CHARACTERIZATION OF BROADLY NEUTRALIZING ANTIBODIES TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)

A THESIS

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA BY

JORDAN ELLIOTT YOUNG

IN PARTIAL FULFILLEMNT OF THE REQUIRMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

MICHAEL P. MURTAUGH, ADVISER

CHERYL M.T. DVORAK, ADVISER

JANUARY 2020

© Jordan Elliott Young 2020

ACKNOWLEDGEMENTS

I would like to thank everyone in the Murtaugh lab, including my advisers Dr. Michael Murtaugh and Dr. Cheryl Dvorak, as well as all the Murtaugh lab members, Suzanne Stone, Diem Gray, Kyra Martin, Shaoyan Tan and Frances Shepard, for their guidance and friendship.

I would also like to acknowledge my committee members, Hinh Ly, Maxim Cheeran, Mark Rutherford and Simon Graham for their input and suggestions that shaped this project into its final form and helped me become a competent scientist.

I am also gracious of the training and hospitality provided to me by everyone at the Pirbright Institute. Special thanks to Simon Graham, David Goldeck and Jack Hayes whose expertise helped develop the initial steps of this thesis.

Thank you to AIMM Therapeutics for providing the retroviral vector and reagents that made this entire project possible. Thank you to CEO John Womelsdorf for agreeing to send us materials and Arjen Bakker for his help in implementing the vector and assistance in navigating the never-ending bureaucracy that is the US Customs.

I am also gracious for the funding provided by USDA NIFA grant #2016-67015-24928 and the Sam Maheswaran Graduate Fellowship, that made the completion of this thesis possible.

Finally, I would like to thank all my family and friends who supported me from beginning to end of this project.

DEDICATION

This thesis is dedicated to the memory of Dr. Michael Murtaugh, who inspired this work and who's passion for research will continue to inspire others for years to come. And to my soon to be wife, Corinne, who always stood by me, and with her love and encouragement, fueled me throughout my time as a graduate student.

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an all too common disease with a devastating impact on pork producers in the US and worldwide. The disease is caused by the PRRS virus (PRRSV) which leads to abortions and other forms of reproductive failure in sows and severe respiratory disease in adults and neonates. Unfortunately, current vaccines against PRRSV provide limited protection and often only protects against closely related viruses. Efficacious vaccine-based prevention of infectious disease is based on antigen-specific long-lived memory B and T cells. Protective, or neutralizing, antibodies produced by memory B cells, that are activated in response to virulent pathogen challenge, are critical to this process. Thus, it is of utmost importance to investigate antibodies with neutralizing potential especially broadly neutralizing antibodies (bnAbs), which possess the ability to neutralize distantly related strains. In this manuscript, I take a reverse approach to the classic neutralization study, starting first with pigs we know are broadly neutralizing and then investigating the identity of the responsible B cells, using a novel technique for the isolation of PRRSV neutralizing antibodies. PBMCs were harvested from pigs sequentially exposed to divergent PRRSV isolates and vaccine. Memory B cells were then transduced, with a proprietary retroviral vector developed by AIMM Therapeutics, containing genes highly expressed by germinal center B cells, creating an immortalized B-cell population. B-cells were then sorted by FACS and five PRRSV-specific B cells were isolated. All identified PRRSV-specific antibodies were found to be broadly binding to all PRRSV-2 isolates tested, but not PRRSV-1. Antibodies against GP5 protein, commonly thought to be the PRRSV neutralizing epitope, were found to be highly abundant, as four out five clonal B cells were GP5 specific. Next, an isolated GP5 clone was discovered to be neutralizing against homologous, but not heterologous PRRSV. Sequencing of this clone's heavy

chain variable region and CDR3 revealed a gene that was heavily mutated compared to germline sequence, suggesting somatic hypermutation playing an essential role in generation of broadly neutralizing antibodies. Further investigation of these antibodies, and others, may lead to the elucidation of conserved neutralizing epitopes that can be exploited for improved vaccine design and lays the groundwork for the study of bnAbs against other porcine pathogens.

Hypothesis: Broad neutralization against PRRSV is a product of antibodies from multiple B-cell populations, producing a polyclonal response, as opposed to a single monoclonal neutralizing antibody.

Aim 1: Isolation and immortalization CD21+, IgG+ memory B-cells that bind PRRSV.

Aim 2: Evaluation and characterization of secreted PRRSV-specific antibodies.

Aim 3: Determination of virus-binding and neutralizing B-cell V(D)J mRNA sequences.

TABLE OF CONTENTS

Acknowledgements **i**
Dedication **ii**
Abstract **iii**
List of Tables **viii**
List of Figures **ix**
Abbreviations **x**
CHAPTER I: Literature review **1**
- **Introduction** **2**
- **PRRSV structure and organization** **2**
- **Porcine humoral immunity** **7**
- **The pig's immune response to PRRSV** **10**
- **Neutralization of PRRSV** **17**
- **Isolation of B cells and antibodies** **22**

CHAPTER II: Isolation and immortalization of PRRSV-specific IgG+ memory B cells **25**
- **Introduction** **26**
- **Methods** **27**
  - Cells and viruses 27
  - Evaluation of serum for PRRSV neutralization 28
  - Flow cytometry and cell sorting 29
  - Isolation and immortalization of IgG+ memory B cells 30
  - PRRSV antigen-baiting of immortalized memory B cells 30
  - Detection of PRRSV specific antibodies via IFA 31
  - Isolation of PRRSV specific clones from PRRSV binding populations 32
- **Results** **33**
  - Identification of swine showing evidence of broad neutralization against PRRSV 33
  - Isolation, immortalization and identification of PRRSV-specific IgG+ memory B cells from porcine PBMCs 36
  - Creation of immortal PRRSV-specific clonal B cell lines 38
  - Characterization of PRRSV-specific clonal B cells 38
- **Discussion** **40**

CHAPTER III: Evaluation and characterization of PRRSV-specific antibodies **45**
- **Introduction** **46**
- **Methods** **47**

Immunofluorescent analysis (IFA) of antibody binding to diverse viral strains 47
Identification of PRRSV protein specific antibodies via ELISA 49
Evaluation of serum and B cell supernatants for PRRSV neutralization 50
PRRSV GP5 N-glycosylation prediction 51
Results 51
Antibody binding to diverse PRRSV strains 51
Evaluation of PRRSV protein specificity of clonal antibodies 52
Neutralization activity of secreted antibodies from immortalized B cell clones 56
Predicted N-glycosylation of PRRSV-2 ATP and VR2332 GP5 viral protein 58
Discussion 58
CHAPTER IV: Determination of virus-binding and neutralizing B cell V(D)J mRNA sequences 64
Introduction 65
Methods 65
Isolation and sequencing of PRRSV-specific B cell V(D)J mRNA 65
Results 66
Identification and characterization of V(D)J regions of neutralizing and non-neutralizing clones 66
Discussion 70
CHAPTER V: General Discussion 75
References 91

LIST OF TABLES

Table 1: Timeline of Pig Vaccination/Virus Challenge and Inoculation 34

Table 2: Primers used for PCR amplification and sequencing of porcine B cell V(D)J regions. 67

LIST OF FIGURES

Figure 1: PRRSV genomic RNA and PRRS viral structure 5

Figure 2: Neutralization of PRRSV-2 ATP and VR2332 and PRRSV-1 SDEU by pigs BNW4 and BNW7 35

Figure 3: Identified PRRSV-specific antibodies 37

Figure 4: Surface marker characterization of BNW7 clones p5c4 and p10c5 39

Figure 5: Whole genome phylogenetic tree of PRRSVs examined for antibody reactivity. 53

Figure 6: Ability of clonal antibodies to bind a diverse panel of PRRSV strains 54

Figure 7: Identification of antibody reactivity against PRRSV proteins 55

Figure 8: Neutralizing activity against PRRSV of antibodies secreted from immortalized B cells. 57

Figure 9: Isolated heavy and light chain PCR products from BNW7 p5c4 and p10c5 V(D)J mRNA 68

Figure 10: Sequence comparison of BNW7 p5c4 V(D)J mRNA to fetal mRNA pvg 24 71

ABBREVIATIONS

Activation-Induced Deaminase	AID
Antibody	Ab
Antibody dependent enhancement	ADE
Antigen presenting cell	APC
B cell receptor	BCR
Broadly neutralizing antibodies	bnAbs
Cluster of differentiation	CD
Complimentary determining region	CDR
Days post infection	dpi
Equine arterivirus	EAV
Fetal bovine serum	FBS
Fluorescent-activated cell sorting	FACS
Green fluorescence protein	GFP
Glycoprotein	GP
Human immunodeficiency virus	HIV
Immunoglobulin	Ig
Interferon	IFN
Interleukin	IL
Iscove's Modified Dulbecco's Medium	IMDM
Lelystad virus	LV
Minimal essential medium	MEM
Non-structural protein	NSP
Open reading frame	ORF
Peripheral blood mononuclear cell	PBMC
Porcine alveolar macrophage	PAM
Porcine reproductive and respiratory syndrome virus	PRRSV
United States Dollars	USD

CHAPTER I: LITERATURE REVIEW

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an all too common disease with a devastating impact on pork producers in the US and worldwide. The disease is caused by PRRS virus (PRRSV) which leads to abortions and other forms of reproductive failure in sows and severe respiratory disease in adults and neonates (Zimmerman et al. 2019). Efficacious vaccine-based prevention of infectious diseases, such as PRRSV, is based on antigen-specific long-lived memory B and T cells. Protective, or neutralizing, antibodies produced by memory B cells, that are activated in response to virulent pathogen challenge, are critical to this process. Viral neutralization is brought about by an antibody blocking a specific viral protein, preventing the virus from infecting its host cell, and flagging it for digestion and destruction by the immune system. In the pig, neutralizing antibodies are a critical component of the immune response and the mechanism by which effective vaccines protect against infection. Even more important for adequate protection is the ability of a pig to develop broadly neutralizing antibodies. These are antibodies that can bind multiple genotypes of related virus and produce a neutralizing effect, despite never being exposed to these exact genotypes previously. Elucidation of conditions leading to consistent development of broadly neutralizing antibodies would allow for design and development of highly effective vaccines against almost any pathogen. In this study, generation of PRRSV-specific B cell lines are described, antigen specific neutralization of PRRSV is explored and other potential antibody mediated mechanisms are discussed.

PRRSV structure and organization

Porcine reproductive and respiratory syndrome, formally mystery swine disease, was first reported and documented in domestic swine in the mid-1980s in North America (Hanada et al. 2005), followed by a similar syndrome in Europe and Asia by 1991

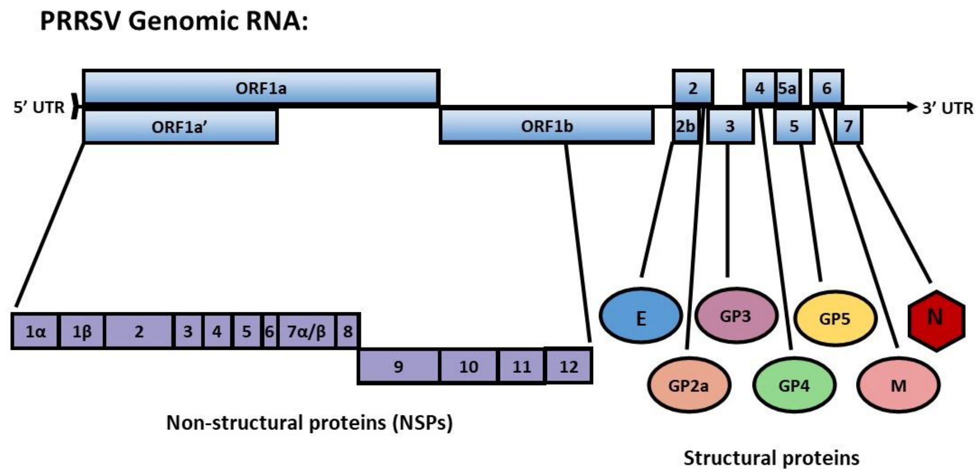
(Albina 1997). In 1991, the virus was isolated in both the Netherlands (Wensvoort et al. 1991) and the United States (Collins et al. 1992), the former where Koch's postulate was first proven for the virus (Terpstra, Wensvoort and Pol 1991). Although the origins of the virus remain unknown, it is thought to have jumped from an unidentified host to swine in 1980 (Hanada et al. 2005). What makes PRRSV devastating is its dual pathologies of respiratory and reproductive disease. The virus can spread rapidly amongst both neonates and adult pigs through multiple routes of transmission, including aerosolization, causing severe respiratory disease that often leads to secondary infections and death in nursery age pigs. In sows it is common to see sudden loss of litters between days 21 and 109 of gestation, meaning spontaneous abortions can occur almost at any point during pregnancy (Zimmerman et al. 2019). The infection can also remain persistent in pigs for several months before it is totally cleared (Horter et al. 2002, Allende et al. 2000). Because of these clinical manifestations and viral persistence, PRRSV remains to be an economically devastating disease in swine herds worldwide, costing an estimated $6.25 to $15.25 USD per marketed pig in North America and Europe or $664 hundred million USD in the USA alone in 2011 (Holtkamp, Kliebenstein and Neumann 2013b, Nathues et al. 2017).

Porcine reproductive and respiratory syndrome virus is a member of the family *Arterivirdae* within the order *Nidovirales.* It is a single stranded, positive sense, enveloped RNA virus, 45-70 nm in diameter, with a genome that averages 15.2 kb in length, encoding for 11 known open reading frames (ORFs) (Lunney et al. 2016). The virus was previously classified into two PRRSV genotypes, PRRSV type 1 (PRRSV-1), the European type viruses, and PRRSV type 2 (PRRSV-2), the American type viruses. However, the names of these genotypes was a misnomer; albeit they were first observed in Europe and North America, both now have worldwide distribution (Fang et

al. 2007, Gao, Guo and Yang 2004). The two genotypes are very dissimilar, with only 50-60% sequence identity (Nelsen, Murtaugh and Faaberg 1999, Forsberg 2005) and have now been designated as two entirely separate species (Adams et al. 2016, Kuhn et al. 2016).

The PRRSV genome is translated into 11 major open reading frames (ORFs), encoding both non-structural and structural proteins. The first two main open reading frames of the virus, ORF1a and ORF1b encode the 14 known non-structural proteins (NSPs)(Fig. 1). These proteins have various known functions, ranging from virus replication to immunosuppression. NSP1α, NSP1β, NSP2 and NSP4 all encode for proteases thought to be involved in the down regulation of the pigs IFNα response (Chen et al. 2010, Li et al. 2010, Beura et al. 2010, Sun et al. 2010). NSP3 and NSP5 are transmembrane proteins thought to be involved in membrane modification and formation of the replication complex (Posthuma et al. 2008). Little is known about the functions of NSP5,6,7,8 and 12, however NSP7 has been shown to be highly antigenic (Brown et al. 2009). NSP9, 10 and 11, within ORF1b, have been found to be highly conserved amongst viruses within the order *Nidovirales* (Gorbalenya et al. 2006). NSP9 encodes for the viral RNA-dependent RNA polymerase, NSP10 encodes for the RNA helicase that contains a putative zinc-binding domain, and NSP11 encodes for a uridylate-specific endoribonuclease, all thought to be part of the PRRSV replication complex (Ulferts and Ziebuhr 2011).

Open reading frames 2 through 6 encode for envelope associated structural proteins in PRRSV (Fig. 1). ORF 5 and 6 encode respectively for glycoprotein 5 (GP5) and matrix (M) viral proteins, the major envelope proteins for PRRSV. GP5 and M together form a heterodimer that is essential for production of viral particles



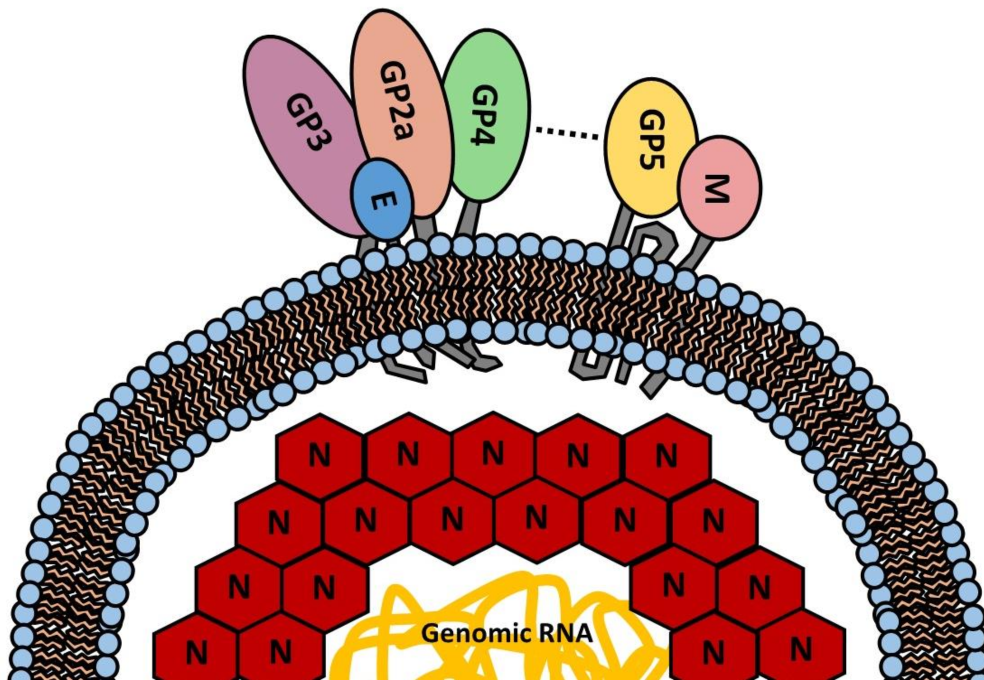


Figure 1: PRRSV genomic RNA and PRRS viral structure

PRRSV genomic RNA and their associated viral proteins are presented at the top of this diagram. The figure at the bottom is a representation of the PRRSV viral envelope and capsid structure. The dotted line between GP4 and GP5 represents a recently discovered strong interaction between the two proteins (Das et al. 2010). Neither graphics are to scale.

(Wissink et al. 2005), however expression of both viral proteins alone is not enough to release viral particles from cells in a closely related virus, equine artevirus (EAV), suggesting other factors are involved in the process (Wieringa et al. 2004). M protein is the mostly highly conserved structural protein in PRRSV with 96-100% identity amongst American and Canadian isolates (Meng et al. 1995), while GP5 appears to be the most highly variable with only 55% sequence identity between European and American isolates (Murtaugh, Elam and Kakach 1995). The GP5/M complex is involved in the first step of PRRSV infection, with a low affinity attachment of the viral protein complex to heparan sulfate (HS) on the porcine alveolar macrophage's (PAMs) surface, its host cell, followed by a high affinity attachment of GP5 to sialic acid-binding protein sialoadhesin (Sn/Siglec-1/CD169) (Delputte, Costers and Nauwynck 2005). It should be noted that the sialoadhesin molecule is not present on the PRRSV permissive cell line, MARC 145, but most viruses can eventually be adapted to infect them (Shi et al. 2015). Once the virus is internalized, such as in a macrophage endosome, uncoating and release of the viral particle into the cytoplasm is dependent on GP2a-GP3-GP4's interactions with the host scavenger receptor cluster of differentiation 163 (CD163) (Calvert et al. 2007). ORF 2, 3 and 4, encode the viral structural proteins GP2a, GP3 and GP4 respectively, and along with viral protein E (from ORF2b), are considered minor envelope proteins. Recent work has shown that GP2a and GP4 interact directly with CD163 (Das et al. 2010). Das et al. showed a strong interaction between GP4 and GP5, possibly facilitating the step between PAM adhesion (GP5/M) and virus internalization (GP2a-GP3-GP4) (Das et al. 2010). The small 73 amino acid long E protein is expressed from ORF2b and is thought to form an ion channel that lowers the internal pH of the virus upon fusion with the endosomal compartment (Lee and Yoo 2006). GP2a, 3 and 4 form a multimeric complex that is associated with E, where each protein is essential for complex formation and

infectivity (Wissink et al. 2005, Welch et al. 2004). ORF5a encodes an additional small structural protein, sharing the same name, ORF5a, that has been shown to be essential for virus viability, however little is known about its function (Sun et al. 2013).

ORF 7 encodes for the single, 123-128 amino acid, nucleocapsid protein (N) (Fig. 1). N protein has been found to be the most abundantly expressed protein (Dea et al. 2000) and the most immunogenic, although antibodies against it have been shown to be non-neutralizing (Murtaugh, Xiao and Zuckermann 2002). N protein is the major component of the viral nucleocapsid where it interacts and packages viral RNA (Dokland 2010). It has also been demonstrated to shuttle to the host nucleus during infection and may affect nuclear processes during viral replication (Yoo et al. 2003). Although it has no apparent role in neutralization, it is commonly used as a serological diagnostic tool.

Porcine humoral immunity

While not the first line of defense against infectious disease, the pig's adaptive immune system is responsible for the eventual clearance and prevention of pathogens. It accomplishes this through the development and identification of pathogen-specific B and T lymphocytes. Commonly referred to as B and T cells, these multifaceted cells establish antigen-specific receptors during their development in either the bone marrow (B cells) or thymus (T cells). When activated, antigen-specific B cells and T cells, bind their specific pathogen associated antigen, stimulating themselves and other immune cells against the pathogen or infected cell. The arm of the adaptive immune system associated with B cells is called the humoral immune system.

B cells begin life as hematopoietic stem cells in the bone marrow; a pluripotent cell capable of transforming into many different immune cells. Through a series of cytokine signals, a pro-B cell is developed. Next, random recombination of the cell's

antigen specific receptor, heavy and light chain immunoglobin, occurs, a process called V(D)J recombination, until a functional B cell receptor (BCR) is developed. This process is independent of antigen stimulation. The B cell randomly rearranges a single gene locus from the variable region (VH), diversity region (DH), and joining region (JH) for its heavy chain. The pig genome contains approximately 30 VH genes, all of which are homologous to the VHIII family in humans (Sun et al. 1994). Of these 30 functional VH genes, only 7 are commonly used to form over 90% of the heavy chain VDJ repertoire from gestation through adult life (Butler et al. 2011). There are five DH genes and five JH genes, however only two DH and a single JH are functional (Eguchi-Ogawa et al. 2010). Despite the lack of genetic variety in the genes encoding the porcine heavy chain, the pig can produce highly diverse immune responses, in most part thanks to somatic hypermutation of the V(D)J region (Butler and Wertz 2012). The pig creates its antibody light chain, containing a single variable and joining region, from one of two loci, lambda and kappa. The lambda locus contains 12-13 functional variable regions and 4 joining regions and the kappa locus contains 14-50 variable regions and 5 joining regions (Schwartz, Lefranc and Murtaugh 2012a, Schwartz, Lefranc and Murtaugh 2012b). Porcine light chain expression is unique in that both kappa and lambda make up 50% of the light chain expression within the B cell population (Skvaril et al. 1976, Sun et al. 2012), while most other ungulates express lambda as the dominant light chain (Butler 1997). Also differing from other mammals, in the pig light chain loci rearrangement precedes heavy chain rearrangement (Sinkora, Sinkorova and Stepanova 2017). Once both heavy and light chain segments are rearranged, they are expressed as a BCR containing two copies of the heavy chain, linked to two copies of the light chain.

The newly randomly developed BCR is specific to a single, unknown antigen, which upon activation will convert the B cell into an antibody secreting plasma cell. The

antibody secreted by this plasmid cell is identical in structure to the BCR and is also specific to the same single, unknown antigen. This newly formed immature B cells BCR is immunoglobulin class IgM, an immature form, that will class switch to a more mature form, such as IgA, IgE or IgG, after contact with its specific antigen. Before it leaves the bone marrow, the B cell's BCR is tested against self-antigens to confirm no auto-reactivity and is then sent out to secondary lymphoid organs, such as lymph nodes, mammary tissue, tonsils or gut-associated lymphoid tissues.

Once the B cell has arrived at a secondary lymphoid organ, it is in a resting state as it waits for interaction with its cognate antigen. The swine lymph node is uniquely structured, with B cell germinal centers developing in the interior of the node as opposed to the cortical region commonly seen in other mammals (Rothkötter 2009). Interaction with the B cell's specific antigen can occur through several means. Most commonly, B cells interact with free antigen or antigen presented on antibodies attached to antigen presenting cells (APCs), such as macrophages or dendritic cells. This alone can activate a B cell, but optimal stimulation involves interactions with activated antigen-specific T cells, most often CD4+ T cells also known as helper T cells (Th cells). Th cells interact and activate the B cell through release of cytokines and contact with costimulatory molecules on the T cells surface, including CD40L. This enhances and invigorates the B cells response to antigen, stimulating B cell mitosis, clonal expansion and antibody class switching. The B cell itself can also act as an APC, engulfing antigen bound to its BCR, then processing and presenting antigen on MHC class II molecules and activating T cells.

Upon activation, the B cell has two common routes, a long-lived memory B cell or a short-lived antibody secreting plasma cell. Both of these outcomes are essential for

developing protective humoral immunity. When initially stimulated, by antigen and cytokines from CD4+ T helper cells, an immature B cell has immunoglobulin class M (IgM) as its surface BCR. The now stimulated B cell goes through rapid mitosis, creating numerous clones of itself. Some of these clones go on to become plasma cells, secreting antigen specific pentameric IgM, designed to bind multiple antigens and cluster associated pathogens. Other IgM B cells, with the stimulatory aid of Th cells, will go on to become long lived memory B cells, which often involves class switching to either IgD, IgG, IgE or IgA immunoglobulin, each of which has unique properties and common areas of circulation. These memory B cells retain their original antigen specific heavy and light chains but switch the Ig class constant region. When these memory B cells are reactivated, by either re-stimulation during the initial infection or subsequent repeat infections, the cells convert to antigen specific plasma cells, of their new Ig class, in a much more efficient manner than the initial antigen exposure. During this time, the heavy and light chain genes of each B cell go through almost constant somatic hypermutation, where, through enzymatic activity by activation-induced deaminase (AID) and error prone DNA replication, base pairs are switched and mutated. This random mutation can, in rare instances, lead to affinity maturation of the BCR and secreted antibody, enhancing the response and binding to antigen. These long-lived memory B cells persist and circulate through the blood and lymphoid organs, often for the entire life of a domestic pig, and are incredibly important for long term protection from repeated exposure to pathogens.

The pig's immune response to PRRSV

Pigs are highly susceptible to PRRSV infection, with viable routes of exposure including, intranasal, intramuscular, oral, intrauterine and vaginal (Zimmerman et al. 2019). Once the virus has crossed primary immune barriers, it infects porcine alveolar

macrophages (PAMs)(Duan, Nauwynck and Pensaert 1997), but has also been shown capable to infect pulmonary intravascular macrophages (PIMs) (Thanawongnuwech, Thacker and Halbur 1997), monocyte-derived macrophages (MDMs)(Duan et al. 1997), and to a lesser extent dendritic cells (DCs) (Loving, Brockmeier and Sacco 2007). The virus is not able to infect every type of macrophage, as peripheral blood monocytes and peritoneal macrophages are resistant to infection (Duan et al. 1997). Macrophages play important roles in tissue remodeling, immunity and immunopathology, so their elimination by PRRSV likely has immunomodulatory effects that benefit the virus's survival. The virus replicates rapidly in both lung and lymphoid tissue. Once PRRSV has bypassed the initial physical and chemical immune barriers, it interacts with the pig's innate immune system.

When a viral infection takes place, a common innate immune response is the upregulation of type 1 interferons (IFNs). IFN-α and IFN-β secretion from infected cells are key in inducing the innate immune system into an anti-viral response. Essential players in this process are antigen presenting cells (APCs), such as macrophages and dendritic cells, who are efficient secretors of type 1 IFNs. Dendritic cells are especially efficient type 1 IFN producers thanks to their ability to detect virus before infection through interactions of toll-like receptor 7 (TLR7) in endosomes interacting with viral RNA, leading to a wave of type 1 IFN production (Baum and García-Sastre 2010). During PRRSV infection however, IFN-α responses appear to be actively suppressed, when compared to responses to other porcine viruses (Albina et al. 1998, Van Reeth et al. 1999). It has been determined that transcription of type 1 IFN genes does occur in alveolar macrophages and dendritic cells during PRRSV infection (Genini et al. 2008), but secretion of functional protein fails (Miller, Lager and Kehrli 2009, Zhang et al. 2012). This suggests that PRRSV somehow controls type 1 IFN expression post-

transcriptionally (Wang and Christopher-Hennings 2012) or that the rapid destruction of alveolar macrophages stunts the response (Weesendorp et al. 2013). This down regulation of IFN-α leads to a delayed interferon gamma (IFN-γ) associated antigen specific T cell response, which requires type 1 IFN and other cytokines (Meier et al. 2003). PRRSV non-structural proteins, NSP1α, NSP1β, NSP2, and NSP4 have all been implicated in the modulation of IFN-mediated host innate immunity (Li et al. 2010, Chen et al. 2010, Beura et al. 2010, Sun et al. 2010).

Natural killer cells (NK cells) play an important role in the innate immune system's early interactions with PRRSV and impact the later adaptive immune response. NK cells normally secrete IFNγ, are cytotoxic to infected cells and those not expressing MHC-I, induce maturation of dendritic cells, and regulate innate immunity (Shekhar and Yang 2015). During initial PRRSV infection, there is a sharp spike in IFNγ levels in serum at day 10 dpi, thought to be attributed to an early NK response (Wesley, Lager and Kehrli 2006). Although this response is too early to effect the T cell response, it may interact with early humoral non-neutralizing antibodies and perform antibody dependent cell-mediated cytotoxicity (ADCC) on PRRSV infected macrophages (Rahe and Murtaugh 2017a). Macrophages have also been shown to produce IFNγ in the presence of PRRSV infection (Thanawongnuwech et al. 2003). Some PRRSV strains have also been demonstrated to actively suppress NK cell cytotoxic activity, without effect on NK cell frequency (Dwivedi et al. 2012, Manickam et al. 2013, Renukaradhya et al. 2010). Once PRRSV has broken through the innate immune response, the pig's adaptive immune response begins to build its own virus specific counteroffensive.

Antibodies and the B cells that make them are thought to be important in the immune response against PRRSV. The pig mounts an antibody response against

PRRSV by 7-9 days post infection (dpi), however the development of neutralizing antibodies is not believed to appear until over 28 dpi (Yoon et al. 1994). PRRSV-specific memory B cells and antibody secreting plasma cells are found in many lymphoid tissues, but are especially prominent in lymph nodes draining from the lungs, genitals, spleen and tonsils (Mulupuri et al. 2008), where virus commonly multiplies and often becomes persistent. PRRSV-specific IgM is the first class of antibody to appear at 7 dpi, with peaks in titer between 14 and 21 dpi, and then quickly decreasing to undetectable levels at 35 to 41 dpi. PRRSV-specific IgG first appears at 7 to 14 dpi, peaks at 21 to 28 dpi and then remain high through the persistent phase of infection (Loemba et al. 1996). The initial immune response has been shown to concentrate on development of antibodies against first E protein (Loemba et al. 1996) at 7 dpi, followed by nucleocapsid (N), M protein and GP5 appearing at 14 to 21 dpi (Nelson, Christopher-Hennings and Benfield 1994). In germfree piglets exposed to PRRSV, polyclonal B cell activation, lymphoid adenopathy and hypergammaglobulinemia were seen, although <1% of B cells were found to be PRRSV specific (Lemke et al. 2004), suggesting PRRSV's involvement in immune dysregulation. However, there should be caution in overinterpreting these results, as the germfree pigs in this study are missing many immune components, such as microflora and maternal antibodies, that are present in conventionally reared animals, making translation of these results to virus in the field dubious.

A cell-mediated T cell response is thought to be an important avenue by which the pig eliminates PRRSV, however there remains many gaps in our current knowledge. The pig's T cell response is characterized by CD3, CD4 and CD8 phenotyped cells that secrete IFN-γ. The pig uniquely has a large population of CD4/CD8 double positive, IFN-γ secreting, T cells that represent memory T cells (Zuckermann and Husmann 1996). It has been determined that, during PRRSV infection, CD4+ T cells decrease in the blood

3-7 dpi, but return to normal levels 7-14 dpi (Nielsen and Bøtner 1997). CD8+ T cells increase by 28-35 dpi (Shimizu et al. 1996), specifically showing increased CD8+ populations in the lungs (Samsom et al. 2000) and lymphoid tissues (Gómez-Laguna et al. 2013). Cytotoxic CD8+ T lymphocytes are thought not to be involved in the clearance of acute infection (Lohse, Nielsen and Eriksen 2004), but rather have been shown to be involved in the clearance of persistent infection (Costers et al. 2009, Lamontagne et al. 2003). PRRSV-specific T cells are first observed at 14 dpi, but there is extensive variation in timing amongst individual pigs (Xiao et al. 2004). Memory recall CD4+ T cells are present 28 dpi and have been shown to remain present for more than 3 months post infection (López Fuertes et al. 1999). It must be noted that the phenotypic differences between effector and memory T cells is not well studied or characterized in swine, so labeling T cells as such is difficult. Overall, the pig shows a delayed and limited T cell response to virus that is not thought to be involved in initial changes in virus load (Xiao et al. 2004), yet is still critical to developing immunity to the virus.

Despite an eventual immune response, PRRSV infection often remains persistent. This persistent infection is referred to as a “chronic persistent” infection in which pigs show little to no clinical signs and the infection is eventually cleared after a long period of time. Infections have long been documented to persist in infected pigs for 105 to 150 dpi in tonsillar and lymph node tissue (Horter et al. 2002, Allende et al. 2000), with viral RNA detectible upwards of 251 dpi by RT-PCR (Wills et al. 2003). PRRSV persistent infection involves continuous low level viral replication as opposed to a steady-state persistent infection (Allende et al. 2000). The mechanism by which PRRSV evades the immune system is not known, however it has been shown that virus goes through very low rates of mutation of ORF5 (GP5 protein) during persistent infections (Chang et al. 2002), suggesting that evasion of immunity through evolution is unlikely.

This evasion and persistence makes PRRSV incredibly difficult to eliminate from domestic herds.

In a recent review, Lunney et al. proposed several possible mechanisms for the delay in development of neutralizing antibodies against PRRSV (Lunney et al. 2016), all of which may enhance immune evasion and influence virus persistence in the pig. One of the proposed mechanisms, that has been widely studied, is the use of glycan shielding by PRRSV's glycoproteins, GP2a, GP3, GP4, and GP5 for immune evasion. Each of the aforementioned GPs possess two to six associated N-glycan moieties that are linked with specific amino acids and may interfere with antibody binding to sites of neutralization. It has been demonstrated that removing N-glycan sites on GP5 improve immunogenicity and pace of developing GP5 specific antibodies, but does not affect the virions sensitivity to neutralization (Faaberg et al. 2006). It appears glycosylation is involved in resistance of virus to the development of neutralization and that glycosylation sites down stream of GP5's neutralizing epitope are involved (Wei et al. 2012). Vu et al. showed similar findings upon investigation of GP3 (Vu et al. 2011). Removing N-glycan shields from these proteins has been proposed as a possible way to enhance protective efficacy in vaccines (Ansari et al. 2006).

A mechanism by which non-neutralizing PRRSV-specific antibodies may increase rates of infection is antibody dependent enhancement (ADE). First proposed to be involved in PRRSV infection by Yoon et al., the theory asserts that PRRSV infects macrophages secondarily though macrophage engulfment of virus laden with non-neutralizing antibody, enhancing overall infectivity of the virus (Yoon et al. 1996, Yoon et al. 1997). ADE has already been shown to occur in several other viruses, including flaviviruses and coronaviruses (Mady et al. 1991, Yip et al. 2016, Takano et al. 2017).

There has even been work showing that macrophage CD16 is involved in PRRSV ADE (Gu et al. 2015). The effect ADE of PRRSV has *in vitro* is well studied, but it's effect *in vivo* are controversial. Two studies have sought to investigate non-neutralizing antibodies role in PRRSV ADE *in vivo.* Yoon showed that pigs given non-neutralizing antibodies before virus inoculation had a 1 to 2 log increase in viremia compared to the virus only group (Yoon et al. 1996). However, Lopez et al. could not replicate these findings and showed non-neutralizing antibodies had no effect on virus production in the pig, but did raise rectal temperatures (Lopez et al. 2007). The difference in *in vitro* and *in vivo* results most likely points toward secondary immune factors present within the live pig, such as NK cells and cytokines, that are not represented in lab assays, leading to the discrepancies seen. Together this paints an unconvincing story of an effect of PRRSV ADE's *in vivo.*

Current vaccines against PRRSV provide limited protection and often only against closely related homologous viruses (Kimman et al. 2009). Within populations of swine, response to the virus, especially the generation of neutralizing antibody, differs from pig to pig (Mateu and Diaz 2008, Plagemann 2006), with some pigs developing a broad neutralizing response, some developing no neutralizing response at all, and many pigs developing solely homologous immunity to exposed virus/vaccine. Cross-protection has been shown to be achievable in pigs, but it does not always occur (Martínez-Lobo et al. 2011). Cross-protection or broad neutralization can rarely be achieved with MLV vaccine alone, but with the addition of heterologous virus challenge, the immune response is enhanced (Scortti et al. 2006). Several studies have reported similar findings, with enhanced neutralization after multiple homologous or heterologous PRRSV exposures (Robinson et al. 2015, Li et al. 2014). The adaptive immune systems development of protective immunity depends upon interactions with the innate immune

system. With PRRSV having a tropism for macrophages, these essential interactions are most likely dampened by elimination of macrophages. To make the situation more complicated, PRRRS virus is highly mutagenic, with a mutation rate of $4.7–9.8 \times 10^{-2}$/site/year, the highest rate of any known RNA virus (Hanada et al. 2005), potentially leading to virus that mutates and escapes neutralization over time. Because of this, development of an early broadly neutralizing immune response in the pig is of utmost importance.

Neutralization of PRRSV

The role of neutralizing antibodies in the clearance of PRRSV infection has historically been controversial. Neutralizing antibody had long been thought to be unimportant in control and prevention of PRRSV infection. Early papers demonstrated that the development of neutralizing antibodies was not rapid enough to prevent the establishment of chronic infection (Nelson et al. 1994) and that PRRSV persists in lymphoid tissues despite the presence of PRRSV-specific antibodies (Allende et al. 2000). Some have even suggested non-neutralizing antibodies enhance PRRSV infection through ADE (Yoon et al. 1996). However, recent studies have pointed toward neutralizing antibodies having a more critical role. PRRSV neutralizing antibodies only appear after the resolution of viremia, but high levels of neutralizing antibodies in serum are a predicter of shorter length and lower levels of viremia (Molina et al. 2008). Passive transfer of neutralizing antibodies has been shown to prevent reproductive disease in sows and inhibit viral transmission of disease to offspring (Osorio et al. 2002). It has also been established that broad neutralizing antibody production develops in some pigs and provides cross-protection against heterologous virus (Robinson et al. 2015, Robinson et al. 2018). These works demonstrate that neutralizing antibodies against PRRSV have an important role in suppression of infection.

The exact epitope responsible for the generation of neutralization against PRRSV in the pig has remained elusive for decades. The majority of historical neutralization studies have concentrated on the GP5 ectodomain (Pirzadeh and Dea 1997, Yang et al. 2000), with recent interest in epitope B (amino acids 36-52), on GP5's ectodomain (Ostrowski et al. 2002, Popescu et al. 2017, Plagemann, Rowland and Faaberg 2002), which has been proposed as an epitope for broad neutralization (Popescu et al. 2017). These works state that GP5 plays a major role in the generation of neutralizing antibodies. Additionally, it has been determined that neutralization of GP5 is based on its confirmation, rather than just a linear epitope. This was first revealed by Pirzadeh and Dea, in which a DNA vaccine encoding for GP5 could elicit neutralizing antibodies in the pig; however if the same GP5 DNA was used to construct the viral protein in bacteria and given to a pig, no neutralizing antibodies were seen (Pirzadeh and Dea 1998). Pirzadeh and Dea suggested that differences in polypeptide folding and post-transcriptional modifications, such as N-glycosylation, between the prokaryotic and native systems may play a role in these differences in protein confirmation and later neutralization (Pirzadeh and Dea 1998). Studies have also confirmed that GP5 ectodomain does not contain a linear neutralizing epitope in either PRRSV-1 (Vanhee et al. 2011) or PRRSV-2 (Li and Murtaugh 2012). These studies suggest that only GP5 produced in mammalian cells is the correct confirmation to elicit neutralizing antibodies, as opposed to a generic linear epitope. Additionally, GP5's conformation may also be influenced by M protein, which GP5 forms a dimer with on the virus envelope. Several studies have also stated that while a GP5 specific antibody response is generated, it is non-neutralizing (Robinson et al. 2013, Leng et al. 2012, Li and Murtaugh 2012), however these studies appear to conflict with previously published works (Jiang et al. 2007b, Kim et al. 2013, Vanhee et al. 2011, Xu et al. 2012, Zhou et al. 2010). It is

important to note that while GP5 is required for the formation of viral particles, it is not required for virus to be infectious (Wissink et al. 2005), suggesting inhibition of the viral protein alone may not lead to full neutralization of the virus. All these studies together suggest that while GP5 specific antibodies hamper PRRSV infectivity and enhance neutralization, they may not be the only PRRSV-specific antibodies involved, and that additional immune responses to other PRRSV proteins may be required for full protection from the virus.

Recent work has determined that the PRRSV GP2a-GP3-GP4 viral protein complex is key to virus entry into the host, through its interactions with CD163 (Das et al. 2010, Calvert et al. 2007, Tian et al. 2012). These studies have renewed interest in the GP2a-GP3-GP4 complex as a target for neutralization. Recently Kimpston-Burkren et al. showed GP2a-GP3-GP4 to contain neutralizing epitopes by exchanging ORF2-4 sequence from a PRRSV-1 strain (SD01-08) with that of a PRRSV-2 strain (FL-12), creating a new chimeric PRRSV-1 SDFL24. When the ORFs were exchanged, antisera raised against FL-12 (PRRSV-2) was able to neutralize the new chimeric virus SDFL24, but was unable to neutralize the parental strain SD01-08 (PRRSV-1) (Kimpston-Burkgren et al. 2017). This suggests that viral proteins encoded by ORF2-4, GP2a-GP3-GP4, are involved in protective immunity. Vanhee et al. also pointed in a similar direction with the identification of neutralizing antigenic regions in GP2, GP3 and GP4, which were confirmed by *in vitro* neutralization tests (Vanhee et al. 2011). Also interesting in this work, the GP2a, GP3, and GP4 antigenic sites were found to be linear epitopes, in contrast to the conformational epitopes theorized for GP5 (Pirzadeh and Dea 1998). Spurred by Calvert et al.'s findings other works have also suggested sites of neutralization on GP4 (Costers et al. 2010a, Costers et al. 2010b, Vanhee et al. 2010) and GP3 (Jiang et al. 2007a, Zhou et al. 2012). Based on Das et al.'s findings that GP2a

and GP4 together bind CD163, GP2a should also be further investigated as a neutralizing epitope (Das et al. 2010). Taken as a whole, all these works point towards GP2a-GP3-GP4 protein complex's involvement in the development of protective immunity against PRRSV.

Other minor PRRSV surface proteins have also been suggested to be involved in neutralization. Matrix (M) protein, the protein that forms a heterodimer with GP5, has shown some promise as a potential neutralizing target (Delputte et al. 2002, Fan et al. 2016, Trible et al. 2015). Trible et al. demonstrated that when Tyr-10 was deleted in M protein, the associated virus was able to escape neutralization by serum known to be broadly neutralizing (Trible et al. 2015). It is possible that changing M protein may have caused conformational change to dimer associated GP5 protein, changing GP5's site of neutralization to evade the established immune response. M protein's close relationship with GP5, and GP5's known conformational dependent neutralization, makes M protein, in combination with GP5, an interesting candidate for future studies on PRRSV neutralization. Other studies have also pointed towards nonstructural proteins (NSPs) as possible sites of neutralization (Leng et al. 2017) and immunogenicity (Lu et al. 2014), potentially through blocking viral proteins involved in virulence or virus replication.

Although neutralization of PRRSV is challenging in the pig, it is not impossible. In fact, some pigs not only neutralize the PRRSV strain they are exposed to (homologous virus), they also neutralize related PRRSV they have not seen (heterologous virus), in a process called cross-protection. Cross-protective immunity is common in herds, although the incidence per pig varies (Plagemann 2006). Most studies look at clinical indications to show cross-protection (Mengeling et al. 2003, Wei et al. 2013), but Robinson et al. established that cross-neutralization was achievable in the pig, based on diagnostic

neutralization tests, to titers in excess of 1/1,024 (Robinson et al. 2015). Robinson et al. later went on to determine that passive transfer of these broadly neutralizing antibodies achieved control of heterologous virus in the pig (Robinson et al. 2018). These works show that although induction of broadly neutralizing antibodies is difficult in the pig, it is possible and further study into the conditions that induce this response are necessary.

A flaw in the approach to the investigation of PRRSV neutralization is the concentration on the search for single viral epitopes and their corresponding single neutralizing antibodies. This ignores that the pig produces numerous other virus binding antibodies and that true neutralization of PRRSV most likely involves multiple viral epitopes. Many of these virus binding antibodies are likely poorly neutralizing, but when combined, they may produce a strong and broadly neutralizing response. Work in HIV in recent decades has shown that many HIV patients with evidence of broad neutralization express an extensive array of HIV specific and broadly neutralizing antibodies (Walker et al. 2011). Some of these patients possess upwards of 50 highly specific and neutralizing anti-HIV antibodies (Scheid et al. 2009). This has shown that the immune systems suppression of HIV is not due to a single “kill-all” antibody, but a variety of virus-binding antibodies, that when combined produce a polyclonal response to HIV and neutralize the virus. It is possible that the pig’s response to PRRSV is similar to HIV, with a strong polyclonal response to virus dominating the immune response of those pigs showing broad neutralization. Recent studies on PRRSV neutralization have pointed towards a polyclonal response that may enhance overall neutralization (Jiang et al. 2008, Popescu et al. 2017).

Most of the PRRSV literature has also ignored the potential role PRRSV-specific non-neutralizing antibodies play in immunity. In recent works in HIV and other viruses it

has been demonstrated that non-neutralizing antibodies are believed to be involved in immunity and elimination of virus (Lu et al. 2016, Holl et al. 2006, von Bredow et al. 2016). All of these works show that secondary antibody effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (CDC) and antibody dependent cellular phagocytosis (ADCP) play a role in the elimination of virus through interactions with the innate immune system. Some PRRSV manuscripts speculate that non-neutralizing antibodies may be involved in the suppression of PRRSV viremia through effector functions not related to neutralization, but at this time there is no concrete evidence (Montaner-Tarbes et al. 2019, Rahe and Murtaugh 2017a, Rahe and Murtaugh 2017c). Further work is needed to investigate the roles both neutralizing and non-neutralizing antibodies play in PRRSV elimination.

Isolation of B cells and antibodies

A major hindrance in the study of PRRSV neutralization has been the inability to isolate and produce PRRSV-specific monoclonal antibodies. Past works have looked at swine serum antibodies as a whole or produced monoclonal antibodies within mice, however no study has been able to isolate anti-PRRSV antibodies which were produced by the pig in response to infection. Two major barriers have prevented advancing this research; 1) Inadequate and/or unavailable B cell specific biomarkers for swine and 2) The inability to maintain and study porcine B cells in culture.

Commonly in B cell research, cluster of differentiation 19 (CD19) is used as a biomarker to isolate B cells. CD19 has been found to be a reliable marker for both mature and immature B cells in humans and mice (Wang, Wei and Liu 2012). However, an antibody specific for porcine CD19 has yet to be developed, meaning an alternate biomarker must be used to isolate pig B cells. Cluster of differentiation 21 (CD21), also

known as complement receptor type 2 (CR2), is a B cell specific surface marker, that interacts with CD19 and CD81 (TAPA-1) (Bradbury et al. 1992) to bind to opsonized antigens, and enhance the B cell immune response (Cherukuri, Cheng and Pierce 2001). In humans and mice, low/negative expression of CD21 is seen on memory B cells (Thorarinsdottir et al. 2016) and mature B cells that have recently left active germinal centers (Lau et al. 2017). However in the pig, CD21 has been shown to be expressed on all mature B cells (Sinkora, Stepanova and Sinkorova 2013). For these reasons as well as the availability of porcine CD21 specific antibodies, CD21 was selected as a B cell marker in the following experiments.

Historically scientist have used hybridoma technology to develop stable antibody secreting immortal B cell lines. In short, this technique involves the isolation of antigen specific B cells and fusing them with myeloma cells through a process called electrofusion (Tomita and Tsumoto 2011). This results in immortal B cells lines that continuously secrete antibody. The downside of this technique is that it is time consuming, difficult to perform, and requires isolation of antigen specific cells beforehand. Recent advances in genetic manipulation using retroviruses has opened new paths by which to create immortal cell lines. Within the last decade, a genetic manipulation technology was developed that allows the creation immortal B cell lines without the use of hybridomas. The technology uses a retrovirus, LZRS-Bcl6-2A-BclxL-IRES-GFP, to insert two antiapoptotic genes, human B cell lymphoma 6 (BCL6) protein and human B cell lymphoma 2 like protein 1 (BCL-xL) protein, with the addition of jellyfish origin green fluorescent protein (GFP) as a marker protein (Kwakkenbos et al. 2010). Immortal B cells generated by this method retain native surface markers while also continuously secreting antibody. This technique has continued to be expanded upon, being used for the generation of antigen specific antibodies from humans

(Kwakkenbos et al. 2014), mice, llama, rabbits, non-human primates (Kwakkenbos et al. 2016) and most recently in swine (Goldeck et al. 2019). In this manuscript, the above retroviral vector was used to isolate and generate immortal PRRSV specific porcine B cells.

Neutralization of PRRSV is complicated. The virus's pathology aids in its quick transmission from pig to pig, where it avoids the pig's immune system with ease and becomes persistent. However, even when presented with this problematic virus, the pig is eventually able to generate an immune response that eliminates the pathogen, albeit often only against homologous virus. Some pigs can generate this immune response quicker and more successfully than other individuals for unknown reasons. Select swine within populations generate broadly neutralizing antibodies; an immune response far stronger and with greater breadth than expected based on virus exposure. In this work, I aimed to uncover answers to some of the mysteries surrounding neutralization of PRRSV. I take the reverse approach to classic neutralization studies and start with pigs that are known broad neutralizers of PRRSV, creating immortal cell lines from their B cells, and investigating their secreted PRRSV-specific antibodies. Through these experiments I shed light on the enigma that is PRRSV neutralization and point towards avenues for better vaccine design.

CHAPTER II: ISOLATION AND IMMORTALIZATION OF PRRSV-SPECIFIC IGG+ MEMORY B CELLS

Introduction

Porcine reproductive and respiratory syndrome virus is a devastating and costly disease with worldwide impact to the pork industry. The pig's immune response to the virus varies in level of protection from individual to individual (Plagemann 2006). The humoral immune response to PRRSV generates a large array of PRRSV-specific antibodies, but these antibodies vary in neutralizing capacity from non-neutralizing, to homologously neutralizing, and potentially broadly neutralizing against PRRSV. Little is known about the characteristics of these antibodies or how they are developed within the pig. Despite the development of several mouse-derived PRRSV-specific monoclonal antibodies, no swine derived PRRSV specific antibodies have been isolated or produced. Isolating these antibodies would allow the evaluation of how the pig neutralizes PRRSV, as opposed to studying PRRSV neutralization in a mouse model. PRRSV-specific antibodies have been demonstrated to neutralize virus *in vivo* (Osorio et al. 2002) and several viral epitopes are suspected to be involved in neutralization (Loving et al. 2015, Rahe and Murtaugh 2017c), but there is a large gap in knowledge on how individual porcine derived antibodies are capable of neutralizing PRRSV. I hypothesize a broad polyclonal antibody response is required to neutralize PRRSV. Therefore, to better evaluate PRRSV neutralization in the pig, a method was developed, with the aid of the Pirbright Institute, to isolate and immortalize CD21+/low, IgG+ memory B cells and identify clonal populations of antibody secreting PRRSV-specific B cells. In this project the reverse of the classic neutralization study approach was performed, starting first with pigs known to broadly neutralize PRRSV and then investigating the identity of the responsible B cells. The process was initiated by isolating IgG+, memory B cells from pigs, who have shown evidence of an incredible capacity to broadly neutralize PRRS, based on conventional neutralization assays, at titers far

beyond what is thought to be necessary for sterile neutralization of PRRSV (Osorio et al. 2002). Next, B cells were immortalized using a proprietary retroviral vector developed by AIMM Therapeutics. The immortalized B cells were then sorted into clonal populations based on PRRSV binding. PRRSV-binding clones were then characterized for their expressed surface markers. Finally, PRRSV-specific antibodies are isolated to further evaluate PRRSV neutralization in the pig. This method is also shown to be repeatable in a second pig. Together I present a method for isolating and immortalizing antigen specific porcine B cells that could be applied to almost any known pathogen within pigs.

Methods

Cells and viruses

To further evaluate PRRSV-specific B cell's response to virus, peripheral blood mononuclear cells (PBMCs), splenocytes and serum were derived from two parity 1 sows from a previous study involving the effect of multiple PRRSV vaccine and virus exposures on long term immunity. Pigs received two Ingelvac ATP vaccinations (Boehringer Ingelheim Vetmedica, St. Joseph, MO), at the recommended dose, and were inoculated with one or two PRRSV-2 field strains over a period of several months (Table 1). Samples for this study were collected 90 days post final live virus exposure.

MARC-145 cells, a green monkey kidney cell line, that is highly permissive to PRRSV infection (Kim et al. 1993), were used for virus production and PRRSV-specific IFAs.

Several MARC-145 adapted PPRS viruses were used in this study to evaluate secreted antibody specificity and neutralization capacity. For this section PRRSV-2 ATP (EF532801.1) and PRRSV-2 VR2332 were used to screen for antibody virus specificity

via IFA and were also used to determine neutralizing capacity of serum from the selected pigs.

AF647 labeled PRRSV-2 VR2332 was created as previously described in dengue virus (Zhang et al. 2010).

CD40L expressing mouse L-cells, recombinant human IL-21, and immortalization vector, GALV pseudotyped LZRS-Bcl6-2A-BclxL-IRES-GFP, were all graciously provided by AIMM Therapeutics (Amsterdam, The Netherlands).

Evaluation of serum for PRRSV neutralization

The neutralizing antibody assay was performed as previously described (Robinson et al. 2015). Basically, MARC 145 cells were plated at 10,000 cells per well in 96 well plates and incubated at 37°C, 5% CO_2, for 48 hours. In separate 96 well round bottom plates, serum was serially diluted from 1:4 to 1:32,768 in complete MEM media with 2% FBS. PRRS virus was added to serum at $2x10^4$ $TCID_{50}$/ml and plates were incubated for 1 hour at 37°C, 5% CO_2. The 100 µl of the virus/serum mixture was then applied to each well of MARC cells and incubated at 37°C, 5% CO_2, for 1 hour. Cells were then washed twice with warm PBS, 200 µl cMEM+10% FBS was added, and the plates were incubated at 37°C, 5% CO_2, for 23 hours. Wells were then washed with 100 µl PBS and fixed with 50 µl of 3.7% formaldehyde for 30 minutes. Plates were washed three times with PBST, permeabilized with 50 µl of 0.1% Triton X-100 per well for 5 minutes, washed again three times with PBST, and blocked for 1 hour with 300 µl of 5% non-fat dry milk (NFDM) in PBST, pH 9.6 per well. Plates were washed three times with PBST and 100 µl of primary antibody SR-30A (mouse anti-PRRSV nucleocapsid antibody, RTI, Brookings, SD) was applied at a 1:10,000 dilution to each well and incubated for 1 hour. Plates were again washed three times with PBST and 100 µl of

secondary detection antibody HRP-conjugated goat anti-mouse IgG (Bethyl Laboratories, Montgomery, TX) was applied at 1:10,000 to each well and incubated for 1 hour. Plates were washed three times with PBST, 100 µl TMB solution was applied to each well for 15 min at room temperature protected from light, and 100 µl of 1M phosphoric acid was added to each well to stop the reaction. Plates were then read at 450 nm on a BioTek Epoch ELISA plate reader. Background subtracted OD values were used to determine the 50% neutralizing titer of the sample by plotting the curve corresponding to the OD value at each dilution and calculating the sample dilution corresponding to a 50% reduction in infected cells as compared to virus only wells.

Flow cytometry and cell sorting

Flow cytometry was used to both sort and characterize B cells based on intracellular and surface markers similar to methods used by Rahe et al. (Rahe, Gustafson and Murtaugh 2018). Live cells were identified by staining with the fixable viability dye eFluor780 (1:200, eBioscience, San Diego, CA). The presence or absence of desired cell surface antigens were examined using the following antibodies: PE mouse anti-porcine CD21 (1:1,000 dilution, Abcam, Cambridge, MA), AF647 mouse anti-porcine IgG (1:1,000 dilution, Cohesion Biosciences, London, UK) and biotinylated goat anti-porcine IgM (1:4,000 dilution, Bethyl Laboratories, Montgomery, TX) with Brilliant Violet 421 streptavidin (1:3,000 dilution, BioLegend, San Diego, CA).

Flow cytometry cell sorting was performed using a BD FACSAriaII flow cytometer cell sorter (BD Biosciences, San Jose, CA) at the University of Minnesota flow cytometry resource (UFCR) facilities.

Flow cytometry monitoring of GFP expression and cell surface characterization of B cell clones was performed using a BD LSRII or Fortessa flow cytometer (BD

Biosciences, San Jose, CA) at the University of Minnesota flow cytometry resource (UFCR) facilities. Generated characterization data was analyzed using FlowJo software v10 (Becton Dickinson, Franklin Lakes, NJ)

Isolation and immortalization of IgG+ memory B cells

In order to create immortal IgG+ memory B cell lines, PBMCs showing evidence of heterologous (broad) neutralization were isolated from porcine whole blood samples following a previously described protocol (Rahe and Murtaugh 2017b) and frozen in liquid nitrogen. 2 to 4 x 10^8 PBMCs were thawed from liquid nitrogen storage and cultured for 24 hr in 24 well tissue culture plates containing 10% FBS IMDM, $5x10^4$ CD40L expressing L-cells, and 50 ng IL-21. PBMCs were then sorted via flow cytometry for live, IgG+, memory B cell traits (live, CD21low/+, IgG+, IgM-) and returned to culture for 36 hours. Sorted B cells were next mixed with the retroviral vector (GALV pseudotyped LZRS-Bcl6-2A-BclxL-IRES-GFP) used for immortalization, following a protocol developed by AIMM Therapeutics and implemented in swine by the Pirbright Institute (Goldeck et al. 2019). B cells were then returned to culture. B cells were monitored for GFP expression (constitutively expressed marker of successful immortalization) via flow cytometry starting three days post transduction. In short, a small sample of B cells are removed from culture, washed with 2% FBS PBS, fixed with 4% PFA, washed with PBS, and taken to a flow cytometer at the UFCR for evaluation of GFP expression.

PRRSV antigen-baiting of immortalized memory B cells

PRRSV-specific B cells were isolated by mixing immortalized IgG+ B cells with fluorescently labeled PRRSV, created based on previously designed protocol (Zhang et

al. 2010). Immortalized B cell populations were removed from culture and washed with 2% FBS in PBS and incubated with AF647 labeled PRRSV-2 VR2332 for 15 min at room temperature. Cells were again washed twice with 2% FBS in PBS and returned to 2% FBS IMDM media. Cells were then sorted based on GFP (marker for immortalization) and AF647 (PRRSV-binding B cell) and double positive cells were sorted as 20 cell populations or single cell population in each well of several 96 well plates containing 100 µl 10%FBS IMDM and 10 ng IL-21. After two days in culture, $2x10^4$ CD40L expressing L-cells and 10 ng IL-21 were added to each well. Twice a week, B cells were supplemented with $2x10^4$ CD40L expressing L-cells and 10 ng IL-21, until B cells grew to 25% confluency on plates. Supernatants were then screened for secreted PRRSV-specific antibodies.

Detection of PRRSV specific antibodies via IFA

An immunofluorescence assay (IFA) was performed to identify PRRSV-specific secreted antibodies from previously sorted immortal B cells. B cells from either single or multi-cell sorts were grown in culture continuously for three or more weeks. Supernatants from these cultures, containing secreted antibodies, were then evaluated for PRRSV specificity. MARC 145 cells were plated at $1x10^4$ cells per well in 96-well plates in complete MEM media (cMEM; 0.075% sodium bicarbonate, 1X MEM non-essential amino acids, 25 mg gentamycin sulfate, and 10mM HEPES) containing 10% FBS. After two days in culture, cells were washed once with warm PBS and 50 µl of $2x10^3$ $TCID_{50}$/ml PRRSV virus was added to each well. MARC 145 cells were infected with either VR2332 or ATP PRRSV-2 virus. Cells were then incubated with virus at 37°C, 5% CO_2, for 1 hour. Next, 150 µl of cMEM media + 10% FBS was added to each well and cells were incubated for an additional 23 hours. Media was removed from the plates

and each well was fixed in 50 µl of ice-cold absolute methanol and placed at -20°C for 10 minutes. Methanol was then removed from the plates and cells were air dried in a sterile hood for 5 to 10 min. Plates were then either used immediately for a PRRSV specific IFA or stored for up to two months at -20°C. For IFA detection of PRRSV-specific antibody containing supernatants, 100 µl of supernatants from each immortalized B cell population were applied directly to each well and incubated at 37°C, 5% CO_2, for 1 hour. Supernatants were then removed by gently decanting off the liquid and washed three times with PBST. Goat anti-pig IgG conjugated to FITC (1:100 dilution, Bethyl Laboratories, Montgomery, TX) was diluted 1:100 in PBST with 4% horse serum, 50 µl was applied to each well, and plates were incubated at 37°C, 5% CO_2, for 1 hour. Plates were again washed three times with PBST. To stain the cell nucleus, 50 µl of 1:1000 Bisbenzimide in PBST with 4% horse serum was added to each well and incubated at room temperature for 20 to 30 minutes followed by three PBS washes. Plates were then examined under a fluorescent microscope, Nikon TE2000 (Nikon, Tokyo, JPN), to evaluate PRRSV antibody binding via the appearance of FITC labeled MARC 145 cells.

Isolation of PRRSV specific clones from PRRSV binding populations

Clonal PRRSV-specific B cell lines were created by resorting previously identified PRRSV-specific multi-cell populations. Multi-cell B cell populations that showed positive PRRSV binding by IFA were transferred to 24 well plates and multiplied in culture until at least 1 x 10^6 B-cells were grown. Immortalized B cell populations were removed from culture and washed with 2% FBS in PBS. Cells were then incubated with anti-Pig IgG (H+L) antibody for 15 min at room temperature. Cells were washed again with 2% FBS in PBS and returned to 2% FBS IMDM media. Cells were then sorted based on GFP

(marker for immortalization) and AF647 (surface IgG) and double positive cells were sorted as single cells in each well of several 96 well plates containing 100 µl 10%FBS IMDM and 10 ng IL-21. After two days in culture, $2x10^4$ CD40L expressing L-cells and 10 ng IL-21 were added to each well. Twice a week, B cells were supplemented with $2x10^4$ CD40L expressing L-cells and 10 ng IL-21. At three weeks post sort, plates were screened for cell growth, live populations were selected, and reconfirmed for PRRSV specificity via IFA.

Results

Identification of swine showing evidence of broad neutralization against PRRSV

Two pigs (BNW4 and BNW7) from a previous study received numerous exposures to both PRRSV vaccine (Ingelvac PRRS ATP, Boehringer Ingelheim Vetmedica, Duluth, GA) and additional live virus inoculation/challenge of a PRRSV RFLP 1-3-4 strain for BNW7 and PRRSV RFLP 1-3-4 and RFLP 1-18-2 strains for BNW4 (Table 1). These pigs were observed to possess broad neutralization against highly diverse PRRSV type 1 (SDEU) and type 2 (MN184 and VR2332), strains of which they had no previous exposure. Pigs were euthanized 90 days after their final live virus challenge and PBMCs, splenocytes and serum were obtained. Serum neutralization titers against two type 2 PRRSV's (ATP and VR2332) were determined, and a strong neuralization capacity against both ATP, the virus they were vaccinated with, and VR2332, a closely related strain that the pigs were not exposed to was demonstrated (Fig. 2). BNW4 exhibited a 50% neutralization titer between 1:4,096 and 1:8,192 for ATP, between 1:2,048 and 1:4,096 for VR2332 and between 1:8 and 1:16 for SDEU (Fig. 2). BNW7 presented a 50% neutralization titer between 1:512 and 1:1,024 for ATP, between 1:64 and 1:128 for VR2332, and between 1:16 and 1:32 for SDEU (Fig. 2).

Table 1: Timeline of Pig Vaccination/Virus Challenge and Inoculation

	Previous ATP vaccination	Arrival at farm	ATP booster	PRRSV 1-18-2 innoculation	PRRSV 1-3-4 challenge	Tissue and whole blood collection
Age (days)	Unknown	175	181	222	356	446
BNW7	Received vaccination		Received booster	No inoculation	Viral challenge	266 days post booster 90 days post challenge
BNW4	Received vaccination		Received booster	Received inoculation	Viral challenge	266 days post booster 224 days post inoculation 90 days post challenge

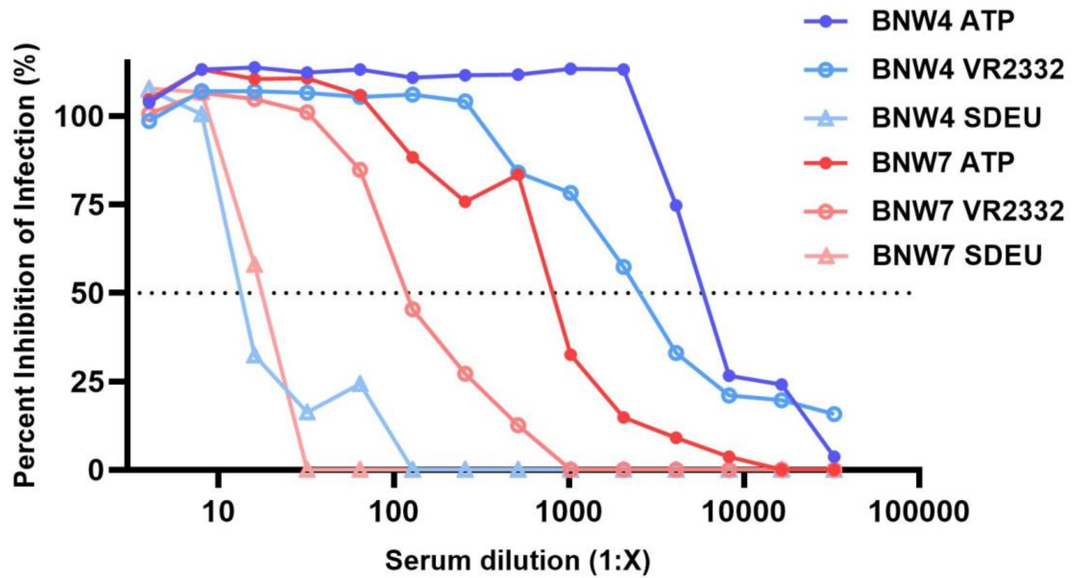


Figure 2: Neutralization of PRRSV-2 ATP and VR2332 and PRRSV-1 SDEU by pigs BNW4 and BNW7

Neutralizing capacity of serum from pigs BNW4 and BNW7 were evaluated for their ability to neutralize homologous PRRSV-2 ATP and heterologous PRRSV-2 VR2332 and PRRSV-1 SDEU.

Isolation, immortalization and identification of PRRSV-specific IgG+ memory B cells from porcine PBMCs

IgG memory B cells from PBMCs from both pigs were isolated and then immortalized using the B cell immortalization vector from AIMM therapeutics. Live memory B cells (live, CD21+/-, IgG+, IgM-) were obtained, cultured, and then immortalized. Cultured immortalized B cells from BNW7 were resorted based on PRRSV-binding into 96-well plates of single cells or multi-cell populations of 20 cells. Five multi-cell plates were created, totaling 480 populations and ten single cell plates totaling 960 cells. After 2-3 weeks in culture all 480 multi-cell populations replicated and by 3-4 weeks 186 single cell populations had visible growth. All single and multi-cell groups that showed visible growth were evaluated for the presence of PRRSV-binding antibodies in their culture supernatants. A total of 4 multi-cell populations were identified as PRRSV-binding by IFA and were named BNW7 population 1 (p1), population 5 (p5), population 9 (p9) and population 10 (p10) (Fig. 3). Interestingly, antibodies in 3 out of 4 PRRSV-binding supernatants stained the cytoplasm, but BNW7 p10 strongly stained around the nucleus or ER region of infected MARC-145 cells with no diffuse cytoplasmic staining observed (Fig. 3).

Immortalization of B cells from PBMCs from pig BNW4 was repeated at a smaller scale to evaluate the repeatability of the process. Instead of resorting immortalized cells based on PRRSV binding, as was performed with BNW7, immortalized BNW4 cells were resorted based on IgG+ and GFP into 20-cell populations in a 96 well plate. After 3 weeks in culture, 86 populations were identified by visible growth, and their supernatants were screened for PRRSV specificity. PRRSV-binding antibodies in cell culture supernatants from the immortalized populations were only observed in a single

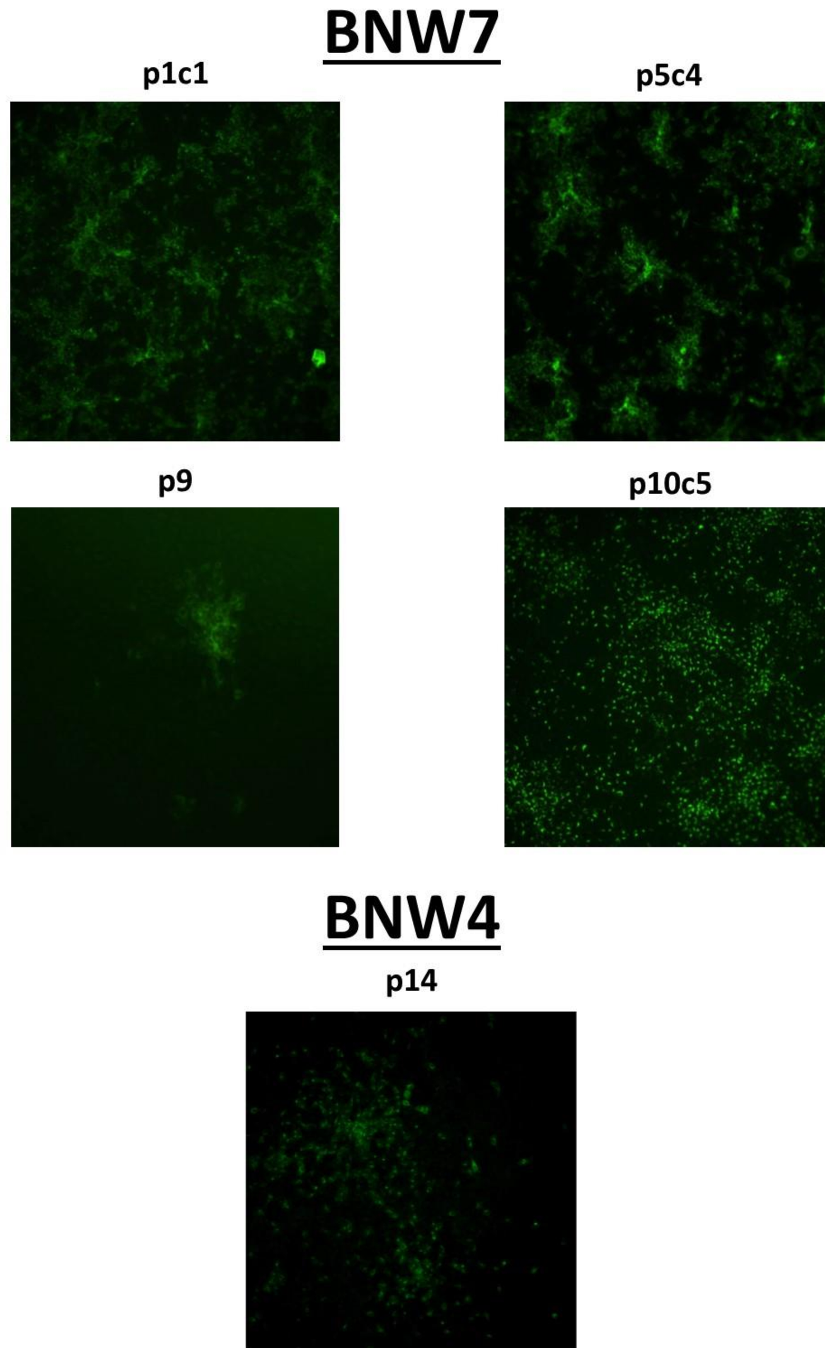


Figure 3: Identified PRRSV-specific antibodies

Supernatants containing secreted antibodies from immortalized B cell clones from pigs BNW4 and BNW7, were analyzed by IFA for PRRSV-binding. One PRRSV-specific population was identified for BNW4 and four populations were identified for BNW7.

population, identified as BNW4 population 14 (p14) and demonstrated similar cytoplasmic staining to that of the majority of the BNW7 populations (Fig. 3).

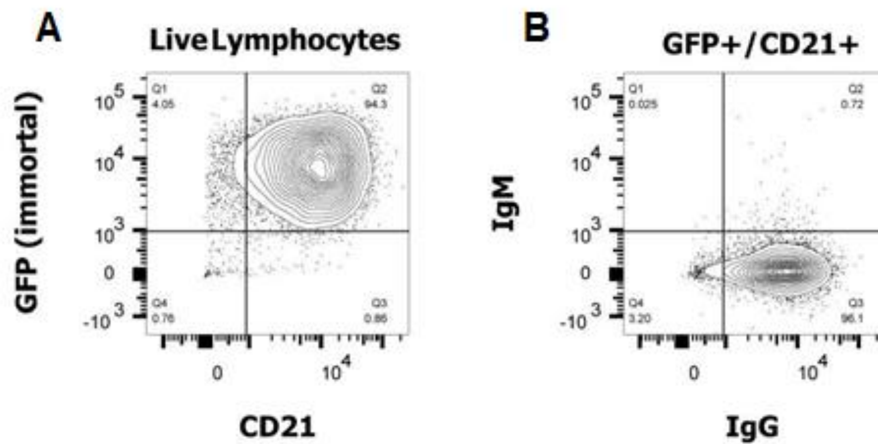
Creation of immortal PRRSV-specific clonal B cell lines

In order to obtain and evaluate a single PRRSV-specific antibody, a clonal population of immortalized B cells producing a single monoclonal antibody needed to be obtained. Thus, the multi-cell B cell populations that produced PRRSV-specific antibodies (BNW7 p1, p5, p9, and p10) were further sorted into single cell populations based on the immortalization marker (GFP) and IgG positivity, grown, and evaluated for secretion of PRRSV-specific antibodies (VR2332 or ATP) by IFA. Single cell clones were obtained for BNW7 p1 (one clone), p5 (six clones) and p10 (nine clones). Population 9 has yet to be isolated as a clonal population and population 1, clone p1c1, was later lost due to contamination, however the clone's secreted antibodies were evaluated briefly. Of the clones isolated, BNW7 p1c1, p5c4 and p10c5, along with the multi-cell populations BNW7 p9 and BNW4 p14, were further evaluated for diversity of PRRSV binding, viral protein specificity and neutralizing capacity in Chapter 3.

Characterization of PRRSV-specific clonal B cells

The p5c4 and p10c5 clonal B cells were characterized for expressed surface markers using flow cytometry. Both clones were examined for expression of GFP and cell surface markers CD21, IgG and IgM (Fig. 4). A total of 20,000 cells were examined for each clone. Over 94% of clone BNW7 p5c4 was found to express high levels of GFP, the marker for successful immortalization, and CD21, a B cell marker. Of the GFP+/CD21+ p5c4 population, the majority of cells expressed surface IgG and no cells were found to express surface IgM. Clone BNW7 p10c5 expression of GFP was similar

BNW7 p5c4



BNW7 p10c5

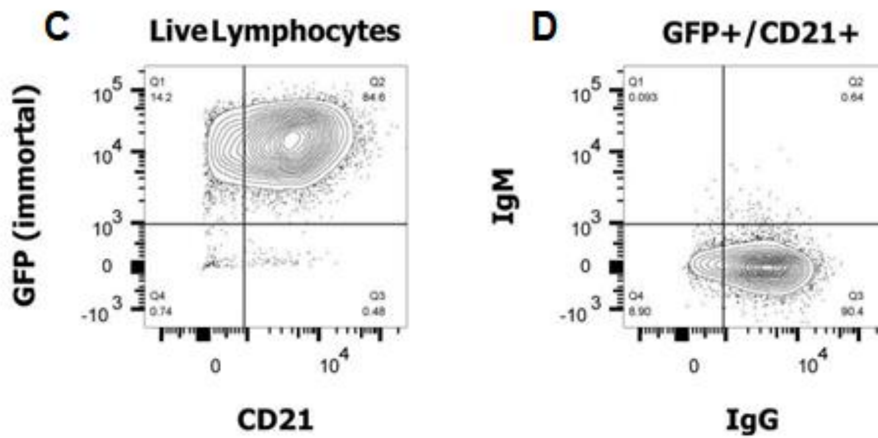


Figure 4: Surface marker characterization of BNW7 clones p5c4 and p10c5

PRRSV-specific BNW7 clones p5c4 and p10c5 were evaluated for expressed cell surface markers via flow cytometry. **A** and **C** examine intracellular GFP (y-axis), the marker for successful immortalization, and surface CD21(x-axis) expression. **B** and **D** examine surface IgM (y-axis) and surface IgG (x-axis) expression.

to p5c4 with over 90% showing high levels of GFP, however expression of CD21 differed with 85% of cells expressing CD21 and 14% showing little to no expression of CD21. Further examination of the GFP+/CD21+ population of p10c5 showed sole expression of surface IgG, with no evidence of IgM, similar to clone p5c4.

Discussion

Neutralization of PRRSV in the pig is poorly understood. It is known that anti-PRRSV antibodies play a critical role in immunity and can provide sterilizing immunity (Osorio et al. 2002), however the humoral immune response to the virus varies and not all pigs exposed to virus develop neutralizing antibodies (Mateu and Diaz 2008, Martínez-Lobo et al. 2011, Plagemann 2006). Interestingly, some animals that lack neutralizing antibodies are still resistant to reinfection (Díaz et al. 2006). We identified two pigs, BNW4 and BNW7, containing incredibly high broad neutralizing antibody titers against PRRSV-2, with surprising cross reactivity to PRRSV-1 (SDEU). Other pigs receiving the same vaccination and virus treatments showed similar evidence of broad neutralization, but not necessarily to the same capacity. Repeated exposure to diverse viral strains has been proposed as a way of eliciting broad neutralization (Robinson et al. 2018) and repeated vaccination and infections are known to enhance immune responses (Scortti et al. 2006). Similar results were seen in this current work, with both pig BNW4 and BNW7 showing marked increases in neutralization titers to both homologous and heterologous virus after multiple vaccine/virus exposures (Fig. 2). Both pigs appear to have retained strong immunity despite the length of time since last exposure to virus, 90 days. Even more surprising is the retention of ATP specific neutralizing antibodies, discussed in chapter 3, even though both pigs had not seen ATP vaccine in over 266 days. Antigen-specific B cells that are not exposed to their antigen

often are known to go through increased anergy and have diminished presence in the immune system (Dal Porto et al. 2002, Shih et al. 2002). The ATP specific B cells remained presence is most likely because of repeated exposure to ATP vaccine and additional exposures of live virus that reinvigorated ATP specific B cells. These results show that strong, long term heterologous immunity to PRRSV is possible in the pig, however it may require repeat exposure to diverse viruses.

A key component of my work was to isolate PRRSV-specific B cells by using fluorescently labeled virus and sorting by flow cytometry. This method has also been attempted by other labs in order to isolate PRRSV specific B cells (Goldeck et al. 2019). However, in this study isolation of PRRSV-specific B cells using fluorescently labeled virus was no more successful than what one would have expected by chance. A total of 4 virus-specific populations were originally isolated out of a total of 480 populations. It is estimated that antigen specific B cells occur at a rate of <0.05% of lymphocytes (Smith et al. 2017). Assuming that all 480 populations contained 20 unique B cells, there were then 9,600 B cells total. If PRRSV antigen specific B cells occur at a rate of 0.05% in lymphocytes, a total of four to five PRRSV specific B cells would be expected to occur by chance, similar to my observation of four PRRSV specific populations within BNW7. If the baiting had been successful, a much larger number of antigen-specific populations would have been expected to be observed by IFA. For the sorting of BNW4, a virus specific sort was not implemented, rather B cells were sorted based on IgG+ and GFP alone. Out of 86 isolated populations, a single PRRSV specific population was isolated, at a rate expected by random chance. This suggests that the virus-baiting had no effect on improving isolation of PRRSV-specific B cells. Other alternative methods should be investigated for future virus-specific B cell isolation experiments and are discussed later in the general discussion, chapter 5.

While performing PRRSV binding IFAs on isolated clones, it was observed that the majority of clones bound PRRSV protein contained within the cytoplasm. This observation was seen in BNW7 clones p1c1, p5c4, p9 and BNW4 clone p14. In chapter 3, BNW7 clone p1c1, p5c4, p9 and BNW4 p14 are later found to be PRRSV GP5 specific. As GP5 is associated with the viral envelope and the virus is packaged around the endoplasmic reticulum (ER) and golgi bodies in the cytoplasm (Yun and Lee 2013), cytoplasmic staining on IFA is expected. However, BNW7 clone p10c5 appears to have a different staining phenotype, concentrating around the outside of the MARC 145 cell nucleus, and no cytoplasmic staining. Previous work has shown that arterivirus non-structural proteins assemble into replication and transcription complexes in the host cell ER (Pedersen et al. 1999). Pedersen et al. present IFA images specific to EAV NSP2 staining that are strikingly similar to my IFA images of p10c5 staining. Based on IFA findings alone, it is likely that p10c5 is specific to a non-structural protein. Possible targets could include non-structural protein 9, 10, or 11, proteins involved in viral replication, which takes place in the ER of host cells (Kappes and Faaberg 2015), but other NSPs, such as NSP2, should also be considered. In chapter 3, the p10c5 antibodies were examined for reactivity against a panel of PRRSV proteins using an ELISA (Fig. 7). Unfortunately, antibodies from the p10c5 supernatants did not specifically bind to any of the viral proteins tested. However, many proteins especially NSPs are missing from this assay. It is also possible that p10c5 binds one of the NSPs included in the assay, but in a different confirmation or with modifications not available in this assay. Further examination of the protein specificity to the antibodies from clone p10c5 needs to be performed to identify the protein to which it binds. Western blotting of whole virus lysate using p10c5 supernatant as the antibody may be a good place to start in the search for its protein specificity.

After PRRSV specific B cell clones were isolated, they were characterized for expressed surface markers. Both BNW7 clones p5c4 and p10c5 were found to express high levels of CD21 and IgG (Fig. 3). Based the pigs last exposure to PRRSV, 90 days before collection of PBMCs, the isolated cells are believed to be long-lived memory B cells. Interestingly, both isolated clones express high levels of CD21, which in the human literature is thought to be a marker associated with more naïve B cells, with low to negative CD21 expression more associated with memory cells (Thorarinsdottir et al. 2016, Lau et al. 2017). However similar work in the pig has shown CD21 expression on all mature B cells (Sinkora et al. 2013). Work in my lab has revealed levels of CD21 to be 10x to 100x higher on porcine B cells expressing surface IgM when compared to IgG BCRs expressing counterparts (data not shown). My findings appear to align with both the pig and human/mouse literature, with lower expression in more mature/memory B cells (IgG+) and higher expression in less mature/naïve B cells (IgM+), while at the same time generalized expression on all isolated mature B cells. Based on this work, CD21 is a viable marker for mature porcine B cells and can be used for the isolation of memory B cells. As for my isolated clones, it would be interesting to compare their CD21 expression to more naïve B cells, to see if the clones' expression of CD21 is lower.

The technique illustrated in this section can be applied to almost any mammalian species and associated pathogens. Previous research using this vector and culture system have been used to generated numerous monoclonal antibodies in humans (Kwakkenbos et al. 2010, Westerhuis et al. 2015, Friesen et al. 2014), some of which are being used to develop therapeutics and vaccines (McLellan et al. 2013, Domachowske et al. 2018). This technique has also been shown to immortalize B cells in rabbits, mice, rats, llamas and non-human primates, in addition to pigs (Goldeck et al. 2019, Kwakkenbos et al. 2016). I found similar success with B cells in this work, further

proving the versatility of this method. There are many other diseases within swine that could benefit from the development of monoclonal antibodies against them for therapeutic and diagnostic uses. Future research using this technique and method should be applied to other production animals, such as cattle, horses, sheep and goats, and the pathogens of concern within those species. Particular areas of concern, such as emerging diseases and prions, could benefit immensely from using this method. Overall, I successfully created immortalized B cells that secrete PRRSV-specific antibodies and this technique can be applied to other hosts and their pathogens.

CHAPTER III: EVALUATION AND CHARACTERIZATION OF PRRSV-SPECIFIC ANTIBODIES

Introduction

Pig serum antibodies have been well studied for their neutralizing capacity against PRRSV (Nelson et al. 1994, Robinson et al. 2015, Osorio et al. 2002, Plagemann 2006, Mengeling et al. 2003). While the pig is capable of developing protective immunity with antibody alone (Osorio et al. 2002), the antibodies responsible for protection have yet to be isolated and characterized. Several viral protein epitopes have been suggested as sites of neutralization, but no single PRRSV protein has been linked to the development of sterile immunity. Despite our lack of knowledge, the pig develops humoral immunity against PRRSV on its own, although it is often only against homologous virus. In rare instances, some pigs can develop heterologous or broad neutralizing antibodies under field or experimental conditions (Robinson et al. 2015, Robinson et al. 2018). Antibodies within these pigs may hold the key to developing next generation vaccines against PRRSV, but the study of these PRRSV-specific antibodies has not yet been performed due to the inability to isolate single antibodies. Therefore, I aimed to fill in these gaps in knowledge by taking my previously isolated PRRSV-specific antibodies from chapter 2 and characterizing them. To elucidate the viral promiscuity of my PRRSV-specific antibodies, the ability to bind diverse PRRSV strains was evaluated. A panel of viruses were selected to test for PRRSV-specific antibody binding based on their genetic diversity, which was determined by whole genome sequencing. To identify potential epitopes of neutralization, the ability of the antibodies to bind a panel of 12 different PRRSV proteins was examined. Previously expressed and purified recombinant PRRSV VR2332 proteins were selected based on availability and antibody reactivity was determined using an ELISA. Finally, to determine the PRRSV neutralizing specificity and capacity of antibodies from two clonal immortalized B cell populations (p5c4 and p10c5), a neutralizing antibody assay was performed using two different PRRSV viruses. The

two PRRSV that were evaluated were ATP, which both pigs were vaccinated against and VR2332, which the pigs were not exposed to, so any ability to neutralize would be due to heterologous reactivity. Through these experiments PRRSV GP5 specific antibodies were identified, with capacity to bind a broad range of PRRSV-2. However, these antibodies were found to only neutralize homologous virus, suggesting that neutralization of PRRSV is much more complicated that previously hypothesized.

Methods

Immunofluorescent analysis (IFA) of antibody binding to diverse viral strains

Immortalized PRRSV-specific B cells were cultured for 3-7 days following protocols in chapter 2. Supernatants from these B cell cultures were obtained and used to determine the ability of the secreted antibodies to bind diverse PRRSV strains using IFA. The following diverse panel of lab adapted PRRSV strains were used to infect cells and test for reactivity to antibodies produced by immortalized B cells: ATP (PRRSV-2, GenBank ID EF532801.1), VR2332 (PRRSV-2), MN 184 (PRRSV-2), 174 (PRRSV-2, GenBank ID MN175677), 144 (PRRSV-2), NC 134 (PRRSV-2), 1-26-2 (PRRSV-2, GenBank ID KF724400), SDEU (PRRSV-1, GenBank ID MN175678) and Lelystad virus (PRRSV-1).

PRRSV-infected cells were produced through infection of MARC 145 cells. Cells were plated at $1x10^4$ cells per well in 96-well plates in complete MEM media (cMEM; 0.075% sodium bicarbonate, 1X MEM non-essential amino acids, 25 mg gentamycin sulfate, and 10mM HEPES) containing 10% FBS. After two days in culture, cells were washed once with warm PBS, 50 µl of $2x10^3$ $TCID_{50}$/ml of each PRRSV tested was added to each well, and incubated at 37°C, 5% CO_2, for 1 hour. An additional 150 µl of cMEM media + 10% FBS was then added to each well and cells were incubated for 23 hours. Media and virus were then removed from the plates and each well was fixed by

adding 50 µl of ice-cold absolute methanol and placed at -20°C for 10 minutes. Methanol was then removed from the plates, plates were air dried for 5 to 10 min, and were either used immediately to test supernatants for reactivity to virus by IFA or stored for up to two months at -20°C.

Antibody reactivity to PRRSV-infected cells was visualized using IFA. Supernatants from each PRRSV-specific immortalized B cell population (100ul) were applied directly to each well containing one of the above viruses, and incubated at 37°C, 5% CO_2, for 1 hour. Supernatants were then removed by gently decanting off the liquid and washed three times with PBST. Goat anti-pig IgG conjugated to FITC (1:100 dilution, Bethyl Laboratories, Montgomery, TX) was diluted in PBST with 4% horse serum, 50 µl was applied to each well, and plates were incubated at 37°C, 5% CO_2 for 1 hour. Plates were again washed three times with PBST. The cell nucleus was stained by adding 50 µl of 1:1000 Bisbenzimide in PBST with 4% horse serum and incubating at room temperature for 20 to 30 minutes followed by three PBS washes. Infected cells detected by the antibodies were then visualized using a Nikon TE2000 microscope (Nikon, Tokyo, JPN) at the University of Minnesota CBS Imaging Center using Elements imaging software. Images were examined using Image J software (Rueden et al. 2017). As a positive control, 200ul of BNW4 and BNW7 serum was examined for reactivity to each of the viruses at a 1:200 dilution in PBST with 4% horse serum. Commercial PRRSV nucleocapsid specific antibody SR30-A (RTI, Brookings, SD) at a dilution of 1: 10,000 in PBST with 4% horse serum was also used as a positive control. Serum from a PRRSV naïve pig was used as a negative control at 1:200 in PBST with 4% horse serum.

Identification of PRRSV protein specific antibodies via ELISA

Immortalized PRRSV-specific B cells were cultured for 3-7 days following protocols in chapter 2. Supernatants from these B cell cultures were obtained and used to determine the ability of the secreted antibodies to bind structural and non-structural PRRSV proteins using ELISA. A total of 5 structural (GP5 total, GP5/M, M 3', M 5' and N) and 7 non-structural (NSP2P, NSP4, NSP7, NSP8, NSP9, NSP10, NSP11) recombinant VR2332 proteins that were previously expressed and purified were examined (Brown et al. 2009, Mulupuri et al. 2008). ELISAs were performed as previously described (Brown et al. 2009, Johnson, Yu and Murtaugh 2007). Basically, 100 ng of PRRSV recombinant protein in carbonate buffer was used to coat high binding polystyrene 96-well plates at 4°C overnight. Plates were washed three times with PBST and blocked with 300 μl of blocking buffer (PBST with 5% NFDM pH 9.6) at room temperature for 2 hours. Plates were again washed three times with PBST, 100ul of PRRSV-binding supernatants from immortalized B cell cultures were applied to each well and incubated for 1 hour at room temperature. Plates were washed three times with PBST and 100ul of goat anti-swine IgG HRP (1:100,000 dilution, Bethyl Laboratories, Montgomery, TX) was applied to each well and incubated for 1 hour at room temperature protected from light. Plates were washed three times with PBST, 100ul of TMB solution was applied to each well, incubated for 15 min at room temperature protected from light, and then 100 μl of 1M phosphoric acid was added to each well to stop the reaction. Plates were then read at 450 nm on a BioTek Epoch ELISA plate reader (BioTek, Winooski, VT). Positive and negative controls were run on each plate. Background reactivity was determined by PRRSV negative serum. Positive signals above a background OD value of 0.4 signified binding of secreted antibody to PRRSV protein.

Evaluation of serum and B cell supernatants for PRRSV neutralization

Immortalized PRRSV-specific B cells were cultured for 3-7 days following protocols in chapter 2. Supernatants from these B cell cultures were obtained and concentrated to examine the ability of antibody to neutralize different PRRSV strains. Supernatants were concentrated by harvesting PRRSV-specific B cells and supernatants, centrifuging at 1,400 rpm for 5 min at room temperature, and decanting supernatants. Supernatants (15ml) were applied to an Amicon Ultra 15 Ultracel 30K MWCO centrifugal filter (Merck Millipore, Cork, Ireland), spun at 4,000 g for 20 min at room temperature. B cell supernatant concentrates were decanted and frozen until used in the neutralizing antibody assay. The concentration of IgG in each concentrated sample was determined using a total IgG ELISA according to manufacturer's protocols (Pig IgG ELISA Quantitation Set, Bethyl Laboratories, Montgomery, TX)

The neutralizing antibody assay was performed as previously described (Robinson et al. 2015). Basically, MARC 145 cells were plated at 10,000 cells per well in 96 well plates and incubated at 37°C, 5% CO_2, for 48 hours. In separate 96-well round bottom plates, serum or concentrated B cell supernatants were serially diluted from 1:4 to 1:32,768 in complete MEM media with 2% FBS. PRRS virus was added to serum/supernatants at $2x10^4$ $TCID_{50}$/ml and plates were incubated for 1 hour at 37°C, 5% CO_2. The 100 µl of the virus/supernatant mixture was then applied to each well of MARC cells and incubated at 37°C, 5% CO_2, for 1 hour. Cells were then washed twice with warm PBS, 200 µl cMEM+10% FBS was added, and the plates were incubated at 37°C, 5% CO_2, for 23 hours. Wells were then washed with 100 µl PBS and fixed with 50 µl of 3.7% formaldehyde for 30 minutes. Plates were washed three times with PBST, permeabilized with 50 µl of 0.1% Triton X-100 per well for 5 minutes, washed again three times with PBST, and blocked for 1 hour with 300 µl of 5% non-fat dry milk (NFDM) in

PBST, pH 9.6 per well. Plates were washed three times with PBST and 100 µl of primary antibody SR-30A (mouse anti-PRRSV nucleocapsid antibody, RTI, Brookings, SD) was applied at a 1:10,000 dilution to each well and incubated for 1 hour. Plates were again washed three times with PBST and 100 µl of secondary detection antibody HRP-conjugated goat anti-mouse IgG (Bethyl Laboratories, Montgomery, TX) was applied at 1:10,000 to each well and incubated for 1 hour. Plates were washed three times with PBST, 100 µl TMB solution was applied to each well for 15 min at room temperature protected from light, and 100 µl of 1M phosphoric acid was added to each well to stop the reaction. Plates were then read at 450 nm on a BioTek Epoch ELISA plate reader. Background subtracted OD values were used to determine the 50% neutralizing titer of the sample by plotting the curve corresponding to the OD value at each dilution and calculating the sample dilution corresponding to a 50% reduction in infected cells as determined by virus only wells.

PRRSV GP5 N-glycosylation prediction

ORF5 sequence was isolated from previously generated full genome sequences of both PRRSV-2 ATP and PRRSV-2 VR2332. ORF5 sequence from both viruses were convert to GP5 amino acid sequence using MEGA-X analysis software (Kumar et al. 2018). N-glycosylation sites within each virus's GP5 protein were next predicted using NetNGlyc 1.0 Server webservice (Blom et al. 2004)

Results

Antibody binding to diverse PRRSV strains

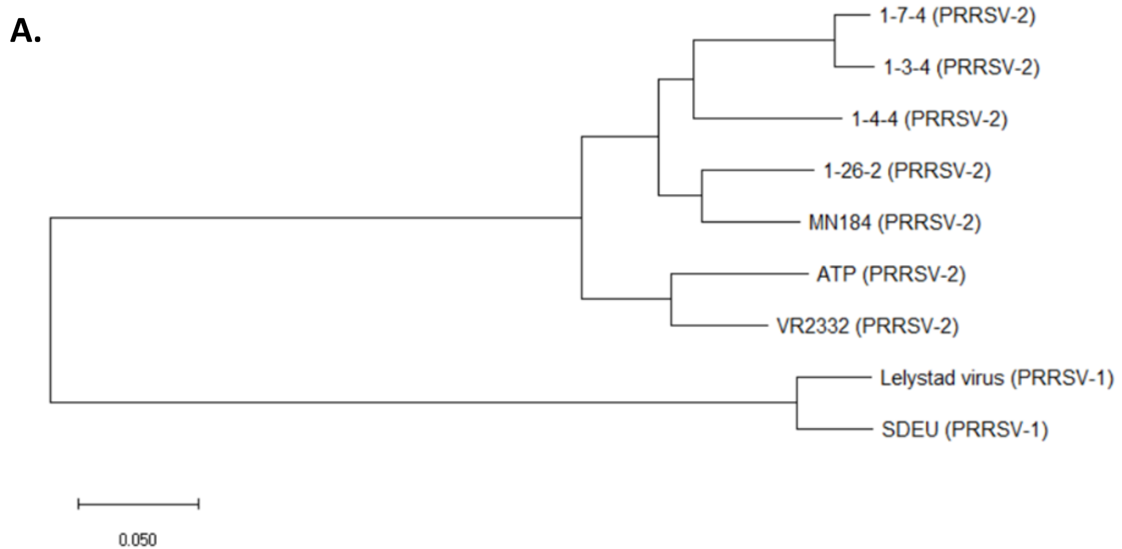
Antibodies secreted by PRRSV-specific immortalized B cells were evaluated for their ability to bind a diverse panel of PRRSV strains. Viruses were selected based on availability of in lab stocks, sequence divergence from other strains, ability of virus to

infect MARC 145 cells, and availability of whole genome sequence. To this end, seven PRRS type-2 viruses, ATP, VR2332, MN 184, 174, 144, NC 134, and 1-26-2 and two PRRS type-1 viruses SDEU and Lelystad virus (LV) were selected (Fig. 5). Supernatants from PRRSV-specific BNW4 p14 and BNW7 p9 multi-cell populations, BNW7 clones p1c1, p5c4 and p10c5, and BNW4 and BNW7 serum samples were obtained and tested for the ability of their secreted antibodies to bind virus. Unsurprisingly, broadly neutralizing, serum from both BNW4 and BNW7 were able to bind all the PRRSV strains examined. Surprisingly, all antibodies from isolated PRRSV-specific clones and populations tested were able to bind every PRRSV-2 virus tested (Fig. 6). However, no antibody from any clone or population was able to bind either of the PRRSV-1 strains tested, despite BNW4 and BNW7 serum being able to do so (Fig. 6). Thus, broad binding of secreted antibodies from immortalized B cell supernatants was observed throughout PRRSV-2 strains, but not against tested PRRS type 1 viruses.

Evaluation of PRRSV protein specificity of clonal antibodies

PRRSV-binding antibodies secreted from immortalized B cells were evaluated for their ability to bind PRRSV VR2332 viral proteins. Supernatants containing antibodies secreted from immortalized B cell populations BNW7 p9 and BNW4 p14 and B cell clones BNW7 p1c1, p5c4, and p10c5 were tested by ELISA for reactivity against 12 VR2332 proteins (Fig. 7). Serum from pig BNW4 and BNW7 were used to evaluate the reactivity of the total antibody population present within both animals. Serum from both animals showed strong reactivity to GP5 total, GP5/M complexed protein, M (matrix protein) 5’ end, N (nucleocapsid protein), NSP2P, NSP7, and NSP8 and weak reactivity to M (matrix protein) 3’ end, NSP4, NSP9 and NSP10. No binding of BNW4 or BNW7

A.



B.

Percent similarity (%) (top-right); Percent difference (%) (bottom-left)

	ATP	VR2332	1-26-2	134	144	174	MN184	SDEU	LV
ATP (PRRSV-2)		90.3	83.2	82.6	82.7	82.8	84.1	42.4	41.9
VR2332 (PRRSV-2)	9.7		84.3	83.1	83.8	83.4	86.0	43.3	42.8
1-26-2 (PRRSV-2)	16.8	15.7		87.7	87.9	87.7	91.2	42.1	42.3
134 (PRRSV-2)	17.4	16.9	12.3		86.9	96.9	87.0	41.6	42.0
144 (PRRSV-2)	17.3	16.2	12.1	13.1		87.0	88.2	42.1	42.9
174 (PRRSV-2)	17.2	16.6	12.3	3.1	13.0		87.2	41.9	42.5
MN184 (PRRSV-2)	15.9	14.0	8.8	13.0	11.8	12.8		42.8	43.0
SDEU (PRRSV-1)	57.6	56.7	57.9	58.4	57.9	58.1	57.2		93.6
Lelystad virus (PRRSV-1)	58.1	57.2	57.7	58.0	57.1	57.5	57.0	6.4	

Figure 5: Whole genome phylogenetic tree of PRRSVs examined for antibody reactivity.

Whole genome sequences were obtained and their genetic relatedness was examined using the neighbor-joining method within MEGA-X software (Kumar et al. 2018). **A.** Phylogenetic tree comparing the identity of the whole genome sequence for each of the strains. **B.** The identity between each of the PRRSV strains was compared for percent difference (bottom-left) and percent similarity (top-right).

Sample	ATP (PRRSV-2)	VR2332 (PRRSV-2)	MN 184 (PRRSV-2)	174 (PRRSV-2)	144 (PRRSV-2)	134 (PRRSV-2)	1-26-2 (PRRSV-2)	SDEU (PRRSV-1)	LV (PRRSV-1)
BNW4 serum	+	+	+	+	+	+	+	+	+
BNW7 serum	+	+	+	+	+	+	+	+	+
BNW4 p14	+	+	+	+	+	+	+	-	-
BNW7 p1c1	+	+	+	+	+	+	+	-	-
BNW7 p5c4	+	+	+	+	+	+	+	-	-
BNW7 p9	+	+	+	+	+	+	+	-	-
BNW7 p10c5	+	+	+	+	+	+	+	-	-
Pos ctrl (commerical antibody)	+	+	+	+	+	+	+	+	+
Neg ctrl (PRRSV naïve serum)	-	-	-	-	-	-	-	-	-

Figure 6: Ability of clonal antibodies to bind a diverse panel of PRRSV strains

Antibodies from VR2332-binding IFA positive immortalized B cell populations were screened for reactivity against a diverse panel of PRRSV-2 and PRRSV-1 viral strains. A "+" indicates antigen-antibody binding and a "-" indicates no visible reactivity.

PRRSV protein	BNW4 Serum	BNW7 Serum	BNW4 p14	BNW7 p1c1	BNW7 p5c4	BNW7 p9	BNW7 p10c5	Naïve Serum
GP5 total	+ (2.93)	+ (2.93)	+ (2.29)	+ (0.86)	+ (3.05)	+ (1.04)	- (0.30)	- (0.11)
GP5/M	+ (2.92)	+ (3.11)	+ (2.56)	+ (0.97)	+ (3.09)	+ (0.72)	- (0.25)	- (0.15)
M 3'	+ (1.06)	+ (0.55)	- (0.15)	- (0.09)	- (0.34)	- (0.12)	- (0.14)	- (0.12)
M 5'	+ (2.49)	+ (2.85)	- (0.05)	- (0.07)	- (0.05)	- (0.04)	- (0.06)	- (0.06)
N	+ (1.61)	+ (1.21)	- (0.05)	- (0.05)	- (0.04)	- (0.04)	- (0.05)	- (0.07)
NSP2P	+ (1.79)	+ (1.89)	- (0.04)	- (0.08)	- (0.04)	- (0.1)	- (0.05)	- (0.08)
NSP4	+ (0.90)	+ (0.73)	- (0.09)	- (0.08)	- (0.35)	- (0.11)	- (0.26)	- (0.06)
NSP7	+ (2.65)	+ (2.53)	- (0.07)	- (0.06)	- (0.15)	- (0.05)	- (0.05)	- (0.09)
NSP8	+ (2.19)	+ (1.62)	- (0.10)	- (0.06)	- (0.25)	- (0.10)	- (0.05)	- (0.07)
NSP9	+ (0.41)	- (0.28)	- (0.09)	- (0.09)	- (0.20)	- (0.09)	- (0.10)	- (0.06)
NSP10	+ (0.92)	+ (0.48)	- (0.17)	- (0.07)	- (0.05)	- (0.18)	- (0.05)	- (0.07)
NSP11	+ (0.41)	- (0.20)	- (0.09)	- (0.07)	- (0.20)	- (0.14)	- (0.04)	- (0.06)

Figure 7: Identification of antibody reactivity against PRRSV proteins

Antibodies secreted from immortalized B cell populations were examined for reactivity against VR2332 proteins using an ELISA. OD_{450} values are indicated in parentheses. BNW4 and BNW7 serum samples were examined as positive controls. A “+” indicates antigen-antibody binding and a “-“ indicates reactivity below OD_{450} cut off of 0.4.

serum was seen against NSP11 and BNW7 was unable to bind NSP9 (Fig. 7). BNW7 population 9 and clones p1c1, p5c4 and BNW4 p14 bound to both the GP5 total and GP5/M complex protein, suggesting GP5 specific binding (Fig. 7). BNW7 clone p10c5 did not bind any of the proteins examined (Fig. 7).

Neutralization activity of secreted antibodies from immortalized B cell clones

Antibodies from BNW7 p5c4 and p10c5 were examined for their capacity to neutralize both homologous and heterologous PRRSV. Animal were vaccinated using ATP virus, thus the presence of antibodies against ATP would be considered homologous neutralization. To test heterologous neutralization, the neutralization activity against the VR2332 strain, which the animals were not exposed to and which has a 10% difference in nucleotide sequence as compared to ATP, was examined. Antibodies from supernatants were concentrated in order to confirm that lack of neutralizing activity was not due to low antibody levels. The antibody concentrations were determined using a total IgG ELISA kit (Bethyl Laboratories, Montgomery, TX) in order to compare concentrations between clones. The highest antibody level tested was 6,000 ng of IgG for BNW7 p5c4. Antibodies were also concentration matched and examined starting at 4,000 ng of IgG per well then diluted 2-fold, 13x, down to a 1:32,768 dilution or approximately 0.5 ng IgG. A 50/50 mixture of antibodies from the two clones, p5c4 and p10c5, was tested for neutralizing activity to study a multi-clonal response to virus. The neutralization assay identified that BNW7 clone p5c4 (GP5-specific) was able to achieve 50% inhibition of viral infection at 2,440 ng for ATP virus (Fig. 8). However, it was unable to similarly inhibit infection of VR2332, suggesting the antibody is only capable of homologous neutralization (Fig. 8). BNW7 clone p10c5 was unable to inhibit infection of either ATP or VR2332, suggesting that despite it being PRRSV-specific, it is non-neutralizing (Fig. 8). The mixed p5c4/p10c5 sample showed similar patterns of

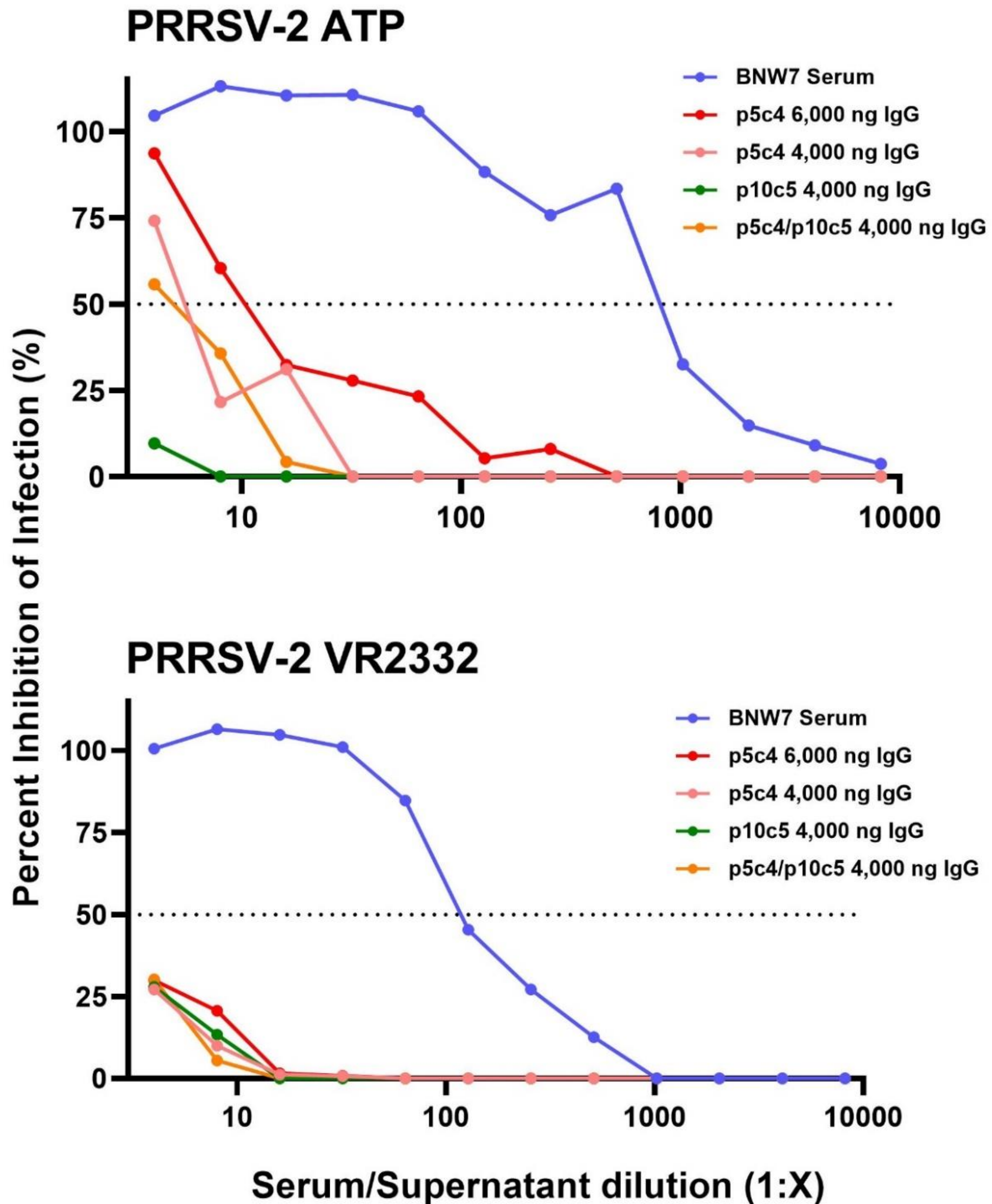


Figure 8: Neutralizing activity against PRRSV of antibodies secreted from immortalized B cells.

Supernatants containing antibodies from immortalized B cell clones BNW7 p5c4 and p10c5 were concentrated, serially diluted and tested for their ability to neutralize homologous PRRSV (ATP) and heterologous PRRSV (VR2332) infection of MARC 145 cells. Activity of a 50/50 mixture of both p5c4 and p10c5 was examined to mimic a polyclonal response. Serum from BNW7 was used as a positive control.

neutralization to p5c4, suggesting that p5c4 was driving inhibition of infection in that sample and p10c5 was not contributing to neutralization (Fig. 8). Together these results show the first ever isolation of a porcine antibody capable of neutralizing PRRSV and of a porcine antibody that is PRRSV-specific but non-neutralizing.

Predicted N-glycosylation of PRRSV-2 ATP and VR2332 GP5 viral protein

Isolated GP5 amino acid sequences of both PRRSV-2 ATP and VR2332 were evaluated for predicted N-glycosylation sites using NetNGlyc 1.0 Server webservice (Blom et al. 2004). For ATP, potential sites of N-glycosylation were identified at amino acids 34, 44 and 51, three sites in total. For VR2332, four potential sites of N-glycosylation were predicted at amino acids, 30, 33, 44 and 51.

Discussion

PRRSV glycoprotein GP5 has long been thought to be the major viral protein involved in neutralization. Numerous neutralization studies have concentrated solely on the PRRSV GP5 protein and our historical classification of the virus, RFLP typing, is based on restriction enzyme digestion of the ORF5 gene, which encodes the GP5 protein. The pig shows a strong immune response to the GP5 protein starting early in infection (Nelson et al. 1994). A similar propensity towards GP5 was seen with isolated PRRSV-specific B cells in this work, with four out five isolated B cells secreting GP5-specific antibodies (Fig. 7), thus pointing toward GP5 being a dominant epitope in the anti-PRRSV immune response. These results are in line with literature that state GP5 specific antibodies are common within PRRSV immune pigs (Nelson et al. 1994). However, the commonality of GP5 specific B cells is not analogous to their involvement in neutralization. PRRSV infected pigs often have strong humoral immune responses to NSP1, NSP2, NSP7 and nucleocapsid (Fang and Snijder 2010) despite these proteins

not appearing to be involved in neutralization. It is also important to note that the important structural proteins and potential neutralizing epitopes of GP2, GP3 and GP4 were not examined in my protein binding panel and B cells specific to these proteins are most likely present in the whole population.

In this manuscript, for the first time, a porcine derived PRRSV neutralizing antibody has been isolated and GP5 had been definitively confirmed to contain a neutralizing epitope. Several studies have claimed that GP5 specific antibodies are non-neutralizing (Li and Murtaugh 2012, Robinson et al. 2013, Leng et al. 2012), however the data generated in this study contradicts these findings. Monoclonal, GP5-specific, porcine antibody BNW7 p5c4 was shown to be able to achieve 50% inhibition of infection at 2,440 ng of PRRSV specific IgG against homologous ATP virus (Fig. 8). Full neutralization was not achieved with this antibody, even with 6,000 ng of IgG-specific GP5 antibody mixed with 2,200 $TCID_{50}$ of ATP virus *in vitro*. Previous research has determined that while GP5 is essential for virus formation, lack of GP5 is not essential for infection (Wissink et al. 2005) and that GP5 is responsible for adhesion to the host cell, but is not the protein associated with viral entry in to the cytoplasm (Delputte et al. 2005, Calvert et al. 2007). Li et al. showed that titers of antibody to the ectodomain of GP5 did not correlate with virus neutralizing antibody titers (Li and Murtaugh 2012), suggesting that GP5 is not solely responsible for neutralization. It has also been demonstrated that pigs lacking sialoadhesin (Sn/Siglec-1/CD169), the host protein GP5 interacts with, are permissive to PRRSV infection (Prather et al. 2013), while pigs lacking CD163, the host protein in which the GP2a-GP3-GP4 complex interacts with, are completely resistant to virus infection (Whitworth et al. 2016). I theorize that based on current knowledge of PRRSV infection, full sterile immunity will not be achievable with this GP5 antibody. What is more likely is that the GP5-specific antibody, p5c4, can inhibit

infection but not prevent it. My theory is that by blocking GP5, p5c4 blocks virus adhesion to the host cell, thus lowering the ability of the virus to bind and infect the cell. However, since the GP2a-GP3-GP4 complex on the virus is still intact and functional, rare viral entry events still occur and infection takes place. This theory on GP5 neutralization would explain some of the confusion and disagreement about the role of GP5 in neutralization. This could also present a reason why the virus persists, as the majority of neutralizing antibodies are directed toward GP5, and not the proteins responsible for viral entry. I propose, based on known virus mechanics and my findings, that GP5-specific antibodies aid in virus neutralization, but are neither essential nor solely responsible for PRRSV neutralization in the pig.

Homologous neutralization of virus is the most common outcome of exposure to PRRSV, either through vaccine or viral infection. In this work it was shown that isolated antibodies from GP5 specific BNW7 clone p5c4 can neutralize homologous virus (ATP), but not heterologous virus (VR2332) (Fig. 8). This outcome is unsurprising, as antibodies against homologous virus are very common in pigs exposed to PRRSV. What make these findings interesting is that despite lack of neutralization against VR2332, the antibody is still able to bind this virus (Fig. 6). Strangely, it appears the isolated antibody, p5c4, has vastly different roles depending on which virus it is bound to; becoming a neutralizing antibody against ATP but a non-neutralizing antibody against VR2332. It is unknown what is causing the discrepancy between the function of the antibody between these two viruses. One cause could be possible differences in GP5 N-glycosylation between the two viruses. In this manuscript, PRRSV-2 ATP and VR2332 were shown to have differing predicted GP5 N-glycosylation sites with three total predicted sites for ATP and four total predicted sites for VR2332. It is possible that the additional predicted N-glycosylation site within VR2332 makes it more difficult for the humoral immune system

to develop a neutralizing antibody against it. However. it should be noted that the initial two predicted glycosylation sites in VR2332, at amino acid 30 and 33, are so close together that potentially only one is functional. Numerous papers have shown that glycan shielding of GP5 influences development of PRRSV-specific antibodies and immunogenicity (Wei et al. 2012, Vu et al. 2011, Faaberg et al. 2006, Ansari et al. 2006). It is possible that the glycan shielding has some effect, but unlikely since most authors conclude that glycan shielding has no effect on overall neutralization, but rather affects the ease by which neutralizing antibodies are developed (Wei et al. 2012, Faaberg et al. 2006). Another possible, and more likely, outcome is that antibody avidity (strength of antibody binding to antigen) to the epitope differs between the viruses, with stronger, long lasting binding to homologous virus and short duration, weak binding to heterologous virus. Longer periods of antibody/antigen interactions should in theory improve elimination of pathogen and increase positive feedback signals to B cells. Very few published works have investigated antibody avidity to PRRSV. Ko et al. observed that vaccinated pigs that produced antibodies with high relative avidity indexes (RAI) had decreased and shortened viremia in response to viral challenge (Ko et al. 2016). Unpublished work from our group demonstrated a strong positive correlation (R^2=0.93) between viremia and avidity, with high initial levels of viremia leading to later development of strong avidity antibodies. Similar results were shown by Islam et al., where longer periods of viremia, as opposed to either quick clearance of virus or rebound infection, were associated with the development of heterologous and broadly neutralizing antibodies (Islam et al. 2013). It is possible that avidity also plays a role in the development of cross-neutralizing antibodies, with antibodies with a high avidity index being more likely to cross-neutralize virus. However, despite weak binding (low

avidity) and a lack of heterologous virus neutralization, these antibodies may still play a role the immune response to PRRSV, as discussed in Chapter 5.

In a recent paper published by Popescu et al., the authors divided the GP5 ectodomain into three immunological domains, Epitope A, Epitope B and Epitope C (Popescu et al. 2017). Epitope A is categorized as an immunodominant non-neutralizing epitope. Its hypervariable region is proposed as an immunological decoy that activates new B cells (Thaa et al. 2013). Epitope B is a highly conserved region that is thought to function as a major site of neutralization (Plagemann et al. 2002) or even broad neutralization (Popescu et al. 2017). Within Epitope B, at amino acid 48, is a site where GP5 forms a disulfide bond with the M protein. Epitope C, a hyper variable region downstream from Epitope B, was proposed by Popescu et al. as a site for homologous neutralization based on mutations seen in escape mutants generated using homologously neutralizing serum (Popescu et al. 2017). It should also be noted that Popescu et al. also detected possible broad and homologous neutralization escape sites in GP2, GP3 and M protein (Popescu et al. 2017). Based on the authors findings, it is possible that the anti-GP5 antibodies generated in this work (p14, p9, p1c1 and p5c4) are all specific to Epitope B of GP5. Based on their broad binding capacity to numerous genotypes of PRRSV-2 (Fig. 6), it is likely that a conserved region of the GP5 protein is being bound by these antibodies. The additional observation that p5c4 is also neutralizing would also point towards the highly conserved Epitope B as opposed Epitope A or C which are hypervariable. However, I showed p5c4 to be only homologously neutralizing, similar to the proposed characteristics of Epitope C, not broadly neutralizing which was a proposed characteristic of Epitope B. Further work is needed to identify the exact neutralizing epitope of antibody p5c4. P5c4 could be used to study how immune pressures change PRRSV evolution in a similar manner to the

experiments performed by Popescu et al., with the exception that we now have the opportunity to use a monoclonal PRRSV neutralizing antibody to apply epitope specific pressure to the virus. Since p5c4 only appears to be partially neutralizing to ATP, it could potentially be used to generate ATP escape mutants with long term exposure to the antibody. The generated resistant viruses could then be sequenced to determine which epitope within GP5 is responsible for neutralization.

The results of this work have established that GP5 contains a confirmed neutralizing epitope within the pig, shown the first ever isolation of a porcine derived monoclonal PRRSV neutralizing antibody, and demonstrated that broad binding, non-neutralizing antibodies may precede those that are broadly neutralizing. While much has been revealed about PRRSV neutralization in these pages, numerous questions arise from this work. What is known is that neutralization of PRRSV is complicated, but not impossible, and the further we investigate its origins, the closer we will be to the elimination of the virus.

CHAPTER IV: DETERMINATION OF VIRUS-BINDING AND NEUTRALIZING B CELL V(D)J MRNA SEQUENCES

Introduction

The pig's humoral immune response relies upon successful recombination of variable, diversity and joining region genes to form functional heavy and light chains for B cell receptors and immunoglobulin. Random rearrangement of these genes and development of a promiscuous panel of antibodies is key to developing an effective humoral immune response to PRRSV. Despite the pig's lack of diversity in its heavy chain VDJ genes, swine can develop an effective humoral response against the virus (Butler and Wertz 2012), which may rely heavily on somatic hypermutation (Butler et al. 2011). In this chapter, I take previously isolated and characterized PRRSV-specific B cells and sequence their mRNA pertaining to expressed antibody V(D)J regions. I concentrate my search on the variable, diversity and joining regions of the heavy chain and the variable and joining regions of the kappa and lambda light chain loci. Through this process, sequence was obtained that allowed for the determination of important genetic elements for PRRSV binding and neutralization. I also show evidence of substantial somatic hypermutation of heavy chain variable regions.

Methods

Isolation and sequencing of PRRSV-specific B cell V(D)J mRNA

RNA was extracted from $5x10^6$ immortalized clonal B cells was extracted using the recommended protocol for the RNeasy Mini Kit (Qiagen, Venlo, NL). Once RNA was isolated, 10 µl of eluted RNA was converted to cDNA following the recommended protocol for the High Capacity cDNA synthesis kit (Applied Biosciences, Beverly Hills, CA) in an S1000 Thermal Cycler (Bio Rad, Hercules, CA). Newly converted cDNA was used as a template for heavy chain, kappa light chain, and lambda light chain PCR amplification using primers based on GenBank IDs: AK405769.1, AK233830.1, AK233855.1 (Table 2) and AccuStart II PCR SuperMix (Quantabio, Beverly, MA)

following manufacturer's protocols on an S1000 Thermal Cycler (Bio Rad, Hercules, CA). To confirm amplification of the correct PCR product, agarose gel electrophoresis was performed on PCR products. Agarose gels were then stained with ethidium bromide for 10 min and visualized using UV light to confirm the size of the amplified PCR product (Table 2). Desired PCR products were isolated by cutting them out of the gel and purified using the QIAquick Gel Extraction kit (Qiagen, Venlo, NL). Prior to sequencing, purified PCR products were treated with ExoSAP-IT PCR product cleanup reagent (Thermo Fisher Scientific, Waltham, MA) and the DNA concentration was determined using a NanoDrop ND-1000 (Thermo Fischer Scientific, Waltham, MA). Samples were then mixed with the desired sequencing primer and sequenced by Sanger sequencing at the University of Minnesota Genomics Center (UMGC). Sequence trace files were examined and aligned using MEGA X alignment software (Kumar et al. 2018) and CLC Genomics Workbench v11.0 (Qiagen, Hilden, Germany). Variable regions and CDRs were identified using IgBLAST (Ye et al. 2013), and matched to known pig variable region sequences in the NCBI non-redundant database using BLASTn (Altschul et al. 1990).

Results

Identification and characterization of V(D)J regions of neutralizing and non-neutralizing clones

V(D)J regions were amplified from BNW7 clones p5c4 and p10c5 by PCR for both IgG heavy and light chains. Agarose gel analysis showed that p5c4's light chain uses the kappa locus and BNW7 clone p10c5 was shown to express both kappa and lambda light chain PCR products (Fig. 9). Good quality sequence was obtained for p5c4's heavy chain and its kappa light chain, covering the entire variable region of both chains. A standard nucleotide BLAST search of the obtained sequence confirmed the

Table 2: Primers used for PCR amplification and sequencing of porcine B cell V(D)J regions.

Primer sequence (5'-3')	Direction	Name	PCR product length (bp)
TCCGGAAAGAACACAGACCAC	Forward	IgG Heavy chain F1	1511
TCAGGTGAGCTCTTGGGACC	Reverse	IgG Heavy chain R1	
ATGGCCTGGACGGTGCTTCTG	Forward	Light chain lambda F2	711
AGGGACCTAGGCGCACTCGGAG	Reverse	Light chain lambda R2	
ATGAGGTTCCCTGCTCAGCTCC	Forward	Light chain kappa F2	720
CTAAGCCTCACACTCGTTCCT	Reverse	Light chain kappa R2	

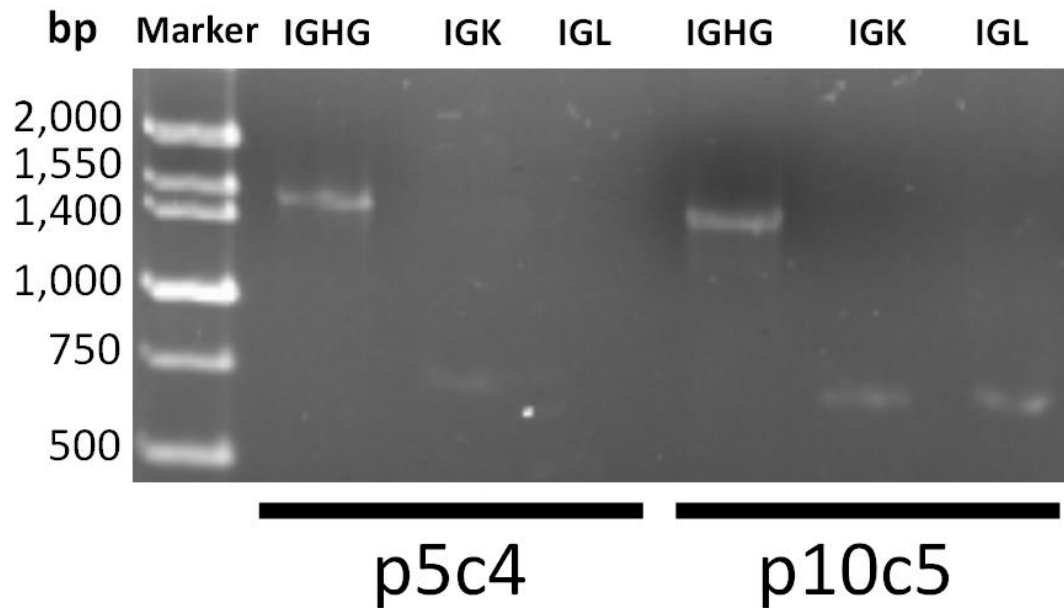


Figure 9: Isolated heavy and light chain PCR products from BNW7 p5c4 and p10c5 V(D)J mRNA

Porcine heavy and light chains were PCR amplified from immortalized clonal B cell populations, p5c4 and p10c5, from pig BNW7. PCR products were separated using agarose gel electrophoresis and visualized by ethidium bromide staining. A nucleotide size marker is shown at the far left to estimate the size of the PCR products. IGHG designates IgG heavy chain, IGK: light chain kappa, and IGL: light chain lambda.

identities of both heavy and light chains as porcine IgG heavy and light chain kappa mRNA. For p10c5, partial sequence was obtained for its heavy chain and full sequence for both lambda and kappa light chains. A standard nucleotide BLAST search of p10c5's heavy chain confirmed that it was pig IgG heavy chain sequence. BLAST searches of both p10c5 kappa and lambda light chains identified both sequences as unique porcine kappa and lambda light chains. As expected, an alignment of both p10c5 kappa and lambda light chains revealed no similarity between the two sequences, identifying both as unique sequence. The p10c5 B cell either expresses both light chain loci or is contaminated with another B cell population.

Using IGBLAST, p5c4's heavy chain variable region was identified to be 295 base pairs in length. The full CDR3 region, which includes the end of the variable region, the joining and diversity region was also identified. Once the variable region was isolated it was compared and aligned using BLASTn with 34 porcine fetal mRNA variable region sequences identified by Sun et al. from fetal pigs (Sun et al. 1994). CDR3 regions were not included in this analysis because these areas were too hypervariable. P5c4's heavy chain variable region was found to have 79.2% to 86.8% sequence identity with an average of 98% query coverage when aligned with the 34 identified fetal variable regions. This suggests that since its fetal origins, p5c4's variable region has gone through somatic hypermutation to change 13.2% to 20.8% of its sequence. Next, full p5c4 heavy chain sequence was compared to its closest related germline variable region sequence, pvg24 (86.8% variable region identity, GenBank ID: U15451.1), with the comparison extending into the full CDR3 region, using CLC Genomics Workbench. When the two V(D)J sequences were compared, including the CDR3, 37 sites of nucleotide exchange were identified, ranging from 1 to 11 base pairs exchanged at each

site, a total of 65 exchanged nucleotides and 82.8% identify between the sequences (Fig 10). Three sites of deletion were identified within the CDR3 of p5c4 (Fig. 10).

Using IGBLAST a full heavy chain variable region identified to be 296 base pairs in length, for clone p10c5. Only the beginning of the CDR3 region was identified, no joining or diversity region sequence were isolated, so this region was not included in analysis. The heavy chain variable region mRNA was compared and aligned using BLASTn to those isolated by Sun et al. (Sun et al. 1994), in a similar manner to p5c4. P10c5's variable region was found to have an 82.3% to 91.1% sequence identify with an average of 98% query coverage when aligned with the 34 identified fetal mRNA variable regions. This suggests a somatic hypermutation exchange of 8.9% to 17.7% of its sequence since germline rearrangement. When compared to its closest related germline variable region sequence, pvm4a (91.1% identity, GenBank ID: U15439.1), 18 sites of nucleotide exchange were identified, ranging from 1 to 3 base pairs exchanged at each site, a total of 26 exchanged nucleotides, spanning the entire heavy chain variable region.

Discussion

Heavy chain variable region sequence was identified for BNW7 clone p5c4 and p10c5. Sequences were compared to known fetal heavy chain variable regions, but neither sequence could be linked to a specific fetal variable region gene. All porcine heavy chain variable region genes are fairly homologous, with Sun et al. finding over 80% homology between all isolated genes (Sun et al. 1994), making identification of a specific germline gene of origin from a heavily somatic hypermutated mRNA difficult. Both heavy chain sequences show evidence of somatic hypermutation, with abundant point mutations within complementarity determining regions (CDR) (Fig. 10). Somatic hypermutation in pig heavy chain variable region CDRs has been suggested as critical

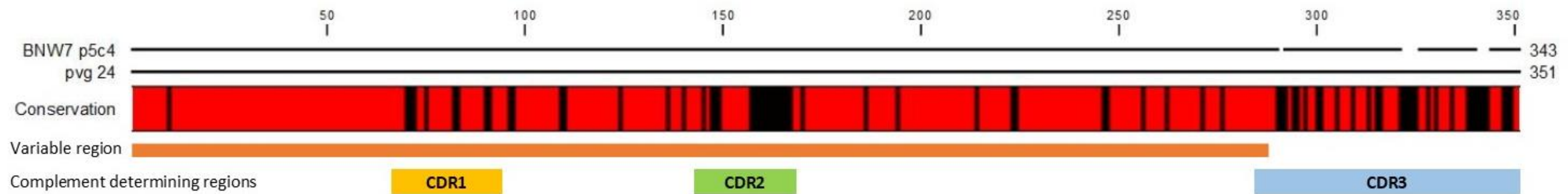


Figure 10: Sequence comparison of BNW7 p5c4 V(D)J mRNA to fetal mRNA pvg 24

BNW7 p5c4 full V(D)J mRNA sequence, including its CDR3, was aligned and compared to historical fetal V(D)J mRNA pvg 24 (GenBank ID: U15451.1). Gaps within p5c4's sequence signify deletions. In the line below the sequences, sites of genetic conservation are noted by red blocks, while sites of differentiation are noted by black blocks. The entire variable region is highlighted in orange, CDR1 in yellow, CDR2 in green, and CDR3 in blue.

component of diversifying variable region genes (Butler, Weber and Wertz 2006). Sun et al. also found all heavy chain variable region genes to be closely related to a single family of human heavy chain variable region genes, IGHVIII (Sun et al. 1994). Both heavy chain variable region sequences in this work were also found to share close homology to the human IGHVIII family according to an IGBLAST search. These sequences, especially from the neutralizing clone p5c4, will be of high interest to researchers investigating variable regions associated with PRRSV specificity and neutralization. However, the pig's repertoire of heavy chain variable region genes is extremely limited compared to other mammalian species, with only ~30 known variable region genes that are all genetically similar and as an adult, pigs predominantly use 7 of these genes (Sun et al. 1994, Butler et al. 2011). Yet, despite its lack of diversity, the pig can respond and form antigen specific immunity to any pathogen it is exposed to. It has been suggested that somatic hypermutation of V(D)J genes is primarily responsible for the diversity in humoral immune response (Butler and Wertz 2012). The sequences I identified were heavily mutated compared to germline genes, with the neutralizing clone p5c4's full V(D)J mRNA, including its CDR3, having an 18.2% sequence mismatch when compared to its closest matched germline mRNA sequence, pvg 24 (Fig. 10). Similar results were shown by Butler et al., where ~90% of variable region genes in piglets were found to by somatically hypermutated after exposure to flu virus (Butler et al. 2011). In HIV, isolated broadly neutralizing B cells show high levels of somatic hypermutation, ranging from 7% to 32% divergence from germline sequence (Sok et al. 2013). It is likely that somatic hypermutation, after exposure to PRRSV, plays a large and critical role in

developing a diverse heavy chain repertoire in the pig and generation of broadly neutralizing antibodies.

AID, the protein responsible for somatic hypermutation, has been shown to be active within B cells produced by the method used in this work (Kwakkenbos et al. 2010), however the rate is much lower than seen *in vivo* (Peled et al. 2008). Both clones have been in culture for approximately 4 months and with a full replication cycle occurring every 36 hours, 80 divisions would be expected. The high end of expected AID-mediated mutation within this culture system is 8.85×10^{-5} mutations per base per cell division (Kwakkenbos et al. 2010) and the length of both variable regions is around 300 base pairs. Based on both clones' estimated divisions, mutation rate, and length of sequence, 1 or 2 of the base pair exchanges seen could be from somatic hypermutation after immortalization, suggesting that the majority of mutations occurred naturally, *in vivo,* prior to isolation and immortalization of B cells.

Swine also have a diverse population of light chain variable regions when compared to the heavy chain variable regions (Schwartz et al. 2012a, Schwartz et al. 2012b), but they are not well studied (Butler and Wertz 2012). P5c4's light chain was identified as kappa based on PCR amplification (Fig. 9). Analysis of p5c4's light chain sequence confirmed its designation as kappa, but its particular gene identity was not elucidated. P10c5's saw PCR amplification of both lambda and kappa light chains and sequencing of both products confirmed their identify as unique lambda and kappa light chains. It is unknown why p10c5 expresses both light chains. It is possible that another immortal B cell population is contaminating the culture, however the heavy chain sequence was good quality and very clean, suggesting it was the only heavy chain sequence present. What is more likely is that p10c5 expresses both light chains,

however it is possible that only one is functional and is translated and integrated into the antibody. Some B cells associated with autoimmunity and myeloma have been shown to express multiple light chain genes (Pelanda 2014, Perfetti et al. 2004). Since the genes within vector used for immortalization are associated with lymphoma, it is possible the dual expression of light chains is associated with insertion of those genes. Further work is needed to confirm this. Light chain rearrangement and expression has been observed to precede heavy chain expression in the pig (Sinkora et al. 2017). Based on these observations, it is a possibility that light chains play a bigger role in antigen specific immunity in swine than in other mammals and the subject warrants further investigation.

CHAPTER V: GENERAL DISCUSSION

The results from this work provide new and intriguing insights into the pig's immune response to PRRSV. I demonstrated that the pig can retain high titers of neutralizing antibodies for months after PRRSV exposure. Pigs selected for this study showed neutralizing titers up to 1:8,192 (Fig. 2) at 90 dpi, far beyond the 1:8 titer that is thought to be required for protective immunity (Osorio et al. 2002, Yoon et al. 1996). I found that PRRSV specific IgG+ memory B cells express CD21 on their surface, confirming previous findings that the marker can be used to identify mature porcine B cells (Sinkora et al. 2013). Although this does not preclude CD21- B cells involvement in memory. All five of the isolated immortalized B-cell populations were found to be broadly binding to PRRSV-2, but not PRRSV-1. GP5 specific immortalized B cell clonal population, BNW7 p5c4, was determined to be homologously neutralizing against PRRSV ATP and broadly binding to all PRRSV-2. I theorize that these antibodies are involved in not only neutralization of homologous virus but also act as non-neutralizing antibodies to heterologous virus that may be involved in reduction of viremia via binding of conserved regions. GP5 is now a confirmed neutralizing epitope of PRRSV, but it is doubtful to be the only protein involved in neutralization. Isolated heavy chain variable region sequences from neutralizing clone p5c4 and non-neutralizing clone p10c5 were found to be heavily mutated. This showed that for the pig to generate a neutralizing response somatic hypermutation of V(D)J genes is critical. These results indicate that humoral immunity's involvement in suppression of PRRSV infection is complicated and critical to elimination of the virus.

Generation of broadly binding/neutralizing antibodies

The development of broadly neutralizing antibodies against PRRSV is rare and difficult to replicate. Based on my findings of that all five isolated B cells secreted broad

binding antibody against a diverse group of PRRS viruses, I propose that these antibodies were generated via repeated positive antigen selection of a conserved region of GP5. Many previous works have shown that varied virus and vaccine exposures lead to improved immunity (Robinson et al. 2015, Robinson et al. 2018, Scortti et al. 2006, Martínez-Lobo et al. 2011) and most likely broad PRRSV binding, as seen in this study. For the sake of simplicity, I will concentrate on GP5 protein driving this selection and ignore that there are numerous other PRRS antigen specific B cells being generated. I will also disregard the neutralizing capacity of the antibody and only consider its ability to bind to the GP5 antigen. I hypothesize that initially, when each of the pigs in this study were vaccinated with ATP, they generated a pool of B cells that were ATP GP5 specific. These B cells were likely spread amongst all the known binding epitopes of GP5 protein from the ATP strain, creating a group of B cells that are highly diverse in the location of their binding sites. From this pool of ATP specific B cells, the pig's first memory B cells specific to PRRSV were generated. When the pig received its second dose of ATP, the GP5 specific memory response was reinvigorated for the entire pool of PRRSV ATP strain-specific B cells, reestablishing and strengthening the anti-GP5 response to ATP. At the same time the diversity of this pool was most likely further expanded against ATP's GP5. Re-diversification of B cells upon booster vaccination has been documented (McHeyzer-Williams et al. 2015). When the pig was next exposed to a new heterologous virus, PRRSV 1-3-4 or 1-18-2 in the case of the pigs in this study, the previously generated memory response to ATP GP5 was once again reinvigorated. From the pool of ATP GP5 specific memory B cells, only those who also bound the new virus were selected and reactivated; the B cells which have a broad binding capacity. Memory B cells have been shown to respond to antigen much faster than naïve B-cells to the same antigen (Seifert et al. 2015), which gives them a competitive edge in reactivation. B cells

that are only ATP specific, and were not reactivated, would go through anergy, and over time eventually be eliminated, leaving behind only recently stimulated broad binding antibody. Similar results have been shown by other authors, where B cells with higher affinity for the presented antigen will proliferate, while those with low or no affinity go through increased cell death (Dal Porto et al. 2002, Shih et al. 2002). Further exposure to heterologous virus most likely had the same effect; selecting for antibodies that bound the new virus from the pool of memory B cells that bound the previous viruses, establishing an array of B cells with broad binding capacity to PRRSV, and leaving previous homologously binding B cells inactivated. Preceding works have suggested that boosting immunity with heterologous antigen enhances overall immunity to pathogens (Kardani, Bolhassani and Shahbazi 2016). In the case of BNW4, these antibodies went through four exposures to virus: twice to ATP, once to PRRSV 1-3-4 and once to PRRSV 1-18-2, with each reactivation selecting for B cells that bound all three viruses. This theory would explain why all isolated PRRSV specific antibodies were broadly binding, as over time only those who bound conserved regions of GP5 remained as immune memory cells. It has been shown previously that additional memory recall events lead to further expansion of antigen specific memory B cell populations dominating lymph node germinal centers (Bende et al. 2007). Recent works in HIV have pointed towards similar conclusions, with evidence that increased and repeated “education” of the immune system to specific epitopes increases immune responses to virus (Agazio and Torres 2019, Saunders et al. 2019, Steichen et al. 2019). This theory would also explain why ATP specific neutralizing antibody BNW7 p5c4 is still present in the blood after 260+ days post final vaccination, as this broadly binding B cell rode a wave of positive selection and continued to exist. It is likely that p5c4 is specific for GP5 epitope B, as this region has been shown to be highly conserved in ORF5 and is thought

to be a site for broad neutralization (Popescu et al. 2017). This may also mean that broadly PRRSV binding B cells precede broadly PRRSV neutralizing B cells and based on my finding of high levels of somatic hypermutation within my neutralizing clone, that random mutation drives the transition from binding to neutralizing. This theory warrants further testing and could be accomplished by isolating PRRS antigen specific B cells at different points during the lifetime of pig that was exposed to a variety of PRRSV and assessing changes in binding capacity and V(D)J sequence with changes in virus exposure.

Potential involvement of PRRSV-specific non-neutralizing antibodies in immunity

In my work I show an interesting dynamic between PRRSV specificity in binding versus the ability to neutralize virus. All antibodies isolated for my study were able to bind a broad range of PRRSV-2, but not PRRSV-1 (Fig. 6). However, binding did not correlate with neutralization, as antibody from BNW7 clone p5c4 was able to bind all PRRSV-2 but only able to effectively neutralize homologous virus (ATP)(Fig. 8). Similarly, antibody from BNW7 clone p10c5 was also able to bind all PRRSV-2 virus but was non-neutralizing. It should be noted that our conventional neutralization assay only investigates a single component of antibody function, the ability of an antibody to neutralize virus by directly interfering with the virus's ability to infect the host cell. Antibodies have various functions in an immune response beyond direct neutralization of virus. Antibodies also flag pathogens and infected cells for digestion via interactions of antibody Fc with immune cell surface Fc receptors. It is possible that non-neutralizing antibodies may aid the process of elimination of PRRSV through processes such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (CDC) and antibody dependent cellular phagocytosis

(ADCP). A recent study of HIV found non-neutralizing antibodies to inhibit infectivity of HIV to macrophages and dendritic cells, via engulfment and digestion of antibody flagged virus by macrophage binding antibody Fc to FcγR (Holl et al. 2006). While these antibodies were not able to neutralize virus, the had an inhibitory effect on infection by marking virus for elimination by immune cell interactions with Fc. Holl et al. referred to these antibodies as non-neutralizing inhibitory antibodies (NNiabs), to distinguish them from neutralizing antibodies that have the ability to both neutralize and inhibit virus (Holl et al. 2006). Another recent paper on HIV saw that broadly neutralizing antibodies aided in the clearance of infected cells *in vivo* by an FcγR-dependent mechanism involving NK cells akin to ADCP or ADCC (Lu et al. 2016). It is possible that the immune response to PRRSV is similar, with what conventional neutralization assays label as non-neutralizing antibodies, aiding the innate immune system in the elimination of virus through Fc/FcγR interactions between infected macrophages and NK cells. The potential role non-neutralizing antibodies play in PRRSV suppression has been discussed in recent reviews (Montaner-Tarbes et al. 2019, Rahe and Murtaugh 2017c, Rahe and Murtaugh 2017a). This hypothesis could explain my findings of the broad diversity of PRRSV binding observed in my isolated monoclonal antibodies and could point toward their involvement in broad neutralization. However, for this to be true the concept of antibody dependent enhancement in PRRSV would have to have little or no effect in the pig. Several studies have shown PRRSV ADE *in vitro* (Yoon et al. 1997, Gu et al. 2015) but none have been able to show the effect convincingly *in vivo* (Yoon et al. 1996, Lopez et al. 2007). In these *in vitro* ADE studies, it is likely that the PAM culture microenvironment, considering cytokines and other immune signals, is highly dissimilar to the activated immune environment *in vivo*. Macrophages *in vivo,* during infection, are much more active and phagocytic than *in vitro* (Morland and Kaplan 1977). These

cultures also did not include NK cells, which may have a larger role than previously thought in the elimination of PRRSV infected macrophages and lowering viremia. It is possible that a large panel of non-neutralizing, broadly binding, anti-PRRSV antibodies, may have a strong enough inhibitory effect to reduce viremia *in vivo* even though they are non-neutralizing. This theory could also explain why neutralizing antibodies are not observed at high levels in the pig until viremia is resolved (Molina et al. 2008, Lopez et al. 2007), as the inhibitory effect of non-neutralizing antibodies could be contributing to the reduction in viremia, as virus specific non-neutralizing antibodies appear at 7 to 14 dpi, while elimination of virus is achieved by the later development of neutralizing antibodies at 28 dpi (Loemba et al. 1996). This also correlates with the early NK cell IFNγ response that peaks at 10 dpi and drops back to baseline at 22 dpi, implicating that NK cells may interact with non-neutralizing antibodies and eliminate PRRSV infected cells through ADCC (Wesley et al. 2006) further reducing viremia. Additional studies are needed to investigate this possible facet of the pig's immune response and its role in PRRSV elimination.

Refocusing the search for PRRSV neutralizing epitopes

In this work I explored avenues for the generation of cross neutralizing antibodies, but the actual mechanism and epitope interactions remain elusive. Previous work has shown that the GP5 gene, ORF5, is highly variable (Murtaugh et al. 1995) and it has been suggested that neutralization of GP5 is conformation dependent (Pirzadeh and Dea 1998). Because of its propensity to mutate and confirmation dependent neutralization, GP5 may often escape neutralization, possibly making homologous neutralization against GP5 common and broad neutralization rare. Neutralization against GP2a-GP3-GP4 on the other hand has been shown to be directed against linear

epitopes (Vanhee et al. 2011), not conformational ones. GP2a-GP3-GP4 has also been shown to be essential for infection (Wissink et al. 2005, Welch et al. 2004) and the key protein complex in virus entry (Das et al. 2010, Calvert et al. 2007). Because of its essential role in virus entry, conserved regions most likely exist within these proteins that could be exploited as sites of broad neutralization. For these reasons, GP2a-GP3-GP4 should be further investigated for its involvement broad neutralization.

Limitations

The data presented in this work should be interpreted considering several limitations. First, all experiments, including IFAs and neutralizations assays, in this manuscript were done in MARC-145 cells, not in the true porcine host cell of PRRSV, PAMs. Although MARC-145 and PAMs are both mammalian cell lines are fairly similar (Kim et al. 1993), there are several differences in cell surface markers involved in PRRSV infection (Shi et al. 2015). This could mean that the neutralization results seen in this work may not translate to infection of PAMs. Second, all the proteins used in ELISAs in this work were created in E. Coli expression systems, not the virus's native mammalian system, so there may be differences in glycosylation and protein confirmation that effect antigen/antibody interactions. This may explain why p10c5's viral protein specificity remains unknown, as the proteins used in the ELISA may not have been in the correct native confirmation.

Future directions

The findings in this work open the doors to numerous future studies. The isolation of a PRRSV neutralizing antibody (BNW7 p5c4) alone is groundbreaking and further work is needed to characterize it. It is still unknown where exactly antibody p5c4 binds on the GP5 protein. It would be fascinating to examine if the antibody bound a

predicted site for neutralization. Most historical literature would suggest co-culturing PRRSV with p5c4 antibody, until an escape mutant virus was generated that the antibody could no longer bind. However, based on my hypothesis that anti-GP5 antibodies are not fully neutralizing, it may be difficult to apply enough pressure to force an escape mutant. X-ray crystallography could also be implemented to identify the site of antibody/antigen interaction. Another approach would be to examine ORF5 of closely related PRRSV that p5c4 can bind but not neutralize, looking for genomic differences that could point toward the site of neutralization. This would take advantage of the interesting dynamic I illustrated, where p5c4 antibody binds both ATP and VR2332 virus, but only neutralizes ATP. It would also be wise to investigate ORF5 of PRRSV-1 virus since p5c4 is unable to bind the PRRSV-1 strain examined, which may identify regions of nucleotide differences possibly involved in binding GP5, although since PRRSV-1 viruses are so distantly related to PRRSV-2 this task may be difficult.

The isolation of these antibodies also could allow us to answer a long-disputed facet of PRRSV infection, antibody dependent enhancement. While previous studies have used non-neutralizing pig serum, my generated monoclonal PRRSV-specific antibodies would allow us to study the effect directly. Antibody p5c4 could be mixed with homologous virus (ATP), which it neutralizes, as well as with heterologous virus (VR2332 or another PRRSV-2), which it is not able to neutralize. These mixtures, of neutralized homologous virus and non-neutralized heterologous virus, would then be used to infect PAM culture in vitro and monitored for virus production over time by RT-PCR and IFA. If the theory of ADE were true, antibody p5c4 that has been mixed with heterologous virus, which it is unable to neutralize, should show increased virus production compared to virus only controls and p5c4 mixed with homologous virus should show decreased virus replication compared to virus only controls. It would also

be prudent to test how activation of macrophages, using cytokines such as IFN-γ or TNF-α (Mosser and Edwards 2008), affected virus production. I earlier hypothesized that activated macrophages and NK cells, similar to the expected immune environment *in vivo* during infection, may have the opposite effect of ADE, actively eliminating virus and virus infected cells marked with PRRSV-specific non-neutralizing antibody. For these reasons, any results generated from a study such as this should be interpreted with caution, as *in vitro* and *in vivo* immune environments differ.

Further work is needed to determine if PRRSV-specific neutralizing antibodies against other viral proteins are produced in the pig following vaccination or challenge. I showed that BNW4 and BNW7 pig serum contains antibodies that bind a wide variety of PRRSV-specific proteins (Fig. 7). Clearly PBMCs from both pigs contain B cells specific for the majority of screened proteins and most likely many others not in the examined ELISA panel. Thus, direct isolation of B cells by sorting based on binding to specific PRRSV proteins should be undertaken to identify these antibody producing B cells. Many of the viral proteins used in my ELISA panel contain HIS-tags that could easily be used to isolate antigen specific cells either through column purification or fluorescent-activated cell sorting (FACS). Our lab has also had success with the development of PRRSV viral protein specific tetramers that could be used to increase isolation efficiency (Rahe et al. 2018). Isolated clones could then be screened for PRRSV antibodies and specificity against PRRSV proteins. Particular interest should be taken in insolating GP2a, GP3 and GP4 specific immortalized B cell clones, which have recently been both theorized and demonstrated to contain PRRSV neutralizing epitopes (Loving et al. 2015, Vanhee et al. 2011, Costers et al. 2010a). This process could also be used to isolate additional GP5 clones and clones specific to M and E proteins.

This work also opens avenues for similar investigations examining other pathogens of swine. Porcine rotavirus is a particularly costly disease for the swine industry worldwide. Rotavirus is the leading cause of acute gastroenteritis in suckling and weaning pigs (Vlasova, Amimo and Saif 2017). Protection from the virus is often passed from sow to piglet through antibody rich colostrum via a process called passive transfer. However, levels of antibody in colostrum vary from sow to sow and this maternal derived immunity is not always effective in piglets, especially against viral strains the sow has not been exposed to (Vlasova et al. 2017). Work on human rotavirus has revealed that antibodies developed after exposure to virus vary from non-neutralizing to highly heterotypic (Nair et al. 2017), suggesting that broadly neutralizing antibodies could potentially be isolated. Isolation of rotavirus broadly neutralizing antibodies could lead to the design of an anti-viral piglet colostrum supplement that would be highly effective at protecting piglets from the virus through passive transfer of the isolated neutralizing antibody.

PRRSV vaccination

The findings in this manuscript lead to questions on how we should vaccinate for PRRSV and if current vaccination strategies are effective for the development of adequate immunity within the pig. Most current vaccines on the market have common problem, they are designed based on PRRSV strains that have not been field relevant in over two decades. Recent work has shown that one of the most commonly used PRRSV vaccines in the US, Igelvac PRRSV MLV (Boehringer Ingelheim, Ingelheim am Rhein, Germany) improves production outcomes in pigs, but did little to reduce viremia and spread of virus, despite 89% similarity to challenge virus (Haiwick et al. 2018). Similar results were shown by Oh et al. where several currently marketed PRRSV vaccines

were used under field conditions. While production parameters, such as average daily gain, were slightly increased in vaccinated animals, viremia and clinical outcomes were similar between vaccinated and unvaccinated groups (Oh et al. 2019). While both works argue that vaccine is efficacious because it improves production outcome, neither works demonstrated that current marketed products can fulfill the purpose of vaccination, to provide immunity to disease. Other works have shown the current vaccines provide limited to weak humoral immunity against PRRSV (Zuckermann et al. 2007, Díaz et al. 2006), that is often only against homologous virus (Charerntantanakul 2012, Roca et al. 2012). Based on findings in this manuscript, I propose several recommendations for future vaccines and vaccination schedules. First, next generation vaccines must represent current field strains and should be updated often to reflect changes in the virus. Current vaccines, based on viruses such as ATP and VR2332, are highly divergent from present field strains (Fig. 5). Second, pigs should be vaccinated with a variety of diverse PRRSV strains, either MLV or killed, and vaccinated often. This would likely lead to higher incidences of cross-protective immunity within herds and repeating these diverse vaccines often would maintain that immunity of long periods of time, similar to the individuals studied in this work (Table 1, Fig. 2). Repeated exposure to virus would also improve the development of broadly binding/neutralizing B cells through positive B cell selection, theorized in this manuscript. Third, vaccine development should focus on MLVs that provide longer periods of vaccine induced viremia. It was discussed earlier, in chapter 3 of this manuscript, that higher viremia correlated with improved avidity and development of cross-protective immunity. Shedding and circulation of MLV vaccine within herds could also have the additional effect of reinvigorating immune responses analogous vaccine boosters. However, under these circumstances, unless the vaccine was a DIVA (differentiation of infected from vaccinated animals) vaccine,

actual field breaks of PRRSV would be difficult to detect. There is also the additional risk of MLVs recombining with field strains and creating new viruses. Taken together, there are many ways we can improve PRRSV vaccination, but to achieve them we must shift our focus from improving production parameters to actual prevention of disease.

Based on the findings within this manuscript and current PRRSV literature, a hypothetical ideal vaccination plan was developed for the maturation of protective immunity and total elimination of virus. The goal of this vaccination plan is not only to improve production parameters, but to also eliminate PRRSV virus from herds and localities. Its implementation would involve a coordinated effort between producers and government agencies, along with vigilant biosecurity and surveillance. At farrowing, gilts and sows should be evaluated for good PRRSV immunity via serological tests, to ensure that transferred maternal immunity to piglets will be protective. If any gilt or sow does not meet a predetermined threshold for protection, their newborn piglets should receive a colostrum supplement containing highly concentrated, porcine derived, broadly neutralizing PRRSV-specific antibodies (IgG, IgM and IgA), within the first 48 hours of life. This supplement could be created by isolating PRRSV neutralizing B cells, immortalizing the cells using the methods within this manuscript and concentrating their secreted antibodies. Next, as piglets enter the nursery, they should be vaccinated with a killed PRRSV vaccine that contains a large pool of diverse PRRSV. Ideally these killed viruses would be autogenous viruses seen within the production system and other closely related viruses that have been reported within the systems geographic region. This vaccination should be repeated when the pig exits the nursery. If the pig is destined for a breeding stock, either as a new gilt or as a boar, they should begin receiving a modified live vaccine (MLV) vaccination every 6 months. The MLV these pigs receive should rotate to a different strain every 6 months, so pigs are exposed to a new virus

and develop cross-protective immunity. The MLV rotation should contain several genetically distinct and diverse MLVs, and companies should update their MLVs every few years, switching out vaccine strains that are less effective for strains that better match field virus. Ideally these MLVs would be DIVA vaccines to retain the ability to differentiate vaccination from virus outbreaks. Pigs that are entering finishing herds should receive a single dose of a few of the aforementioned MLVs, to boost protection while the pig gains weight for eventual slaughter. Selection of the MLVs given to finishers should be made based on field virus present in the locality. While this hypothesized ideal vaccination plan is complicated and expensive, it will likely provide excellent lifelong immunity to pigs within it. In this work I demonstrated that a combination of neutralizing and even non-neutralizing antibodies potentially leads to long term broad protection from PRRSV. Further isolation of similar antibodies to those described in this manuscript could lead to a better understanding of pig's immune response to PRRSV and better vaccine design. Compared to the current annual costs the disease inflicts on producers, over 650 million US dollars a year (Holtkamp, Kliebenstein and Neumann 2013a), implementing this elimination plan would have immense long-term benefits. While good protection and increased production would likely be seen with this plan, the ultimate goal is to eradicate all traces of the virus. Thus, involvement of all pig producers and government agencies within the area this plan is implemented is key. Only through a vigorous and concerted effort by all parties, government and industry, will PRRSV ever be completely eliminated.

Correlations between HIV and PRRSV

Investigating the literature on broad neutralization to human immunodeficiency virus (HIV) should be further examined to gain insights on the development of broad

neutralization to PRRSV in the pig. I argue, that despite large differences in genome and structure, there are many core features to the virus and the host's immune response to the disease that are similar between HIV and PRRSV. Both viruses infect and destroy immune cells, be it T cells (Fanales-Belasio et al. 2010) or macrophages (Koppensteiner, Brack-Werner and Schindler 2012) in HIV and PAMs in PRRSV (Zimmerman et al. 2019), leading to immune deficiencies and susceptibility to other infections. Both viruses mutate rapidly, leading to immune escape, repeat infections and highly diverse viruses within populations (Ndung'u and Weiss 2012, Hanada et al. 2005). Both viruses persist in the host leading to long term, chronic infections, that aid in spreading the virus (Churchill et al. 2016, Horter et al. 2002, Allende et al. 2000). Both viruses have neutralizing epitopes that are heavily glycosylated structural proteins and neutralization seems to be confirmation dependent (Behrens and Crispin 2017, Ansari et al. 2006, Wei et al. 2012, Vu et al. 2011, Pirzadeh and Dea 1998). Development of broad neutralization to both viruses is rare in populations that have circulating disease (Robinson et al. 2015, Scheid et al. 2009, Walker et al. 2009, Walker et al. 2011). Broadly neutralizing antibodies against HIV often show high levels of somatic hypermutation (Klein et al. 2013, Sok et al. 2013). I hypothesized that PRRSV broadly neutralizing antibodies are similarly heavily mutated. It may also be advantageous to examine HIV vaccination strategies as a model for PRRSV vaccination as noted in Rahe et al (Rahe and Murtaugh 2017c). For these reasons and others, strategies for the investigation of HIV neutralization should be examined as a conceptual model for work in PRRSV neutralization.

Conclusion

In this study I aimed to gain insights on neutralization of PRRSV and the development of broadly neutralizing antibodies. I isolated, for the first time, porcine monoclonal antibodies capable of neutralizing PRRSV. I presented a PRRSV GP5 specific antibody that is capable of neutralizing homologous virus, cementing GP5's involvement in PRRSV neutralization within the pig. I hypothesize that while GP5 is involved in neutralization it is not the sole responsible antigen and that other PRRSV proteins are most likely involved. Anti-GP5 B cells and antibodies were found to be abundant in the pig's long-term memory response to PRRSV, with four out of five B cells isolated being GP5 specific. Isolated antibodies, regardless of neutralizing capacity, were able to bind a wide variety of PRRSV-2 but not PRRSV-1. I suspect that this is thanks to repeated positive selection for broadly binding antibodies by the pig's immune system over time. I theorized that strength of antibody/antigen interactions and non-neutralizing antibodies, through interactions with the innate immune system, play a more important role in PRRSV immunity than previously thought. I also showed the heavy chain variable regions from these clones were highly mutated when compared to historical germline sequences, suggesting the critical role of somatic hypermutation in generating PRRSV-specific and broadly neutralizing antibodies. My work sheds new light on neutralization to PRRSV in the pig, but much is still unknown.

REFERENCES

Adams, M. J., E. J. Lefkowitz, A. M. King, B. Harrach, R. L. Harrison, N. J. Knowles, A. M. Kropinski, M. Krupovic, J. H. Kuhn, A. R. Mushegian, M. Nibert, S. Sabanadzovic, H. Sanfaçon, S. G. Siddell, P. Simmonds, A. Varsani, F. M. Zerbini, A. E. Gorbalenya & A. J. Davison (2016) Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2016). *Arch Virol,* 161**,** 2921-49.

Agazio, A. & R. M. Torres (2019) Ushering along B cells to neutralize HIV. *Science,* 366**,** 1197-1198.

Albina, E. (1997) Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. *Vet Microbiol,* 55**,** 309-16.

Albina, E., L. Piriou, E. Hutet, R. Cariolet & R. L'Hospitalier (1998) Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol,* 61**,** 49-66.

Allende, R., W. W. Laegreid, G. F. Kutish, J. A. Galeota, R. W. Wills & F. A. Osorio (2000) Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *J Virol,* 74**,** 10834-7.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman (1990) Basic local alignment search tool. *J Mol Biol,* 215**,** 403-10.

Ansari, I. H., B. Kwon, F. A. Osorio & A. K. Pattnaik (2006) Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol,* 80**,** 3994-4004.

Baum, A. & A. García-Sastre (2010) Induction of type I interferon by RNA viruses: cellular receptors and their substrates. *Amino Acids,* 38**,** 1283-99.

Behrens, A. J. & M. Crispin (2017) Structural principles controlling HIV envelope glycosylation. *Curr Opin Struct Biol,* 44**,** 125-133.

Bende, R. J., F. van Maldegem, M. Triesscheijn, T. A. Wormhoudt, R. Guijt & C. J. van Noesel (2007) Germinal centers in human lymph nodes contain reactivated memory B cells. *J Exp Med,* 204**,** 2655-65.

Beura, L. K., S. N. Sarkar, B. Kwon, S. Subramaniam, C. Jones, A. K. Pattnaik & F. A. Osorio (2010) Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. *J Virol,* 84**,** 1574-84.

Blom, N., T. Sicheritz-Pontén, R. Gupta, S. Gammeltoft & S. Brunak (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics,* 4**,** 1633-49.

Bradbury, L. E., G. S. Kansas, S. Levy, R. L. Evans & T. F. Tedder (1992) The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J Immunol,* 149**,** 2841-50.

Brown, E., S. Lawson, C. Welbon, J. Gnanandarajah, J. Li, M. P. Murtaugh, E. A. Nelson, R. M. Molina, J. J. Zimmerman, R. R. Rowland & Y. Fang (2009) Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin Vaccine Immunol,* 16**,** 628-35.

Butler, J. E. (1997) Immunoglobulin gene organization and the mechanism of repertoire development. *Scand J Immunol,* 45**,** 455-62.

Butler, J. E., X. Sun, N. Wertz, K. M. Lager, K. Chaloner, J. Urban, D. L. Francis, P. L. Nara & G. J. Tobin (2011) Antibody repertoire development in fetal and neonatal piglets XXI. Usage of most VH genes remains constant during fetal and postnatal development. *Mol Immunol,* 49**,** 483-94.

Butler, J. E., P. Weber & N. Wertz (2006) Antibody repertoire development in fetal and neonatal piglets. XIII. Hybrid VH genes and the preimmune repertoire revisited. *J Immunol,* 177**,** 5459-70.

Butler, J. E. & N. Wertz (2012) The porcine antibody repertoire: variations on the textbook theme. *Front Immunol,* 3**,** 153.

Calvert, J. G., D. E. Slade, S. L. Shields, R. Jolie, R. M. Mannan, R. G. Ankenbauer & S. K. Welch (2007) CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J Virol,* 81**,** 7371-9.

Chang, C. C., K. J. Yoon, J. J. Zimmerman, K. M. Harmon, P. M. Dixon, C. M. Dvorak & M. P. Murtaugh (2002) Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. *J Virol,* 76**,** 4750-63.

Charerntantanakul, W. (2012) Porcine reproductive and respiratory syndrome virus vaccines: Immunogenicity, efficacy and safety aspects. *World J Virol,* 1**,** 23-30.

Chen, Z., S. Lawson, Z. Sun, X. Zhou, X. Guan, J. Christopher-Hennings, E. A. Nelson & Y. Fang (2010) Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. *Virology,* 398**,** 87-97.

Cherukuri, A., P. C. Cheng & S. K. Pierce (2001) The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J Immunol,* 167**,** 163-72.

Churchill, M. J., S. G. Deeks, D. M. Margolis, R. F. Siliciano & R. Swanstrom (2016) HIV reservoirs: what, where and how to target them. *Nat Rev Microbiol,* 14**,** 55-60.

Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison & H. S. Joo (1992) Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J Vet Diagn Invest,* 4**,** 117-26.

Costers, S., D. J. Lefebvre, B. Goddeeris, P. L. Delputte & H. J. Nauwynck (2009) Functional impairment of PRRSV-specific peripheral CD3+CD8high cells. *Vet Res,* 40**,** 46.

Costers, S., D. J. Lefebvre, J. Van Doorsselaere, M. Vanhee, P. L. Delputte & H. J. Nauwynck (2010a) GP4 of porcine reproductive and respiratory syndrome virus contains a neutralizing epitope that is susceptible to immunoselection in vitro. *Arch Virol,* 155**,** 371-8.

Costers, S., M. Vanhee, W. Van Breedam, J. Van Doorsselaere, M. Geldhof & H. J. Nauwynck (2010b) GP4-specific neutralizing antibodies might be a driving force in PRRSV evolution. *Virus Res,* 154**,** 104-13.

Dal Porto, J. M., A. M. Haberman, G. Kelsoe & M. J. Shlomchik (2002) Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. *J Exp Med,* 195**,** 1215-21.

Das, P. B., P. X. Dinh, I. H. Ansari, M. de Lima, F. A. Osorio & A. K. Pattnaik (2010) The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. *J Virol,* 84**,** 1731-40.

Dea, S., C. A. Gagnon, H. Mardassi, B. Pirzadeh & D. Rogan (2000) Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch Virol,* 145**,** 659-88.

Delputte, P. L., S. Costers & H. J. Nauwynck (2005) Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. *J Gen Virol,* 86**,** 1441-5.

Delputte, P. L., N. Vanderheijden, H. J. Nauwynck & M. B. Pensaert (2002) Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol,* 76**,** 4312-20.

Dokland, T. (2010) The structural biology of PRRSV. *Virus Res,* 154**,** 86-97.

Domachowske, J. B., A. A. Khan, M. T. Esser, K. Jensen, T. Takas, T. Villafana, F. Dubovsky & M. P. Griffin (2018) Safety, Tolerability and Pharmacokinetics of MEDI8897, an Extended Half-life Single-dose Respiratory Syncytial Virus Prefusion F-targeting Monoclonal Antibody Administered as a Single Dose to Healthy Preterm Infants. *Pediatr Infect Dis J,* 37**,** 886-892.

Duan, X., H. J. Nauwynck & M. B. Pensaert (1997) Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol,* 142**,** 2483-97.

Dwivedi, V., C. Manickam, B. Binjawadagi, D. Linhares, M. P. Murtaugh & G. J. Renukaradhya (2012) Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. *Virol J,* 9**,** 45.

Díaz, I., L. Darwich, G. Pappaterra, J. Pujols & E. Mateu (2006) Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology,* 351**,** 249-59.

Eguchi-Ogawa, T., N. Wertz, X. Z. Sun, F. Piumi, F. Puimi, H. Uenishi, K. Wells, P. Chardon, G. J. Tobin & J. E. Butler (2010) Antibody repertoire development in fetal and neonatal piglets. XI. The relationship of variable heavy chain gene usage and the genomic organization of the variable heavy chain locus. *J Immunol,* 184**,** 3734-42.

Faaberg, K. S., J. D. Hocker, M. M. Erdman, D. L. Harris, E. A. Nelson, M. Torremorell & P. G. Plagemann (2006) Neutralizing antibody responses of pigs infected with natural GP5 N-glycan mutants of porcine reproductive and respiratory syndrome virus. *Viral Immunol,* 19**,** 294-304.

Fan, B., X. Liu, J. Bai, T. Zhang, Q. Zhang & P. Jiang (2016) Influence of the amino acid residues at 70 in M protein of porcine reproductive and respiratory syndrome virus on viral neutralization susceptibility to the serum antibody. *Virol J,* 13**,** 51.

Fanales-Belasio, E., M. Raimondo, B. Suligoi & S. Buttò (2010) HIV virology and pathogenetic mechanisms of infection: a brief overview. *Ann Ist Super Sanita,* 46**,** 5-14.

Fang, Y., P. Schneider, W. P. Zhang, K. S. Faaberg, E. A. Nelson & R. R. Rowland (2007) Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. *Arch Virol,* 152**,** 1009-17.

Fang, Y. & E. J. Snijder (2010) The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. *Virus Res,* 154**,** 61-76.

Forsberg, R. (2005) Divergence time of porcine reproductive and respiratory syndrome virus subtypes. *Mol Biol Evol,* 22**,** 2131-4.

Friesen, R. H., P. S. Lee, E. J. Stoop, R. M. Hoffman, D. C. Ekiert, G. Bhabha, W. Yu, J. Juraszek, W. Koudstaal, M. Jongeneelen, H. J. Korse, C. Ophorst, E. C. Brinkman-van der Linden, M. Throsby, M. J. Kwakkenbos, A. Q. Bakker, T. Beaumont, H. Spits, T. Kwaks, R. Vogels, A. B. Ward, J. Goudsmit & I. A. Wilson (2014) A common solution to group 2 influenza virus neutralization. *Proc Natl Acad Sci U S A,* 111**,** 445-50.

Gao, Z. Q., X. Guo & H. C. Yang (2004) Genomic characterization of two Chinese isolates of porcine respiratory and reproductive syndrome virus. *Arch Virol,* 149**,** 1341-51.
Genini, S., P. L. Delputte, R. Malinverni, M. Cecere, A. Stella, H. J. Nauwynck & E. Giuffra (2008) Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. *J Gen Virol,* 89**,** 2550-64.
Goldeck, D., D. M. Perry, J. W. P. Hayes, L. P. M. Johnson, J. E. Young, P. Roychoudhury, E. L. McLuskey, K. Moffat, A. Q. Bakker, M. J. Kwakkenbos, J. P. Frossard, R. R. R. Rowland, M. P. Murtaugh & S. P. Graham (2019) Establishment of Systems to Enable Isolation of Porcine Monoclonal Antibodies Broadly Neutralizing the Porcine Reproductive and Respiratory Syndrome Virus. *Front Immunol,* 10**,** 572.
Gorbalenya, A. E., L. Enjuanes, J. Ziebuhr & E. J. Snijder (2006) Nidovirales: evolving the largest RNA virus genome. *Virus Res,* 117**,** 17-37.
Gu, W., L. Guo, H. Yu, J. Niu, M. Huang, X. Luo, R. Li, Z. Tian, L. Feng & Y. Wang (2015) Involvement of CD16 in antibody-dependent enhancement of porcine reproductive and respiratory syndrome virus infection. *J Gen Virol,* 96**,** 1712-22.
Gómez-Laguna, J., F. J. Salguero, M. Fernández de Marco, I. Barranco, I. M. Rodríguez-Gómez, M. Quezada & L. Carrasco (2013) Type 2 Porcine Reproductive and Respiratory Syndrome Virus infection mediated apoptosis in B- and T-cell areas in lymphoid organs of experimentally infected pigs. *Transbound Emerg Dis,* 60**,** 273-8.
Haiwick, G., J. Hermann, M. Roof, B. Fergen, R. Philips & A. Patterson (2018) Examination of viraemia and clinical signs after challenge with a heterologous PRRSV strain in PRRS Type 2 MLV vaccinated pigs: A challenge-dose study. *PLoS One,* 13**,** e0209784.
Hanada, K., Y. Suzuki, T. Nakane, O. Hirose & T. Gojobori (2005) The origin and evolution of porcine reproductive and respiratory syndrome viruses. *Mol Biol Evol,* 22**,** 1024-31.
Holl, V., M. Peressin, T. Decoville, S. Schmidt, S. Zolla-Pazner, A. M. Aubertin & C. Moog (2006) Nonneutralizing antibodies are able to inhibit human immunodeficiency virus type 1 replication in macrophages and immature dendritic cells. *J Virol,* 80**,** 6177-81.
Holtkamp, D., J. Kliebenstein & E. Neumann (2013a) Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *J Swine Health Prod.,* 21**,** 72-84.
--- (2013b) **Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers** *J Swine Health Prod.,* 21**,** 72-84.
Horter, D. C., R. M. Pogranichniy, C. C. Chang, R. B. Evans, K. J. Yoon & J. J. Zimmerman (2002) Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. *Vet Microbiol,* 86**,** 213-28.
Islam, Z. U., S. C. Bishop, N. J. Savill, R. R. Rowland, J. K. Lunney, B. Trible & A. B. Doeschl-Wilson (2013) Quantitative analysis of porcine reproductive and respiratory syndrome (PRRS) viremia profiles from experimental infection: a statistical modelling approach. *PLoS One,* 8**,** e83567.
Jiang, W., P. Jiang, Y. Li, X. Wang & Y. Du (2007a) Analysis of immunogenicity of minor envelope protein GP3 of porcine reproductive and respiratory syndrome virus in mice. *Virus Genes,* 35**,** 695-704.
Jiang, W., P. Jiang, X. Wang, Y. Li & Y. Du (2007b) Influence of porcine reproductive and respiratory syndrome virus GP5 glycoprotein N-linked glycans on immune responses in mice. *Virus Genes,* 35**,** 663-71.

--- (2008) Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. *Virus Res,* 136**,** 50-7.

Johnson, C. R., W. Yu & M. P. Murtaugh (2007) Cross-reactive antibody responses to nsp1 and nsp2 of Porcine reproductive and respiratory syndrome virus. *J Gen Virol,* 88**,** 1184-95.

Kappes, M. A. & K. S. Faaberg (2015) PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. *Virology,* 479-480**,** 475-86.

Kardani, K., A. Bolhassani & S. Shahbazi (2016) Prime-boost vaccine strategy against viral infections: Mechanisms and benefits. *Vaccine,* 34**,** 413-423.

Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo & M. L. Frey (1993) Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch Virol,* 133**,** 477-83.

Kim, W. I., J. J. Kim, S. H. Cha, W. H. Wu, V. Cooper, R. Evans, E. J. Choi & K. J. Yoon (2013) Significance of genetic variation of PRRSV ORF5 in virus neutralization and molecular determinants corresponding to cross neutralization among PRRS viruses. *Vet Microbiol,* 162**,** 10-22.

Kimman, T. G., L. A. Cornelissen, R. J. Moormann, J. M. Rebel & N. Stockhofe-Zurwieden (2009) Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine,* 27**,** 3704-18.

Kimpston-Burkgren, K., I. Correas, F. A. Osorio, D. Steffen, A. K. Pattnaik, Y. Fang & H. L. X. Vu (2017) Relative contribution of porcine reproductive and respiratory syndrome virus open reading frames 2-4 to the induction of protective immunity. *Vaccine,* 35**,** 4408-4413.

Klein, F., R. Diskin, J. F. Scheid, C. Gaebler, H. Mouquet, I. S. Georgiev, M. Pancera, T. Zhou, R. B. Incesu, B. Z. Fu, P. N. Gnanapragasam, T. Y. Oliveira, M. S. Seaman, P. D. Kwong, P. J. Bjorkman & M. C. Nussenzweig (2013) Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell,* 153**,** 126-38.

Ko, S. S., S. W. Seo, S. Y. Sunwoo, S. J. Yoo, M. H. Kim & Y. S. Lyoo (2016) Efficacy of commercial genotype 1 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine against field isolate of genotype 2 PRRSV. *Vet Immunol Immunopathol,* 172**,** 43-9.

Koppensteiner, H., R. Brack-Werner & M. Schindler (2012) Macrophages and their relevance in Human Immunodeficiency Virus Type I infection. *Retrovirology,* 9**,** 82.

Kuhn, J. H., M. Lauck, A. L. Bailey, A. M. Shchetinin, T. V. Vishnevskaya, Y. Bào, T. F. Ng, M. LeBreton, B. S. Schneider, A. Gillis, U. Tamoufe, J. e. D. Diffo, J. M. Takuo, N. O. Kondov, L. L. Coffey, N. D. Wolfe, E. Delwart, A. N. Clawson, E. Postnikova, L. Bollinger, M. G. Lackemeyer, S. R. Radoshitzky, G. Palacios, J. Wada, Z. V. Shevtsova, P. B. Jahrling, B. A. Lapin, P. G. Deriabin, M. Dunowska, S. V. Alkhovsky, J. Rogers, T. C. Friedrich, D. H. O'Connor & T. L. Goldberg (2016) Reorganization and expansion of the nidoviral family Arteriviridae. *Arch Virol,* 161**,** 755-68.

Kumar, S., G. Stecher, M. Li, C. Knyaz & K. Tamura (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol,* 35**,** 1547-1549.

Kwakkenbos, M. J., A. Q. Bakker, P. M. van Helden, K. Wagner, E. Yasuda, H. Spits & T. Beaumont (2014) Genetic manipulation of B cells for the isolation of rare therapeutic antibodies from the human repertoire. *Methods,* 65**,** 38-43.

Kwakkenbos, M. J., S. A. Diehl, E. Yasuda, A. Q. Bakker, C. M. van Geelen, M. V. Lukens, G. M. van Bleek, M. N. Widjojoatmodjo, W. M. Bogers, H. Mei, A. Radbruch, F. A. Scheeren, H.

Spits & T. Beaumont (2010) Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nat Med,* 16**,** 123-8.
Kwakkenbos, M. J., P. M. van Helden, T. Beaumont & H. Spits (2016) Stable long-term cultures of self-renewing B cells and their applications. *Immunol Rev,* 270**,** 65-77.
Lamontagne, L., C. Pagé, R. Larochelle & R. Magar (2003) Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of CD8high T cells. *Viral Immunol,* 16**,** 395-406.
Lau, D., L. Y. Lan, S. F. Andrews, C. Henry, K. T. Rojas, K. E. Neu, M. Huang, Y. Huang, B. DeKosky, A. E. Palm, G. C. Ippolito, G. Georgiou & P. C. Wilson (2017) Low CD21 expression defines a population of recent germinal center graduates primed for plasma cell differentiation. *Sci Immunol,* 2.
Lee, C. & D. Yoo (2006) The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. *Virology,* 355**,** 30-43.
Lemke, C. D., J. S. Haynes, R. Spaete, D. Adolphson, A. Vorwald, K. Lager & J. E. Butler (2004) Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs. *J Immunol,* 172**,** 1916-25.
Leng, C., W. Zhang, H. Zhang, Y. Kan, L. Yao, H. Zhai, M. Li, Z. Li, C. Liu, T. An, J. Peng, Q. Wang, Y. Leng, X. Cai, Z. Tian & G. Tong (2017) ORF1a of highly pathogenic PRRS attenuated vaccine virus plays a key role in neutralizing antibody induction in piglets and virus neutralization in vitro. *Virol J,* 14**,** 159.
Leng, C. L., T. Q. An, J. Z. Chen, D. Q. Gong, J. M. Peng, Y. Q. Yang, J. Wu, J. J. Guo, D. Y. Li, Y. Zhang, Z. X. Meng, Y. Q. Wu, Z. J. Tian & G. Z. Tong (2012) Highly pathogenic porcine reproductive and respiratory syndrome virus GP5 B antigenic region is not a neutralizing antigenic region. *Vet Microbiol,* 159**,** 273-81.
Li, H., Z. Zheng, P. Zhou, B. Zhang, Z. Shi, Q. Hu & H. Wang (2010) The cysteine protease domain of porcine reproductive and respiratory syndrome virus non-structural protein 2 antagonizes interferon regulatory factor 3 activation. *J Gen Virol,* 91**,** 2947-58.
Li, J. & M. P. Murtaugh (2012) Dissociation of porcine reproductive and respiratory syndrome virus neutralization from antibodies specific to major envelope protein surface epitopes. *Virology,* 433**,** 367-76.
Li, X., A. Galliher-Beckley, L. Pappan, B. Trible, M. Kerrigan, A. Beck, R. Hesse, F. Blecha, J. C. Nietfeld, R. R. Rowland & J. Shi (2014) Comparison of host immune responses to homologous and heterologous type II porcine reproductive and respiratory syndrome virus (PRRSV) challenge in vaccinated and unvaccinated pigs. *Biomed Res Int,* 2014**,** 416727.
Loemba, H. D., S. Mounir, H. Mardassi, D. Archambault & S. Dea (1996) Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Arch Virol,* 141**,** 751-61.
Lohse, L., J. Nielsen & L. Eriksen (2004) Temporary CD8+ T-cell depletion in pigs does not exacerbate infection with porcine reproductive and respiratory syndrome virus (PRRSV). *Viral Immunol,* 17**,** 594-603.
Lopez, O. J., M. F. Oliveira, E. A. Garcia, B. J. Kwon, A. Doster & F. A. Osorio (2007) Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. *Clin Vaccine Immunol,* 14**,** 269-75.

Loving, C. L., S. L. Brockmeier & R. E. Sacco (2007) Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus. *Immunology,* 120**,** 217-29.
Loving, C. L., F. A. Osorio, M. P. Murtaugh & F. A. Zuckermann (2015) Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. *Vet Immunol Immunopathol,* 167**,** 1-14.
Lu, C. L., D. K. Murakowski, S. Bournazos, T. Schoofs, D. Sarkar, A. Halper-Stromberg, J. A. Horwitz, L. Nogueira, J. Golijanin, A. Gazumyan, J. V. Ravetch, M. Caskey, A. K. Chakraborty & M. C. Nussenzweig (2016) Enhanced clearance of HIV-1-infected cells by broadly neutralizing antibodies against HIV-1 in vivo. *Science,* 352**,** 1001-4.
Lu, W., B. Sun, J. Mo, X. Zeng, G. Zhang, L. Wang, Q. Zhou, L. Zhu, Z. Li, Q. Xie, Y. Bi & J. Ma (2014) Attenuation and immunogenicity of a live high pathogenic PRRSV vaccine candidate with a 32-amino acid deletion in the nsp2 protein. *J Immunol Res,* 2014**,** 810523.
Lunney, J. K., Y. Fang, A. Ladinig, N. Chen, Y. Li, B. Rowland & G. J. Renukaradhya (2016) Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci,* 4**,** 129-54.
López Fuertes, L., N. Doménech, B. Alvarez, A. Ezquerra, J. Domínguez, J. M. Castro & F. Alonso (1999) Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. *Virus Res,* 64**,** 33-42.
Mady, B. J., D. V. Erbe, I. Kurane, M. W. Fanger & F. A. Ennis (1991) Antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies against cell surface molecules other than Fc gamma receptors. *J Immunol,* 147**,** 3139-44.
Manickam, C., V. Dwivedi, R. Patterson, T. Papenfuss & G. J. Renukaradhya (2013) Porcine reproductive and respiratory syndrome virus induces pronounced immune modulatory responses at mucosal tissues in the parental vaccine strain VR2332 infected pigs. *Vet Microbiol,* 162**,** 68-77.
Martínez-Lobo, F. J., F. Díez-Fuertes, I. Simarro, J. M. Castro & C. Prieto (2011) Porcine Reproductive and Respiratory Syndrome Virus isolates differ in their susceptibility to neutralization. *Vaccine,* 29**,** 6928-40.
Mateu, E. & I. Diaz (2008) The challenge of PRRS immunology. *Vet J,* 177**,** 345-51.
McHeyzer-Williams, L. J., P. J. Milpied, S. L. Okitsu & M. G. McHeyzer-Williams (2015) Class-switched memory B cells remodel BCRs within secondary germinal centers. *Nat Immunol,* 16**,** 296-305.
McLellan, J. S., M. Chen, M. G. Joyce, M. Sastry, G. B. Stewart-Jones, Y. Yang, B. Zhang, L. Chen, S. Srivatsan, A. Zheng, T. Zhou, K. W. Graepel, A. Kumar, S. Moin, J. C. Boyington, G. Y. Chuang, C. Soto, U. Baxa, A. Q. Bakker, H. Spits, T. Beaumont, Z. Zheng, N. Xia, S. Y. Ko, J. P. Todd, S. Rao, B. S. Graham & P. D. Kwong (2013) Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. *Science,* 342**,** 592-8.
Meier, W. A., J. Galeota, F. A. Osorio, R. J. Husmann, W. M. Schnitzlein & F. A. Zuckermann (2003) Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology,* 309**,** 18-31.
Meng, X. J., P. S. Paul, P. G. Halbur & M. A. Lum (1995) Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. *Arch Virol,* 140**,** 745-55.

Mengeling, W. L., K. M. Lager, A. C. Vorwald & D. F. Clouser (2003) Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. *Vet Microbiol,* 93**,** 25-38.

Miller, L. C., K. M. Lager & M. E. Kehrli (2009) Role of Toll-like receptors in activation of porcine alveolar macrophages by porcine reproductive and respiratory syndrome virus. *Clin Vaccine Immunol,* 16**,** 360-5.

Molina, R. M., S. H. Cha, W. Chittick, S. Lawson, M. P. Murtaugh, E. A. Nelson, J. Christopher-Hennings, K. J. Yoon, R. Evans, R. R. Rowland, W. Wu & J. J. Zimmerman (2008) Immune response against porcine reproductive and respiratory syndrome virus during acute and chronic infection. *Vet Immunol Immunopathol,* 126**,** 283-92.

Montaner-Tarbes, S., H. A. Del Portillo, M. Montoya & L. Fraile (2019) Key Gaps in the Knowledge of the Porcine Respiratory Reproductive Syndrome Virus (PRRSV). *Front Vet Sci,* 6**,** 38.

Morland, B. & G. Kaplan (1977) Macrophage activation in vivo and in vitro. *Exp Cell Res,* 108**,** 279-88.

Mosser, D. M. & J. P. Edwards (2008) Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

Mulupuri, P., J. J. Zimmerman, J. Hermann, C. R. Johnson, J. P. Cano, W. Yu, S. A. Dee & M. P. Murtaugh (2008) Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. *J Virol,* 82**,** 358-70.

Murtaugh, M. P., M. R. Elam & L. T. Kakach (1995) Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch Virol,* 140**,** 1451-60.

Murtaugh, M. P., Z. Xiao & F. Zuckermann (2002) Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol,* 15**,** 533-47.

Nair, N., N. Feng, L. K. Blum, M. Sanyal, S. Ding, B. Jiang, A. Sen, J. M. Morton, X. S. He, W. H. Robinson & H. B. Greenberg (2017) VP4- and VP7-specific antibodies mediate heterotypic immunity to rotavirus in humans. *Sci Transl Med,* 9.

Nathues, H., P. Alarcon, J. Rushton, R. Jolie, K. Fiebig, M. Jimenez, V. Geurts & C. Nathues (2017) Cost of porcine reproductive and respiratory syndrome virus at individual farm level - An economic disease model. *Prev Vet Med,* 142**,** 16-29.

Ndung'u, T. & R. A. Weiss (2012) On HIV diversity. *AIDS,* 26**,** 1255-60.

Nelsen, C. J., M. P. Murtaugh & K. S. Faaberg (1999) Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol,* 73**,** 270-80.

Nelson, E. A., J. Christopher-Hennings & D. A. Benfield (1994) Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. *J Vet Diagn Invest,* 6**,** 410-5.

Nielsen, J. & A. Bøtner (1997) Hematological and immunological parameters of 4 1/2-month old pigs infected with PRRS virus. *Vet Microbiol,* 55**,** 289-94.

Oh, T., H. Kim, K. H. Park, J. Jeong, S. Yang, I. Kang & C. Chae (2019) Comparison of four commercial PRRSV MLV vaccines in herds with co-circulation of PRRSV-1 and PRRSV-2. *Comp Immunol Microbiol Infect Dis,* 63**,** 66-73.

Osorio, F. A., J. A. Galeota, E. Nelson, B. Brodersen, A. Doster, R. Wills, F. Zuckermann & W. W. Laegreid (2002) Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology,* 302**,** 9-20.

Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio & O. J. Lopez (2002) Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol,* 76**,** 4241-50.

Pedersen, K. W., Y. van der Meer, N. Roos & E. J. Snijder (1999) Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. *J Virol,* 73**,** 2016-26.

Pelanda, R. (2014) Dual immunoglobulin light chain B cells: Trojan horses of autoimmunity? *Curr Opin Immunol,* 27**,** 53-9.

Peled, J. U., F. L. Kuang, M. D. Iglesias-Ussel, S. Roa, S. L. Kalis, M. F. Goodman & M. D. Scharff (2008) The biochemistry of somatic hypermutation. *Annu Rev Immunol,* 26**,** 481-511.

Perfetti, V., M. C. Vignarelli, G. Palladini, V. Navazza, C. Giachino & G. Merlini (2004) Insights into the regulation of immunoglobulin light chain gene rearrangements via analysis of the kappa light chain locus in lambda myeloma. *Immunology,* 112**,** 420-7.

Pirzadeh, B. & S. Dea (1997) Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. *J Gen Virol,* 78 (Pt 8)**,** 1867-73.

--- (1998) Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. *J Gen Virol,* 79 (Pt 5)**,** 989-99.

Plagemann, P. G. (2006) Neutralizing antibody formation in swine infected with seven strains of porcine reproductive and respiratory syndrome virus as measured by indirect ELISA with peptides containing the GP5 neutralization epitope. *Viral Immunol,* 19**,** 285-93.

Plagemann, P. G., R. R. Rowland & K. S. Faaberg (2002) The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. *Arch Virol,* 147**,** 2327-47.

Popescu, L. N., B. R. Trible, N. Chen & R. R. R. Rowland (2017) GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) as a target for homologous and broadly neutralizing antibodies. *Vet Microbiol,* 209**,** 90-96.

Posthuma, C. C., K. W. Pedersen, Z. Lu, R. G. Joosten, N. Roos, J. C. Zevenhoven-Dobbe & E. J. Snijder (2008) Formation of the arterivirus replication/transcription complex: a key role for nonstructural protein 3 in the remodeling of intracellular membranes. *J Virol,* 82**,** 4480-91.

Prather, R. S., R. R. Rowland, C. Ewen, B. Trible, M. Kerrigan, B. Bawa, J. M. Teson, J. Mao, K. Lee, M. S. Samuel, K. M. Whitworth, C. N. Murphy, T. Egen & J. A. Green (2013) An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. *J Virol,* 87**,** 9538-46.

Rahe, M. C., K. L. Gustafson & M. P. Murtaugh (2018) B Cell Tetramer Development for Veterinary Vaccinology. *Viral Immunol,* 31**,** 1-10.

Rahe, M. C. & M. P. Murtaugh (2017a) Effector mechanisms of humoral immunity to porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol,* 186**,** 15-18.

--- (2017b) Interleukin-21 Drives Proliferation and Differentiation of Porcine Memory B Cells into Antibody Secreting Cells. *PLoS One,* 12**,** e0171171.

--- (2017c) Mechanisms of Adaptive Immunity to Porcine Reproductive and Respiratory Syndrome Virus. *Viruses,* 9.

Renukaradhya, G. J., K. Alekseev, K. Jung, Y. Fang & L. J. Saif (2010) Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. *Viral Immunol,* 23**,** 457-66.

Robinson, S. R., M. C. Figueiredo, J. E. Abrahante & M. P. Murtaugh (2013) Immune response to ORF5a protein immunization is not protective against porcine reproductive and respiratory syndrome virus infection. *Vet Microbiol,* 164**,** 281-5.

Robinson, S. R., J. Li, E. A. Nelson & M. P. Murtaugh (2015) Broadly neutralizing antibodies against the rapidly evolving porcine reproductive and respiratory syndrome virus. *Virus Res,* 203**,** 56-65.

Robinson, S. R., M. C. Rahe, D. K. Gray, K. V. Martins & M. P. Murtaugh (2018) Porcine reproductive and respiratory syndrome virus neutralizing antibodies provide in vivo cross-protection to PRRSV1 and PRRSV2 viral challenge. *Virus Res,* 248**,** 13-23.

Roca, M., M. Gimeno, S. Bruguera, J. Segalés, I. Díaz, I. J. Galindo-Cardiel, E. Martínez, L. Darwich, Y. Fang, J. Maldonado, R. March & E. Mateu (2012) Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. *Vet J,* 193**,** 92-6.

Rothkötter, H. J. (2009) Anatomical particularities of the porcine immune system--a physician's view. *Dev Comp Immunol,* 33**,** 267-72.

Rueden, C. T., J. Schindelin, M. C. Hiner, B. E. DeZonia, A. E. Walter, E. T. Arena & K. W. Eliceiri (2017) ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics,* 18**,** 529.

Samsom, J. N., T. G. de Bruin, J. J. Voermans, J. J. Meulenberg, J. M. Pol & A. T. Bianchi (2000) Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and respiratory syndrome virus: a role for CD8(+) cells. *J Gen Virol,* 81**,** 497-505.

Saunders, K. O., K. Wiehe, M. Tian, P. Acharya, T. Bradley, S. M. Alam, E. P. Go, R. Scearce, L. Sutherland, R. Henderson, A. L. Hsu, M. J. Borgnia, H. Chen, X. Lu, N. R. Wu, B. Watts, C. Jiang, D. Easterhoff, H. L. Cheng, K. McGovern, P. Waddicor, A. Chapdelaine-Williams, A. Eaton, J. Zhang, W. Rountree, L. Verkoczy, M. Tomai, M. G. Lewis, H. R. Desaire, R. J. Edwards, D. W. Cain, M. Bonsignori, D. Montefiori, F. W. Alt & B. F. Haynes (2019) Targeted selection of HIV-specific antibody mutations by engineering B cell maturation. *Science,* 366.

Scheid, J. F., H. Mouquet, N. Feldhahn, M. S. Seaman, K. Velinzon, J. Pietzsch, R. G. Ott, R. M. Anthony, H. Zebroski, A. Hurley, A. Phogat, B. Chakrabarti, Y. Li, M. Connors, F. Pereyra, B. D. Walker, H. Wardemann, D. Ho, R. T. Wyatt, J. R. Mascola, J. V. Ravetch & M. C. Nussenzweig (2009) Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature,* 458**,** 636-40.

Schwartz, J. C., M. P. Lefranc & M. P. Murtaugh (2012a) Evolution of the porcine (Sus scrofa domestica) immunoglobulin kappa locus through germline gene conversion. *Immunogenetics,* 64**,** 303-11.

--- (2012b) Organization, complexity and allelic diversity of the porcine (Sus scrofa domestica) immunoglobulin lambda locus. *Immunogenetics,* 64**,** 399-407.

Scortti, M., C. Prieto, I. Simarro & J. M. Castro (2006) Reproductive performance of gilts following vaccination and subsequent heterologous challenge with European strains of porcine reproductive and respiratory syndrome virus. *Theriogenology,* 66**,** 1884-93.

Seifert, M., M. Przekopowitz, S. Taudien, A. Lollies, V. Ronge, B. Drees, M. Lindemann, U. Hillen, H. Engler, B. B. Singer & R. Küppers (2015) Functional capacities of human IgM memory B cells in early inflammatory responses and secondary germinal center reactions. *Proc Natl Acad Sci U S A,* 112**,** E546-55.

Shekhar, S. & X. Yang (2015) Natural killer cells in host defense against veterinary pathogens. *Vet Immunol Immunopathol,* 168**,** 30-4.
Shi, C., Y. Liu, Y. Ding, Y. Zhang & J. Zhang (2015) PRRSV receptors and their roles in virus infection. *Arch Microbiol,* 197**,** 503-12.
Shih, T. A., E. Meffre, M. Roederer & M. C. Nussenzweig (2002) Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol,* 3**,** 570-5.
Shimizu, M., S. Yamada, K. Kawashima, S. Ohashi, S. Shimizu & T. Ogawa (1996) Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. *Vet Immunol Immunopathol,* 50**,** 19-27.
Sinkora, M., J. Sinkorova & K. Stepanova (2017) Ig Light Chain Precedes Heavy Chain Gene Rearrangement during Development of B Cells in Swine. *J Immunol,* 198**,** 1543-1552.
Sinkora, M., K. Stepanova & J. Sinkorova (2013) Different anti-CD21 antibodies can be used to discriminate developmentally and functionally different subsets of B lymphocytes in circulation of pigs. *Dev Comp Immunol,* 39**,** 409-18.
Skvaril, F., S. Barandun, F. Kuffer & M. Probst (1976) [Changes of kappa/lambda ratio of human serum immunoglobulins in the course of development]. *Blut,* 33**,** 281-4.
Smith, M. J., T. A. Packard, S. K. O'Neill, R. M. Hinman, M. Rihanek, P. A. Gottlieb & J. C. Cambier (2017) Detection and Enrichment of Rare Antigen-specific B Cells for Analysis of Phenotype and Function. *J Vis Exp*.
Sok, D., U. Laserson, J. Laserson, Y. Liu, F. Vigneault, J. P. Julien, B. Briney, A. Ramos, K. F. Saye, K. Le, A. Mahan, S. Wang, M. Kardar, G. Yaari, L. M. Walker, B. B. Simen, E. P. St John, P. Y. Chan-Hui, K. Swiderek, S. H. Kleinstein, G. Alter, M. S. Seaman, A. K. Chakraborty, D. Koller, I. A. Wilson, G. M. Church, D. R. Burton & P. Poignard (2013) The effects of somatic hypermutation on neutralization and binding in the PGT121 family of broadly neutralizing HIV antibodies. *PLoS Pathog,* 9**,** e1003754.
Steichen, J. M., Y. C. Lin, C. Havenar-Daughton, S. Pecetta, G. Ozorowski, J. R. Willis, L. Toy, D. Sok, A. Liguori, S. Kratochvil, J. L. Torres, O. Kalyuzhniy, E. Melzi, D. W. Kulp, S. Raemisch, X. Hu, S. M. Bernard, E. Georgeson, N. Phelps, Y. Adachi, M. Kubitz, E. Landais, J. Umotoy, A. Robinson, B. Briney, I. A. Wilson, D. R. Burton, A. B. Ward, S. Crotty, F. D. Batista & W. R. Schief (2019) A generalized HIV vaccine design strategy for priming of broadly neutralizing antibody responses. *Science,* 366.
Sun, J., I. Kacskovics, W. R. Brown & J. E. Butler (1994) Expressed swine VH genes belong to a small VH gene family homologous to human VHIII. *J Immunol,* 153**,** 5618-27.
Sun, L., Y. Li, R. Liu, X. Wang, F. Gao, T. Lin, T. Huang, H. Yao, G. Tong, H. Fan, Z. Wei & S. Yuan (2013) Porcine reproductive and respiratory syndrome virus ORF5a protein is essential for virus viability. *Virus Res,* 171**,** 178-85.
Sun, X., N. Wertz, K. Lager, M. Sinkora, K. Stepanova, G. Tobin & J. E. Butler (2012) Antibody repertoire development in fetal and neonatal piglets. XXII. λ Rearrangement precedes κ rearrangement during B-cell lymphogenesis in swine. *Immunology,* 137**,** 149-59.
Sun, Z., Z. Chen, S. R. Lawson & Y. Fang (2010) The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. *J Virol,* 84**,** 7832-46.
Takano, T., M. Nakaguchi, T. Doki & T. Hohdatsu (2017) Antibody-dependent enhancement of serotype II feline enteric coronavirus infection in primary feline monocytes. *Arch Virol,* 162**,** 3339-3345.

Terpstra, C., G. Wensvoort & J. M. Pol (1991) Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q,* 13**,** 131-6.

Thaa, B., B. C. Sinhadri, C. Tielesch, E. Krause & M. Veit (2013) Signal peptide cleavage from GP5 of PRRSV: a minor fraction of molecules retains the decoy epitope, a presumed molecular cause for viral persistence. *PLoS One,* 8**,** e65548.

Thanawongnuwech, R., A. Rungsipipat, S. Disatian, R. Saiyasombat, S. Napakanaporn & P. G. Halbur (2003) Immunohistochemical staining of IFN-gamma positive cells in porcine reproductive and respiratory syndrome virus-infected lungs. *Vet Immunol Immunopathol,* 91**,** 73-7.

Thanawongnuwech, R., E. L. Thacker & P. G. Halbur (1997) Effect of porcine reproductive and respiratory syndrome virus (PRRSV) (isolate ATCC VR-2385) infection on bactericidal activity of porcine pulmonary intravascular macrophages (PIMs): in vitro comparisons with pulmonary alveolar macrophages (PAMs). *Vet Immunol Immunopathol,* 59**,** 323-35.

Thorarinsdottir, K., A. Camponeschi, N. Cavallini, O. Grimsholm, L. Jacobsson, I. Gjertsson & I. L. Mårtensson (2016) CD21(-/low) B cells in human blood are memory cells. *Clin Exp Immunol,* 185**,** 252-62.

Tian, D., Z. Wei, J. C. Zevenhoven-Dobbe, R. Liu, G. Tong, E. J. Snijder & S. Yuan (2012) Arterivirus minor envelope proteins are a major determinant of viral tropism in cell culture. *J Virol,* 86**,** 3701-12.

Tomita, M. & K. Tsumoto (2011) Hybridoma technologies for antibody production. *Immunotherapy,* 3**,** 371-80.

Trible, B. R., L. N. Popescu, N. Monday, J. G. Calvert & R. R. Rowland (2015) A single amino acid deletion in the matrix protein of porcine reproductive and respiratory syndrome virus confers resistance to a polyclonal swine antibody with broadly neutralizing activity. *J Virol,* 89**,** 6515-20.

Ulferts, R. & J. Ziebuhr (2011) Nidovirus ribonucleases: Structures and functions in viral replication. *RNA Biol,* 8**,** 295-304.

Van Reeth, K., G. Labarque, H. Nauwynck & M. Pensaert (1999) Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci,* 67**,** 47-52.

Vanhee, M., S. Costers, W. Van Breedam, M. F. Geldhof, J. Van Doorsselaere & H. J. Nauwynck (2010) A variable region in GP4 of European-type porcine reproductive and respiratory syndrome virus induces neutralizing antibodies against homologous but not heterologous virus strains. *Viral Immunol,* 23**,** 403-13.

Vanhee, M., W. Van Breedam, S. Costers, M. Geldhof, Y. Noppe & H. Nauwynck (2011) Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use of peptide-specific serum antibodies. *Vaccine,* 29**,** 4794-804.

Vlasova, A. N., J. O. Amimo & L. J. Saif (2017) Porcine Rotaviruses: Epidemiology, Immune Responses and Control Strategies. *Viruses,* 9.

von Bredow, B., J. F. Arias, L. N. Heyer, B. Moldt, K. Le, J. E. Robinson, S. Zolla-Pazner, D. R. Burton & D. T. Evans (2016) Comparison of Antibody-Dependent Cell-Mediated Cytotoxicity and Virus Neutralization by HIV-1 Env-Specific Monoclonal Antibodies. *J Virol,* 90**,** 6127-6139.

Vu, H. L., B. Kwon, K. J. Yoon, W. W. Laegreid, A. K. Pattnaik & F. A. Osorio (2011) Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. *J Virol,* 85**,** 5555-64.

Walker, L. M., M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J. P. Julien, S. K. Wang, A. Ramos, P. Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A. Olsen, P. Phung, S. Fling, C. H. Wong, S. Phogat, T. Wrin, M. D. Simek, W. C. Koff, I. A. Wilson, D. R. Burton, P. Poignard & P. G. P. Investigators (2011) Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature,* 477**,** 466-70.

Walker, L. M., S. K. Phogat, P. Y. Chan-Hui, D. Wagner, P. Phung, J. L. Goss, T. Wrin, M. D. Simek, S. Fling, J. L. Mitcham, J. K. Lehrman, F. H. Priddy, O. A. Olsen, S. M. Frey, P. W. Hammond, S. Kaminsky, T. Zamb, M. Moyle, W. C. Koff, P. Poignard, D. R. Burton & P. G. P. Investigators (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science,* 326**,** 285-9.

Wang, K., G. Wei & D. Liu (2012) CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Exp Hematol Oncol,* 1**,** 36.

Wang, X. & J. Christopher-Hennings (2012) Post-transcriptional control of type I interferon induction by porcine reproductive and respiratory syndrome virus in its natural host cells. *Viruses,* 4**,** 725-33.

Weesendorp, E., N. Stockhofe-Zurwieden, D. J. Popma-De Graaf, H. Fijten & J. M. Rebel (2013) Phenotypic modulation and cytokine profiles of antigen presenting cells by European subtype 1 and 3 porcine reproductive and respiratory syndrome virus strains in vitro and in vivo. *Vet Microbiol,* 167**,** 638-50.

Wei, Z., T. Lin, L. Sun, Y. Li, X. Wang, F. Gao, R. Liu, C. Chen, G. Tong & S. Yuan (2012) N-linked glycosylation of GP5 of porcine reproductive and respiratory syndrome virus is critically important for virus replication in vivo. *J Virol,* 86**,** 9941-51.

Wei, Z., J. Zhang, J. Zhuang, Z. Sun, F. Gao & S. Yuan (2013) Immunization of pigs with a type 2 modified live PRRSV vaccine prevents the development of a deadly long lasting hyperpyrexia in a challenge study with highly pathogenic PRRSV JX143. *Vaccine,* 31**,** 2062-6.

Welch, S. K., R. Jolie, D. S. Pearce, W. D. Koertje, E. Fuog, S. L. Shields, D. Yoo & J. G. Calvert (2004) Construction and evaluation of genetically engineered replication-defective porcine reproductive and respiratory syndrome virus vaccine candidates. *Vet Immunol Immunopathol,* 102**,** 277-90.

Wensvoort, G., C. Terpstra, J. M. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten & F. Wagenaar (1991) Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q,* 13**,** 121-30.

Wesley, R. D., K. M. Lager & M. E. Kehrli (2006) Infection with Porcine reproductive and respiratory syndrome virus stimulates an early gamma interferon response in the serum of pigs. *Can J Vet Res,* 70**,** 176-82.

Westerhuis, B. M., K. S. Benschop, G. Koen, Y. B. Claassen, K. Wagner, A. Q. Bakker, K. C. Wolthers & T. Beaumont (2015) Human Memory B Cells Producing Potent Cross-Neutralizing Antibodies against Human Parechovirus: Implications for Prevalence, Treatment, and Diagnosis. *J Virol,* 89**,** 7457-64.

Whitworth, K. M., R. R. Rowland, C. L. Ewen, B. R. Trible, M. A. Kerrigan, A. G. Cino-Ozuna, M. S. Samuel, J. E. Lightner, D. G. McLaren, A. J. Mileham, K. D. Wells & R. S. Prather (2016) Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nat Biotechnol,* 34**,** 20-2.

Wieringa, R., A. A. de Vries, J. van der Meulen, G. J. Godeke, J. J. Onderwater, H. van Tol, H. K. Koerten, A. M. Mommaas, E. J. Snijder & P. J. Rottier (2004) Structural protein requirements in equine arteritis virus assembly. *J Virol,* 78**,** 13019-27.

Wills, R. W., A. R. Doster, J. A. Galeota, J. H. Sur & F. A. Osorio (2003) Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. *J Clin Microbiol,* 41**,** 58-62.

Wissink, E. H., M. V. Kroese, H. A. van Wijk, F. A. Rijsewijk, J. J. Meulenberg & P. J. Rottier (2005) Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. *J Virol,* 79**,** 12495-506.

Xiao, Z., L. Batista, S. Dee, P. Halbur & M. P. Murtaugh (2004) The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J Virol,* 78**,** 5923-33.

Xu, X. G., Z. S. Wang, Q. Zhang, Z. C. Li, L. Ding, W. Li, H. Y. Wu, C. D. Chang, L. H. Lee, D. W. Tong & H. J. Liu (2012) Baculovirus as a PRRSV and PCV2 bivalent vaccine vector: baculovirus virions displaying simultaneously GP5 glycoprotein of PRRSV and capsid protein of PCV2. *J Virol Methods,* 179**,** 359-66.

Yang, L., M. L. Frey, K. J. Yoon, J. J. Zimmerman & K. B. Platt (2000) Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. *Arch Virol,* 145**,** 1599-619.

Ye, J., N. Ma, T. L. Madden & J. M. Ostell (2013) IgBLAST: an immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res,* 41**,** W34-40.

Yip, M. S., H. L. Leung, P. H. Li, C. Y. Cheung, I. Dutry, D. Li, M. Daëron, R. Bruzzone, J. S. Peiris & M. Jaume (2016) Antibody-dependent enhancement of SARS coronavirus infection and its role in the pathogenesis of SARS. *Hong Kong Med J,* 22**,** 25-31.

Yoo, D., S. K. Wootton, G. Li, C. Song & R. R. Rowland (2003) Colocalization and interaction of the porcine arterivirus nucleocapsid protein with the small nucleolar RNA-associated protein fibrillarin. *J Virol,* 77**,** 12173-83.

Yoon, I. J., H. S. Joo, S. M. Goyal & T. W. Molitor (1994) A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. *J Vet Diagn Invest,* 6**,** 289-92.

Yoon, K. J., L. L. Wu, J. J. Zimmerman, H. T. Hill & K. B. Platt (1996) Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. *Viral Immunol,* 9**,** 51-63.

Yoon, K. J., L. L. Wu, J. J. Zimmerman & K. B. Platt (1997) Field isolates of porcine reproductive and respiratory syndrome virus (PRRSV) vary in their susceptibility to antibody dependent enhancement (ADE) of infection. *Vet Microbiol,* 55**,** 277-87.

Yun, S. I. & Y. M. Lee (2013) Overview: Replication of porcine reproductive and respiratory syndrome virus. *J Microbiol,* 51**,** 711-23.

Zhang, H., X. Guo, E. Nelson, J. Christopher-Hennings & X. Wang (2012) Porcine reproductive and respiratory syndrome virus activates the transcription of interferon alpha/beta (IFN-α/β) in monocyte-derived dendritic cells (Mo-DC). *Vet Microbiol,* 159**,** 494-8.

Zhang, S. L., H. C. Tan, B. J. Hanson & E. E. Ooi (2010) A simple method for Alexa Fluor dye labelling of dengue virus. *J Virol Methods,* 167**,** 172-7.

Zhou, J. X., J. D. Xue, T. Yu, J. B. Zhang, Y. Liu, N. Jiang, Y. L. Li & R. L. Hu (2010) Immune responses in pigs induced by recombinant canine adenovirus 2 expressing the glycoprotein 5 of porcine reproductive and respiratory syndrome virus. *Vet Res Commun,* 34**,** 371-80.

Zhou, L., Y. Y. Ni, P. Piñeyro, B. J. Sanford, C. M. Cossaboom, B. A. Dryman, Y. W. Huang, D. J. Cao & X. J. Meng (2012) DNA shuffling of the GP3 genes of porcine reproductive and

respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. *Virology,* 434**,** 96-109.

Zimmerman, J. J., L. A. Karriker, A. Ramirez, K. J. Schwartz, G. W. Stevenson & J. Zhang. 2019. *Diseases of Swine, 11th Edition*. Wiley-Blackwell.

Zuckermann, F. A., E. A. Garcia, I. D. Luque, J. Christopher-Hennings, A. Doster, M. Brito & F. Osorio (2007) Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol,* 123**,** 69-85.

Zuckermann, F. A. & R. J. Husmann (1996) Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology,* 87**,** 500-12.