addition, an upregulation of IDO promotes naïve T cells to regulatory T cells, rather than cytotoxic CD8+ T cells, and impairs the function of Natural Killer cells by limiting the amount of tryptophan locally, which inhibits the proliferation of NK cells (Chiesa et al., 2006; Fallarino et al., 2006). This encourages the immune system towards a more regulatory role. The upregulation of IDO handicaps the T cell-driven cell-mediated immune system by creating unstable cell environments. The over expression of IDO influences the role of antigen-presenting cells from an immunogenic role to a tolerogenic role (Fallarino et al., 2006). Excessive expression of IDO has been shown to correlate with poor clinical outcomes in various cancers, such as breast and ovarian cancers (Savas et al., 2015).

Kozuma et al. (2018) studied the impact of IDO expression on the prognosis and severity of lung adenocarcinoma. Minimal IDO expression (1% and above) was present in 60.9% of resected lung adenocarcinomas, and high expression of IDO (50% and above) was expressed in 14.8% resected lung adenocarcinomas (Kozuma et al., 2018). This may indicate that even very low levels of IDO expression are positively correlated to higher prevalence of advanced forms of cancers. Furthermore, Kozuma et al. (2018) also found that patients that expressed IDO in their lung adenocarcinoma had a shorter "disease-free survival time" and lower overall survival. Additionally, Kozuma et al. (2018) studied the impact of upregulation of immunosuppression factor PD-1 and IDO together. All patients that were found to have higher expression of PD-1 (≥50%) were also found to express IDO in resected lung adenocarcinoma (Kozuma et al., 2018). This indicates that high expression of both IDO and PD-1 together may indicate an aggressive form of lung adenocarcinomas, as prevalence of expression of both is consistently found in resected lung adenocarcinomas.

Bovine Respiratory Disease

Bovine respiratory disease (BRD) is complex and caused by a combination of viral and bacterial pathogens. It is usually most prevalent in times of stress such as weaning, transportation, and temperature extremes. The viral pathogens associated most with BRD are bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV3), bovine viral diarrhea virus (type 1 and 2), and bovine herpes virus 1 (that causes infectious bovine rhinotracheitis or IBR). The bacteria associated with BRD are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Mycoplasma bovi* (Zoetis, 2022). These viral and bacterial pathogens interact to produce full-blown disease, which causes stress on the bovine respiratory tract. Respiratory symptoms of BRD present as rapid or shallow breathing, coughing, and excessive salivation (Zoetis, 2022).General symptoms include fever, depression, lack of appetite, and dullness (Zoetis, 2022). Bovine respiratory diseases contributes to $750 million to $1 billion financial losses annually for the cattle industry due to costs of treatment, prevention measures such as metaphylaxis, morbidity, and mortality (National Animal Health Monitoring System, 2013). When cattle are treated for BRD, the specific pathogen of initial infliction is not typically known. The pathogen of focus for this thesis is Bovine Viral Diarrhea Virus and its impacts on the immune system.

Bovine Viral Diarrhea Virus

Bovine Viral Diarrhea Virus (BVDV) is a member of the genus *Pestivirus* (Fulton, 2015). The BVD virus is largely divided into two species, BVDV 1 and BVDV 2. There have been 21 BVDV 1 subgenotypes identified and 4 BVDV 2 subgenotypes identified (Falkenberg et al., 2021). Within these distinct subgenotypes, there are distinct subgenotypes, with the most common subgenotype in U.S. production settings being BVDV1b (Fulton et al., 2002). The other

principle subgenotypes found widely in production settings are BVDV1a and BVDV2a (Ridpath et al., 2010). For several years, the three most recognized subgenotypes have been BVDV1a, 1b, and 2a with BVDV1b the most prominent (Snowder et al., 2006). These subgenotypes vary genetically, but also antigenically as well. These antigenic differences were revealed analyzing monoclonal antibodies from cattle that received modified live vaccines (MLV) and killed vaccines (KV) (Fulton, 2015). The control of BVDV remains difficult as many persistently infected cattle will continue to infect susceptible cattle in the herd, as they consistently shed the virus throughout their life (Palomares et al., 2014c). The current best practices for BVDV control include biosecurity, stringent surveillance for infected cattle, and thorough vaccination (Liebler-Tenorio et al., 2003; Ridpath et al., 2007; Downey-Slinker et al., 2016).

Bovine viral diarrhea virus remains a prevalent issue within beef and dairy operations across the world. Bovine viral diarrhea virus negatively impacts the efficiency and productivity of cattle, impacting the economic viability of breeding herds, stocker operations and feedlots (Glew et al., 2003; Platt et al., 2009). This virus impacts cattle by disrupting normal respiratory functions, as well as negatively impacting digestive tract and reproductive functions (Fulton et al., 2006; Palomares et al., 2014a; Downey-Slinker et al., 2016).

Martin et al., (1999) found seroconversion to BVDV in 72% of calves infected with BRD (Martin et al., 1999). Martin et al., (1999) also found that higher titers to BVDV upon cattle arrival to the feedlot were associated with higher levels of weight gain, however a subsequent study indicated that high BVDV titers were related to lower weight gain in feedlot cattle in Alberta, Canada (Martin et al., 1999). This may indicate that higher levels of titers may offer more protection from infection, and in turn the animal in able to gain more efficiency. The contradictory results in the subsequent study may indicate that higher titers may indicate more

incidence of previous infection, which does not create an environment for efficient weight gain. This relationship was only significant when BRD incidence was not controlled for, controlling for BRD made the association between BVDV titers and weight gain insignificant (Martin et al., 1999). Bovine viral diarrhea virus has the ability to cause immunosuppression in infected cattle, which leads to respiratory infections, digestive tract infections, mucosal disease, reproductive tract infections, which may lead to PI, or persistently infected, calves (Fulton, 2013). Both the innate and adaptive immune system appear to suffer immunosuppression from BVDV infection as evidenced by NK cell death T and B cell depletion (Fulton, 2013; Ridpath, 2013).

Vaccination

Vaccination to control BVDV is exceptionally complex, due to the species, subgenotypes, and genetic diversity of the virus, as well as immunosuppression and ability to create persistent infections within cattle (Fulton, 2013). Current control of BVDV relies on the removal of persistently infected cattle from the herd, biosecurity, and an appropriate vaccination protocol (Fulton, 2015). Within vaccination protocol, producers have a wide array of vaccines to choose from. Modified live virus (MLV) vaccination, and killed (KV), which incorporates an inactivated virus, vaccinations encompass several species and subgenotypes of immunogens for BVDV in an attempt to offer more complete protection from infection (Fulton, 2015). Producers in North America utilize BVDV vaccinations that contain BVDV1a, BVDV1b, and BVDV2a subgenotypes (Ridpath et al., 2010). Vaccine studies in both MLV and KV trials measuring serum antibodies in seronegative calves often do not take samples as early as day 7, so the day of actual onset of immune system response is unknown (Ridpath et al., 2010). Cattle receiving killed or MLV BVDV vaccines experienced a dramatic decline in antibodies from days 42 to 140 (Ridpath et al., 2010).

One variable in vaccine efficacy is the strain of BVDV utilized in the vaccine. Fulton et al. (2020) investigated immune response to six different BVDV vaccines by measuring antibodies to BVDV1a, BVDV1b, BVDV2a, and BVDV2c (Fulton, 2013). This study found that the subgenotype in the vaccine appeared to make a drastic difference with vaccines that incorporated the Singer strain inducing higher VN titers to BVDV1a and BVDV1b than a vaccine that contained the NADL strain, which contained subgenotype BVDV 1a (Fulton et al., 2020). Sozzi et al. (2020) studied whether animals immunized with one of four commercial BVDV vaccines developed humoral immunity against other viral subgenotypes than that included in the selected vaccine. This study found that between different subgenotypes, notably BVDV1a and BVDV1b, cross reactivity in virus-neutralizing antibody titers could be significantly low (Sozzi et al., 2020). There was also no evidence of cross-immunity against any sub-genotype of BVDV1 (Sozzi et al., 2020).

The modified live virus (MLV) and killed vaccines have different strengths and weaknesses. Modified live vaccines have traditionally been administered in one dose and are thought to provide protection faster than a killed vaccine (Fulton, 2015). Killed vaccines require two doses to be effective, and must include an adjuvant to stimulate the innate immune response (Fulton, 2015). Most BVDV vaccines in the United States, both MLV and KV, have BVDV1a and BVDV2a (Fulton, 2015). Fulton et al. (2003) found detectable antibodies in calves given a MLV BVDV vaccine 14 days post vaccination. The protection against infection gained from the MLV vaccine at this rate was likely accredited to the result of the MLV vaccine enhancing the ability of the innate immune system against BVDV. The MLV vaccine also excels in cross-reactivity, which is the reaction of an antibody or antigen receptor specific for one antigen, with a second antigen, this occurs when the two antigens share an epitope (Tizard, 2018). Sozzi et al.

(2020) found that MLV vaccines containing the Singer strain of BVDV1a induced a significant level of BVDV1b antibodies, less than the level of antibodies to 1a, but indicating a larger, more expansive immune response (Sozzi et al., 2020).

Downey-Slinker et al. (2016), found differences in titers depending on the vaccine type used. When utilizing the modified-live vaccine (MLV), 34.68% of cattle did not present pyrexia post challenge, while there was no difference observed in protection between the killed vaccine and the non-vaccinated control group (Downey-Slinker et al., 2016). The MLV group had the highest incidence of total cattle with detectable antibody titers (53% of all animals) for all evaluation times of 3, 7. 10, 14,28, and 42 days post challenge, in healthy animals, but no difference in pyrexia was detected between antibody levels (Downey-Slinker et al., 2016). It has been thought that modified-live vaccines rely more on the processes of the cell-mediated immune system, compared to killed vaccines (Platt et al., 2009; Stevens et al., 2009; Falkenberg et al., 2020), so immune protection may not be clear based on antibody titers. The greatest anamnestic response, or secondary response, was observed in one study that utilized a killed vaccine, and found that on day 42 post-challenge, there was no difference between vaccine groups for antibody titers (Downey-Slinker et al., 2016). This may indicate that antibody titers may be an indication of exposure, rather than a measurement of protection (Downey-Slinker et al., 2016). Modified-live vaccines have been shown to upregulate the expression of CD25+, which is the receptor for IL-2, a cell-mediated immunity focused cytokine (Platt et al., 2006). Walz et al. (2017, 2018) found through *in vitro* data that both MLV and KV for BVDV induced immune responses, however *in vivo* data would suggest there is a benefit to utilizing MLV vaccines, as it is better able to activate a cell-mediated response against the virus (Walz et al., 2018).

Genetic Considerations for Immune Response

In cattle, and other species, several major genes dictate the innate and adaptive immune responses, which offers the possibility of breeding cattle for resistance to various diseases (Lewin, 1989). The chromosomal region known as the major histocompatibility complex is thought to contain major genes that control the humoral and cell-mediated immune responses (Ellman et al., 1970; Lewin, 1989). Engle et al. (1999) indicated that breed may account for variations in immune response. When newly weaned Angus and Simmental cattle were challenged with bovine herpesvirus type 1 (BHV-1), temperature response to the challenge was higher in Angus cattle. The calves were then injected with a pig red blood cell (PRBC) suspension. All immunoglobulin titers, including IgM titers, were higher in Angus calves, indicating a stronger humoral response (Engle et al., 1999). Cell mediated immune response also appeared to differ by breed, as the peripheral lymphocyte blastogenic response to phytohemagglutinin was also greater in the Angus calves (Engle et al., 1999). Despite having different immune responses, average daily gain, feed intake, and feed efficiency were not impacted by breed. These results are indicative that immune response of cattle may differ due to genetic background.

Runyan et al. (2017) investigated sire line differences in reactions to a BVDV-1b challenge under feedlot industry-like production conditions. Sire by vaccine strategy impacted feed intake and in turn, average daily gain. This indicated the different coping responses may exist across genetic lines to the same pathogen, and across sire lines. This shows potential interactions of sire lines, and therefore possibly breed differences, with BRD vaccine strategies, or, immune response to vaccine treatments on weight gain and feed intake (Runyan et al., 2017). However, the genetic tie may reside more in the reaction to the challenge or disease, as sire x

vaccine strategy did not result in a difference in rectal temperature, but sire alone related to differences in rectal temperature. Genetic investigations of BRD incidence have indicated low heritability estimates (Snowder et al., 2006), but this may be due in part to inaccuracy in diagnosis using visual indicators and some animals and sire lines having different physiologic responses to the same pathogen (Runyan et al., 2017). Recent findings have identified various genomic regions that impact the incidence of BRD, or more so the animal's response to BRD infection.

Neibergs et al. (2014) found common genomic regions associated with BRD susceptibility in pre-weaned Holstein calves hand noted a moderate heritability estimate of 0.21 within individual populations of California and New Mexico pre-weaned Holstein calves (Neibergs et al., 2014). For both groups of calves, genes on BTA15 and BTA23 were the most prevalent across all tests implemented (Neibergs et al., 2014). This research, along with further investigation may provide ways to identify animals that may have different physiological responses to pathogens due to genetics.

Bos taurus and *Bos indicus* cattle have been shown to vary in immune reactions. Piper et al. (2009) performed a study to evaluate how cellular and antibody components of the immune system differed between Brahman (*Bos indicus*) and Holstein-Friesian (*Bos taurus*) cattle when challenged with the cattle tick *R. microplus*. The Brahman cattle carried significantly less ticks than the Holstein-Friesian cattle. Piper et al. (2009) found that the Brahman cattle had higher counts of CD4+ cells and CD25+ cells than that of their Holstein-Friesian counterparts (Piper et al., 2009). Holstein-Friesian cattle had higher incidence of macrophage cells in circulation. The difference in breeds was more pronounced in cell-mediated immunity actions, as there was no significant difference in the percentages of B cells in circulation between the breeds. The *Bos*

indicus cattle were able to develop a stable cell-mediated immune response, whereas the *Bos taurus* cattle reacted to the infestation with a sustained innate, inflammatory response (Piper et al., 2009). However, elevated *R. microplus*- specific IgG1 titers indicated that the *Bos taurus* animals did express some kind of humoral immune response. *Bos taurus* cattle experience a sustained inflammatory response to an infestation of parasites, whereas *Bos indicus* cattle develop a T cell-mediated favored response to an infestation of parasites (Piper et al., 2009).

Reciprocal cross differences

In many tropical and subtropical regions *Bos indicus* and crosses of *Bos indicus* and *Bos taurus* are utilized due to the *Bos indicus*' cattle tolerance to heat and parasite challenges. This particular thesis studies the F_1 cross of *Bos taurus* (Angus) and *Bos indicus* (Brahman) crosses with both reciprocal crosses. The difference in birth weight among reciprocal crosses utilizing *Bos indicus* and *Bos taurus* breeds has been well documented. When utilizing *Bos taurus* sires on *Bos indicus* dams, Brown et al. (1993) found smaller birthweights in the Angus- sired calves than calves resulting from the reciprocal cross (Brahman-sired). The Brahman-sired heifer calves were 7.4 kg heavier but the Brahman-sired bull calves were 13.7 kg heavier (Brown et al., 1993). This confirms work done by Ellis et al. (1965) that found Brahman (*Bos indicus*) x Hereford (*Bos taurus*) F_1 crosses had higher birth weights than Hereford x Brahman F1 crosses by 8.9 kg (Ellis et al., 1965). Thallman et al. (1993) found that this trend remained through one year of age in Brahman and Simmental (*Bos taurus*) crosses with Brahman-sired calves significantly heavier than Simmental-sired calves (Thallman et al., 1993).

Summary

In conclusion, the immune system is vastly complex and interconnected, with less known about the cell-mediated immune system, and limited ways to study and determine protection

afforded by the cell-mediated immune system. Antibody titers, which have been historically used as a way to measure immune protection, fail to give indication to the protection provided by the cell-mediated branch of the adaptive immune system. The cytokine interferon-γ is widely tied to cell-mediated immune system, and may be a viable option as an adjuvant to stimulate the cell-mediated immune response. Bovine viral diarrhea virus, and consequently bovine respiratory disease, are widely harmful to the beef industry, so discovering unique ways to combat susceptibility or provide protection is necessary. Research has found genetic influence in the differing immune responses generated to challenge and host susceptibility to disease. The reciprocal cross difference phenomena has been consistently documented in birth weight where *Bos indicus*-sired F_1 calves have substantially larger birth weight compared to contemporary *Bos taurus*-sired F_1 calves when the same breeds are involved; however, this pattern has not been investigated for immune responses. Therefore, the objectives of this study were to investigate potential reciprocal F_1 cross effects for T cell responses from *in vitro* exposure to bovine viral diarrhea virus following modified live BRD vaccination. Trial 1 examined T cell frequency for BVDV presence and Interferon-γ. Trial 2 examined T cell frequency of expression for PD-1 and IDO. Both trials had the goal to improve understanding of cattle immune system responses in typical production settings.

CHAPTER III

ANTIBODY AND CELL-MEDIATED RESPONSES ASSOCIATED WITH BVDV AND INTERFERON-γ IN RECIPROCAL ANGUS-BRAHMAN F_1 CROSSES

Introduction

Bovine viral diarrhea virus is widely prevalent in the cattle industry globally, and is commonly a precursor to other ailments (Richter et al., 2017), and leads to depression in activity in various immune-associated mechanisms (Alpay and Yeşilbağ, 2015). Fulton et al., (2000) indicated the connection between BVDV infection and BRD incidence by isolating BVDV type 1 from affected cattle, BVDV type 1 found in pneumonic lungs, and active infection of BVDV types 1 and 2 in paired sera. Some research has suggested that genetic composition of cattle may account for variations in immune response (Neibergs, 2020), however more extensive research must be done to strengthen the understanding of genetic influence over immunity (Van Anne et al., 2018). Additional research has evaluated genetic correlations with diseases such as BRD with a genome-wide association analyses identifying 324 SNPs that were associated with Bovine Respiratory Disease (BRD) susceptibility when two calf populations from New Mexico and California were evaluated together (Neibergs et al., 2014). This indicates that host susceptibility to BRD, could be possible (Neibergs, 2020). Antibody titers have historically been utilized to evaluate levels of potential protection against microbes or disease, however this method is no holistic view of immunity and gives little insight into the level of protection afforded through cell-mediated immune functions (Alpay and Yeşilbağ, 2015; Neill et al., 2019). Interferon-γ is a cytokine that promotes cytotoxic activity related to cell-mediated immunity (Tizard, 2018), and may be an indicator of cell-mediated immunity. Additionally, it has been identified as a possible adjuvant for existing vaccines in order to boost the cell-mediated immune response from

vaccination (Schijns et al., 2000; Burakova et al., 2018). Reciprocal cross differences in birth weight, where calves sired by *Bos indicus* bulls out of *Bos taurus* cows have larger birth weights than calves sired by *Bos taurus* bulls out of *Bos indicus* cows have been well documented (Roberson et al., 1986; Brown et al., 1993). Investigation of this phenomenon in immune response traits is needed.

Objectives

The objectives of this study were to evaluate relationships among antibody titers and cell-mediated immune responses to BVDV and performance traits in reciprocal cross Brahman-Angus F_1 cattle that were injected with a modified-live virus (MLV) vaccine for respiratory pathogens and managed in industry-like settings. This study also investigated interferon-γ and its potential relationships pertaining to antibody response and performance traits. Potential differences due to reciprocal cross type (Angus-sired vs. Brahman-sired) and animal sex that have been widely reported for birth weight were of particular interest.

Materials and Methods

Health attributes, utilizing antibody titers to bovine viral diarrhea virus (BVDV), as well as interferon-γ levels, were evaluated using reciprocal cross Brahman-Angus F_1 cattle that were raised and held at the McGregor Research Center of Texas A&M University. All animal procedures were approved by the Agricultural Animal Care and Use Committee under AUP number 2018-006A. Analyses in this chapter were based on data provided to the student. Cattle utilized were born in the spring of 2020 at the McGregor Research Center. To create calves for this study, cows were bred through natural service during an approximately 70-day breeding season. Calves were ear tagged when birth weight was recorded, within 24 to 48 hours of birth. Calves were not implanted or tested to determine if they were persistently infected with BVDV.

When calves averaged approximately 70 days of age, they were given a primary clostridial vaccine (Covexin 8, Merck Animal Health), and approximately 1 month before weaning the cattle were given Triangle 5 (a killed vaccine, Boehringer Ingelheim Animal Health containing BVDV strains Singer-1a and 5912-2a). Before weaning, at 2 to 3 months of age, the steers were castrated surgically. Calves were then weaned at approximately 7 months of age, and given a clostridial vaccine booster (Covexin 8, Merck Animal Health) and given Titanium 5 (a MLV vaccine, Elanco Animal Health containing BVDV strains C24V-1a and 296-2a), and weights were recorded. After weaning, steers and heifers were managed separately.

Heifers entered a forage-based growth program to be used as replacements in the future breeding herd of the McGregor Research Center. All steers in this study were housed together, and all heifers were housed together, respectively. The steers were grown on pasture at the McGregor Research Center until mid-February, at which point they were shipped to a commercial finishing feedlot in Gonzales, Texas. The steers were harvested at a commercial processing plant in November on a single day. Carcass traits were collected by trained meat science personnel 48 hours following harvest

Blood was collected on all animals via jugular venipuncture into 8.5 mL evacuated glass blood collection tubes acid citric dextrose (ACD) for the PrimeFlow assay and associated cell culture and into 8.5 mL evacuated serum separator tubes containing a polymer gel for the antibody titer assays at weaning (7 months of age), to record pre-MLV vaccination information. The ACD tubes were packaged with insulation upon collection to minimize temperature loss and were shipped overnight. Tubes for serum were allowed to cool to room temperature, centrifuged at 4000 rpm for 8 minutes and were refrigerated until serum was isolated 24 hours later. Serum was stored in 0 C freezer until VN ELISA were performed. Frozen serum was shipped overnight

to the laboratory. Samples were balanced for collection time, breed type and sex across ELISA assay plates. This was repeated in late January of 2021 (at 9 months of age), to ensure at least 8 weeks post MLV vaccination. A subset of animals (n = 48) born earlier during the calving season was identified for study of cell-mediated immune response assays. Animals that were born toward the beginning of the 2020 calving season were targeted for this subset to utilize those with potentially more developed immune systems due to increased age and further removed from influence of remaining circulating maternal antibodies. Whole blood and serum were sent to the USDA-ARS National Animal Disease Center, Ames, IA for antibody titer and whole cell assays. Samples were investigated for antibodies utilizing virus neutralization ELISA and for cell-mediated aspects through PrimeFlow RNA assay (Falkenberg et al., 2017). The PrimeFlow RNA assay allows for identification of BVDV in peripheral blood mononuclear cells (PMBCs) in the midst of BVDV stimulation (Falkenberg et al., 2017; Falkenberg et al., 2019). This assay assessed differences in viral distribution of BVDV and IFN-γ within subpopulations of PBMCs (Falkenberg et al., 2019). Using RNA probes specific for BVDV-1a (PI34) and BVDV-2a (PI28) strains from NADC laboratory, number of cell populations with BVDV RNA were found and recorded quantitatively. Because the number of PMBCs were variable across animals, the frequency of cells expressing BVDV RNA (deemed probe-positive) and frequency of cells expressing IFN-γ were the two main variables analyzed. Cells were evaluated in four stimulant treatment groups for BVDV-1a, BVDV-2a, mitogen (as a positive control), and a non-stimulated negative control. Animals were evaluated at two time points, before and after receiving MLV vaccine containing BVDV types 1 and 2. This design provided for 8 observations per animal across 2 time points and 4 stimulants. The number of calves that could be evaluated per collection time for the cell culture and PrimeFlow assay was limited to 24.

Mixed model procedures of SAS were utilized for analyses through a repeated measures approach where individual calf ID was included in the model as a random effect. Models to investigate antibody titers and calf growth included fixed effects of F_1 cross type (Angus-sired vs. Brahman-sired), calf sex, date, and the 2-way interactions between all fixed effects. Models to study the frequency of cells positive for antigen, or frequency of cells expressing IFN-γ, additionally included stimulant and all potential two-way and three-way interactions of fixed effects of breed type, calf sex, vaccination timing, and stimulant. Tendencies were investigated for *F*-tests where $P < 0.10$, and differences were investigated when $P < 0.05$. Backward selection was used and interactions that had $P < 0.20$ were kept in the statistical model. Least squares means for frequency of cells positive for antigen, or frequency of cells expressing IFN-γ, were compared following a significant F-test through two-tailed t-tests.

Models for birth weight and weaning weight included breed type, calf sex classification, age of dam, and immune response category (low, medium, high) for antibody titers and BVDV positive cell frequency, based on being 0.5 standard deviations below the mean (low), within 0.5 standard deviations of the mean (medium, or 0.5 standard deviations above the mean (high). Models for carcass traits were similar, but did not include sex because only steers were slaughtered, whereas heifers remained in the herd as potential future producers. All models investigated potential two-way interactions among main effects. Correlations involving all performance traits and immune traits were also investigated using the correlation procedure in SAS. The data are presented in terms of frequencies of lymphocytes that were found positive for Bovine Viral Diarrhea Virus antigen by the BVDV+ Probe+, and frequencies of lymphocytes that expressed interferon-γ (IFN-γ). Virus-neutralizing antibody titers were represented in log base 2.

Results and Discussion

Table 3.1 presents the summary statistics for the continuous traits evaluated in this study. Table 3.2 presents the findings from models evaluating frequency of BVDV probe-positive cells and IFN-γ probe-positive cells across lymphocyte populations.

Table 3.1. Summary statistics for immune related frequencies evaluated by cell type.

Variable	n	Mean	Standard deviation	Coefficient of variation	Minimum	Maximum
BVDV Probe+ total cells	384	0.119	0.190	158.8	0	0.754
IFN-γ Probe+ total cells	384	0.016	0.026	163.5	9.23E-05	0.133
BVDV Probe+ CD2+ cells	384	0.124	0.202	162.9	0	0.803
IFN-γ Probe+ CD2+ cells	384	0.021	0.035	165.2	0.000	0.179
BVDV Probe+ CD4+ cells	384	0.149	0.242	162.4	0	0.859
IFN-γ Probe+ CD4+ cells	384	0.017	0.028	164.7	0.000	0.160
BVDV Probe+ CD8+ cells	384	0.115	0.185	161.1	0	0.805
IFN-γ Probe+ CD8+ cells	384	0.028	0.044	160.0	0	0.220
BVDV Probe+ CD335+ cells	384	0.058	0.097	168.1	0	0.481
IFN-γ Probe+ CD335+ cells	384	0.145	0.242	167.1	0	0.918

All values represent the frequency of cells that were probe-positive for BVDV or expressed IFN-γ respectively.

The value for n represents 4 observations at 2 different time periods for 48 animals.

Table 3.2. Statistical significance for frequency of BVDV Probe + and IFN-γ Probe+ across cell types and their respective models.

	BVDV+					IFN-γ +					
	Cell type										
Effect	CD2+	CD4+	CD8+	CD335+	Total cells	CD2+	CD4+	CD8+	CD335+	Total cells	Number of single cells
Stimulant	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Breed type	0.700	0.789	0.914	0.454	0.754	0.002	0.004	0.008	0.081	0.003	0.067
Sex	0.011	0.004	0.013	0.038	0.006	0.152	0.061	0.614	0.170	0.158	0.913
Vaccination timing	<0.001	<0.001	<0.001	<0.001	<0.001	0.834	0.018	0.260	<0.001	0.759	0.041
Stimulant x Breed type	0.879	0.987	0.901	0.598	0.956	<0.001	<0.001	<0.001	0.001	<0.001	0.099
Sex x Breed type	0.661	0.223	--	--	--	0.091	0.048	--	--	0.195	--
Stimulant x Sex	0.001	0.000	0.002	0.008	0.000	0.000	<0.001	0.233	0.205	0.000	0.075
Sex x Vaccination timing	<0.001	<0.001	<0.001	0.000	<0.001	0.575	0.364	0.334	0.144	--	<0.001
Breed type x Vaccination	0.001	0.001	0.001	0.016	0.000	0.540	0.512	0.301	0.089	--	<0.001
Stimulant x Vaccination	<0.001	<0.001	<0.001	<0.001	<0.001	0.655	0.005	0.185	<0.001	0.514	--

Bovine Viral Diarrhea Virus Positive Cells

The least squares means for frequencies for the appropriate lymphocyte populations derived from the RNA PrimeFlow assay that were BVDV Probe+ are presented below. Tables 3.3 through 3.5 provide the least squares means for the frequencies of cells positive for BVDV and differences for main effects.

Table 3.3. Least squares means for frequencies of BVDV Probe+ cells by breed type.

	Cell type				
Breed type	CD2+	CD4+	CD8+	CD335+	Total cells
Angus x Brahman	0.126 [a]	0.151 [a]	0.115 [a]	0.060 [a]	0.121 [a]
Brahman x Angus	0.122 [a]	0.148 [a]	0.114 [a]	0.055 [a]	0.118 [a]
% A x B vs. B x A[1]	103.7	102.2	101.0	109.7	102.6
SEM	0.008	0.009	0.007	0.005	0.007

[a, b] Indicate differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 103.7 indicates A x B were 3.7% higher, etc. Each breed type classification included 24 animals.

Table 3.4. Least squares means for frequencies of BVDV Probe+ by sex.

	Cell type				
Sex	CD2+	CD4+	CD8+	CD335+	Total cells
Heifer (H)	0.109 [a]	0.132[a]	0.101 [a]	0.050 [a]	0.106 [a]
Steer (S)	0.138 [b]	0.167 [b]	0.128 [b]	0.065 [b]	0.133 [b]
% H vs. S[1]	78.9	78.9	79.3	77.2	79.3
SEM	0.008	0.009	0.007	0.005	0.007

[a, b] Indicate differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of heifers to steers, where 78.9 indicates heifers were 21.1% lower, etc. Each sex classification included 24 animals.

Table 3.5. Least squares means for frequencies of BVDV Probe+ by stimulant.

	Cell type				
Stimulant	CD2+	CD4+	CD8+	CD335+	Total cells
BVDV1 (strain PI34-1a)	0.076 [a]	0.082[a]	0.077 [a]	0.050 [a]	0.077 [a]
BVDV2 (strain PI28-2a)	0.418[b]	0.513[b]	0.378[b]	0.178 [b]	0.400 [b]
Mitogen	0.001[c]	0.001 [c]	0.002[c]	0.001 [c]	0.000 [c]
No stimulant	0.000 [c]	0.001 [c]	0.001[c]	0.001 [c]	0.000 [c]
SEM	0.009	0.010	0.008	0.005	0.008

[a, b, c] Indicate differences ($P < 0.05$) within cell type.

Main effects. Many of the main effects were involved in significant two-way interactions, but are discussed here for completeness.

Breed type. Breed type was not significant when evaluated as a main effect (Table 3.3). All lymphocyte cell types from Angus-sired calves ranked higher for frequency of BVDV-positive cells presenting antigen.

Sex. There were distinct differences between frequencies of lymphocytes taking up antigen between the sexes (Table 3.4). Lymphocytes from steer calves had higher frequencies of presenting antigen on lymphocytes than heifer counterparts. The lymphocyte population that differed the most by sex was the CD335+ population, where lymphocytes from steer calves were positive for antigen at a 22.8% higher frequency than lymphocytes from their heifer counterparts. Future research on the differences in cytotoxic activity of the CD335+ between castrated males and females would offer insight into the function of lymphocytes in the sexes. This may unveil potential Th1 or Th2 biases due to sex effect.

Stimulant. There were differences in the mean frequencies of antigen uptake by lymphocytes in culture between stimulants as expected (Table 3.5). Lymphocytes contained BVDV1 and for BVDV2 at vastly different rates from each other and from the 2 control treatments. Lymphocytes were positive BVDV2 at an exceptionally higher rate than BVDV1.

The positive control stimulant, mitogen (PMA/ionomycin), did not differ in antigen presentation from the non-stimulated cultures, and both controls were not different from zero. Differences between genotypes of BVDV on immune function have been investigated. Archambault et al. (2000) found a non-cytopathic strain of BVDV2 (field strain 24515) resulted in high mortality and morbidity, and reduced antigen surface presentation on MHC II on the plasma membrane. In vivo, the effect of BVDV infection on circulating cell types, is dependent on the type of the BVDV strain, with non-cytopathic BVDV2 type genotype, showing 50-70% decrease in circulating cells (Archambault et al., 2000). This indicates that the BVDV2 genotype decreased immune function, and has ability to replicate in cells without interference.

Two-way interactions. All two-way interactions that were statistically significant for the different lymphocyte cell types were included in final models for completeness. However, not all of these interactions will be discussed in this thesis because those involving breed type, sex, and vaccine timing were of most interest for this study.

Table 3.6. Least squares means for frequencies of BVDV Probe+ for breed type by MLV vaccination timing.

	Pre-MLV vaccination					Post- MLV vaccination				
	Cell type									
Breed type	CD2+	CD4+	CD8+	CD335+	Total cells	CD2+	CD4+	CD8+	CD335+	Total cells
Angus x Brahman	0.079 [a,x]	0.098 [a,x]	0.069 [a,x]	0.028 [a,x]	0.075 [a,x]	0.174 [a,y]	0.204 [a,y]	0.162 [a,y]	0.092 [a,y]	0.167 [a,y]
Brahman x Angus	0.101 [a,x]	0.124 [a,x]	0.093 [a,x]	0.034 [a,x]	0.097 [b,x]	0.142 [b,y]	0.171 [b,y]	0.135 [b,y]	0.075 [a,y]	0.139 [b,y]
% A x B vs. B x A[1]	77.7	78.9	73.7	82.4	77.5	122.0	119.1	119.8	122.1	120.2
SEM	0.009	0.011	0.009	0.006	0.008					

[a,b] Indicates breed differences within cell types ($P < 0.05$).
[x,y] Indicates vaccination timing differences within cell types ($P < 0.05$).
[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 77.7 indicates Angus-sired were 22.3% lower, etc.

Table 3.7. Least squares means for frequencies of BVDV Probe+ for sex by MLV vaccination timing.

	Pre-MLV vaccination					Post-MLV vaccination				
	Cell type									
Sex	CD2+	CD4+	CD8+	CD335+	Total cells	CD2+	CD4+	CD8+	CD335+	Total cells
Heifer (H)	0.094 [a,x]	0.113 [a,x]	0.083 [a,x]	0.032 [a,x]	0.089[a,x]	0.125 [a,y]	0.150 [a,y]	0.119 [a,y]	0.068 [a,y]	0.122[a,y]
Steer (S)	0.086 [a,x]	0.109 [a,x]	0.078 [a,x]	0.030 [a,x]	0.083[a,x]	0.191 [b,y]	0.225 [b,y]	0.178 [b,y]	0.099 [b,y]	0.183[b,y]
% H vs. S[1]	109.6	103.8	105.9	107.5	107.4	65.2	66.8	67.5	68.1	66.7
SEM	0.010	0.011	0.009	0.006	0.008					

[a, b] Indicates sex differences ($P < 0.05$) within cell type.

[x, y] Indicates vaccination timing differences ($P < 0.05$) within cell type.

[1]Percent relative difference compares values of heifers to steers, where 109.6 indicates heifers were 9.6% higher, etc.

Breed by MLV vaccination timing for BVDV Probe+. The interaction of breed type by vaccination timing was significant in models for CD2+, CD4+, CD8+, CD335+ lymphocytes, as well as total frequency of lymphocytes positive for antigen (Table 3.6).

Prior to MLV vaccination, there were no differences in frequency of BVDV Probe positivity between breed types in any lymphocyte population. Pre-MLV vaccination, although not statistically significant, Brahman-sired calves consistently ranked higher for frequency of BVDV Probe positivity, regardless of lymphocyte classification by approximately 23% when compared to the Angus-sired calves. The frequency of CD8+ cell positivity indicated a trend for the most relative difference between breed types at pre-MLV vaccination, although not statistically significant ($P = 0.062$). This may indicate that the Brahman-sired animals may a more developed innate immune response and were able to recognize antigen more efficiently.

Post-MLV vaccination, CD2+, CD4+, CD8+ lymphocytes, and overall BVDV Probe+ frequency across total cells differed by breed type ($P < 0.05$). For every cell type post-MLV vaccination, those from Angus-sired calves had approximately 20% higher frequency of cells be BVDV Probe+ than Brahman-sired counterparts. For both breed types, CD4+ lymphocytes had the highest frequency of cells be BVDV Probe+. The CD2+ lymphocytes demonstrated the greater differences in frequency of BVDV Probe positivity between breed types, with Angus-sired calves demonstrating 22% higher frequency. Previous studies have shown that vaccination reactions can vary by breed type. Mutugi et al. (1991) found that the reaction of crossbred cattle (Zebu-European) and the purebred Jersey (European) cattle varied where 14.7% of the purebred Jersey cattle exhibited reactions to East Coast Fever vaccinations, but 0.6% of the Zebu crossbred cattle showed clinical reactions, a marked difference (Mutugi et al., 1991). Although

that study was not regarding BRD, it identified differences between *Bos taurus* versus *Bos indicus* immune response.

Regardless of breed type or lymphocyte classification, MLV vaccination impacted the frequency of lymphocytes with BVDV in the present study. Every lymphocyte type within both F_1 crosses demonstrated an increase from pre-MLV vaccination to post-MLV vaccination.

Sex by MLV vaccination timing for BVDV Probe+. The interaction between sex (heifer vs. steer) and vaccination timing (pre-MLV vaccination vs. post-MLV vaccination) was significant in models for lymphocytes cell types CD2+, CD4+, CD8+, and CD335+, as well as the frequency of total lymphocytes that were positive for the BVDV probe.

Pre-MLV vaccination, there were no significant differences in frequency of BVDV Probe+ between the two sexes. Pre-MLV vaccination, CD2+ lymphocytes showed the biggest difference between sexes, with lymphocytes from heifer calves being BVDV Probe+ at a frequency of 9.6% higher than CD2+ lymphocytes from steer calves when stimulated. Heifer calves consistently ranked higher for frequency of BVDV Probe positivity when stimulated prior to vaccination, although not statistically different.

Post-MLV vaccination, BVDV Probe positivity differed by sex; steer calves had higher BVDV Probe positivity in every cell type, as well as total cells. Consistently, heifer calves had 35% less BVDV Probe+ across cell types post-MLV vaccination when stimulated than steer calves. Post-MLV vaccination, as seen in pre-vaccination, CD2+ lymphocytes showed the biggest difference between sexes, with steer calves BVDV Probe+ frequency in CD2+ cells at 34.8% higher than heifer calves. Previous studies in other species have shown that innate and adaptive immune cells can vary by sex. Early in life, pre-puberty, it has been found that male rats had larger thymocyte (cells originating in the thymus) counts, due to having larger thymuses than

females (Leposavić et al., 1996). Post puberty, into adulthood, female humans, and other mammals, have more B cells than males, which may favor a humoral response to antigen, whereas males have higher levels of CD8+ T cells, which would favor cell-mediated immune response (Klein and Flanagan, 2016). Although rodent and cattle immune systems are quite different, these patterns were similar to results in this study, which indicated a large difference of BVDV Probe positivity in the CD8+ cells between steers and heifers.

Vaccination timing (pre-MLV vs. post-MLV) impacted each cell type, regardless of sex with significant increase in frequency of BVDV Probe+ after MLV vaccination in each cell type. Both sex classifications saw significant increase in frequency of BVDV Probe+ following MLV vaccination.

Evidence indicates role of T cells in vaccination and antigen uptake is important for BVDV immunity, even in the absence of antibodies (Ridpath et al., 2003; Downey-Slinker et al., 2016). Memory T cells are created to protect against future viral challenges. In addition, both CD4+ and CD8+ T cell responses are stimulated by BVDV1 (Rhodes et al., 1999).

Table 3.8. Least squares means for frequencies of BVDV Probe+ for sex by stimulant.

	BVDV1 (strain PI34-1a)					BVDV2 (strain PI28-2a)				
	Cell type									
Sex	CD2+	CD4+	CD8+	CD335+	Total cells	CD2+	CD4+	CD8+	CD335+	Total cells
Heifer (H)	0.057 [a,x]	0.059 [a,x]	0.060 [a,x]	0.038 [a,x]	0.058 [a,x]	0.379 [a,y]	0.465 [a,y]	0.342 [a,y]	0.159 [a,y]	0.364 [a,y]
Steer (S)	0.095 [b,x]	0.105 [b,x]	0.094 [b,x]	0.062 [b,x]	0.096 [b,x]	0.458 [b,y]	0.561 [b,y]	0.414 [b,y]	0.196 [b,y]	0.436 [b,y]
% H vs S[1]	60.0	56.5	64.0	60.6	60.6	82.8	82.9	82.7	81.4	83.4
SEM	0.013	0.014	0.012	0.008	0.011					

[a, b] Indicate sex differences ($P < 0.05$) within cell type.
[x, y] Indicate stimulant differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of heifers to steers, where 60.0 indicates heifers were 40% lower, etc.

Table 3.9. Least squares means for frequencies of BVDV Probe+ for breed by sex.

	Heifer		Steer	
	Cell type			
Breed type	CD2+	CD4+	CD2+	CD4+
Angus x Brahman	0.114 [a,x]	0.141 [a,x]	0.138 [a,x]	0.161 [a,x]
Brahman x Angus	0.105 [a,x]	0.123 [a,x]	0.139 [a,y]	0.173 [a,y]
% A x B vs. B x A[1]	109.1	114.7	99.6	93.3
SEM	0.012	0.012		

[a, b] Indicate breed differences ($P < 0.05$) within cell type.

[x, y] Indicate sex differences ($P < 0.05$) within cell type.

[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 109.1 indicates Angus-sired were 9.1% higher, etc.

Stimulant by sex for BVDV Probe+. Table 3.8 presents the frequency of BVDV Probe positivity in the interaction of calf sex with stimulants.

Every cell type differed in frequency of BVDV Probe positive cells between the sexes with the largest differences seen with the BVDV type 1 stimulant. When stimulated with BVDV1, the cell type that differed the most between the sexes was the CD4+ lymphocytes with CD4+ cells from steer calves being BVDV1 positive at almost twice the rate than in heifer calves. Consistently, steers were BVDV Probe+ at a frequency approximately 40% higher than heifers.

The difference was less pronounced between sexes when utilizing the BVDV type 2 strain as the stimulant, however all lymphocyte populations still showed a significant difference ($P < 0.05$) between steer and heifers in BVDV Probe+ with steer calves consistently BVDV Probe+ at a higher frequency than heifer calves. When stimulated the BVDV2, the CD335+ cell type showed the largest difference between sexes with steers being BVDV Probe+ at of 18.6% higher.

Seong et al. (2013) stimulated 5 calves with noncytopathic (ncp) BVDV1 genotype and ncp BVDV2 types and monitored lymphocyte decline through infection. Calves infected with ncp BVDV2 experienced the largest decline in lymphocyte count and apoptosis has been documented to be higher in BVDV2 stimulated samples when compared to BVDV1 (Ridpath et al., 2006; Seong et al., 2013). In this study, noncytopathic subgenotypes of BVDV were used so cells would not undergo apoptosis so they could be studied. The results in Table 3.8 indicate that frequency of cells BVDV Probe+ were higher when stimulated with BVDV2, which may be a precursor to lymphocyte apoptosis, though this was not measured in this study.

There was a significant difference ($P < 0.05$) in the frequency of BVDV Probe+ cells due to the stimulant used. Lymphocytes from both Steer and Heifer calves were positive for BVDV2 antigen at a higher rate than BVDV1. Cells were positive for BVDV type 2 antigen at approximately four times higher frequency than BVDV Probe positive for the BVDV type 1 antigen.

Sex by breed for BVDV Probe+. Table 3.9 presents the frequency of BVDV Probe+ lymphocytes by F_1 breed type and sex. There were no significant differences in frequency of BVDV Probe+ for any cell types between the breed types. Although not significant, Angus-sired heifers ranked higher frequencies of BVDV Probe+ than Brahman-sired heifers. In contrast, Brahman-sired steers ranked higher for frequencies of BVDV Probe+ than Angus-sired steers. This pattern was consistent but likely was not significantly different because of limited sample size.

There was a significant difference ($P < 0.05$) between Brahman-sired steers and heifers for BVDV Probe+, but no significant difference ($P > 0.10$) between Angus-sired steers and heifers. Brahman-sired steers were BVDV Probe+ at a higher frequency than Brahman-sired heifers for both cell types. Hulbert et al. (2013) found Brahman heifers had greater levels of basal concentrations of cortisol than Brahman bulls when challenged with corticotropin-releasing hormone, which impacted immune regulatory cytokines such as IFN-γ, TNF-α, and IL-6. This suggests that heifers have an increased priming of the immune system following stressors, and are able to handle stressors more efficiently (Hulbert et al., 2013). Stress response has been studied as a way to evaluate immune function between Angus cattle and Brahman x Angus cattle. Blecha et al., (1984) studied shipping as a stressor on cellular immune reactivity. After shipping stress was induced Brahman x Angus steers displayed lower phytohaemagglutinin

(PHA) skin test reactions than Angus steers, which may be an indication that different breeds do not respond the same to stressors or immunization protocols (Blecha et al., 1984). A greater lymphocyte proliferation act in response to PHA may be indicative to greater function of adaptive immunity (Engle et al., 1999). There was a difference in sex effects between the two F_1 breed types. Cross types may not only differ in response to stressors (Blecha et al., 1984), but also have different impacts on the sex effect.

Interferon-γ Positive cells

The least squares means for frequencies of IFN-γ positive cells within each lymphocyte population that was derived from the RNA PrimeFlow assay IFN-γ Probe+ are presented below.

Main effects. Tables 3.10 through 3.12 present the least square means for the frequencies of cells expressing IFN-γ for main effects. Main effects of breed type, sex, and stimulant were included in all statistical models regardless of *P*-values because these factors were the basis of the experimental design; all interactions were evaluated, but included only based on statistical significance.

Breed type. Frequency of interferon-γ expression varied by breed type (Table 3.10) for the CD2+, CD4+, and CD8+ cell types, as well as the total cells. The CD335+ lymphocyte did not vary significantly ($P > 0.10$) between breed types. Angus-sired calves had a higher frequency of cells express IFN-γ at an approximately 50% higher rate than Brahman-sired calves. The most pronounced difference between breed types was in the CD8+ lymphocyte population. Carroll et al., (2011) studied genetic differences between two diverse *Bos taurus* breeds (Angus and Romosinuano) in response to an endotoxin challenge, in which the pro-inflammatory cytokine IFN-γ and cortisol was measured. Angus steers had higher serum cortisol levels than the Romosinuano steers, but there was no difference seen in IFN-γ levels (Carroll et al., 2011). The

differences presented in Table 3.10 may indicate a difference in *Bos taurus* sire lines and *Bos indicus* sire lines in terms of frequency of cells expressing IFN-γ. This may indicate that there may be a way to select for cattle that express IFN-γ at higher frequencies, which may indicate selection for more affinity for a cell-mediated immune response.

Table 3.10. Least squares means for frequencies of cells expressing IFN-γ by breed type.

	Cell type				
Breed type	CD2+	CD4+	CD8+	CD335+	Total cells
Angus x Brahman	0.025 [a]	0.020 [a]	0.033 [a]	0.155[a]	0.019 [a]
Brahman x Angus	0.017 [b]	0.014 [b]	0.022 [b]	0.134[a]	0.013[b]
% A x B vs. B x A[1]	147.5	146.0	150.0	115.6	149.2
SEM	0.002	0.002	0.003	0.008	0.001

[a, b] Indicate differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 147.5 indicates Angus-sired were 47.5% higher, etc.

Table 3.11. Least squares means for frequencies of cells expressing IFN-γ by sex.

	Cell type				
Sex	CD2+	CD4+	CD8+	CD335+	Total cells
Heifer (H)	0.019 [a]	0.015 [a]	0.027 [a]	0.137[a]	0.015 [a]
Steer (S)	0.023 [a]	0.019 [a]	0.029 [a]	0.153[a]	0.018 [a]
% H vs. S[1]	83.6	78.3	92.8	89.2	83.1
SEM	0.002	0.002	0.003	0.008	0.001

[a, b] Indicate differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of heifers to steers, where 83.6 indicates heifers were 16.4% lower, etc.

Table 3.12. Least squares means for frequencies of cells expressing IFN-γ by stimulant.

	Cell Type				
Stimulant	CD2+	CD4+	CD8+	CD335+	Total cells
BVDV1	0.004 [a]	0.004 [a]	0.008 [a]	0.026[a]	0.003 [a]
BVDV2	0.006 [a]	0.005 [a]	0.010 [a]	0.029[a]	0.004 [a]
Mitogen	0.072 [b]	0.056 [b]	0.088 [b]	0.515[b]	0.054 [b]
No stimulant	0.002 [a]	0.002 [a]	0.004 [a]	0.010[a]	0.002 [a]
SEM	0.002	0.002	0.003	0.011	0.001

[a, b] Indicate differences ($P < 0.05$) within cell type

Sex. Frequency of IFN-γ expression did not differ by sex classification when sex was evaluated as a main effect (Table 3.11). All cell types from steer calves consistently ranked higher for frequency of cells expressing IFN-γ than heifer calves. These differences were not significant ($P > 0.10$), as analysis is limited by sample size.

Hulbert et al., (2013) found that Brahman heifers had greater concentrations of IFN-γ following a challenge with corticotropin-releasing hormone than Brahman bulls. Hulbert et al., (2013) found that heifers expressed IFN-γ at 14.90 pg/ml and bulls expressed IFN-γ at 7.64 pg/ml. In contrast, our results indicated no significant difference, but it is important to note that we evaluated the frequency of cells expressing IFN-γ, not level of expression.

Stimulant. Frequency of IFN-γ expression from evaluated cell types did not differ between BVDV antigens (Table 3.12). The positive control, mitogen, is the only stimulant that induced any differences in the frequency of cells that expressed IFN-γ. This indicates that the type of BVDV would have little expected impact on frequency of IFN-γ expression. The BVDV stimulants, regardless of type, did not vary from the cells that were not stimulated, which may indicate that the BVDV stimulation is not connected to frequency of IFN-γ expression.

Two-way interactions. All two-way interactions were evaluated for significance in statistical models for the different lymphocyte cell types, and were included in models for completeness. Table 3.13 presents the breed type by vaccination timing interaction for frequency of cells expressing IFN-γ, and Table 3.14 presents the sex by vaccination timing interaction for frequency of cells expressing IFN-γ. Figure 3.1 illustrates the sex by breed type interaction for frequency of cells expressing IFN-γ.

Table 3.13. Least squares means for frequencies of cells expressing IFN-γ for breed type by MLV vaccination timing.

	Pre-MLV vaccination				Post-MLV vaccination			
	Cell type							
Breed type	CD2+	CD4+	CD8+	CD335+	CD2+	CD4+	CD8+	CD335+
Angus x Brahman	0.026 [a,x]	0.018 [a,x]	0.031 [a,x]	0.124 [a,x]	0.025 [a,x]	0.022 [a,y]	0.036 [a,x]	0.187 [a,y]
Brahman x Angus	0.017 [b,x]	0.013 [b,x]	0.022 [a,x]	0.120 [a,x]	0.018 [b,x]	0.015 [b,x]	0.022 [b,x]	0.148 [b,x]
%A x B vs. B x A[1]	155.3	143.3	139.3	102.7	140.3	148.3	160.8	126.1
SEM	0.002	0.002	0.003	0.011				

[a,b] Indicates breed differences within cell types ($P < 0.05$).
[x,y] Indicates vaccination timing differences within cell types ($P < 0.05$).
[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 155.3 indicates Angus-sired were 55.3% higher, etc.

Table 3.14. Least squares means for frequencies of cells expressing IFN-γ for sex by MLV vaccination timing.

	Pre-MLV vaccination				Post-MLV vaccination			
	Cell type							
Sex	CD2+	CD4+	CD8+	CD335+	CD2+	CD4+	CD8+	CD335+
Heifer (H)	0.020 [a,x]	0.014 [a,x]	0.026 [a,x]	0.121 [a,x]	0.019 [a,x]	0.016 [a,x]	0.027 [a,x]	0.151 [a,y]
Steer (S)	0.023 [a,x]	0.017 [a,x]	0.026 [a,x]	0.123 [a,x]	0.024 [a,x]	0.021 [b,y]	0.031 [a,x]	0.183 [b,y]
% H vs. S[1]	86.9	82.7	100.7	98.9	80.4	74.9	86.0	82.7
SEM	0.002	0.002	0.003	0.011				

[a, b] Indicates sex differences ($P < 0.05$) within cell type.
[x, y] Indicates vaccination timing differences ($P < 0.05$) within cell type.
[1]Percent relative difference compares values of heifers to steers, where 86.9 indicates heifers were 13.1% lower, etc.

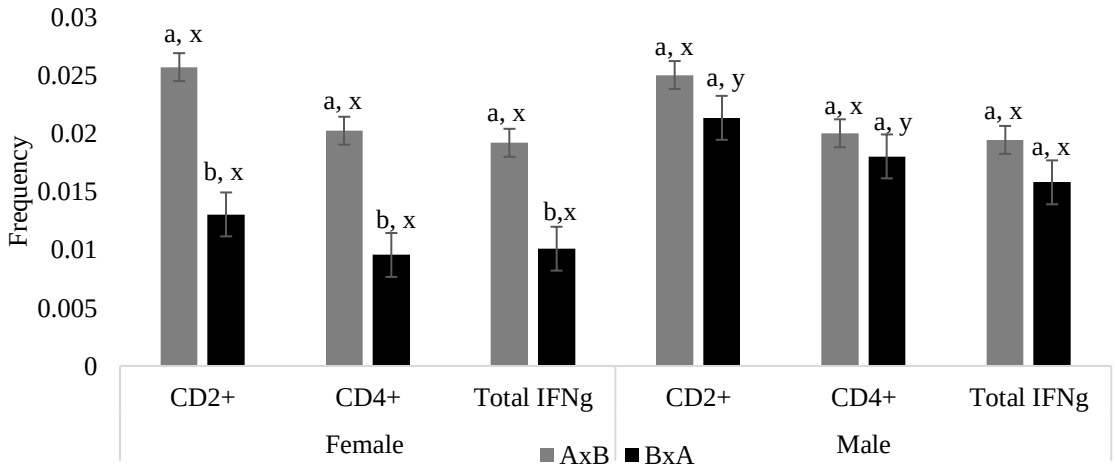

Figure 3.1.Least squares means for frequency of IFN- γ expression for breed type by sex.

[a, b] Indicates breed type differences ($P < 0.05$) within cell type.

[x, y] Indicates sex differences ($P < 0.05$) within cell type

Breed type by MLV vaccination timing for IFN-γ Probe+. Prior to MLV vaccination, frequency of interferon-γ expression differed significantly by breed type in cell types CD2+ and CD4+. In these lymphocyte populations, Angus-sired calves expressed IFN-γ at a higher frequency than Brahman-sired calves. Although differences were not significant ($P < 0.10$), CD335+ lymphocytes expressed IFN-γ at a higher frequency than any other lymphocyte population. Interferon-γ is mainly produced by NK cells and CD8+ cells (Tizard, 2018), as these two cell populations are heavily responsible for cytotoxic activities within the immune system. This confirms our results as the frequency of IFN-γ expression was the highest in these two populations in our study.

Post-MLV vaccination, all lymphocyte populations indicated significant differences in frequency of IFN-γ expression by breed type. Consistently, lymphocytes from Angus-sired calves ranked higher for frequency of IFN-γ expression than Brahman-sired calves. As seen in

cell populations pre-MLV vaccination, the CD335+ cells expressed IFN-γ at a higher frequency than any of the other lymphocyte populations.

Lymphocyte populations CD4+ and CD335+ from Angus-sired calves had significant differences in frequency of IFN-γ expression ($P < 0.05$) pre-MLV vaccination to post-MLV vaccination. Least squares mean differences in Table 3.13 did not indicate that MLV vaccination had a significant effect on the frequency of interferon-γ expression of other lymphocytes populations.

Sex by MLV vaccination timing for IFN-γ Probe+. Pre-MLV vaccination, there were no significant differences between the sexes in the frequency of expression of IFN-γ. Post-vaccination, CD4+ and CD335+ cell types differed by sex, with lymphocytes from steer calves exhibiting higher frequency of IFN-γ expression than heifer calves.

There was limited change in the frequency of IFN-γ expression from pre-MLV vaccination to post-MLV vaccination. Cell populations CD4+ and CD335+ from steer calves had a significant ($P < 0.05$) difference in frequency of IFN-γ expression after MLV vaccination. The CD335+ cell population in Heifers indicated a significant ($P < 0.05$) change in frequency of IFN-γ expression post- MLV vaccination. Least squares mean differences in Table 3.14 did not indicate that MLV vaccination had a significant impact on the frequency of IFN-γ expression of lymphocytes. An advantage of the MLV vaccination is stimulating the animal to elicit an immune response similar to one induced by natural infection, which would include a cell-mediated immune response. Table 3.14 indicating that MLV vaccination did not impact frequency of cells expressing IFN-γ may indicate that the MLV vaccination did not properly stimulate cytotoxic activity.

Reber et al. (2006) studied the development of immunity to vaccination utilizing three varying strategies evaluated over an 8-week time period, but found no significant difference

between three vaccination protocols for level of IFN-γ. The three vaccination protocols were a killed vaccination with a killed boost, a MLV vaccine and a killed vaccine boost, and then a MLV vaccine and MLV booster. Reber et al. (2006) also found no significant effect of time after vaccination on IFN-γ release, which was also noted in our study (Table 3.13 and Table 3.14). The data in Table 3.13 and Table 3.14, as well as work done by Reber et al. (2006), do not show IFN-γ expression is impacted by vaccination. Previous studies indicate that BVDV downregulates T helper 1 cells, which demonstrate a cell-mediated and IFN-γ bias (Charleston et al., 2002). This may be why we saw no significant change in frequency of IFN-γ pre-MLV vaccination to post-MLV vaccination.

Sex by breed type for IFN-γ Probe+. There were significant differences between Angus-sired heifers and Brahman-sired heifers in cell frequency of IFN-γ expression (Figure 3.1). Consistently, cell types from Angus-sired heifers expressed IFN-γ at a significantly higher cell frequency than Brahman-sired heifers, for CD2+ and CD4+ cell types, as well as total cells. There was also significant ($P < 0.05$) differences between the sexes of the Brahman-sired calves where Brahman-sired heifers consistently had lower frequency of IFN-γ expression than Brahman-sired steers. Notably, this sex difference was not observed in Angus-sired calves.

Although immune response to parasites is different than immune response to viral pathogens, potential genetic responses in IFN-γ associated with challenge are of interest. McGuire et al. (2004) evaluated breed-specific differences during challenge from parasite *Theileria annulate* and did not find any differences in cytokine production directly tied to breed. Notably, other pro-inflammatory cytokines were studied, but IFN-γ was not recorded. Breed type differences were seen within Figure 3.1, indicating potential differences in the cellular activity due to how F_1 crosses were generated.

Total number of single cells. Tables 3.15 and 3.16 indicate the analysis of the total cell number of peripheral blood mononuclear cells (PBMCs) and lymphocytes analyzed via flow and are an indication of how well the cells would survive in the cell culture. Cell survivability is important as infection studies of cells *in vitro* rely on culture media.

There were no statistically significant differences for total cell numbers due to sex or breed type when evaluated as main effects (Table 3.15), but MLV vaccination timing indicated the total number of single cells were significantly lower ($P < 0.05$) post-MLV vaccination. When stimulant was evaluated as a main effect, there was no statistical difference between the BVDV1 and the BVDV2 environment, however mitogen and no stimulant were both different ($P < 0.05$) with no stimulant having the highest count of PMBCs. This was to be expected because cell growth is not as negatively stimulated, as compared to the other stimulants. Mitogen has the lowest cell count as this stimulant causes the most negative impact on cell proliferation, which is followed by both BVDV types. The mitogen effect was exceptionally hard on cell types and creates a challenging environment for cell survival (Falkenberg et al., 2020).

Table 3.16 presents the relevant interactions that were included in the model for the total number of single cells. Pre-MLV vaccination there was a significant difference ($P < 0.05$) between breed types with Brahman-sired animals displaying a higher count of total single cells pre-MLV vaccination than Angus-sired counterparts. This may be an indication of a difference in levels of innate immunity. Vaccination significantly ($P < 0.05$) increased the count of single cells in Angus-sired animals, whereas vaccination decreased the count of single cells in Brahman-sired animals.

Post-MLV vaccination, there was no difference between breed types. Breed type had no significant impact on stimulant. Stimulant followed a similar trend in both breed types that was seen when stimulant was evaluated as a main effect.

Pre-MLV vaccination, there was no significant difference between sexes. Interactions of sexes, both steers and heifer, with MLV vaccination timing is notable. Heifers decreased in total single cell count post-MLV vaccination, whereas steers increased in total cell count post MLV vaccination. Sex had no significant impact on stimulant. Stimulant followed a similar trend in both sexes that was seen when stimulant was evaluated as a main effect.

Table 3.15. Least squares means for number of single cells for the main effects.

	Main effect									
	Breed type		Sex		Vaccination timing		Stimulant			
	Angus-sired	Brahman-sired	Heifer	Steer	Pre	Post	BVDV1	BVDV2	Mitogen	No stimulant
Single Cells	62961 [a]	70901[a]	66696[a]	67167[a]	68547[a]	65315[b]	76526[a]	74485[a]	31892[b]	84821[c]
SEM	3029.04	3076.57	3076.57	3029.04	2298.17		2548.25			

a, b, c Indicate differences within main effect factors

Table 3.16 Least squares means for number of single cells for breed type and sex by vaccination timing and stimulant, respectively.

	MLV vaccination timing				Stimulant				
	Pre-MLV	Post-MLV	% Change in post-MLV vs pre-MLV	SEM	BVDV1	BVDV2	Mitogen	No stimulant	SEM
Angus-sired	58605 [a, x]	67317 [a, y]	114.9	3230.89	71829 [a, x]	70667 [a, x]	25497 [a, y]	83853 [a, z]	3583.58
Brahman-sired	78489 [b, x]	63313 [a, y]	80.7	3269.19	81224 [a, x]	78303 [a, x]	38288 [a, y]	85789 [a, z]	3623.84
% A x B vs B x A	74.7	106.3							
Sex									
Heifer (H)	72494 [a, x]	60897 [a, y]	84.0	3269.19	77448 [a, x]	72961 [a, x]	29074 [a, y]	87300 [a, z]	3623.84
Steer (S)	64600 [a, x]	69734 [a, y]	107.9	3230.89	75605 [a, x]	76009 [a, x]	34711 [a, y]	82342 [a, z]	3583.58
% H vs S	112.2	87.3							

[a, b] Indicate differences in breed type, or sexes, respectively.

[x, y, z] Indicate differences due to vaccination timing or stimulant, respectively.

Virus neutralizing antibody titers. Table 3.17 indicates the differences in virus neutralizing (VN) antibody titers of BVDV types 1 and 2 via ELISA. The only significant effect in this model was vaccination timing, and there were no significant interactions. The increase in VN titers was expected as virus neutralizing antibodies equip the body to fight antigen effectively which is the fundamental basis of vaccination. Virus neutralizing antibodies block infection at the cellular level by blocking the interaction of cellular receptors with viruses, preventing entry of viruses into the cell (Murphy et al., 2005).

Table 3.17. Virus neutralization titers log base 2 by MLV vaccination timing.

	BVDV genotype	
MLV vaccination timing	BVDV1	BVDV2
Pre-MLV, weaning	2.24[a]	1.13[a]
Post-MLV	9.68[b]	8.73[b]
SEM	0.247	0.271

[a, b] Indicates differences within genotypes due to vaccination timing

Simple correlations of BVDV1 stimulated traits to BVDV1 VN titers. The simple correlations presented in Table 3.18 represent the selected BVDV1-stimulated traits in correlation to each other as well as the virus neutralizing antibody titers to BVDV1. Table 3.18 shows the correlation of traits stimulated with BVDV1 across all animals (n=48 without reference to sex or breed type). The respective BVDV+ Probe positive indicators were correlated with other BVDV+ Probe positive indicators, and IFN-γ positive traits correlated with IFN-γ positive traits. The IFN-γ+ traits were not associated with the BVDV+ Probe+ traits, even within the same cell type. There were no interactions between the traits measuring BVDV Probe+ or IFN-γ with the virus neutralizing antibody titers when there was no separation by F_1 breed type or sex. When sorted by breed type, the Angus-sired calves were consistent with the correlation patterns found in Table 3.18. Table 3.19 shows the simple correlations of BVDV+ Probe+, IFN-

γ+ selected trait, and VN titers amongst each other, for Brahman-sired calves. In many regards, Brahman-sired calves followed a consistent pattern that was observed in Table 3.18, as BVDV+ Probe+ traits were correlated with other BVDV+ Probe+ traits, and IFN-γ+ traits correlated with other IFN-γ+ traits. However, the correlation between VN titers and the IFN-γ+ traits indicated a connection not seen in the model with no sort for breed type or sex. Total frequency of cells expressing IFN-γ, frequency of IFN-γ+ expression in CD4+ cells, and frequency of IFN-γ+ expression in CD335+ cells had a significant ($P < 0.05$) positive correlation to BVDV1 log 2 VN titers. This indicates a connection between humoral and cell-mediated immune functions, in the Brahman-sired cattle and this was only found among Brahman-sired calves. However, this trend was not found when data were separated by calf sex. This trend was surprising as IFN-γ has been shown to have an immense Th1 bias, meaning the humoral system, represented by antibody titers, would not be as prevalent when IFN-γ is upregulated (Tizard, 2018).

Table 3.18. Correlations of BVDV1 stimulated traits to BVDV1 VN titers including all sexes and breed types following vaccination.

	BVDV1 log 2 VN titers	Total cells IFN-γ+	IFN-γ+ CD2+	IFN-γ+ CD4+	IFN-γ+ CD8+	IFN-γ+ CD335+	Total cells BVDV1+	BVDV1+ CD2+	BVDV1+ CD4+	BVDV1+ CD8+
Total cells IFN-γ+	0.099									
	0.502									
IFN-γ+ CD2+	0.090	**0.972**								
	0.541	< 0.001								
IFN-γ+ CD4+	-0.017	**0.794**	**0.833**							
	0.908	< 0.001	< 0.001							
IFN-γ+ CD8+	0.116	**0.914**	**0.915**	**0.639**						
	0.433	< 0.001	< 0.001	< 0.001						
IFN-γ+ CD335+	0.141	**0.744**	**0.728**	**0.404**	**0.757**					
	0.339	< 0.001	< 0.001	0.004	< 0.001					
Total cells BVDV1+	0.017	0.100	0.104	0.070	0.134	0.193				
	0.910	0.497	**0.482**	0.634	0.362	0.189				
BVDV1+ CD2+	-0.021	0.011	0.031	0.057	0.058	0.073	**0.975**			
	0.885	0.938	0.835	0.703	0.698	0.622	< 0.001			
BVDV1+ CD4+	-0.037	0.034	0.692	0.137	0.053	0.041	**0.957**	**0.990**		
	0.801	0.821	0.833	0.354	0.720	0.781	< 0.001	< 0.001		
BVDV1+ CD8+	-0.015	0.065	0.079	0.073	0.126	0.159	**0.962**	**0.972**	**0.954**	
	0.921	0.659	0.594	0.624	0.395	0.281	< 0.001	< 0.001	< 0.001	
BVDV1+ CD335+	0.029	0.234	**0.290**	0.094	**0.757**	**0.414**	**0.808**	**0.792**	**0.741**	**0.813**
	0.846	0.109	0.046	0.523	< 0.001	0.003	< 0.001	< 0.001	< 0.001	< 0.001

Upper row= r correlations, significant correlations are in bold. *P*-values are second row.
N = 48 animals were equally balanced across sex and F_1 breed type

Table 3.19. Correlations of BVDV1 stimulated cell traits to BVDV1 VN titers of Brahman-sired calves following vaccination.

	BVDV1 log 2 VN Titers	Total cells IFN-γ+	IFN-γ+ CD2+	IFN-γ+ CD4+	IFN-γ CD8+	IFN-γ CD335+	Total cells BVDV1+	BVDV1+ CD2+	BVDV1+ CD4+	BVDV1+ CD8+
Total cells IFN-γ+	**0.427**									
	0.038									
IFN-γ+ CD2+	**0.378**	**0.982**								
	0.068	< 0.001								
IFN-γ+ CD4+	**0.406**	**0.783**	**0.754**							
	0.049	< 0.001	< 0.001							
IFN-γ+ CD8+	0.330	**0.879**	**0.901**	**0.491**						
	0.115	< 0.001	< 0.001	0.015						
IFN-γ+ CD335+	**0.472**	**0.752**	**0.794**	**0.653**	**0.683**					
	0.020	< 0.001	< 0.001	0.001	0.000					
Total cells BVDV1+	-0.137	0.213	0.247	0.292	0.194	0.088				
	0.522	0.317	0.245	0.166	0.365	0.683				
BVDV1+ CD2+	-0.176	0.087	0.138	0.212	0.122	0.017	**0.967**			
	0.412	0.687	0.520	0.319	0.569	0.938	< 0.001			
BVDV1+ CD4+	-0.204	0.051	0.091	0.210	0.051	-0.015	**0.966**	**0.991**		
	0.339	0.814	0.674	0.326	0.812	0.946	< 0.001	< 0.001		
BVDV1+ CD8+	-0.130	0.147	0.210	0.263	0.192	0.100	0.955	**0.978**	**0.954**	
	0.544	0.494	0.325	0.214	0.369	0.641	< 0.001	< 0.001	< 0.001	
BVDV1+ CD335+	-0.084	0.355	**0.445**	0.325	**0.488**	0.323	**0.841**	**0.871**	**0.823**	**0.897**
	0.697	0.089	0.029	0.122	0.016	0.124	< 0.001	< 0.001	< 0.001	< 0.001

Upper row= r correlations, significant correlations are in bold. *P*-values are second row in parentheses.

N = 24 animals equally balanced across sex.

Table 3.20. Correlations of BVDV2 stimulated traits to BVDV2+ VN titers including all sexes and breed types following vaccination.

	BVDV2 log 2 VN titers	Total cells IFN-γ+	IFN-γ+ CD2+	IFN-γ CD4+	IFN-γ CD8+	IFN-γ CD335+	Total cells BVDV2+	BVDV2+ CD2+	BVDV2+ CD4+	BVDV2+ CD8+
Total cells IFN-γ+	0.055									
	0.710									
IFN-γ+ CD2+	0.031	**0.963**								
	0.834	< 0.001								
IFN-γ+ CD4+	-0.003	**0.869**	**0.858**							
	0.985	< 0.001	< 0.001							
IFN-γ+ CD8+	0.004	**0.841**	**0.847**	**0.700**						
	0.978	< 0.001	< 0.001	< 0.001						
IFN-γ+ CD335+	0.046	**0.790**	**0.819**	**0.678**	**0.742**					
	0.756	< 0.001	< 0.001	< 0.001	< 0.001					
Total cells BVDV2+	-0.194	**0.334**	**0.351**	**0.340**	**0.366**	**0.375**				
	0.187	0.020	0.014	0.018	0.011	0.009				
BVDV2+ CD2+	-0.177	**0.312**	**0.336**	**0.342**	**0.367**	**0.359**	**0.983**			
	0.228	0.031	0.020	0.018	0.010	0.012	< 0.001			
BVDV2+ CD4+	-0.198	**0.306**	**0.322**	**0.386**	**0.328**	**0.292**	**0.946**	**0.967**		
	0.178	0.034	0.026	0.007	0.023	0.044	< 0.001	< 0.001		
BVDV2+ CD8+	-0.234	0.261	**0.294**	**0.332**	**0.329**	**0.323**	**0.946**	**0.967**	**0.933**	
	0.109	0.074	0.043	0.021	0.022	0.025	< 0.001	< 0.001	< 0.001	
BVDV2+ CD335+	-0.016	**0.394**	**0.472**	**0.397**	**0.450**	**0.584**	**0.806**	**0.802**	**0.717**	**0.777**
	0.913	0.006	0.001	0.005	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Upper row= r correlations, significant correlations are in bold. *P*-values are second row in parentheses.
N = 48 animals are equally balanced across breed type and sex.

Simple correlations of BVDV2 stimulated traits to BVDV2 VN titers. The simple correlations in Table 3.18 represent the selected BVDV2-stimulated traits in correlation to each other as well as the virus neutralizing antibody titers to BVDV2 when evaluated across all 48 animals (no reference for F_1 breed type or sex).

There were no significant correlations between the selected BVDV2-stimulated traits to the virus neutralizing antibody titers of BVDV2. Notably, within BVDV2+ Probe+ traits and frequency of IFN-γ expression traits, there were several significant correlations that were not seen previously when BVDV1 was used as the stimulant.

When BVDV2 was used as the stimulant, IFN-γ+ traits were consistently tied to the BVDV+ Probe positive traits. This may indicate a connection between various functions from differing branches of the cell-mediated immune system.

Seong et al., (2013) demonstrated how ncp BVDV type 1 and BVDV type 2 attack the immune system and found that more severe leukopenia was observed in calves stimulated with ncp BVDV2 than in calves stimulated in ncp BVDV1. Additionally, lymphocyte apoptosis was more pronounced and lasted longer in calves stimulated with ncp BVDV2 (Seong et al., 2013). In terms of this study, the positive correlations between IFN-γ+ traits and BVDV+ traits when stimulated by BVDV2 may come from severe depression of lymphocyte counts, at which they expressed the pro-inflammatory cytokine IFN-γ at a higher frequency in order to stimulate more proliferation of cellular immunity linked lymphocytes. Decrease in lymphocyte circulation was not measured in the scope of this study, but cells were positive for BVDV2+ at the higher frequency than BVDV1 stimulant. This measures the ability of mRNA of BVDV2 to replicate within the cell.

The same pattern was not seen when BVDV1 was used as the stimulant. The simple correlations when utilizing BVDV1 as the stimulant would indicate that an increase in frequency of BVDV+ Probe positivity in various cell types had no effect on the frequency of expression of IFN-γ from these cell types. When BVDV2 was used as the stimulant, an increase in antigen positivity as indicated by the BVDV+ Probe+, would be correlated to an increase in the frequency of IFN-γ expression. Typically, the immune system experiences bias towards Th1 or Th2 driven immunity when faced with challenges (Tizard, 2018). The results of the simple correlations would indicate the connection of two measures of cell-mediated immunity, an increase in antigen positivity was positively correlated to an increase in frequency of cells expressing IFN-γ.

The correlations seen in Table 3.20 were consistent when investigated separately for subsets of breed type and sex stimulated with BVDV type 2. Amongst both steers and heifers, as well as Brahman-sired and Angus-sired, frequency of BVDV+ Probe+ cells, was positively correlated with frequency of IFN-γ expression. This would indicate that BVDV type 2 antigen interacts with the cell-mediated immune system differently than BVDV type 1 antigen, and connects the two functions of the immune system differently. The most prevalent BVDV subgenotype is a BVDV1b subgenotype (Fulton et al., 2003; Falkenberg et al., 2019). The BVDV1 genotype in this study does not appear to infiltrate cells as the same rate as BVDV2.

Evaluation of performance traits. Selected performance traits were evaluated for connection to evaluated BVDV+ Probe+ traits and IFN-γ+ traits. Carcass traits were only evaluated on the steers. Birth weight and weaning weight were evaluated on both steers and heifers. Table 3.21 provides the summary of statistical significance of effects and the relevant

interactions for the performance trait models. Models were also evaluated with BVDV2 as the simulant, but indicated no differences from traits when BVDV1 was the stimulant.

Table 3.21. Statistical significance of effects and interactions in performance trait models

	Performance trait			
Effect	Birth weight	Weaning weight	REA[1]	HCW[1]
Breed type	**<0.001**	**0.004**	0.078	0.272
Sex	0.102	**<0.001**	--	--
Age of dam	0.752	**0.034**	**0.005**	0.172
Kill age	--	--	**0.020**	**0.044**
BVDV1 VN titer	--	0.056	--	--
Frequency of BVDV+	--	--	0.412	0.883
Frequency of IFN-γ +	0.135	--	--	--
Breed type by BVDV1 VN titer	--	0.118	--	--
Breed type by Frequency of BVDV+	--	--	**0.039**	0.069
Breed type by Frequency of IFN-γ+	0.096	--	--	--
Breed type by sex	**0.042**	0.193	--	--

[1]REA= ribeye area, HCW= hot carcass weight.

Table 3.22. Least squares means for birth and weaning weight for categorical variables.

Effect		Birth weight (kg)	SEM	Weaning weight (kg)	SEM
Breed type	Angus-sired	33.1[a]	2.06	266.1[a]	11.06
	Brahman-sired	42.4[b]	2.13	236.9[b]	10.80
Sex	Heifer	36.7[a]	1.93	234.8[a]	10.20
	Steer	38.7[a]	1.90	268.2[b]	10.44
Age of dam, years	3	37.8[a]	2.60	237.1[a]	13.52
	4	37.0[a]	3.09	260.4[b]	16.66
	5	37.3[a]	2.76	244.8[b]	15.52
	≥6	38.8[a]	2.87	263.7[b]	15.91
Frequency of IFN-γ+, category	High	39.4[a]	3.12	--	--
	Medium	36.0[a]	2.01	--	--
	Low	37.7[a]	2.11	--	--
BVDV1 VN titers, category	High	--	--	260.7[a]	11.83
	Medium	--	--	254.0[a]	12.31
	Low	--	--	239.9[b]	14.21
Breed by sex interaction	Angus-sired heifer	33.4[a,x]	2.67	254.0[a,x]	14.83
	Angus-sired steer	32.8[a,x]	2.85	278.2[a,y]	15.59
	Brahman-sired heifer	40.1[b,x]	2.89	215.7[b,x]	14.55
	Brahman-sired steer	44.6[b,y]	2.80	258.2[a,y]	15.83
Breed by frequency of IFN-γ+	Angus-sired, High IFN-γ	33.1[a,x]	4.44	--	--
	Angus-sired, Med. IFN-γ	31.2[a,x]	2.72	--	--
	Angus-sired, Low IFN-γ	35.0[a,x]	3.35	--	--
	Brahman-sired, High IFN-γ	45.7[b,x]	4.60	--	--
	Brahman-sired, Med. IFN-γ	40.9[b,y]	3.44	--	--
	Brahman-sired, Low IFN-γ	40.4[b,y]	2.55	--	--
Breed by BVDV1 VN titers	Angus-sired, High Titers	--	--	285.4[a,x]	18.67
	Angus-sired, Med. Titers	--	--	267.3[a,x]	16.62
	Angus-sired, Low Titers	--	--	245.5[a,y]	22.25
	Brahman-sired, High Titers	--	--	236.0[b,x]	17.17
	Brahman-sired, Med. Titers	--	--	240.7[b,x]	18.15
	Brahman-sired, Low Titers	--	--	234.2[a,x]	19.32

[a, b] Indicate differences between main effects, as well as breed differences when breed is included in the interaction.
[x, y] Indicate sex differences in interactions and differences in immune trait levels when included in the interaction.

Birth weight. Table 3.22 presents the differences in least squares means of birth weight with various main effects and the relevant interactions. Evaluated BVDV1+ Probe+ and IFN-γ+ traits and the relevant interactions were included in the model based on relevance.

Main effects. When F_1 breed type was evaluated as a main effect, there was a significant ($P < 0.05$) difference between breed types. This difference was expected due to various studies done previously. It is commonly expected that calves sired by *Bos indicus* sires on *Bos taurus*

dams have higher birth weights than calves sired by *Bos taurus* sires on *Bos indicus* dams. Brown et al. (1993) found that bull calves that were Brahman-sired were 13.7 kg heavier than the bull calves that resulted from the Angus-sired cross. In this model, birth weight did not vary by the age of dam.

Frequency of IFN-γ expression was split into levels (high, medium, and low) and included as a main effect in the birth weight model. Although there were no significant differences, interestingly calves that fell into the "high" category of IFN-γ had the highest mean birth weight. There may be no significant differences due to the sample size. Further research should investigate the connection between birth weight and frequency of IFN-γ expression to understand if frequency of IFN-γ is predetermined and inherently allows for larger birth weights, or if there was undocumented maternal influence.

Interactions. The interaction of F_1 breed type by sex was relevant in the birth weight model. As expected, the *Bos indicus* sired calves were heavier than the *Bos taurus* sired calves, within each sex. The difference between breeds was larger in males than in heifers. The F_1 breed type by sex interaction observed for birth weight follows the historical pattern reported in multiple studies (Roberson et al., 1986; Brown et al., 1993). The difference in breed types when evaluating heifer calves was not significant ($P > 0.10$), but was significant in male calves. In the interaction of breed type and frequency of IFN-γ expression, each level (high, medium, low) of frequency of IFN-γ expression showed a difference between breed types. This difference may be due differences in breed, rather than differences in the level of frequency of IFN-γ expression as Angus-sired calves had no significant differences in birth weights between levels of frequency of IFN-γ expression. The Brahman-sired calves showed differences between the levels of frequency of IFN-γ expression. There was a significant difference between birth weights of Brahman-sired

animals with the highest level of frequency of IFN-γ expression and the medium and lowest levels of frequency of IFN-γ expression. The highest level of frequency of IFN-γ expression had the highest birthweights in Brahman-sired animals. The Brahman-sired animals with high frequency of IFN-γ expression averaged 5 kg heavier at birth than Brahman-sired animals with medium or low frequency of IFN-γ expression.

Weaning weight. Table 3.22 presents the differences in least squares means of weaning weight with various main effects and the relevant interactions. Health traits and the relevant interactions were included in the model based on relevance.

Main effects. There was a difference in weaning weight when breed was evaluated as a main effect. Angus-sired animals had significantly ($P < 0.05$) higher weaning weights than the Brahman-sired calves (Table 3.22) which agrees with Roberson et al. (1986) who found that weaning weights from Brahman dams (Hereford-sired) had larger weaning weights than Hereford dams (Brahman-sired). In our study, Angus-sired animals had a weaning weight 29.2 kg higher than Brahman-sired animals which may be a function of preweaning gain, which again agrees with Roberson et al. (1986) who found that Brahman direct additive effect on preweaning gain was 17.7 kg less than Hereford (Roberson et al., 1986). As expected, steer calves had a significantly higher weaning weight than heifer calves. This has been well documented.

Age of dam impacted weaning weight. Calves with dams that were three years of age had significantly smaller weaning weights than calves with dams older than the age of three. This may be due differences mothering ability or milking ability of young first-calf heifers. However, we did not measure mothering or milking ability

The humoral immunity trait of BVDV1 VN titer level was included in the model based on previous analysis. Within Angus-sired animals, there was a difference in weaning weight

between the highest level of BVDV1 VN titers and the other two levels, medium and low in Angus-sired animals. Notably, weaning weight decreased as level of VN titer decreased.

Interactions. The breed type by sex interaction was relevant for the weaning weight model, which was to be expected. There was a breed type difference when evaluating heifer weaning weights, with Angus-sired heifers having significantly ($P < 0.05$) higher weaning weights than Brahman-sired heifers. There was not a significant difference between breeds when evaluating steer weaning weights, however Angus-sired steers had a higher weaning weight than Brahman-sired steers, consistent with Angus-sired heifers. There was a sex difference, with steers having higher weaning weights in both breed types as expected.

The breed type by level of BVDV1 VN titers was also a significant interaction in the weaning weight model. When evaluating the highest level of BVDV1 VN titers and the medium level of BVDV1 VN titers, there was a significant ($P < 0.05$) difference between breeds in weaning weight. This difference may be more due to differences in genetic composition of breed type, rather than level of BVDV1 VN titers, as the Angus-sired animals were heavier than the Brahman-sired animals for each level. There was not a significant difference between the breed types when evaluating the lowest level of BVDV1 VN titers. When evaluating the levels of BVDV1 VN titers within the Angus-sired breed type, there was a significant difference between the higher two levels of BVDV1 VN titers and the lowest level of BVDV1 VN titers.

The lowest weaning weight for both breed types was seen with calves that fell in the lowest level of BVDV1 VN titers, although not significant. This observation may not be significant due to sample size. This may show a connection of level of humoral immunity with weaning weight and growth characteristics in F_1 Brahman-Angus calves but further research with these types of crosses is required to verify this.

Silva et al. (2022) evaluated the impact of avian-derived antibodies against *Strepococcus bovis, Fusobacterium necrophorum*, and *lipopolysaccharides*, utilizing Angus crossbred heifers at 360 kg at the beginning of the study, and steers at 386 kg at the beginning of the study. It was found that the added polyclonal antibody preparation successfully improved growth performance in Angus crossbred cattle from days 0-14. Although, the study evaluated antibody preparations and not naturally occurring virus neutralizing antibodies, similarities can be drawn between high VN titer levels indicating higher weaning weights (Silva et al., 2022).

Hot carcass weight. Table 3.23 presents the differences in least squares means of hot carcass weight with various main effects and the relevant interactions. Health traits and the relevant interactions were included in the model based on relevance.

Main effects. Breed, age of dam, and level (high, medium, low) of BVDV1 VN titers were included as main effects in the model. Sex was excluded as carcass data was only recorded on steer calves.

There were no significant differences between breed types in hot carcass weight when evaluated as a main effect. Differences in carcass traits due to type of reciprocal cross have not been widely investigated or reported. Amen et al. (2007b) evaluated reciprocal backcrosses involving Angus and *Bos indicus* found no significant difference due to reciprocal backcrosses (Angus x F_1 vs. F_1 x Angus, etc). In this study as Brahman-sired steers ranked lower for carcass weight than Angus-sired steers, but were not different (Table 3.23). When evaluating age of dam as a main effect, hot carcass weights of steers from dams age five were significantly ($P < 0.05$) higher than any of the other dam ages.

Table 3.23. Least squares means for hot carcass weight and ribeye area within relevant effects and interactions.

Effect		Hot carcass weight (kg)	SEM	Ribeye area (cm^2)	SEM
Breed	Angus-sired	382.7[a]	15.76	80.3[a]	0.22
	Brahman-sired	368.6[a]	18.17	75.4[a]	0.29
Age of dam	3	375.3[a]	17.50	83.8[a]	0.28
	4	356.6[a]	21.75	71.8[b]	0.29
	5	396.1[b]	24.23	80.9[a]	0.31
	≥6	374.6[a]	31.90	75.0[b]	0.43
BVDV1 VN Titers	High	374.7[a]	16.24	--	--
	Medium	378.8[a]	16.66	--	--
	Low	373.5[a]	20.62	--	--
Frequency of BVDV+ Probe	High	--	--	78.6[a]	0.34
	Medium	--	--	76.0[a]	0.27
	Low	--	--	78.9[a]	0.18
Breed by BVDV1 VN Titer	Angus-sired, High Titers	395.8[a,x]	28.57	--	--
	Angus-sired, Med. Titers	371.1[a,x]	22.85	--	--
	Angus-sired, Low Titers	381.2[a,x]	25.94	--	--
	Brahman-sired, High Titers	353.6[b,x]	20.45	--	--
	Brahman-sired, Med. Titers	386.5[a,y]	36.90	--	--
	Brahman-sired, Low Titers	365.8[a,x]	36.90	--	--
Breed by Freq. of BVDV+ Probe	Angus-sired, High Freq.	--	--	80.3[a,x]	0.34
	Angus-sired, Med. Freq.	--	--	75.6[a,x]	0.42
	Angus-sired, Low Freq.	--	--	85.0[a,y]	0.33
	Brahman-sired, High Freq.	--	--	77.0[a,x]	0.64
	Brahman-sired, Med. Freq.	--	--	76.4[a,x]	0.36
	Brahman-sired, Low Freq.	--	--	72.7[b,x]	0.24

[a, b] Indicate differences between main effects, as well as breed differences when breed is included in the interaction.
[x, y] Indicate sex differences within categories in interactions and differences in immune trait levels when included in the interaction.

Age of dam has long been accepted as a point of importance in performance traits of cattle. Koch and Clark (1955) demonstrated that the maternal environment provided by the dam, usually improved after the first calf, contributed to performance traits (Koch and Clark, 1955). In prime reproductive years, (5-10), cows are ideally going to create the best maternal environment possible leading to higher weaning weights, and therefore higher hot carcass weights. Results

from our study agreed with the largest hot carcass weight being was from calves from dams 5 years old (Table 3.23).

There were no significant differences between levels of BVDV1 VN titers in hot carcass weight. However, the lowest hot carcass weight was seen in calves within the lowest level of BVDV1 VN titers. This may indicate lower levels of protection from disease, meaning animals were more prone to illness and gain less efficiently.

Interactions. The only relevant interaction included in the hot carcass weight model was between breed type and level of BVDV1 VN titers (high, medium, low). The difference between breeds was seen at the highest level of BVDV1 VN titers. Angus-sired calves had significantly higher hot carcass weights than Brahman-sired animals at the highest level of BVDV1 VN titers. There was no significant difference between breeds at the other BVDV1 VN titer levels. Of the Angus-sired calves, those with the lowest level of BVDV1 VN titers had the lowest hot carcass weight even though it was not statistically significant. This interesting association of VN titers with hot carcass weight warrants future study to determine the impact of VN titers on growth characteristics.

Ribeye area. Table 3.23 shows the differences in least squares means of ribeye area with various main effects and the relevant interactions. Health traits and the relevant interactions were included in the model based on relevance.

Main effects. Breed, age of dam, and level (high, medium, low) of frequency of cells BVDV Probe positivity were included as main effects in this model. There were no significant ($P > 0.10$) differences in ribeye area between breed types when evaluated as a main effect. When evaluating age of dam as a main effect, ribeye areas of steers from dams ages six and higher were significantly lower than any of the other dam ages.

There were no significant differences in ribeye area between frequency of cells BVDV Probe+ levels. However, it is of interest that the lowest level of frequency cells that were BVDV Probe positive had the largest ribeye area mean when evaluated as a main effect.

Interactions. The only relevant interaction included in the ribeye area model was breed type by frequency of cells that BVDV Probe+ level. When evaluating Angus-sired steers, there was a significant difference between the lowest level of frequency of cells BVDV+ and middle and highest frequency of BVDV+ levels. It is important to note that the lowest level of frequency of cells BVDV+ had the highest mean ribeye area of all Angus-sired steers. However, the opposite is true in the Brahman-sired steers, where the lowest level of frequency of BVDV+ had the smallest ribeye area. This may indicate differences in the cell-mediated immune system between the two breed types, and how it impacts the performance traits of the animals but further research is needed.

Table 3.24. Correlations of steer performance traits to BVDV1 stimulated cell traits and BVDV1 VN titers.

	Total cells IFN-γ+	IFN-γ+ CD4+	IFN-γ+ CD8+	Total cells BVDV+	BVDV+ CD4+	BVDV+ CD8+	BVDV1 log 2 VN titers	Birth weight	Weaning weight	Hot carcass weight	Marbling
IFN-γ+ CD4+	**0.961**										
	< 0.001										
IFN-γ+ CD8+	**0.954**	**0.904**									
	< 0.001	< 0.001									
Total cells BVDV+	-0.082	-0.058	0.006								
	0.703	0.788	0.977								
BVDV+ CD4+	-0.280	-0.228	-0.216	**0.950**							
	0.185	0.284	0.312	< 0.001							
BVDV+ CD8+	-0.156	-0.114	-0.051	**0.943**	**0.948**						
	0.468	0.595	0.813	< 0.001	< 0.001						
BVDV1 log 2 VN Titers	0.189	0.174	0.171	0.033	-0.038	0.025					
	0.376	0.416	0.425	0.879	0.859	0.909					
Birth weight	0.288	0.278	0.210	-0.262	-0.222	-0.200	0.236				
	0.183	0.200	0.336	0.228	0.309	0.360	0.279				
Weaning weight	-0.213	-0.038	-0.114	0.351	0.308	0.289	0.104	**-0.576**			
	0.330	0.862	0.606	0.101	0.153	0.181	0.638	0.004			
Hot carcass weight	0.200	0.107	0.290	0.244	0.103	0.166	-0.094	-0.029	0.264		
	0.371	0.635	0.191	0.275	0.647	0.461	0.678	0.900	0.247		
Marbling	0.067	0.063	0.014	-0.180	-0.211	-0.246	-0.292	0.207	-0.056	**0.425**	
	0.768	0.780	0.952	0.422	0.346	0.270	0.187	0.369	0.809	0.049	
Ribeye area	0.261	0.185	0.286	**0.497**	0.393	**0.447**	-0.099	-0.424	0.064	0.406	0.027
	0.240	0.411	0.197	0.019	0.070	0.037	0.661	0.055	0.784	0.061	0.905

Upper row= r correlations, significant correlations are in bold. *P*-values are second row in parentheses.

Table 3.25. Correlations for steer performance traits to BVDV2 stimulated cell traits and BVDV2 VN titers.

	Total cells IFN-γ+	IFN-γ+ CD4+	IFN-γ+ CD8+	Total cells BVDV+	BVDV+ CD4+	BVDV+ CD8+	BVDV2 log 2 VN Titers	Birth weight	Weaning weight	Hot carcass weight	Marbling
IFN-γ+ CD4+	**0.953**										
	< 0.001										
IFN-γ+ CD8+	**0.915**	**0.869**									
	< 0.001	<0.001									
Total cells BVDV+	0.083	0.046	0.199								
	0.701	0.831	0.352								
BVDV+ CD4+	-0.025	-0.058	0.044	**0.918**							
	0.909	0.786	0.839	< 0.001							
BVDV+ CD8+	0.002	-0.052	0.101	**0.952**	**0.913**						
	0.993	0.808	0.639	< 0.001	< 0.001						
BVDV2 log 2 VN Titers	0.127	0.122	0.109	-0.143	-0.126	-0.155					
	0.554	0.571	0.611	0.506	0.559	0.469					
Birth weight	0.117	0.135	-0.037	-0.241	-0.141	-0.167	0.219				
	0.596	0.540	0.868	0.269	0.522	0.446	0.316				
Weaning weight	-0.239	-0.094	-0.103	0.297	0.200	0.299	0.005	**-0.576**			
	0.273	0.670	0.641	0.168	0.360	0.166	0.983	0.004			
Hot carcass weight	0.264	0.223	0.193	0.221	0.071	0.264	0.159	-0.029	0.264		
	0.236	0.320	0.389	0.322	0.753	0.235	0.479	0.900	0.247		
Marbling	0.034	0.048	-0.128	-0.241	-0.225	-0.214	-0.059	0.207	-0.056	0.425	
	0.881	0.833	0.570	0.280	0.313	0.340	0.795	0.369	0.809	0.049	
Ribeye area	0.334	0.253	0.351	0.395	0.265	0.399	-0.024	-0.424	0.064	0.406	0.027
	0.129	0.256	0.109	0.069	0.233	0.066	0.916	0.055	0.784	0.061	0.905

Upper row= r correlations, significant correlations are in bold. *P*-values are second row in parentheses.

Steer performance traits and immune traits. Tables 3.24 and 3.25 present simple correlations of various immune traits to selected performance traits gathered from the 2020 steer calves when stimulated by BVDV1 and BVDV2, respectively. The immune traits were selected on their associated functions of cell-mediated and humoral immune systems that might have relationships with important beef cattle industry performance traits.

The cell type CD4+ gives insight to the humoral immune system, whereas the CD8+ cell type potentially gives insight to the cell-mediated immune system.

There were no significant correlations of carcass traits with our measured markers of cell meditated or antibody immune responses for BVDV1 or BVDV2 as a stimulant in these animals. This is encouraging as an alteration to immune traits, at least as measured by CD8+ and CD4+ cells, would appear not to negatively affect animal performance.

Summary

Reciprocal cross and sex differences were prevalent in the vaccine-induced immune responses to Bovine Viral Diarrhea Virus in these Brahman-Angus F_1 cattle. Differences in response also appeared due to BVDV genotypes (Type 1 vs. Type 2) used to stimulate cell cultures. Modified-live vaccination appeared to impact F_1 breed types differently for several T cell populations. For pre-MLV vaccination, the studied T cells from Brahman-sired animals consistently ranked approximately 20% higher for BVDV+ positivity than those from Angus-sired animals; however post-MLV vaccination, approximately 20% more cells from Angus-sired animals were significantly BVDV+ positive across cell types. Angus-sired animals also had 20 to 60% higher frequencies of T cells expressing IFN-γ. Post-MLV steers consistently had 30 to 35% higher frequencies of T cells were positive for BVDV following stimulation; steers also had higher frequencies of CD4+ and CD 335+ cells expressing IFN-γ, and ranked higher for CD2+

and CD8+ cells as well. There were no reciprocal or sex differences for VN antibody titer level or total number of PMBCs. The BVDV genotype impacted how the immune system responded to the virus. Steers had approximately 40% more cells BVDV-positive than heifers when stimulated with BVDV1, but had approximately 18% more cells BVDV-positive when stimulated with BVDV2. When stimulated with BVDV2, many simple correlations between cell frequencies of BVDV presence and IFN-γ expression were positive, but these correlations were not significant when cells were stimulated with BVDV1. This may indicate that BVDV genotype effect the immune lymphocyte populations differently in *Bos indicus-Bos taurus* crossbred cattle. With both BVDV types used for cell stimulation, no significant correlations were seen between VN antibody titers and cell-mediated traits. Immune response category for cell frequency of BVDV presence or IFN-γ expression did not influence birth weight or weaning weight. An interaction in carcass ribeye area ($P = 0.039$) due to BVDV positivity level and breed type was identified with Brahman-sired steers having the lowest percentage of cells BVDV positive with the smallest ribeye area. No major correlations between immune responses and weight or carcass traits were observed. This study found adaptive immune system functions differed in Angus-sired and Brahman-sired F_1 crosses and the sex effect was not the same in these two crosses. These findings may make cattle producers consider different health management strategies based on the combination of sex and reciprocal cross type.

CHAPTER IV

IMMUNE REGULATION PROTEINS EXPRESSION IN RESPONSE TO IN VITRO BVDV STIMULATION IN RECPRICAL ANGUS-BRAHMAN F_1 CROSSES

Introduction

Programmed cell death-1 (PD-1) and Indoleamine2,3-dioxygenase-1 (IDO) are important immune regulation proteins. The PD-1 protein is an inhibitory immune protein that is expressed in response to immune challenges, however it is severely downregulated in response to acute challenges (Okazaki and Honjo, 2007; Bally et al., 2016), which allows for typical immune activity to occur. Programmed cell death-1 mediates immune tolerance, and is closely related to immune exhaustion (Zhang et al., 2007). After acute antigen stimulation, PD-1 is upregulated to ensure that uncontrolled T cell activation, and subsequent tissue damage, does not occur (Okazaki and Honjo, 2007; Allie et al., 2011). Prolonged upregulation of PD-1 leads to immune system exhaustion, and is commonly seen in chronic infections (Okazaki and Honjo, 2007). Chronic exposure to viruses has been connected to CD8+ T cell exhaustion due to an upregulation of PD-1 (Bally et al., 2016). Eventually, T cell exhaustion will lead to a decrease in cell-mediated immune function, as the ability of T cells to proliferate and produce cytokines will decrease (Francisco et al., 2010; Sharpe and Pauken, 2017). Indoleamine2,3-dioxygenase-1is also an immune reaction regulation effector expressed on antigen presenting cells, such as macrophages and dendritic cells (Mellor, 2005), and typically only expressed in tissue types that experience chronic inflammation, as well as lymphoid tissue (Taylor and G.Feng, 1991; Mellor, 2005). The IDO-dependent T cell suppression plays a significant role in the regulation of cell-mediated immune response, which if left unchecked, leads to severe tissue damage. The IDO protein induces T cell apoptosis and dysfunction, as well as creating bias for naïve T cells to

differentiate into regulatory T cells, rather than cytotoxic T cells (Mellor, 2005). The IDO protein also hinders the function of natural killer cells (CD335+ cells) which perform cytotoxic activity within the innate immune system. High expression of IDO has been heavily correlated to poor outcomes in various cancers (Théate et al., 2015). Kozuma et al. (2018) studied the relationship between PD-1 and IDO to determine the connection between these two immune checkpoint factors and found that all patients that had strong expression of PD-1 (≥50%) were also positive for IDO. Their findings suggest that expression of both IDO and PD-1 may indicate a more aggressive form of lung adenocarcinoma (Kozuma et al., 2018). The immune system regulating proteins PD-1 and IDO have not been investigated in cattle regarding Bovine Respiratory Disease or Bovine Viral Diarrhea Virus in particular.

Objectives

The objectives of this study were to evaluate PD-1 and IDO as immune reaction suppression factors in reciprocal cross Brahman-Angus F_1 cattle following modified-live vaccination. This study also investigated the impact of sex classification (bull versus steer) on these traits. The scope of this chapter is to evaluate the differences in PD-1 and IDO expression in various lymphocyte types.

Materials and Methods

Immune suppression factors PD-1 and IDO were studied using reciprocal cross Brahman-Angus F_1 cattle that were raised at the McGregor Research Center of Texas A&M University. All animal procedures were approved by the Agricultural Animal Care and Use Committee under AUP numbers 2018-006A and 2021-001A. Data analyses were the only work conducted by the student for this chapter. Cattle utilized were born in the Spring of 2021 (n = 19) at the McGregor Research Center, resulting from a 70-day natural service breeding season. The

majority of males were surgically castrated at weaning, however a subset of 2 of each F_1 type (Angus-sired and Brahman-sired) were left intact as potential future sires. When calves averaged approximately 70 days of age they were vaccinated with Covexin 8 (Merck Animal Health) against clostridial diseases. At approximately 6 months of age and 1 month prior to weaning, calves were provided One-shot (Zoetis) for *Mannheimia (Pasteurella) haemolytica* Type A1 and Cattlemaster Gold FP5 (Zoetis) for BRD viral diseases (its BVDV component is killed and includes BVDV strains 5960-1a and 53637-2a). At weaning, calves were provided booster injections with Covexin 8 and Cattlemaster Gold FP5. After weaning (the last week of October) until mid-May 2022 steers and bulls were fed a growing diet in the McGregor Center feedlot. Steers and bulls were fed the same diet, but housed in separate pens. On May 16, 2022 steers and bulls were administered the MLV Titanium 5 (Elanco Animal Health containing BVDV strains C24V-1a and 296-2a). Blood samples were via jugular venipuncture collected on July 18, 2022 to evaluate post-MLV immune traits. There were 15 steers and 4 bulls evaluated in this study.

The PrimeFlow assay, a method to study cell-mediated factors, was utilized in the lab of Dr. Shollie Falkenberg at Auburn University to evaluate PMBCs of post-MLV blood samples to identify specific mRNA in peripheral blood mononuclear cells (PMBCs) following exposure to BVDV (Falkenberg et al., 2017; Falkenberg et al., 2019). This assay uses flow cytometry to isolate subpopulations of PMBCs (Falkenberg et al., 2017; Falkenberg et al., 2019). Cell type subpopulations were stimulated with BVDV-1b (AU526) and BVDV-2a (PI28) strains, along with mitogen as a positive control and a non-stimulated negative control. Number of T cell populations with PD-1 and IDO mRNA were identified and recorded quantitatively, and the frequencies of cells expressing these proteins were evaluated.

Mixed model procedures of SAS were utilized for analyses through a repeated measures approach (4 stimulant treatments per animal) where individual calf ID was included in the model as a random effect. Models to study frequency of immune suppressant factors included stimulant, F_1 cross type (Angus-sired vs. Brahman-sired), calf sex, and potential two-way and three-way interactions. Tendencies were investigated for *F*-tests where $P < 0.10$, and differences were investigated when $P < 0.05$. Interactions were included in the model $P < 0.20$, utilizing a backward selection process. Least squares means for responses were compared following a significant F-test through two-tailed t-tests.

Results and Discussion

The following data are presented as frequencies of lymphocytes that were found to express the IDO protein, which was determined by the IDO+ Probe+, and found to express the PD-1 protein, as found by the PD-1+ Probe+. The following data on frequency of cells expressing the proteins do not automatically equate to the overall levels of expression.

Table 4.1 presents the summary statistics for the continuous traits that were evaluated in this chapter. Table 4.2 presents the summary of statistical significance for cell frequency of IDO and PD-1 expression across evaluated lymphocyte populations and their respective statistical models.

Immune regulation factors, IDO and PD-1.

The least squares means for frequencies for the appropriate lymphocyte populations derived from the IDO+ Probe+ and PD-1+ Probe+ are presented below. Tables 4.3 to 4.5 present the least squares means for the cell frequencies of lymphocytes expressing IDO and PD-1 respectively, when evaluating cross type (Angus-sired vs. Brahman-sired), sex classification based on castration status (Bull vs. Steer), and stimulant as main effects, respectively.

Table 4.1 Summary statistics for immune related frequencies evaluated by cell type, and frequency of lymphocyte classifications.

Variable	n	Mean	Standard deviation	Coefficient of variation	Minimum	Maximum
IDO Probe CD4+ cells	68	0.150	0.112	74.2	0.014	0.586
PD-1 Probe CD4+ cells	68	0.259	0.201	77.5	0.023	0.893
Frequency of CD4+ cells	68	0.238	0.141	59.0	0.034	0.980
IDO Probe CD8+ cells	68	0.162	0.075	46.2	0.009	0.365
PD-1 Probe CD8+ cells	68	0.150	0.136	91.0	0.021	0.640
Frequency of CD8+ cells	68	0.121	0.044	36.7	0.027	0.218
IDO Probe CD335+ cells	68	0.716	0.201	28.1	0.197	0.973
PD-1 Probe CD335+ cells	68	0.202	0.171	84.7	0.039	0.697
Frequency of CD335+ cells	68	0.080	0.053	65.8	0.019	0.314
IDO probe- All cells	68	0.137	0.080	58.2	0.009	0.407
PD-1 Probe- All cells	68	0.148	0.115	77.5	0.020	0.566

All values represent the frequency of cells that expressed IDO and PD-1, as well as frequency of lymphocyte type from total lymphocytes.
The n value represents 4 samples per animal, with 19 animals included.

Table 4.2 Statistical significance for frequency of IDO+ Probe+ cells, frequency of PD-1+ Probe+, and frequency of cell types

	IDO probe				PD-1 probe				Lymphocyte frequencies			
	Cell type											
Effect	CD4+	CD8+	CD335+	Total cells	CD4+	CD8+	CD335+	Total cells	CD4+	CD8+	CD335+	Total cells
Stimulant	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0018	<0.001	<0.001	<0.001
Breed type	0.200	0.784	0.001	0.018	0.215	0.254	0.688	0.825	<0.001	0.084	0.032	0.983
Sex	0.089	0.075	0.058	0.012	<0.001	0.355	0.056	0.171	0.025	0.246	0.106	0.364
Stimulant x breed type	--	0.028	--	--	--	--	--	--	--	0.033	0.107	--
Stimulant x sex	0.002	0.196	--	--	<0.001	0.082	0.086	--	--	0.011	0.074	--
Breed type x sex	--	--	--	--	0.009	0.013	0.025	0.125	0.013	0.133	0.256	--

Table 4.3 Least squares means for frequencies of IDO+ probe and PD-1 probe by breed type across lymphocytes.

Trait	IDO probe								PD-1 probe							
	Cell type															
	CD4+		CD8+		CD335+		Total cells		CD4+		CD8+		CD335+		Total cells	
Breed type	LSM	SEM	LSM	SEM	SEM	LSM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
Angus-sired	0.191[a]	0.029	0.171[a]	0.018	0.802[a]	0.031	0.182[a]	0.019	0.293[a]	0.020	0.133[a]	0.021	0.212[a]	0.022	0.150[a]	0.019
Brahman-sired	0.137[a]	0.034	0.181[a]	0.021	0.634[b]	0.037	0.115[b]	0.022	0.256[a]	0.022	0.169[a]	0.023	0.199[a]	0.024	0.156[a]	0.021
% AxB vs BxA[1]	139.6		96.0		126.4		158.1		114.5		78.4		106.5		95.9	

[a, b] Indicate breed type differences ($P < 0.05$) within cell type.

[1]Percent relative difference compares values of Angus-sired F_1 to Brahman-sired F_1, where 139.6 indicates Angus-sired were 39.6% higher.

Table 4.4 Least squares means for frequencies of IDO+ probe traits and PD-1 probe by sex across lymphocytes.

Trait	IDO Probe								PD-1 Probe							
	Cell Type															
	CD4+		CD8+		CD335+		Total cells		CD4+		CD8+		CD335+		Total cells	
Sex class	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
Bull	0.205[a]	0.041	0.205[a]	0.026	0.768[a]	0.044	0.188[a]	0.026	0.336[a]	0.025	0.166[a]	0.027	0.237[a]	0.028	0.173[a]	0.025
Steer	0.123[a]	0.024	0.151[a]	0.015	0.668[a]	0.026	0.108[b]	0.016	0.213[b]	0.015	0.136[a]	0.016	0.174[a]	0.017	0.133[a]	0.015
% Bull vs Steer[1]	166.5		136.0		114.89		173.5		158.2		121.7		136.4		130.0	

[a, b] Indicate differences in sex ($P < 0.05$) within cell types.

[1]Percent relative difference compares values of bulls to steers, where 166.5 indicates bulls were 66.5% higher.

Table 4.5 Least squares means for frequencies of IDO+ probe traits and PD-1 probe by stimulant across lymphocytes.

	IDO probe				PD1 probe			
				Cell type				
Stimulant	CD4+	CD8+	CD335+	Total cells	CD4+	CD8+	CD335+	Total cells
BVDV1	0.175[a]	0.214[a]	0.846[a]	0.185[a]	0.182[a]	0.129[a]	0.149[a]	0.116[a]
BVDV2	0.156[a]	0.174[a]	0.831[a]	0.169[a]	0.168[a]	0.098[a]	0.109[a]	0.107[a]
Mitogen	0.231[b]	0.195[a]	0.521[b]	0.126[b]	0.612[b]	0.328[b]	0.474[b]	0.310[b]
No stimulant	0.094[c]	0.128[b]	0.673[c]	0.112[b]	0.136[a]	0.049[ac]	0.090[a]	0.078[a]
SEM	0.029	0.020	0.035	0.018	0.023	0.028	0.024	0.018

[a, b, c] Indicate differences in stimulant ($P < 0.05$) within cell type.
BVDV1 = strain AU526-1b, BVDV2 = strain PI28-2a.

Main effects. Main effects of F_1 breed type, sex, and stimulant were included in all statistical models, regardless of *P*- values because these factors were the basis of the experimental design; however, interactions were included only based on statistical significance. Many of the main effects were involved in significant two-way interactions, and are discussed here for completeness.

Breed type. When breed type was evaluated as a main effect (Table 4.3), there was not a significant difference between breed types in almost any lymphocyte population, except for the CD335+ cell and total cells when evaluating the frequency of IDO expression. Within the CD335+ lymphocyte population, Angus-sired calves expressed IDO at a 26.4% higher rate than Brahman-sired counterparts. CD335+ cell populations expressed IDO at an exceptionally higher rate than other cell types. The IDO receptor has been found to hinder the cytotoxic activity of the CD335+ cells (Natural Killer cells (NK cells)) (Tizard, 2018) as a form of immune reaction suppression (Chiesa et al., 2006). Higher frequency of IDO expression on CD335+ cells may indicate a decrease in CD335+ cell efficiency. Chiesa et al., (2006) found that an IDO-generated catabolite impacted the process of NK cell activation, which in turn impacts the ability of NK

cells to kill certain target cells, therefore making the CD335+ cells less efficient. Frequency of total cells expressing IDO varied when breed type was evaluated as a main effect. Total frequency of cells varied by 58.1%, with Angus-sired calves expressing IDO at a higher rate overall. This value does not indicate the level of expression, however higher frequency of cell expression may indicate prolonged expression of IDO, which would not be beneficial to immune function.

There was no significant ($P > 0.10$) difference in PD-1 expression for breed type as a main effect. When evaluating the PD-1 immune regulation factor, CD4+ cells expressed PD-1 at a higher rate than other lymphocyte populations. Cattle breed comparisons for this protein are scarce in the scientific literature. Ikebuchi et al., (2010) evaluated the PD-1 gene in the Holstein-Friesian and Japanese Black breeds, and found the PD-1 gene to be identical in sequence, which was also found to be identical to that of Hereford cattle (derived from the NCBI database as reported by Ikebuchi et al. 2010). The sequence of DNA does not equate gene expression, but may give insight to breed differences (Ikebuchi et al., 2010). However, all breeds compared were of *Bos taurus* descent, and comparison to *Bos indicus* breeds in future projects would be useful.

Sex class. Several lymphocyte populations did not differ when sex class was evaluated as a main effect for frequency of IDO expression (Table 4.4). However total frequency of cells expressing IDO differed significantly ($P < 0.05$) across sex class with bulls having higher frequency of expression than steers by 73.5%. This analysis is limited in scope as there are very few (n = 4) bulls. Pozzo et al., (2018) compared IDO-1 expression between PMBCs from human males and females before and following cortisol exposure *in vitro*, and found males had higher levels in basal conditions. When stimulated with physiological cortisol concentrations, female PMBCs experienced no change in IDO mRNA expression, whereas male PMBCs expressed IDO

mRNA at a significantly ($P < 0.05$) higher rate of approximately 1.5 times the change compared to females following physiological cortisol exposure. When exposed to the highest concentration of cortisol, Heifer PMBCs significantly ($P < 0.05$) increased IDO-1 gene expression, whereas human male PMBCs significantly ($P < 0.05$) decreased IDO-1 gene expression (Pozzo et al., 2018).

When evaluating the frequency of PD-1 expression based on castration status as a main effect, the CD4+ lymphocyte population differed significantly ($P < 0.05$) between bulls and steers. Bulls expressed PD-1 at a 58.2 % higher rate than steer counterparts and in fact bulls consistently ranked higher than steers across all cell types. Although rodent and cattle immune systems are quite different. Wang et al. (2009) studied the effect of sex hormones on the expression of PD-1 in experimental autoimmune encephalomyelitis mice *in vitro* and found E2 (estradiol) boosted the expression of PD-1 in mice. This could potentially indicate that an upregulation of PD-1 stimulates the sex hormone estradiol and estradiol-mediated protection if expression is supplemental. The present study could not evaluate females vs. males.

In the current study, bulls ranked higher than steers for cell frequency of expression for both IDO and PD-1 across all cell types, although not statistically significant. These differences may not be significant because of the low sample size. Kozuma et al. (2018) found that high expression of PD-1 and IDO together are associated with more aggressive forms of cancers. Higher cell frequencies of expression does not guarantee higher levels of overall expression, but higher frequencies consistently across IDO and PD-1 traits may indicate immune regulation tendencies. This may indicate that bulls are able to return to homeostasis after challenge more efficiently, or may indicate that bulls are more prone to experience CD8+ T cell exhaustion.

Stimulant. Frequency of IDO and PD-1 expression differed when stimulant was evaluated as a main effect (Table 4.5). Consistently, there was no differences ($P > 0.10$) in frequency of cells expressing IDO or PD-1 between cell cultures stimulated with BVDV1 or BVDV2. This would indicate the genotype of BVDV did not impact the frequency of expression for the immune reaction suppressors factors IDO and PD-1. When evaluating the IDO Probe, expression was lower for non-stimulated cells compared to those stimulated with BVDV.

When cells were not stimulated, the lowest value of frequency of IDO expression was seen, excluding the CD335+ cell type in which cells stimulated with the positive control mitogen had the lowest frequency of IDO expression. This would indicate that these two types of BVDV were stimulating the frequency of IDO expression. Palomares et al. (2014b) studied the differences between low virulence and high virulence BVDV strains on various T cell regulation factors on 30 beef calves at 7 months of age, all free of BVDV. Changes in IDO mRNA expression utilizing the CT method found the low virulence strain at 5.80. The control group at 10.21 CT value, which indicates exceptional upregulation in IDO mRNA in the group challenged with a low virulence strain in the trachea-bronchial lymph nodes when compared to the control group (Palomares et al., 2014b). Evaluating the effect of a highly virulent compared to low virulent strain of BVDV, the low virulence strain had a significantly ($P < 0.05$) higher expression of IDO mRNA in the trachea-bronchial lymph nodes (Palomares et al., 2014b). However, when evaluating the IDO mRNA expression in the spleen, there was no significant ($P > 0.10$) difference between either BVDV genotype and the control (Palomares et al., 2014b).This may indicate that location of the cells plays a role in regulation of IDO transcription, along with the strain of virus.

The PD-1 Probe traits indicated the cell cultures stimulated with the positive control mitogen consistently had the highest cell frequency of PD-1 expression for each cell type. There were no significant differences ($P > 0.10$) in PD-1 expression between cells that were stimulated with BVDV1 or BVDV2. The positive control of mitogen is exceptionally hard on lymphocytes, so as mitogen causes more harm, PD-1 would be expressed to signal cell death (Okazaki and Honjo, 2007). Stimulation with BVDV2 has been found to be harder on immune cells. The immunosuppression factors PD-1 and IDO would be expected to be down-regulated when stimulated with BVDV2 in order to allow for a large cell-mediated immune response to form.

Two-way interactions. All two-way interactions that were statistically significant for the different lymphocyte cell types were included in final models for completeness. However, not all of these interactions are discussed in this thesis because those involving breed type, sex, and stimulant were of most interest for this study. Some significant interactions included in respective models are not portrayed in tables if the interaction was included in a single cell type alone, or only in one factor, as it does not aide in comparing the two immune reaction suppression factors.

Sex class by stimulant interaction IDO Probe. The interaction between sex class (bull vs. steer) and stimulant (BVDV1, BVDV2, mitogen, and no stimulant) was significant in models for CD4+ and CD8+ lymphocyte types (Table 4.6).

Consistently, throughout cell types that included the interaction sex class by stimulant in the model, bull calves were found to express IDO at a higher frequency than steer counterparts across all stimulants. The difference between sexes were not significant ($P > 0.10$) for any stimulant, except for the positive control of mitogen.

When utilizing the positive control mitogen as the stimulant, the cells derived from the bulls expressed IDO at almost a three-fold higher frequency than steers within the CD4+ cells. Within the CD8+ cells, bulls expressed IDO at a 65.1% higher frequency than steers. The mitogen positive control created an environment for IDO to be expressed at a significantly ($P < 0.05$) higher rate than other stimulant treatments in bulls. Within the steers, the mitogen positive control did not have significantly ($P > 0.10$) higher frequency of IDO expression than other stimulants. This would indicate that the positive control mitogen might potentially have different effects on the cells related to sex hormone levels, but this would need to be formally evaluated on future studies.

Table 4.6 Least squares means for sex class by stimulant of IDO+ Probe frequencies.

Stimulant	BVDV1		BVDV2		Mitogen		No stimulant		SEM	
						Cell type				
Sex class	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Bull	$0.201^{a,x}$	$0.253^{a,x}$	$0.171^{a,x}$	$0.187^{a,x}$	$0.341^{a,y}$	$0.243^{a,x}$	$0.108^{a,x}$	$0.137^{a,xy}$	0.050	0.034
Steer	$0.150^{a,x}$	$0.176^{a,x}$	$0.141^{a,x}$	$0.161^{a,x}$	$0.122^{b,x}$	$0.147^{b,x}$	$0.080^{a,xy}$	$0.119^{a,xy}$	0.029	0.020
%Bull vs. Steer	133.8	144.0	121.9	116.2	278.4	165.1	135.6	115.0		

$^{a, b}$ Indicate sex differences ($P < 0.05$) within cell types.

$^{x, y, z}$ Indicate differences in stimulant ($P < 0.05$) within cell types.

Table 4.7 Least squares means for sex class by stimulant interaction for the PD1 Probe frequencies.

Stim	BVDV1			BVDV2			Mitogen			No stimulant			SEM		
								Cell type							
Sex class	CD4+	CD8+	CD335+	CD4+	CD8+	CD335+	CD4+	CD8+	CD335+	CD4+	CD8+	CD335+	CD4+	CD8+	CD335+
Bull	$0.228^{a,x}$	$0.136^{a,x}$	$0.187^{a,x}$	$0.195^{a,x}$	$0.073^{a,x}$	$0.113^{a,x}$	$0.760^{a,y}$	$0.401^{a,y}$	$0.548^{a,y}$	$0.163^{a,x}$	$0.054^{a,x}$	$0.101^{a,x}$	0.039	0.049	0.042
Steer	$0.135^{b,x}$	$0.122^{a,x}$	$0.110^{a,x}$	$0.142^{a,x}$	$0.124^{a,x}$	$0.105^{a,x}$	$0.464^{b,y}$	$0.256^{b,y}$	$0.400^{b,y}$	$0.109^{a,x}$	$0.043^{a,z}$	$0.080^{a,x}$	0.023	0.028	0.024
%Bull vs Steer	168.4	111.9	170.0	137.1	58.4	107.0	163.9	156.8	137.0	148.7	123.6	125.9			

$^{a, b}$ Indicate sex differences ($P < 0.05$) within cell types.

$^{x, y, z}$ Indicate differences in stimulant ($P < 0.05$) within cell types.

This study could not compare males versus females, but is of further interest for future research. Pozzo et al. (2018) found that larger concentrations of cortisol impacts PMBCs from intact male and female mice differently, with physiological levels of cortisol not impacting female PMBCs, but increasing IDO mRNA expression in male PMBCs. However, when the highest level of cortisol concentration (0.40 μg/ml) was administered, it had inverse effects on male and female PMBCs. At the highest concentration, female PMBCs significantly increased IDO mRNA expression, and male PMBCs significantly decreased IDO mRNA expression at the same concentration (Pozzo et al., 2018). Fertan et al., (2019) studied the effect of an IDO enzyme inhibitor on the impact of male and female mice to treat the effects of Alzheimer's Disease. Tryptophan, the amino acid that the enzyme IDO is charged to breakdown, is metabolized through the kynurenine pathway and can be induced by IDO activity (Chen and Guillemin, 2009). Fertan et al., (2019) found a sex difference in the IDO blockage function, which would indicate differences in enzymatic activity of IDO itself, and male and female hormones impact the kynurenine pathway in different ways (Fertan et al., 2019). Rodent and cattle immune systems are quite different, and how well these results might correspond to cattle needs further investigation.

Sex class by stimulant PD-1 Probe. The interaction of sex class (bull vs. steer) with stimulant (BVDV1, BVDV2, mitogen, and no stimulant) was included in models evaluating frequency of PD-1 expression was significant in models for CD4+, CD8+, and CD335+ cells.

Within the BVDV1 stimulant, the CD4+ cell type alone was significantly ($P < 0.05$) different between the sex classes, with bulls expressing PD-1 at a 68.4% higher frequency than steers. Although not statistically significant ($P > 0.10$), all cell types (CD8+ and CD335+) from bulls consistently ranked higher for cell frequencies expressing PD-1.

When stimulated with BVDV2, lymphocytes of the CD4+ and CD335+ classification from bulls ranked higher for PD-1 expression cell frequency than steers, although not statistically significant ($P > 0.10$). However, when stimulated with BVDV2, CD8+ cells from steers ranked higher than bulls at a 41.6% higher rate, although not statistically significant ($P > 0.10$). This trend seen throughout the rest of the data. If replicated on a larger scale with more samples from bulls, this could indicate that the different genotypes of BVDV impact cell types differently, and impact the castration status in different ways. This may be a random result associated with this one measurement or a result of the small sample size of bulls used in this study, but bears further study.

Utilizing the positive control mitogen as the stimulant, PD-1 was expressed at significantly ($P < 0.05$) higher frequencies across all cell types, versus when other stimulants were used. Between the sexes, when the positive control was utilized as the stimulant, lymphocytes from bulls expressed antigen at significantly ($P < 0.05$) higher rates than lymphocytes from steers.

Polanczyk et al. (2007) found that estrogen receptor knockout mice showed a significant reduction in the level of PD-1 expression among Treg cells, which in turn lead to a decrease in immune reaction suppression. This would indicate that less presence of estrogen leads to less immune regulatory function of PD-1. Which may also indicate linkage of sex hormones to PD-1 (Polanczyk et al., 2007). Scientific literature comparing intact bulls versus castrated steers expression of PD-1 is scarce. This study indicated that there may be differences due to castration status in immune function.

Table 4.8 Least squares means for the breed by sex class interaction for the PD-1 probe frequencies.

	Bull								Steer							
	Cell types															
	CD4+		CD8+		CD335+		Total cells		CD4+		CD8+		CD335+		Total cells	
Breed	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
Angus-sired	0.315^{a,x}	0.036	0.107^{a,x}	0.038	0.206^{a,x}	0.039	0.147^{a,x}	0.035	0.271^{a,x}	0.017	0.159^{a,x}	0.018	0.218^{a,x}	0.018	0.152^{a,x}	0.016
Brahman-sired	0.358^{a,x}	0.036	0.225^{b,x}	0.038	0.268^{a,x}	0.039	0.198^{a,x}	0.035	0.154^{b,y}	0.025	0.114^{a,y}	0.027	0.130^{b,y}	0.027	0.114^{a,y}	0.025
%AxB vs BxA	87.9		47.4		76.9		74.3		176.2		139.7		167.9		133.6	
SEM	0.036		0.038		0.039		0.035		0.025		0.027		0.028		0.025	

^{a,b} Indicate differences in breed type ($P < 0.05$) within cell type.

$^{x, y}$ Indicate differences in sex class ($P < 0.05$) within cell type.

Breed by sex class interaction PD-1 Probe. The interaction between breed (Angus-sired vs. Brahman-sired) and sex class (bull vs. steer) was significant in models when evaluating PD-1 Probe+ traits within cell types CD4+, CD8+, and CD335+.

Evaluating cell types derived from bulls, cells from Brahman-sired animals consistently ranked at a higher frequency for PD-1 than cells from Angus-sired animals, although this difference was only significant ($P < 0.05$) in CD8+ cell types. Within the CD8+ cell type, Brahman-sired animals expressed PD-1 at 52.6% higher rate than Angus-sired animals. When PD-1 is upregulated on CD8+ cells, this usually indicates returning the immune system back to a normal equilibrium, or leads to T cell exhaustion in which case immune function would falter (Zhang et al., 2007). This value does not indicate level of PD-1 expression, but higher frequency of expression may indicate an upregulation of PD-1 and subsequently the impact of upregulation of PD-1.

Evaluating cell types from steers, cells from Angus-sired animals consistently ranked higher for PD-1 cell frequency expression than cells from Brahman-sired animals, although this difference was only significant ($P < 0.05$) in cell types CD4+ and CD335+. Within the CD4+ cell type among steers, Angus-sired steers expressed PD-1 at a 76.2% higher frequency than Brahman-sired steers, and within the CD335+ cell type, Angus-sired steers expressed PD-1 at a 67.9% higher frequency than Brahman-sired steers.

It is important to note that breed type trends changed rank between the sex classes. The cells from Brahman-sired bulls expressed PD-1 at a higher frequency across all cell types. However, the cells from Angus-sired steers expressed PD-1 at a higher frequency across all cell types. This may indicate that PD-1 may be tied to sex hormones as reported in rodents (Wang et

al., 2009), and may differ due to which parent breeds are used to generate the F_1 crosses, but additional studies with larger sample sizes are needed to confirm this in cattle.

Table 4.9 Least squares means for frequencies of lymphocytes, and total lymphocyte number.

		CD4+		CD8+		CD335+		Total cells	
		LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
Breed type	A x B	0.189[a]	0.021	0.143[a]	0.013	0.112[a]	0.016	40030[a]	4310.0
	B x A	0.308[b]	0.023	0.109[a]	0.014	0.061[b]	0.017	39891[a]	4774.8
	%A x B vs B x A	61.5		131.1		184.6		100.4	
Sex class	Bull	0.212[a]	0.027	0.137[a]	0.016	0.106[a]	0.020	37014[a]	5513.4
	Steer	0.285[b]	0.016	0.115[a]	0.010	0.061[a]	0.012	42907[a]	3313.2
	% Bull vs Steer	74.5		119.7		156.8		86.3	
Stimulant	BVDV1	0.272[a]	0.027	0.131[a]	0.011	0.078[a]	0.013	40662[a]	3747.3
	BVDV2	0.286[a]	0.027	0.134[a]	0.011	0.070[a]	0.013	53597[b]	3747.3
	Mitogen	0.157[b]	0.027	0.094[b]	0.011	0.132[b]	0.013	6617[c]	3747.3
	No stimulant	0.279[a]	0.027	0.145[a]	0.011	0.065[a]	0.013	58966[b]	3747.3
Interactions									
Sex by Stim.	Bull, BVDV1	--	--	0.153[j,x]	0.020	0.096[j,x]	0.022	--	--
	Bull, BVDV2	--	--	0.154[j,x]	0.020	0.085[j,x]	0.022	--	--
	Bull, Mitogen	--	--	0.084[j,y]	0.020	0.167[j,y]	0.022	--	--
	Bull, No stim	--	--	0.159[j,x]	0.020	0.075[j,x]	0.022	--	--
	Steer, BVDV1	--	--	0.110[j,x]	0.012	0.060[j,x]	0.013	--	--
	Steer, BVDV2	--	--	0.115[j,x]	0.012	0.056[j,x]	0.013	--	--
	Steer, Mitogen	--	--	0.105[j,x]	0.012	0.097[k,y]	0.013	--	--
	Steer, No stim	--	--	0.130[j,x]	0.012	0.055[j,x]	0.013	--	--
Breed type by Stim.	A x B, BVDV1	--	--	0.160[a,x]	0.015	0.109[a,x]	0.017	--	--
	A x B, BVDV2	--	--	0.157[a,x]	0.015	0.101[a,x]	0.017	--	--
	A x B, Mitogen	--	--	0.096[a,y]	0.015	0.144[a,y]	0.017	--	--
	A x B, No stim	--	--	0.159[a,x]	0.015	0.094[a,x]	0.017	--	--
	B x A, BVDV1	--	--	0.103[b,x]	0.017	0.047[b,x]	0.019	--	--
	B x A, BVDV2	--	--	0.111[b,x]	0.017	0.040[b,x]	0.019	--	--
	B x A, Mitogen	--	--	0.093[a,x]	0.017	0.120[a,y]	0.019	--	--
	B x A, No stim	--	--	0.130[a,y]	0.017	0.035[b,x]	0.019	--	--
Breed type by Sex	A x B, Bull	0.193[a,j]	0.038	0.169[a,j]	0.023	0.145[a,j]	0.028	--	--
	B x A, Bull	0.231[a,j]	0.038	0.106[a,j]	0.023	0.067[a,j]	0.028	--	--
	% A x B vs. B x A	83.6		159.9		217.4			
	A x B, Steer	0.185[a,j]	0.018	0.117[a,k]	0.011	0.080[a,k]	0.013	--	--
	B x A, Steer	0.384[b,k]	0.027	0.113[a,j]	0.016	0.055[a,j]	0.020	--	--
	% A x B vs. B x A	48.2		104.1		145.0			

[a,b] Indicate differences within cell type within main effects, and differences between breeds when included in interactions.

[x, y, z] Indicate differences in stimulant within cell type when stimulant is included in the model.

[j,k] Indicate differences in sex within cell type when sex is included within interactions.

Total frequency of each cell type. Table 4.9 presents the analysis of the total frequency of each cell type of the total lymphocyte population, as well as total lymphocyte number. Analysis of these frequencies, and total lymphocyte number, gives insight to the cell population of the animal, which may give insight to the immune functions within different populations. Each cell type model included the main effects regardless of significance, where interactions were evaluated for significance before being included in their respective models.

Breed type as a main effect. When evaluating the frequency of lymphocytes that were classified as CD4+, breed type indicated significant differences when evaluated as a main effect. Brahman-sired animals had a higher portion of cells classified as CD4+ cells when compared to Angus-sired counterparts. The CD4+ cell is usually classified as the T helper cell, which can indicate higher levels of humoral immunity, and less of a bias towards cytotoxic activity (Tizard, 2018). The difference in CD4+ proportion between the breed types may indicate different levels of cell-mediated immunity, or differences in how challenges are handled by the immune system. This is complimented by the CD8+ cell analysis. This could indicate a potential "trade-off" relationship across types of responses, indicating some animals may have a cell-mediated bias, where others have a humoral bias. Biases to one branch of adaptive immunity does exist (Johnson et al., 2008; Tizard, 2018), but this difference in proportion of cell type may indicate a role of breed composition in these biases. In terms of total lymphocytes, Angus-sired animals and Brahman-sired animals were very comparable, just the classifications of the lymphocytes themselves differed. This may indicate that breed type does not impact the level of immunity, perhaps just the bias to Th1 or Th2 supplemented immunity.

Within the CD8+ cell model, Angus-sired animals ranked higher for proportion of CD8+ cells, but they did not differ significantly. Angus-sired animals had a higher proportion of their

lymphocytes classified as CD8+ cells and a lower proportion of CD4+ cells. This may be an indication of a cytotoxic, or cell-mediated immunity bias, as the CD8+ cells are the main effector of cytotoxic activity (Tizard, 2018). Angus-sired animals had a significantly higher proportion ($P < 0.05$) of their lymphocytes classified as CD335+ cells. The CD335+ cells, commonly known as Natural Killer cells, are known to be the effector of cytotoxic activity of the innate immune system (Tizard, 2018). This may further suggest that Angus-sired animals have more of a bias towards a cytotoxic, cell-mediated, immune response, but additional studies with larger sample sizes are needed to confirm this possibility.

Sex class as a main effect. When evaluating sex class as a main effect, there were no significant differences between the castration status in frequency of lymphocyte population for any cell type. The biggest difference between the sexes was observed within the CD335+ cell type. Bulls had 56.8% more of their lymphocytes be classified in the CD335+ category than the steer counterparts. This could be important as the CD335+ cells perform cytotoxic activity within the bounds of the innate immune system (Tizard, 2018) and higher proportions of CD335+ could indicate a bias towards cell-mediated activity. CD335+ cells also stimulate the production of IFN-γ, which further produces a Th1 bias (Johnson et al., 2008). However, it must also be acknowledged that only 4 bulls, 2 of each F_1 breed type, were evaluated. An additional study of a more balanced set of steers versus bulls is needed to confirm this. Total lymphocyte number did not differ significantly by sex, yet many cell frequencies differed, indicating potential cell activity may not be the same in steers versus bulls.

Sex class by stimulant interaction. The sex class by stimulant interaction was relevant in models for the CD8+ and CD335+ cell types. As this was a model to measure the frequency of cell types across total lymphocytes, extreme differences were not expected. Among the CD8+,

no significant differences between the sexes were observed. The only observed difference was among bulls when stimulated with the positive control of mitogen in the CD8+ and CD335+ cell types. This value was lower than the other stimulants, which may be due to the fact that mitogen is exceptionally hard on cells and breaks down cells at a much faster rate.

Breed by stimulant interaction. The breed by stimulant interaction was relevant in models for the CD8+ and the CD335+ cell types. When evaluating the breed by stimulant interaction for both BVDV1 and BVDV2 genotype, there was a significant ($P < 0.05$) difference between breeds. When stimulated with BVDV1, there were more CD4+ lymphocytes in Angus-sired animals than Brahman-sired animals. This trend remained when stimulated with BVDV2. When evaluating the CD335+ cell type, Angus-sired animals also had more cells classified as CD335+ than Brahman-sired animals when stimulated with both BVDV1 and BVDV2. This indicates that the stimulants may impact these F_1 crosses differently, but further research into this relationship is needed.

Breed by sex class interaction. The breed by sex interaction was relevant in models for the CD4+, CD8+, and the CD335+ cell types. When evaluating the model for CD4+ cell type, there was no difference in frequency of CD4+ cell type between Angus-sired bulls and Brahman-sired bulls. However, when evaluating the breed difference between the steers, Brahman-sired steers had 51.8% higher frequency of CD4+ cells than Angus-sired steers. CD4+ cells are known as regulatory T cells and could create a more humoral biased immune system (Tizard, 2018). There was a sex difference observed within the Brahman-sired animals for CD4+ frequency of total lymphocytes. Brahman-sired steers had significantly higher ($P < 0.05$) frequency of CD4+ cells than Brahman bulls. This analysis may be limited because of the limited number of bulls included in the model. However, this may indicate an impact of sex in Th1 versus Th2 biases. An

additional study is necessary in which the number of steers and bulls is more similar to confirm this.

Within the CD8+ model, there was no significant difference due to breed type within the bulls but there was a sex difference observed among the Angus-sired animals. Angus-sired bulls appeared to have a higher concentration of CD8+ cells than Angus-sired steers. The CD8+ cell is the main component of cytotoxic activity within the cell-mediated immune system, so higher frequency of CD8+ cells may indicate higher levels of cytotoxic activity, which is necessary to fight viruses (Tizard, 2018). However, too many active CD8+ cells could lead to an overactive cell-mediated immune response and eventual tissue damage (Tizard, 2018). This trend remained when evaluating the CD335+ cell model. The CD335+ model mirrors the CD8+ cell model so symmetry between these two frequencies was expected.

Summary

Programmed cell death-1 (PD-1) and Indoleamine 2,3-dioxygenase-1 (IDO) proteins are crucial to immune regulation, however are harmful when expressed at high levels for extended periods of time. There were reciprocal cross differences in frequency of IDO expression due to F_1 breed type when evaluated as a main effect when evaluating CD335+ cells and the frequency of expression in total cells. Total cell frequency of IDO expression also differed by castration status. The interaction of sex class by stimulant indicated that bulls ranked higher than steers for frequency of IDO expression and PD-1 expression, which may indicate higher levels of immune suppression. Proportion of different lymphocyte types differed by F_1 breed type. Angus-sired animals had an 85% significantly higher proportion of CD335+ cells, while having 38% significantly lower proportion of CD4+ cells. This may indicate that immune system biases (Th1 versus Th2) may be in part due to breed differences. In the breed type by sex class interaction,

Angus-sired animals consistently had a higher proportion of CD8+ and CD335+ cells, while having a lower proportion of CD4+ cells. Angus-sired bulls consistently ranked lower for PD-1 expression frequency than Brahman-sired bulls across cell types, but Angus-sired steers consistently ranked higher for PD-1 expression frequency across cell types. This may further strengthen the theory that how these F_1 crosses were generated (Angus x Brahman vs. Brahman x Angus) may influence their cell-mediated immune responses, and therefore types of immune response biases; however, additional studies with larger sample sizes are needed to confirm the results seen here.

CHAPTER V

SUMMARY

This thesis evaluated T cell responses to *in vitro* exposure to Bovine Viral Diarrhea Virus (BVDV) among reciprocal cross Brahman-Angus F_1 cattle and sex classifications following modified live vaccination for bovine respiratory viral pathogens. The phenomenon of birth weight differences where *Bos indicus*-sired calves out of *Bos taurus* cows produce much heavier calves than the reciprocal cross of *Bos taurus*-sired calves out of *Bos indicus* cows is well documented but this potential effect has not been investigated for immune responses. Differences due to F_1 cross type and sex classification in several immune response traits were identified].

In trial 1, prior to MLV vaccination, cells from Brahman-sired animals consistently ranked higher for BVDV+ positivity after exposure than those from Angus-sired animals; however post-MLV vaccination, cells from Angus-sired animals were higher for BVDV+ presence following exposure across cell types. Angus-sired animals also ranked higher frequencies of T cells expressing IFN-γ. Differences in response also occurred due to BVDV genotypes (Type 1 vs. Type 2) used to stimulate cell cultures. When stimulated with BVDV2, many simple correlations between cell frequencies of BVDV presence and IFN-γ expression were significant. The *Bos indicus-Bos taurus* crossbred cattle. With both BVDV types used for cell stimulation, no significant correlations were seen between antibody titers and cell mediated traits. Immune response category for cell frequency of BVDV presence or IFN-γ expression did not influence birth weight or weaning weight. This may indicate adaptive immune system functions may differ in Angus-sired and Brahman-sired F_1 crosses. This immune response also differed by stimulant.

In trial 2 there were reciprocal cross differences in frequency of IDO expression due to F_1 breed type when evaluated as a main effect when evaluating CD335+ cells and the frequency of expression in total cells. Angus-sired animals expressed IDO at a higher frequency than Brahman-sired animals in CD335+ cells and total frequency overall. Total cell frequency of IDO expression also differed by sex. The interaction of sex by stimulant indicated that bull calves ranked higher for frequency of IDO expression and PD-1 expression, which may indicate higher levels of immune suppression. This may further strengthen the theory that F_1 breed type may influence immune cell population, and therefore types of immune response biases. These results indicate that how these F_1 crosses were generated (Angus x Brahman vs. Brahman x Angus) may influence their cell-mediated immune responses.

Some of these results may relate to genomic imprinting that has been proposed to influence birth weight in reciprocal *Bos indicus-Bos taurus* crosses. Future research should further investigate the impact of reciprocal cross type and sex classification in combination with performance traits and involve larger sample sizes. Producers need to know if generating different reciprocal cross types impact immune responses and performance traits. Future investigation of level of expression of IDO, PD-1, and IFN-γin addition to cell expression frequency is needed.

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