Fall 9-2017

# Cross-reactive Immunity to Porcine Reproductive and Respiratory Syndrome Virus and its Contribution to Protection

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# CROSS-REACTIVE IMMUNITY TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND ITS CONTRIBUTION TO PROTECTION

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Integrative Biomedical Sciences

Under the Supervision of Professor Fernando A. Osorio

Lincoln, Nebraska

September, 2017

# CROSS-REACTIVE IMMUNITY TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND ITS CONTRIBUTION TO PROTECTION

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University of Nebraska, 2017

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Due to the vast geographical distribution and significant economic losses generated, porcine reproductive and respiratory syndrome virus (PRRSV) can be considered the most important swine pathogen of contemporary times. Current control and eradication strategies against PRRSV have difficulty succeeding because of their complex nature and the absence of an effective vaccine. A major obstacle for PRRSV vaccine development is the broad heterogeneity of the virus, both at the genetic and antigenic level, its rapid evolution, and an incomplete knowledge of the immune responses responsible for clearing the virus from the host. Specifically, how known correlates of protection against PRRSV—neutralizing antibodies and T cells—cross-react with heterologous isolates and mediate cross-protection is inadequately understood. The objectives of this dissertation were (i) to determine the extent of cross-reactivity of immune responses against PRRSV, and (ii) to ascertain how cross-reactive immune responses mediate protection against heterologous isolates. T cell responses were found to be cross-reactive among PRRSV-2 isolates, but extremely variable among individual animals, while the neutralizing antibody response induced by a single infection with PRRSV was deemed to be solely self-

neutralizing. Sequential exposure to heterologous PRRSV-2 isolates elicited neutralizing antibodies to the isolates used for infection and challenge, as well as other heterologous PRRSV-2 isolates. Furthermore, prior exposure to PRRSV afforded cross-protection against heterologous challenge, with reduction in viremia, tissue viral load and the extent of microscopic lung lesions; however, protection was still suboptimal. T cell cross-reactivity between PRRSV-1 and PRRSV-2 was evaluated at the structural protein level and was deemed to be feeble or absent. Prior exposure to PRRSV-1 did not prime the T cell response against the PRRSV-2 structural proteins after PRRSV-2 challenge. Collectively, the results in this dissertation contribute to furthering the understanding of immune responses against PRRSV and may be used in the development of a better vaccine.

*Dedicated to my parents*

*for their love and unwavering support*

# ACKNOWLEDGEMENTS

I am deeply thankful to my advisor, Dr. Fernando A. Osorio, for his support, constructive feedback, and scientific leadership. His mentorship was instrumental in the completion of this dissertation and my professional development. I would also like to thank Dr. Hiep L. X. Vu for his advice and thought-provoking guidance. His passion for science is contagious, and a constant driver for excellence. I would further like to thank my Supervisory Committee members, Dr. Gustavo A. Delhon, Dr. Qingsheng Li, and Dr. Asit K. Pattnaik for their suggestions and assistance.

I am indebted to my colleagues, Dr. Kathryn A. Kimpston-Burkgren and Dr. Haiyan Sun, for their friendship, patience, and cooperation. I would like to thank former and visiting members of Dr. Osorio's lab, M. Mercedes Abeyá, Dr. B.J. Kwon, and Dr. Cinta M. Prieto Suárez, for their camaraderie, guidance, and help. Additionally, I would like to recognize current and former graduate students at the Nebraska Center for Virology, Arun S. Annamalai, Dr. Anshuman Das, Anna T. Lampe, Annabel T. Olson, Aryamav Pattnaik, and Bikash R. Sahoo, for their fellowship, collaboration, and scientific insight.

I am grateful to my first mentors, Dr. Daniel M. Lombardo and Dr. Ana C. Bratanich, for sharing their knowledge and advice, and encouraging me to pursue a career in science. To Gonzalo M. Bazgán, Gonzalo López Aldazabal, Mónica S. Lopez, Luciano Miccio, Lucía Viña, and M. Sol Yaceszen, I am thankful for your unconditional friendship that withstood the test of time—and distance.

I would also like to thank the faculty and staff of the School of Veterinary Medicine and Biomedical Sciences and the Nebraska Center for Virology for providing scientific, technical, and administrative assistance, and the staff at the Life Sciences Annex and the

Nebraska Veterinary Diagnostic Center for the work performed in support of this dissertation.

I was extremely lucky to have met my best friend and partner Roy during my time in graduate school. I am forever grateful for his devoted support, companionship, and help in the past years. You inspire me to be a better man. I would like to thank my family, my parents, Ignacio and Marina, my brother Benjamín, my grandfather Bebe, aunts, uncles, and cousins, whose encouragement was instrumental in completing this work and achieving many other goals in my life. Lastly, I would like to acknowledge my late grandmother Maisie, without whose steadfast support nothing would have been possible.

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# ABBREVIATIONS

| | |
|---|---|
| ADE | Antibody-dependent enhancement |
| ANOVA | Analysis of variance |
| AUC | Area under the curve |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CMI | Cell-mediated immunity |
| CREB | cAMP response element-binding protein |
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| DIVA | Differentiating infected from vaccinated animals |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DMV | Double-membraned vesicles |
| DP | Double positive |
| DPC | Days post challenge |
| DPI | Days post infection |
| EAV | Equine arteritis virus |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISpot | Enzyme-linked immunospot |
| ER | Endoplasmic reticulum |

| | |
|---|---|
| FBS | Fetal bovine serum |
| GP | Glycoprotein |
| IFA | Indirect fluorescence assay |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IRF | Interferon regulatory factor |
| ISG | Interferon stimulated gene |
| kb | Kilobase |
| kDa | Kilodalton |
| KV | Killed vaccine |
| LaDV | Lactate dehydrogenase elevating virus |
| LTH | Leader transcription regulatory sequence hairpin |
| M-MLV RT | Moloney murine leukemia virus reverse transcriptase |
| MAVS | Mitochondrial antiviral-signaling protein |
| MLV | Modified live vaccine |
| mRNA | Messenger RNA |
| NendoU | Nidoviral endoribonuclease, specific for U |
| NF-κB | Nuclear factor kappa B |
| NK | Natural killer |
| nsp | Nonstructural protein |
| ORF | Open reading frame |
| PAM | Porcine alveolar macrophages |

| | |
|---|---|
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PC | Post challenge |
| PCR | Polymerase chain reaction |
| pDC | Plasmacytoid dendritic cell |
| PI | Post infection |
| PLGA | Poly(lactic-*co*-glycolyc) acid |
| PLP | Papain-like protease |
| PMA | Phorbol myristate acetate |
| pp | Polyprotein |
| PRF | Programmed ribosomal frameshift |
| PRRS | Porcine reproductive and respiratory syndrome |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PRV | Pseudorabies virus |
| PVDF | Polyvinylidene difluoride |
| $R^2$ | Coefficient of determination |
| RBC | Red blood cell |
| RdRp | RNA-dependent RNA polymerase |
| RIG-I | Retinoic acid-inducible gene I |
| RNA | Ribonucleic acid |
| RT | Reverse transcription |
| RTC | Replication and transcription complexes |
| S/P | Sample-to-positive |

| | |
|---|---|
| SC | Secreting cells |
| sg | Subgenomic |
| SHFV | Simian hemorrhagic fever virus |
| SLA | Swine leukocyte antigen |
| SP | Serine protease |
| STAT1 | Signal transducer and activator of transcription 1 |
| SVN | Serum virus neutralization |
| $TCID_{50}$ | Tissue culture infectious dose 50 |
| TNF | Tumor necrosis factor |
| TRS | Transcription regulatory sequence |
| USDA | United States Department of Agriculture |
| UTR | Untranslated region |

# GENERAL INTRODUCTION

Since its emergence approximately three decades ago, PRRSV has made its way to almost all large swine producing economies in the world. As the cost associated with the disease continue to rise, significant progress in vaccine research and development is yet to come. A major hurdle for developing a better vaccine against PRRSV is the high genetic and antigenic variability of the virus, which, coupled with its rapidly evolving nature, manifests as multiple isolates circulating and emerging within a region, farm, or animal. Furthermore, how the known correlates of protection against PRRSV, T cells and neutralizing antibodies, may cross-react and mediate protection against heterologous isolates is incompletely understood. The principal objectives of this dissertation were (i) to determine the extent of cross-reactivity of immune responses against PRRSV, and (ii) to ascertain how cross-reactive immune responses mediate protection against heterologous isolates. Overall, the purpose was to contribute to the understanding of immune responses against PRRSV that could be used to improve the development of vaccines. This dissertation consists of five chapters. Chapter I reviews the literature on PRRSV centering on the virus, its biology and diversity, the disease it causes, how the immune system combats it, and the current strategies for control and prevention, including the latest advances in vaccine development. Chapter II describes the materials and methods utilized throughout this dissertation. Chapter III reports on the evolution of T cell responses after PRRSV infection, the cross-reactivity of immune responses observed in infected animals, the relation of genetic divergence and cross-reactivity, and how cellular and humoral immune responses against PRRSV associate. Chapter IV describes the cell- and antibody-

mediated immune responses against PRRSV in previously infected or naïve animals, before and after challenge, and the quality of protection achieved by prior PRRSV exposure. Finally, Chapter V explores the cross-reactivity between the newly re-classified PRRSV species, PRRSV-1 and PRRSV-2, at the structural protein level.

# CHAPTER I

# LITERATURE REVIEW

## 1. Overview of porcine reproductive and respiratory syndrome

The late 1980's saw the emergence of a new swine disease in the United States characterized by severe reproductive losses, respiratory disease, reduction in growth, and increased mortality (Keffaber, 1989). Similar outbreaks began to be reported in Europe shortly after, and in 1991 the causative agent—a previously unidentified enveloped RNA virus—was identified in the Netherlands and named Lelystad virus (Terpstra et al., 1991; Wensvoort et al., 1991). The virus was first isolated and characterized in the United States later that year and called VR-2332 (Benfield et al., 1992; Collins et al., 1992). The disease, initially named "mystery swine disease", "swine infertility and respiratory syndrome" and "porcine epidemic abortion and respiratory syndrome" was finally designated porcine reproductive and respiratory syndrome or PRRS, and its etiological agent porcine reproductive and respiratory syndrome virus or PRRSV.

PRRSV infects pigs of all ages. An array of clinical signs can be observed in PRRSV-infected pigs, which are most severe in sows and young pigs, and the disease is characterized by persistent transmissible infections (Zimmerman et al., 2012). Although it was first described in 1987, retrospective studies of swine sera found PRRSV-seropositive animals as early as 1979 in Canada and 1986 in the United States (Carman et al., 1995; Yoon et al., 1992). The first estimates of PRRSV herd seroprevalence in the United States placed it at 33% in 1990 (Bautista et al., 1993b), while the latest available data, from 2006,

showed that 71.1% of unvaccinated herds and 49.8% of unvaccinated animals were PRRSV seropositive (USDA, 2009).

An assessment conducted in 2005 revealed that swine producers in United States were estimated to lose $560 million each year due to PRRSV-associated declines in reproductive health, increase in deaths, and reductions in the rate and efficiency of growth (Neumann et al., 2005). Although improvements were made in dealing with the disease in growing pigs, by 2013 the economic impact of PRRS had climbed to $664 million in annual losses, mainly due to increased costs in the breeding herd (Holtkamp et al., 2013). In perspective, these assessments almost double the estimated pre-eradication annual burden attributable to classical swine fever virus, at $364 million, and pseudorabies virus (PRV), at $36 million, when adjusted to 2004 dollars (Neumann et al., 2005).

## 2. Porcine reproductive and respiratory syndrome virus

### *2.1. Taxonomy*

Until recently, PRRSV was considered one of four species within the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales.* Other species in the genus included *Equine arteritis virus* (EAV), the prototype species, *Lactate dehydrogenase-elevating virus* (LaDV), and *Simian hemorrhagic fever virus* (SHFV) (Cavanagh, 1997; Faaberg et al., 2012). A significant reorganization and expansion of the family *Arteriviridae* was recently accepted in order to update the nomenclature and include newly discovered arteriviruses. Newly established pairwise sequence comparison of complete coding genome regions and open reading frame (ORF) 1b phylogeny were used to determine taxon-specific sequence cut-offs (Kuhn et al., 2016). As a result, the family *Arteriviridae* now includes five genera,

*Equartevirus*, *Porartevirus*, *Simartevirus*, *Nesatervirus*, and *Dipatervirus*. EAV, *Wobbly possum disease virus*, and *African pouched rat virus 1* are the sole members of the genera *Equartevirus*, *Dipartevirus*, and *Nesartevirus*, respectively, while SHFV and other non-primate arteriviruses belong to the genera *Simartevirus*. The species *Porcine reproductive and respiratory syndrome virus* was split to accommodate the divergence between the European or type 1 and North American or type 2 genotypes, which are now considered separate species, PRRSV-1 and PRRSV-2, respectively, within the genus *Porartevirus*, which also includes LaDV and *Rat arterivirus 1* (Adams et al., 2016; Adams et al., 2017).

### *2.2. Genome organization and replication*

The PRRSV genome consists of a 14.9–15.5 kilobase (kb) single positive strand of RNA that is 3'-polyadenylated and encodes multiple non-structural and structural proteins. The virus employs a complex array of replication and expression mechanisms which include the rearrangement of host membranes to establish viral replication and transcription complexes (RTC), synthesis and expression of genomic RNA, and synthesis and expression of subgenomic (sg) messenger RNA (mRNA).

#### *2.2.1. Genome organization*

The PRRSV genome (Figure 1.1) encodes 11 ORFs flanked by 5' and 3' untranslated regions (UTR). The large overlapping replicase ORF1a/b occupies the 5'-proximal three-quarters of the genome, which gives rise to four distinct polyprotein (pp) products that are co-translationally and post-translationally processed into 16 distinct non-structural proteins (nsps) by virally-encoded proteinases (Kappes and Faaberg, 2015).

The autocatalytic processing of pp1a, encoded by ORF1a, yields ten nsps: nsp1α, nsp1β, nsp2, nsp3, nsp4, nsp5, nsp6, nsp7α, nsp7β, and nsp8 (Li et al., 2012). ORF1b translation requires a programmed -1 ribosomal frameshift (PRF) that allows for the 3' extension of ORF1a into ORF1b to generate pp1ab, which in turn yields the ten nsps encoded in pp1a plus nsp 9 through 12 (Snijder et al., 2013). Of the four virally encoded proteases described for PRRSV, three are papain-like cysteine proteinases (PLPs) residing in nsp1α (PLP1a), nsp1β (PLP1b), and nsp2 (PLP2), and one is a serine proteinase (SP) in nsp4. PLP1a cleaves the nsp1α-nsp1β junction and PLP1b cleaves the nsp1β-nsp2 junction, whereas PLP2 is responsible for cleaving nsp2 from nsp3 and the main SP processes all remaining nsp products (Li et al., 2015).

A recently described PRF site located within the nsp2 coding region is responsible for generating ORF1a', which yields two additional nonstructural protein products: a -2 PRF produces an nsp2-related transframe protein designated nsp2-TF, and a -1 PRF yields a truncated nsp2 variant named nsp2-N (Fang et al., 2012; Li et al., 2014). A highly conserved putative RNA-binding motif located within the PLP1b domain of nsp1β is responsible for the transactivation of this PRF, which otherwise lacks any obvious stimulatory RNA secondary structure (Li et al., 2014).

The PRRSV structural proteins are encoded by eight overlapping ORFs contained within a set of six sg mRNAs that are generated via negative-strand intermediates from the 3' portion of the genome. Regardless of their polycistronic nature, most sg mRNA are functionally monocistronic (Meng, 2000; Meng et al., 1996).

Flanking the protein coding regions, the 5'UTR and 3'UTR of arteriviruses hold conserved RNA structures considered essential components for viral replication and

protein translation. The 5'UTR is likely to contain a cap structure, and contains the leader transcription regulatory sequence (TRS) hairpin that is essential for sg mRNA synthesis (Sagripanti et al., 1986; van den Born et al., 2004).

### *2.2.2. Viral replication sites*

A hallmark of all positive-stranded RNA virus replication is the formation of organelle-like structures to replicate their genome using host membrane modifications (Romero-Brey and Bartenschlager, 2014). It has been proposed that the association of viral RNA synthesis with dedicated membranes affords viruses three advantages: it confines viral RNA synthesis to compartments where viral proteins and precursors can be optimally concentrated, it spatially distributes and coordinates the various processes of the infectious cycle, and it shields double stranded RNA species preventing or delaying their recognition by the host's innate immune responses (van der Hoeven et al., 2016). For EAV, the formation of these dedicated membranes—called double membrane vesicles (DMVs)—was shown to require the presence of both nsp2 and nsp3 (Snijder et al., 2001).

### *2.2.3. Genome replication*

Upon entry into the cell, the PRRSV genome acts as template for the synthesis of the replicase polyproteins encoded in ORF1a/b, which is presumably initiated by ribosomal scanning (van den Born et al., 2005). ORF1b encodes the nsps responsible for PRRSV genome replication: nsp9, the RNA-dependent RNA polymerase (RdRp), nsp10, the RNA helicase, and nsp11, the nidovirus uridylate-specific endoribonuclease (NendoU) (Snijder et al., 2013).

The RdRp, the catalytic subunit within the RTC, is able to initiate de novo RNA synthesis in the absence of other viral or cellular proteins in a template-specific and primer-independent manner; however, it was postulated that the RdRp might require additional viral or cellular co-factors to perform effectively (Beerens et al., 2007). Unlike other members of the order *Nidovirales*, the arterivirus RdRp lacks 3' proofreading capacity, which contributes to a high rate of random mutations (Kappes and Faaberg, 2015). The RdRp is responsible for synthesizing a genome-length minus strand, or anti-genome, that will subsequently serve as template for the generation of new viral genomes.

*2.2.4. Synthesis of subgenomic messenger RNAs*

PRRSV structural proteins are encoded by a set of six 3'-co-terminal nested sg mRNAs (Meng et al., 1996). The viral RdRp is responsible for synthesizing sg mRNAs that contain the 5'UTR and the polyadenylated 3'UTR and one or more ORFs from the 3' region of the genome, but lack the large replicase ORF1a/b (den Boon et al., 1996). Arteriviruses use a form of discontinuous RNA transcription, a mechanism resembling copy-choice RNA recombination, where through base pairing of short conserved TRSs the 5'UTR (leader TRS) fuses to one of many downstream 3' sites (body TRS). The process first requires the generation of negative strand sg RNAs which will be subsequently used to synthesize sg mRNAs (Pasternak et al., 2001; van Marle et al., 1999). The abundance of sg mRNAs can be correlated with the stability of the duplex between leader and body TRS sites (Pasternak et al., 2004).

### *2.3. Viral structure and assembly*

The PRRSV virion is enveloped, pleomorphic, roughly spherical or oval with an average diameter of 58 nm (Spilman et al., 2009). The PRRSV genome is encapsidated by the N protein and the viral envelope contains two major proteins— glycoprotein (GP) 5 and M—and five minor proteins, GP2, GP3, GP4, E, and the recently discovered ORF5a protein (Dokland, 2010; Johnson et al., 2011). The viral particles are smooth, probably due to the small ectodomains of the major envelope proteins; yet, a few 10-15 nm protrusions can be observed, likely corresponding to the less abundant minor glycoproteins (Spilman et al., 2009). The buoyant density of the particle is 1.19 in cesium chloride, 1.14 in sucrose, and 1.19 g/cm in a glycerol-tartrate gradient (Wensvoort et al., 1992).

#### *2.3.1. Nucleocapsid*

The nucleocapsid core of the PRRSV particle is separated from the envelope by a 3 nm gap and consists of a 10-11 nm thick two-layered shell that surrounds a hollow central cavity averaging 13-14 nm in diameter (Spilman et al., 2009). Although the conformation of the nucleocapsid is yet unknown, it has been suggested that it may adopt a helical or loosely organized filamentous structure, resembling that of coronaviruses (Dokland, 2010).

The 15-kDa N protein, encoded by ORF7, is highly basic, interacts with the viral RNA to form the viral nucleocapsid, and constitutes about 20-40% of the protein content of the virion (Bautista et al., 1996). The N protein is phosphorylated and is incorporated into virions a disulfide-linked homodimer, and both protein oligomerization and direct RNA binding motifs have been implicated in RNA binding (Wootton et al., 2002; Wootton and Yoo, 2003). Although the PRRSV life cycle occurs in the cytoplasm, the N protein has

been found to localize in the nucleus and nucleolus of infected cells (Rowland et al., 1999a).

*2.3.2. Major envelope proteins*

The non-N-glycosylated 16-kDa M protein, encoded by ORF6, and the N-glycosylated 25-kDa GP5, encoded by ORF5, form a disulfide-linked heterodimer on the PRRSV envelope that is deemed crucial for viral particle assembly and budding (Mardassi et al., 1996; Wissink et al., 2005). GP5 is the most variable structural protein of PRRSV, sharing only ~50% amino acid homology between PRRSV-1 and PRRSV-2. Strains and isolates within each species share ~88% and 89-94% GP5 amino acid identity, respectively for PRRSV-1 and PRRSV-2 (Andreyev et al., 1997; Meng et al., 1995b; Suarez et al., 1996). The number of N-glycosylation sites of GP5 is variable. Ansari et al. (2006) showed that of the predicted N-glycosylation sites of GP5—located on sites 34, 44, and 51—the one mapped to reside 44 was essential for viral infectivity; however, Wei et al. (2012a) later demonstrated that mutation of individual N-glycosylation sites in GP5—mapped to residues 30, 34, 44, and 51—did not have such an effect.

*2.3.3. Minor glycoprotein heterotrimer*

The PRRSV minor envelope glycoproteins GP2 (29–30-kDa), GP3 (45–50-kDa), and GP4 (31–35-kDa) are encoded by ORF2a, ORF3, and ORF4, and contain two, seven, and four potential N-glycosylation sites, respectively (Meulenberg and Petersen-den Besten, 1996; van Nieuwstadt et al., 1996). Although they are considered essential for viral infectivity, they were found not to be essential for viral particle assembly (Wissink et al.,

2005). All three minor glycoproteins are incorporated into the virion in smaller proportions than the major envelope proteins and the N protein (de Lima et al., 2009; van Nieuwstadt et al., 1996).

Wissink et al. (2005) first reported that GP2, GP3, GP4, and the E protein were assembled into virions as a multimeric complex that jointly migrated through the Golgi complex and, unless expressed together, were retained in the endoplasmic reticulum (ER). It was later demonstrated that all three minor glycoproteins interact with each other, GP2 and GP4 interact with the major envelope glycoprotein GP5, and GP4 plays a central role in the generation of the heterotrimer and its interaction with GP5 (Das et al., 2010).

Das et al. (2011) established that to produce infectious PRRSV particles N-glycosylation at position 184 of GP2, positions 42, 50, and 131 of GP3, and any three of the four sites of GP4 were essential; however, Wei et al. (2012b) later demonstrated that the lack of N-glycosylation at the aforementioned positions had no effect on virus recovery, and that the absence of one or two N-glycosylation sites on GP4 were not lethal, but the absence of three was. Moreover, it was demonstrated that both N-glycosylation sites on GP2 were deemed dispensable for particle assembly and infectivity of PRRSV-1 (Wissink et al., 2004).

*2.3.4. Small envelope (E) and ORF5a proteins*

Encoded by ORF2b, the 10-kDa E protein is dispensable for PRRSV virion assembly but essential for viral infectivity, and has been implicated in promoting the uncoating of the virion and release of the viral genome into the cytoplasm (Lee and Yoo, 2006; Wu et al., 2001). The ORF5a transmembrane protein (5–6 kDa) is encoded by the

same RNA sequence that encodes the hypervariable glycosylation-rich ectodomain region of GP5, and has been found to be essential for virus viability and to interact with GP4 and the E protein (Johnson et al., 2011; Robinson et al., 2013; Sun et al., 2013; Sun et al., 2015).

#### *2.3.5. Assembly and release*

Little is known about the morphogenesis and assembly of PRRSV particles. The arterivirus-induced DMVs and the ER form a reticulovesicular network which intertwines with tubules abundant in N protein, and it has been hypothesized that morphogenesis and assembly are coordinated in said space and that genome encapsidation is initiated at the site of RNA synthesis (Knoops et al., 2012; Tijms et al., 2002). PRRSV nucleocapsids were shown to bud from the smooth ER and accumulate in the lumen of the ER or Golgi vesicles, and it has been suggested that the N protein self-associative properties likely provide the basis for nucleocapsid assembly (Dea et al., 1995; Pol et al., 1997; Wootton and Yoo, 2003). Wieringa et al. (2004) proposed that the newly synthesized nucleocapsids are likely to bind to exposed domains of the envelope proteins in the process of viral budding, and Dea et al. (1995) showed that the viral particles accumulated in the infected cells are released by exocytosis.

### ***2.3. Virus-host interactions***

#### *2.3.1. Tropism, receptors, and entry*

PRRSV has a very limited tropism of cells and hosts, primarily replicating in porcine alveolar macrophages (PAM) and macrophages of lymphoid tissues of pigs (Duan et al., 1997b; Mardassi et al., 1994; Teifke et al., 2001). Macrophage precursor cells, such

as bone marrow cells, peripheral blood monocytes, and peritoneal macrophages are largely refractory to PRRSV infection (Duan et al., 1997a, b). Loving et al. (2007) demonstrated that monocyte-derived dendritic cells (DCs)—that may still retain specific characteristics of monocytes/macrophages—are permissive to PRRSV infection, but primary lung DCs are not. The MA-104 African green monkey kidney cell line, and its derivatives MARC-145 and CL 2621 cells, can support PRRSV replication and are widely used in research (Bautista et al., 1993a; Benfield et al., 1992; Kim et al., 1993).

Heparan sulfate, vimentin, CD151, CD163, sialoadhesin (SIGLEC1 or CD169), and DC-SIGN have all been implicated as potential cellular receptors for PRRSV (Van Breedam et al., 2010).

CD163 was identified by direct functional screening of a complementary DNA (cDNA) expression library derived from PAM, and it conferred PRRSV-permissiveness to otherwise non-permissive cell lines (Calvert et al., 2007). GP2 and GP4 have been shown to mediate the interaction with certain domains of CD163, and their N-glycosylation status was shown to be of cardinal importance (Das et al., 2010; Das et al., 2011; Van Gorp et al., 2010; Wei et al., 2012b). Tian et al. (2012) demonstrated that a chimeric PRRSV encoding the GP2-4 and E protein of EAV acquired the broad tropism typical of EAV, and Whitworth et al. (2016) showed that CD163-knockout pigs were completely refractory to PRRSV infection. Thus, it is widely accepted that CD163 is the major cellular receptor for PRRSV, and its interaction with the minor envelope glycoproteins is responsible for viral tropism. Nonetheless, the sole presence of CD163 in many cell lines does not grant permissiveness to PRRSV infection, and it has been suggested that other molecules, including sialoadhesin, could be also involved in this process (Welch and Calvert, 2010).

Recently, it was reported that PRRSV-1 and PRRSV-2 interact with different motifs of CD163 (Wells et al., 2017). Virions enter the cell through receptor-mediated, clathrin-dependent endocytosis, followed by fusion of endocytic vesicles with endosomes, where low pH is required for virus uncoating (Kreutz and Ackermann, 1996; Nauwynck et al., 1999).

*2.3.2. Viral modulation of innate immune responses*

Type I interferons (IFNs) IFN-α and IFN-β—a critical component of the innate immune response—are potent antiviral cytokines that induce cellular antiviral proteins, enhance antigen presentation, and promote adaptive immune responses (McNab et al., 2015). IFN-α response during PRRSV infection can be characterized as meager or null, which suggests that the virus may actively suppress type I IFN production. Six proteins of PRRSV have been implicated in type I IFN antagonism: nsp1α, nsp1β, nsp2, nsp4, nsp11, and N (Albina et al., 1998a; Beura et al., 2010; Sagong and Lee, 2011).

Type I IFN suppression has been extensively demonstrated for nsp1 and its two subunits (nsp1α and nsp1β). Beura et al. (2010) were the first to report that five PRRSV nsps (nsp1α, nsp1β, nsp2, nsp4, and nsp11) had strong to moderate inhibitory effect on the activation of the IFN-β promoter. Blocking of the type I IFNs response by nsp1α has been shown to occur through CREB-binding protein degradation, inhibition of nuclear factor (NF)-kB signaling, and inhibition of the tumor necrosis factor (TNF)-α promoter (Han and Yoo, 2014; Ke and Yoo, 2017). The mechanisms by which nsp1β suppresses type I IFNs include inhibition of IFN regulatory factor (IRF) 3 phosphorylation, NF-κB nuclear translocation, STAT1 phosphorylation and nuclear translocation, IFN-stimulated gene

(ISG) factor 3 nuclear translocation, and suppression of the TNF-α promoter, among others (Han and Yoo, 2014; Sun et al., 2012). It was recently demonstrated that nsp1α triggers the degradation of the swine leukocyte antigen (SLA) class I in a ubiquitin proteasome dependent fashion, further interfering with the host's immune responses (Du et al., 2015).

The PLP2 domain of nsp2 antagonizes type I IFN induction by interfering with the NF-κB signaling pathway and inhibiting the phosphorylation and nuclear translocation of IRF3 and the antiviral function of ISG15 (Wang and Zhang, 2014). The SP domain of nsp4 antagonizes NF-κB, while the NendoU domain of nsp11 blocks the phosphorylation and nuclear translocation of IRF3, inhibits NF-κB signaling, and suppresses the expression of the cytoplasmic antiviral receptors MAVS and RIG-I (Ke and Yoo, 2017; Sun et al., 2012) While the N protein was found to suppress type I IFN production by inhibiting IRF3 phosphorylation and nuclear translocation, it was also found to activate NF-κB (Sagong and Lee, 2011; Sun et al., 2012).

PRRSV infection reduces or suppresses IFN-α and pro-inflammatory cytokine production in plasmacytoid dendritic cells (pDCs)—a DC subset capable of producing large amounts of IFN-α—by downregulating the expression of interferon regulatory genes, and was shown to repress natural killer (NK) cell cytotoxicity independent of NK cell frequency (Calzada-Nova et al., 2011; Dwivedi et al., 2012). Because cell-mediated immunity relies on IFN-α and pDCs for maturation, the meager IFN-α response to PRRSV is expected to negatively impact the host's adaptive immune responses (Loving et al., 2015).

*2.3.3. Mechanisms of cell injury*

Four mechanisms of cell injury have been studied for PRRSV: apoptosis of infected macrophages and surrounding cells, production of pro-inflammatory and immunomodulatory cytokines, polyclonal B cell activation, and reduced phagocytosis and killing of bacteria by macrophages.

In PRRSV-infected animals' apoptosis was shown to occur in both lung and lymphoid tissue; however, it was observed mostly in PRRSV-uninfected cells, suggesting that PRRSV-mediated apoptosis affects bystander cells through an indirect mechanism (Sirinarumitr et al., 1998; Sur et al., 1998). PRRSV-induced apoptosis was shown to be dependent on the activation of caspase-8 and caspase-9—signaling through both the extrinsic and intrinsic pathway—and multiple and complex pathways have been implicated in this process (Lee and Kleiboeker, 2007).

The synthesis and presence of the pro-inflammatory cytokines interleukin (IL)-1 and IL-6, that cause pyrexia, inflammation, and promote the infiltration and activation of leukocytes, was found to be upregulated in the lungs of PRRSV-infected pigs (Liu et al., 2010; Van Reeth et al., 1999). The immunomodulatory cytokine IL-10—responsible for inhibiting the production of pro-inflammatory cytokines—was also shown to be upregulated in pigs infected with PRRSV (Suradhat and Thanawongnuwech, 2003). Nonetheless, even though it is widely accepted that PRRSV suppresses IFN-α and TNF-α production in infected animals, certain PRRSV isolates have been shown to enhance it (Liu et al., 2010).

### *2.4. Phenotype and genotype diversity*

Due to a concurrent emergence and similar disease syndromes, until recently, PRRSV-1 and PRRSV-2 were considered two genotypes of the same virus species. However, PRRSV-1 and PRRSV-2 are antigenically distinct viruses that share common antigenic epitopes with moderately conserved genomes (Murtaugh et al., 1995; Nelsen et al., 1999; Nelson et al., 1993).

RNA polymerase infidelity is primarily responsible for the ever-increasing diversity of PRRSV. PRRSV calculated nucleotide substitution rates are very high; however, no biochemical data is available on the base incorporation specificity for the viral replication complex (Murtaugh et al., 2010). PRRSV diversity can also be attributed to the contribution of genomic recombination, which has been demonstrated computationally and experimentally (Murtaugh et al., 2001; Murtaugh et al., 2002; van Vugt et al., 2001).

Only about 60% nucleotide similarity exists between PRRSV-1 and PRRSV-2 (Murtaugh et al., 2010). Nine lineages have been proposed and defined using ORF5 phylogeny for PRRSV-2, seven of which include predominantly North American isolates, and two contain exclusively East Asian isolates (Shi et al., 2010b). For PRRSV-1, genetic and biological studies support its subdivision into three subtypes considering ORF5 and ORF7 genetic diversity (Stadejek et al., 2013).

Canada has been suggested as the potential origin of PRRSV-2, due to having the earliest record of a positive serum sample (Carman et al., 1995; Shi et al., 2010a), while Eastern Europe has been hypothesized to be the origin of PRRSV-1 (Stadejek et al., 2006). Hanada et al. (2005) proposed that PRRSV-1 and PRRSV-2 diverged not long before emergence, somewhere between the early 1970's and mid 1980's, which was followed by

an extremely high substitution rate; nevertheless, this scenario was immediately questioned due to inappropriate methodology and the use an uninformative data set (Forsberg, 2005). Alternatively, and using an expanded ORF3 dataset, Forsberg (2005) proposed an early divergence between PRRSV-1 and PRRSV-2, around the late 1800's, followed by a long independent evolution in the two continents.

Two hypothesis have been suggested for the origin of PRRSV. Plagemann (2003) maintains that PRRSV had an LaDV-like ancestor circulating in rodents that was adapted to Eurasian wild boars. Wild boars from Europe were introduced into the United States in the early 1900's, and it has been hypothesized that PRRSV evolved separately in the wild boar population before its introduction into the domestic pig population; however, PRRSV prevalence studies in wild boars argue against this theory (Plagemann, 2003; Reiner et al., 2009). Alternatively, Murtaugh et al. (2010) suggest a single Eurasian origin of PRRSV, followed by a translocation of infected swine to North America, after which the virus evolved independently in both Eurasia and North America for an indeterminate period giving rise to PRRSV-1 and PRRSV-2, respectively.

**3. Epidemiology and pathogenesis**

PRRSV is distributed worldwide, including the three largest swine producing regions in the world: China, the European Union, and the United States. Because available serology tests do not differentiate virus-exposed from vaccinated animals, PRRSV seroprevalence is difficult to estimate; however, 71.1% of unvaccinated herds and 49.8% of unvaccinated animals in the United States were PRRSV seropositive in 2006 (USDA,

2009). Although PRRSV infects only domestic and feral pigs, seroprevalence studies show a very low prevalence in wild boars (Saliki et al., 1998; Wyckoff et al., 2009).

### *3.1. Transmission, infection, and shedding*

PRRSV infection can occur both directly and indirectly. PRRSV horizontal transmission has been described for the intranasal, intramuscular, oral, and vaginal routes, and the probability that a given dose will infect an animal differs by the route of exposure and isolate (Benfield et al., 2000; Cutler et al., 2011; Hermann et al., 2005; Hermann et al., 2009; Yoon et al., 1999). Husbandry practices, such as ear notching, tail docking, and teeth clipping could lead to PRRSV exposure, and percutaneous (parenteral) exposure is the route with the lowest minimum infectious dose (Pileri and Mateu, 2016). Otake et al. (2002) demonstrated that transmission of PRRSV could be achieved through contaminated needles and Bierk et al. (2001) suggested that biting and fighting between animals could result in PRRSV exposure. PRRSV transplacental infection occurs in late-term gestation and results in fetal death or birth of infected pigs that are weak or appear normal (Karniychuk and Nauwynck, 2013).

Transmission of PRRSV between herds occurs through the introduction of animals, semen, and—less likely—aerosol transmission (Goldberg et al., 2000; Mortensen et al., 2002). Upon entering a naïve herd, an epidemic phase lasting 1–5 months ensues, where all pigs get infected with PRRSV, after which infection becomes endemic, usually when a majority of animals achieve a protective immunity (Pileri and Mateu, 2016). PRRSV tends to circulate within a herd indefinitely. The continual availability of susceptible animals, either through birth, purchase, or loss of protective immunity, sustains persistence PRRSV,

and is more likely to occur as the herd size increases and when gilts are not properly isolated from sows (Evans et al., 2010; Nodelijk et al., 2000).

PRRSV infection is chronic and persistent. Viremia can be detected up to a month post infection (PI) or more, while viral replication can be detected up to 150 days post infection (DPI) or more in lymphoid tissue (Allende et al., 2000; Horter et al., 2002; Wills et al., 1997b). PRRSV persistence has been described in pigs exposed in utero, as a young animal and as an adult, and seems to last for 3–4 months PI, after which it is cleared by the host (Bierk et al., 2001; Christopher-Hennings et al., 1995; Rowland et al., 1999b; Wills et al., 2003).

PRRSV-infected animals shed virus in saliva, nasal secretions, urine, semen, and less frequently in milk and feces (Christianson et al., 1993; Rossow et al., 1994; Swenson et al., 1994; Wagstrom et al., 2001; Wills et al., 1997a). Cho et al. (2006) reported that shedding of virus from infected animals is dependent on the pathogenicity of the isolate.

### *3.2. Pathogenesis, clinical signs, and lesions*

Immediately after exposure to PRRSV, viral replication occurs in local permissive macrophages after which the virus spreads to lungs, lymphoid and other tissues. Viremia can be detected as early as 12 hours PI—most pigs develop viremia by 24 hours PI—and peaks at 7–14 DPI (Rossow et al., 1995). After peaking, viremia decreases rapidly, and most pigs are not viremic by 28 DPI; however, persistent infection continues in tonsil and/or lymph nodes for extended periods of time, where virus is produced by a low level of continuous replication (Allende et al., 2000; Wills et al., 2003).

Different clinical presentations of PRRSV infection can be observed depending on the age of the pig, pregnancy status, and stage of gestation of the sow or gilt. Reproductive failure occurs due to late-term exposure of pregnant sows or gilts, and litters are composed of any combination of normal pigs, weak variably sized pigs, and dead pigs that are fresh stillborn, autolytic, partially mummified, or completely mummified fetuses (Christianson et al., 1992; Mengeling et al., 1998). Sows have delayed return to estrus, low conception rate, and, infrequently, agalactia (Hopper et al., 1992). In suckling pigs—infected in utero or at (or shortly after) birth—PRRS is characterized by severe dyspnea and tachypnea and high pre-weaning mortality, while weaning and grower pigs experience anorexia, lethargy, dyspnea (or hyperpnea), reduction in average daily gain, and an increase in mortality due to concurrent bacterial infections (Hopper et al., 1992; Stevenson et al., 1993). PRRSV-infected boars may lack libido and have variable reduction in semen quality, in addition to anorexia, lethargy, and respiratory clinical signs (Done et al., 1996; Prieto et al., 1996). In breeding herds, endemic PRRS is sustained by subclinical infections, with occasional small outbreaks of clinical PRRS in gilts and, less commonly, in sows (Dee and Joo, 1994; Hopper et al., 1992).

Gross lung lesions associated with PRRSV vary from none to multifocal tan-mottled consolidation, and are commonly complicated by lesions arising from associated bacterial infections (Done and Paton, 1995; Halbur et al., 1995b). In young pigs, lymph nodes are markedly enlarged and vary from solid to polycystic (Halbur et al., 1995a; Rossow et al., 1994). Aborted fetuses due to PRRSV are late term and the body condition ranges from fresh to autolyzed (Christianson et al., 1992; Lager and Mengeling, 1995). Microscopically, lung lesions are characterized by septal thickening, alveoli lined by

hypertrophied and hyperplastic type II pneumocytes, hypertrophy of peribronchial lymphoid tissue, and lymphoplasmacytic perivascular cuffing (Halbur et al., 1993; Rossow et al., 1995). Lymphoid tissue lesions include germinal center hypertrophy and hyperplasia, germinal center necrosis, and multiple cystic spaces with polykaryocytes (Halbur et al., 1995b; Rossow et al., 1994). Other microscopic findings include vasculitis varying in severity, myocarditis, and encephalitis (Halbur et al., 1995b; Rossow et al., 1994). Rarely, PRRSV-induced severe meningoencephalitis has been found to occur (Rossow et al., 1999).

## 4. Adaptive immune responses

Although PRRSV infection is chronic and persistent, most pigs successfully clear the virus 3–4 months PI (Allende et al., 2000). Protective immunity against PRRSV can be achieved, and both antibody-mediated and cell-mediated immune responses have been studied in the context of PRRSV infection; however, the details behind the significant amount of time required for clearance and disease resolution are still elusive.

### *4.1. Antibody-mediated immune responses*

Antibodies against PRRSV can be detected as early as 7–9 DPI, but early antibodies have been proven to lack virus neutralizing activity both *in vitro* and *in vivo* (Lopez et al., 2007; Yoon et al., 1994). PRRSV-neutralizing antibodies can be detected no earlier than 28 DPI, and up to 6 months PI or more; however, PRRSV still manages to persist in tissues and continues to be shed in their presence (Allende et al., 2000; Bierk et al., 2001; Meier et al., 2003; Nelson et al., 1994; Yoon et al., 1994).

*4.1.1. Non-neutralizing antibodies*

PRRSV-specific immunoglobulin (Ig) M against GP5 and the M and N proteins is first detected at 7 DPI, peaks at 14–21 DPI, and becomes undetectable by 35–42 DPI, while PRRSV-specific IgG peaks at 21–28 DPI and remains high through the persistent phase of infection (Loemba et al., 1996; Nelson et al., 1994). Long-lasting antibody responses targeted towards nsp1, nsp4, nsp7, and—to a lesser extent—nsp8 have been described (Brown et al., 2009).

Non-neutralizing antibodies against PRRSV have been linked to antibody-dependent enhancement (ADE) of infectivity. *In vitro*, sub-neutralizing amounts of PRRSV-specific IgG were able to increase PRRSV yields and infection rates; however, significant differences were found between isolates (Gu et al., 2015; Yoon et al., 1997; Yoon et al., 1996). In contrast, Delputte et al. (2004) were unable to demonstrate ADE for PRRSV-1 *in vitro*. *In vivo*, passive transfer of non-neutralizing or sub-neutralizing IgG followed by infection with PRRSV showed greater duration of viremia, increased rectal temperatures, and interstitial pneumonia (Lopez et al., 2007; Yoon et al., 1996); nonetheless, Lopez et al. (2007) argued that the increased rectal temperature and interstitial pneumonia they observed could be attributed to pro-inflammatory cytokines co-salted out with the IgG fractions.

*4.1.2. Neutralizing antibodies*

Albina et al. (1992) were the first to describe the presence of neutralizing antibodies in PRRSV-infected animals. Passive transfer studies proved that homologous neutralizing

antibodies could protect sows and weaned pigs from viremia and, in certain cases, afford them sterilizing immunity (Lopez et al., 2007; Osorio et al., 2002). The breadth and contribution to cross-protection of heterologous neutralizing antibody response against PRRSV is unclear. Kim et al. (2007) showed that sera from pigs inoculated with diverse PRRSV-2 isolates effectively neutralized homologous virus, but meager cross-neutralization was observed. Choi et al. (2016) further demonstrated the absence of cross-neutralizing antibodies between PRRSV-1- and PRRSV-2-infected animals. Using a panel of 30 hyperimmune monospecific sera and 39 PRRSV-1 isolates it was established that certain PRRSV-1 isolates were more prone to cross-neutralization than others (Martinez-Lobo et al., 2011). Recently, broadly cross-neutralizing antibodies have been described in commercial sows after multiple PRRSV exposures (Robinson et al., 2015).

Targets of neutralizing antibodies have been mapped to GP3, GP4, GP5, and the M protein (Cancel-Tirado et al., 2004; Costers et al., 2010; Delputte et al., 2004; Ostrowski et al., 2002; Plagemann et al., 2002; Vanhee et al., 2011; Zhou et al., 2012). Several mechanisms have been proposed for PRRSV evasion of neutralizing antibodies. The absence of N-linked glycosylation in GP3 and GP5 was determined to enhance both the sensitivity of PRRSV to neutralization and its ability to rapidly elicit robust neutralizing antibodies *in vivo* (Ansari et al., 2006; Vu et al., 2011). A decoy epitope in GP5, described by Ostrowski et al. (2002), was shown to induce a rapid non-neutralizing antibody response in detriment of neutralizing antibodies targeting a nearby epitope.

### *4.2. Cell-mediated immune responses*

A transient reduction in CD4+ T cells, at 3–7 DPI, followed by a return to normal levels by 7–14 DPI, can be observed in PRRSV-infected animals, while the number of CD8+ T cells is increased in both blood and lung (Albina et al., 1998b; Nielsen and Botner, 1997; Samsom et al., 2000). Furthermore, γδ T cells are elevated in PRRSV-infected animals from 14 DPI through 70 DPI and increases in both CD8+ and CD4+/CDα+ double-positive (DP) T cells can be observed in lymphoid tissue (Gomez-Laguna et al., 2009; Olin et al., 2005). Correspondingly, piglets infected *in utero* with PRRSV exhibit a reduced number of CD4+ T cells at birth that lasts for two weeks, and a substantial increase in the number of CD8+ T cells (Feng et al., 2002; Nielsen et al., 2003).

Bautista and Molitor (1997) showed that the PRRSV-specific T cell proliferation response could be first detected at four weeks PI, peaked at seven weeks PI, and declined after 11 weeks PI. Antigen-induced proliferation of γδ T cells showed similar kinetics (Olin et al., 2005). Lymphoprolipheration studies showed that the PRRSV GP5, M, and N proteins were the strongest inducers of cell-mediated immunity (Bautista et al., 1999).

The cell-mediated immunity to PRRSV, measured by the frequency of PRRSV-specific IFN-γ secreting cells (SC) in peripheral blood mononuclear cells (PBMC), is weak and late to appear, but is prolonged and relatively stable once established (Meier et al., 2003). PRRSV-specific IFN-γ SC are undetectable until three weeks PI, stay moderately low for the subsequent ten weeks, followed by a gradual and steady surge (Meier et al., 2003). Xiao et al. (2004) were able to detect antigen-specific IFN-γ SC as early as two weeks PI, and observed that PRRSV-specific T cells in peripheral blood showed substantial variation over time and among animals. They further proved the absence of any correlation

between PRRSV load and PRRSV-specific T cell frequencies in lung and lymphoid tissue both at the acute and chronic stages of the disease (Xiao et al., 2004). Phenotypic analysis performed by Meier et al. (2003) revealed that the majority of PRRSV-specific IFN-γ SC were CD4+/CDα+ DP T cells, with a smaller proportion (~20%) of CD4-/CD8αβ+ cytotoxic T cells. Porcine CD4+/CD8+ DP T cells have been associated with immune memory function and shown to have B cell helper function (Zuckermann, 1999). T cell epitopes have been mapped to nsp2, nsp9, and nsp10, and the structural proteins GP4, GP5, M, and N (Burgara-Estrella et al., 2013; Diaz et al., 2009; Parida et al., 2012; Vashisht et al., 2008; Wang et al., 2011).

Together with neutralizing antibodies, cell-mediated immunity has been hypothesized to be a correlate of protection against PRRSV. Variable levels of protection can be achieved in the absence of neutralizing antibody titers, which leads to the belief that cell-mediated immunity contributes to protection against PRRSV (Roca et al., 2012; Trus et al., 2014; Zuckermann et al., 2007). Charerntantanakul et al. (2006) determined the existence of robust correlations between the percentage of IFN-γ+ cells in PBMC and the reduction of lung lesions and viremia after PRRSV challenge.

## 5. Prevention and control

### *5.1. Diagnosis*

PRRSV should be suspected in any herd with reproductive diseases in breeding swine and/or respiratory disease in pigs of any age; however, the absence of clinical signs should not rule out PRRSV infection. Differential diagnosis of PRRSV include classical swine fever virus, cytomegalovirus, hemagglutinating encephalomyelitis virus,

leptospirosis, parvovirus, porcine circovirus 2, pseudorabies virus, swine influenza virus, and teschovirus (Zimmerman et al., 2012).

For optimum isolation of PRRSV from clinical samples, both PAM and MARC-145 cells are recommended (Yoon et al., 2003). Microscopically, PRRSV antigen can be visualized in tissue sections of infected animals, such as lung, by either immunohistochemistry or immunofluorescence using monoclonal antibodies targeting the N protein (Halbur et al., 1994; Rossow et al., 1995). Commercially available real time reverse transcription (RT)-polymerase chain reaction (PCR) can be employed for the detection of PRRSV in serum, semen, tissues, and oral fluids (Prickett et al., 2008; Rovira et al., 2007).

Serological diagnosis of PRRSV can be accomplished by indirect fluorescent antibody (IFA) assay, enzyme-linked immunosorbent assay (ELISA), and serum virus neutralization (SVN) assay. Of these, commercial ELISAs are deemed sensitive, specific, and easy to use (Rossow, 1998; Yoon et al., 2003).

### *5.2. Prevention, control, and eradication strategies*

PRRSV prevention strategies are geared towards impeding the entry of the virus into negative herds and the introduction of new viral variants into infected herds. To achieve this purpose, swine producers may implement one or more protocols, including the use of quarantine facilities and testing protocols for incoming breeding stock, sanitation and drying protocols for transport vehicles and incoming suppliers, personnel entry protocols, and insect control programs (Dee, 2003; Pitkin et al., 2009).

PRRSV control strategies aim to limit the deleterious effects of PRRSV infection in the various stages of production. Gilt acclimatization—a process by which replacement gilts develop immunity to PRRSV prior to their introduction into the breeding herd—can be achieved by direct contact with infected animals, intentional exposure, or vaccination. Gilts that undergo the acclimatization process are introduced into the herd when they are no longer viremic (FitzSimmons and Daniels, 2003). Because vaccine-induced protection to PRRSV has been deemed weak and/or inconsistent, intentional exposure of gilts can be achieved by inoculation with serum obtained from viremic animals (FitzSimmons and Daniels, 2003). Other control strategies include partial depopulation, all-in/all-out pig flow, and vaccination (Dee, 2003).

Several approaches have been proposed for PRRSV eradication, including whole herd depopulation-repopulation, test and removal, herd closure, and partial depopulation (Dee, 2003). Herd closure relies on the ability of the animal to effectively mount an immune response against—and subsequently eliminate—PRRSV. Due to the persistent and chronic nature of PRRSV, an extended period of time of at least 200 days is recommended for herd closure, during which no replacement gilts are allowed entry into the breeding herd (Linhares et al., 2014; Torremorell et al., 2002).

### *5.3. Vaccines*

Commercial vaccines against PRRSV have been available since 1994. Both killed vaccines (KVs) and modified-live vaccines (MLVs) can be found in the market; however, only MLVs were shown to provide effective, albeit variable, levels of protective immunity against PRRSV (Zuckermann et al., 2007). Due to the insufficient efficacy afforded by

current vaccines, extensive research has gone into developing novel PRRSV vaccines capable of eliciting consistent and broad protective immune responses.

*5.3.1. Killed, subunit, and vectored vaccines*

In the young pig model, a PRRSV-1 KV was shown to afford no protection against infectious challenge, although the vaccine and challenge strains were 99% homologous at the level of ORF5 gene sequence (Zuckermann et al., 2007). Furthermore, a PRRSV-1 KV failed to protect pregnant gilts from the appearance of viremia and clinical signs and the transplacental infection of piglets after challenge with a heterologous PRRSV-1 strain (Scortti et al., 2007). Finally, KV-immunized boars showed no change in the onset, level, and duration of viremia and shedding of virus in semen after infectious PRRSV challenge (Nielsen et al., 1997). Recently, vaccination with a PLGA nanoparticle-entrapped PRRSV KV administered intranasally with *Mycobacterium tuberculosis* whole-cell lysate as an adjuvant was shown to reduce viremia and viral burden and lesions in lung after heterologous PRRSV challenge (Binjawadagi et al., 2014).

The efficacy of immunization with baculovirus-expressed GP3, GP5, and N protein was evaluated by the number of piglets born alive and healthy at the time of weaning. GP3- and GP5-immunized sows showed partial protection to homologous challenge, while N protein-immunized sows did not (Plana Duran et al., 1997). Piglets inoculated with a recombinant fowlpox-vectored vaccine encoding both ORF3 and ORF5 of PRRSV showed lower temperature, viremia, and virus load in tonsil, lymph node and lung after homologous challenge (Shen et al., 2007). Likewise, piglets inoculated with a recombinant adenovirus vaccine encoding both ORF3 and ORF5 of PRRSV exhibited reduced clinical signs,

viremia, and lung lesions after homologous challenge (Wang et al., 2009). Both recombinant attenuated PRV- and transmissible gastroenteritis virus-vectored vaccines expressing PRRSV GP5 or GP5 and M protein, respectively, have been shown to confer partial protection against homologous PRRSV challenge in young piglets (Cruz et al., 2010; Qiu et al., 2005).

Pirzadeh and Dea (1998) demonstrated that while immunization with a plasmid encoding ORF5 of PRRSV protected piglets from viremia and the development of macroscopic lung lesions after homologous challenge, vaccination with *Escherichia coli*-expressed recombinant ORF5-encoded protein did not. Contrarily, Diaz et al. (2013) later determined that DNA vaccination with a plasmid encoding both ORF5 and ORF6 of PRRSV could be responsible for an exacerbation of the clinical signs observed after challenge.

*5.3.2. Modified-live vaccines*

Benefits associated with PRRSV MLV vaccination include reduction of clinical signs, rescue in body weight loss, reduced lung lesions, and reduced viral shedding (Cano et al., 2007; Dwivedi et al., 2011; Linhares et al., 2012). Vaccination of young piglets with a PRRSV-2 MLV was shown to afford variable levels of protection against heterologous PRRSV-2 challenge and PRRSV-1 challenge (Park et al., 2015; Park et al., 2014). Nonetheless, a PRRSV-1 MLV was also proven to provide incomplete cross-protection against challenge with a homologous virus, suggesting that the degree of genetic similarity—in this case based on ORF5 genetic sequences—between MLV strain and challenge isolate may not be a good predictor of vaccine efficacy (Prieto et al., 2008).

Overall, although MLVs have been shown to protect against clinical disease, they still fail to prevent PRRSV infection.

PRRSV MLVs are considered ineffective for control and eradication of the virus and severe PRRSV outbreaks have been shown to occur in MLV-vaccinated farms (Vu et al., 2017; Wang et al., 2015). Another major limitation of the current MLVs is that they do not allow serological discrimination between naturally infected and vaccinated animals—also known as DIVA (differentiating infected from vaccinated animals)—a condition *sine qua non* for the control and eradication of most animal diseases (Vu et al., 2013). Furthermore, the extreme genetic and antigenic variability of the virus coupled with its ability to rapidly evolve and subvert the innate immune system are a major obstacle for developing a broadly-protective vaccine against PRRSV (Meng, 2000; Vu et al., 2017). Finally, because MLVs replicate in the host, there is always a potential for reversion to virulence (Nielsen et al., 2001).

### *5.3.3. Approaches to increase the efficacy of vaccines*

Several strategies have been attempted to increase the efficacy of PRRSV vaccines. Mengeling et al. (2003) demonstrated that vaccination with five attenuated strains of PRRSV-2 did not deliver better heterologous protection than vaccination with a single strain MLV. A chimeric virus carrying structural proteins from the genetically distinct PRRSV strains VR-2332 and JA-142 was shown to afford protection against challenge with both its parental strains. It still remains to be proven that it can protect against strains or isolates genetically distinct from its parental strains (Sun et al., 2016a). Furthermore, vaccination with an MLV containing randomly recombined DNA sequences of ORF3,

ORF4, ORF5, and ORF6 from multiple PRRSV-2 strains did not confer better heterologous protection than the original MLV (Tian et al., 2015).

Vu et al. (2015) have developed a centralized or consensus PRRSV-2 immunogen based on 59 full-genome sequences and demonstrated that the immunization by infection with this synthetic virus was able to accord broader levels of cross-protection than their reference strain. Sun et al. (2016b) later showed that—unlike other PRRSV strains or isolates—infection with the consensus PRRSV-2 strain induced type I IFN *in vitro*. Recently, a serially-passaged attenuated strain of this consensus PRRSV-2 immunogen was revealed to maintain the type I IFN-induction phenotype while exhibiting promising safety and efficacy profiles (Sun et al., 2017).

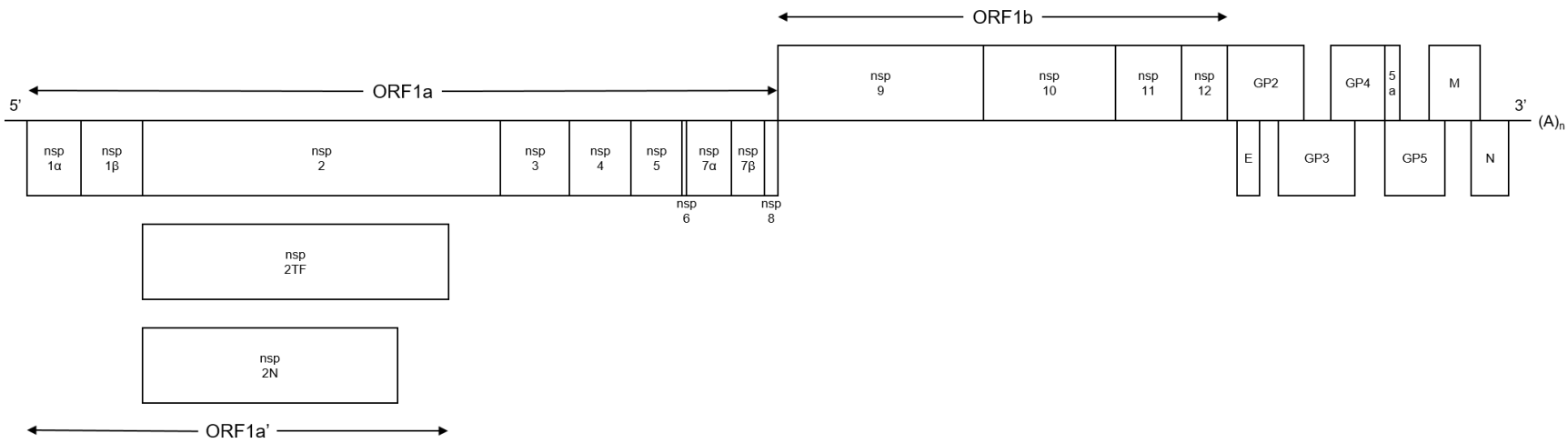
ORF1b
ORF1a
5'
3'
(A)n
nsp 1α
nsp 1β
nsp 2
nsp 3
nsp 4
nsp 5
nsp 6
nsp 7α
nsp 7β
nsp 8
nsp 9
nsp 10
nsp 11
nsp 12
GP2
E
GP3
GP4
5a
GP5
M
N
nsp 2TF
nsp 2N
ORF1a'

Figure 1.1. Schematic representation of the PRRSV genome.

# CHAPTER II

# MATERIALS AND METHODS

## 1. Cells

MARC-145 monkey kidney cells (Kim et al., 1993) were used to propagate and titrate PRRSV and measure serum viral neutralizing activity. Cells were cultured at 37° C with 5% $CO_2$ in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 Units/mL of Penicillin, and 100 µg/mL of Streptomycin (Life Technologies, Carlsbad, CA).

PBMC were cultured at 37° C with 5% $CO_2$ in complete RPMI (cRPMI) media consisting of RPMI-1640 (GE Healthcare, Little Chalfont, United Kingdom) supplemented with 10% FBS, 1X of GlutaMAX-I (Life Technologies), 100 Units/mL of Penicillin, and 100 µg/mL of Streptomycin.

## 2. Antibodies and reagents

The PRRSV-specific monoclonal antibody was purchased from the National Veterinary Services Laboratory (Ames, IA), the Alexa Fluor 488-conjugated goat anti-mouse antibody was purchased from Invitrogen (Eugene, OR), and the mouse anti-pig IgG antibody was purchased from BD Biosciences (San Jose, CA).

The pig IFN-γ enzyme-linked immunospot (ELISpot) assay capture (clone P2G10) and detection (clone P2C11) antibodies were purchased from BD Pharmingen (San Diego, CA), the streptavidin-conjugated alkaline phosphatase, Streptavidin-AP, was purchased

from SouthernBiotech (Birmingham, AL), and the substrate, Vector Blue Alkaline Phosphatase Substrate, was purchased from Vector Laboratories (Burlingame, CA).

**3. Viruses and peptides**

The PRRSV strain FL12 was derived from isolate NVLS 97-7895 and recovered from a full-length infectious cDNA clone (Truong et al., 2004). The PRRSV-1 strain SD01-08 was recovered from a cDNA clone kindly provided by Y. Fang, Kansas State University (Fang et al., 2006). Isolates 1692-98, 21599-00, 46517-00, 16244B (Allende et al., 1999), 3805-00, 43807-00, 18565-01,18066-04 were obtained from the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) and the Nebraska Veterinary Diagnostic Center at the University of Nebraska–Lincoln (Lincoln, NE). The isolate MN184C was kindly provided by K.S. Faaberg, National Animal Disease Center (Wang et al., 2008). All viruses had been adapted to grow in MARC-145 cells.

PRRSV full-genome coding sequences for strains FL12 and SD01-08 and isolates 16244B and MN184C were obtained from GenBank. Full-genome coding sequences for isolates 1692-98, 21599-00, 46517-00, 3805-00, 43807-00, 18565-01, and 18066-04 were kindly provided by W. Laegreid, University of Wyoming and submitted to GenBank. Strains or isolates with their respective GenBank accession numbers are presented in Table 2.1.

Synthetic 20-mer peptides with overlapping 10-mer encompassing the entire sequence of the FL12 structural proteins GP2-5, M, and N were kindly provided by Dr. B. Kaltenboeck, Auburn University.

**4. Virus propagation and titration**

Confluent two-day old MARC-145 cells in 100 mm tissue culture dishes were infected with PRRSV at a multiplicity of infection of 0.1 in 2 mL inoculums and incubated for one hour with intermittent rocking every 10 minutes. Subsequently, 10 mL of low glucose DMEM supplemented with 5% FBS, 100 Units/mL of Penicillin, and 100 µg/mL of Streptomycin were added. All strains and isolates were grown until approximately 80% of the cells exhibited significant cytopathic effect (2–4 days), released from the cells by one freeze-thaw cycle at -80° C, clarified by centrifugation at 3,000 *x g* for 5 minutes, and frozen at -80° C in 1 mL aliquots.

Virus titers were determined by endpoint dilution assay in MARC-145 cells. Virus stocks were diluted 10-fold serially in low glucose DMEM. Each virus dilution was separately inoculated into eight wells of a 96-well plate containing confluent two-day old MARC-145 cells at 100 µL per well and incubated for three to four days. IFA was performed on the cell monolayers. Virus titers were calculated following Reed and Muench's method (Reed and Muench, 1938) and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per mL.

**5. Indirect fluorescence assay**

For IFA, MARC-145 monolayers were washed once with phosphate buffered saline (PBS) and fixed in a cold mixture of acetone/methanol (1:1 v/v) for 10 minutes at room temperature and air dried. Next, the cell monolayers were washed once with PBS and incubated with 30 µL of a 1:500 working dilution of the PRRSV-specific monoclonal antibody in PBS for one hour at 37° C. Cell monolayers were washed three times with PBS,

followed by incubation with 30 µL of a 1:1000 working dilution of Alexa Fluor 488-conjugated goat anti-mouse antibody for one hour at 37° C. After three washes with PBS, the cell monolayers were examined under an inverted fluorescence microscope for specific cytoplasmic fluorescence.

**6. Animal experiments**

All animals were housed and handled following protocols approved by the Institutional Animal Care Committee of the University of Nebraska–Lincoln. Pigs were obtained from a swine herd with certified records of absence of PRRSV infection and were kept in an isolated biosafety level 2 facility. One week acclimation was allowed between the arrival of the pigs to the facility, at three weeks of age, and the initial inoculation. All inoculations with PRRSV were performed intramuscularly with $10^{5.0}$ $TCID_{50}$ of virus in 2 mL of FBS-free low glucose DMEM. Blood samples were collected from all animals before infection, and periodically after infection. Serum samples were stored in 1-mL aliquots at -80° C.

**7. Isolation of peripheral blood mononuclear cells**

Whole blood was collected into a BD Vacutainer tube with sodium citrate (Pharmingen, San Diego, CA). 15 mL of whole blood were thoroughly mixed with 15 mL of PBS+2% FBS and subsequently layered on top of 15 mL of Lymphoprep separation media (StemCell Technologies, Vancouver, Canada) in a 50-mL SeppMate Tube (StemCell Technologies) and centrifuged at 2,500 *x g* at room temperature for 20 minutes. The PBMC-containing interphase was then transferred into a 50-mL polypropylene

centrifuge tube and washed once with PBS+2% FBS, after which contaminating red blood cells (RBCs) were lysed with 5 mL of RBC lysis buffer. Isotonicity was restored with 15 mL of PBS+2% FBS. Next, PBMC were centrifuged at 250 *x g* for 8 minutes and resuspended in 10 mL of RPMI-1640 media. A 10 µL aliquot was mixed with 10 µL of Cellometer AOPI Staining Solution in PBS (Nexcelom Biosciences, Laurence, MA), and viable cells counted on a disposable hemocytometer with an inverted fluorescent microscope. Finally, PBMC were centrifuged at 250 *x g* for 8 minutes and adjusted to 1-1.5x$10^7$ viable cells/mL in RPMI 1640+40% FBS+10% DMSO and frozen in 1-mL aliquots at -80° C.

**8. Enzyme-linked immunospot assay**

The ELISpot assay was performed as previously described (Meier et al., 2003). PVDF membrane 96-well plates were activated with 35% ethanol for 30 seconds, washed twice with distilled water and once with PBS. Subsequently, wells were coated overnight with 0.5 µg of anti-pig IFN-γ antibody in 50 µL of PBS. Plates were then washed three times with PBS and blocked for 2 hours with cRPMI media.

PBMC were rapidly thawed at 37° C and resuspended in 10 mL of warm RPMI-1640 media. Viable PBMC were counted using the Cellometer AOPI Staining Solution in PBS, re-suspended to a concentration of 5x$10^6$ cells/mL in cRPMI media, and plated at 5x$10^5$ cells/well. For whole virus re-stimulation, PRRSV was diluted to 5,000,000 $TCID_{50}$/mL in cRPMI media and 100 µL added to each well (final concentration 500,000 $TCID_{50}$ per well). For peptide re-stimulation, peptide pools were diluted 1:100 in cRPMI and 100 µL added to each well (final concentration 0.04 nmol per well). Two replicates of

each sample were plated for each strain/isolate or peptide, and each animal. Mock-infected MARC-145 supernatant and DMSO diluted 1:100 in cRPMI were used as negative control for PRRSV and peptide re-stimulation, respectively, and PMA (10 ng/mL) and ionomycin (1 μg/mL) as positive control. Cells were incubated for 17–20 hours.

Plates were washed with PBS+0.05% Tween-20 (PBS-T20) for 10 minutes, and further washed three times with PBS-T20. Next, 50 μL of a streptavidin-conjugated anti-pig IFN-γ antibody was added at a concentration of 2 μg/mL for one hour at room temperature. Plates were washed six times with PBS-T20 and 50 μL of a working dilution of 1 μL/mL of Streptavidin-AP was added for 45 minutes at room temperature. Plates were washed six times with PBS-T20 and multiple times under running distilled water after which they were developed with a solution of Vector Blue Alkaline Phosphatase Substrate prepared according to manufacturer instructions for 7–10 minutes. Spots were counted and analyzed using a CTL ImmunoSpot counter (Cellular Technology Limited, Shaker Heights, OH).

**9. Enzyme-linked immunosorbent assay**

PRRSV antibodies in serum were determined at the Nebraska Veterinary Diagnostic Center using the commercial ELISA IDEXX PRRS X3 Ab Test (IDEXX Laboratories, Westbrook, ME) according to the manufacturer's instructions. The status of each sample was evaluated by the sample-to-positive (S/P) ratio. An S/P ratio ≥ 0.4 was considered positive.

**10. Serum IFA**

MARC-145 cells were seeded into 96-well plates, incubated for 48 hours, after which half of the rows on the plate were infected with 100 $TCID_{50}$/well of PRRSV and half were left uninfected. After 48 hours, plates were washed once with PBS and fixed with acetone/methanol (1:1 v/v) for 10 minutes, air dried, and stored at -20° C.

Test sera was diluted to 1:20 in PBS and 30 µL of this dilution was transferred to a PRRSV-infected well and an uninfected well. Plates were incubated for one hour at 37° C and washed three times with PBS. Next, 30 µL of a 1:500 working dilution of mouse anti-pig IgG antibody was added and incubated at 37° C for one hour. Plates were washed three times with PBS and 30 µL of a 1:1000 working dilution of Alexa Fluor 488-conjugated goat anti-mouse antibody was added and incubated for one hour at 37° C. Finally, plates were washed three times with PBS and examined on an inverted fluorescence microscope for specific cytoplasmic fluorescence. A titer ≥ 1:20 was considered to be positive (Nelson et al., 1994).

**11. Serum virus neutralization assay**

Neutralizing antibody titers of serum samples against specific strains or isolates of PRRSV were determined using a fluorescent focus SVN assay as previously described (Wu et al., 2001). Heat-inactivated sera were diluted two-fold serially in 50 µL of low glucose DMEM supplemented with 5% FBS on a 96-well plate and incubated with an equal volume of media containing 100 $TCID_{50}$ of PRRSV for one hour at 37° C. The contents of each plate were transferred to a new 96-well plate containing two-day old confluent MARC-145 cells and further incubated for three to four days. The presence of PRRSV was determined

by IFA. The end point titer was expressed as the reciprocal of the highest serum dilution that neutralized PRRSV in 2 replicate wells. Absence of neutralizing antibody titer was considered as 0 for statistical analysis.

**12. RNA extraction**

RNA from serum was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

For isolation of RNA from tissue samples, 300 mg of lymph node, tonsil, or lung tissue were homogenized in 3 mL of TRIzol reagent (Life Technologies) in a Bullet Blender 5 Storm (Next Advance, Averill Park, NY) for five minutes at 4° C. Subsequently, 500 µL of the homogenized sample were mixed with 500 µL of TRIzol reagent and incubated for 10 minutes at 25° C. Next, 200 µL of chloroform were added and the sample shaken vigorously for 15 seconds, after which it was incubated for 2-3 minutes at 25° C. The sample was centrifuged at 12,000 *x g* for 15 minutes at 4° C. Following, 500 µL of the aqueous phase were transferred to a new microcentrifuge tube, 500 µL of isopropyl alcohol were added and incubated for 10 minutes, and centrifuged at 12,000 *x g* for 10 minutes at 4° C. The supernatant was discarded and the RNA pellet was washed once with 70% ethanol, centrifuged at 7,500 *x g* for 5 minutes at 4° C and left to air dry. Finally, the sample was resuspended in 100 µL of distilled water and incubated at 55–60° C for 10–15 minutes in a heated block. RNA was quantified using a NanoDrop ND-1000 (Thermo Fischer Scientific, Waltham, MA) and adjusted to 200 ng/µL.

**13. Real-time RT-PCR**

PRRSV viral RNA in plasma and tissue samples was quantified by real-time RT-PCR as previously described (Beura et al., 2012). The specific primers and probes are presented in Table 2.3 and were synthesized by Sigma-Aldrich (Woodland, TX). Real-time RT-PCRs were performed in 25 µL reaction mixtures containing 5.6 µL of distilled water, 12.5 µL of RT-PCR master mix (Affymetrix, Santa Clara, CA), 1 µL of each primer (final concentration = 400 nM), 0.5 µL of probe (final concentration = 250 nM), 0.5 µL of M-MLV RT (Affymetrix), 0.2 µL of RNAse inhibitor (Affimetryx), and 5 µL of template. The thermal conditions were as follows: 1 cycle at 50° C for 30 minutes, 1 cycle at 95° C for 2 minutes, and 45 cycles at 95° C for 15 seconds and 60° C for 60 seconds. Sets of viral RNA templates with known copy numbers were used to establish the standard curves from which the RNA copy numbers in the test samples were calculated. Results were reported as $\log_{10}$ copies per mL for serum or $\log_{10}$ copies per µg of total RNA for tissue samples. For statistical purposes, samples that had undetectable viral RNA levels were assigned a value of 0 $\log_{10}$ RNA copies.

**14. Lung pathology**

Microscopic lung lesions were evaluated blindly by a pathologist. Briefly, sections were taken from the right apical, cardiac, and dorsal lung lobes for histopathologic examination. Tissues were fixed in 10% neutral buffered formalin for 1–7 days and routinely processed and embedded in paraffin in an automated tissue processor. Sections were cut and stained with hematoxylin and eosin. Lung sections were blindly examined and given an estimated score of the severity of the interstitial pneumonia: 0 = no

microscopic lesions, 1 = mild perivascular interstitial pneumonia, 2 = mild perivascular to diffuse interstitial pneumonia, 3 = moderate diffuse interstitial pneumonia, 4 = severe diffuse interstitial pneumonia, 5 = severe diffuse interstitial pneumonia with collapse and filling of the alveoli.

**15. Statistical analysis**

Differences between the means of two data sets was determined by Student's t-test. One-way analysis of variance (ANOVA) was used to evaluate differences between the means of three or more data sets. Two-way ANOVA was used compare the mean differences between data sets encompassing two independent variables. Tukey's multiple comparison test was employed to evaluate which means amongst a set of means statistically differ from the rest.

Area under the curve (AUC) was approximated using the trapezoidal rule. The relationship between two variables was calculated using linear regression analysis. The coefficient of determination ($R^2$) was used to determine goodness of fit.

Statistical analyses were performed and graphs were generated using GraphPad Prism version 7 for Mac (GraphPad Software, La Jolla, CA).

**Table 2.1. PRRSV strains and isolates with GenBank accession number.**

| Strain/Isolate | GenBank Accession No. |
|---|---|
| FL12 | AY545985 |
| 1692-98 | KY348847 |
| 21599-00 | KY348850 |
| 46517-00 | KY348852 |
| 16244B | AF046869 |
| 3805-00 | KY348853 |
| 43507-00 | KY348851 |
| 18565-01 | KY348849 |
| MN184C | EF488739 |
| 18066-04 | KY348848 |
| SD01-08 | DQ489311 |

**Table 2.2. Primers for real-time RT-PCR.**

| Target | Primer/Probe | Sequence (5’ → 3’) |
|---|---|---|
| PRRSV-2 3’UTR | 3UTR44F | ATGTGTGGTGAATGGCACTG |
| | 3UTR141R21 | GCATGGTTCTCGCCAATTAAA |
| | 3UTR84P | TCACCTATTCAATTAGGGCGACCG |
| Isolate 16244B | 16244B15262F | GGCTGGCATTCTTGAGGCAT |
| | 16244B15369R | CACGGTCGCCCTAATTGAATA |
| | 16244B15323P | CAGTGCCATTCACCACACATTCTTCC |

# CHAPTER III

# CROSS-REACTIVITY OF IMMUNE RESPONSES AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

The studies in this chapter were published in Vaccine, 2017, Vol. 35, Issue 5, Page 782–788.

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Key words: Cell-mediated immunity; Heterologous immunity; Interferon-γ; Porcine reproductive and respiratory syndrome virus (PRRSV); T cell cross-reactivity.

## 1. Abstract

Because PRRSV exhibits extensive genetic variation among field isolates, characterizing the extent of cross-reactivity of immune responses, and most importantly cell-mediated immunity (CMI), could help in the development of broadly cross-protective vaccines. We infected 12 PRRSV-naïve animals with PRRSV strain FL12 and determined the number of IFN-γ SC by enzyme-linked immunospot (ELISpot) assay using ten PRRSV-2 and one PRRSV-1 isolates as recall antigens. The number of IFN-γ SC was extremely variable among animals, and with exceptions, late to appear. Cross-reactivity of IFN-γ SC among PRRSV-2 isolates was broad, and we found no evidence of an association between increased genetic distance between isolates and the intensity of the CMI response. Comparable to IFN-γ SC, total antibodies evaluated by IFA were cross-reactive; however, neutralizing antibody titers could only be detected against the strain used for infection. Finally, we observed a moderate association between homologous IFN-γ SC and neutralizing antibodies.

## 2. Introduction

PRRSV is the etiological agent of the most important infectious disease of swine worldwide, causing late-term reproductive failure in sows, sperm abnormalities in boars, and respiratory illness in young pigs (Collins et al., 1992; Wensvoort et al., 1991). Widespread in most swine producing countries, PRRSV causes substantial financial losses to swine producers. In the United States alone, PRRSV-associated losses were estimated to be at least $664 million (Holtkamp et al., 2013).

PRRSV is an enveloped, positive-sense, single-stranded RNA virus classified within the genus *Porartevirus*, family *Arteriviridae*, order *Nidovirales* (Adams et al., 2016; Adams et al., 2017). PRRSV encompasses two species, PRRSV-1, former European or type 1, and PRRSV-2, former North American or type 2, that share ~65% genomic sequence identity (Allende et al., 1999; Nelsen et al., 1999).

The PRRSV genome, of approximately 15 kb, encodes 11 ORFs. ORF1a and ORF1b encode two polyproteins that, when cleaved, yield 14 nsps (Snijder et al., 2013). Of these, nsp1α, nsp1β, nsp2, nsp4, and nsp11 have been involved in the modulation of the innate immune response and suppression of type I IFN signaling (Beura et al., 2010). The structural ORFs ORF2a, ORF3, ORF4 encode the minor surface glycoproteins GP2, GP3, GP4, respectively, that form a heterotrimer and interact with the cell surface receptor CD163 (Calvert et al., 2007; Das et al., 2010). Encoded by ORF5, the major glycoprotein GP5 forms a heterodimer with the M protein encoded in ORF6 (Mardassi et al., 1996).

Although PRSSV viremia can last up to one month PI or more, and persistent continuous low levels of viral replication in lymphoid tissues can be detected up to 150 DPI or more, the virus is eventually cleared by the host (Allende et al., 2000). This reveals

that later in the course of infection the pig immune response is competent in removing the virus from the animal, demonstrating that an appropriate adaptive immune response has been mounted (Loving et al., 2015). Notwithstanding, the components of the adaptive immune response that are responsible for such clearance being partially or ill-defined. The humoral immune response against PRRSV can be detected as early as 7 DPI, when abundant non-neutralizing antibodies appear. These antibodies are cross-reactive against heterologous PRRSV isolates (Yoon et al., 1994). Serum neutralizing antibodies only appear on or after 28 DPI, and have been shown to provide full protection against homologous challenge when such antibodies attained appropriate concentrations (i.e. titer) in the circulation; however, titers of cross-neutralizing antibodies are meager and frequently rare (Lopez et al., 2007; Meier et al., 2003; Osorio et al., 2002; Vu et al., 2011; Yoon et al., 1994). On the other hand, the PRRSV-specific T cell response has been shown to be variable over time and among individual pigs, appearing as early as two weeks' post infection, but showing a fairly low initial onset (Meier et al., 2003; Xiao et al., 2004). Previous studies have evaluated the importance of the IFN-γ SC response and its correlation to protective immunity, and several authors have concluded that an adequate correlation exists between the IFN-γ SC response and protective immunity (Charerntantanakul et al., 2006; Lowe et al., 2005; Meier et al., 2004; Zuckermann et al., 2007). With exceptions, only homologous strains have been used to evaluate CMI responses, and a comprehensive evaluation of the cross-reactivity of T cell responses against PRRSV is still lacking.

PRRSV genetic heterogeneity has been thoroughly documented, and it has been hypothesized that antigenic relatedness of the strains or isolates used for immunization and

challenge plays a major role in determining their immunogenic effectiveness, thus constituting a central factor towards the development of more broadly protective vaccines (Meng, 2000; Murtaugh et al., 2010). Because a combination of neutralizing antibody and T cell responses seems to be responsible for protective immunity against PRRSV, we were interested in evaluating how genetic diversity, and hence antigenic diversity, played into the cross-reactivity of cell-mediated and humoral immune responses.

To that effect, we synchronously infected a group of 12 animals with our reference pathogenic strain FL12 and assessed the kinetics of the PRRSV immune response throughout an appropriate period of convalescence (Fig. 3.1). In parallel, 12 animals were left uninfected. We measured total and neutralizing antibodies as well as IFN-γ SC responses sequentially assessed in all cases against the homologous infecting strain as well as against an array of increasingly divergent (thus, increasingly heterologous) PRRSV isolates (Table 3.1). The study allowed us to evaluate and compare the homologous (i.e. against infecting strain) response in each of the 12 animals and characterize the cross-reactivity towards heterologous divergent isolates, thus providing a description of how these parameters may associate.

## 3. Results

### *3.1. Variability of T cell responses*

FL12-specific IFN-γ SC were detected as early as 14 DPI in some animals, with most animals showing a highly variable number of IFN-γ SC at 28 and 42 DPI, after which variability could still be observed, but at a reduced rate. We observed fundamentally distinct IFN-γ SC kinetics among the FL12-infected animals (Fig. 3.2A).

Two animals (301 and 314) showed an increase of PRRSV-specific IFN-γ SC peaking at 42 DPI in very high numbers, after which the numbers slowly declined. While the number of IFN-γ SC of animal 314 was low throughout the first 28 DPI, animal 301 showed an earlier cell-mediated response, starting at 14 DPI. Another group of animals (299, 304, 323, and 358) showed a weaker biphasic response, with a first peak occurring at 28 DPI and a second peak at 63 DPI. A third subset of animals (317, 321, 325, and 330) demonstrated an even weaker response, in most cases only present in very small numbers at 28 and 42 DPI, but with a steady climb, peaking at 77 DPI. The CMI kinetics of two animals (333 and 315) did not fit any of the aforementioned patterns, animal 315 showed a steady climb of IFN-γ SC until 63 DPI that diminished by 77 DPI, while animal 333 experienced two peaks at 42 and 77 DPI. With the exception of animal 301, and regardless of the pattern observed, all animals at 77 DPI appeared to reach a similar number of IFN-γ SC.

To further evaluate the overall variability over the entire duration of the experiment, we calculated the AUC for the number of IFN-γ SC from 0 to 77 DPI (Fig. 3.2B). The two animals with the fastest and strongest responses (301 and 314) had, in turn, the highest calculated AUC, while the four animals with the slowest and weakest kinetics had the smallest calculated AUC. The remaining animals, including those representative of the biphasic response, had a calculated AUC in between the aforesaid animals.

Similar variability of T cell responses could also be observed when we evaluated the IFN-γ SC response using nine other PRRSV-2 isolates as recall antigen, but not with the PRRSV-1 strain SD01-08 (Fig. 3.3). We did not detect any IFN-γ SC in uninfected animals throughout the course of our study.

### *3.2. Nucleotide pairwise distance between FL12 and heterologous strains and isolates*

Coding genome-wide sequences of FL12, nine heterologous PRRSV-2 isolates, and the PRRSV-1 strain SD01-08 were aligned using MUSCLE (Edgar, 2004). Calculated nucleotide pairwise distances are presented in Table 3.1. Using MEGA7, a phylogenetic tree was inferred using the maximum likelihood method and is presented in Figure 3.4 (Kumar et al., 2016; Tamura and Nei, 1993).

Three PRRSV-2 isolates exhibited a low calculated nucleotide pairwise distance to FL12: 1692-98 and 21599-00 at 5.02%, and 46517-00 at 6.36%. Three PRRSV-2 isolates had a moderate calculated nucleotide pairwise distance to FL12: 16244B at 9.66%, 3805-00 at 9.71%, and 43807-00 at 10.11%. Another three PRRSV-2 isolates had a high calculated nucleotide pairwise distance to FL12: 18565-01 at 12.65%, MN184C at 14.32%, and 18066-04 at 14.48%. Finally, the PRRSV-1 isolate SD01-08 had a genome-wide calculated nucleotide pairwise distance of 36.72% to FL12.

### *3.3. Cross-reactivity of T cell responses*

To evaluate the cross-reactivity of T cell responses against PRRSV we used the homologous strain FL12, nine other PRRSV-2 isolates of varying genetic distance, and one PRRSV-1 isolate as recall antigen. A two-way ANOVA was performed to determine the effect of PRRSV isolate used as recall antigen on the number of IFN-γ SC (Fig. 3.5A). Although there was a statistically significant interaction between the effects of PRRSV isolate and the number of IFN-γ SC, simple main effects analysis showed that PRRSV-2 isolates induced significantly stronger T cell responses than the PRRSV-1 strain SD01-08, but no significant differences were detected among PRRSV-2 isolates.

Further analyses demonstrated no significant differences between SD01-08 and all PRRSV-2 isolates at 14 and 28 DPI (with the exception of MN184C at 28 DPI) and, starting at 28 DPI, and through every other time point, all PRRSV-2 isolates produced a significantly higher number of IFN-γ SC than SD01-08. We were able to observe that FL12-, MN184-, and 16244B-re-stimulated PBMC showed a number of IFN-γ SC significantly higher than other PRRSV-2 isolates at 42 and 63 DPI. Finally, FL12- and MN184C-re-stimulated PBMC had higher numbers of IFN-γ SC than the isolates 3805-00 and 18565-01 at 77 DPI.

To additionally evaluate T cell cross-reactivity, we calculated the mean and standard error of the AUC for each isolate used as recall antigen and performed a one-way ANOVA (Fig. 3.5B). There was a significant difference between PRRSV isolates used as recall antigens; however, there was no significant difference among PRRSV-2 isolates. PRRSV-2 isolates recalled a higher number of IFN-γ SC than the PRRSV-1 strain SD01-08. Extensive cross-reactivity was not only observed when we evaluated as a group the mean number of IFN-γ SC against each of the ten PRRSV-2 isolates (including FL12), but also while examining the T cell responses of each individual animal (Fig. 3.6).

### *3.4. Isolate genetic distance and T cell responses*

To study the relationship between isolate genetic distance and T cell responses we determined the mean IFN-γ SC number for each PRRSV-2 isolate and fit a linear regression model against the previously calculated nucleotide pairwise distance of said isolate to FL12 (Fig. 3.7A–E). Our results indicate that changes in genetic distance are not associated with changes in the mean number of isolate-specific IFN-γ SC at 14, 28, 42, 63 and 77 DPI.

To further evaluate this relationship over the entire course of the assay we determined the mean AUC of IFN-γ SC for each PRRSV-2 isolate and fitted a linear regression model with the calculated nucleotide pairwise distance of said isolate to FL12 (Fig. 3.7F). No significant association could be found between the mean AUC of IFN-γ SC and calculated nucleotide pairwise distance of the isolate.

### *3.5. Total and neutralizing antibody responses*

Five of the ten PRRSV-2 isolates were selected to evaluate the total and neutralizing antibody response. Together with the homologous strain FL12, we included low distance isolates 1692-98 and 21599-00, medium distance isolate 16244B and high distance isolate 18565-01. The total antibody responses, evaluated by IFA, was deemed negative at 0 DPI for all animals (< 1:20) and all isolates tested. Starting at 28 DPI, and at 63 and 77 DPI all animals were positive by IFA (≥ 1:20) against all five isolates (Fig. 3.8A).

Neutralizing antibodies against isolate FL12 were determined by SVN and were absent at 0 and 28 DPI. Homologous titers could be detected starting at 63 DPI and continued to rise until 77 DPI (Fig. 3.8B). Subsequently, we sought to determine whether neutralizing antibody titers could be obtained against the four heterologous isolates. We tested the sera obtained at 77 DPI and found that, with very few exceptions, no neutralizing antibody titers could be detected against 1692-98, 21599-00, 16244B and 18565-01 (Fig. 3.8C). A weak (1:4) neutralizing antibody titer was found in one pig against isolate 21599-00 and in another pig against 16244B.

### *3.6. Association between T cell and neutralizing antibody responses*

As both neutralizing antibody and IFN-γ SC seem to appear at later time points of the PRRSV infection, we were interested in determining whether there was an association between these two variables. At 63 and 77 DPI, we paired the homologous neutralizing antibody titer against FL12 for each animal with its respective number of FL12-specific IFN-γ SC and fitted a linear regression (Fig. 3.9). Our results indicate that at 63 DPI 34.86% of the variation of neutralizing antibody titers can be explained by the variation in the number of IFN-γ SC, while this value increases to 51.64% at 77 DPI. In the absence of neutralizing antibody titers, both at earlier time points and against heterologous isolates, no further calculations could be conducted.

## 4. Discussion

Since PRRSV was first reported in the late 1980's, our understanding of its pathogenesis and immunology have grown steadily; however, we are still lacking a broadly cross-protective PRRSV vaccine. Our past research has demonstrated that appropriate concentrations of neutralizing antibodies can provide sterilizing immunity against PRRSV challenge, but these neutralizing antibodies are seldom cross-reactive (Lopez et al., 2007; Osorio et al., 2002; Vu et al., 2011). The cross-protection afforded by currently available commercial vaccine strains is at least that of the field isolates from which they were derived, but it has become clear that there is a great need for improvement in the breadth of this cross-protection (Renukaradhya et al., 2015; Shi et al., 2010a). For any vaccine, sterilizing immunity is the ultimate goal, and in PRRSV this can be accomplished through neutralizing antibodies. Due to our limited knowledge on the nature and kinetics of

neutralizing antibodies, and how T cell help contributes to their appearance, we believe this work could provide new directions for the design of cross-protective PRRSV vaccines.

Because our understanding of CMI against PRRSV is scarce, we were not only interested in determining how PRRSV-specific T cells could associate with neutralizing antibodies, but also to determine how T cells cross-reacted against other field isolates, and whether the isolate heterogeneity, determined by its genome-wide calculated nucleotide pairwise distance to the challenge isolate, could be linked to varying levels of cross-reactivity. We have recently shown that minimizing the calculated nucleotide pairwise distance between immunization and challenge isolates provided an unprecedented level of cross-protection (Vu et al., 2015), hence we hypothesized that increased calculated nucleotide pairwise distance as a determinant of antigenic variability and heterogeneity of field isolates could be negatively associated to CMI.

Our study demonstrates that an outbred population of pigs infected with a PRRSV-2 isolate shows a highly variable IFN-γ SC response among individual animals. In apparent contrast to what was previously described for PRRSV-2 infection by Xiao et al. (2004), half of our 12 FL12-infected animals had high homologous T cell responses at 28 DPI (>115 IFN-γ SC/$10^6$ PBMC). Furthermore, we observed that one of our animals (301) had high homologous and heterologous T cell responses at 14 DPI. Authors have described the PRRSV-specific T cell response as weak and slow; however, our findings reveal that individual animals can potentially achieve high numbers of PRRSV-specific T cell responses as early as 14 DPI. It was previously shown that inoculation with virulent PRRSV elicits a higher number of IFN-γ SC than inoculation with a modified live vaccine both in piglets and finisher pigs (Klinge et al., 2009). When comparing our results to

virulent PRRSV inoculation, and those of commercially available MLV immunization, we further appreciate the fact that virulent PRRSV seems to elicit a stronger CMI than MLV (Meier et al., 2003; Xiao et al., 2004; Zuckermann et al., 2007). Moreover, it could be hypothesized that the extreme variability observed among CMI responses in individual animals could be responsible for the variable protection observed against PRRSV challenge. The host and pathogen factors behind this variability are yet to be understood.

In our study we evaluated CMI cross-reactivity against an array of genetically diverse PRRSV-2 isolates and one PRRSV-1 isolate. Our results indicate that T cells from FL12-infected animals can recognize other PRRSV-2 isolates and secrete IFN-γ in response to them. The overall kinetic of the T cell responses to all PRRSV-2 isolates were not significantly different between PRRSV-2 isolates; however, SD01-08, a PRRSV-1 strain, elicited a significantly lower T cell response. Such cross-reactivity is not surprising, as T cell cross-reactivity has been documented for other pathogens. McMaster et al. (2015) have demonstrated the existence of cross-reactive T cells against influenza A virus, while these cross-reactive T cells were shown to provide cross-protection against heterologous challenge, with reduced morbidity and mortality in mice, they did so in the presence of a limited neutralizing antibody cross-reactivity. Much alike what has been previously hypothesized for PRRSV by Zuckermann et al. (2007), the authors claim that protection from influenza A virus challenge was afforded by T cells. T cell cross-reactivity was also reported at the virus, protein, and peptide level between members of the *Alphaherpersvirinae* subfamily, while T cell cross-reactive peptides have been described between unrelated viruses such as human papillomavirus and coronavirus and *M. bovis* bacillus Calmette-Guérin and poxviruses (Jing et al., 2016; Mathurin et al., 2009; Nilges

et al., 2003). Because the number of potential peptide antigens surpasses the number of T cell receptors available by many orders of magnitude, it can be argued that T cells are only able to provide comprehensive immune coverage if each one of them is capable of recognizing many peptides (Sewell, 2012). However, our results are in sharp contrast to what has been previously described for PRRSV-1. When a virulent PRRSV-1 isolate was inoculated into pigs and PBMC isolated and stimulated in vitro with the homologous isolate and a heterologous isolate of calculated nucleotide pairwise distance of 12.5%, a significant reduction of IFN-γ SC was found to occur in the presence of the heterologous virus stimulation, when compared to the homologous virus. The authors argue that this could be due to the different ability of these strains to inhibit IFN-γ T cell responses, or due to different antigenicity of T cell epitopes (Diaz et al., 2012).

Our findings regarding antibody-mediated immunity against PRRSV confirm what other authors have described: the total antibody response against PRRSV is broadly reactive and early to occur; however, it is known that these antibodies do not mediate protection against infection (Lopez and Osorio, 2004). We were not able to detect any neutralizing antibodies at 28 DPI; conversely, at 63 DPI all but one animal showed neutralizing antibody titers against FL12, and at 77 DPI all animals did, and in many cases, these titers were higher. We were not able to detect the presence of neutralizing antibodies against heterologous PRRSV isolates. Our observation is different from what Martinez-Lobo et al. (2011) reported for PRRSV-1, where the authors describe the presence of cross-reactive neutralizing antibody titers. This divergent observation might be due to the nature of the antisera used in the neutralization assay, while Martinez-Lobo et al. (2011) used

hyperimmune antisera obtained from pigs that were repeatedly immunized with PRRSV, we used convalescent antisera obtained from pigs that were exposed only once to the virus.

IFN-γ SC as measured by the ELISpot assay exhibit a behavior comparable to that of the total antibody response against PRRSV. Neutralizing antibodies are a subset of the total antibody response, similarly, it is possible that a T cell subset could be primarily responsible for providing protection against PRRSV infection. Because we evaluated the PRRSV CMI using the widely accepted IFN-γ ELISpot assay, our results are not without limitations. The techniques available in the field of swine immunology are not as comprehensive or precise as those available for other species, and there is a known necessity to develop a swine T cell biology toolkit (Loving et al., 2015). Further characterization of T cell subsets and cytotoxicity will require the usage of multi-color flow cytometry, already implemented for the study of classical swine fever and influenza A virus in swine, in combination with tetramer staining, previously shown for foot and mouth disease virus (Franzoni et al., 2013a; Franzoni et al., 2013b; Gerner et al., 2015; Patch et al., 2011; Talker et al., 2015; Talker et al., 2016). Further understanding of the major histocompatibility molecules of swine, the SLA, including the distribution within an outbred population and the contribution to adaptive immune responses, will also be vital to further evaluate swine immunity against PRRSV.

Finally, we demonstrate a low to moderate association between the number of IFN-γ SC and the magnitude of the neutralizing antibody response. This association could only be evaluated for the homologous strain FL12, as other isolates failed to elicit any neutralizing antibody titers. However, our data suggests that between 30 and 50% of the variation of neutralizing antibody titers can be explained by variation in IFN-γ SC. Due to

the lack of cross-reactivity of neutralizing antibody responses, the value of this contribution cannot be stated, as even animals with homologous neutralizing antibody titers of 1:64 or 1:128 showed no ability of cross-neutralizing genetically related isolates. It is extensively documented that antigen-activated B cells establish interactions in the lymph node that allow them to receive helper signals from antigen-activated CD4+ T cells. Such interactions allow for class switch and affinity maturation (De Silva and Klein, 2015; Kurosaki et al., 2015). Notwithstanding, studies conducted in measles-vaccinated patients demonstrated the independence between humoral and cellular immune responses (Dhiman et al., 2005; Jacobson et al., 2012). As previously discussed, further analysis into the subtypes of T cells involved in PRRSV immunity could provide a stronger biological or mathematical association between the appearance and progression of neutralizing antibodies and T cells.

Overall, our results could be interpreted in two distinct ways. On the one hand, it could be argued that due to the number of IFN-γ SC being not significantly different amongst PRRSV-2 isolates, T cells play no major role in mediating cross-protection. On the other hand, it could be hypothesized that, similarly to what has been described for influenza, T cells react against a broad spectrum of PRRSV-2 PRRSV isolates, contributing to partial levels of cross-protection against heterologous isolate infection (Sridhar, 2016). We prefer the latter hypothesis. Furthermore, we favor the idea that, very much like universal influenza vaccines, broadly protective PRRSV vaccines could rely on the concept of “heterosubtypic” immunity, in which T cell-mediated immune responses targeting conserved PRRSV epitopes would confer protection against infection and disease.

**Acknowledgements**

This research was supported by the Agriculture and Food Research Initiative competitive grants 2016-67015-24922 (to H.L.X.V.) and 2013-01035 (to F.A.O.) of the USDA National Institute for Food and Agriculture.

We would like to thank Dr. William Laegreid at the University of Wyoming for sequence analysis of the PRRSV isolates and the staff of the University of Nebraska–Lincoln Life Sciences Annex and the Nebraska Veterinary Diagnostic Center for the care of animals and provision of samples.

**Table 3.1. Calculated nucleotide pairwise distance (%) between PRRSV strains and isolates.**

| | FL12 | 1692-98 | 21599-00 | 46517-00 | 16244B | 3805 | 43807 | 18565-01 | MN184C | 18066-04 | SD01-08 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FL12 | - | 5.02 | 5.02 | 6.36 | 9.66 | 9.71 | 10.11 | 12.65 | 14.32 | 14.48 | 36.72 |
| 1692-98 | 5.02 | - | 5.2 | 6.28 | 9.61 | 9.57 | 10.01 | 12.46 | 14.07 | 14.2 | 36.59 |
| 21599-00 | 5.02 | 5.2 | - | 5.42 | 10.01 | 10.05 | 10.59 | 12.61 | 14.27 | 14.47 | 36.47 |
| 46517-00 | 6.36 | 6.28 | 5.42 | - | 10.1 | 9.79 | 10.65 | 12.4 | 14.33 | 14.48 | 36.64 |
| 16244B | 9.66 | 9.61 | 10.01 | 10.1 | - | 5.75 | 3.64 | 11.96 | 13.35 | 13.49 | 36.37 |
| 3805-00 | 9.71 | 9.57 | 10.05 | 9.79 | 5.75 | - | 6.07 | 12 | 13.44 | 13.54 | 36.71 |
| 43807-00 | 10.11 | 10.01 | 10.59 | 10.65 | 3.64 | 6.07 | - | 12.28 | 13.77 | 13.9 | 36.47 |
| 18565-01 | 12.65 | 12.46 | 12.61 | 12.4 | 11.96 | 12 | 12.28 | - | 8.31 | 8.55 | 36.94 |
| MN184C | 14.32 | 14.07 | 14.27 | 14.33 | 13.35 | 13.44 | 13.77 | 8.31 | - | 0.52 | 36.92 |
| 18066-04 | 14.48 | 14.2 | 14.47 | 14.48 | 13.49 | 13.54 | 13.9 | 8.55 | 0.52 | - | 36.95 |
| SD01-08 | 36.72 | 36.59 | 36.47 | 36.64 | 36.37 | 36.71 | 36.47 | 36.94 | 36.92 | 36.95 | - |

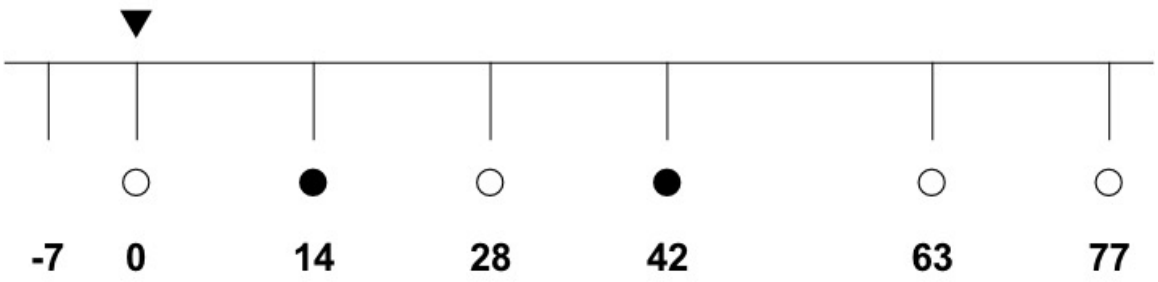

-7
0
14
28
42
63
77

Figure 3.1. Experimental design of cross-reactivity study. Chronology of animal experiment. After an acclimatization period of one week, animals were infected with PRRSV-2 strain FL12 (n=12) at 0 DPI (▼) or left uninfected. White circles (○) indicate times at which humoral and cellular immune responses were assessed. Black circles (●) indicate times at which only cellular immune responses were determined.

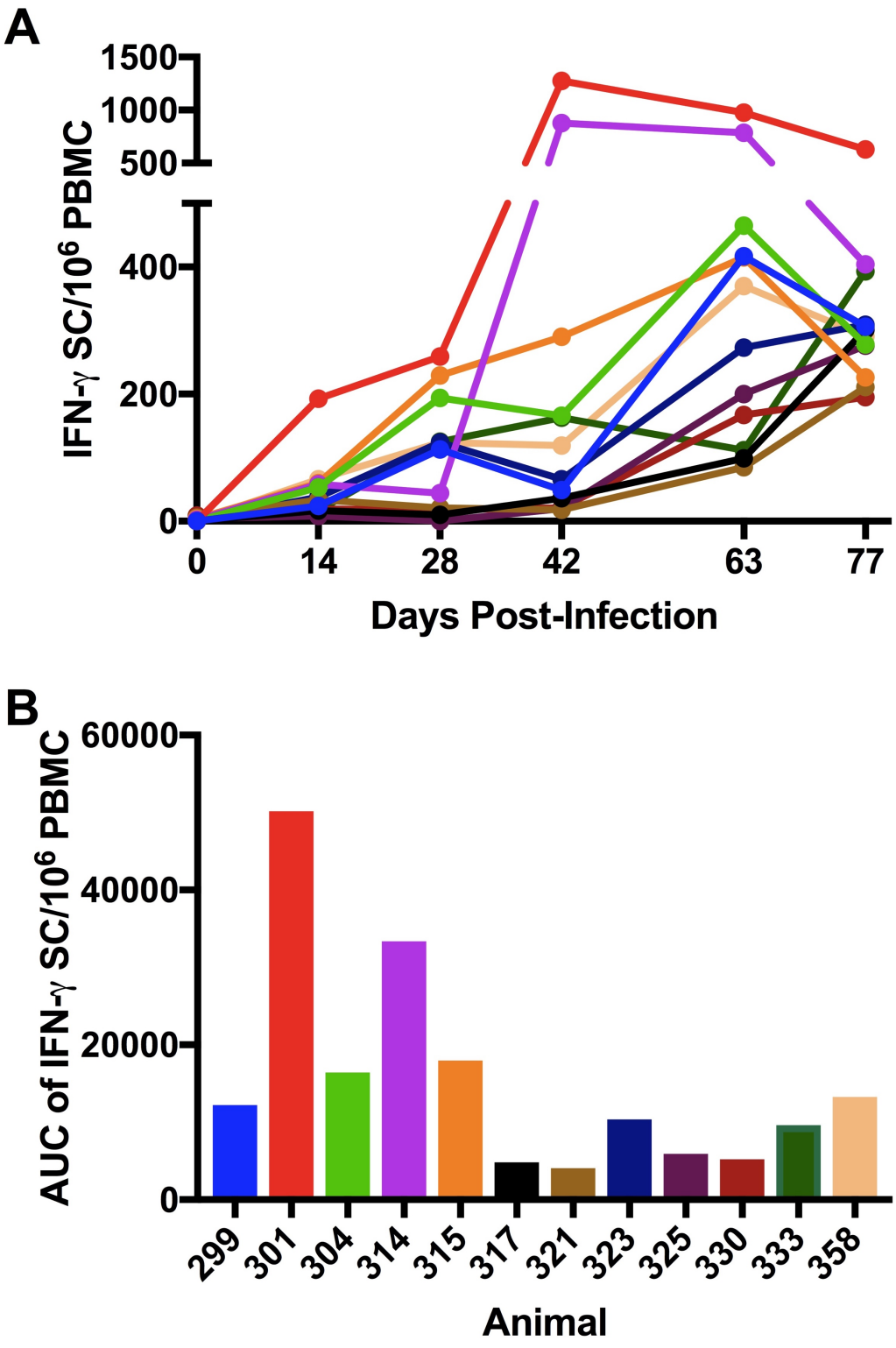

A
1500
1000
500
400
200
0
IFN-γ SC/10^6 PBMC
0
14
28
42
63
77
Days Post-Infection
B
60000
40000
20000
0
AUC of IFN-γ SC/10^6 PBMC
299
301
304
314
315
317
321
323
325
330
333
358
Animal

Figure 3.2. Kinetics of homologous IFN-γ SC in individual animals infected with PRRSV strain FL12. (A) FL12-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times. (B) AUC of IFN-γ SC were calculated using the trapezoidal rule.

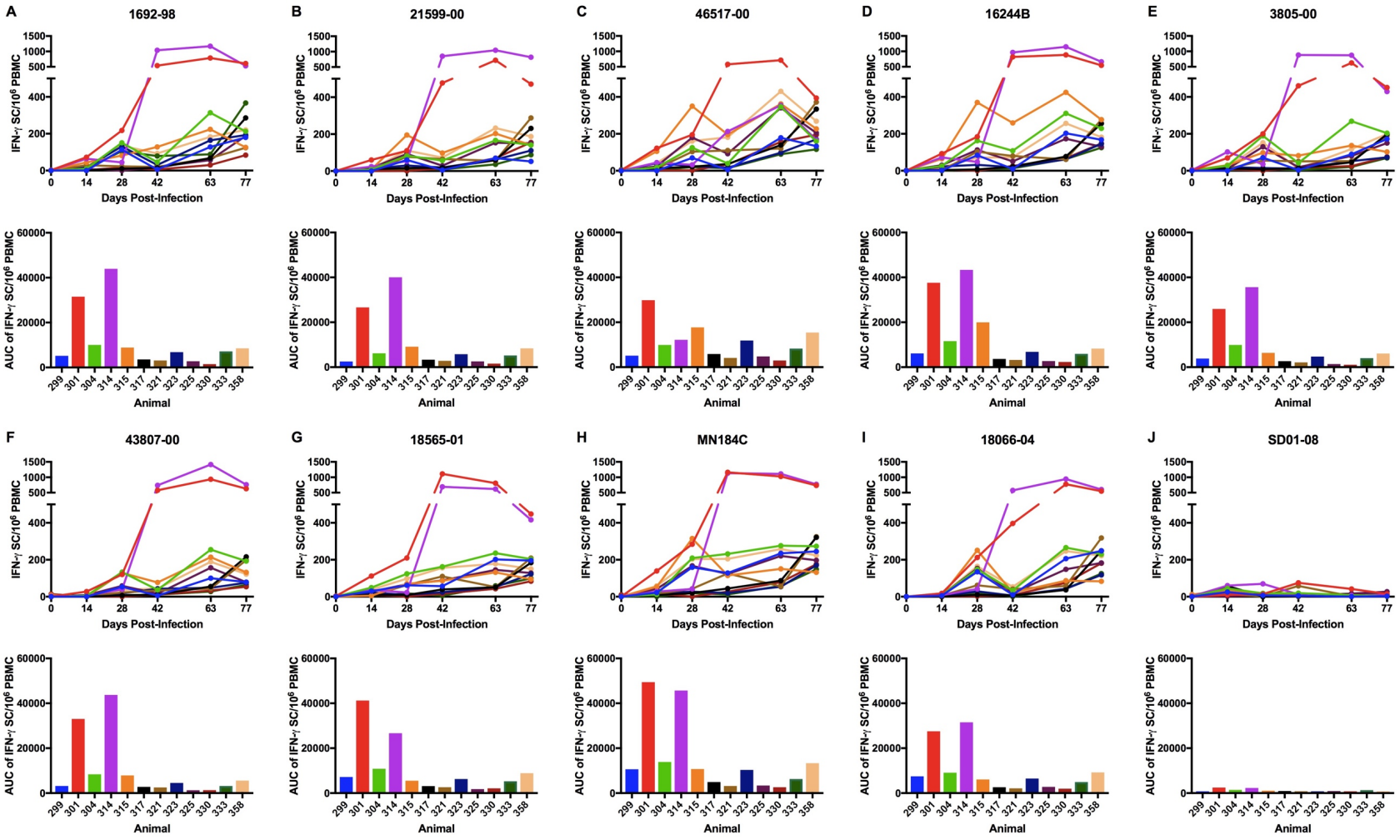

A
1692-98
B
21599-00
C
46517-00
D
16244B
E
3805-00
F
43807-00
G
18565-01
H
MN184C
I
18066-04
J
SD01-08
IFN-γ SC/10^6 PBMC
Days Post-Infection
AUC of IFN-γ SC/10^6 PBMC
Animal
0
14
28
42
63
77
299
301
304
314
315
317
321
323
325
330
333
358

Figure 3.3. Kinetics of IFN-γ SC responses against heterologous PRRSV strains and isolates in individual animals. (A–J, Top) Isolate-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times. (A–J, Bottom) AUC of IFN-γ SC were determined using the trapezoidal rule.

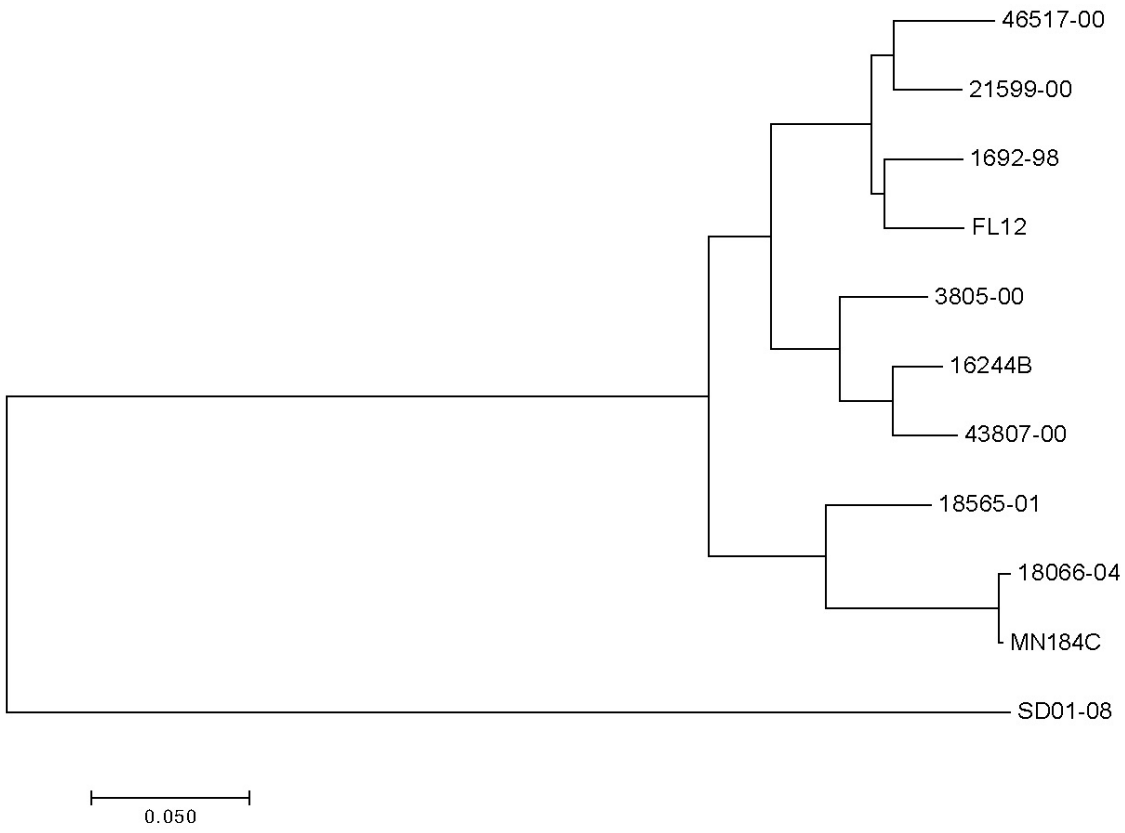

46517-00
21599-00
1692-98
FL12
3805-00
16244B
43807-00
18565-01
18066-04
MN184C
SD01-08
0.050

Figure 3.4. Phylogenetic tree of PRRSV strains and isolates. The phylogenetic tree was inferred using the maximum likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

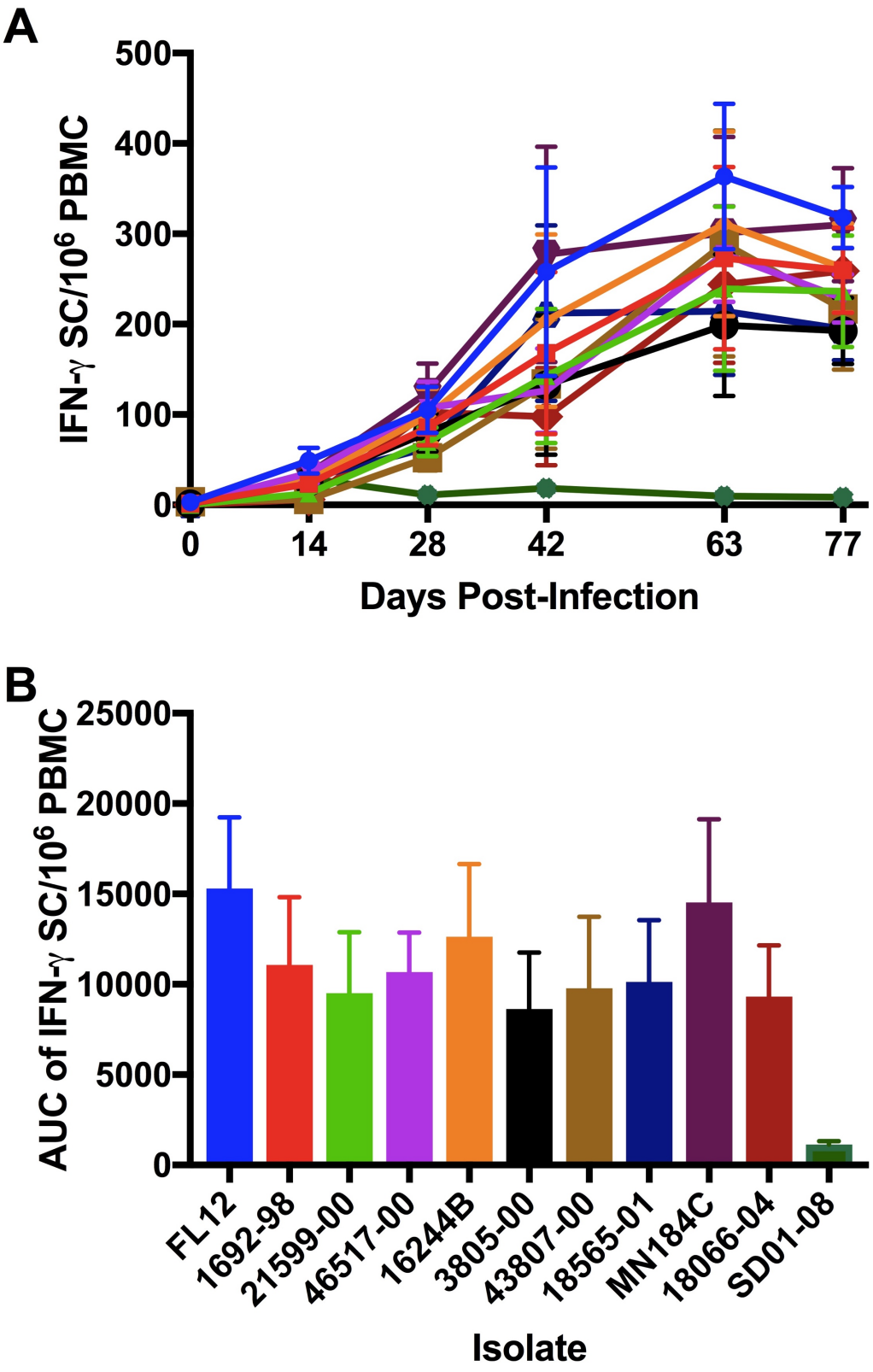

A
500
400
300
200
100
0
IFN-γ SC/10^6 PBMC
0
14
28
42
63
77
Days Post-Infection
B
25000
20000
15000
10000
5000
0
AUC of IFN-γ SC/10^6 PBMC
FL12
1692-98
21599-00
46517-00
16244B
3805-00
43807-00
18565-01
MN184C
18066-04
SD01-08
Isolate

Figure 3.5. Evaluation of IFN-γ SC cross-reactivity in the context of PRRSV infection. (A) The mean and standard error IFN-γ SC number was calculated for each PRRSV strain or isolated used as recall antigen and the kinetics analyzed by repeated measures two-way ANOVA. (B) The IFN-γ SC AUC mean and standard error for each isolate was calculated and analyzed by one-way ANOVA.

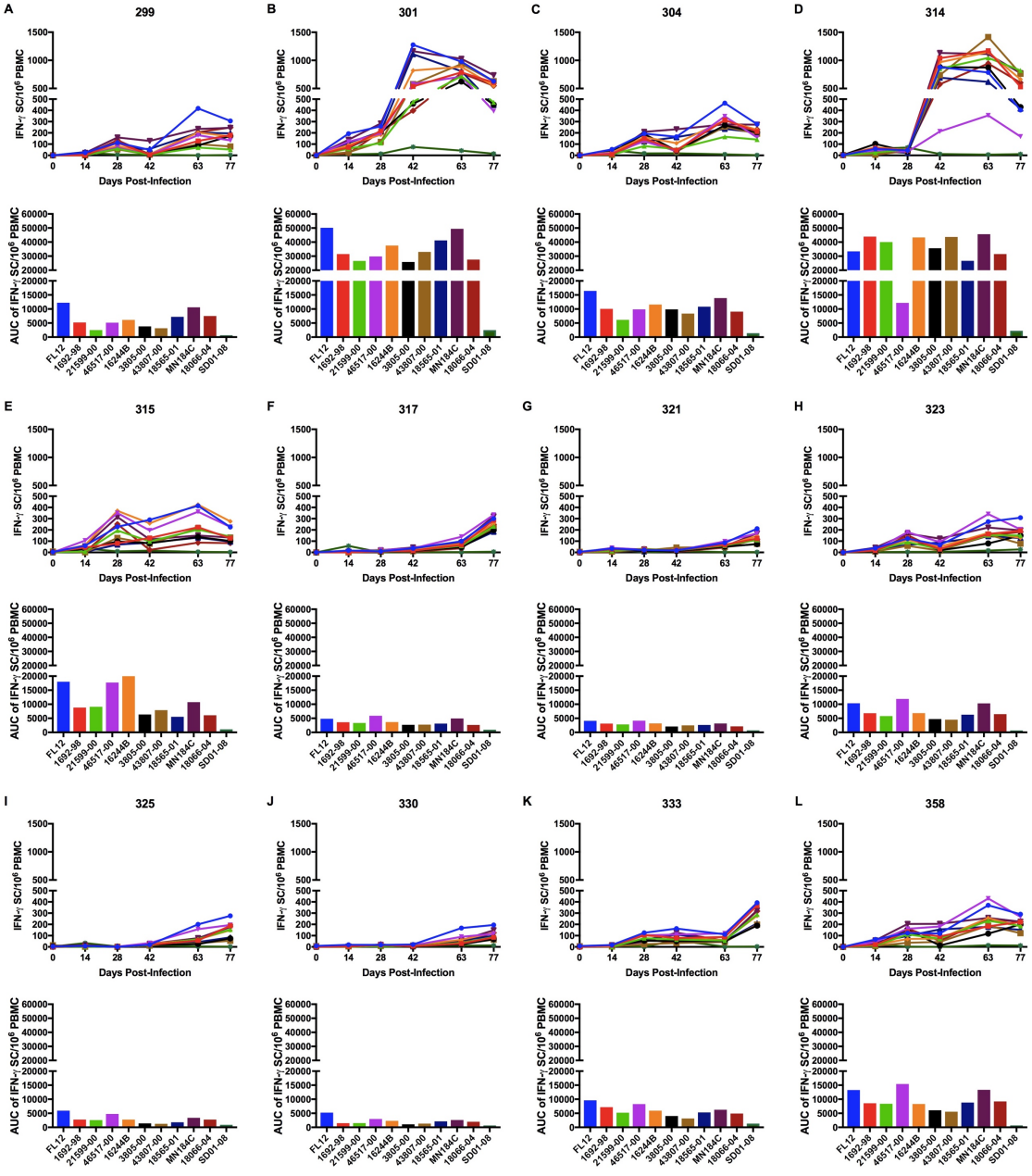

A
299
B
301
C
304
D
314
E
315
F
317
G
321
H
323
I
325
J
330
K
333
L
358
IFN-γ SC/10^6 PBMC
Days Post-Infection
AUC of IFN-γ SC/10^6 PBMC
FL12
1692-98
21599-00
46517-00
16244B
3805-00
43807-00
18565-01
MN184C
18066-04
SD01-08

Figure 3.6. Kinetics of IFN-γ SC responses against heterologous PRRSV strains and isolates in individual animals. (A–L, Top) Isolate-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times. (A–L, Bottom) AUC of IFN-γ SC were determined using the trapezoidal rule.

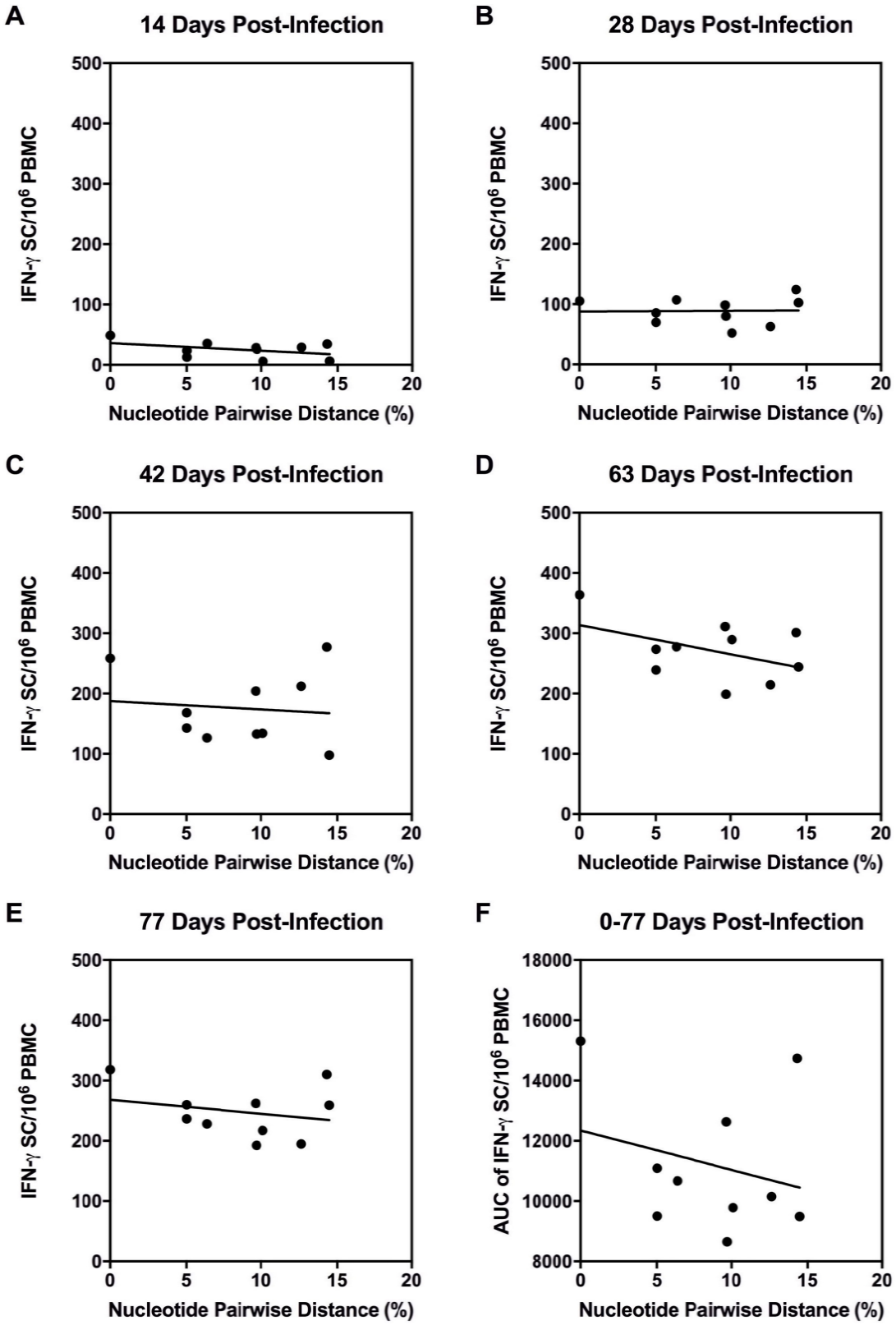

A
14 Days Post-Infection
B
28 Days Post-Infection
C
42 Days Post-Infection
D
63 Days Post-Infection
E
77 Days Post-Infection
F
0-77 Days Post-Infection
IFN-γ SC/10^6 PBMC
AUC of IFN-γ SC/10^6 PBMC
Nucleotide Pairwise Distance (%)
0
5
10
15
20
100
200
300
400
500
8000
10000
12000
14000
16000
18000

Figure 3.7. Analysis of calculated nucleotide pairwise distance to FL12 and IFN-γ SC responses. (A–E) For every time point the mean IFN-γ SC for each PRRSV-2 isolate was calculated and fitted a linear regression against the calculated nucleotide pairwise distance of said isolate to FL12. (F) A linear regression was fitted between the mean AUC for each PRRSV-2 isolate and its calculated nucleotide pairwise distance to FL12.

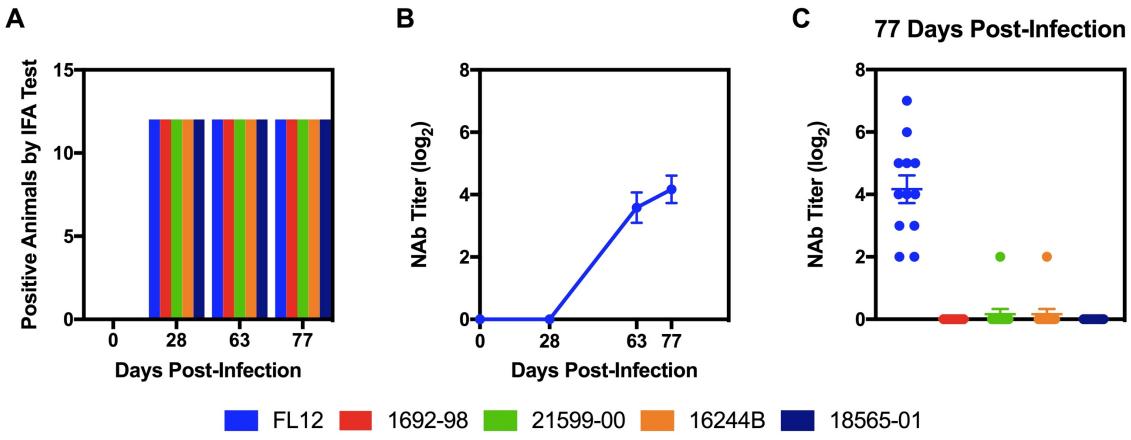

A
Positive Animals by IFA Test
15
10
5
0
0
28
63
77
Days Post-Infection
B
NAb Titer (log2)
8
6
4
2
0
0
28
63
77
Days Post-Infection
C
77 Days Post-Infection
NAb Titer (log2)
8
6
4
2
0
FL12
1692-98
21599-00
16244B
18565-01

Figure 3.8. Evaluation of humoral immune responses after infection with PRRSV. (A) Antibody responses against FL12, 1692-98, 21699-00, 16244B, and 18565-01 were evaluated by IFA test. A titer ≥ 1:20 was considered positive. (B) Neutralizing antibody titers against FL12 were determined by SVN and results are expressed as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of virus in cell culture. (C) Neutralizing antibody titers against 1692-98, 21599-00, 16244B, and 18565-01 were determined at 77 DPI.

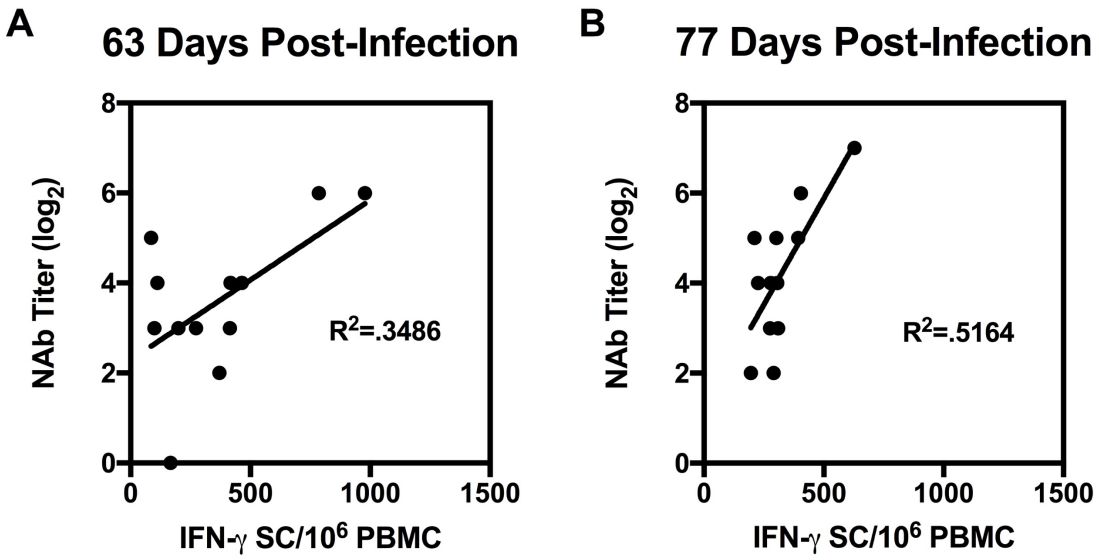

A
63 Days Post-Infection
NAb Titer (log2)
IFN-γ SC/10^6 PBMC
R2=.3486
B
77 Days Post-Infection
NAb Titer (log2)
IFN-γ SC/10^6 PBMC
R2=.5164

Figure 3.9. Correlation between neutralizing antibody titers and the number of IFN-γ SC against FL12. A linear regression was fitted between the FL12-specific IFN-γ SC for each animal, and their respective neutralizing antibody titers at 63 (A) and 77 (B) DPI. $R^2$ values are indicated.

# CHAPTER IV

# INFECTION OF NAÏVE PIGS WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AFFORDS CROSS-PROTECTION TO SUBSEQUENT CHALLENGE WITH A HETEROLOGOUS ISOLATE

## 1. Abstract

PRRSV genetic and antigenic variation are considered a major cause behind the incomplete—sometimes limited—cross-protection observed in vaccinated or infected animals to posterior challenge. To circumvent this, many swine producers implement replacement gilt acclimation protocols infecting naïve animals with live virus obtained from viremic animals in their respective farms. To ascertain the contribution of the humoral and cell-mediated immune responses to cross-protection we infected 12 naïve animals with PRRSV-2 strain FL12 and challenged them 77 days later with the heterologous isolate 16244B. A control group of 12 animals was left uninfected until 77 DPI and challenged in a similar fashion. After challenge, the IFN-γ SC response in FL12-infected animals experienced a modest cross-reactive boost. Neutralizing antibody titers against 16244B were not detected prior to challenge, but rapidly emerged by 14 days post-challenge (DPC). Cross-reactive neutralizing antibodies against other heterologous PRRSV-2 isolates were also detected. Good cross-protection to challenge was observed, viremia post-challenge (PC) was greatly reduced, and tissue viral quantification, and microscopic lung lesions were modestly, albeit significantly, reduced too. The previously uninfected control group showed a quick and cross-reactive cell-mediated response against PRRSV-2, but no neutralizing antibodies after challenge were identified in these animals.

## 2. Introduction

PRRSV, a disease characterized by reproductive failure in sows and respiratory illness in young pigs, appeared in the late 1980's and early 1990's in North America and Europe (Collins et al., 1992; Wensvoort et al., 1991). Since its emergence, it has spread to most swine-producing countries, causing significant financial losses to the swine industry. PRRSV-associated financial losses in the United States alone are estimated to be at least $664 million per year (Holtkamp et al., 2013).

Historically, PRRSV was classified into two distinct genotypes: type 1 or European and type 2 or North American; however, the new taxonomy of the family *Arteriviridae* classifies PRRSV within the genus *Porartevirus* and divides it into two different species, PRRSV-1 and PRRSV-2, encompassing the previous genotypes (Adams et al., 2016; Adams et al., 2017). Genome-wide analysis reveals that only 55-70% nucleotide and 50-80% amino acid similarity is shared between PRRSV-1 and PRRSV-2 (Forsberg, 2005).

The PRRSV virion is enveloped, pleomorphic, with an average diameter of 58 nm, and its genome consists of a single positive strand of RNA of approximately 15 kb (Spilman et al., 2009). Of the 11 PRRSV ORFs, the large replicase polyprotein ORF1a/b (and its truncated version ORF1a'), located in the 5'-proximal three-quarters of the genome, encodes four distinct pp products that are co-translationally and post-translationally processed into 16 distinct nsps by virally-encoded proteases (Kappes and Faaberg, 2015). The PRRSV structural proteins are encoded by the eight 3'-proximal ORFs contained within six sg mRNAs (Snijder et al., 2013).

Infection with PRRSV is chronic and persistent: viremia can be detected up to a month PI or more, and viral replication in lymphoid tissue can be detected up to 150 PDI

or more; nonetheless, the host eventually eliminates the virus (Allende et al., 2000; Wills et al., 1997b). Herd stabilization and closure protocols, in which PRRSV-positive herds eliminate the virus by preventing the entry of PRRSV-naïve animals for extended periods of time further illustrate the existence of an efficacious anti-PRRSV immune response (Linhares et al., 2014; Torremorell et al., 2002).

Significant effort has gone into understanding the immune mechanisms involved in clearing PRRSV from the host. Non-neutralizing antibodies against PRRSV can be detected as early as 7 DPI. While it is widely accepted that these early non-neutralizing antibodies do not mediate protection against the virus, their role in mediating ADE both *in vitro* and *in vivo* is controversial (Delputte et al., 2004; Yoon et al., 1994; Yoon et al., 1997; Yoon et al., 1996). Neutralizing antibodies, which only appear on or after 28 DPI, have been shown to deliver full homologous protection against PRRSV (Lopez et al., 2007; Meier et al., 2003; Osorio et al., 2002). Nevertheless, PRRSV continues to replicate in lymphoid tissue even in the presence of neutralizing antibodies (Lopez and Osorio, 2004). Although cross-neutralizing antibodies have seldom been detected, and in very low titers, it has been proved that hyperimmunization or multiple exposures to PRRSV can induce broadly neutralizing antibodies against PRRSV (Kim et al., 2007; Martinez-Lobo et al., 2011; Robinson et al., 2015; Vu et al., 2011). While T cell responses, particularly IFN-γ SC responses, against PRRSV are slow and weak to appear, they are deemed an important mediator of heterologous cross-protection (Charerntantanakul et al., 2006; Lowe et al., 2005; Meier et al., 2003; Xiao et al., 2004; Zuckermann et al., 2007).

Solid homologous protection can be achieved against PRRSV; however, the highly variable nature and constant evolution of the PRRSV genome constitute an obstacle for

achieving effective cross-protective immunity (Lager et al., 1997; Lopez et al., 2007; Vu et al., 2017). Whole-genome genetic difference between PRRSV-1 and PRRSV-2 is approximately 40%. Diversity, based on ORF5 sequence identity, can be as large as 21%, within PRRSV-2 and 30% within PRRSV-1 (Murtaugh et al., 2010). Incomplete cross-protection is offered by MLVs, which have been shown to reduce clinical signs, body weight loss, lung lesions, and viral shedding, but not prevent infection (Cano et al., 2007; Dwivedi et al., 2011; Linhares et al., 2012). Because of the moderate effectiveness of MLVs, live virus inoculation—obtained from viremic animals within the herd—may be used for gilt acclimatization (FitzSimmons and Daniels, 2003). Due to the genetic and antigenic variability of PRRSV, we were interested in assessing the level of cross-protection afforded by previous PRRSV infection against heterologous challenge, while evaluating the cellular and humoral immune responses.

To determine the level of heterologous cross-protection afforded by prior exposure to PRRSV we infected a group of three-week old pigs with PRRSV strain FL12 (n=12) while a group of pigs (n=12) was left uninfected. Subsequently, 77 days later we challenged all animals with the heterologous PRRSV isolate 16244B (Fig. 4.1). Before and after challenge, we measured total and neutralizing antibodies as well as IFN-γ SC responses sequentially assessed in all cases against the original infection strain, FL12, the challenge isolate, 16244B, and an array of divergent PRRSV isolates. To assess the level of cross-protection afforded by prior exposure to PRRSV strain FL12 we determined the level of viremia for 14 days PC, the tissue viral load at 14 days DPC, and the microscopic lung pathology at 14 DPC. The study allowed us to evaluate the cross-protection afforded by

prior PRRSV exposure and characterize the cellular and humoral immune responses that mediate it.

## 3. Results

### *3.1. Cellular immune responses after heterologous challenge in FL12- infected animals*

Immediately before challenge, at 77 DPI, the number of FL12-specific IFN-γ SC for most animals was between 200 and 400, apart from animal 301, which was slightly higher (Fig 4.2A). 16244-specific IFN-γ SC were between 100 and 300 for most animals, except 301 and 314 which were higher (Fig. 4.2B). Four animals (314, 315, 321, and 299) had increased numbers of FL12- and 16244B-specific IFN-γ SC by 7 DPC, and while three experienced a slight decline at 14 DPC, those of animal 299 continued to climb. On the contrary, five animals (304, 323, 325, 333, and 358) showed a reduction in FL12- and 16244B-specific IFN-γ SC by 7 DPC, followed by an increase at 14 DPC. Animal 301—that unexpectedly died 7 DPC due to causes not associated with this study—exhibited an abrupt decline in both FL12- and 16244B-specific IFN-γ SC by 7 DPC. Animal 317 displayed a continuous decline at 7 and 14 DPC of FL12-specific IFN-γ SC; however, the number of 16244B-specific IFN-γ SC showed a slight increase by 14 DPC. Finally, although animal 330 displayed a static number of FL12-specific IFN-γ SC from 0 through 14 DPC, the number of 16244B-specific IFN-γ SC was down at 7 DPC and back up again by 14 DPC. Equivalent variability was observed in all animals when we determined the number of isolate-specific IFN-γ SC using eight heterologous PRRSV-2 isolates and one PRRSV-1 strain (Fig. 4.3).

To determine the extent of cross-reactivity after challenge, individual data sets for each animal and each strain or isolate were combined (Fig 4.4A). Two-way ANOVA of these data showed that in most cases the isolate-specific IFN-γ SC number was not significantly different among PRRSV-2 strains and isolates. The number of IFN-γ SC specific for of SD-01-08 was shown to be significantly lower than that of FL12, 16244B, 18565-01, and MN184, but not the remaining PRRSV-2 isolates. When evaluating the isolate-specific AUC of IFN-γ SC after challenge (Fig 4.4B), FL12 was found to recall higher IFN-γ SC responses than 21599-00, 43807-00, and 18066-04. Furthermore, MN184C was found to recall higher IFN-γ SC responses than 3805-00, and both FL12 and 46517-00 were found to recall a greater number of IFN-γ SC than SD01-08.

### *3.2. Humoral immune responses after heterologous challenge in FL12-infected animals*

The presence of anti-PRRSV antibodies was assessed with the commercially available ELISA IDEXX PRRS X3 Ab Test. All animals were PRRSV seropositive (i.e. an S/P ratio $\geq$ 0.4) prior to challenge, at 0 DPC, and after challenge, at 14 DPC (Fig. 4.5A).

Neutralizing antibodies against FL12 were present in variable quantities in all animals at 0 DPC (Fig. 4.5B), but only one animal had a meager 1:4 cross-neutralizing antibody titer against isolate 16244B immediately before challenge (Fig. 4.5C). Two weeks after challenge we observed increased anti-FL12 neutralizing antibody titers in all animals, but also that nine out of 11 animals had developed neutralizing antibodies against isolate 16244B. Of these, six had neutralizing antibody titers of 1:4, while three had titers of 1:8 or more. Moreover, of the nine animals that developed neutralizing antibodies against isolate 16244B, five also developed titers against isolate 1692-98, and four against isolate

21599-00 (Fig. 4.5D–E). These titers oscillated between 1:4 and 1:32. Finally, we detected a neutralizing antibody titer of 1:16 in one animal against isolate 18565-01 (Fig 4.5F).

### *3.3. Cross protection against heterologous challenge*

After challenge, nine animals in the FL12-infected group showed low levels of viremia at 3 DPC and only one animal remained viremic by 7 DPC (Fig. 4.6A). At 10 and 14 DPC all animals in the FL12-infected group were viremia free. All 12 animals in the initially uninfected group were viremic starting at 3 DPC and through 14 DPC. Uninfected control animals developed high viremia, peaking at 7 DPC, and still present by 14 DPC. A significant reduction in viremia in the FL12-infected group was demonstrated by repeated measures two-way ANOVA. Additionally, the AUC of viremia, calculated using the trapezoidal rule, was significantly reduced in the FL12-infected group (Fig. 4.6B).

When compared to the uninfected control group, viral RNA quantification in lymph node, tonsil, and lung were significantly lower in the FL12-infected group after challenge (Fig. 4.7). Additionally, 16244B-specific viral RNA could be detected in only two animals in tonsil, and in no animals when evaluated in lymph node (data not show). Microscopic lung scores of the severity of the PRRSV-induced interstitial pneumonia in apical, cardiac, and dorsal lung lobes were significantly reduced in the FL12-infected group after challenge when assessed against those of the uninfected control animals (Fig. 4.8).

### *3.4. Cellular and humoral responses after challenge in uninfected control animals*

Following challenge with isolate 16244B, uninfected control animals developed variable 16244B-specific IFN-γ SC by 14 DPC, with values as low as 25 and as high as

676 (Fig 4.9A). While at 7 DPC five out of 12 animals were PRRSV seronegative (S/P ratio < 0.4), all animals had seroconverted by 14 DPC (Fig. 4.9B). Nonetheless, homologous neutralizing antibody titers were absent by 14 DPC (Fig 4.9C).

To evaluate the cross-reactivity of T cell responses against PRRSV we performed the IFN-γ ELISpot with PBMC obtained at 14 DPC with nine PRRSV-2 isolates, including FL12, and one PRRSV-1 strain (Fig. 4.10). We determined that there was a statistical difference among isolate-specific IFN-γ SC by one-way ANOVA. Simple main effects analysis showed that 1692-98, 18066-04 and SD01-08 recalled a significantly lower number of IFN-γ SC than the challenge isolate 16244B. Moreover, the number of SD01-08-specific IFN-γ SC was significantly lower than that of FL12, 3805-00, 18565-01, and MN184C, but not the remaining PRRSV-2 isolates tested.

**4. Discussion**

Since its emergence, PRRSV has become one of the most important pathogens of swine. Unlike other relevant viral pathogens of swine—such as classical swine fever and pseudorabies virus—an effective and broadly-protective vaccine not yet commercially available (Blome et al., 2017; Freuling et al., 2017). Protection afforded by currently-available commercial PRRSV MLVs is sub-optimal. While reduction in viremia, viral shedding, and transmission have been observed in MLV-vaccinated animals, outbreaks still occur in vaccinated herds (Pileri et al., 2015; Wang et al., 2015). Thus, one of the biggest goals for improving PRRSV vaccines is increasing their cross-protective efficacy.

The starting point for this experiment was the conclusion of our previous cross-reactivity study where we demonstrated that T cells of FL12-infected pigs were broadly

cross-reactive when assessed against a panel of nine progressively divergent PRRSV-2 isolates ranging from 5.02% to 14.48% genome-wide genetic distance. Furthermore, we proved that the antibody response against PRRSV-2 isolates was also cross-reactive; however, with few exceptions, we failed to detect any cross-neutralizing antibodies. Finally, we failed to prove any association between genetic (and antigenic) relatedness of the recall isolate to FL12 and the strength of the recall T cell response, in line with the observation of Prieto et al. (2008) that genetic homology between vaccine (in this case infection) and challenge isolate may not be a predictor of vaccine efficacy.

After a period of convalescence of 77 days, FL12-infected and uninfected control animals were challenged with the heterologous isolate 16244B. We chose this isolate because of the medium genome-wide genetic distance to FL12, of 9.66%, and the variability of 16244B-specific IFN-γ SC at 63 DPI. Charerntantanakul et al. (2006) demonstrated a correlation between the percentage of homologously-stimulated IFN-γ+ T cells in PRRSV-vaccinated animals and reduction in both viremia and lung lesion scores after heterologous challenge. Hence, we hypothesized that in our FL12-infected animals the number of 16244B-specific IFN-γ SC would be associated with reduced viremia and microscopic lung lesions.

On the day of the challenge, 77 days after initial infection, most FL12-infected animals exhibited 200–400 FL12-specific and 100–300 16244B-specific IFN-γ SC. Surprisingly, at 7 DPC we observed that for half of the animals the number of isolate-specific IFN-γ SC was significantly reduced in comparison to pre-challenge levels. Although we can not offer a biological explanation for this decline, a similar phenomenon was observed in PRRSV MLV-vaccinated animals after infectious challenge (Zuckermann

et al., 2007). It is also possible that this cell-mediated response contraction could be unrelated to the challenge, as T cell responses in MLV-vaccinated animals have been shown to fluctuate signficantly during the first three months PI (Meier et al., 2003). In most animals T cells were up again by 14 DPC, in certain cases up to pre-challenge levels, or even higher. Contrarily, a small number of animals showed an increase of IFN-γ SC by 7 DPC, followed by a decline by 14 DPC. Of the two animals that exhibited significantly higher T cell responses during the initial FL12 infection, by 7 DPC animal 301 experienced a drastic reduction of isolate-specific IFN-γ SC, while animal 314 showed modest to major increases. While animal 301 died at 7 DPC, animal 314 continued to maintain ~500 isolate-specific IFN-γ SC by 14 DPC. Furthermore, at 14 DPC two other animals, 299 and 304, also reached ~500 isolate-specific IFN-γ SC for certain PRRSV-2 isolates. Through the 14 days that followed the heterologous challenge we observed great variability of T cell responses against all PRRSV-2 isolates tested. These data, together with the data from our cross-reactivity study, leads us to believe that the cell-mediated response against PRRSV can be erratic, and that host factors must play a significant role in determining its overall kinetic.

As we previously described in our preceding study, T cell cross-reactivity was extensive among PRRSV-2 isolates during the 77 days following initial FL12 infection; however, this did not extend to the PRRSV-1 isolate SD01-08. After heterologous challenge with a PRRSV-2 isolate this continued to be the case, and the mean isolate-specific IFN-γ SC number for each isolate was, in most cases, increased by 14 DPC. Interestingly, FL12- nor 16244B-specific IFN-γ SC were the highest by 14 DPC. Instead, MN184C, a highly pathogenic PRRSV-2 isolate, highly heterologous to both FL12

(14.32%) and 16244B (13.35%) when genome-wide genetic distances are analyzed seemed to recall the highest number of isolate-specific IFN-γ SC (Wang et al., 2008). Certain PRRSV-2 isolates were deemed to have significantly higher IFN-γ SC responses than the PRRSV-1 strain SD01-08 after challenge, and some significant differences were observed among PRRSV-2 isolates; nevertheless, we were not able to draw any association between the isolate genetic distance to the infection strain FL12 or the challenge isolate 16244B, and the intensity of the T cell response.

All animals were seropositive, as deemed by a commercial PRRSV ELISA, both at 0 and 14 DPC. Only a minor increase in S/P ratios was observed, likely due to our samples being in the upper portion of the usefull response range of the assay. All FL12-infected animals had developed neutralizing antibody titers against FL12 previous to challenge, ranging from 1:4 to 1:128, but only one animal showed a 1:4 neutralizing antibody titer against 16244B. After heterologous challenge we observed that the neutralizing antibody titers against FL12 had increased. This could be explained by the natural progression of the humoral immune response against the original challenge infection or due to a boosting interaction provided by the heterologous challenge. We observed that nine out of 11 animals had 16244B-neutralizing antibody titers at 14 DPC, which shows that prior exposure to PRRSV may prime the immune system to generate a much rapid neutralizing antibody response after challenge. Infectious challenge has also been shown to elicit a rapid neutralizing antibody response in MLV-vaccinated animals (Trus et al., 2014). Most interesting were the results indicating that FL12-infected and 16244B-challenged animals developed neutralizing antibodies to the heterologous isolates 1692-98 and 21599-00. When considering genome-wide genetic distance, these two isolates are more related to

FL12, 5.02% genetic distance, than to 16244B, 9.61% genetic distance to 1682-98 and 10.01% to 21599-00. Yet, neutralizing antibody titers of up to 1:32 were observed in five animals. The same five animals developed neutralizing antibodies against FL12, 16244B, 1692-98, and 21599-00, but no association could be found between the respective titers (i.e. quantitatively), or between the isolate-specific titers and the number of isolate-specific IFN-γ SC. Furthermore, one of these animals also developed a neutralizing antibody titer of 1:16 against isolate 18565-01. Only recently have Robinson et al. (2015) demonstrated the existence of broadly neutralizing antibody responses against PRRSV in sows that have endured multiple infections.

Viremia after challenge in the FL12-infected group was short lived and negligible. Although most of the animals had low, albeit quantifiable, viremia at 3 DPC, only one animal was viremic at 7 DPC, and all animals were viremia-negative both at 10 and 14 DPC. In sharp contrast, when challenged, our uninfected control group developed viremia by 3 DPC that peaked at 7 DPC. All uninfected control animals were viremic through the 14 days of duration of the challenge experiment. The reduction of viremia we observed in the FL12-infected group was significant both when analyzed by repeated measures two-way ANOVA and one-way ANOVA of the AUC. The level of protection afforded by prior exposure to FL12 was very good, with a seven-fold reduction of the AUC of viremia when compared to uninfected controls. In an analogous experiment, Vu et al. (2015) observed that the viremia in FL12-infected pigs challenged 56 DPI with isolate 16244B was reduced when compared to PBS inoculated animals; however, the viremia in FL12-infected animals was higher and longer lasting than what we observed in our challenge experiment. We hypothesize that a longer period of convalescence may explain this observation. In our

experiment, we did not observe sterilizing immunity in FL12-infected animals. While Zuckermann et al. (2007) demonstrated that pigs vaccinated with a PRRSV-1 MLV and challenged with a 7% heterologous strain did not develop viremia, Park et al. (2014) failed to demonstrate that a PRRSV-2 MLV elicited sterilizing immunity after heterologous PRRSV challenge. Sterilizing immunity has also been proved in the context of homologous PRRSV challenge (Lager et al., 1997).

Viral load in PRRSV target tissues—lymph node, tonsil, and lung—was significantly reduced in FL12-infected animals when compared to uninfected controls. Furthermore, 16244B-specific RNA was detected in no lymph node samples, and only in two tonsil samples. Thus, we assume that the viral RNA detected in the remaining tissue samples belongs to the ongoing chronic and persistent replication of FL12 from the initial infection. Although our viral RNA quantification results are in line with those presented by Vu et al. (2015), the levels of 16244B-specific viral RNA in lymph node and tonsil appear significantly reduced in our FL12-infected animals. Again, we hypothesize that a longer interval between infection and challenge could account for this difference. MLV vaccination was also shown to produce a significant reduction in viral RNA in tonsil, and a total elimination of viral RNA from lung, the latter in contrast to our observations (Zuckermann et al., 2007). Microscopic lung scores were significantly reduced in FL12-infected animals when compared to uninfected control animals. Regardless, many of these animals still developed diffuse to moderate diffuse interstitial pneumonia after challenge in all examined lung lobes.

The uninfected control animals developed a solid viremia after challenge—which began to decline by 10 DPC—with severe diffuse interstitial pneumonia accompanied in

some cases with collapse of the alveoli. Unlike our four-week old pigs in the cross-reactivity study, these pigs developed a rapid 16244B-specific IFN-γ SC response by 14 DPC. Moreover, in our experiment 75% of 16244B-challenged animals developed high heterologous IFN-γ SC responses (> 115 IFN-γ SC/$10^6$ PBMC) by 14 DPC, while in a previous experiment only 12% of MLV-vaccinated piglets did so by 28 DPI (Xiao et al., 2004). We presume that the differences observed between these three studies are associated with the age of the animals at the time of infection or challenge, and the ability of the strain or isolate to stimulate the T cell response. This we had already suggested in our previous cross-reactivity study, as natural infection seems to elicit higher IFN-γ SC responses than MLV vaccination. Although it was previously shown that natural infection may elicit more potent cell-mediated immune responses, no difference was observed between the T cell responses in piglets and finisher pigs (Klinge et al., 2009).

The humoral immune responses in the uninfected control group were characteristic for PRRSV infection. Many animals were seropositive by ELISA by 7 DPC, and all had seroconverted by 14 DPC. Nonetheless, and in contrast with the sharp induction of IFN-γ SC, no homologous neutralizing antibody titers were detected. It is widely accepted that neutralizing antibodies against PRRSV only appear 28 DPI or later, while cell-mediated immune responses can be detected as early as 14 DPI (Lopez and Osorio, 2004; Meier et al., 2003; Xiao et al., 2004). Analogous to what we describe in our cross-reactivity study (before and after challenge), the IFN-γ SC response in the uninfected control animals challenged with isolate 16244B was broadly cross-reactive. By 14 DPC the isolate-specific number of IFN-γ SC was not significantly different amongst most PRRSV-2 strains and isolates tested, and was significantly higher for many PRRSV-2 isolates when compared

to the PRRSV-1 strain SD01-08. This further cements the notion that T cells are broadly cross-reactive between PRRSV-2 isolates.

Our results demonstrate that prior infection with PRRSV affords solid protection to heterologous challenge. Unlike homologous challenge, or—potentially—certain MLVs, prior infection did not provide sterilizing levels of immunity; however, prior infection does seem to reduce the magnitude and duration of viremia, the viral burden in PRRSV target tissues, and the extent and severity of interstitial pneumonia. The data presented is of relevance, due to the wide use of live virus infection to inoculate naïve replacement gilts in herd closure and stabilization protocols. Live virus inoculation is preferred in many cases because it affords better protection against viruses circulating in the herd (i.e. homologous) (Dee, 2003; FitzSimmons and Daniels, 2003). Here we demonstrate that live virus inoculation also elicits a cross-protective immune response that may be as effective, or even better, than that afforded by MLVs. Herd closure and stabilization with live virus inoculation is an effective and established method to eradicate PRRSV from a herd or farm; yet, it should be noted that live virus inoculation is not without risks, and that risks and benefits should be weighed before pursuing this control and eradication strategy.

**Acknowledgements**

This research was supported by the Agriculture and Food Research Initiative competitive grants 2016-67015-24922 (to H.L.X.V.) and 2013-01035 (to F.A.O.) of the USDA National Institute for Food and Agriculture.

We would like to thank the staff of the University of Nebraska–Lincoln Life Sciences Annex and the Nebraska Veterinary Diagnostic Center for the care of animals and provision of samples.


-7
0
77
91

Figure 4.1. Experimental design of cross-protection study. Chronology of animal experiment. After an acclimatization period of one week, animals were infected with PRRSV-2 strain FL12 (n=12) at 0 DPI (▼) or left uninfected (n=12). All animals were challenged with PRRSV-2 isolate 16244B at 77 DPI (▽) and humanely euthanized and necropsied at 91 DPI (▲).

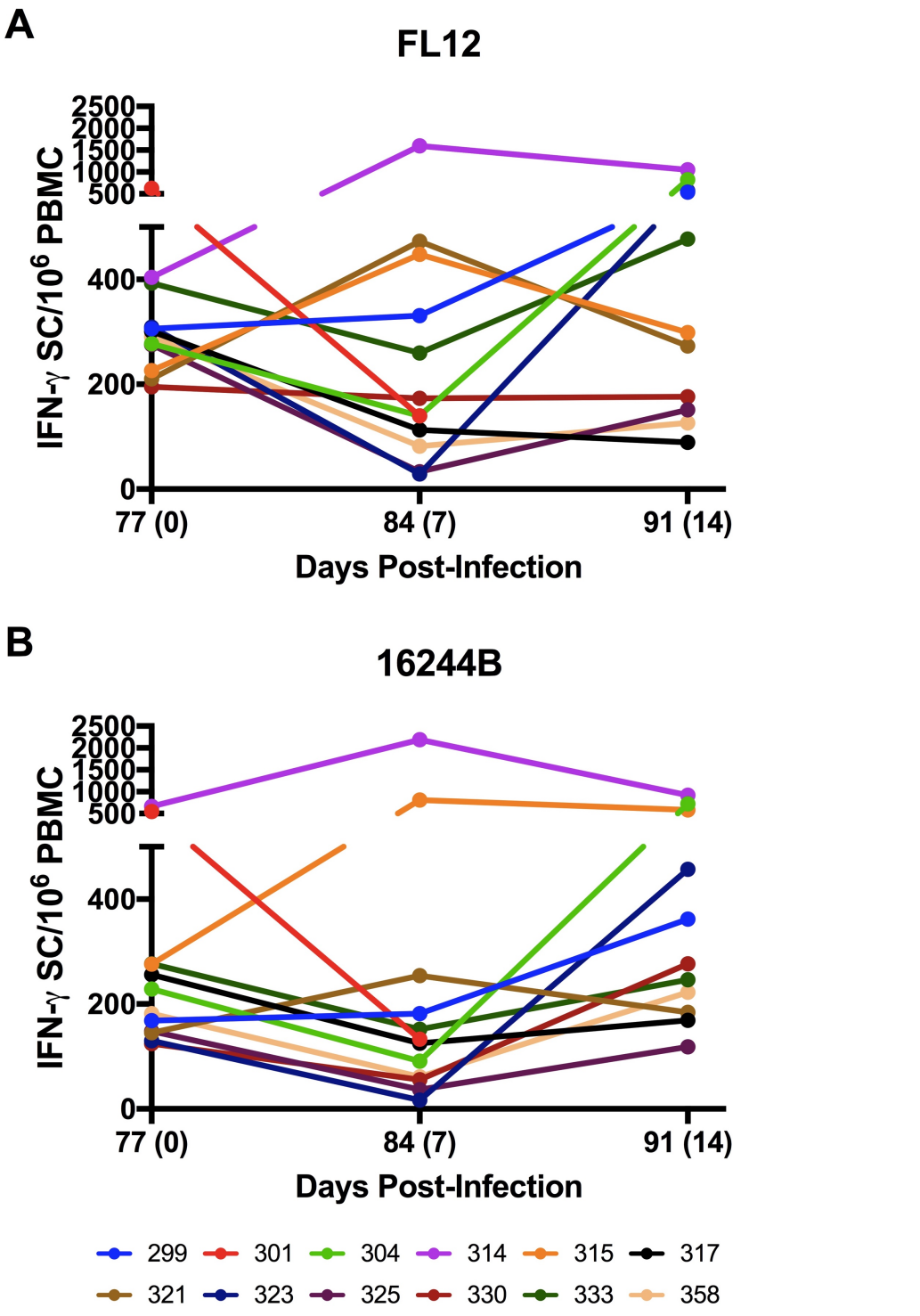

A
FL12
IFN-γ SC/10^6 PBMC
2500
2000
1500
1000
500
400
200
0
77 (0)
84 (7)
91 (14)
Days Post-Infection
B
16244B
IFN-γ SC/10^6 PBMC
2500
2000
1500
1000
500
400
200
0
77 (0)
84 (7)
91 (14)
Days Post-Infection
299
301
304
314
315
317
321
323
325
330
333
358

Figure 4.2. FL12- and 16244B-specific IFN-γ SC responses in FL12-infected animals after challenge. FL12-specific (A) and 16244B-specific (B) IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times.

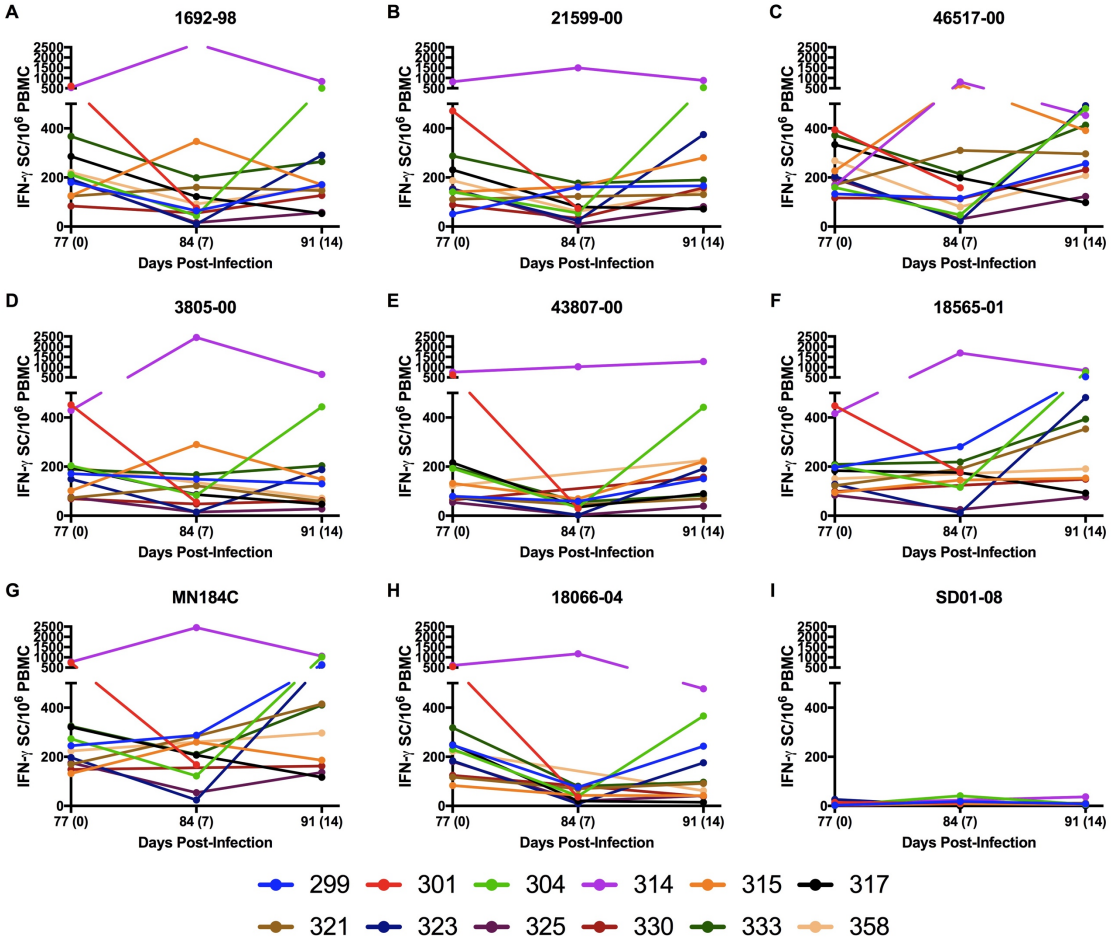
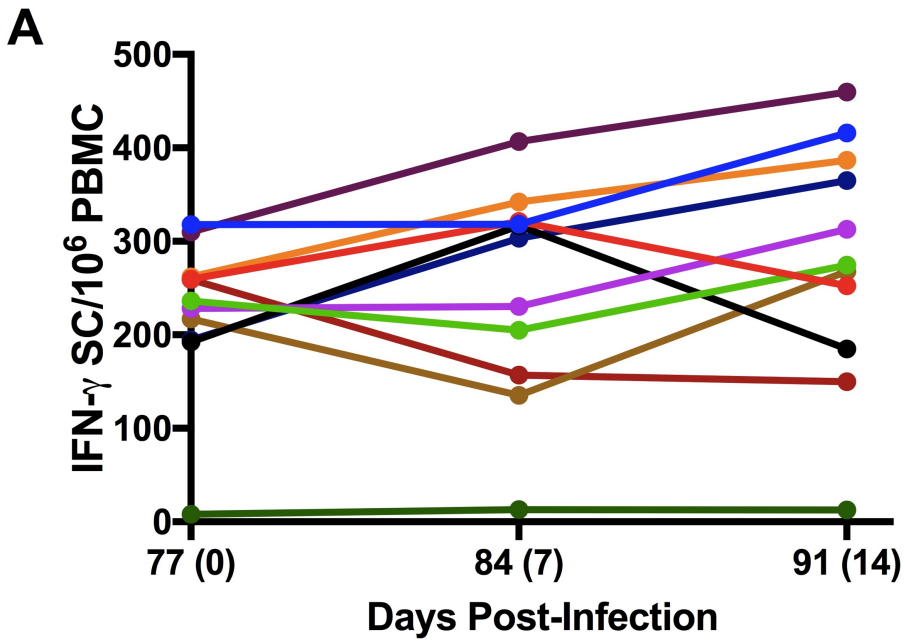

A
1692-98
B
21599-00
C
46517-00
D
3805-00
E
43807-00
F
18565-01
G
MN184C
H
18066-04
I
SD01-08
IFN-γ SC/10^6 PBMC
0
200
400
500
1000
1500
2000
2500
77 (0)
84 (7)
91 (14)
Days Post-Infection
299
301
304
314
315
317
321
323
325
330
333
358

Figure 4.3. Heterologous strain- and isolate-specific IFN-γ SC responses in FL12-infected animals after challenge. Isolate-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times (A–I).


A
500
400
300
200
100
0
IFN-γ SC/10^6 PBMC
77 (0)
84 (7)
91 (14)
Days Post-Infection

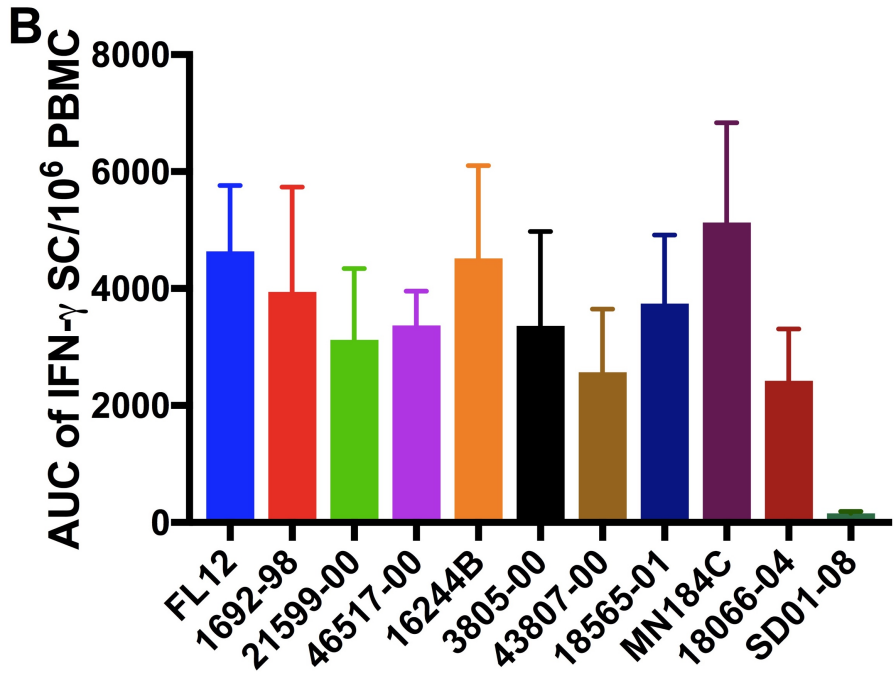

B
8000
6000
4000
2000
0
AUC of IFN-γ SC/10^6 PBMC
FL12
1692-98
21599-00
46517-00
16244B
3805-00
43807-00
18565-01
MN184C
18066-04
SD01-08

Figure 4.4. IFN-γ SC cross-reactivity in FL12-infected animals after challenge. (A) The mean and standard error IFN-γ SC number was calculated for each PRRSV isolate used as recall antigen. (B) The IFN-γ SC AUC mean and standard error for each isolate was calculated.

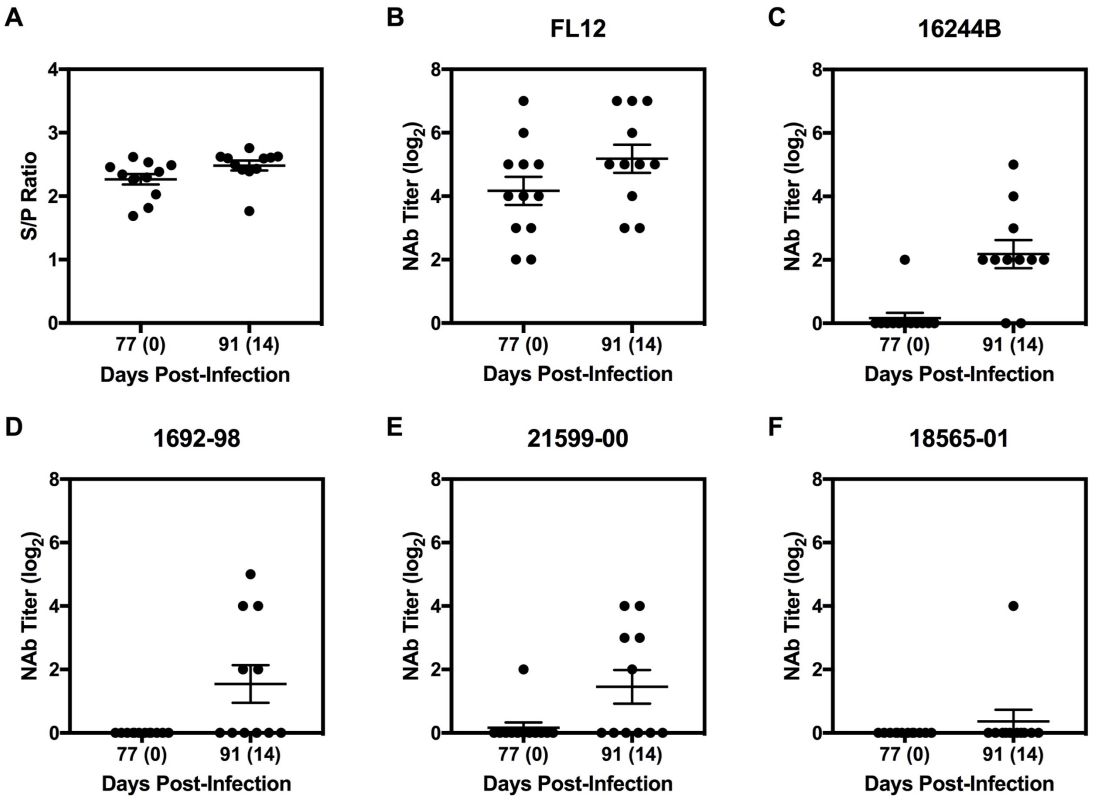

A
S/P Ratio
0
1
2
3
4
77 (0)
91 (14)
Days Post-Infection
B
FL12
NAb Titer (log2)
0
2
4
6
8
77 (0)
91 (14)
Days Post-Infection
C
16244B
NAb Titer (log2)
0
2
4
6
8
77 (0)
91 (14)
Days Post-Infection
D
1692-98
NAb Titer (log2)
0
2
4
6
8
77 (0)
91 (14)
Days Post-Infection
E
21599-00
NAb Titer (log2)
0
2
4
6
8
77 (0)
91 (14)
Days Post-Infection
F
18565-01
NAb Titer (log2)
0
2
4
6
8
77 (0)
91 (14)
Days Post-Infection

Figure 4.5. Humoral immune responses in FL12-infected animals after challenge. (A) Antibody responses against PRRSV were evaluated by ELISA, an S/P ratio ≥ 0.4 was considered positive. (B–F) Isolate-specific neutralizing antibody titers were determined by SVN and results are expressed as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of virus in cell culture.

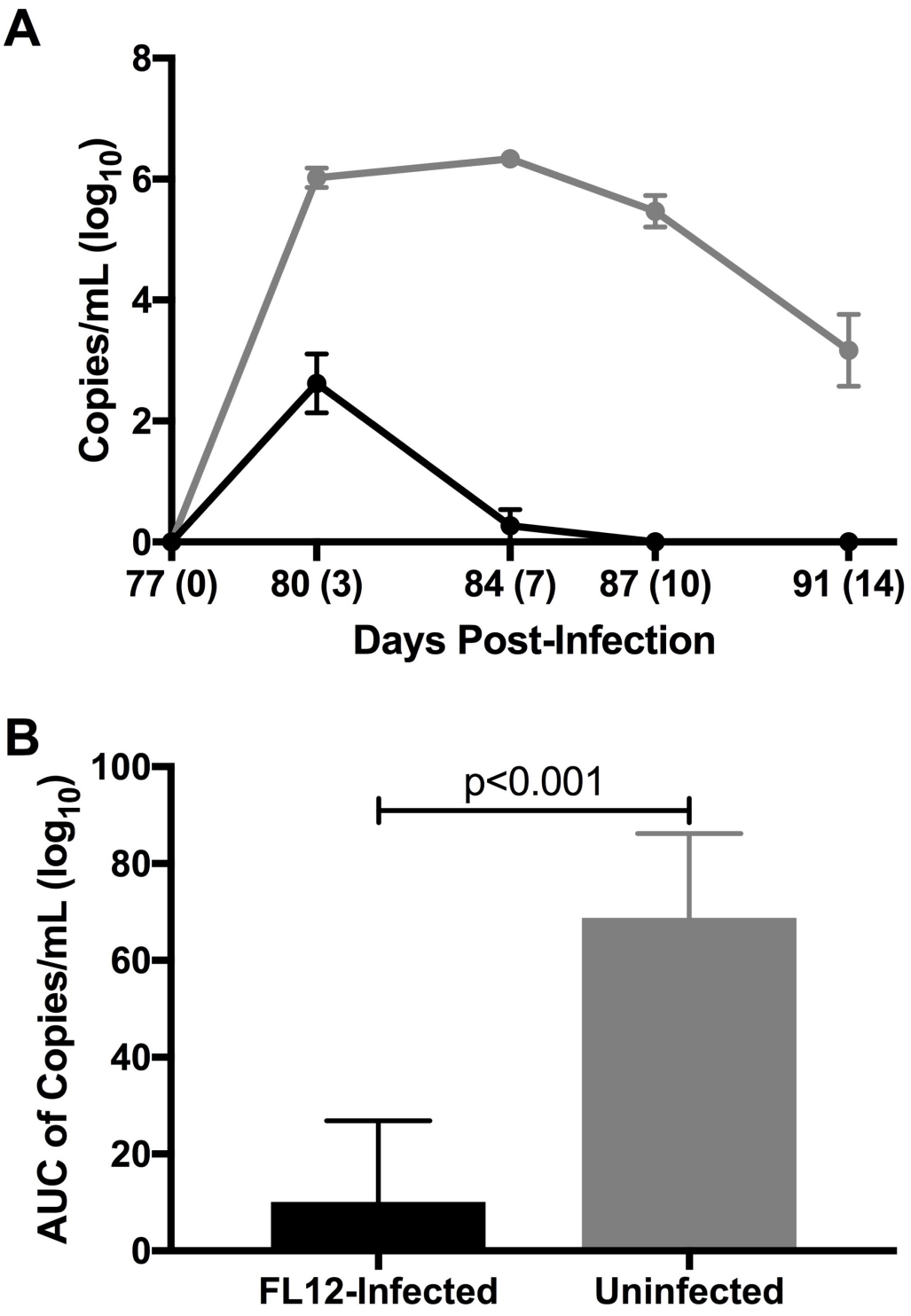

A
8
6
4
2
0
Copies/mL (log10)
77 (0)
80 (3)
84 (7)
87 (10)
91 (14)
Days Post-Infection
B
100
80
60
40
20
0
AUC of Copies/mL (log10)
p<0.001
FL12-Infected
Uninfected

Figure 4.6. Viremia after challenge. (A) Viremia was determined by quantitative real time RT-PCR at the indicated time points. (B) The viremia AUC was calculated using the trapezoidal rule.

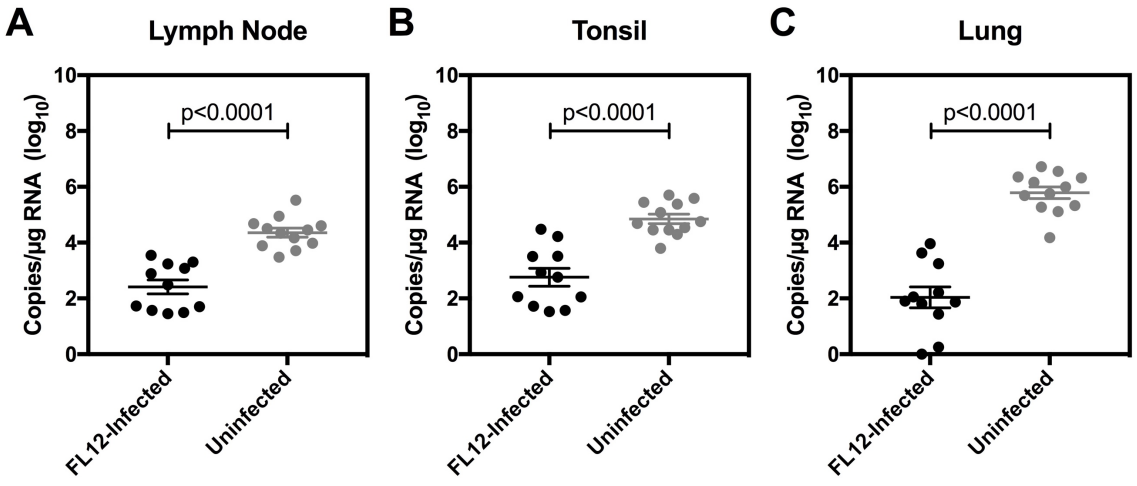

A
Lymph Node
B
Tonsil
C
Lung
Copies/µg RNA (log10)
p<0.0001
FL12-Infected
Uninfected

Figure 4.7. Viral RNA quantification in target tissues after challenge. Viral RNA copies per μg of RNA were determined by quantitative real time RT-PCR at 91 DPI in lymph node (A), tonsil (B), and lung (C).

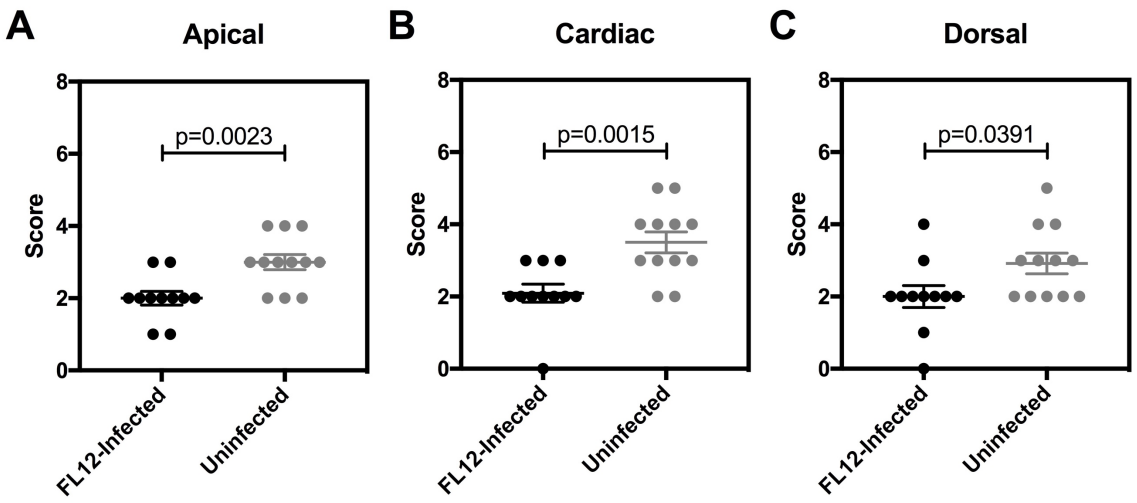

A
Apical
B
Cardiac
C
Dorsal
Score
p=0.0023
p=0.0015
p=0.0391
FL12-Infected
Uninfected

Figure 4.8. Microscopic lung scores after challenge. Sections of the apical (A), cardiac (B), and dorsal (C) lung lobes were blindly examined by a pathologist and given an estimated score of the severity of the interstitial pneumonia.

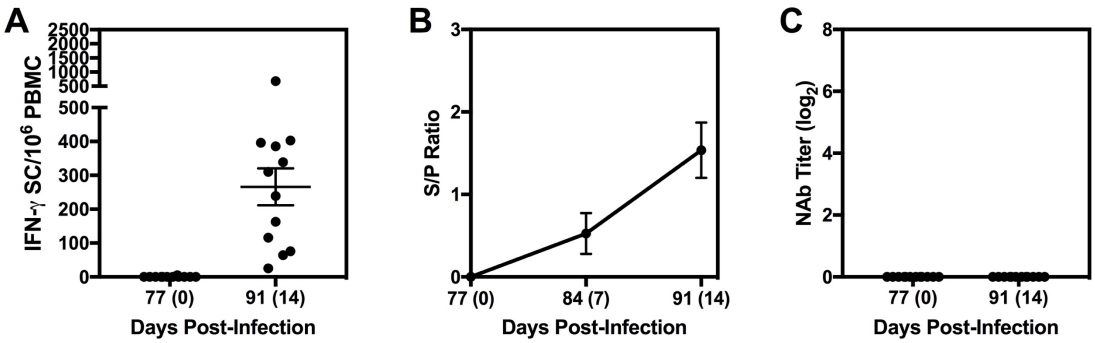

A
IFN-γ SC/10^6 PBMC
2500
2000
1500
1000
500
500
400
300
200
100
0
77 (0)
91 (14)
Days Post-Infection
B
S/P Ratio
3
2
1
0
77 (0)
84 (7)
91 (14)
Days Post-Infection
C
NAb Titer (log2)
8
6
4
2
0
77 (0)
91 (14)
Days Post-Infection

Figure 4.9. Homologous cellular and humoral immune responses in uninfected animals after challenge. (A) 16244B-specific IFN-γ SC were detected in PBMC by ELISpot assay at the indicated times. (B) Antibody responses against PRRSV were evaluated by ELISA, an S/P ratio ≥ 0.4 was considered positive. (C) Neutralizing antibody titers against 16244B were determined by SVN and results are expressed as the $\log_2$ of the reciprocal of the largest dilution of serum that inhibited the development of virus in cell culture.

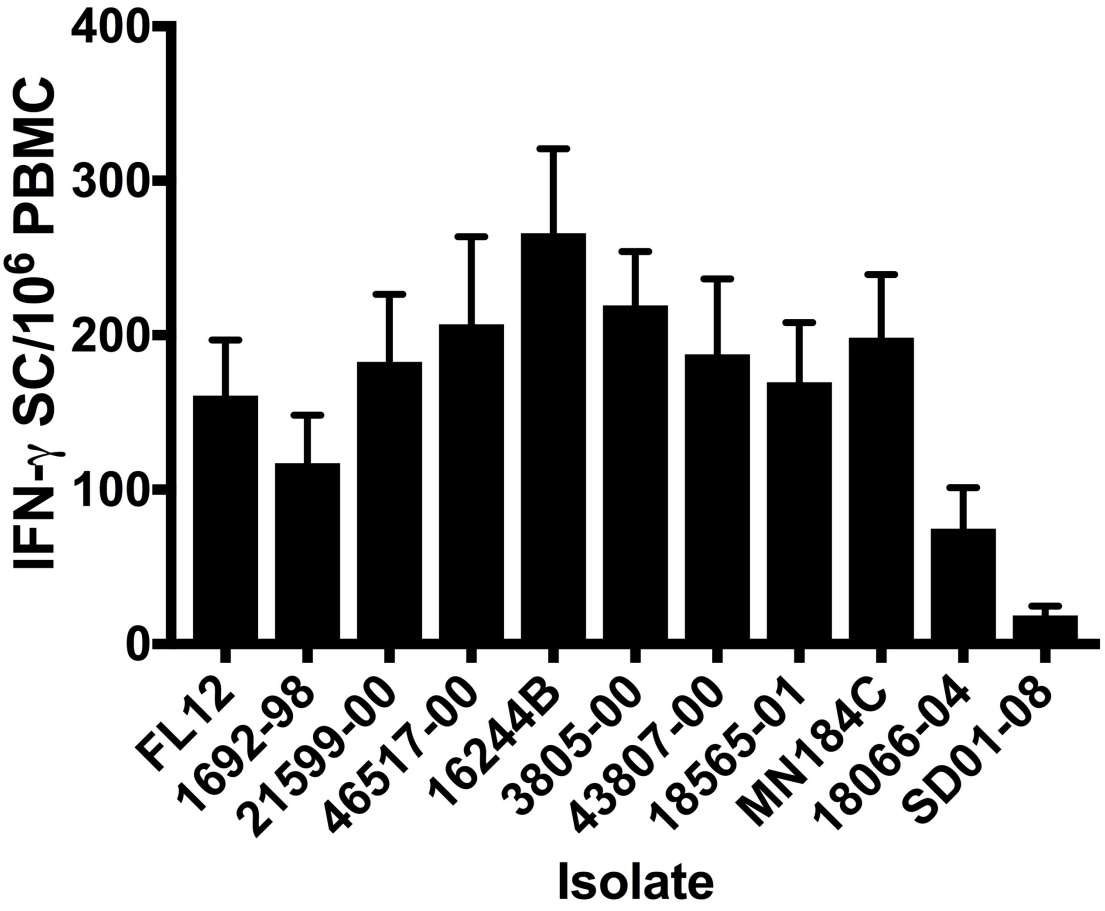

400
300
200
100
0
IFN-γ SC/10^6 PBMC
FL12
1692-98
21599-00
46517-00
16244B
3805-00
43807-00
18565-01
MN184C
18066-04
SD01-08
Isolate

Figure 4.10. Cross-reactivity of IFN-γ SC responses in uninfected animals after challenge. The mean and standard error IFN-γ SC number at 91 DPI was calculated for each PRRSV isolate used as recall antigen.

# CHAPTER V

# CROSS-SPECIES REACTIVITY OF T CELL RESPONSES OF THE STRUCTURAL PROTEINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Portions of the studies in this chapter were published in Vaccine, 2017, Vol. 35, Issue 34, Page 4408–4413.

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Key words: PRRSV; minor glycoproteins; chimeric virus; immunization-challenge experiment.

## 1. Abstract

The two species of PRRSV—previously considered genotypes of the same taxon—have only a ~60% genetic homology. The protection afforded by MLVs of one species against the other species is usually worse than the already incomplete protection observed within each species. Nevertheless, it has recently been shown that certain PRRSV MLVs may afford better cross-protection against opposite species challenge. In this context, weakly cross-reactive IFN-γ SC responses were observed. In our previous experiments we described cross-species reactive IFN-γ SC in equivalent magnitudes. Because the structural proteins of PRRSV have been shown to contain several immunodominant, and sometimes conserved, T cell epitopes, we evaluated the levels of T cell cross-reactivity in SD01-08-infected animals, a PRRSV-1 strain, against the structural proteins of FL12, a PRRSV-2 strain. When compared to mock-infected control animals, the cell-mediated immune response in SD01-08-infected animals was not significantly increased against the structural proteins of FL12. Moreover, prior infection with PRRSV-1 did not seem to prime the cell-mediated immune response against the PRRSV-2 structural proteins, as the magnitude of IFN-γ SC numbers between the mock-infected control group and the SD01-08-infected group was not significantly different after challenge with FL12.

## 2. Introduction

The first reports of PRRSV can be tracked to the non-peer reviewed literature in the late 1980's (Keffaber, 1989). Since then, PRRSV has spread to most swine-producing countries, and in the United States alone the disease is estimated to generate upwards of $664 million in economic losses. PRRSV causes reproductive failure in pregnant sows and respiratory disease in young pigs, belongs to the family *Arteriviridae*, and is an enveloped, positive-sense, single-stranded RNA virus (Rossow, 1998; Snijder et al., 2013).

PRRSV was previously divided into two genotypes, type 1 or European and type 2 or North American, but the current taxonomy classifies each former genotype into a new species, PRRSV-1 and PRRSV-2, respectively (Adams et al., 2016; Adams et al., 2017). Although the extraordinary genetic and antigenic variability between these viruses was demonstrated decades ago, recent analysis of coding-complete genomes and ORF1b phylogeny precipitated the separation into two distinct species (Kuhn et al., 2016; Murtaugh et al., 1995; Nelsen et al., 1999; Nelson et al., 1993). About 40% genetic difference exists between PRRSV-1 and PRRSV-2 (Murtaugh et al., 2010).

Through mechanisms that include the use of PRFs and sg mRNAs, the otherwise short PRRSV genome, at 14.9–15.5 kb, encodes 11 ORFs which, once processed, yield up to 24 distinct protein products (Kappes and Faaberg, 2015). The PRRSV structural proteins are encoded by ORFs contained within a set of six sg mRNAs that are generated via negative-strand intermediates from the 3'-proximal portion of the genome (Meng et al., 1996). GP2-4 associate to form a heterotrimer on the surface of the virion and mediate interaction with the PRRSV major cellular receptor CD163 (Das et al., 2010). GP5 and M form a disulfide-linked heterodimer on the PRRSV envelope that is essential for virus

structure and assembly, with a questioned role in mediating attachment to the host cells (Van Breedam et al., 2010; Wissink et al., 2005). The N protein forms the nucleocapsid core of the virion with a potential responsibility in viral assembly and budding (Dokland, 2010; Spilman et al., 2009). The antigenic homology between the structural ORFs of PRRSV-1 and PRRSV-2 is variable, with amino acid sequence identities ranging from 54% for GP5 to 91% for the M protein (Meng et al., 1995a; Meng et al., 1995b; Murtaugh et al., 1995).

Among the multiple proteins of PRRSV, structural protein peptides from GP2, GP3, GP4, GP5, M, and N were shown to be recognized by T cells of PRRSV-infected animals (Mokhtar et al., 2014). Immunodominant T cell epitopes have been mapped to most structural proteins—GP4, GP5, M, and N—and appear to be moderately conserved within each species, and to a lesser extent across PRRSV-1 and PRRSV-2 (Diaz et al., 2009; Vashisht et al., 2008; Wang et al., 2011).

Cross-protection between PRRSV-1 and PRRSV-2 was assumed to be very limited, as PRRSV-2 MLVs have shown weak efficacy after PRRSV-1 infectious challenge (Labarque, 2003; Labarque et al., 2000; van Woensel et al., 1998). On the contrary, Park et al. (2015) demonstrated that a PRRSV-2 MLV afforded significant cross-protection against PRRSV-1 challenge, as measured by reduction in viremia, viral shedding, and microscopic lung lesions. Equivalent results were also observed with a PRRSV-1 MLV (Kim et al., 2015). How cross- protection between PRRSV species occurs is not yet fully understood. Pigs infected or MLV-vaccinated with either PRRSV-1 or PRRSV-2 strains were shown to recall small numbers of IFN-γ SC against the opposite species, and cross-neutralizing antibodies were absent (Burgara-Estrella et al., 2013; Choi et al., 2016; Kim

et al., 2015). In our cross-reactivity and cross-protection experiments we observed extremely weak IFN-γ SC recall responses against PRRSV-1 strain SD01-08 in PRRSV-2-infected animals. Hence, to gain a better understanding of how cross-species protection may arise, and to further examine the immunodominance of structural proteins, we planned to evaluate the cross-reactivity of IFN-γ SC responses in SD01-08-infected animals against the structural proteins of FL12.

In this experiment, we infected a group of six four-week old pigs with the North American PRRSV-1 strain SD01-08 and six animals were mock-infected with PBS (Fig. 5.1). At 56 DPI, all animals were challenged with PRRSV-2 strain FL12 and humanely euthanized 14 days later. Cell-mediated immune responses were assessed at 0, 56 and 70 DPI against peptide pools representing the structural proteins of FL12: GP2, GP3, GP4, GP5, M, and N. This allowed us to evaluate the T cell cross-reactivity between PRRSV-1 and PRRSV-2 at the structural protein level.

## 3. Results

### *3.1. Nucleotide and amino acid pairwise distance between the structural proteins of SD01-08 and FL12*

Genome-wide coding nucleotide pairwise distance between SD01-08 and FL12 was calculated at 36.72% and ORF-level nucleotide and amino acid sequences were aligned using MUSCLE (Edgar, 2004). Nucleotide and amino acid calculated pairwise distances by ORF are presented in Table 5.1.

The highest calculated nucleotide pairwise distance between the structural ORFs of SD01-08 and FL12 belongs to ORF5, which codes for GP5, at 34.80%. ORF7, that codes

for the N protein, has the lowest calculated nucleotide pairwise distance between these two strains at 29.44%. Amino acid-wise, ORF5 continues to be the most distant between these two strains, with a calculated amino acid pairwise distance of 42.54%, while the M protein, encoded by ORF6, is the most conserved, at 21.39%.

### *3.2. Cell-mediated responses against the structural proteins of FL12 in SD01-08-infected animals*

SD01-08-infected animals were allowed 56 days to reach convalescence before determining the level of IFN-γ SC recalled by structural peptides of the heterologous PRRSV-2 strain FL12. The number of IFN-γ SC recalled by GP2, GP3, GP4, GP5, M, and N peptide pools was not significantly different between the SD01-08-infected and mock-infected groups as determined by an unpaired t-test (Fig. 5.2). The IFN-γ SC recall responses of individual animals against these peptides was negligible, in most cases under 10 IFN-γ SC/$10^6$ PBMC. When PBMC were re-stimulated with an M protein peptide pool two animals exhibited higher IFN-γ SC recall responses, at 31 and 54 IFN-γ SC/$10^6$ PBMC. A moderately higher IFN-γ SC recall response was also observed in one of these animals against an N protein peptide pool, at 19 IFN-γ SC/$10^6$ PBMC.

### *3.3. Cell-mediated responses against the structural proteins of FL12 in SD01-08-infected animals after challenge*

At 56 DPI, all animals were challenged with the PRRSV-2 strain FL12 and the number of IFN-γ SC recalled by peptide pools of the structural proteins of FL12 were determined 14 DPC (Fig. 5.3). One of the animals in the mock-infected PBS group

unexpectedly died due to causes not associated with this study. Unpaired t-test analysis failed to demonstrate significance between the SD01-08- and mock-infected groups for all six structural ORFs evaluated. IFN-γ SC recall responses were marginally diminished in the SD01-08-infected group against the minor glycoproteins (GP2-4) and the N protein, but slightly increased against the major glycoprotein, GP5, and the M protein, when compared to the mock-infected group.

**4. Discussion**

The established paradigm that PRRSV was a sole, albeit extremely variable, species within the genus *Arterivirus* was recently replaced by the introduction of new molecular phylogeny analysis tools (Kuhn et al., 2016). While the former European or type 1 strains and isolates remained in the former PRRSV taxon, now renamed PRRSV-1, the North American or type 2 strains and isolates were re-classified into a newly-created PRRSV-2 taxon. These taxa join the prototype species LaDV in the newly-formed genus *Porartevirus* of the family *Arteriviridae* (Adams et al., 2016; Adams et al., 2017).

The recent demonstration that vaccination with either a PRRSV-1 or a PRRSV-2 MLV can reduce both viremia and microscopic lesions after opposite species challenge disputed the notion that PRRSV MLVs did not afford significant cross-species or cross-genotype protection (Kim et al., 2015; Labarque et al., 2000; Park et al., 2015; van Woensel et al., 1998). Furthermore, it was shown that vaccination or infection with one species of PRRSV elicited a weak cross-species reactive IFN-γ SC response (Burgara-Estrella et al., 2013; Choi et al., 2016; Kim et al., 2015). Our prior results clearly demonstrated that IFN-γ SC responses were broadly cross-reactive between PRRSV-2 isolates, but when PBMC

from FL12-infected animals were re-stimulated with a PRRSV-1 strain the number of IFN-γ SC recalled was, although present, significantly lower.

In this experiment we further assessed the cross-reactivity between PRRSV-1 and PRRSV-2 at the structural ORF level. Although immunodominant T cell epitopes have been mapped to both structural and non-structural proteins, the former seem to consistently elicit higher T cell responses in a broader number of animals (Diaz et al., 2009; Mokhtar et al., 2014; Parida et al., 2012). In the current study PBMC obtained from pigs infected with the North American PRRSV-1 strain SD01-08 failed to recall a significant number of IFN-γ SC against peptide pools representing the structural proteins of the PRRSV-2 strain FL12, when compared to mock-infected animals. Our results show that, with few exceptions, the six animals in the SD01-08-infected group only presented meager T cell responses against GP2-5, M, and N peptide pools. A notable exception occurred when PBMC were re-stimulated with peptide pools corresponding to the M protein, in which case we observed that two animals had improved T cell responses. We hypothesize that the conserved nature of the M protein, at 21.39% calculated amino acid pairwise distance, could be responsible for this observation. The distribution of SLA molecules in an outbred pig population cannot be underestimated, as this observation could only be explained if these animals had distinct haplotypes, and thus, different abilities to present and recognize peptides in the context of their respective SLA molecules.

We were also interested in evaluating if prior exposure to a PRRSV species had a priming effect after challenge with the opposite species. To that effect, we challenged all animals with the PRRSV-2 strain FL12, and 14 days later evaluated their cell-mediated immune responses. Once again, our results showed no statistically significant difference

between the group of animals that had been previously infected with SD01-08 and that mock-infected. This was true for all the structural proteins tested. Hence, prior exposure to PRRSV-1 does not seem to prime the cell-mediated immune response against challenge with PRRSV-2.

The experimental data here presented regarding cross-species reactivity between the North American PRRSV-1 strain SD01-08 and PRRSV-2 strain FL12 conforms with those data previously presented for both our cross-reactivity and cross-protection studies. Although we failed to prove any statistical difference between PRRSV-1-infected and mock-infected animals, we did observe that individual animals may have IFN-γ SC recall responses in the context of PRRSV-2 peptides. In a smaller magnitude, our findings are in line with the recent reports of cross-protection and weak cross-reactivity between PRRSV-1 and PRRSV-2 (Burgara-Estrella et al., 2013; Choi et al., 2016; Kim et al., 2015; Park et al., 2015).

Certain bacterial and viral vaccines have been shown to afford cross-species protection. A *Brucella suis* MLV was shown to protect against *B. suis* and cross-protect against *B. abortus* and *B. mellitenesis* challenge in mice; however, these are considered closely related strains (Halling et al., 2005; Zhu et al., 2016). Although vaccination with a bovine herpesvirus-1 MLV was proven to protect against heterologous bubaline herpesvirus-1 challenge, these two viruses have an almost identical glycoprotein B, with 96.6% nucleotide homology, which is a major target of neutralizing antibodies (Alves Dummer et al., 2014; Montagnaro et al., 2014). Approximately 35% genetic difference exists between measles virus and canine distemper virus, both members of the genus *Morbillivirus*, family *Paramyxoviridae*. Cross-species protection has been observed in

dogs and non-human primates vaccinated with measles virus and challenged with canine distemper virus. Cross-neutralizing cellular immune responses were assumed to mediate the observed cross-protection, and while cross-neutralizing antibodies were not detected prior to challenge, the presence cross-reactive virus neutralizing epitopes was suggested (de Vries et al., 2014). T cell-mediated immune responses—CD4+ IFN-γ+ or IFN-γ+/TNF-α+ cells—triggered by both homologous or heterologous re-stimulation has been observed for both *Chlamydia suis* and *C. trachomatis* infection (Kaser et al., 2017). Again, *C. suis* and *C. trachomatis* are closely related bacterial strains (Hadfield et al., 2017).

Although our results do not reveal a significant cell-mediated immune response against the PRRSV-2 structural proteins in PRRSV-1-infected animals, they still demonstrate that certain individuals may be able to weakly recognize cross-reactive epitopes between both species. Other authors have confirmed the existence of cross-protective immunity between both PRRSV species. In the absence of neutralizing antibodies—the best characterized correlate of protection for PRRSV—the question of whether these limited cross-reactive T cell responses could be responsible for the observed reduced levels of viremia, viral shedding, and microscopic lung lesions remains unanswered. The extraordinary genetic variability of PRRSV, coupled with the immense variability of SLA haplotypes, constitute a great obstacle for identifying immunodominant T cell epitopes. Nonetheless, evidence for non-sterilizing cross-protection in the absence of neutralizing antibodies, both within and between PRRSV species, continues to push forward the concept that T cells are a major component of the cross-reactive immune response against PRRSV.

**Acknowledgements**

This research was supported by the Agriculture and Food Research Initiative competitive grants 2016-67015-24922 (to H.L.X.V.) and 2013-01035 (to F.A.O.) of the USDA National Institute for Food and Agriculture.

We would like to thank Dr. Bernhard Kaltenboeck at Auburn University for the FL12 peptides and the staff of the University of Nebraska–Lincoln Life Sciences Annex and the Nebraska Veterinary Diagnostic Center for the care of animals and provision of samples.

**Table 5.1. Calculated nucleotide and amino acid pairwise distance (%) between the ORFs of SD01-08 and FL12.**

| ORF (Protein) | Nucleotide | Amino Acid |
|---|---|---|
| ORF1a (nsp1-8) | 37.62 | 50.00 |
| ORF1b (nsp9-12) | 34.57 | 30.89 |
| ORF2a (GP2) | 32.84 | 34.17 |
| ORF3 (GP3) | 32.23 | 39.04 |
| ORF4 (GP4) | 33.40 | 29.78 |
| ORF5 (GP5) | 34.80 | 41.54 |
| ORF6 (M) | 31.03 | 21.39 |
| ORF7 (N) | 29.44 | 34.17 |


-7
0
56
70

Figure 5.1. Experimental design of cross-species reactivity study. Chronology of animal experiment. After an acclimatization period of one week, animals were infected with PRRSV-1 strain SD01-08 (n=6) at 0 DPI (▼) or mock-infected with PBS (n=6). All animals were challenged with PRRSV-2 strain FL12 at 56 DPI (▽) and humanely euthanized at 70 DPI.

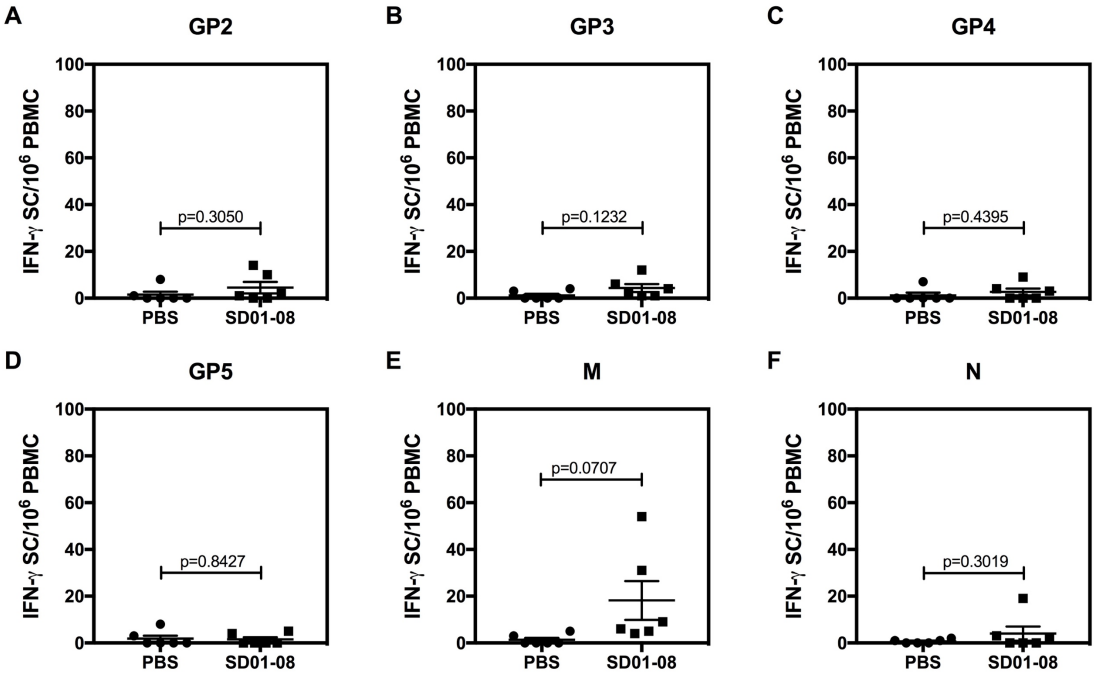
A
GP2
B
GP3
C
GP4
D
GP5
E
M
F
N
IFN-γ SC/10^6 PBMC
100
80
60
40
20
0
p=0.3050
p=0.1232
p=0.4395
p=0.8427
p=0.0707
p=0.3019
PBS
SD01-08

Figure 5.2. IFN-γ SC recall responses in SD01-08-infected animals against FL12 structural protein peptide pools. PBMC obtained at 56 DPI from SD01-08- and mock-infected animals were re-stimulated with peptide pools representing the structural proteins of FL12 and IFN-γ SC determined by ELISpot assay. Data were analyzed by unpaired t-test, and p-values are presented.

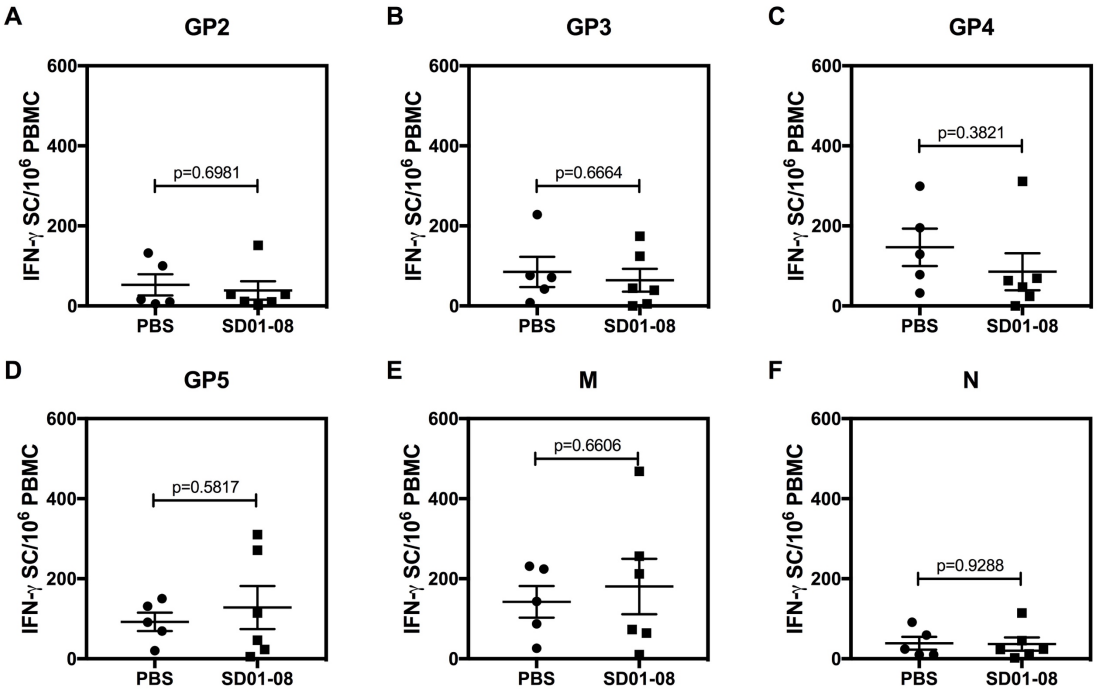
A
GP2
B
GP3
C
GP4
D
GP5
E
M
F
N
IFN-γ SC/10^6 PBMC
600
400
200
0
p=0.6981
p=0.6664
p=0.3821
p=0.5817
p=0.6606
p=0.9288
PBS
SD01-08

Figure 5.3. IFN-γ SC recall responses in SD01-08-infected and challenged animals against FL12 structural protein peptide pools. PBMC obtained at 70 DPI (14 DPC) from SD01-08- and mock-infected animals were re-stimulated with peptide pools representing the structural proteins of FL12 and IFN-γ SC determined by ELISpot assay. Data were analyzed by unpaired t-test, and p-values are presented.

# GENERAL DISCUSSION AND CONCLUSIONS

The economic burden associated with PRRSV infection is on the rise, and both PRRSV-1 and PRRSV-2 are present in Asia, North America, and Europe, where most of the world's swine production is concentrated. The genetic and antigenic variability of the virus and its outstanding capacity to evolve are a major hurdle for controlling and eliminating PRRSV. Nonetheless, several strategies have been demonstrated to be effective against it. While gilt acclimatization is commonly used to reduce the incidence of PRRSV-associated disease in naïve animals, herd stabilization and closure are the most widely used method for eliminating PRRSV from a farm (Corzo et al., 2010; Linhares et al., 2014; Torremorell et al., 2002). The effectiveness of the hosts' immune response against the virus can be illustrated by the success of herd stabilization and closure protocols, where PRRSV negative status can be achieved by limiting the introduction of susceptible animals while allowing those already infected to eliminate the virus. The success of these protocols is reliant on long periods of time where animals are not introduced into the herd (Loving et al., 2015). The absence of DIVA vaccines against PRRSV, the suboptimal protection afforded by MLV vaccination—outbreaks have been documented in well-vaccinated herds—and the extended periods of time required by current eradication strategies may make unrealistic a long-term and large-scale eradication of PRRSV (Vu et al., 2017; Wang et al., 2015). Thus, there is a pressing need to improve PRRSV vaccines, fundamentally in regard to their cross-protective efficacy which, in turn, would make PRRSV eradication much more feasible.

Immunity against PRRSV is complete and sterilizing in the presence of appropriate titers of neutralizing antibodies against homologous virus challenge (Lager et al., 1997; Osorio et al., 2002). Nonetheless, commercially available MLVs are poor inducers of neutralizing antibodies, and cross-neutralizing antibodies against heterologous isolates are rare to occur (Kim et al., 2007; Lopez and Osorio, 2004). Recently, broadly neutralizing antibodies have been shown to occur in sows with multiple exposures to PRRSV (Robinson et al., 2015). Partial protection against heterologous challenge is afforded by MLV vaccination, and it is assumed to be mediated by T cells (Zuckermann et al., 2007). The extent to which T cells may cross-react with heterologous PRRSV isolates is unknown. Thus, if protection can be afforded by cell-mediated responses, there is a necessity to characterize how these behave against the extensive genetic and antigenic array of PRRSV. Chapter III described the extensive variability observed in cell-mediated immune responses in PRRSV-infected animals against both homologous and heterologous isolates. When individual animal T cell responses against PRRSV were evaluated over a period of 11 weeks, a significant variation was observed. While certain animals had robust T cell responses within two to four weeks after infection, others required over eight weeks to attain modest levels of PRRSV-specific IFN-γ SC. Most importantly, cell-mediated responses in PRRSV-2-infected animals were shown to be broadly cross-reactive against other PRRSV-2 isolates, but not against a PRRSV-1 strain, and that increased genetic heterology between PRRSV-2 isolates was not associated with reduced T cell responses. Although cell- and antibody-mediated responses were cross-reactive, no cross-reactive neutralizing antibodies were detected, regardless of genetic distance to the infection isolate. Finally, a moderate correlation between homologous T cell and neutralizing antibody

responses was described. Taken together, these results demonstrate that T cell responses against PRRSV-2 are cross-reactive to other PRRSV-2 isolates regardless of genetic distance, and may be mediate partial protection against heterologous challenge.

Because Charerntantanakul et al. (2006) demonstrated that a higher presence of PRRSV-specific IFN-γ+ SC in MLV vaccinated animals was associated with reduction in viremia and microscopic lung lesions after challenge, the relative contribution of IFN-γ SC in FL12-infected animals against heterologous challenge was evaluated. To assess the extent of protection afforded by prior exposure to PRRSV, previously uninfected control animals were concurrently challenged. Immediately before challenge, the homologous (against strain FL12) T cell responses were higher than heterologous (against isolate 16244B) ones, with the latter ranging between 100 and 300 IFN-γ SC/$10^6$ PBMC. During the 14 days following challenge an array of divergent patterns were observed. While some animals experienced a boost in both homologous and heterologous T cell responses at 7 DPC, others experienced a decline. At 14 DPC this pattern repeated, with PRRSV-specific T cells in certain animals bouncing back, and in others going down. Overall, the mean T cell response for both homologous and heterologous virus was slightly boosted after challenge. As in the previous chapter, extensive cross-reactivity between PRRSV-2 isolates was observed. Furthermore, FL12-infected animals developed 16244B neutralizing antibodies within 14 days after challenge, and more importantly, cross-neutralizing antibodies against other heterologous isolates. Although the challenge data showed strong reduction of viremia, the viral load in lymph node and microscopic lung lesion were modestly, albeit significantly, reduced. 16244B-specific RNA in lymph node and tonsil was not detected, thus, the viral RNA quantified in those tissues corresponds to the ongoing

chronic infection of the primary inoculation with FL12. In the uninfected animals, a relatively homogeneous rapid development of T cell responses against homologous (in this case 16244B) and heterologous PRRSV-2 isolates was observed as early as 14 DPC, in sharp contrast to what occurred in our initial cross-reactivity study. This supports the hypothesis that the development T cell responses may be influenced by the age of the animal. Overall, these data demonstrate that previous infection with PRRSV affords protection against heterologous challenge, that manifests as solid reduction of viremia post challenge, as well as significant reduction in tissue viral load and microscopic lung lesions. Furthermore, it continues to advance the notion that T cells mediate heterologous protection against PRRSV. Finally, it proves that broadly neutralizing antibody responses can be achieved against PRRSV.

Chapter V describes the efforts to better characterize the cross-reactivity of T cell responses between PRRSV-1 and PRRSV-2 at the structural protein level. Cell-mediated responses against the structural proteins of PRRSV are strong, and several T cell epitopes have been mapped to them, some of which are conserved (Bautista et al., 1999; Diaz et al., 2009; Vashisht et al., 2008; Wang et al., 2011). Re-stimulating PBMC of SD01-08-infected animals, a PRRSV-1 strain, with peptide pools representing the structural proteins of FL12, a PRRSV-2 strain, did not elicit a significant T cell responses when compared to those of PBMC from mock-infected animals. Nevertheless, isolated episodes in which certain animals had apparent cross-reactive T cells against the M and N proteins were detected, though in a very limited capacity. Moreover, prior exposure to PRRSV-1 did not seem to prime the T cell response against PRRSV-2 challenge, as SD01-08-infected animals challenged with FL12 showed similar numbers of IFN-γ SC against the structural proteins

of FL12 than mock-infected and challenged animals. Therefore, and in unison with the results presented in the two previous chapters, there doesn't seem to be a cross-reactive cell-mediated immune response between the structural proteins of PRRSV-1 and PRRSV-2.

Overall, the results presented in this dissertation further our understanding of humoral, but fundamentally cell-mediated immunity against PRRSV, and continues to advance our knowledge of swine immunology. Furthermore, these results can contribute to the ongoing efforts of developing broadly-protective vaccines against PRRSV that will in turn advance the eradication efforts against the virus.

# APPENDICES

## A.1. Phosphate buffered saline (PBS)

| | |
|---|---|
| NaCl | 140 mM |
| KCl | 2.7 mM |
| Na2HPO4 | 10 mM |
| KHPO4 | 1.8 mM |

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