Improved vaccine platform for safe and effective control of Bovine Viral Diarrhea Virus

By

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
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Abstract

Bovine Viral Diarrhea Virus (BVDV), a *Pestivirus* with single stranded viral genome, is prevalent in cattle. Commercial BVDV vaccines have many side effects and are not completely protective because BVDV strains are highly diverse. Biosecurity control measures coupled with BVDV vaccination are practiced to minimize incidences of BVDV infection within herds, but BVDV still persists in cattle herds in many places in the world. Hence, the objective of the first study was to address the lack of a safe and efficacious BVDV vaccine. A prototype subunit vaccine comprising of mosaic BVDV antigens, envelop (E2) and non-structural (NS2-3) antigens, from BVDV type 1a, 1b, and 2 along with unique as well as shared B cell and $CD4^+$ T cell epitopes was generated. The mosaic BVDV antigens were shown to be highly immunogenic in calves and elicited broadly neutralizing antibody responses against diverse BVDV-1a, -1b, and -2a strains. The mosaic antigens also induced strong BVDV-1 and -2 cross-reactive bovine $CD4^+$ T cell responses. Following BVDV-1b challenge, the prototype vaccine conferred better protection than a commercial killed virus (KV) vaccine as judged by significant ($p < 0.05$) reduction in viremia and BVD disease in the vaccinated calves. The results from this study therefore indicated that a rationally designed multi-epitope BVDV subunit vaccine can offer broader protection than a commercial BVDV vaccine containing inactivated BVDV-1a, -1b, and -2a strains. In the second study, twenty-eight novel $CD8^+$ T cell epitopes were identified from various BVDV antigens. The identified IFN-γ-inducing $CD8^+$ T cell epitopes were conserved across >200 BVDV-1 and -2 strains. These highly conserved $CD8^+$ T cell epitopes can prime broadly reactive $CD8^+$ T cells against multiple BVDV strains in cattle. Future analyses of the novel well-conserved BVDV epitopes for the induction of cytotoxic $CD8^+$ T lymphocyte (CTL) responses will lead to the identification of protective determinants that can be included in a subunit BVDV vaccine.

Altogether, the knowledge generated by the studies outlined in this thesis will form a basis for development of more efficacious contemporary BVDV vaccines capable of conferring broad protection against diverse BVDV strains.

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Dedication

I would like to dedicate this work to my parents, Arun and Sarita, and my brother, Aditya. Their love and encouragement made this possible.

Chapter 1- Latest Advances in Next-Generation Bovine Viral Diarrhea Virus Vaccines

1.1 Bovine Viral Diarrhea Virus (BVDV)

Bovine Viral Diarrhea Virus (BVDV) is a *Pestivirus* that belongs to the *Flaviviridae* family and this pathogen circulates in global cattle population notably, in European, North American, South American and Asian countries (Becher et al., 2003; Scharnbock et al., 2018). The BVDV polyprotein, encoded by a 12.5 kb long viral RNA genome, is processed by the combination of viral and host proteases to generate four structural proteins (Capsid, E^{rns}, E1, and E2) and seven non-structural proteins (N^{pro}, p7, NS2-3, NS4A-B, and NS5A-B) (Figure 1.1) (Neill, 2013). The E1 and E2 glycoproteins as heterodimers form the outer envelope of BVDV whereby, the E2 glycoprotein acts as viral receptor for host cell entry (Neill, 2013). CD46, a complement inhibition receptor expressed by most nucleated cells, is the proposed host cell receptor for BVDV (Merwaiss et al., 2019). The BVDV genome is packaged inside the virus with the capsid protein whereas the non-structural proteins such as p7, NS2-3, NS4A-B, and NS5A-B are involved in proteolytic cleavage of viral polyprotein and viral replication (Neill, 2013). During BVDV infection, the E^{rns} RNase and N^{pro} autoprotease inhibit Type 1 interferon (IFN) responses by degrading the viral RNA and interferon regulatory factor-3 (IRF-3), respectively (Darweesh, Rajput, Braun, Rohila, & Chase, 2018; Meyers et al., 2007). Hence, BVDV is an immunosuppressive virus that engenders opportunistic secondary infections and therefore, bovine respiratory disease (BRD) in the infected cattle (Ackermann, Derscheid, & Roth, 2010; J. Ridpath, 2010). The BVDV is highly virulent and is usually disseminated within herds by the persistently infected (PI) animals (Chernick et al., 2018; Fulton, 2013). In cattle, BVDV gains entry by ingestion or inhalation where it infects

gastrointestinal, respiratory and reproductive tracts, and causes a range of symptoms such as fever, diarrhea, pneumonia, congenital defects and abortion (J. Ridpath, 2010). Due to high morbidity and mortality rates among BVDV infected cattle, dairy and beef industries incur significant financial damages (Givens & Newcomer, 2015; Johnson & Pendell, 2017).

There are two genotypes of BVDV, BVDV-1 and -2, and currently, there are twenty-one BVDV-1 (BVDV-1a to -1u) and four BVDV-2 (BVDV-2a to -2d) sub-genotypes (Kalaycioglu, 2007; Yesilbag, Alpay, & Becher, 2017). Some BVDV strains have lytic effect on the infected cells hence, BVDV strains are also identified as either cytopathic (cp) or non-cytopathic (ncp) strains (J. F. Ridpath, 2005). Thus, BVDV is an extremely diverse pathogen (J. F. Ridpath, 2005; Yesilbag et al., 2017).

1.2 Current BVDV Vaccines

BVDV vaccines were first generated almost sixty years ago when attenuation of BVDV-1a Oregon C24V strain was achieved (Coggins L Fau - Gillespie et al.; van Oirschot, 1999). Since then, modified-live virus (MLV) and killed virus (KV) vaccines from commercial vendors have been available (Griebel, 2015; Newcomer, Chamorro, & Walz, 2017; Wittum, 2001). Vaccination in conjunction with biosecurity measures such as identification and elimination of PI animals are the major control strategies against BVDV in North America and some European countries (Brock, 2004; Scharnbock et al., 2018; Sozzi et al., 2020). Multivalent MLV and KV are formulated to comprise two or more BVDV strains based on prevalent sub-genotype(s) (Fulton, Cook, Payton, Burge, & Step, 2020; Sozzi et al., 2020). Together, the diversity of endemic strains and the strain composition of available vaccines impact the overall efficacy of current BVDV vaccines (Fulton et al., 2020; J. F. Ridpath, 2005; Sozzi et al., 2020). The MLV and KV vaccines are administered

subcutaneously in cattle to prevent the birth of PI calves, reduce viremia, alleviate morbidity, and protect against BRD (Newcomer et al., 2017).

1.3 Immune Responses Elicited by the BVDV Vaccines

Although MLV and KV vaccines offer protection, they differ markedly in terms of efficacy and vaccine-induced immune responses (Griebel, 2015; Reber et al., 2006). The MLV vaccines, on one hand, confers protection by rapidly generating BVDV neutralizing antibodies, and BVDV-specific IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells in the vaccinated animals (Bittar et al., 2020; Janice J. Endsley, Roth, Ridpath, & Neill, 2003; Zimmerman, Boots, Valli, & Chase, 2006). The KV vaccines, on the other hand, requires formulation in a strong adjuvant such as Quil A for induction of neutralizing antibody and $CD4^+$ T cell responses against BVDV (R. Platt, Coutu, Meinert, & Roth, 2008; P. H. Walz et al., 2018). The antibody and T cell responses induced by MLV vaccines are driven towards the structural as well as non-structural BVDV antigens, whereas KV vaccine-induced immune responses are directed solely against structural BVDV antigens (Griebel, 2015). Notably, induction of BVDV-specific cytotoxic $CD8^+$ T lymphocyte (CTL) responses by either MLV or KV vaccines have not been demonstrated. Since PI animals act as BVDV reservoirs, one of the major uses of BVDV vaccine is for the prevention of PI in breeding cattle and therefore, the birth of PI calves (Fulton, 2013; P. H. Walz et al., 2018; Xue, Mattick, & Smith, 2011). When it comes to providing fetal protection in pregnant dams against BVDV, the MLV vaccines perform better than the KV vaccines (Fairbanks, Rinehart, Ohnesorge, Loughin, & Chase, 2004; P. H. Walz et al., 2018; Xue et al., 2011; Zimmer et al., 2002).

High titers of cross-neutralizing antibodies (1:256 to 1:1024) are typically detected in calves against homologous BVDV sub-genotypes, but sub-optimal to low levels (1:4 to 1:128) of cross-

neutralizing antibodies against heterologous sub-genotypes are also generated (Fulton et al., 1997). This indicates that diverse BVDV strains have unique as well as shared neutralizing epitopes (Botton, da-Silva, Brum, Weiblen, & Flores, 1998). Due to the antigenic diversity, commercial BVDV vaccines in the United States are often bivalent (containing BVDV-1a, and -2a strains) or trivalent (containing BVDV-1a, -1b, and -2a strains) (P. H. Walz et al., 2020). In vaccinated cattle, these vaccines generate high titers of neutralizing antibodies and are efficacious against the homologous strains (Fulton et al., 1997; Sozzi et al., 2020). Depending on the strain composition, commercial BVDV vaccines also induce cross-neutralizing antibodies against heterologous strains (Fulton et al., 2020; Xue, Mattick, Smith, Umbaugh, & Trigo, 2010).

1.4 Limitations Associated with the Current BVDV Vaccines

In spite of having access to licensed vaccines, some countries prefer biosecurity control measures over vaccination because BVDV vaccines are not 100% efficacious (Moennig, Houe, & Lindberg, 2005; Scharnbock et al., 2018; Ståhl & Alenius, 2012). Notably, several European countries, such as Austria, Denmark, Finland, Sweden and Norway, do not allow BVDV vaccination (Moennig et al., 2005; Scharnbock et al., 2018). The combination of BVDV strains in the current vaccines limits their coverage and efficacy against heterogeneous strains (Fulton et al., 2020; Newcomer et al., 2017). While there is a lack of evidence for a direct link between BVDV vaccine strains and the circulating strains in an endemic region, in the United States, most of the commercial vaccines only include BVDV-1a and -2a strains and as a result, BVDV-1b has emerged as the most prevalent sub-genotype (Fulton et al., 1997; Julia F. Ridpath et al., 2011; Rodning et al., 2010). Usually, vaccines induce neutralizing antibodies against heterologous strains from an included sub-genotype, however, this does not guarantee complete protection (Downey-Slinker, Ridpath, Sawyer, Skow, & Herring, 2016). This gap in the vaccine-induced immunity and efficacy can be

attributed to the high antigenic as well as genetic diversity within a sub-genotype (Pecora et al., 2014). In addition, with the recent emergence of diverse strains from BVDV-2b and -2c sub-genotypes in the United States, it is clear that the current vaccines are unreliable and will likely fail against the newer emerging strains (Fulton et al., 2020; Neill et al., 2019).

BVDV vaccines are also associated with a variety of safety issues (J. F. Ridpath, 2013). The MLV vaccine can be contaminated with adventitious wild-type virus leading to inadvertent spread of BVDV within the vaccinated herds (Palomares, Marley, Givens, Gallardo, & Brock, 2013). Besides the risk of contamination, the MLV vaccine, like the wild-type BVDV, can be immunosuppressive and cause post-vaccination mucosal disease (Chase, Hurley, & Reber, 2008; J. F. Ridpath, 2013). The MLV vaccine has been reported to infect reproductive organs where BVDV antigen from a cytopathic strain was detected in ovaries for as long as 30 days post-vaccination which raises the concern for reduced fertility in the vaccinated heifers (Grooms, Brock, & Ward, 1998). Moreover, similar to the wild-type BVDV, the MLV can infect fetus by crossing placenta and is shed by the vaccinated animals, thus contaminating the environment (Kelling, 2004; van Oirschot, 1999).

The BVDV viruses in MLV and KV vaccines, are traditionally grown in Madin-Darby Bovine Kidney (MDBK) cells. Because of the use of this production system, BVDV vaccines can contain bioprocess impurities originating from MDBK cells such as bovine cell antigens including major histocompatibility complex (MHC) I molecules (Foucras et al., 2011). Hence, dams vaccinated with a KV vaccine formulated in a strong adjuvant can develop alloantibodies against bovine MHC I molecules (Assad, 2012; Deutskens et al., 2011; Foucras et al., 2011). When the maternal alloantibodies induced by the vaccines are transmitted to young calves via colostrum, they have high chances of developing adverse reactions involving spontaneous bleeding and severe anemia with bone

marrow aplasia (Assad, 2012; Bell, 2013; Friedrich et al., 2011). This fatal hemorrhagic syndrome is known as bovine neonatal pancytopenia (BNP) and can be reproduced in healthy calves by transferring sera containing the alloantibodies (Foucras et al., 2011; Friedrich et al., 2011). A commercial BVDV vaccine was recalled from the European market after several cases of BNP were reportedly linked to a KV vaccine formulated in Quil A adjuvant (Benedictus, Rutten, & Koets, 2016).

1.5 BVDV Antigen Targets

During infection or vaccination, antibodies are raised against BVDV E^{rns}, E1, E2 and NS2-3 antigens (Steven R. Bolin, 1993). However, the E2 antigen is the only protective immunogen that contains neutralizing epitopes (Table 1.1) (Dirk Deregt, van Rijn, Wiens, & van den Hurk, 1998). The N^{pro}, E^{rns}, E2, and NS2-3 antigens also elicit $CD4^+$ T cell responses which help in conferring protection against BVDV infection (Table 1.1) (Trevor Collen & Morrison, 2000). Depletion of bovine $CD4^+$ T cells delays the resolution of acute BVDV infection in cattle (Howard, 1992). Importantly, defined MHC *DR*-restricted T cell epitopes have been identified from the conserved regions of the E2 and NS2-3 antigens (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). It is worth noting that the immune responses, specifically $CD4^+$ T cells and CTLs, elicited by the E1, NS4A-B, and NS5A-B antigens haven't been evaluated (Table 1.1). In *Flaviviruses*, such as Hepatitis C virus (HCV), Zika virus and Yellow Fever virus (YFV), CTL responses generated by the structural and non-structural antigens confer protection (Ahlén et al., 2005; Bullard, Corder, Gorman, Diamond, & Weaver, 2018; Co, Terajima, Cruz, Ennis, & Rothman, 2002; Wen, Tang, et al., 2017). CTLs developed in cattle during BVDV infection recognize and kill autologous BVDV-infected cells, but the specific CTL determinants within the BVDV polyprotein are unknown (Table 1.1) (Beer, 1997). Since the current vaccines are poorly efficacious and unsafe, there has been an ongoing effort to develop the next generation of BVDV vaccines that are safer and better at inducing neutralizing

antibody and protective T cell immunity. Over the years, several candidate BVDV vaccines such as recombinant subunit, DNA plasmid, viral-vectored, chimeric, and mutant virus vaccines have been successfully developed and tested in cattle.

1.6 Recombinant BVDV E2 Subunit Vaccines

The subunit vaccine platform is the safest platform for developing a novel vaccine (Moyle & Toth, 2013). Recombinant E2 antigen, when mixed with an adjuvant is highly immunogenic and elicits high titers of neutralizing antibodies against its cognate BVDV strain (Toth, Nettleton, & McCrae, 1999). The soluble ectodomain of E2 antigen can be produced using different expression platforms (Bhuyan et al., 2018; Toth et al., 1999). Monovalent E2-based subunit vaccines generate BVDV neutralizing antibodies, however, the level of protection conferred in the vaccinated cattle varies significantly depending on the expression platform used to generate the E2 antigen (Chimeno Zoth et al., 2007; Pecora et al., 2012). While the E2 antigen produced using insect cells induces BVDV neutralizing antibody responses, it doesn't confer complete protection (S. R. Bolin & Ridpath, 1996; Chimeno Zoth et al., 2007). Interestingly, mammalian-expressed BVDV E2 antigen (expressed in HEK293, MDBK, or CHO cells) confers protection at a dose as low as 5 µg per calf (Pecora et al., 2012; Sadat, Snider, Garg, Brownlie, & van Drunen Littel-van den Hurk, 2017; C. Thomas, Young, Heaney, Collins, & Brownlie, 2009). The E2 subunit vaccine produced in mammalian cells is superior to the insect cell-expressed E2 antigen likely due to the difference in the post-translation modification (C. Thomas et al., 2009).

An acute BVDV infection is resolved by neutralizing antibody response, which can be enhanced with $CD4^+$ T cell help, and CTLs that clear the BVDV-infected cells (Chase, 2013). A formulation of mammalian cell-expressed recombinant E2 antigen in an adjuvant containing a toll-like receptor (TLR)

3 agonist and a Th1/Th2 enhancer induced BVDV neutralizing antibody, E2-specific $CD4^+$ and $CD8^+$ T cell responses (Snider, Garg, Brownlie, van den Hurk, & Hurk, 2014). Following challenge with a virulent BVDV-2 strain, the elicited Th1 and Th2 immune responses prevented viremia in the vaccinated animals (Sadat et al., 2017). Similarly, a biomimetic particle containing encapsulated NS3 antigen and a TLR 3 agonist with E2 antigen displayed on the surface was generated as a prototype BVDV vaccine and compared to a commercial KV vaccine (Riitho et al., 2017). The particles mimicking wild-type BVDV induced antibody and T cell responses in cattle. However, upon challenge, it did not control viremia as well as the KV vaccine, perhaps because non-mammalian systems were used to produce the BVDV antigens included in the particles (Riitho et al., 2017).

In another recombinant subunit vaccine, the BVDV E2 antigen was fused with a single-chain antibody that binds an invariant epitope on the β-chain of the MHC II DR molecule (Perez Aguirreburualde et al., 2013). The chimeric E2 antigen was designed to target MHC II-expressing antigen presenting cells (APCs) to stimulate $CD4^+$ T cells and enhance the antibody response. The multivalent MHC II-targeted vaccine containing E2 antigens from BVDV-1a, -1b, and -2a strains, elicited high neutralizing antibody titers against the three cognate strains (Bellido et al., 2020; Pecora et al., 2015). When the vaccinated animals (n=4) were separately challenged with a heterologous BVDV-1b strain (n=2) and a BVDV-2b strain (n=2), the multivalent vaccine conferred complete and partial protection, respectively (Pecora et al., 2015). In 2017, this recombinant vaccine was approved for field application in Argentina and hence, it is the first licensed BVDV subunit vaccine (Bellido et al., 2020). This commercial subunit vaccine produced at a large scale in insect cells has an enhanced immunogenicity and was 100% protective against one of the two tested BVDV strains (Bellido et al., 2020; Pecora et al., 2015). However, the breadth of its protective efficacy against more diverse BVDV strains in a larger number of animals remains to be determined.

1.7 DNA-based BVDV Vaccines

Earlier, it was observed that a DNA plasmid encoding the E2 antigen from a BVDV-1a strain partially protected calves against a BVDV-1b strain (Harpin, Hurley, Mbikay, Talbot, & Elazhary, 1999). DNA vaccines elicit strong immune responses in small animals such as mice or guinea pigs, but in large animals, they are usually less potent (Cui, 2005). Hence, to improve their potency different approaches were tested, such as DNA vaccine delivery via electroporation or inclusion of a DNA plasmid encoding either interleukin 2 (IL-2) or granulocyte macrophage colony stimulating factor (GM-CSF), which led to reduced viremia in the vaccinated cattle after BVDV challenge (Nobiron, 2003; van Drunen Littel-van den Hurk et al., 2010). Complete protection was observed against a virulent BVDV challenge, when calves were primed with the DNA plasmid encoding E2 and then boosted with the recombinant E2 antigen (Liang et al., 2008). It has been shown that calves immunized with DNA plasmids encoding a Retinoic acid-inducible gene I (RIG I) agonist along with E2 and NS3 antigens from BVDV-1a developed cross-neutralizing antibodies against BVDV-1a strains (LM, Thomas, Luke, J, & Brownlie, 2015). In recent years, molecular vaccine technology has become more advanced and is now a popular platform because of high scalability (Liu, 2019). Thus, an efficacious DNA- or RNA-based BVDV vaccine is no longer unattainable.

1.8 Live viral vectored BVDV Vaccines

Recombinant viral vectors that express BVDV antigen(s) generate strong BVDV-specific humoral and cellular immune responses in vaccinated animals (Lokhandwala et al., 2017; Rosas et al., 2007). A recombinant equine herpesvirus-1 (EHV-1) encoding BVDV-1b Capsid, E^{ms}, E1, and E2 antigens induced neutralizing antibody responses and following BVDV-1b challenge, reduced viremia in peripheral blood and viral shedding in the nasal secretion of the vaccinated cattle (Rosas et al., 2007).

Similarly, intranasal immunization of cattle with a recombinant bovine herpesvirus-4 (BoHV-4) [a viral vector developed from the avirulent and non-oncogenic BoHV-4] that expressed BVDV E2 antigen induced BVDV neutralizing antibodies (Williams et al., 2019). Venezuelan Equine Encephalitis virus-derived replicon was also tested in cattle as a delivery vector for BVDV-1b E2 antigen and some level of protection was observed against the homologous BVDV-1b challenge (Loy et al., 2013). The EHV-1- and alphavirus-vectored BVDV-1b E2 antigen also elicited BVDV-2 cross-neutralizing antibody responses. However, BVDV-specific T cell responses induced by these prototype vaccines were not evaluated. High levels of protective BVDV-specific $CD4^+$ T cell responses along with broad BVDV-1 and -2 neutralizing antibody titers were developed in calves vaccinated with a cocktail of recombinant adenoviruses expressing mosaic N^{pro}, E2, and NS2-3 antigens from BVDV-1 and -2 (Lokhandwala et al., 2017). The adenovirus-vectored BVDV mosaic antigens performed better than a commercial MLV vaccine and completely protected the vaccinated calves against a virulent BVDV-2 challenge (Lokhandwala et al., 2017). These live-vectored prototype BVDV vaccines that have been shown to reliably induce cross-protective immunity can be viable alternatives to MLV vaccines for protecting herds against the diverse BVDV strains.

1.9 Chimeric BVDV Vaccines

Since BVDV is a component of Bovine Respiratory Disease complex, a chimeric vaccine against more than one bovine viral pathogen is an ideal candidate for mitigation of BRD in herds (Williams et al., 2019). Vaccines containing BVDV and BoHV-1 components have been developed to confer dual protection against the two co-circulating immunosuppressive BRDC pathogens (Chowdhury et al., 2021; Williams et al., 2019). When tested in cattle, a chimeric BoHV-1 vectored BVDV-2 E2 vaccine induced BVDV-1 and -2 cross-reactive T cells as well as BVDV-2 and BoHV-1 neutralizing antibodies that were recalled during BVDV-2 challenge (Chowdhury et al., 2021). The BVDV-1 and

-2 strains are often considered as two distinct species and therefore, a dual vaccine was constructed where E1 and E2 antigens from a BVDV-2 strain were inserted into the backbone of a BVDV-1b strain that contained a deletion within N^{pro} for attenuation, and a substituted heterologous E^{rns} from Bungowannah virus (a *Pestivirus*) for differentiation of infected from vaccinated animals (DIVA) (Koethe et al., 2020). The chimeric dual vaccine was effective against BVDV-1 and -2 infection in the vaccinated calves (Koethe et al., 2020). Experimental chimeric viral vaccines that are DIVA compatible have successfully been developed, but their efficacy and safety need to be assessed before they can be considered for field application (Luo et al., 2012).

1.10 Mutant BVDV Virus Vaccines

An attenuated BVDV containing defined mutations is considered to be a safer MLV vaccine compared to a spontaneously mutated attenuated BVDV. Wild-type BVDV strains were genetically modified via deletions within 5' untranslated region (UTR), N^{pro}, Capsid, E^{rns}, and E1 genes to generate attenuated BVDV mutants (Makoschey et al., 2004; Meyers et al., 2007; Reimann, Semmler, & Beer, 2007; Zemke, Konig, Mischkale, Reimann, & Beer, 2010). Deletion mutant-based live BVDV vaccines have been demonstrated to be stable, highly immunogenic and efficacious against multiple BVDV strains in cattle (Makoschey et al., 2004; Zemke et al., 2010). Genetically engineered BVDV can be quality controlled for phenotypic and genetic stability. As a result, a N^{pro} and E^{rns} double mutant BVDV called Bovela was licensed in 2014 for commercial use in Germany (Wernike et al., 2018).

1.11 Conclusion

Commercial MLV and KV vaccines have been unable to control prevalence of the BVDV pathogen in herds for the past 60 years. Studies have consistently demonstrated that the limited protective efficacy and the associated safety concerns are current vaccines' greatest shortcomings.

Hence, BVDV eradication programs don't rely solely on current vaccines. Data from experimental BVDV vaccine studies have provided the much-needed knowledge to develop a contemporary vaccine. Consequently, the development of a novel BVDV vaccine that is capable of safely inducing durable, broadly protective BVDV-specific B cells, $CD4^+$ and $CD8^+$ T cells will improve BVDV management and therefore, cattle productivity.

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Table 1.1 BVDV antigens and their immune responses

BVDV Antigen	Immune Responses			References
	Neutralizing Antibody	$CD4^+$ T cell	Cytotoxic $CD8^+$ T cells	
N^{pro}	-	Yes	Unknown	(Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Lokhandwala et al., 2017)
Capsid	-	Unknown	Unknown	-
E^{rns}	-	Yes	Unknown	(Beer, 1997; Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002)
E1	-	Unknown	Unknown	-
E2	Yes	Yes	Unknown	(Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Dirk Deregt et al., 1998; Lokhandwala et al., 2017)
p7	-	Unknown	Unknown	-
NS2-3	-	Yes	Unknown	(Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; LM et al., 2015; Lokhandwala et al., 2017; Riitho et al., 2017)
NS4A	-	Unknown	Unknown	-
NS4B	-	Unknown	Unknown	-
NS5A	-	Unknown	Unknown	-
NS5B	-	Unknown	Unknown	-

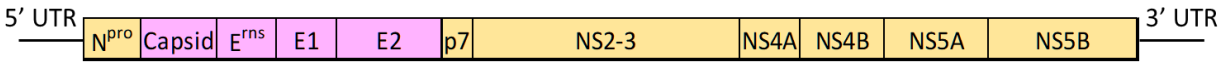

Figure 1.1 BVDV Genome Organization. Single-stranded RNA BVDV genome encodes for four structural proteins: Capsid protein, E^{rns}, E1 and E2 glycoproteins, and seven non-structural proteins: N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A and NS5B. Viral genome is flanked with 5' and 3' untranslated regions (UTR) that are involved in RNA replication and translation (Neill, 2013).

Chapter 2- Mosaic Bovine Viral Diarrhea Virus Antigens Elicit Cross-protective Immunity in Calves

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Abstract

Bovine Viral Diarrhea Virus (BVDV) is an important pathogen that plays a significant role in initiating Bovine Respiratory Disease Complex (BRDC) in cattle. The disease causes a billion dollar losses globally due to high calf mortality and increased morbidity leading to heavy use of antibiotics. Current commercial vaccines provide limited cross-protection with several drawbacks such as safety, immunosuppression, potential reversion to virulence, and induction of neonatal pancytopenia. This study evaluates two prototype vaccines containing multiple rationally designed recombinant mosaic BVDV antigens for their potential to confer cross-protection against diverse BVDV strains. Genes encoding three novel mosaic antigens, designated $E2^{123}$, NS2-3^{1}, and NS2-3^{2}, were designed *in silico* and expressed in mammalian cells for the formulation of a prototype protein-based vaccine. The mosaic antigens contain highly conserved protective epitopes from BVDV-1a, -1b, and -2, and included unique neutralizing epitopes from disparate strains to broaden coverage. We tested immunogenicity and protective efficacy of Expi293^{TM}-expressed mosaic antigens (293F-$E2^{123}$, 293F-NS2-3^{1}, and 293F-NS2-3^{2}), and baculovirus-expressed $E2^{123}$ (Bac-$E2^{123}$) mosaic antigen in calves. The Expi293^{TM}-expressed antigen cocktail induced robust BVDV-specific cross-reactive IFN-γ responses, broadly neutralizing antibodies, and following challenge with a BVDV-1b strain, the calves had significantly ($p < 0.05$) reduced viremia and clinical BVD disease compared to the calves vaccinated with a commercial killed vaccine. The Bac-$E2^{123}$ antigen was not as effective as the Expi293^{TM}-expressed antigen cocktail, but it protected calves from BVD disease better than the commercial killed vaccine. The findings support feasibility for development of a broadly protective subunit BVDV vaccine for safe and effective management of BRD.

2.1 Introduction

Bovine Viral Diarrhea Virus (BVDV) is a single-stranded RNA virus from the genus *Pestivirus* in the family *Flaviviridae* with a 12.5 kb genome that encodes N^{pro}; capsid; the E^{rns}, E1, and E2 glycoproteins; NS2-3; NS4A-B; and NS5A-B proteins (Chernick & van der Meer, 2017; Neill, 2013). The BVDV is grouped into antigenically distinct genotypes 1 and 2, and cytopathic (CP) and non-cytopathic (NCP) biotypes based on the effect of virus on infected cell cultures (J. F. Ridpath, 2005). Both genotypes are further divided into various sub-genotypes and in the United States BVDV-1b is the predominant sub-genotype (Julia F. Ridpath et al., 2011). The BVDV is one of the major players in causing Bovine Respiratory Disease Complex (BRDC) in cattle worldwide. The damage caused to the cattle industry by the disease every year is estimated to be worth more than a billion dollar due to high calf mortality, increased treatment costs and production loses (Smith, 2009). In cattle, BVDV infection can be acute or persistent with a range of clinical symptoms such as fever, diarrhea, pneumonia, immunosuppression, congenital malformation and abortion (Saliki, Fulton, Hull, & Dubovi, 1997; Smith, 2009). Persistently infected (PI) cattle are chronic virus shedders and therefore, if not diagnosed and culled, they are the main source of BVDV within a herd (Newcomer et al., 2017).

Currently, two types of commercial BVDV vaccines are available in the United States, modified-live virus [MLV] and killed virus [KV] vaccines (Wittum). Although majority of the commercial vaccines contain representative BVDV-1 and -2 strains, cross-protective efficacy of the MLV and KV vaccines against heterologous BVDV strains is still limited (Fulton et al., 2020; Newcomer et al., 2017). The MLV vaccines can confer protection after a single vaccination by inducing neutralizing antibody along with $CD4^+$ T cell and $CD8^+$ cytotoxic T lymphocyte [CTL] responses (Griebel, 2015; J. F. Ridpath, 2013; Xue et al., 2011; Xue et al., 2010). However, there are safety

concerns associated with MLV such as immunosuppression, wild-type BVDV contamination of MLV vaccine and potential reversion to virulence (Palomares et al., 2013; J. F. Ridpath, 2013). The KV vaccines on the other hand do not offer the same level of protective immunity as MLV vaccines without booster dose and require strong adjuvants which may lead to induction of bovine neonatal pancytopenia (Deutskens et al., 2011; Janice J. Endsley et al., 2003; Reber et al., 2006; Rodning et al., 2010).

BVDV is widespread in the United States and Canada, where diverse strains circulate in cattle (Chernick & van der Meer, 2017; Neill et al., 2019; J. F. Ridpath, 2005). Despite BVDV vaccination coverage of nearly 80% of the cattle population, prevalence of PI cattle over the years in North America has remained constant implying that the current vaccines are inefficient in eliminating and controlling BVDV infection (Scharnbock et al., 2018). Limited strain composition of available vaccines has not kept pace with new genetically and antigenically distinct sub-genotypes arising and circulating in cattle herds (Fulton et al., 2020; Neill et al., 2019). Thus, there is a need for a more coherent and contemporary proactive vaccine approach to eradicate BVDV since it is evident that the traditional vaccines have been inadequate in providing broad protection.

BVDV-specific $CD4^+$ and $CD8^+$ T cells have been detected in the infected and protected animals (Janice J. Endsley et al., 2003; R. Platt, Kesl, Guidarini, Wang, & Roth, 2017; Sadat et al., 2017). Apart from neutralizing antibodies, $CD4^+$ T cells are critical for enhancing the BVDV-specific antibody response and for clearance of infected cells, whereas $CD8^+$ T cells can be directly cytotoxic for BVDV-infected cells (Beer, 1997; Chase, 2013; T. Collen, Douglas, Paton, Zhang, & Morrison, 2000; Trevor Collen & Morrison, 2000). The BVDV E2 and NS2/3 are immunodominant antigens that drive the majority of neutralizing antibody and T cell responses and hence, are frequently selected targets for BVDV subunit vaccine development (LM et al., 2015; Lokhandwala et al.,

2017; J. F. Ridpath, 2013; Riitho et al., 2017). Experimental subunit BVDV vaccines involving recombinant E2 glycoprotein can provide some protection in cattle by limiting pyrexia, weight loss, leucopenia and viremia (Pecora et al., 2012; Riitho et al., 2017; Sadat et al., 2017; Snider et al., 2014; C. Thomas et al., 2009). However, the protective immunity generated by the monovalent E2 subunit vaccines are mostly against homologous BVDV strains (Riitho et al., 2017; Sadat et al., 2017; Snider et al., 2014; C. Thomas et al., 2009). In contrast, a multivalent E2 subunit vaccine can provide some level of cross-protection against BVDV-1 and -2 strains (Pecora et al., 2015). The focus of vaccine development efforts has been chiefly on inducing neutralizing antibody response by E2 glycoprotein (Pecora et al., 2015; Sadat et al., 2017; C. Thomas et al., 2009). Some of the latest reports have highlighted the importance of inclusion of NS2 and NS3 in vaccine for induction of protective BVDV-specific T cell responses (LM et al., 2015; Lokhandwala et al., 2017; Riitho et al., 2017). MHC *DR*-restricted T cell epitopes identified from the highly conserved regions of E2 and NS3 are suitable for inclusion in a subunit vaccine (Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). Given the heterogeneity of protective antigens among diverse BVDV isolate, mosaic antigens designed based on consensus protein sequences from circulating strains and addition of unique epitopes from disparate strains is likely to result in a chimeric antigen capable of eliciting broad protection (Barouch et al., 2013; Lokhandwala et al., 2017; Yusim et al., 2010).

Contemporary computational techniques were used to design three novel mosaic polypeptides consisting of structural and non-structural antigens that are well-conserved among BVDV genotypes along with an array of well characterized epitopes. These epitopes include defined protective neutralizing epitopes, defined and predicted IFN-γ-inducing $CD4^+$ T cell and CTL determinants highly conserved across BVDV-1a, -1b, and -2 strains. In addition, unique strain-

specific protective epitopes from disparate BVDV strains were included in order to broaden coverage.

In a previous proof-of-concept study, we found that an adenovirus-vectored prototype vaccine conferred better protection than a commercial multivalent MLV vaccine upon challenge with a BVDV-2a strain (Lokhandwala et al., 2017). In the antigen validation study, three novel mosaic antigens, designated $E2^{123}$, NS2-3^{1}, and NS2-3^{2} were designed for immunization of calves. The antigens expressed in replication-deficient adenoviruses elicited significantly higher BVDV-specific antibody and T cell responses compared to a commercial MLV vaccine (Lokhandwala et al., 2017). However, since adenovirus vector is a BSL-2 agent with associated safety concerns, we developed recombinant protein-based prototype vaccines as a safer alternative for eliciting cross-protective immune responses in cattle. Two experimental vaccine formulations, one containing mammalian-expressed $E2^{123}$, NS2-3^{1}, and NS2-3^{2} mosaic antigens; and another one containing $E2^{123}$ antigen expressed in insect cells were evaluated for their immunogenicity as well as protective efficacy. The immunized calves were challenged with a BVDV-1b [CA401186a] strain that is prevalent in United States and is considered to be a preferable BVDV-1 strain for vaccine efficacy challenge studies (J. F. Ridpath, Neill, & Peterhans, 2007). The CA401186a is a non-cytopathic BVDV-1b strain which persistently infects cattle leading to pyrexia and immunosuppression, but it doesn't cause severe gastrointestinal and respiratory disease (Downey-Slinker et al., 2016; J. F. Ridpath et al., 2007). *In vitro* virus neutralization against representative BVDV-1 and -2 strains was used to evaluate potential for broad protection.

2.2 Materials and Methods

2.2.1 Generation of BVDV Mosaic Antigen Expression Constructs

Synthetic genes encoding novel BVDV mosaic antigens were designed and validated as previously described (Lokhandwala et al., 2017). Briefly, previously defined protective B- and T-cell epitopes as well as E2 and NS2-3 polypeptide sequences from BVDV strains whose genome sequences were available were aligned to generate consensus polypeptides (Supplementary Figure 2.1). Where there was no consensus, the most common amino acid was selected and where there was none, a residue from the BVDV-1b strain, the most prevalent isolate, was selected. The data was utilized to design a mosaic chimeric antigen, designated $E2^{123}$, which comprised of consensus sequences of E2 glycoprotein from BVDV-1a, -1b, and -2. In addition, the chimera included unique strain specific neutralizing B cell and T cell epitopes, and a FLAG tag.

Two mosaic chimeric antigens, NS2-3^1 and NS2-3^2, representing diverse NS2-3 antigen repertoire from BVDV-1 and -2, respectively, were similarly designed with a FLAG tag as described above. The novel mosaic polypeptide sequences were used to generate synthetic genes codon-optimized for protein expression in mammalian cells [GenScript]. A gene encoding an irrelevant antigen, designated TMSP [*Theileria Parva* Modified Sporozoites Protein], was also generated and used as a negative control. Expression and authenticity of the proteins encoded by the synthetic genes were validated using BVDV-specific sera, mAbs, and T cells (Lokhandwala et al., 2017).

2.2.2 Expression and Purification of Recombinant BVDV Mosaic Antigens

The three *flag*-tagged synthetic genes encoding $E2^{123}$, NS2-3^1, and NS2-3^2 mosaic antigens were subcloned into pcDNA3.1+ mammalian expression vector (Invitrogen™), which had been

modified by addition of a CD5 secretory signal sequence (C. P. Edwards & Aruffo, 1993). Positive clones for each construct were identified by PCR screening, sequence-verified and subsequently used for recombinant protein expression in the mammalian Expi293™ Expression System (Gibco) as per manufacturer's protocol and as previously described (Jain et al., 2017). Briefly, Expi293 cell suspension cultures were transfected with pcDNA3.1+ constructs expressing the mosaic antigens whereby, cell lysate and culture supernatant were combined for purification of $E2^{123}$, whereas NS2-3^{1} and NS2-3^{2} were purified from cell lysate. Anti-FLAG M2 affinity gel (Sigma, A2220) was used for affinity purification of the recombinant mosaic antigens. The gene encoding TMSP was used to similarly generate a FLAG-tagged negative antigen.

The synthetic gene encoding the $E2^{123}$ mosaic antigen was also subcloned into pFastBac™/HBM-TOPO® vector for baculovirus protein expression [Bac-to-Bac® HBM TOPO® Secreted Expression System, Invitrogen™]. Selected positive clones were verified by DNA sequencing and then used to assemble recombinant baculovirus for recombinant protein production using High Five™ insect cell suspension cultures according to manufacturer's protocol. The antigen was affinity purified from High Five™ cell lysate and culture supernatant using anti-FLAG M2 affinity gel as described above.

2.2.3 Validation of Purified BVDV Mosaic Antigens

The affinity purified antigens were quality control validated by SDS-PAGE and Western Blotting. The baculovirus-expressed $E2^{123}$, Expi293™-expressed $E2^{123}$, and NS2-3^{1} antigens were resolved in a NuPAGE® Bis-Tris gel (Invitrogen™, NP0322) by denaturing electrophoresis. The gel was then stained with Imperial™ Protein Stain (Invitrogen™, 24615) for visualization of the protein bands. The antigens were resolved on gel as above and transferred to Amersham™ Protran™

0.45µm Nitrocellulose Membrane (GE Healthcare Life Science, 10600114) by electrophoresis for Western Blot analysis. After transfer, the blot was incubated in blocking buffer, 10 % non-fat dry milk in TBST, overnight at 4°C, and then probed for 1 hr. with anti-BVDV polyclonal sera (Porcine origin, Cat. #210-70-BVD, VMRD, Inc) diluted at 1:3000 in blocking buffer. Following 3X washes with TBST, the blot was incubated with peroxidase-conjugated goat anti-porcine IgG (Jackson ImmunoResearch, Cat. #114-035-003) diluted at 1:5000 in blocking buffer. SuperSignal West Pico PLUS substrate (Thermo Scientific, Prod #34577) was used for protein band visualization on immunoblot by chemiluminescence.

2.2.4 Immunization of Calves

Twenty, four-month old Holstein calves were determined as BVDV sero-negative using the standard serum neutralization assay against BVDV-1 and -2 [Kansas State Veterinary Diagnostic Lab.]. The calves were then randomly divided into four groups A-D (n=5) as shown in Table 2.1. Following acclimatization for 10 days, the treatment and control calves were primed at day 0 and then boosted on day 21 with doses as shown in Table 2.1. Each calf in group A was immunized intramuscularly in the neck area with a cocktail of the Expi293^{TM}-expressed E2^{123}, NS2-3^{1}, and NS2-3^{2} formulated in MONTANIDE^{TM} ISA 201 VG adjuvant (Seppic). Similarly, calves in group B received the baculovirus-expressed E2^{123} formulated in the same adjuvant. Calves in group C served as positive controls and were immunized with a commercial BVDV Killed Vaccine [Vira Shield^{TM} 6; Disclaimer: The commercial vaccine was used off label as booster dose was administered at day 21 instead of at day 28-35], whereas calves in group D served as negative controls and were immunized intramuscularly with an irrelevant antigen, TMSP formulated in the MONTANIDE^{TM} ISA 201 VG adjuvant. During immunization, calves were housed together in outdoor pens.

2.2.5 Evaluation of BVDV-specific IFN-γ Responses

Antigen-specific IFN-γ responses by peripheral blood mononuclear cells (PBMCs) isolated from blood collected at two weeks post-prime and one week post-boost were evaluated by Enzyme-linked immunospot (ELISPOT) assay using Bovine IFN-γ ELISpot^{BASIC} (ALP) kit (Mabtech; product code: 3119-2A) as per manufacturer's instruction and as previously described (Njongmeta et al., 2012). Briefly, 0.125×10^6 PBMCs were seeded in triplicate wells of MultiScreen-IP plates (MilliporeSigma™ MAIPS4510) and incubated at 37°C for 48 hr. with 2.5 µg/ml of affinity-purified mosaic antigens, defined BVDV $CD4^+$ T cell epitope peptides, representative whole heat-killed BVDV-1b (CA0401186a, TGAC), or BVDV-2a (A125, 1373) strains in a final volume of 100 µl complete RPMI 1640 medium. The positive control was 2.5 µg/ml ConA, whereas medium alone was used as a negative control. The spots were counted with an ELISpot reader [Cellular Technology Limited (CTL) ImmunoSpot® S6 Analyzer] and the results were presented as the mean number of IFN-γ^+ spot-forming cells (SFC) per 10^6 PBMCs after background medium counts were deducted.

2.2.6 Evaluation of Antibody Responses

Antigen-specific IgG responses were determined by indirect ELISA using sera from blood collected before immunization, two weeks post-prime, and three weeks post-boost. Briefly, triplicate wells in polystyrene 96-well microplates were coated overnight at 4°C with 100 µl of affinity purified antigens diluted at 5 µg/ml in bicarbonate coating buffer. Expi293^{TM}-expressed mosaic antigens ($E2^{123}$, NS2-3^1, and NS2-3^2) were used to evaluate antibody responses in the calves immunized with the Expi293^{TM}-expressed antigen cocktail, the Vira Shield^{TM} 6 vaccine, and the negative controls, whereas baculovirus-expressed $E2^{123}$ antigen was used to test sera from

calves immunized with the cognate antigen. The plates were washed with PBS containing 0.1% Tween 20 and incubated with 200 µl of 10% sodium caseinate blocking buffer for 1 hr. at 37°C. 100 µl of sera diluted in blocking buffer (1:500 dilution for pre-bleed and post-prime sera, and 1:5000 for post-boost sera) were added in triplicates and then incubated at 37°C for 1 hr. After washing, 100 µl of 1:5000 dilution of peroxidase-conjugated goat anti-bovine IgG (Jackson ImmunoResearch, Cat #101-035-003) was added. Plates were incubated for another hr. at 37°C, washed and then developed with Sure Blue Reserve TMB substrate (KPL, Cat# 53-00-02). 1N Hydrochloric acid was used to stop the reactions and the plates were read at 450 nm in BioTek microplate reader (Synergy H1 Multi-mode reader). Antigen-specific IgG responses in sera from the Vira Shield^{TM} 6 vaccinees and the negative controls were also tested at 1:250 dilution for pre-bleed and post-prime sera, and at 1:2500 for post-boost sera as described above. Antigen-specific IgG responses were presented as mean OD (Optical Density) absorbance for each treatment and control groups.

2.2.7 Virus Neutralization Assays

Sera from blood collected at three weeks post-boost were tested to determine BVDV-1 and -2 neutralizing antibody titers using BVDV-1 strains (BJ, CA401186a, Singer, NADL, and TGAC) and BVDV-2a strains (A125, 890, 1373, 296 C, and 296 NC) as previously described (S. Edwards, 1990; Lokhandwala et al., 2017). Briefly, sera was heat-inactivated at 56° C for 30 min, and 50 µl of each serum was serially diluted two-fold in 96-well microtiter plates using minimum essential media (MEM). 50 µl of stock BVDV virus containing 300 $TCID_{50}$/ml was added to each test well. In each test, a positive control serum was also included. The serum/virus mixture was incubated for 1 hr. at 37°C followed by addition of MDBK cells, and the plates were incubated at 37° C for 72 hr. The cells were monitored daily for signs of CPE in cells exposed to cytopathic strains,

whereas the presence of non-cytopathic virus strains was detected by immuno-peroxidase assay (Fulton et al., 1997). The results were presented as virus neutralization titers (VNT).

2.2.8 Animal Challenge

At day 21 post-boost (day 42 post-prime), all the calves were challenged intranasally with BVDV-1b CA0401186a strain. Each calf received 5 ml of 1×10^6 $TCID_{50}$/ml of the virus in 0.9% saline (J. F. Ridpath et al., 2007). The inoculum [2.5 ml] was delivered in each nostril using LMA® MAD Nasal™ Intranasal Mucosal Atomization Device (Teleflex; Item number: MAD100). The animals were monitored for reaction to the challenge virus and post-challenge rectal temperatures were recorded daily. Challenge study was conducted in ABSL-2 facility where calves were segregated in pens according to their assigned groups (Table 2.1).

2.2.9 Determination of Viremia and WBC Counts

Post-challenge, blood samples were collected in vacutainer tubes containing EDTA on day 0, 2, 3, 6, 9, 13, and 15 for evaluation of viremia and white blood cell (WBC) counts. Blood samples were lysed by freeze-thawing, centrifuged, and the lysate was used for BVDV isolation to determine viral titer by alkaline phosphatase monolayer immunostaining as previously described (Brock, Grooms Dl Fau - Ridpath, Ridpath J Fau - Bolin, & Bolin; Saliki et al., 1997; Paul H. Walz et al., 2001). Briefly, serial 10-fold dilutions of sample lysate were prepared in Dulbecco's modified Eagle medium (DMEM) and 50 µl were added to 96-well plate containing fresh MDBK cells. Following incubation at 37° C for 72 hr., the cells were fixed for staining with anti-BVDV E2 mAb (Cat. # 348, VMRD) and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Cat #115-055-146). BVDV titers in blood samples were reported as the lowest dilution at which the lysate exposed MDBK cells stained positive for BVDV E2. The profile of

CBC in each blood sample was evaluated by HESKA Veterinary Hematology System (RTI LLC, Brookings, SD 57006, USA) with counting parameters set for bovine WBC, HGB, RBC, and PLT. The counts for platelets, WBC, and RBC and WBC morphology were verified microscopically.

2.2.10 Statistical Analysis

The significance of the differences between the treatments and the controls was determined by ordinary one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. Post-immunization, the significance of the differences in BVDV-specific immune responses (IFN-γ responses, IgG responses, and VN titers) were compared among all groups. Post-challenge, mean viral titers for blood viremia were also analyzed by performing comparisons among all groups. However, post-challenge clinical outcomes: mean rectal temperatures and WBCs change ratios were analyzed by performing comparisons between the treatments (293F-Cocktail, Bac-E2^{123}, and Vira Shield^{TM} 6), and the negative control group (TMSP sham treatment) by ordinary one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical analysis was performed using GraphPad Prism 7 (Version 7.04, GraphPad Software, Inc. La Jolla, CA) and a significance level of $p < 0.05$ was used for all analyses.

2.3 Results

2.3.1 Design and Expression of Novel Recombinant BVDV Mosaic Antigens

Three pCDNA3 constructs encoding novel BVDV mosaic antigens [E2^{123}; NS2-3^{1}; and NS2-3^{2}] were used to express recombinant antigens by transient transfection of Expi293F cells (Figure 2.1A). Baculovirus encoding the E2^{123} mosaic polypeptide was also used to generate recombinant protein using High Five™ cells. Affinity-purified recombinant antigens were validated by SDS-

PAGE (Figure 2.1B) and Western Blot using BVDV-1- and -2-specific polyclonal serum (Figure 2.1C).

2.3.2 Mosaic BVDV Antigens induced Strong IFN-γ Responses

Immunogenicity and protective efficacy of immunogens formulated using the recombinant mosaic antigens was evaluated in calves following prime-boost immunization (Table 2.1). After priming, antigen-specific IFN-γ responses were detected in calves immunized with the 293F-expressed antigen cocktail [$E2^{123}$, NS2-3^1, and NS2-3^2] and in the calves immunized with the baculovirus-expressed $E2^{123}$antigen (Figure 2.2A). Notably, the Bac-$E2^{123}$-immunized calves had a significantly higher ($p < 0.05$) post-prime $E2^{123}$-specific IFN-γ response compared to the calves immunized with the 293F-expressed antigen cocktail and the calves immunized with the Vira Shield™ 6 commercial vaccine. Strong IFN-γ response against NS2-3^1 was only detected in the calves immunized with the 293F-expressed antigen cocktail and the response was significantly higher ($p < 0.05$) than the response detected in the calves immunized with the Vira Shield™ 6 vaccine (Figure 2.2A). The calves immunized with the 293F-expressed antigen cocktail also had a high mean NS2-3^2-specific IFN-γ response, but this response was not significantly different from the other treatment groups (Figure 2.2A). Following priming, no $E2^{123}$- and NS2-3^1-specific IFN-γ responses were detected in the calves immunized with the Vira Shield™ 6 vaccine and only 1/5 calf in this group had IFN-γ response against NS2-3^2 (Figure 2.2A).

Boosting expanded antigen-specific IFN-γ responses in the calves immunized with the 293F-expressed antigen cocktail and the calves immunized with the Bac-$E2^{123}$ antigen (Figure 2.2B). The calves in both treatment groups had high levels of $E2^{123}$-specific IFN-γ responses, but only the mean response of the calves immunized with the 293F-expressed antigen cocktail was

significantly higher ($p < 0.05$) than the response detected in the Vira Shield™ 6 and the sham control groups (Figure 2.2B). The calves immunized with the 293F-expressed antigen cocktail also had the strongest NS2-3^1- and NS2-3^2-specific IFN-γ responses (Figure 2.2B). Significantly higher NS2-3^1-specific IFN-γ response was detected in the calves immunized with the 293F-expressed antigen cocktail compared to the responses detected in the calves immunized with the Vira Shield™ 6 vaccine ($p < 0.05$) and the sham treatment ($p < 0.01$) (Figure 2.2B). Boosting with the 293F-expressed antigen cocktail significantly expanded NS2-3^2-specific response compared to the Vira Shield™ 6 vaccine ($p < 0.01$) and the sham treatment ($p < 0.001$) (Figure 2.2B). Overall, post-boost antigen-specific responses were significantly low in the calves immunized with the Vira Shield™ 6 vaccine with only 2/5, 3/5, and 2/5 calves responded to E2^{123}, NS2-3^1, and NS2-3^2, respectively (Figure 2.2B).

IFN-γ responses were also analyzed using a peptide pool containing previously defined BVDV CD4$^+$ T cell epitopes (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002) (Figure 2.2). Calves immunized with the 293F-expressed antigen cocktail and the Bac-E2^{123} exhibited high levels of post-prime CD4$^+$ T cell epitope-specific IFN-γ responses (Figure 2.2A). The Bac-E2^{123} antigen elicited significantly higher epitope-specific responses compared to Vira Shield™ 6 vaccine ($p < 0.05$) and sham treatment ($p < 0.01$) (Figure 2.2A). The epitope-specific IFN-γ responses were evidently amplified post-boost in the calves immunized with either the 293F-expressed antigen cocktail or the Bac-E2^{123} antigen (Figure 2.2B). However, the calves immunized with the 293F-expressed antigen cocktail had significantly higher ($p < 0.05$) response than the other treatment and control groups (Figure 2.2B). Interestingly, post-boost IFN-γ responses against the defined BVDV CD4$^+$ T cell epitopes induced by Vira-Shield™ 6 were

considerably lower, with only 2/5 calves responding, than the responses induced by the prototype vaccines.

2.3.3 Mosaic Antigens Induced Robust Cross-reactive BVDV-specific IFN-γ Responses

The 293F-expressed antigen cocktail and the Bac-E2^{123} antigen, but not the Vira Shield^{TM} 6 vaccine, primed and expanded IFN-γ responses that were recalled in the majority of the vaccinees by representative BVDV-1b and -2a strains (Figure 2.3). Following priming, the Bac-E2^{123} antigen, but not the 293F-expressed antigen cocktail, elicited IFN-γ memory responses that were consistently recalled by representative BVDV-1b strains (CA0401186a and TGAC) and BVDV-2a strains (A125 and 1373) (Figure 2.3A). However, following boosting, the memory responses expanded by the 293F-expressed antigen cocktail and the Bac-E2^{123} antigen, but not the Vira Shield^{TM} 6 vaccine, were consistently recalled by the representative BVDV-1b and -2a strains and in addition, the magnitude of the recall responses were similar (Figure 2.3B). These post-boost responses recalled by the representative BVDV-1b and -2a strains were significantly ($p < 0.05$) higher than the recall responses detected in calves immunized with the Vira Shield^{TM} 6 vaccine (Figure 2.3B). The IFN-γ memory responses induced by the Vira Shield^{TM} 6 vaccine were low and poorly recalled by the representative BVDV-1b and -2a strains whereby, recall responses were only detected in 2/5 for CA0401186a, 1/5 for TGAC, 2/5 for A125, and none for 1373 (Figure 2.3B).

2.3.4 Mosaic Antigens Induced BVDV Cross-neutralizing Antibodies

Immunization of calves with the recombinant antigens induced strong antibody responses that were significantly amplified after boosting (Figure 2.4). After priming, all the calves immunized with the prototype vaccines seroconverted (Figure 2.4A and 2.4B). Both the 293F-expressed

antigen cocktail and Bac-E2^{123} antigen primed high antigen-specific IgG responses, but the mean IgG response induced by Bac-E2^{123} antigen was significantly higher ($p < 0.05$) than the response induced by the Vira Shield^{TM} 6 vaccine and the sham treatment (Figure 2.4B). Post-boost IgG responses recalled in the calves immunized with the 293F-expressed antigen cocktail ($p < 0.01$) and the Bac-E2^{123} antigen ($p < 0.001$) were significantly higher than the responses recalled in calves immunized with the Vira Shield^{TM} 6 vaccine and the sham treatment calves (Figure 2.4C).

Virus neutralizing (VN) antibodies against BVDV-1 strains were detected in the immunized calves three weeks post-boost (Figure 2.5). The 293F-expressed antigen cocktail elicited the highest levels of VN titers (1:128 to 1:8192) against the five BVDV-1 strains that were detected in all the vaccinees (Figure 2.5). However, compared to the other treatment groups, mean VN titer for the 293F-expressed antigen cocktail group was significantly higher ($p < 0.05$) for two BVDV-1b strains, CA0401186a and TGAC (Figure 2.5). The Bac-E2^{123} antigen elicited BVDV-1-specific VN titers (1:8 to 1:256) in most of the vaccinees against BVDV-1a NADL, BVDV-1a Singer, BVDV-1b BJ and BVDV-1b TGAC, but there was no detectable BVDV-1b CA0401186a-specific neutralizing antibody response (Figure 2.5). On the other hand, all calves immunized with the Vira Shield^{TM} 6 vaccine had detectable but low VN titers (1:16 to 1:256) against BVDV-1a Singer and BVDV-1b BJ, the BVDV-1 included in the Vira Shield™ 6 vaccine. Additionally, VN titers (1:8 to 1:32) induced by the Vira Shield^{TM} 6 vaccine against BVDV-1a NADL (2/5 calves) and BVDV-1b TGAC (3/5 calves) were lower compared to the responses induced by the 293F-expressed antigen cocktail and the Bac-E2^{123} antigen (Figure 2.5). The Vira Shield^{TM} 6 vaccine did not induce detectable neutralizing antibody response against BVDV-1b CA0401186a, which was similar to the outcome observed in the calves immunized with the Bac-E2^{123} antigen (Figure

2.5). Altogether, the 293F-expressed antigen cocktail induced broader and consistent VN antibody responses against BVDV-1 strains.

The 293F-expressed antigen cocktail elicited VN antibodies against all five BVDV-2a strains in either 2/5 or 3/5 vaccinees, but the responses were inconsistent and generally low [except two responses against A125 and 890 strains] compared to the responses against BVDV-1 strains (Figure 2.6). Surprisingly, the Bac-E2^{123} antigen did not induce detectable VN antibodies against any of the BVDV-2a strains (Figure 2.6). The Vira Shield^{TM} 6 vaccine induced VN antibodies against 890 (3/5 calves), 1373 (1/5 calves), and 296 C (1/5 calves) strains, however the responses were poor except the response by one calf (1:512) mounted against the 890 strain (Figure 2.6). Like the BVDV-1 specific VN responses, BVDV-2 VN antibody responses induced by the 293F-expressed antigen cocktail were better than the responses induced by the Bac-E2^{123} antigen and the Vira Shield^{TM} 6 vaccine.

2.3.5 Mosaic Antigens conferred Protection against BVDV-1b

Three days post-challenge, the calves immunized with the 293F-expressed antigen cocktail or the Bac-E2^{123} antigen had lower mean BVD virus titers compared to the Vira Shield^{TM} 6 vaccinees and the negative controls (Figure 2.7A). Specifically, the 293F-expressed antigen cocktail vaccinees had significantly lower mean viremia compared to the Vira Shield^{TM} 6 vaccinees ($p < 0.05$) as well as the negative controls ($p < 0.01$) (Figure 2.7A). The mean viremia for the 293F-expressed antigen cocktail vaccinees was also lower than that observed in the calves immunized with the Bac-E2^{123} antigen, but the difference was not significant (Figure 2.7A). Notably, 3/5 of the calves immunized with the 293F-expressed antigen cocktail and 2/5 of calves immunized with the Bac-E2^{123} antigen had no viremia three days post-challenge (Figure 2.7A). But thereafter, all

the calves had viremia on days 6-13 (data not shown) and even though the mean viremia for all the treatment groups and the negative controls was higher on day 15 post-challenge, the trend was consistent with the outcome observed on day 3 post-challenge (Figure 2.7B). There was no difference in mean viremia between the Vira Shield^{TM} 6 vaccinees and the negative controls at 3 and 15 days post-challenge (Figure 2.7).

Following challenge, all the calves had fever but there were overt differences in temperature fluctuation patterns between the treatment groups (Figure 2.8A). Notably, the calves immunized with the 293F-expressed antigen cocktail or the Bac-E2^{123} antigen had delayed temperature peak whereby the highest mean temperature peaked on days 9 and 8, respectively (Figure 2.8A). However, no significant difference in post-challenge mean temperatures were detected among the treatment and control groups (Figure 2.8A). The calves immunized with the Vira Shield^{TM} 6 vaccine had biphasic pyrexia typical of BVDV infection with first peak in mean body temperature on day 3 followed by a higher peak on day 7 (Figure 2.8A). The negative control calves also had fever and their mean body temperature peaked on day 7.

The most dramatic outcome, post-challenge, was the observation that the calves immunized with the 293F-expressed antigen cocktail never experienced leucopenia and had increased mean WBCs counts that were significantly higher than the negative control group on day 3 ($p < 0.0001$), day 6 ($p < 0.01$), and day 9 ($p < 0.05$) (Figure 2.8B). In contrast, the calves in all the other treatment groups as well as the negative controls had leukopenia 3 days post-challenge (Figure 2.8B). The calves immunized with the Bac-E2^{123} antigen recovered by day 6, whereas the Vira Shield^{TM} 6 vaccinees recovered by around day 8, but the negative controls had not recovered by day 13(Figure 2.8B).

2.4 Discussion

There is still a need for safe and more efficacious vaccines for protection of cattle against diverse BVDV strains. Three novel mosaic BVDV polypeptides designated $E2^{123}$, NS2-3^{1}, and NS2-3^{2}, which consist of immunogenic antigens highly conserved among BVDV-1 and -2 strains were generated and evaluated for their potential to induce cross-protection against diverse BVDV strains. This approach has previously been pursued to generate cross-protective vaccine candidates for pathogens with heterogeneous circulating strains or subtypes (Barouch et al., 2013; Lokhandwala et al., 2017; Yusim et al., 2010). In a previous proof-of-concept study, the mosaic polypeptides induced BVDV-specific antibody and T cell responses and conferred protection against a BVDV-2 strain following immunization with adenovirus expression constructs (Lokhandwala et al., 2017). In the current study, the genes encoding the three mosaic polypeptides were used to express recombinant proteins in Human Embryonic Kidney Expi293F cells [$E2^{123}$, NS2-3^{1}, and NS2-3^{2}] or High Five™ insect cells [$E2^{123}$] and authenticity of the affinity purified antigens was confirmed using polyclonal serum generated against BVDV-1 and -2 strains (Figure 2.1) (Lokhandwala et al., 2017). Immunogenicity and protective efficacy of two prototype vaccines formulated using a cocktail of the 293F-expressed antigens or the Bac-$E2^{123}$ antigen was evaluated by prime-boost immunization of calves (Table 2.1) followed by challenge with a BVDV-1b strain, the predominant sub-genotype in United States (Julia F. Ridpath et al., 2011).

Both prototype vaccines, but not the Vira Shield™ 6 commercial vaccine, primed strong IFN-γ responses against the immunizing antigens and the induced memory was recalled by peptides generated using well characterized *DRB*-restricted BVDV $CD4^{+}$ T cell epitope sequences (Figure 2.2A) (Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). This suggests that priming with a single dose of the prototype vaccines could

generate memory responses that can be recalled upon BVDV infection. This outcome was further supported by the observation that, the IFN-γ memory induced by the 293F-expressed antigen cocktail underwent strong recall after boosting (Figure 2.2B). Moreover, these outcomes were consistent with the previous findings where the same mosaic antigens expressed by recombinant adenoviruses elicited IFN-γ responses of similar magnitude that were strongly recalled by BVDV $CD4^+$ T cell epitopes in the immunized calves but not in the commercial MLV vaccinees (Lokhandwala et al., 2017).

Experimental BVDV subunit vaccines induce strong IFN-γ responses against the immunizing antigens (Riitho et al., 2017; Sadat et al., 2017; Snider et al., 2014) however, there is very limited evidence as to whether these responses are recalled against BVDV (Lokhandwala et al., 2017; Pecora et al., 2015). The antigen-specific IFN-γ responses elicited by the prototype vaccines were strongly and consistently recalled by all the representative BVDV-1b (CA0401186a and TGAC) and BVDV-2a (A125 and 1373) strains (Figure 2.3). The BVDV-1b and -2a strain-specific IFN-γ recall responses detected in the calves immunized with the prototype vaccines were also significantly ($p<0.05$) higher than the recall responses detected in the Vira Shield™ 6 vaccinees (Figure 2.3B). The pro-inflammatory anti-viral response of IFN-γ is crucial for limiting BVDV infection in cattle (Seong, Oem, & Choi, 2013; Smirnova et al., 2014). Therefore, the potential of 293F-expressed antigen cocktail and the Bac-$E2^{123}$ antigen to elicit strong BVDV-specific IFN-γ memory responses is of great significance as it could result in improved vaccine efficacy.

Cattle infected with BVDV develop neutralizing antibodies against the virus (Dirk Deregt et al., 1998; Rajput, Darweesh, Braun, Mansour, & Chase, 2020). The prototype vaccines generated high levels of BVDV antigen-specific IgG responses in the immunized calves (Figure 2.4). The elicited IgG responses, especially by the 293F-expressed antigen cocktail, contributed towards BVDV-1a,

-1b, and -2a neutralization as demonstrated by the detected VN titers against diverse strains (Figures 2.5 and 2.6). Importantly, the calves immunized with the 293F-expressed antigen cocktail developed exceptionally high VN titers [in the range of 1:1024 to >1:8192] against the representative BVDV-1 strains (Figure 2.5). Neutralizing antibody titers of this magnitude are usually achieved with MLV vaccination or multiple BVDV exposures (Fulton et al., 2020; Fulton et al., 1997). Additionally, these titers were higher than the BVDV-1 neutralizing antibody titers that were generated in calves immunized with recombinant adenoviruses expressing the mosaic antigens (Lokhandwala et al., 2017). In contrast, the Bac-E2^{123} antigen and Vira Shield^{TM} 6 vaccine induced moderate to very low BVDV-1 neutralizing antibody titers in calves (Figure 2.5). The 293F-expressed antigen cocktail induced BVDV-2a neutralizing antibodies in a few calves and the overall titers were inferior compared to the BVDV-1 neutralizing antibody titers (Figure 2.6). This outcome was similar to a previous finding in which, following boosting with recombinant adenoviruses expressing the mosaic antigens, low BVDV-2a neutralizing antibody titers were detected in 3/5 immunized calves but all 5/5 calves were completely protected upon challenge with a BVDV-2a strain (Lokhandwala et al., 2017). The Vira Shield^{TM} 6 vaccine elicited either no or very low BVDV-2a neutralizing antibody titers, whereas the Bac-E2^{123} antigen failed to elicit BVDV-2a neutralizing antibodies in calves (Figure 2.6). It is worth noting that altogether, the prototype vaccines induced higher neutralizing antibody titers against BVDV-1 strains than BVDV-2 strains. Recombinant adenoviruses expressing the mosaic antigens had also induced better BVDV-1 neutralizing antibody titers than BVDV-2 neutralizing antibody titers (Lokhandwala et al., 2017). Since the three components of the E2^{123} mosaic antigen were generated using E2 from BVDV-1a, -1b, and -2, epitope coverage was biased towards BVDV-1 genotype, and thus it can be deduced that the skewed neutralizing antibody response towards BVDV-1 strains

is likely due to the presence of higher number of BVDV-1 neutralizing epitopes than the BVDV-2 neutralizing epitopes in the mosaic antigens. The data from *in vitro* virus neutralization demonstrate that the prototype vaccine containing the 293F-expressed antigen cocktail is better than the Vira Shield^{TM} 6 vaccine in eliciting broadly neutralizing antibodies and therefore, has the potential to bridge the gap between the protective immunity conferred by the MLV and KV vaccines (Downey-Slinker et al., 2016; Fulton et al., 2020; Reber et al., 2006).

Potency of the immune responses elicited in calves by the prototype vaccines was reflected by clinical outcomes following challenge with BVDV-1b. Compared to the Vira Shield^{TM} 6 vaccine, the immune responses induced by the 293F-expressed antigen cocktail had significant (p<0.05) effect on the onset of viremia as indicated by the absence (3/5) and very low titers (2/5) of virus in the vaccinees on day 3 post-challenge (Figure 2.7A). Although not as effective as the immune responses induced by the 293F-expressed antigen cocktail, the immune responses elicited by the Bac-E2^{123} antigen also inhibited the onset of BVDV infection in 2/5 calves (Figure 2.7A). All the calves had BVDV later during the challenge, but the 293F-expressed antigen cocktail significantly (p<0.05) reduced viremia in calves compared to the Vira Shield^{TM} 6 vaccine (Figure 2.7B). The Vira Shield^{TM} 6 vaccine, concurrent with previous reports (Rodning et al., 2010; P. H. Walz et al., 2018), was unsuccessful in limiting viremia and therefore, the vaccinated calves had similar level of viremia as the negative control calves (Figure 2.7).

The two prototype vaccines also protected calves from BVD disease better than the Vira Shield^{TM} 6 vaccine by significantly reducing the disease outcomes. There was no fever detected in the calves immunized with the 293F-expressed antigen cocktail until day 9 post-challenge, compared to fever peaking on day 8 in calves immunized with the Bac-E2^{123} antigen, whereas fever in calves vaccinated with the Vira Shield^{TM} 6 vaccine peaked on day 7 concurrently with the negative

control calves (Figure 2.8A). Most importantly, following challenge, the calves immunized with the 293F-expressed antigen cocktail were protected from immunosuppression (Figure 2.8B). In comparison, the calves immunized with the Bac-E2^{123} antigen had mild leukopenia, whereas the calves immunized with the Vira Shield^{TM} 6 vaccine had significant decrease in WBC counts from day 3 until around day 8 post-challenge (Figure 2.8B). This outcome indicates that the 293F-expressd antigen cocktail can elicit better immune responses for improved BRD management in cattle.

In this study, rationally designed prototype BVDV vaccines performed significantly better compared to a traditional commercial vaccine by effectively priming broad BVDV-specific IFN-γ and neutralizing antibody responses that were strongly recalled upon boost. However, the 293F-expressed antigen cocktail and the Bac-E2^{123} antigen conferred strikingly different levels of protection in cattle. Non-structural antigen NS3 when used as an immunogen by itself confers protection in cattle by significantly reducing viremia (LM et al., 2015; Young, Thomas, Thompson, Collins, & Brownlie, 2005) since it induces T cell responses important for controlling the BVDV infection (T. Collen et al., 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Riitho et al., 2017). Moreover, NS3 in Flavivirus is a highly conserved non-structural antigen critical for inducing protective T cell responses and this could explain why the 293F-expressed antigen cocktail, which contained the NS2-3 antigen, was more efficacious at reducing the viral burden as well as preventing immunosuppression in vaccinees compared to the Bac-E2^{123} antigen (Folgori et al., 2006; Wen, Elong Ngono, et al., 2017; Wen, Tang, et al., 2017).

The cross-neutralizing antibody responses elicited by the two prototype vaccines were also significantly different. The 293F-expressed antigen cocktail induced antibody responses that were better at *in vitro* BVDV cross-neutralization. Compared to the 293F-expressed antigen cocktail,

the Bac-E2^{123} antigen, despite inducing high E2-specific IgG responses, elicited lower BVDV-1 neutralizing antibody titers with no BVDV-2 neutralization. The n-glycosylation pattern in Pestivirus E2 glycoprotein is relevant to the protein structure and function, and hence, to the infectivity of virus (Asfor, Wakeley, Drew, & Paton, 2014; Pande et al., 2005; Risatti et al., 2007). Similar to our findings, BVDV E2 antigen produced in insect cells have been previously demonstrated to elicit BVDV neutralizing antibodies however, it doesn't confer complete protection in cattle (S. R. Bolin & Ridpath, 1996; Chimeno Zoth et al., 2007; Pecora et al., 2015; C. Thomas et al., 2009). Therefore, it could be concluded that the inherently simpler post-translational modifications offered by the insect cells influence the antigenicity of candidate vaccine in a manner that potentially leads to misrepresentation of some, if not all, key conformational neutralizing epitopes (Clarke et al., 2017; Du et al., 2009; C. Thomas et al., 2009). Mammalian expression system, on the other hand, generates more authentic post-translationally modified antigen and thereby is more suitable for the production of an efficacious subunit vaccine (Du et al., 2009; Pecora et al., 2012; Sadat et al., 2017; C. Thomas et al., 2009).

In conclusion, the results presented here demonstrate that the mosaic BVDV antigens conferred broader and better protection than a current commercial vaccine. Therefore, a targeted approach of designing a computationally optimized vaccine for broader coverage can be developed and deployed to improve management of BVDV in cattle. Furthermore, this study highlights and reinforces the impact of the non-structural antigens on vaccine efficacy. The Flavivirus non-structural antigens, which are relatively more conserved compared to E2, are known to be rich in broadly protective T cell epitopes that have been exploited in order to improve vaccine efficacy (Barnes et al., 2012; Co et al., 2002; Folgori et al., 2006; Hickman & Pierson, 2017). Therefore, in future the BVDV non-structural antigens apart from NS2-3, need to be investigated to identify

other protective determinants for inclusion in a contemporary subunit vaccine. The outcomes from this pilot study also provide insight into the gaps in current vaccines' efficacy that warrants future BVDV vaccine upgrades.

Abbreviations

293F- Expi293^{TM}-expressed

Bac- Baculovirus-expressed

$E2^{123}$- Mosaic antigen containing E2 glycoprotein from BVDV-1a, -1b and -2.

$NS2\text{-}3^{1}$- Mosaic nonstructural 2-3 antigen from BVDV-1

$NS2\text{-}3^{2}$- Mosaic nonstructural 2-3 antigen from BVDV-2

TMSP- Theileria Modified Sporozoites Protein

Ethics Statement: Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Institutional Animal Care and Use Committees (IACUC registration # 3929) and Institutional Biosafety Committee (IBC registration # 1201).

Author Contributions: WM and SDW designed and oversaw the development and characterization of the experimental vaccines. The vaccines were generated in lab by NS, WH, RR, LM, SL and JB. Animal immunization, sample collection and challenge experiments were done by NS, SL, WM, RR, MM, HS and JY. KWA and CC conducted serum neutralization assays. NS and BF performed IFN-γ ELISPOT and viremia assays. NS and WM were involved in data analysis, result interpretation and drafted the initial manuscript.

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Table 2.1 Calf Immunization Protocol.

Groups	Calf ID	Immunogen	Prime-Boost Dose/Calf
A: 293F-Cocktail	39 56 60 65 93	Expi293^{TM}-expressed mosaic antigens: E2^{123}; NS2-3^{1} and NS2-3^{2}	293F-E2^{123}: 250 µg 293F-NS2-3^{1}: 75 µg 293F-NS2-3^{2}: 50 µg
B: Bac-E2^{123}	99 1 16 9 11	Baculovirus-expressed mosaic antigen: Bac-E2^{123}	250 µg
C: Vira Shield^{TM} 6	90 92 24 86 6	Commercial BVDV KV vaccine: Vira Shield^{TM} 6	5 ml
D: Sham	58 76 21 22 31	Expi293^{TM}-expressed irrelevant antigen: TMSP	250 µg

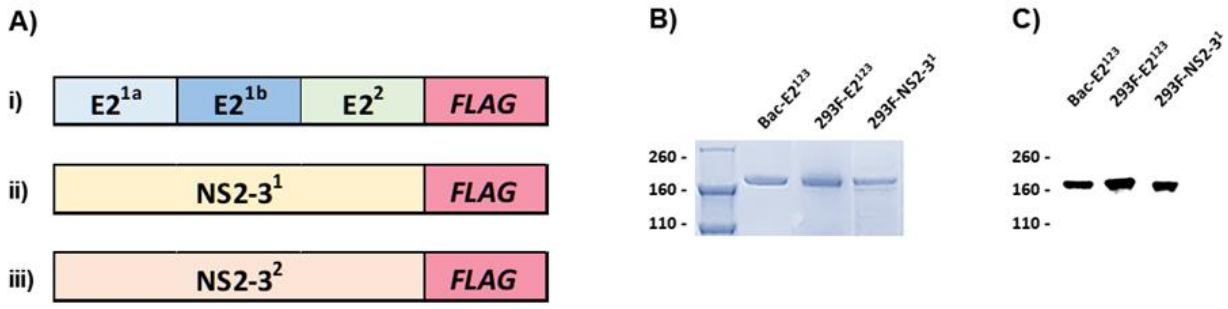

Figure 2.1 Recombinant BVDV Mosaic Antigens. (A) Schematic diagram of codon-optimized synthetic genes encoding novel BVDV mosaic antigens: i) $E2^{123}$ contains mosaic genes: $E2^{1a}$, $E2^{1b}$ and $E2^{2}$ encoding consensus sequences of E2 glycoprotein from BVDV-1a, -1b and -2, respectively; ii) NS2-3^{1}; and iii) NS2-3^{2} encodes for mosaic BVDV-1 and -2 nonstructural proteins 2-3, respectively. A gene encoding the FLAG tag was added in-frame at the end of the synthetic genes for affinity purification of the recombinant antigens; **(B)** SDS-PAGE; and **(C)** Western Blot analyses of the affinity-purified baculovirus-expressed $E2^{123}$ [Bac-$E2^{123}$], Expi293^{TM}-expressed $E2^{123}$ [293F-$E2^{123}$] and NS2-3^{1} [293F-NS2-3^{1}] antigens probed with anti-BVDV polyclonal serum generated against BVDV-1 and -2 strains. The molecular weights are expressed in kDa. The Expi293^{TM}-expressed NS2-3^{2} [293F-NS2-3^{2}] is not shown.

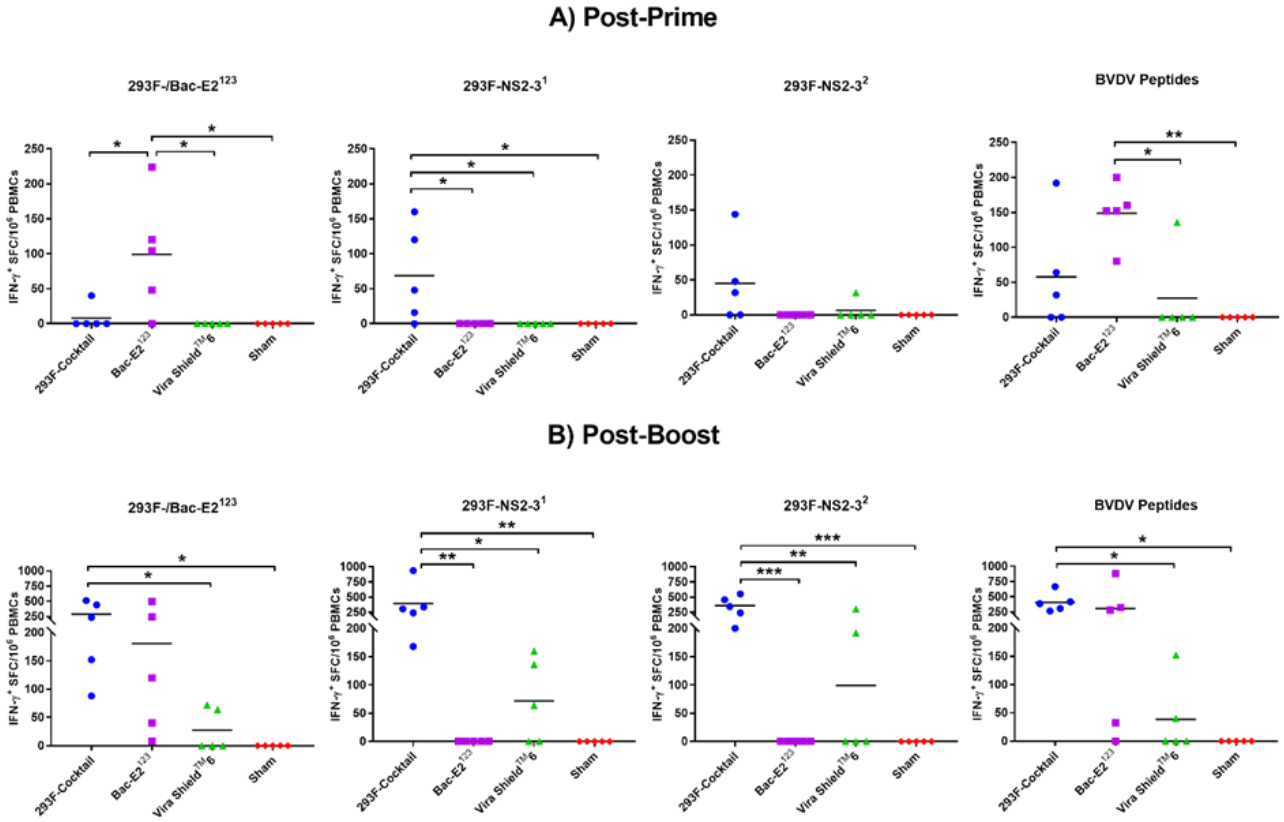

Figure 2.2 BVDV mosaic antigen-specific IFN-γ responses. IFN-γ secreting PBMC responses against recombinant BVDV mosaic antigens and defined BVDV $CD4^+$ T cell epitopes were determined at two weeks post-prime **(A)** and one week post-boost **(B)** by IFN-γ ELISPOT assays. The response is presented as IFN-γ^+ SFC/10^6 PBMCs. For $E2^{123}$ antigen-specific IFN-γ readouts, PBMCs from 293F-Cocktail, Vira Shield™ 6 and Sham groups were stimulated with 293F-$E2^{123}$ antigen whereas PBMCs from Bac-$E2^{123}$ group were stimulated with the Bac-$E2^{123}$ antigen (Table 2.1). Medium alone served as the negative control and the data shown is minus media background counts. The group mean is represented by a bar. Asterisks denote statistically significant differences between the groups (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

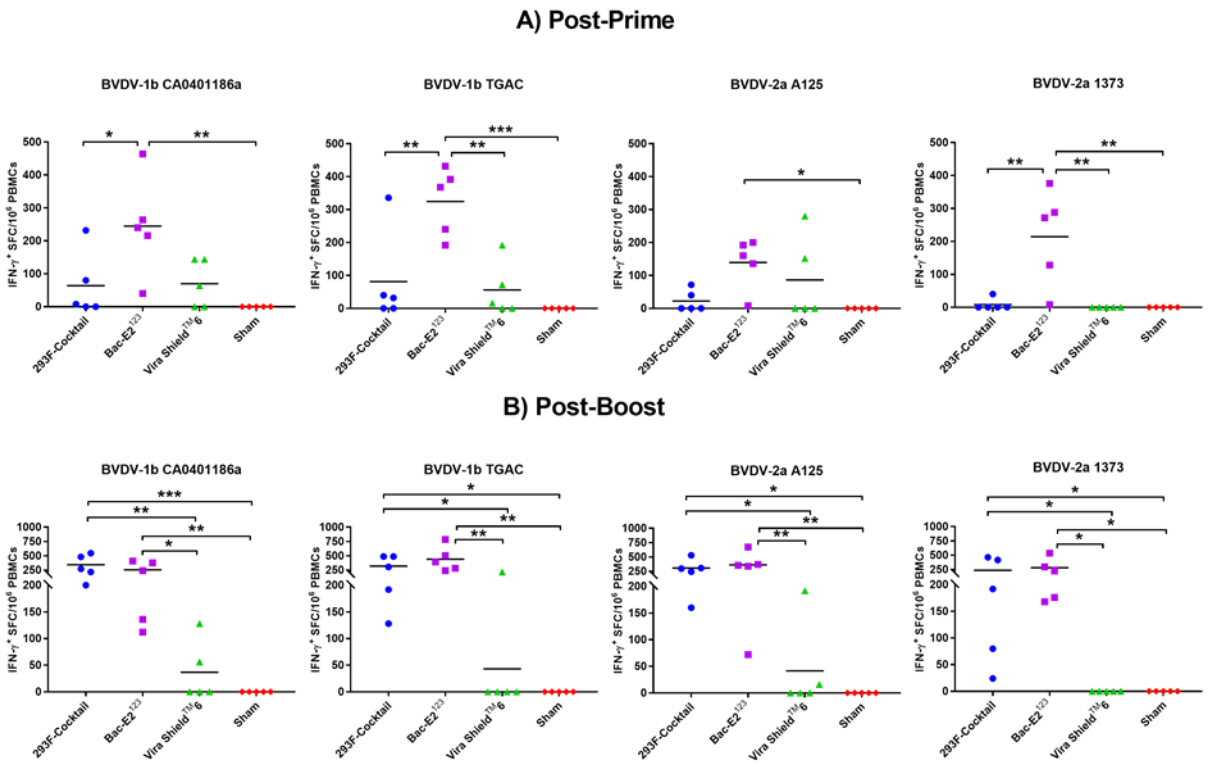

Figure 2.3 BVDV-1 and -2 specific IFN-γ responses. IFN-γ secreting PBMC responses against BVDV-1b and -2a strains were determined at two weeks post-prime **(A)** and one week post-boost **(B)** by IFN-γ ELISPOT assays. The response is presented as IFN-γ^+ SFC/10^6 PBMCs. Medium alone served as the negative control and the data shown is minus media background counts. The group mean is represented by a bar. Asterisks denote statistically significant differences between the groups (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

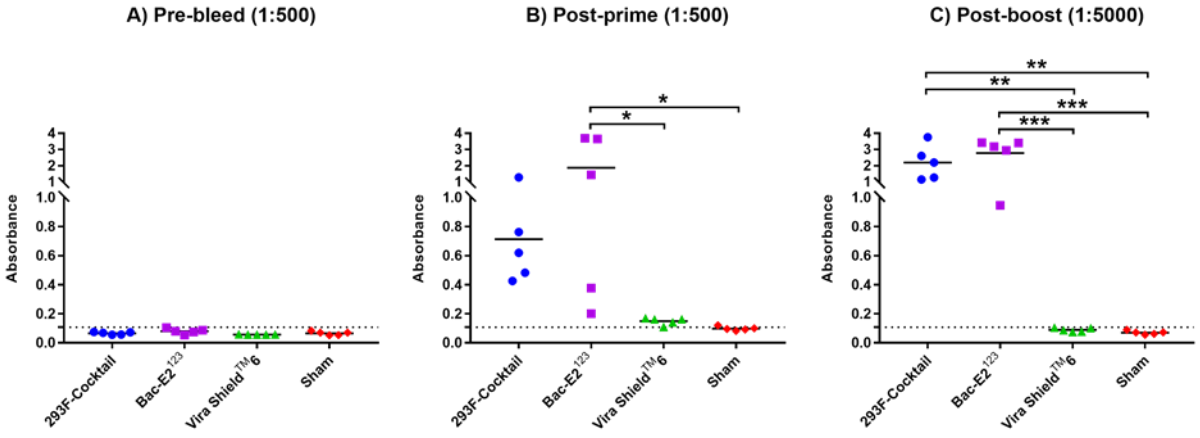

Figure 2.4 BVDV mosaic antigen-specific IgG responses. IgG responses against recombinant BVDV mosaic antigens were determined using serum samples collected prior to immunization **(A)**, at two weeks post-prime **(B)** and at three weeks post-boost **(C)** by ELISA. The group mean is represented by a bar. Asterisks denote statistically significant differences between the groups (*p < 0.05, **p < 0.01 and ***p < 0.001). IgG responses in Vira Shield™ 6 group were also determined at lower dilutions: 1:250 and 1:2500 for post-prime and post-boost respectively where the average absorbance detected were 0.509 and 0.410 respectively (not shown in figure).

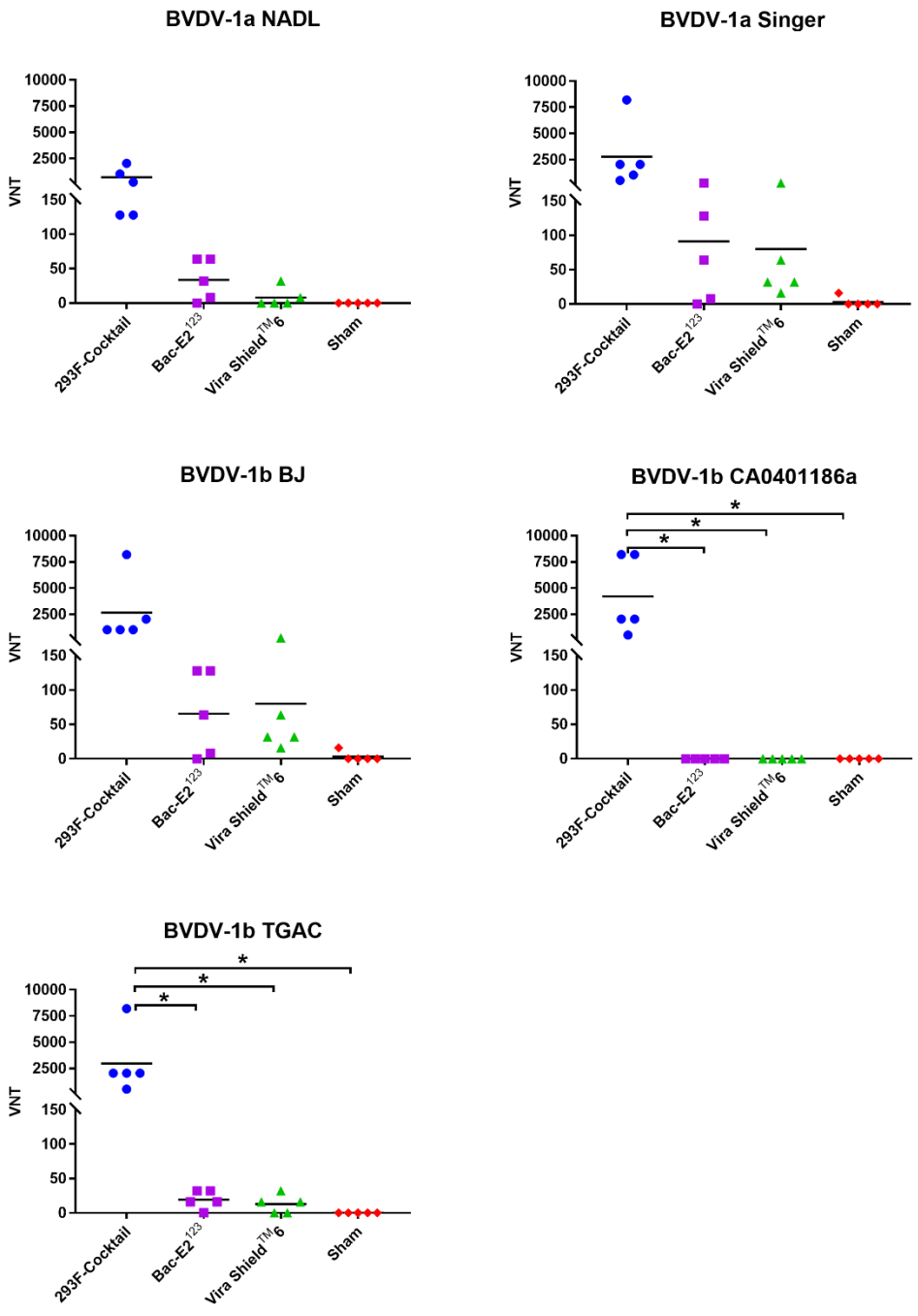

Figure 2.5 BVDV-1 specific neutralizing antibody titers. Virus neutralization assays were used to evaluate BVDV-1-specific neutralization titers in immunized calves at three weeks post-boost against the representative BVDV-1a and -1b strains. Mean group virus neutralization titers (VNT) are represented by the bars. Asterisks denote statistically significant differences between the groups (*$p < 0.05$).

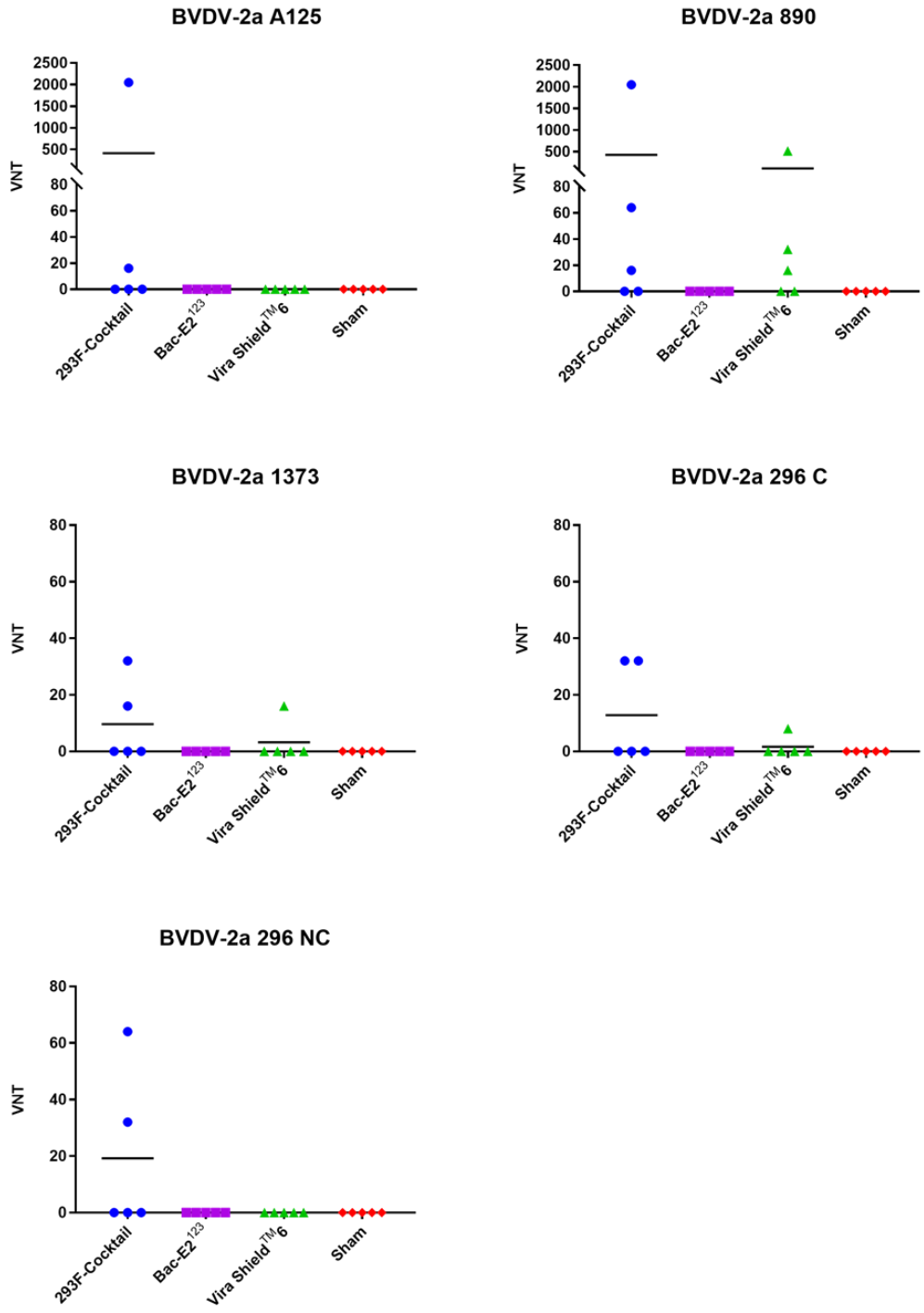

Figure 2.6 BVDV-2-specific neutralizing antibody titers. Virus neutralization assays were used to evaluate BVDV-2-specific neutralization titers in immunized calves at three weeks post-boost against the representative BVDV-2a strains. Mean group virus neutralization titers (VNT) are represented by the bars.

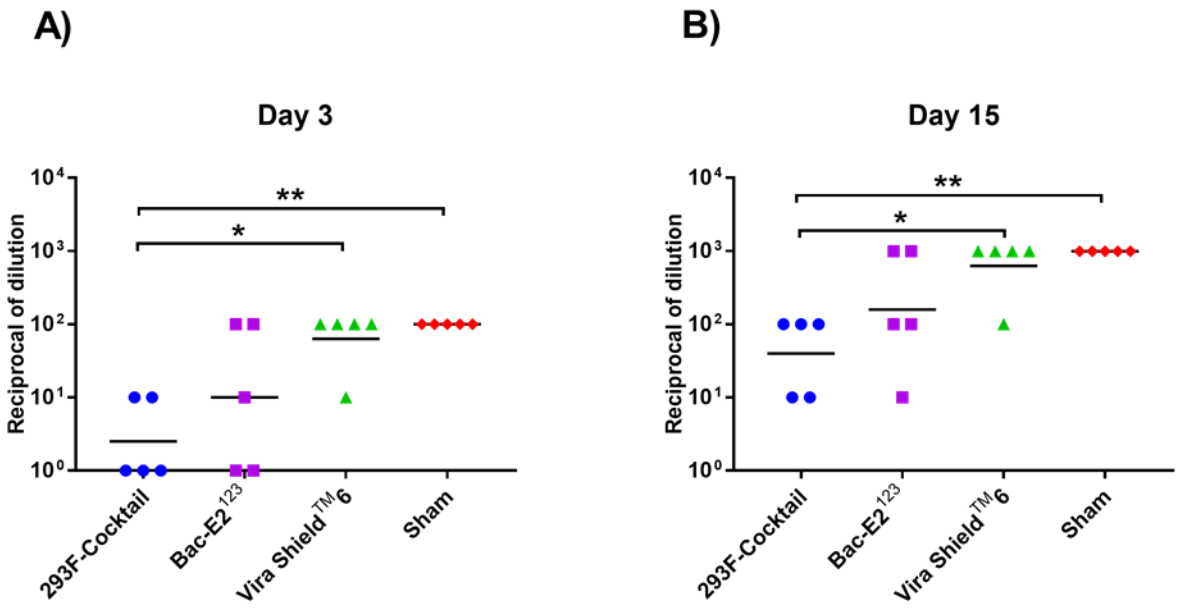

Figure 2.7 Post-challenge viremia in calves challenged with BVDV-1b [CA0401186a]. Viremia detected in blood samples collected from calves on day 3 **(A)** and day 15 **(B)** post-challenge. Mean group dilutions are represented by the bars. Asterisks denote statistically significant differences between the groups (*$p < 0.05$ and **$p < 0.01$).

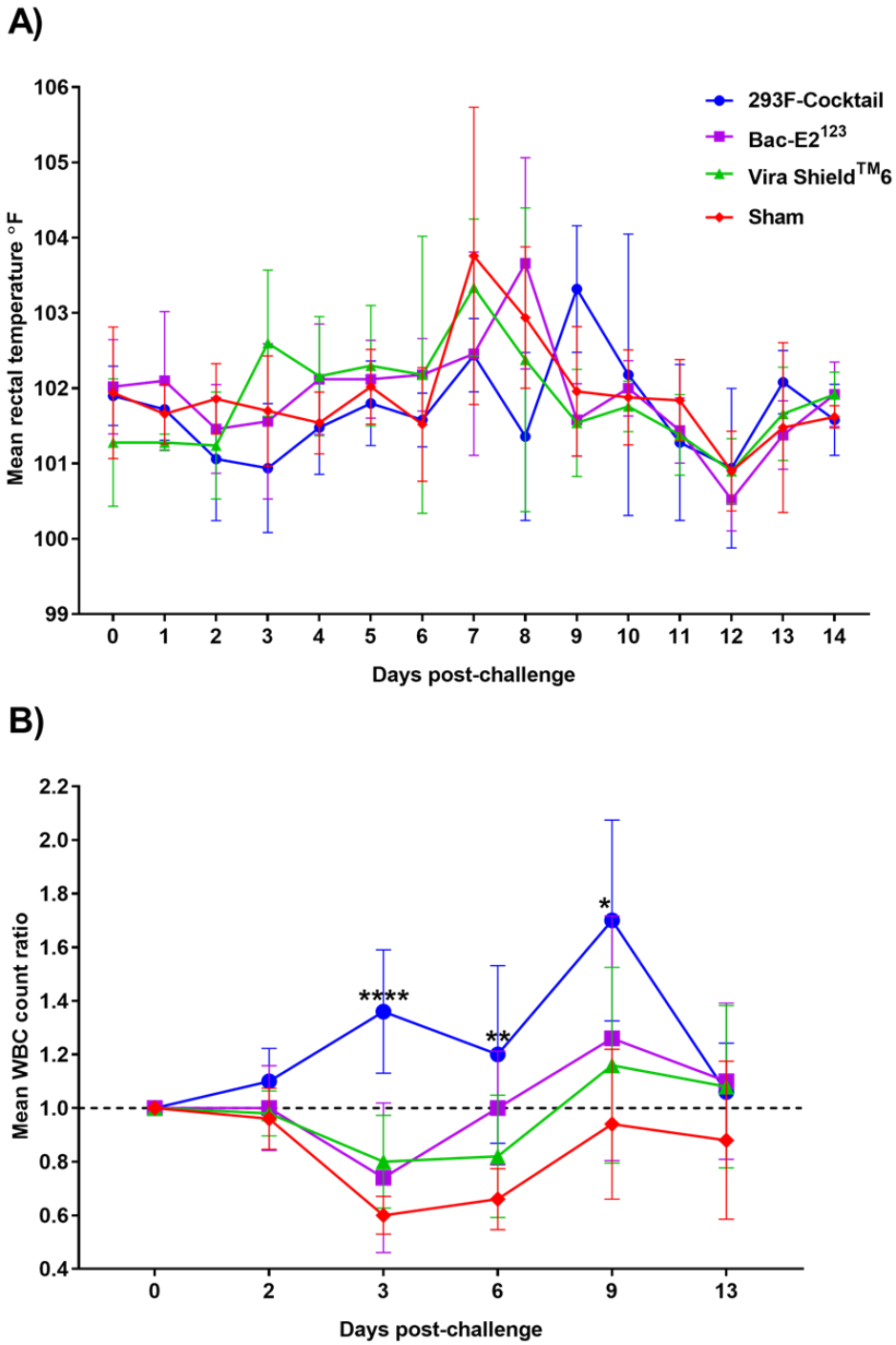

Figure 2.8 Post-challenge clinical outcomes. (A) Mean rectal temperature fluctuation. **(B)** Mean change ratios of white blood cell counts in treatment and control groups. Asterisks denote statistically significant differences as compared to the negative controls (*$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$).

Supplementary Table 2.1 BVDV genomes used for designing the mosaic BVDV antigens.

Genotype	BVDV Strain	Genbank Accession Number
BVDV-1a	Singer	DQ088995.2
	NADL	M31182.1
	6010	JN380080.1
	8844	HQ174293.1
	180	HQ174292.1
	08GB44-1	JQ418633.1
	SD-1	M96751.1
	Oregon C24V	AF091605.1
	Nose	AB033752.1
BVDV-1b	Hercules	JX297517.1
	RK-13	JX419398.1
	Powder	JN380089.1
	PJ	JN380088.1
	CC13B	KF772785.1
	8824	HQ174295.1
	6151	JN380083.1
	12F004	KC963967.1
	8830	HQ174296.1
	CP7	U63479.1
	Columba	JX297514.1
	Mars	JX297520.1
	Osloss	M96687.1
	NY-1	AY027671.1
	Hastings	AF083349.1
BVDV-2	PI99	JN380086.1
	AM1	JN380085.1
	NRW 19-13-8_Dup(-)	HG426489.1
	NRW 19-13-1_Dup(-)	HG426487.1
	D37-13-2_Dup(-)	HG426479.1
	NRW 12-13_Dup(-)	HG426483.1
	NRW 14-13_Dup(-)	HG426485.1
	D75-13-609_Dup(-)	HG426481.1
	VOE 4407	HG426495.1
	Parker	AF145971.1
	296nc	AF145969.1

	125c	AF083345.1
	Potsdam 1600	HG426491.1
	C413	AF002227.1
	1373	AF145967.2
	New York'93	AF502399.1
	SH-28	HQ258810.1
	NRW 19-13-8_Dup(+)	HG426490.1
	NRW 12-13_Dup(+)	HG426484.1
	37621	HQ174303.1
	793	HQ174302.1
	IAF-103	HQ174301.1
	890	U18059.1
	SH2210-14	HG426492.1
	p24515	AY149216.1

Chapter 3- Novel Potent IFN-γ-inducing CD8$^+$ T cell Epitopes Conserved among Diverse Bovine Viral Diarrhea Virus Strains

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Sangewar, N., S. D. Waghela, J. Yao, H. Sang, J. Bray, and W. Mwangi. 2021. Novel potent IFN-γ-inducing CD8$^+$ T cell epitopes conserved among diverse Bovine Viral Diarrhea Virus strains. *The Journal of Immunology*. Accepted for publication.

Abstract

Studies of immune responses elicited by Bovine Viral Diarrhea Virus (BVDV) vaccines have primarily focused on the characterization of neutralizing B cell and $CD4^+$ T cell epitopes. Despite the availability of commercial vaccines for decades, BVDV prevalence in cattle have remained largely unaffected. There is limited knowledge regarding the role of BVDV-specific $CD8^+$ T cells in immune protection and indirect evidence suggests that they play a crucial role during BVDV infection. In this study, the presence of BVDV-specific $CD8^+$ T cells that are highly cross-reactive in cattle was demonstrated. Most importantly, novel potent IFN-γ-inducing $CD8^+$ T cell epitopes were identified from different regions of BVDV polyprotein. Eight $CD8^+$ T cell epitopes were identified from structural BVDV antigens: E^{rns}, E1, and E2 glycoproteins. In addition, from nonstructural BVDV antigens: N^{pro}, NS2-3, NS4A-B, and NS5A-B, twenty $CD8^+$ T cell epitopes were identified. The majority of these IFN-γ-inducing $CD8^+$ T cell epitopes were found to be highly conserved among more than two hundred strains from BVDV-1 and -2 genotypes. These conserved epitopes were also validated as cross-reactive since they induced high recall IFN-γ^+CD8^+ T cell responses, *ex vivo*, in purified bovine $CD8^+$ T cells isolated from BVDV-1- and -2-immunized cattle. Altogether, twenty-eight bovine MHC I-binding epitopes were identified from key BVDV antigens that can elicit broadly reactive $CD8^+$ T cells against diverse BVDV strains. The data presented here will lay the groundwork for the development of a contemporary $CD8^+$ T cell-based BVDV vaccine capable of addressing BVDV heterogeneity more effectively than current vaccines.

3.1 Introduction

Bovine Viral Diarrhea Virus (BVDV) is an immunosuppressive viral pathogen that triggers multifactorial Bovine Respiratory Disease (BRD) in feedlot cattle and therefore, has a huge economic impact on various aspects of cattle industry (Newcomer et al., 2017; Smith, 2009). The 12.5 kb long single-stranded RNA genome of BVDV encodes four structural antigens, capsid, E^{rns}, E1, and E2; and seven nonstructural antigens, N^{pro}, p7, NS2-3, NS4A-B, and NS5A-B (Neill, 2013). The BVDV, a *Pestivirus* belonging to the *Flaviviridae* family, is a heterogeneous pathogen that is categorized into two antigenically distinct genotypes, BVDV-1 and -2, which are further subdivided into various sub-genotypes (Neill, 2013; J. F. Ridpath, 2013). BVDV strains are also classified into two biotypes, cytopathic and non-cytopathic strains (J. F. Ridpath, 2005). The BVDV causes transient or persistent infection (PI) in cattle often making them susceptible to secondary pathogens associated with BRD which, in turn, causes increased morbidity and mortality (J. Ridpath, 2010). Thus, management of BRD prevalence through deployment of effective counter-measures is expected to benefit the cattle industry (Johnson & Pendell, 2017). In the United States, modified-live virus (MLV) and killed virus (KV) BVDV vaccines have been in the market for almost six decades (Griebel, 2015; J. F. Ridpath, 2013). Although commercial BVDV vaccines are widely used as part of the BRD management strategy in the United States, BVDV remains widespread in herds (Newcomer et al., 2017; Scharnbock et al., 2018). For the MLV and KV vaccines, along with the safety-related issues, diversity of BVDV strains continues to be a challenge especially, as new variants emerge in endemic areas (Deutskens et al., 2011; Fulton et al., 2020; Neill et al., 2019; Palomares et al., 2013; J. F. Ridpath, 2005). Therefore, a more efficacious, broadly protective BVDV vaccine is needed for better BRD management.

MLV and KV provide different levels of protection whereby, they mostly elicit BVDV-specific antibody and $CD4^+$ T cell responses to protect cattle (Fulton et al., 2020; Griebel, 2015; Newcomer et al., 2017; Reber et al., 2006; Rodning et al., 2010). Unlike KV, the MLV also induces BVDV-specific $CD8^+$ T cells which is one of the key features that makes MLV more efficacious (Griebel, 2015; Ratree Platt, Burdett, & Roth, 2006). BVDV-specific $CD4^+$ and $CD8^+$ T cells are also elicited in cattle during infection and in the absence of BVDV neutralizing antibody response, BVDV-specific T cell responses provide protection (Beer, 1997; Trevor Collen & Morrison, 2000; J. J. Endsley, Ridpath, Neill, Sandbulte, & Roth, 2004; Janice J. Endsley et al., 2003). Additionally, there are defined MHC-*DR*-restricted epitopes within E2 and NS3 that drive BVDV-specific $CD4^+$ T cells (T. Collen et al., 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). However, cytotoxic $CD8^+$ T lymphocytes (CTLs) targets have not been identified in BVDV. CTLs against Classical Swine Fever Virus (CSFV), another *Pestivirus* from *Flaviviridae* family, are elicited by E2 and NS3 antigens which have been found to contain broadly reactive $CD8^+$ T cell epitopes (Ceppi et al., 2005; S. P. Graham et al., 2012). Structural and nonstructural antigens from *Flavisviruses*, such as Hepatitis C Virus (HCV) and Zika Virus, have been used to develop T cell-based vaccine candidates that expand the breadth of protective cellular immunity against heterologous infections (Barnes et al., 2012; Bullard et al., 2018; Folgori et al., 2006). Hence, considering the undermining effects of hypervariable neutralizing epitopes on current BVDV vaccines' efficacy, a CTL-based vaccine capable of priming potent and sustained cross-protective $CD8^+$ T cells can help overcome BVDV antigenic diversity strains (Boppana, Fiore-Gartland, Bansal, & Goepfert, 2020; Fulton et al., 2020). Subunit vaccines that contain E2 and NS3 antigens tend to be more efficacious than a vaccine that contains only E2 antigen, which suggests that NS3-specific T cell responses have synergistic role in providing BVDV-specific immunity (LM et al.,

2015; Riitho et al., 2017; Sangewar N, 2020). Thus, besides E2 and NS3 antigens, inclusion of additional T cell targets, specifically CTL determinants from other structural and non-structural BVDV antigens which comprise ~75% of BVDV polyprotein, can markedly boost protective efficacy of a CTL-based BVDV vaccine.

Recent advances in immunoinformatics, rapid genome sequencing, and the availability of prediction algorithms have revolutionized the once labor-intensive epitope discovery as putative epitopes can be identified by proteome-wide computational analysis (Backert & Kohlbacher, 2015; Kumar Pandey, Ojha, Mishra, & Kumar Prajapati, 2018; Panda et al., 2020). This approach has transformed subunit vaccine development by enabling rapid identification of T cell epitopes from emerging human pathogens (Samad et al., 2020; Wen, Tang, et al., 2017; L. Zhang, 2018). Similarly, these tools can be applied to identify novel major histocompatibility complex (MHC) class I-restricted $CD8^+$ T cell epitopes from economically significant livestock pathogens (Liao, Lin, Lin, & Chung, 2013; Pandya et al., 2015; Srikumaran & Hegde, 1997). Usually, $CD8^+$ T cell epitope mapping focusses on few epitopes that bind a single prevalent MHC I allele (Assmus et al., 2020; Co et al., 2002; W. Zhang, Li, Lin, & Tian, 2013). But given the diversity among the highly polymorphic MHC I genes, wider array of MHC I alleles along with promiscuous epitopes should be considered for the investigation of $CD8^+$ T cell repertoire at population level (Babiuk et al., 2007; Frahm et al., 2007; Lehmann et al., 2019).

In this study, the full-length BVDV polyprotein was screened for bovine MHC I-binding 9-mers to identify putative novel $CD8^+$ T cell epitopes using NetMHCpan2.8. The top two-hundred peptides that were predicted as the strongest binders for the available bovine leukocyte antigen (BoLA) I alleles were selected for further *ex vivo* screening. The cross-reactivity of $CD8^+$ T cells against heterologous *Flaviviruses* is well known and expansion of these broad spectrum responses

can be achieved by multiple heterologous immunizations (Brehm et al., 2002; Singh et al., 2010; Wen, Tang, et al., 2017). Therefore, using this as an experimental model, outbred cattle were first infected with a BVDV-1b strain (CA401186a) and after recovery, the cattle were given multiple immunizations of either an irradiated heterologous BVDV-1b (TGAC) or -2a (A125) strain. Since irradiated virus retains the ability to infect host cells like the live virus, the cattle were immunized with gamma-irradiated BVDV to ensure the presentation of BVDV antigens by BoLA I for amplification of BVDV-specific $CD8^+$ T cells *in vivo* (Chen et al.). Purified $CD8^+$ T cells from splenocytes of these BVDV hyper-immunized cattle were used to screen the predicted 9-mer peptides by IFN-γ enzyme-linked immunospot (ELISPOT) assay. As a result, novel $CD8^+$ T cell epitopes were identified from BVDV structural and nonstructural antigens. Most of these bovine MHC I-binding epitopes, which recalled IFN-γ-secreting $CD8^+$ T cells in BVDV-1 and -2 immunized cattle, are highly conserved across the two genotypes. These findings strongly support the hypothesis that, a contemporary vaccine that targets highly conserved BVDV-specific $CD8^+$ T cell responses will confer broad protection and reduce prevalence which will potentially lead to BVDV eradication.

3.2 Materials and Methods

3.2.1 BVDV 9-mer Peptide Prediction and Synthesis

A BVDV-1b strain was chosen for BVDV $CD8^+$ T cell epitope mapping since it's the predominant sub-genotype in the United States (Julia F. Ridpath et al., 2011). For epitope prediction, the BVDV-1b polyprotein sequence (GenBank: AGG54029.1) was used as the input sequence and 9-mer peptide length along with all the available BoLA I alleles in the NetMHCpan2.8 database (http://www.cbs.dtu.dk/services/NetMHCpan-2.8/) were selected. The predicted 9-mers were then

sorted by their prediction scores. Overall, two-hundred candidate epitopes were selected that were predicted as strong binders for their corresponding predicted BoLA I alleles (Table 3.1). The two-hundred peptide sequences were used to generate a library of crude synthetic 9-mer peptides (Peptide 2.0, Inc.). Each synthetic peptide was re-constituted at a concentration of 10 mg/ml in ultrapure sterile water with 25% DMSO.

3.2.2 Inactivation of BVDV by Gamma-Irradiation

BVDV-1b TGAC and BVDV-2a A125 were inactivated by gamma-irradiation at The Kansas State University TRIGA Mark II nuclear reactor facility, as described previously (F. C. Thomas et al., 1981). Briefly, 1 ml (1.5 X 10^{10} $TCID_{50}$) of each virus was irradiated with an estimated dose of 200 krad using Californium-252 source. To ensure inactivation of BVDV, the viability of the gamma-irradiated viruses was tested by infecting MDBK cells and the presence of virus progenies was evaluated using BVDV-specific antibodies. Briefly, following 72 h incubation at 37°C, the cells were observed for CPE and the culture supernatant were collected. Fresh MDBK cells were then exposed to the collected supernatant and were incubated for another 72 h. For detection of rescued viral particles, after fixing, the cells were stained with anti-BVDV polyclonal sera (Porcine origin, Cat# 210-70-BVD, VMRD, Inc) and alkaline phosphatase conjugated goat anti-porcine IgG (Jackson ImmunoResearch, Cat# 114-055-003) whereby no BVDV-positive cells were detected (data not shown).

3.2.3 Infection and Immunization of Steers

Eight, seven-eight months old, BVDV-1 and -2 seronegative steers were infected intranasally with BVDV-1b CA0401186a strain (J. F. Ridpath et al., 2007). After four weeks, following recovery, the steers were randomly allocated into two groups A-B (n=4) (Table 3.2). Steers in both groups

were boosted six times every four weeks with gamma-irradiated BVDV-1b TGAC or BVDV-2a A125 (Table 3.2). Gamma-irradiated BVDV mixed with MONTANIDE^{TM} ISA 201 VG adjuvant (Seppic) was administered intramuscularly in the neck region. During immunization, weekly sera and peripheral blood mononuclear cells (PBMCs) samples were collected. At four weeks after the last boost, the steers were bled, and spleens were collected after the animals were euthanized.

3.2.4 CD8$^+$ T cell and Autologous CD14$^+$ Monocyte Isolation

For all BVDV-immunized steers, positively selected CD8$^+$ T cells and autologous CD14$^+$ monocytes were purified using MACS LS columns (Miltenyi Biotec, Cat# 130-042-401) in accordance with vendor's protocol and as previously described (Guzman et al., 2014). Anti-bovine CD8α mAb [7C2B clone, IgG2a isotype; WSU Monoclonal Antibody Center (WSUMAC), Item# BOV2019] and goat anti-mouse IgG microbeads (Miltenyi Biotec, Cat# 130-048-402) were used for isolation of CD8$^+$ T cells from splenocytes. Similarly, anti-bovine CD14 mAb (MM61A clone, IgG1 isotype; WSUMAC, Item# BOV2109), along with goat anti-mouse IgG microbeads, was used for the isolation of CD14$^+$ monocytes from autologous PBMCs. The purity of the isolated subsets were determined to be 95-98% by flow cytometry (data not shown). Purified cell subsets were re-suspended in complete RPMI 1640 medium at appropriate dilution for IFN-γ ELISPOT assay.

3.2.5 Evaluation of BVDV-specific CD8$^+$ T cell Responses

IFN-γ responses in purified CD8$^+$ T cells from the BVDV-immunized steers were evaluated by ELISPOT assay (Bovine IFN-γ ELISpot BASIC ALP kit, Mabtech, Cat# 3119-2A) in accordance with vendor's protocol and as previously described (Lokhandwala et al., 2017; Sangewar N, 2020). Briefly, for all eight steers, 0.2 x 10^6 CD8$^+$ T cells were co-cultured with 0.4 x 10^5 autologous

$CD14^+$ monocytes that were pulsed with 2.5 μg/ml of gamma-irradiated BVDV-1b TGAC or BVDV-2a A125 in a total volume of 100 μl complete RPMI 1640 medium in triplicate wells of MultiScreen-IP plates (MilliporeSigma™, Cat# MAIPS4510). Similar co-cultures incubated with 2.5 μg/ml of ConA or the medium alone served as positive and negative controls, respectively. The plates were incubated at 37°C for 48 h and following processing, IFN-γ spots were enumerated using ELISPOT reader [ImmunoSpot® S6 Analyzer, Cellular Technology Limited]. The responses were reported as spot forming cells (SFC) per million $CD8^+$ T cells after the background spot counts from negative control triplicates were deducted (Sadat et al., 2017).

3.2.6 *Ex vivo* Screening of Predicted Bovine MHC I-binding BVDV Peptides

To screen the two-hundred predicted peptides, twenty pools of 10 peptides were generated and each peptide was diluted to a final concentration of 2.5 μg/ml in complete RPMI 1640 medium (Table 3.1). The peptide pools were tested for non-specific IFN-γ responses using PBMCs collected from naïve steers and no background responses were detected (data not shown). Two steers (2539 and 2599) immunized with TGAC and one steer (2593) immunized with A125 had the highest number of TGAC- and A125-specific IFN-γ^+CD8^+ T cells, respectively, (Figure 3.1) and thus, $CD8^+$ T cells and autologous $CD14^+$ monocytes purified from these steers were used to screen the 20 peptide pools by ELISPOT assay as described above. Additionally, a pool of previously defined BVDV $CD4^+$ T cell epitope peptides (2.5 μg/ml of each peptide) was used as negative control (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Sangewar N, 2020). Peptide pools 4-7, 9, 10, and 18, stimulated IFN-γ^+CD8^+ T cells in the three steers and thus, individual peptides were tested to identify T cell epitopes. Peptides that generated IFN-γ^+CD8^+ T cell responses in 6-8 BVDV-immunized steers were reported as IFN-γ-inducing $CD8^+$ T cell epitopes (Table 3.3).

3.2.7 IFN-γ-inducing $CD8^+$ T cell Epitope Sequences Analyses

Amino acid sequences of IFN-γ-inducing $CD8^+$ T cell epitopes were evaluated for conservation across the two BVDV genotypes using National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (Table 3.3). $CD8^+$ T cell epitopes derived from N^{pro}, E^{rns}, and E1 antigens were analyzed across six BVDV-1a, seven BVDV-1b, and nine BVDV-2a strains using available genome data in NCBI server. Similarly, for $CD8^+$ T cell epitopes derived from E2, NS2-3, NS4A-B, and NS5A-B antigens, sequences were analyzed using the latest available BVDV genomes and published amino acid sequences of the individual BVDV antigens from different isolates whose full genomes have not been sequenced. Sequences from forty-four [44] BVDV-1a, fifty-one [51] BVDV-1b, and one hundred and twelve [112] BVDV-2 (all available BVDV-2 sub-genotypes were included) strains were used for the analyses of E2- and NS2-3-derived epitopes (Table 3.3). In the case of epitopes from NS4A-B and NS5A-B antigens, sequences from seventy-seven [77] BVDV-1 and one hundred and one [101] BVDV-2 strains from diverse sub-genotypes were analyzed (Table 3.3).

3.2.8 MHC I Blocking ELISPOT Assay

The identified $CD8^+$ T cell epitopes were tested for bovine MHC I-restriction by ELISPOT assay as above, but peptide binding was blocked with anti-bovine MHC I mAbs, H58A (IgG2a isotype; WSUMAC, Item# BOV2001) and PT85A (IgG2a isotype; WSUMAC, Item# BOV2002), at 1.0 μg/ml concentration (Abdellrazeq et al., 2020). Six IFN-γ inducing $CD8^+$ T cell epitopes, $N^{pro}_{95\text{-}103}$, $E^{rns}_{493\text{-}501}$, $E1_{610\text{-}618}$, $E2_{999\text{-}1007}$, $NS4B_{2585\text{-}2593}$, and $NS5A_{2783\text{-}2791}$ [Peptides 61, 86, 56, 100, 37, and 64, respectively (Table 3.3)], were selected for this assay. Co-cultures of $CD8^+$ T cells and autologous $CD14^+$ monocytes from one TGAC-immunized steer (2539) and one A125-immunized

steer (2593), were incubated with 2.5 μg/ml of peptide in the presence of either the two anti-bovine MHC I mAbs or IgG2a isotype control. The IFN-γ^+CD8^+ T cell responses in the presence or absence of anti-bovine MHC I mAbs were reported as SFC per million $CD8^+$ T cells described as above.

3.2.9 Statistical Analysis

The results from MHC I blocking ELISPOT assay were analyzed by Wilcoxon test in GraphPad Prism 7 (Version 7.04, GraphPad Software, Inc. La Jolla, CA). The significance of the difference in peptide-specific IFN-γ^+CD8^+ T cell responses in the absence or presence of anti-bovine MHC I mAbs was determined by a non-parametric test and the level of significance was set at $p < 0.05$.

3.3 Results

3.3.1 Gamma-irradiated BVDV Primed and Expanded Strong Cross-reactive IFN-γ^+CD8^+ T cells in Steers

Steers that had previously recovered from BVDV-1b CA401186a infection were hyper-immunized with gamma-irradiated BVDV-1b TGAC or BVDV-2a A125 (Table 3.2). All the steers had high levels of BVDV-specific IFN-γ-secreting $CD8^+$ T cells in their splenocytes (Figure 3.1). Strong TGAC-specific IFN-γ^+CD8^+ T cell responses were detected in the spleens of all the steers immunized with TGAC or A125 (Figure 3.1A), whereas A125-specific IFN-γ^+CD8^+ T cells were detected in 4/4 TGAC vaccinees and in 3/4 A125 vaccinees (Figure 3.1B). Multiple boosts of the steers with the gamma-irradiated BVDV-1 or -2 successfully expanded robust BVDV-specific $CD8^+$ T cells. These cells were cross-reactive as judged by strong recall of IFN-γ responses against TGAC as well as A125 BVDV strains.

3.3.2 Predicted Bovine MHC I-binding BVDV Peptides Stimulated IFN-γ^+CD8^+ T cells

Pools of predicted bovine MHC I-binding 9-mer peptides from BVDV-1b polyprotein (Table 3.1) were tested for their ability to stimulate IFN-γ secretion by $CD8^+$ T cells from the BVDV immunized steers (Figure 3.2). IFN-γ responses against various peptide pools were detected in purified $CD8^+$ T cells from three steers (two TGAC-immunized steers and one A125-immunized steer) that had the highest number of TGAC- as well as A125-specific IFN-γ^+CD8^+ T cells (Figure 3.2). Among peptide pools 1 to 20, high levels of IFN-γ-secreting $CD8^+$ T cells were stimulated by peptide pools: 4-7, 9, 10, and 18 in all the three steers (Figure 3.2). As expected, the pool of defined BVDV $CD4^+$ T cell epitopes did not recall IFN-γ^+CD8^+ T cells in steers since these epitopes are MHC-*DR*-restricted (Figure 3.2) (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). Consequently, individual peptides from the peptide pools that stimulated IFN-γ^+CD8^+ T cell responses were further evaluated to identify IFN-γ-inducing BVDV $CD8^+$ T cell epitopes.

3.3.3 Structural BVDV Antigens Contain Novel IFN-γ-inducing $CD8^+$ T cell Epitopes

Eight bovine MHC I-binding epitopes were identified from BVDV-1b structural antigens: E^{rns}, E1 and E2 (Table 3.3), which were recognized by $CD8^+$ T cells isolated from the spleens of BVDV-1b and -2a immunized steers (Figure 3.3). $E^{rns}_{363-371}$, $E^{rns}_{488-496}$, $E^{rns}_{493-501}$, and $E^{rns}_{496-504}$, stimulated IFN-γ^+CD8^+ T cell responses in most of the TGAC and A125 vaccinees (Figure 3.3). The $E^{rns}_{488-496}$, $E^{rns}_{493-501}$, and $E^{rns}_{496-504}$ are overlapping epitopes and interestingly, $E^{rns}_{488-496}$ and $E^{rns}_{493-501}$ were predicted as binders for the same BoLA I allele (Table 3.3). However, unlike the $E^{rns}_{488-496}$ epitope, the $E^{rns}_{493-501}$ epitope stimulated IFN-γ^+CD8^+ T cell responses in 4/4 TGAC- and A125-immunized steers (Figure 3.3). Overall, out of the four E^{rns}-derived epitopes, the $E^{rns}_{363-371}$

stimulated the highest number of IFN-γ^+CD8^+ T cells in 4/4 steers from both groups (Figure 3.3). All BVDV-immunized steers also had IFN-γ^+CD8^+ T cell responses against the three epitopes from E1, $E1_{552\text{-}560}$, $E1_{610\text{-}618}$, and $E1_{628\text{-}636}$ (Figure 3.3). Among the predicted epitopes from the most immunogenic BVDV E2 antigen, only one IFN-γ-inducing $CD8^+$ T cell epitope, $E2_{999\text{-}1007}$, was identified (Figure 3.3). Notably, this epitope stimulated very high levels of IFN-γ^+CD8^+ T cell responses in 4/4 TGAC- as well as A125-immunized steers (Figure 3.3).

Upon analysis, it was determined that the E^{rns}-derived epitopes ($E^{rns}_{363\text{-}371}$, $E^{rns}_{488\text{-}496}$, $E^{rns}_{493\text{-}501}$, and $E^{rns}_{496\text{-}504}$) are present in BVDV-1a, -1b, and -2a strains (Table 3.3). Hence, cross-reactive BVDV-specific $CD8^+$ T cells were recalled by the epitopes in steers (Figure 3.3). Similarly, $E1_{610\text{-}618}$ recalled cross-reactive $CD8^+$ T cells in immunized steers because it is present in BVDV-1a, -1b, and -2a strains (Figure 3.3, and Table 3.3). $E1_{552\text{-}560}$ epitope is specific to BVDV-1a and -1b strains, whereas $E1_{628\text{-}636}$ epitope is only present in BVDV-1b strains (Table 3.3). Surprisingly, these two epitopes from E1 recalled $CD8^+$ T cell responses in BVDV-2a immunized steers (Figure 3.3). Since these steers were infected with BVDV-1b CA401186a prior to immunization, the two epitopes apparently recalled the E1-specific $CD8^+$ T cell memory responses primed during infection (Figure 3.3). Importantly, $E2_{999\text{-}1007}$ was highly conserved across the 207 strains from BVDV-1a, -1b, and -2 genotypes (Table 3.3) and therefore, can prime bovine $CD8^+$ T cells against diverse BVDV isolates (Figure 3.3).

3.3.4 Nonstructural BVDV Antigens Contain Multiple Novel Broadly Reactive $CD8^+$ T cell Epitopes

Novel T cell epitopes from the nonstructural antigens: N^{pro}, NS2, NS3, and NS4A stimulated recall IFN-γ^+ T cell responses in $CD8^+$ T cells from BVDV-immunized steers (Figure 3.4). $N^{pro}_{95\text{-}103}$

epitope recalled higher mean IFN-γ^+CD8^+ T cells in TGAC and A125 vaccinees than $N^{pro}_{106-114}$, but $N^{pro}_{106-114}$ epitope-specific recall responses were detected in all vaccinees (Figure 3.4). The two N^{pro}-derived epitopes are cross-reactive since they are present in BVDV-1a, -1b, and -2a strains (Table 3.3). Four IFN-γ-inducing $CD8^+$ T cell epitopes were identified from NS2 antigen among which, $NS2_{1195-1203}$ and $NS2_{1407-1415}$ are conserved across 95 BVDV-1a and -1b strains, while $NS2_{1291-1299}$ and $NS2_{1373-1381}$ are conserved across the 207 BVDV-1a, -1b, and -2 strains (Table 3.3). In the TGAC treatment group, 3/4 steers had $NS2_{1195-1203}$- and $NS2_{1407-1415}$-specific recall of IFN-γ^+CD8^+ T cell responses (Figure 3.4). On the other hand, $NS2_{1195-1203}$- and $NS2_{1407-1415}$-specific IFN-γ^+CD8^+ T cells were recalled in 4/4 and 3/4 A125 vaccinees, respectively, evidently due to the memory responses induced during BVDV-1b infection (Figure 3.4). IFN-γ^+CD8^+ T cells were recalled by the two cross-reactive epitopes, $NS2_{1291-1299}$ and $NS2_{1373-1381}$, in 4/4 steers from both groups (Figure 3.4). Likewise, $NS3_{2010-2018}$, an NS3-derived $CD8^+$ T cell epitope which is also highly conserved across the 207 BVDV strains (Table 3.3), recalled IFN-γ^+CD8^+ T cells in 4/4 TGAC and A125 vaccinees (Figure 3.4). An NS4A-derived epitope, $NS4A_{2291-2299}$, conserved among 178 BVDV-1 and -2 strains (Table 3.3), recalled IFN-γ^+CD8^+ T cells in 4/4 TGAC and 3/4 A125 vaccinees (Figure 3.4).

Various $CD8^+$ T cell epitopes, which recalled IFN-γ^+CD8^+ T cell responses in BVDV-1b- and -2a-immunized steers, were located within NS4B, NS5A, and NS5B antigens (Figure 3.5). With the exception of $NS5A_{3067-3075}$, these epitopes were well-conserved across the 77 BVDV-1 and the 101 BVDV-2 strains (Table 3.3). The five NS4B-derived epitopes ($NS4B_{2555-2563}$, $NS4B_{2568-2576}$, $NS4B_{2585-2593}$, $NS4B_{2620-2628}$, and $NS4B_{2664-2672}$) recalled IFN-γ^+CD8^+ T cells in all vaccinees except for one TGAC vaccinee, which had no detectable response against $NS4B_{2620-2628}$ (Figure 3.5). $NS5A_{2783-2791}$, $NS5A_{2992-2930}$, and $NS5A_{3038-3046}$, like NS4-derived epitopes, induced IFN-γ

recall responses in $CD8^+$ T cells from the majority of BVDV-immunized steers (Figure 3.5). The $NS5A_{3067\text{-}3075}$ epitope was present only in the BVDV-1 strains (Table 3.3) however, it recalled IFN-γ^+CD8^+ T cells in 4/4 TGAC and A125 vaccinees (Figure 3.5). As observed for the other BVDV-1 specific $CD8^+$ T cell epitopes, the $NS5A_{3067\text{-}3075}$ epitope likely recalled BVDV-1b-specific memory responses in A125 vaccinees (Figure 3.5). The $CD8^+$ T cell epitopes from NS5B, [$NS5B_{3273\text{-}3281}$, $NS5B_{3434\text{-}3442}$, and $NS5B_{3673\text{-}3681}$], recalled high numbers of IFN-γ^+CD8^+ T cells in most of the BVDV-immunized steers (Figure 3.5), and were broadly reactive against BVDV-1 and -2 strains (Table 3.3).

3.3.5 Novel BVDV $CD8^+$ T cell Epitopes are Bovine MHC I-restricted

IFN-γ^+CD8^+ T cells were consistently recalled by $N^{pro}_{95\text{-}103}$, $E^{rns}_{493\text{-}501}$, $E1_{610\text{-}618}$, $E2_{999\text{-}1007}$, $NS4B_{2585\text{-}2593}$, and $NS5A_{2783\text{-}2791}$ [Peptides 61, 86, 56, 100, 37, and 64 respectively (Table 3.3)], in a TGAC (2539) and an A125 vaccinee (2593) (Figure 3.6). However, the recall responses by the six BVDV $CD8^+$ T cell epitopes in the presence of anti-bovine MHC I mAbs were significantly reduced (* $p < 0.05$) (Figure 3.6). The inhibition of epitope-specific $CD8^+$ T cell recall responses in BVDV-immunized steers due to MHC I blockade therefore confirmed that the novel defined BVDV epitopes are bovine MHC I-restricted.

3.4 Discussion

Although the presence of BVDV-specific $CD8^+$ T cells in the vaccinated and infected cattle have been documented, identification of $CD8^+$ T cell epitopes and evaluation of their importance for mediating protective immunity against BVDV is not well studied (Beer, 1997; Trevor Collen & Morrison, 2000; J. J. Endsley et al., 2004; R. Platt et al., 2017). Previously defined BVDV neutralizing epitopes [from E2] and MHC-*DR*-restricted $CD4^+$ T cell epitopes [from E2 and NS3]

were recently used to generate a rationally designed BVDV subunit vaccine which conferred significantly better cross-protection in cattle than the traditional MLV and KV vaccines (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; D. Deregt, 1998; Dirk Deregt et al., 1998; Lokhandwala et al., 2017; Sangewar N, 2020). Unlike the hypervariable neutralizing B cell epitopes, *Flavivirus*-specific $CD8^+$ T cell epitopes, from both structural and nonstructural antigens, tend to be highly conserved and therefore, are broadly reactive against heterologous strains (Hickman & Pierson, 2017; Sarobe et al., 2001; Wen, Elong Ngono, et al., 2017; Wen, Tang, et al., 2017). To increase vaccine coverage and efficacy, discovery of novel BVDV $CD8^+$ T cell determinants is paramount (Lokhandwala et al., 2017; Sangewar N, 2020; Yusim et al., 2010). Hence with that goal in mind, we integrated *in silico* epitope prediction (NetMHCpan2.8) with the *ex vivo* validation of the predicted epitopes using outbred steers to identify defined BVDV $CD8^+$ T cell epitopes. Steers were infected with BVDV-1 and then boosted multiple times with gamma-irradiated BVDV-1 or -2 to facilitate MHC I-restricted presentation of BVDV antigens which subsequently, primed, and expanded BVDV-specific $CD8^+$ T cells. For the first time, BVDV-specific $CD8^+$ T cells elicited in steers were demonstrated in the present study and were shown to be highly cross-reactive. The $CD8^+$ T cells from these steers were then employed to screen pools of predicted bovine MHC I-binding peptides that recalled high levels of IFN-γ-secreting $CD8^+$ T cell responses.

Individual peptides from the positive pools were analyzed for recalling IFN-γ^+CD8^+ T cell responses in BVDV-1- and -2-immunized steers in order to determine the extent of cross-reactivity in the responding $CD8^+$ T cell repertoires (data not shown). Several predicted peptides from the positive pools were identified as strong IFN-γ-inducers that are highly conserved and are located within BVDV structural and nonstructural antigens. E^{rns}, which helps BVDV in establishing

persistent infection by its RNase activity, elicits BVDV-specific T cell responses, but defined T cell epitopes from E^{rns} have not been reported (Trevor Collen & Morrison, 2000; Matzener, Magkouras, Rumenapf, Peterhans, & Schweizer, 2009; Meyers et al., 2007; Riitho et al., 2017; Wang et al., 2020). In this study, defined IFN-γ-inducing $CD8^+$ T cell epitopes that are conserved across BVDV-1 and -2, were identified from E^{rns} ($E^{rns}_{363-371}$, $E^{rns}_{488-496}$, $E^{rns}_{493-501}$, and $E^{rns}_{496-504}$). E1 and E2 heterodimers form the outer envelope of BVDV (Li, Wang, Kanai, & Modis, 2013). While E2 is a protective antigen against BVDV, E1 has not been studied for its contribution to protective immunity (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; D. Deregt, 1998; Riitho et al., 2017). Three IFN-γ-inducing $CD8^+$ T cell epitopes were identified within E1. Remarkably, two E1-derived epitopes ($E1_{552-560}$ and $E1_{628-636}$) which are present only in BVDV-1 strains, induced IFN-γ responses in $CD8^+$ T cells from BVDV-2-immunized steers. Since the immunized steers had previously recovered from a BVDV-1 infection, these responses observed in BVDV-2-immunized steers indicate that the two epitopes are likely immunodominant and have the potential to prime strong memory $CD8^+$ T cells against BVDV-1 strains. *Flavivirus* E2 antigen contains $CD8^+$ T cell epitopes that induce T cell responses against heterogeneous viruses (Singh et al., 2010; Wen, Tang, et al., 2017). In Classical Swine Fever Virus [CSFV], E2 is one of the major CTL targets (Ceppi et al., 2005). In the current study, one potent IFN-γ-inducing $CD8^+$ T cell epitope was discovered from E2 ($E2_{999-1007}$). In all likelihood, this sole E2-derived epitope, which is highly conserved in more than 200 BVDV strains, could be a broadly protective CTL determinant and therefore, it needs to be further investigated along with its cognate BoLA I haplotype(s) (Simon P. Graham et al., 2006; S. P. Graham et al., 2008).

Other than E^{rns}, N^{pro}, the first non-structural antigen encoded by the viral genome, is another BVDV antigen responsible for causing immunosuppression and persistent infection (Darweesh,

Rajput, Braun, Rohila, & Chase, 2018; Gottipati, Holthauzen, Ruggli, & Choi, 2016; Meyers et al., 2007). While N^{pro} is an important $CD4^+$ T cell target, it is not known whether it elicits $CD8^+$ T cell response during BVDV infection (Trevor Collen & Morrison, 2000; Lokhandwala et al., 2017). Two novel $CD8^+$ T cell epitopes predicted from N^{pro} ($N^{pro}_{95\text{-}103}$ and $N^{pro}_{106\text{-}114}$) were shown to be inducers of strong cross-reactive IFN-γ response. BVDV NS2/3 antigens are also targets for $CD4^+$ T cells and are often included in experimental subunit vaccines (T. Collen et al., 2000; Trevor Collen & Morrison, 2000; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; LM et al., 2015; Lokhandwala et al., 2017; Riitho et al., 2018; Riitho et al., 2017; Sangewar N, 2020). Subunit vaccine comprising only of NS3, protects BVDV-infected cattle by alleviating viral burden (LM et al., 2015; Young et al., 2005). Clearly, apart from BVDV-specific $CD4^+$ T cells, NS3 also stimulates $CD8^+$ T cell responses which help in eliminating BVDV-infected cells. Moreover, NS2/3-derived CTL epitopes have been identified in CSFV and in other *Flaviviruses* (Armengol et al., 2002; Co et al., 2002; Wen, Tang, et al., 2017). From BVDV NS2, two $CD8^+$ T cell epitopes ($NS2_{1195\text{-}1203}$- and $NS2_{1407\text{-}1415}$) that are conserved in 95 BVDV-1a and -1b strains, were identified. Most notably, broadly reactive $CD8^+$ T cell epitopes, conserved among more than 200 BVDV-1 and -2 strains, were discovered to have originated from NS2/3 ($NS2_{1291\text{-}1299}$, $NS_{21373\text{-}1381}$, and $NS3_{2010\text{-}2018}$).

The significance of broadly reactive T cell responses mounted by the nonstructural antigens [NS2, NS3, NS4A-B, and NS5A-B], which constitute about 75% of the viral polyprotein, have been emphasized and utilized for designing T cell-based vaccines against key global pathogens that are notorious for their heterogeneity (Folgori et al., 2006; Wen, Tang, et al., 2017). Thus unsurprisingly, besides NS2/3, multiple BVDV cross-reactive $CD8^+$ T cell epitopes from NS4 ($NS4A_{2291\text{-}2299}$, $NS4B_{2555\text{-}2563}$, $NS4B_{2568\text{-}2576}$, $NS4B_{2585\text{-}2593}$, $NS4B_{2620\text{-}2628}$, and $NS4B_{2664\text{-}2672}$) and

NS5 ($NS5A_{2783-2791}$, $NS5A_{2992-2930}$, $NS5A_{3038-3046}$, $NS5B_{3273-3281}$, $NS5B_{3434-3442}$, and $NS5B_{3673-3681}$) were identified and these are conserved among 178 strains from BVDV-1 and -2 genotypes. However, there was one IFN-γ-inducing $CD8^+$ T cell epitope from NS5A ($NS5A_{3067-3075}$) which is only present in BVDV-1 genotype.

The results presented here are unique, especially in the context of BVDV and *Flaviviruses*, since this study sought to identify novel $CD8^+$ T cell epitopes from various regions of the BVDV polyprotein. The outcome also corroborates that high frequencies of long-term BVDV-specific memory $CD8^+$ T cells created during infection are localized in the spleen. This was made apparent by the consistent recall responses detected in the BVDV-2-immunized steers, which had undergone BVDV-1 infection prior to the immunization, against the epitopes that were conserved only in BVDV-1. Undeniably, within the BVDV polyprotein, there are numerous conserved as well as sub-genotype-specific T cell epitopes that can impart long-term protective T cell immunity and thereby, mitigate BVDV infection prevalence in herds. Hence, BVDV vaccination strategy should aim to incorporate divergent and conserved T cell epitopes for protection against diverse circulating BVDV strains. Furthermore, comprehensive assessment of IFN-γ-inducing $CD8^+$ T cell epitopes will certainly yield novel protective determinants which will reshape the landscape of BVDV vaccine immunology and advance the BVDV eradication programs (Simon P. Graham et al., 2006; S. P. Graham et al., 2008).

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Ethics Statement: Animal study was conducted in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Institutional Animal Care and Use Committees (IACUC registration # 4412) and Institutional Biosafety Committee (IBC registration # 1201).

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Table 3.1 Bovine MHC I-binding 9-mer peptides from BVDV-1b polyprotein predicted using NetMHCpan version 2.8.

Pool 1		Pool 2		Pool 3		Pool 4		Pool 5	
Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence
1	FQRGVNRSL	11	YLPYATSAL	21	YQDYKGPVY	31	AQFGAGEIV	41	RQAAVDLVV
2	VQYTARGQL	12	YMAGRDTAV	22	LMISYVTDY	32	SEVLLLSVV	42	LQLQTRTSL
3	LTIPNWRPL	13	KQMSLTPLF	23	AENALLIAL	33	LEQEVQVEI	43	IMFEAFELL
4	VASLFISAL	14	AMVEYSYIF	24	YEMKALRNV	34	YETATVLVF	44	KAVAFSFLL
5	YILDLIYSL	15	FAPETASVL	25	YEYSDGLQL	35	QEYSGFVQY	45	FEEASMCEI
6	LLMYSWNPL	16	QQYMLKGEY	26	WQMVYMAYL	36	RQLGILGKK	46	FEIAVSDVL
7	GEYQYWFDL	17	YQYWFDLEI	27	SQFLDIAGL	37	SEQKRTLLM	47	YAASPYCEV
8	REMNYDWSL	18	YMAYLTLDF	28	RTYKRVRPF	38	REHNKWILK	48	TAATTTAFL
9	TAFFGVMPR	19	YMLKGEYQY	29	YKRWIQCVL	39	AMAVLTLTL	49	WPYETATVL
10	SALATYTYK	20	NMMDKLTAF	30	RDYFAESLL	40	ALRDFNPEL	50	IPNWRPLTF
Pool 6		**Pool 7**		**Pool 8**		**Pool 9**		**Pool 10**	
Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence
51	LAALHTRAL	61	GPVYHRAPL	71	YHIIVMHPL	81	KTARNINLY	91	MLNVLTMMY
52	NPLVRRICL	62	YAIAKNDEI	72	QLFLRNLPI	82	ISSKWQMVY	92	YTARGQLFL
53	ITYASYGYF	63	SVMLGVGAI	73	KLANLNLSL	83	ISSKTGHLY	93	SSAENALLI
54	YTMKLSSWF	64	YYDDNLNEI	74	APVRFPTAL	84	KSWLGGLDY	94	YLKPGPLFY
55	SVIQDTAHY	65	FVNEDIGTI	75	YIPDKGYTL	85	ITLATGAGK	95	KVVEPALAY
56	GSVWNLGKY	66	ARRVKIHPY	76	VILSTTIYK	86	FGAYAASPY	96	ETASVLYLV
57	SVYQYMRLK	67	LRRLRVLLM	77	ATVTTWLAY	87	KGYNSGYYY	97	WADFLTLIL
58	STQTTYYYK	68	DTYENYSFL	78	ISALATYTY	88	KSKTWFGAY	98	RVIAALIEL
59	WTAATTTAF	69	VMSRVIAAL	79	VAFSFLLMY	89	RYYETAIPK	99	ALFEAVQTI
60	NSMLNVLTM	70	GHMASAYQL	80	KVLKWVHNK	90	SRDERPFVL	100	YFEPRDNYF
Pool 11		**Pool 12**		**Pool 13**		**Pool 14**		**Pool 15**	
Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence
101	YGMPKVVTI	111	FGPGVDAAM	121	TTATVRELL	131	AGNSMLNVL	141	LTLDFMYYM
102	VVTYFLLLY	112	WRPLTFILL	122	ENALLIALF	132	IGPLGATGL	142	EGRRFVASL
103	YSYIFLDEY	113	YSFLNARKL	123	VTTWLAYTF	133	CTFNYTRTL	143	RGLETGWAY
104	KIMGAISDY	114	LLPLIRATL	124	TPSDERIRL	134	DSIEVVTYF	144	IGNPLRLIY
105	IAYEKAVAF	115	ATPEQLAVI	125	HPYEAYLKL	135	DSKLYHIYV	145	TTTAFLVCL
106	VTGSDSKLY	116	VTIIRACTL	126	RGKFNTTLL	136	RGDFKQITL	146	VSVGISVML
107	VTASGTPAF	117	FGYVGYQAL	127	KGWSGLPIF	137	LGPIVNLLL	147	TTLLNGPAF
108	ATTVVRTYK	118	YNIEPWILL	128	HGWCNWYNI	138	MTATPAGSV	148	DTKSFHEAI
108	TSMNRGDFK	119	DNYFQQYML	129	AGVFLIRSL	139	NSYEVQVPV	149	SLTPLFEEL
110	KGPVSGIYL	120	MVYMAYLTL	130	TYFLLLYLL	140	ESGEGVYLF	150	KIHPYEAYL
Pool 16		**Pool 17**		**Pool 18**		**Pool 19**		**Pool 20**	
Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence	Peptide ID	Sequence
151	KNFSFAGIL	161	KLLEIFHTI	171	ALRNVSGSL	181	REALEALSL	191	VNYRVTKYY
152	KSFNRVARI	162	GTAKLTTWL	172	MEILSQNPV	182	LTPLFEELL	192	AMFQRGVNR
153	YHRAPLELF	163	LAQGNWEPL	173	IEFCSHTPV	183	GEIVMMGNL	193	LSSAENALL
154	LLAWAILAL	164	YLERVDLSF	174	KEHDCTSVI	184	SEKHLVEQL	194	ALRYVAGPI
155	KLMSGIQTV	165	WSDNTSSYM	175	AESLLVIVV	185	YELVKLYYL	195	GIYLKPGPL
156	RRFVASLFI	166	VIPGSVWNL	176	LMNKTQANL	186	SQNPVSVGI	196	GENITQWNL
157	KMLLATDKW	167	MMDKLTAFF	177	ALSKRHVPM	187	ITGAQGFPY	197	RECAVTCRY
158	IYLKPGPLF	168	YMRLKHPSI	178	AMDDKLGPM	188	ALIELNWTM	198	GRHKRVLVL
159	YEKAVAFSF	169	LLRRLRVLL	179	GLWGTHTAL	189	RETRYLAAL	199	ILLQGAPVL
160	ALLGGRYVL	170	VQKFINSLI	180	GEDLYDCAL	190	GVFLIRSLK	200	ASYGYFCQM

Table 3.2 Immunization of steers that were previously exposed to BVDV-1b CA401186a.

Groups	Steer ID and Figure Legend	Immunogen	Immunization Dose/Steer	No. of Immunizations
A: TGAC	2539 ● 2565 ▲ 2599 ■ 2609 ♦	Gamma irradiated BVDV-1b TGAC	200 µg	6
B: A125	2593 ○ 2556 Δ 2601 □ 2611 ◊	Gamma irradiated BVDV-2a A125	200 µg	6

Table 3.3 IFN-γ-inducing $CD8^+$ T cell epitopes from BVDV.

Peptide ID	Peptide Name*	Sequence	Conserved in BVDV Genotypes (number of strains)	Predicted BoLA I Allele	Predicted 1-log50k(aff)
61	**N^{pro}_{95-103}**	GPVYHRAPL	1a (6), 1b (7), 2a (9)	BoLA-2*03001	0.474
45	**$N^{pro}_{106-114}$**	FEEASMCEI	1a (6), 1b (7), 2a (9)	BoLA-1*07401	0.317
176	**$E^{rns}_{363-371}$**	LMNKTQANL	1a (6), 1b (7), 2a (9)	BoLA-6*01501	0.305
88	**$E^{rns}_{488-496}$**	KSKTWFGAY	1a (6), 1b (7), 2a (9)	BoLA-2*04601	0.366
86	**$E^{rns}_{493-501}$**	FGAYAASPY	1a (6), 1b (7), 2a (9)	BoLA-2*04601	0.388
47	**$E^{rns}_{496-504}$**	YAASPYCEV	1a (6), 1b (7), 2a (9)	BoLA-2*00501	0.344
32	**$E1_{552-560}$**	SEVLLLSVV	1a (6), 1b (7)	BoLA-1*01901	0.523
56	**$E1_{610-618}$**	GSVWNLGKY	1a (6), 1b (7), 2a (9)	BoLA-2*00801	0.292
34	**$E1_{628-636}$**	YETATVLVF	1b (7)	BoLA-1*07401	0.292
100	**$E2_{999-1007}$**	YFEPRDNYF	1a (44), 1b (51), 2 (112)	BoLA-2*06001	0.141
39	**$NS2_{1195-1203}$**	AMAVLTLTL	1a (44), 1b (51)	BoLA-1*04901	0.490
97	**$NS2_{1291-1299}$**	WADFLTLIL	1a (44), 1b (51), 2 (112)	BoLA-2*05601	0.213
82	**$NS2_{1373-1381}$**	ISSKWQMVY	1a (44), 1b (51), 2 (112)	BoLA-2*04301	0.434
69	**$NS2_{1407-1415}$**	VMSRVIAAL	1a (44), 1b (51)	BoLA-2*02601	0.343
87	**$NS3_{2010-2018}$**	KGYNSGYYY	1a (44), 1b (51), 2 (112)	BoLA-2*04601	0.408
177	**$NS4A_{2291-2299}$**	ALSKRHVPM	1 (77), 2 (101)	BoLA-6*01501	0.327
172	**$NS4B_{2555-2563}$**	MEILSQNPV	1 (77), 2 (101)	BoLA-6*01401	0.450
63	**$NS4B_{2568-2576}$**	SVMLGVGAI	1 (77), 2 (101)	BoLA-2*01801	0.257
37	**$NS4B_{2585-2593}$**	SEQKRTLLM	1 (77), 2 (101)	BoLA-1*04201	0.509
99	**$NS4B_{2620-2628}$**	ALFEAVQTI	1 (77), 2 (101)	BoLA-2*05701	0.246
43	**$NS4B_{2664-2672}$**	IMFEAFELL	1 (77), 2 (101)	BoLA-1*06701	0.362
64	**$NS5A_{2783-2791}$**	YYDDNLNEI	1 (77), 2 (101)	BoLA-2*01802	0.247
65	**$NS5A_{2992-2930}$**	FVNEDIGTI	1 (77), 2 (101)	BoLA-2*01802	0.218
81	**$NS5A_{3038-3046}$**	KTARNINLY	1 (77), 2 (101)	BoLA-2*04501	0.403
40	**$NS5A_{3067-3075}$**	ALRDFNPEL	1 (77)	BoLA-1*04901	0.448
38	**$NS5B_{3273-3281}$**	REHNKWILK	1 (77), 2 (101)	BoLA-1*04201	0.421
89	**$NS5B_{3434-3442}$**	RYYETAIPK	1 (77), 2 (101)	BoLA-2*04701	0.278
173	**$NS5B_{3673-3681}$**	IEFCSHTPV	1 (77), 2 (101)	BoLA-6*01401	0.454

* Peptide name represents the BVDV antigen and amino acid position for the predicted peptide within BVDV polyprotein.

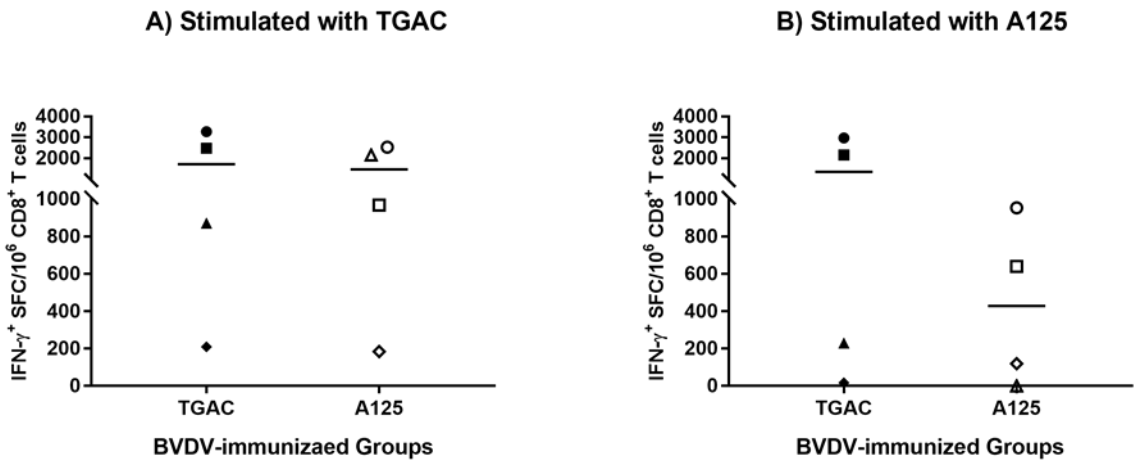

Figure 3.1 BVDV cross-reactive CD8$^+$ T cell responses in immunized steers. IFN-γ secretion by CD8$^+$ T cells isolated from splenocytes of BVDV-immunized steers (Table 3.2) [TGAC-immunized: 2539 (●), 2565 (▲), 2599 (■), and 2609 (♦); A125-immunized: 2593 (○), 2556 (Δ), 2601 (□), and 2611 (◊)] were evaluated by ELISPOT assay. Co-cultures of purified CD8$^+$ T cells and autologous CD14$^+$ monocytes were incubated with gamma-irradiated **(A)** BVDV-1b TGAC or **(B)** BVDV-2a A125. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells minus media background counts and bars represent the mean responses for the two groups.

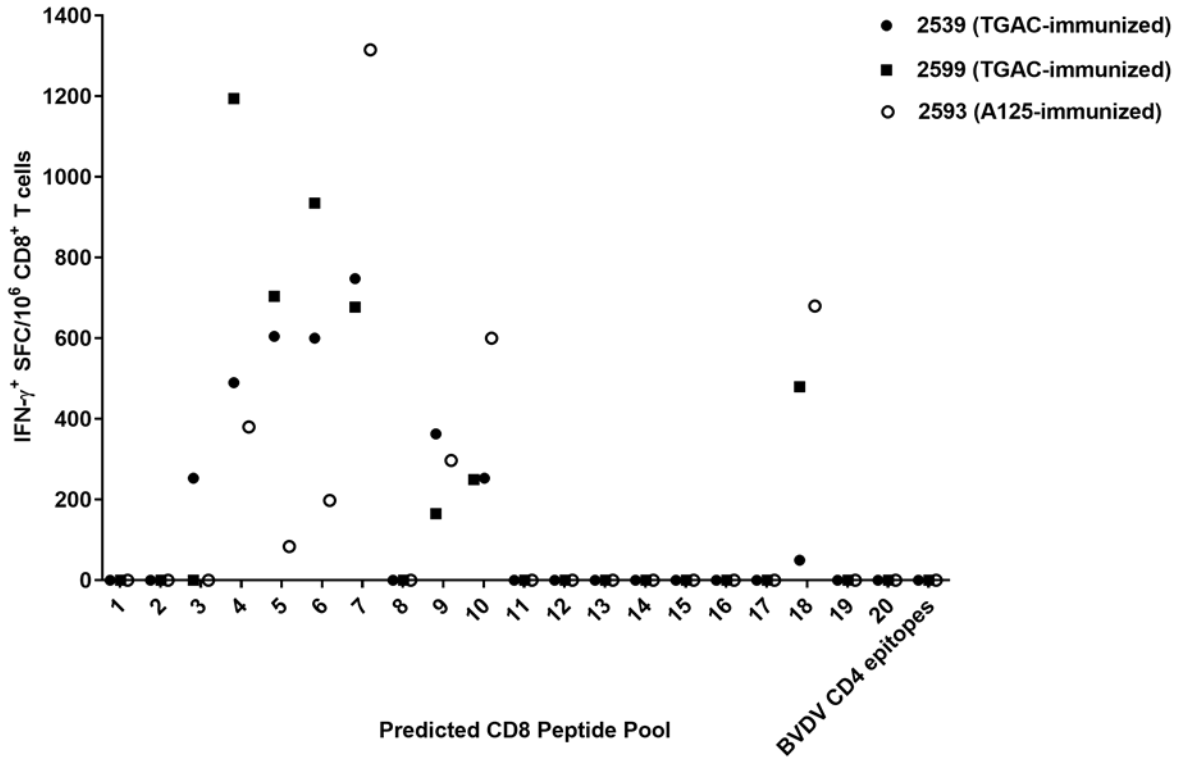

Figure 3.2 IFN-γ$^+$CD8$^+$ T cell responses by the predicted BVDV-1b peptide pools. CD8$^+$ T cells from two steers that were immunized with BVDV-1b TGAC (2539 and 2599) and one steer immunized with BVDV-2a A125 (2593) (Table 3.2) were used to screen pools of predicted bovine MHC I-binding BVDV-1b peptides (Table 3.1) by IFN-γ ELISPOT. CD8$^+$ T cells and autologous CD14$^+$ monocytes were incubated with peptide pools [Pools 1 to 20] where, each pool contained 10 predicted peptides (Table 3.1). A pool of previously defined BVDV CD4$^+$ T cell epitopes was included as a negative control. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells after the background media counts were deducted.

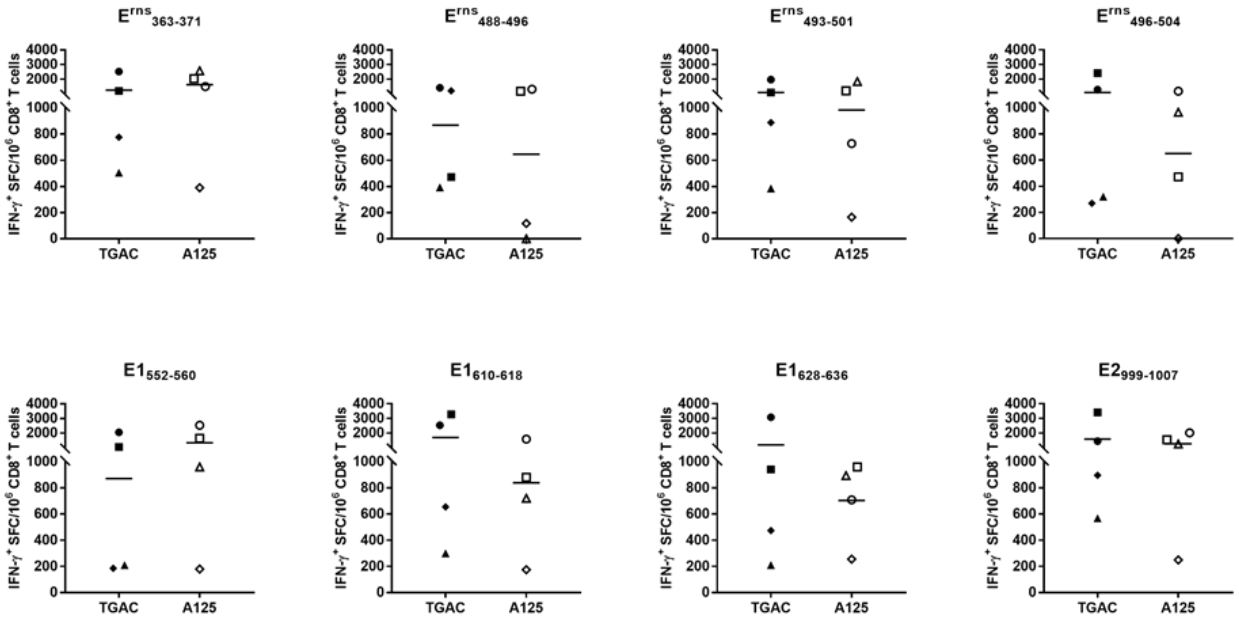

Figure 3.3 IFN-γ-inducing CD8$^+$ T cell epitopes from structural BVDV antigens. The predicted bovine MHC I-binding epitopes from BVDV-1b E^{rns}, E1, and E2 stimulated IFN-γ responses in CD8$^+$ T cells from BVDV-immunized steers [TGAC-immunized: 2539 (●), 2565 (▲), 2599 (■), and 2609 (♦); A125-immunized: 2593 (○), 2556 (Δ), 2601 (□), and 2611 (◊)]. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells minus media background counts and bars represent the mean responses for the two groups.

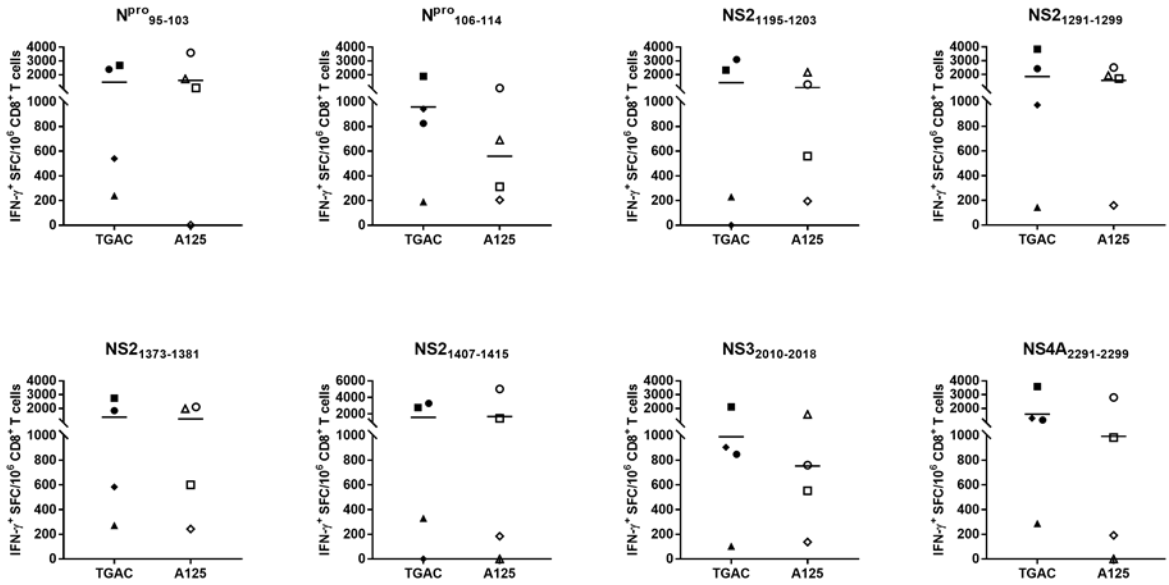

Figure 3.4 CD8$^+$ T cell epitopes from BVDV non-structural N^{pro}, NS2, NS3, and NS4A antigens. IFN-γ^+CD8$^+$ T cell responses were stimulated in BVDV-immunized steers [TGAC-immunized: 2539 (●), 2565 (▲), 2599 (■), and 2609 (♦); A125-immunized: 2593 (○), 2556 (Δ), 2601 (□), and 2611 (◊)] by epitopes predicted from BVDV-1b N^{pro}, NS2, NS3, and NS4A non-structural antigens. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells minus media background counts and bars represent the mean responses for the two groups.

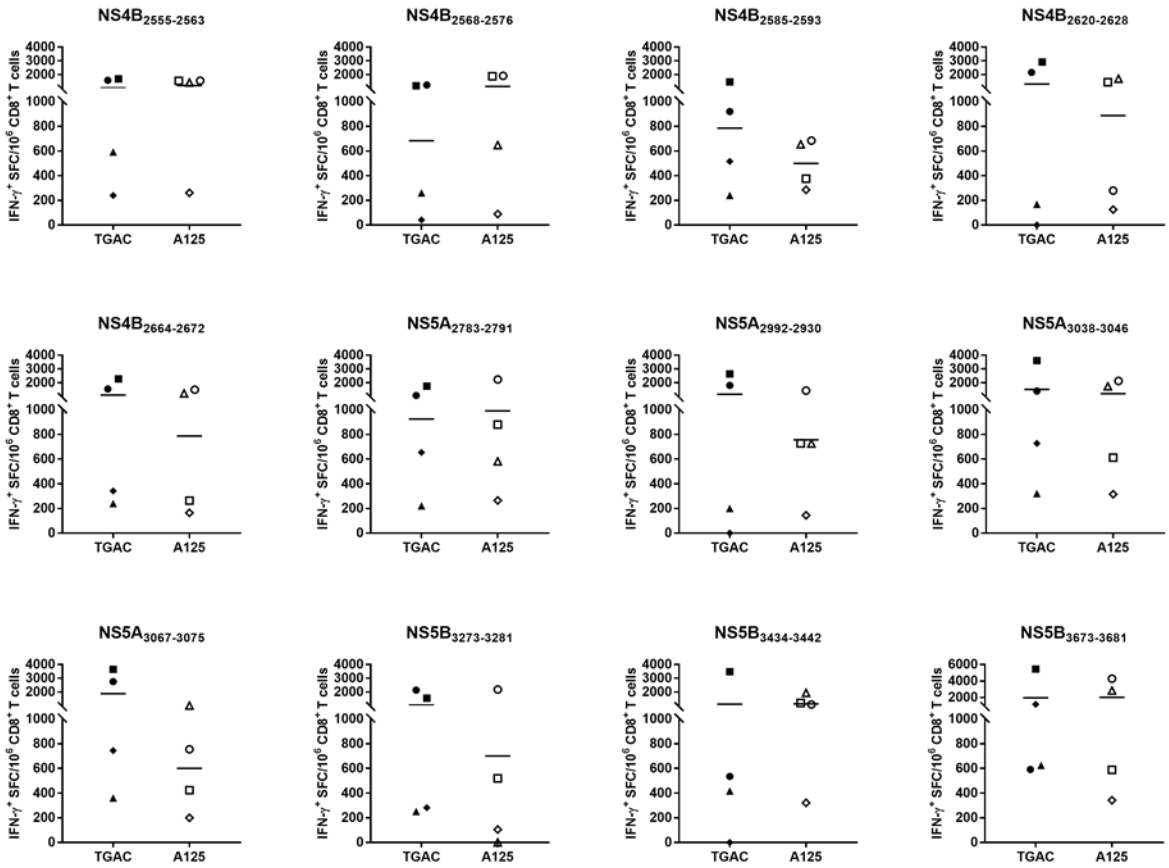

Figure 3.5 BVDV NS4B-, NS5A- and NS5B-derived broadly reactive CD8$^+$ T cell epitopes. CD8$^+$ T cells from BVDV-immunized steers [TGAC-immunized: 2539 (●), 2565 (▲), 2599 (■), and 2609 (♦); A125-immunized: 2593 (○), 2556 (Δ), 2601 (□), and 2611 (◊)] recognized various highly conserved bovine MHC I-binding epitopes predicted from BVDV-1b NS4B, NS5A, and NS5B. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells minus media background counts and bars represent the mean responses for the two groups.

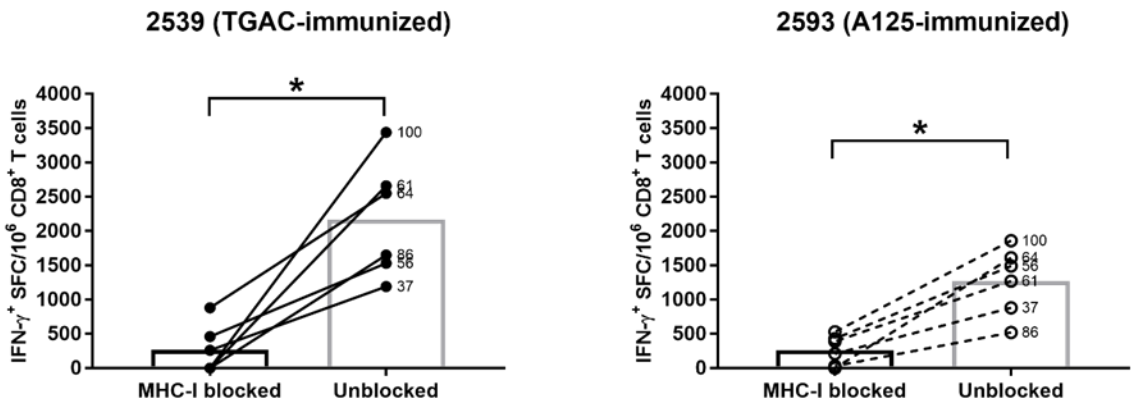

Figure 3.6 Predicted CD8$^+$ T cell epitopes from BVDV are bovine MHC I-restricted. Anti-bovine MHC I mAbs reduced IFN-γ^+CD8$^+$ T cell responses in two BVDV-immunized steers (2539 and 2593) against IFN-γ-inducing epitopes, $N^{pro}_{95\text{-}103}$, $E^{rns}_{493\text{-}501}$, $E1_{610\text{-}618}$, $E2_{999\text{-}1007}$, $NS4B_{2585\text{-}2593}$, and $NS5A_{2783\text{-}2791}$ [Peptides 61, 86, 56, 100, 37, and 64 respectively (Table 3.3)]. CD8$^+$ T cells and autologous CD14$^+$ monocytes were incubated with the individual peptides either in the presence or absence of anti-bovine MHC I mAbs. Responses are presented as spot forming cells (SFC) per million CD8$^+$ T cells minus media background counts and bars represent the mean responses for the two steers. Statistically significant differences between the responses of steers due to MHC I blockade is indicated by asterisks (* $p < 0.05$).

Chapter 4- Conclusion

Commercial vaccines are inadequate against diverse BVDV strains and their safety is questionable (Fulton et al., 2020). Studies have shown that the BVDV neutralizing antibody titer of 1:128 in vaccinated cattle is sufficient to protect against BVDV (Fulton et al., 2020; Fulton et al., 1997). MLV and KV vaccines induce >1:128 titer of neutralizing antibodies against strains from the included sub-genotypes and yet they are not efficacious (Downey-Slinker et al., 2016; Fulton et al., 2020). Studies also indicate that the BVDV-specific $CD4^+$ and $CD8^+$ T cell responses contribute towards protection (Beer, 1997; T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Lokhandwala et al., 2017). In addition, the difference in the protective efficacy of MLV and KV points at the protective role of $CD8^+$ T cell responses (Chase, 2013; P. H. Walz et al., 2018; Xue et al., 2011). Human *Flavivirus*es have highly conserved regions within their structural and non-structural antigens which contain protective T cell epitopes (Hickman & Pierson, 2017). In fact, a prototype T cell-based HCV vaccine was demonstrated to be protective against heterologous HCV strains in chimpanzees (Folgori et al., 2006). Hence, highly variable pathogens capable of escaping humoral immunity can be intercepted by vaccines that elicit robust T cell responses directed at more conserved epitopes. We know that BVDV E2 and NS2-3 contain defined $CD4^+$ T cell epitopes (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002). However, it is important to include and examine other BVDV antigens as well for protective T cell targets, specifically for the identification of cytotoxic $CD8^+$ T lymphocyte (CTL) epitope. Undoubtedly, there are two major gaps in the field of BVDV vaccine immunology: 1) The lack of an efficacious and safe vaccine; and 2) The lack of knowledge about the BVDV-specific $CD8^+$ T cell responses.

Therefore, our aim in the first study was to address the critical need for a better and safer BVDV vaccine. Experimental monovalent subunit vaccines are effective against cognate BVDV strain, but a broadly protective multivalent subunit vaccine is yet to be developed (Pecora et al., 2012; C. Thomas et al., 2009). We generated a mosaic subunit vaccine containing E2, NS2-3 antigens, and well-defined epitopes from diverse BVDV strains for conferring protection against BVDV-1 and -2 strains (Lokhandwala et al., 2017). The mosaic antigens were specifically designed to include relevant neutralizing B cell epitopes and defined IFN-γ-inducing $CD4^+$ T cell epitopes that are presented by MHC *DR* to BVDV-specific $CD4^+$ T cells (T. Collen, V. Carr, K. Parsons, B. Charleston, and W. I. Morrison., 2002; Lokhandwala et al., 2017). Our findings demonstrate that a rationally designed mosaic vaccine can provide protection against multiple BVDV-1 and -2 strains far better than a commercial KV vaccine. The novel mosaic vaccine successfully induced BVDV cross-neutralizing antibody and cross-protective T cell responses in cattle. Furthermore, upon challenge with a prevalent BVDV-1b strain from the United States, the prototype vaccine significantly outperformed the commercial vaccine in protecting the vaccinated cattle (Sangewar N, 2020). The findings from this study provide a foundation for the optimization and deployment of a broadly protective multi-epitope BVDV vaccine.

While BVDV neutralizing and $CD4^+$ T cell epitopes have been reported, information on defined CTL epitopes in BVDV is limited (Beer, 1997). Therefore, in the second study, we sought to identify novel BVDV $CD8^+$ T cell epitopes that can be incorporated in subunit vaccines to increase coverage and improve efficacy. In previous studies, low levels of bovine $CD8^+$ T cell activities were reported whereby pathogen-specific $CD8^+$ T cells were typically expanded *in vitro* via multiple rounds of re-stimulation (Beer, 1997; Simon P. Graham et al., 2006). In our study, we followed a hyper-immunization protocol using irradiated BVDV strains to prime high frequencies

of BVDV-specific bovine $CD8^+$ T cells *in vivo* (Singh et al., 2010). Consequently, we were able to demonstrate that the hyper-immunized cattle had high levels of IFN-γ^+CD8^+ T cell responses that were highly cross-reactive against BVDV-1 and -2 strains. We screened computationally predicted synthetic bovine MHC I-binding BVDV peptides *ex vivo* using the purified bovine $CD8^+$ T cells from the spleens of hyper-immunized animals. Hence, we identified novel IFN-γ-inducing $CD8^+$ T cell epitopes, from structural and non-structural BVDV antigens, that are highly conserved in more than 200 BVDV-1 and -2 strains. The outcome from this study offers a strong premise needed for the prospective research for BVDV CTL epitope(s) identification.

The primary goal of this work was to generate knowledge that will potentially lead to the development of an efficacious universal BVDV vaccine. Ideally, such a vaccine will incorporate shared as well as unique neutralizing, $CD4^+$ T cell, and CTL epitopes, and will elicit broad protective immunity against diverse strains. Such a vaccine will also require an optimized antigen delivery platform for optimal priming and expansion of effector and memory lymphocytes (Riitho, Strong, Larska, Graham, & Steinbach, 2020). A safe live vector that can rapidly induce and augment protective immunity after single immunization will be a cost-effective delivery platform. Additionally, an appropriate adjuvant will be needed for potentiating a balanced humoral and cellular immune responses (Sadat et al., 2017). An increased localization of $CD4^+$ and $CD8^+$ T cell populations in lungs was observed during primary BVDV infection in cattle and during secondary infection, the two T cell subsets were recalled in lungs at higher frequencies (Chase, 2013; Silflow, Degel, & Harmsen, 2005). Moreover, intranasal immunization with BVDV MLV can induce secretory IgA responses in cattle (Hill, Hunsaker, Townsend, van Drunen Littel-van den Hurk, & Griebel, 2012). Mucosal IgA responses are first line of defense against respiratory pathogens and have been demonstrated as modestly cross-protective against heterotypic Influenza A virus strains

(van Riet, Ainai, Suzuki, & Hasegawa, 2012). Since vaccinating herds of large animals via injections can be challenging, an intranasal BVDV vaccine conferring cross-protective mucosal immunity will be very advantageous. A single dose, easy to administer, universal BVDV vaccine which can reliably protect herds against various circulating strains will make a huge impact on global BVDV eradication programs.

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