Novel Approaches Towards Vaccine Developments Against Porcine Circovirus Type 2 And Porcine Reproductive And Respiratory Syndrome Virus

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the causative agent of porcine circovirus-associated disease (PCVAD). Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV). Both PCV2 and PRRSV have caused devastating diseases in the swine industry worldwide, resulting in immense economic losses. One of the most common co-infections in the swine industry is PCV2 and PRRSV. The aim of this dissertation research is to explore different experimental approaches to develop novel vaccines against the two major pathogens affecting swine production and study the basic mechanisms that may be involved in viral pathogenesis.

Two types of porcine circovirus (PCV), PCV1 and PCV2, have been identified thus far. PCV1, first identified as a contaminant of the PK-15 cell line, is non-pathogenic and has a low prevalence in swine herds. PCV2 is highly prevalent in most swine-producing countries and is associated with clinical PCVAD. The non-pathogenic PCV1 shares similar genomic organization with PCV2. Previously, it has been demonstrated that a genetically modified infectious chimeric PCV1-2a virus can tolerate up to a 27 aa insertion in the C-terminus of the ORF2 without affecting infectivity and produce a dual immune response against PCV2cap and the inserted epitope tag. Therefore, we evaluated the use of the non-pathogenic PCV1 wild-type (wt) virus and chimeric PCV1-2a vaccine virus (vs) to express four known B-cell epitopes of PRRSV. Peptide epitopes of PRRSV-VR2385, including GP2II (aa 40–51, ASPSHVGWWSFA), GP3I (aa 61–72, QAAAEAYEPGRS), GP5I (aa 35–46, SSSNLQLIYNLT), and GP5IV (aa 187–200,
TPVTRVSAEQWGRP) were inserted in frame into the C-terminus of the ORF2 of PCV1wt as well as the PCV1-2av. Four PCV1-PRRSV$_{EPI}$ chimeric viruses and four PCV1-2a-PRRSV$_{EPI}$ chimeric viruses were successfully rescued and shown to be infectious in vitro and co-expressed PCV1cap or PCV2cap with each specific PRRSV epitope. Two independent animal studies were conducted to evaluate whether the non-pathogenic PCV1 can serve as a vaccine delivery vector and whether the PCV1-2a vaccine virus can be used to develop a bivalent vaccine against both PCV2 and PRRSV. We demonstrated that three PCV1-PRRSV$_{EPI}$ chimeric viruses and two PCV1-2a-PRRSV$_{EPI}$ chimeric viruses were infectious in pigs. Importantly, we demonstrated that the PCV1-PRRSV$_{EPI}$ and PCV1-2a-PRRSV$_{EPI}$ chimeric viruses not only induced specific PCV1 or PCV2 IgG antibody but also specific anti-PRRSV epitope antibody responses as well. Regardless of the PCV backbone used, we showed that the PCV-PRRSV chimeric viruses elicited neutralizing antibodies against PRRSV-VR2385. These results provided a proof of concept for the potential use of the non-pathogenic PCV1 as a vaccine delivery system for PRRSV or other swine pathogens and the use of PCV1-2a vaccine virus to generate a bivalent vaccine against both PCV2 and PRRSV.

PRRSV causes a persistent infection and immunosuppression. Immunomodulation of the host immune system is caused by modulation of numerous interleukins, such as type I interferons, tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-12 (IL-12) in infected pigs. Antigen-presenting cells (APCs) are the first line of defense, and their infection plays an important role in innate-mediated immune regulation during early immune responses. Among the APCs, pulmonary alveolar macrophages (PAMs), pulmonary interstitial macrophages (PIMs), and dendritic cells (DCs) are the main targets for PRRSV replication. The role of PRRSV-DCs interaction is not fully understood, and current research focuses on the
production and regulation of interferons through DC-SIGN receptors. In this study, we evaluated the immunomodulation of MoDCs by PRRSV through interactions with the pDC-SIGN receptor, by blocking pDC-SIGN with recombinant hICAM-3-Fc or anti-pDC-SIGN mAb. Our results indicate that recombinant hICAM-3-Fc enhances mRNA expression of proinflammatory cytokines and that anti-pDC-SIGN mAb inhibits mRNA expression of TNF-α and IL-1α and enhances the expression of IL-12 induced by PRRSV in MoDCs. The results will help understand the molecular mechanisms of PRRSV pathogenesis.
DEDICATION

This dissertation is entirely dedicated to my family:

Maria Matilde, we finally made it one more time. I can’t thank you enough. As I did before, thanks for being my partner in this journey. We started this journey long time ago and has been rough, but here we are. I could not make it without your love and support. Thank you.

Dear son, thank you for bring light to my life.

To my Mom and sisters: Susana, Cami and Sole, for their unconditional love. Love you guys

Sin ustedes esto no hubiese sido posible. Los amo

He never said so, but with his example he taught me three important things:

Work hard, love what you do, and believe that there are no impossible dreams.

This dissertation is dedicated to the memory of my father, Jose Piñeyro.
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ATTRIBUTION

Several colleagues aided in the research behind one or two of my chapters presented as part of this dissertation. Other colleagues aided with the research or in vivo study for Chapter 2, 3 and 4. A brief description of their contribution is included here.

Chapter 2: Evaluation of the use of non-pathogenic porcine circovirus type 1 as a vaccine delivery virus vector to express antigenic epitopes of porcine reproductive and respiratory syndrome virus.

Scott P. Kenney, PhD: Is currently a Research Assistant Professor (Department of Biomedical Sciences and Pathobiology, Virginia Tech). Dr Kenney was a co-author in this paper and helped to develop the chimeric infectious clones and to perform the in vitro characterization.

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Chapter 3: Expression of antigenic epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) in a porcine circovirus type 2 (PCV2) modified-live vaccine virus (PCV1-2a) as a potential bivalent vaccine against both PCV2 and PRRSV.

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Chapter 4: Modulation of pro-inflammatory cytokines in monocyte-derived dendritic cells (MoDCs) by PRRSV through porcine intercellular-adhesion-molecule-3 (ICAM-3)-grabbing non-integrin (pDC-SIGN) interaction.

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C. Lynn Heffron, B.S: Is a laboratory Specialist Senior (Department of Biomedical Sciences and Pathobiology, Virginia Tech). Ms. Heffron was a co-author in this paper and helped with the development and hands on genes expression RT-PCR.
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CHAPTER 1

Literature Review

Introduction

Porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) are responsible for porcine multisystemic and wasting syndrome (PMWS) and porcine reproductive and respiratory syndrome (PRRS) in pigs, respectively. Both viruses, individually or during co-infection, have caused devastating diseases in the swine industry worldwide. Currently, there are commercial vaccines available against both viruses, although the vaccines against PRRSV are not effective against heterologous protection. These two entities are still clinically present and cause severe economic loses. The heterogeneity of PRRSV field strains and the emergence of new variants of PCV2 make necessary a constant review of the epidemiology and virology features related to immunogenicity and current approaches for vaccine strategies against these two agents.

PORCINE CIRCOVIRUS

General history and epidemiology

Porcine circovirus (PCV) was first described as a contaminant of porcine kidney cell line PK-15 in 1974 [1]. Serological studies showed that antibodies against PCV were circulating in Canada [2, 3], Germany [4], Great Britain [5], and Northern Ireland [6] demonstrating that PCV was widespread. However, despite multiple attempts to experimentally induce clinical disease with the PK-15 derived-PCV demonstrate, this virus was found to be non-pathogenic [7]. Studies using PK-15 derived-PCV failed to reproduce clinical disease in mini pigs, 1-day-old colostrum-
deprived pigs [7] and infections in gnotobiotic pigs also failed to produce lesions and clinical disease [8]. These observations lead to the conclusion that PCV was ubiquitous in nature and non-pathogenic in pigs. The first publication describing the complete genomic sequence of PCV reveals a 1759 nucleotides (nt), single-stranded DNA virus arranged in a circular genome with 11 open reading frames (ORFs) [1, 9]. During the early 90’s, reports from Canada described outbreaks of severe porcine multisystemic wasting syndrome (PMWS) [10]. Reports about this new entity rapidly arose from most pig producing countries around the world and were linked to the presence of PCV [11]. However, it was not until 1998 the clinical disease was associated with a new variant of PCV [12]. These findings also indicated that pigs were concomitantly infected with two phylogenetically-related but antigenically- and phenotypically- unrelated viruses. Therefore, a new classification was proposed to establish the non-pathogenic contaminate of PK-15 as PCV1 and the variant strain associated with PMWS as PCV2 [11]. Nowadays, PCV1 is still not implicated in any clinical disease, however PCV2 has been linked not only to PMWS but also to reproductive failure, respiratory disease, renal failure and congenital tremors and myocarditis in newborn piglets. All of these clinical conditions were designated as porcine circovirus diseases (PCVD) [13] or porcine circovirus associated diseases (PCVAD) [14, 15].

**Taxonomy and genomic organization**

Both PCV1 and PCV2 belong to the family *Circoviridae* which includes three genera: *Circovirus, Gyrovirus* and the newly proposed *Cyclovirus* [16]. The only known member of the genus *Gyrovirus* is the *chicken anemia virus* (CAV). Numerous circular PCV-like particles have been detected in humans and other farm animals, and it is believed that they can cross species barriers [17]. These PCV-like particles lack one intergenic region and differ in their stem loop compared to members of the *Circovirus* genus. Based on the genomic and phylogenetic
differences, it has been proposed for their inclusion in the new genus *Cyclovirus* [16]. Members of the genus *Circovirus* are host specific and currently known to affect a wide variety of birds [18-21], pigs [22] and dogs [23].

Two types of porcine circovirus (PCV), PCV1 and PCV2, have been characterized. Both are nonenveloped, single stranded circular DNA viruses approximately 1.7 kb in size. The genome is packaged in an icosahedral capsid of 17 nm in diameter and is considered the smallest virus infecting mammals. Analysis of the PCV2 genome predicts the existence of 11 potential ORFs with predicted coding capacity ranging from 2 to 36 KDa [24]. However, only two majors ORFs are necessary to complete the basic functions of the virus: ORF1 encodes for the full length replicase (Rep) (314 amino acids) and the truncated, spliced Rep’ protein (178 amino acids), and ORF2 encodes the capsid protein (233 amino acid). The ORF1 and ORF2 genes are oriented in opposite directions resulting in ambisense orientation. Between the 5’ end of ORF1 and ORF2, there is a small intergenic region containing the origin of the virus replication (Ori) characterized by a stem loop structure [25]. Another protein encoded by ORF3 in the antisense region of ORF1 has been reportedly associated with induction of apoptosis [26]. This protein specifically interacts with pPirh2 (porcine p53-induced RING-H2), an E3 ubiquitin ligase, increasing p53 expression and resulting in apoptosis [27]. Although, *in vivo* studies demonstrate that ORF3 is indispensable for replication in pigs, the real association of ORF3 with apoptosis and viral pathogenesis could not be experimentally verified [28]. Recently it has been demonstrated that ORF3 interacts with RGS16 and leads to upregulation and secretion of IL-6 and IL8 mRNA in PK-15 cells through NFκβ translocation into the nucleus [29]. The putative ORF4 is located within ORF3 in the same direction. The identification and functional analysis of a peptide (59 aa) produced by ORF4 demonstrated that this gene is not required for viral replication *in vitro* but suppresses caspase 3
and 8 activity and regulates CD4+ and CD8+ T lymphocytes during PCV2 infection [30]. The newly recognized putative ORF5 has been described at the transcriptional and translational level in PCV2 productive infection of porcine alveolar macrophages. The ORF5 is 180bp and overlaps completely with ORF1 when read in the same direction. The biological importance of ORF5 is still under investigation but there are data suggesting that this ORF5 protein upregulates IL-6 and IL8 [31].

Two main PCV2 groups have been described, PCV2a and PCV2b containing 8 clusters (1A to 1C and 2A to 2E). They have a slight difference in their genome size 1,768 nt for PCV2a and 1767 nt for PCV2b, and differ in a signature motif located between nt 1472 and 1486 of ORF2, which correlates to a six amino acid difference in the capsid protein [32]. Although there is a structural difference, PCV2a and PCV2b do not have differences in pathogenicity [33]. Due to the appearance and identification of new PCV2 variants, a unified nomenclature for PCV2 genotypes has been proposed. Using this methodology, the PCV2 ORF2 sequences are assigned to different genotypes when the genetic distance between them is 0.035 [34]. Based on this new classification, additional less prevalent PCV2 subtypes were identified including PCV2c which has been found in archived samples from pigs in Denmark and feral pig in Brazil [35, 36], and PCV2d and PCV2e which have been described in China and Thailand [37, 38]. Moreover, a recent global genetic analysis of known PCV2 strains showed that the four proposed subtypes are widespread worldwide and there has been an increase in prevalence of PCV2d in North America and China [39]. During the last few years, the emergence of a new mutated PCV2 (mPCV2) has been linked to clinical cases of PCVAD in vaccinated herds in the United States [40]. This new strain has an elongation of ORF2 and a mutation of the stop codon leading to the expression of an extra amino acid. These mutations have been observed in cases in United States [41] and China [38, 42] and the viruses
seem to be more virulent than the classic PCV2a and PCV2b. Theoretically, PCV2 nucleotide substitution is the highest among the single-stranded DNA viruses (1.2 × 10⁻³ substitutions/site/year), which is comparable with single-stranded RNA viruses [43]. The emergence of chimeric viruses containing combination of multiple PCV2 subtypes has been described. Thus, recombination of the intergenic region results in new recombinant variants. All these observations might support the idea that PCV2 undergoes genetic shift with the potential emergence of new immunogenic and pathogenic genotypes.

Clinical Presentation

Since the first description of PCV2 as the causative agent of post weaning multisystemic wasting (PMWS) [11], this virus has been associated with numerous disease syndromes [44]. International consensus is still needed regarding a nomenclature that can integrate all these clinical manifestations associated with PCV2. Collectively, in Europe, the term “porcine circovirus diseases (PCVDs)” is used. In October of 2006 the American Association of Swine Veterinarians (AASV) proposed the name “porcine circovirus associated diseases (PCVADs)” (http://www.aasp.org/aasv/position-PCVAD-htm) (accessed November 30, 2014). Although the main focus of research has been systemic PCVAD, also known as PMWS, PCV2 can also be subclinical and has also been implicated as a potential causative agent of porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC) [45], necrotizing pneumonia (PNP) [46], enteritis [47], reproductive failure [48, 49] and neuropathy [50].

Subclinical infection

The high serological prevalence without clinical presentation of systemic PCVAD [44], and the detection of PCV2 in retrospective studies, even before the first description of PMWS [51],
suggest that PCV2 subclinical infection is highly prevalent. Due to the low prevalence of clinical cases compared to the high prevalence of PCV2 detection; three criteria are necessary for the PCVAD diagnosis: compatible clinical signs, characteristic microscopic lesions and presence of PCV2 within the pathological lesions [52].

The data about interference with vaccine efficacy in subclinically-infected animals is still controversial. It has been proposed that the efficacy of PCV2 vaccines is impaired in subclinically-infected animals [53] but no detrimental effect has been observed against pseudorabies vaccine [54].

**Systemic porcine circovirus associated diseases (S-PCVAD)**

S-PCVAD or PMWS was one of the first described and recognized manifestations of PCV2 infection [12]. This PCV2 presentation affects mainly grower-finisher pigs from 5 to 16 weeks of age [55]. Morbidity varies from 4 to 60% and mortality can range from 4 to 20% depending on farm type, husbandry and co-infections. This epidemiological distribution is believed to be the result of declining levels of maternally-derived antibodies [56]. S-PCVAD-affected animals are characterized by weight loss, skin pallor or jaundice, enlarged lymph nodes, dyspnea and diarrhea. Less frequent clinical signs also include coughing, pyrexia, and sudden death. Gross changes include enlarged superficial lymph nodes, non-collapsed lungs, gastric ulcers, colonic edema, and white spotted kidneys are commonly observed [57-59]. The histological features are a characteristic diagnostic criteria of S-PCVAD. Lymph nodes present a marked follicular and paracortical lymphoid depletion associated with infiltration of histiocytes and/or multinucleated giant cells. Lymphoid depletion associated with granulomatous inflammation can be seen in most of the lymphoid tissues; including Peyer patches, spleen, thymus, and tonsils [59]. Histiocytes in granulomatous lesions can also have basophilic intracytoplasmic botryoid inclusion bodies [58].
It has been described that severity of the lymphoid depletion is correlated with the amount of PCV2 detected in affected tissues [60]. Lymphoid depletion caused by PCV2 has been linked to lymphopenia, specifically depletion of peripheral CD8+ and CD4+-CD8+ double positive T lymphocytes and B lymphocytes [60, 61]. Other histological changes include multifocal to coalescing lymphoplasmacytic interstitial nephritis, lymphoplasmacytic periportal hepatitis and interstitial pneumonia [58]. Depending on the lesion’s chronicity, PCV2 antigen or nucleic acid can be detected by immunohistochemistry (IHC) or in situ hybridization (IHS) in affected tissues [14, 62].

**Porcine dermatitis and nephropathy syndrome (PDNS)**

PDNS primarily affects growers and finisher pigs but can also affect adult animals. The prevalence of PDNS is 1-2%, but the mortality is higher, reaching 100% in pigs more than 3 month-old and 40-50% in growers [63]. Clinical signs are non-specific; anorexia, weight loss, depression, and normal temperature to mild pyrexia. The onset of the clinical symptoms is characterized by well demarcatied to coalescing red-to-purple macules and papules located on the hind limbs and perineal region. As the disease progresses, lesions have a generalized distribution of dark-red depressed centers covered by serocellular crusts that fade in 2-3 weeks in animals that survive the disease, leaving cutaneous scars [64]. The kidneys are normally enlarged and have cortical petechia. Other gross abnormalities include subcutaneous edema and enlarged and hemorrhagic lymph nodes. Histological lesions are characterized by systemic fibrinonecrotic vasculitis, necrotizing glomerulitis and interstitial nephritis with occasional intratubular proteinaceous casts [65]. Glomerular sclerosis, interstitial fibrosis and tubular atrophy can also be seen in chronically-affected animals. Skin lesions are characterized by dermal and epidermal necrosis associated with vascular necrosis, thrombosis and lymphoplasmacytic perivasculitis [64].
The attribution of the pathogenic role of PCV2 has not been totally demonstrated, however PCV2 seems to be necessary but not sufficient for inducing PDNS manifestation [45]. Moreover, it has been demonstrated that PCV2 viral load is not a decisive factor for the presence of PDNS. Previous studies showed animals with PDNS or mild lesions of systemic-PCVAD had no difference in PCV2 viral load [66]. Although, the pathogenesis is not totally understood, the presence of immunoglobulin and complement factors in affected vessels and glomeruli suggest a typical type III hypersensitivity reaction. It has been observed that kidneys from PDNS-affected animals have increased levels of IgG1 + IgG2 and IgM, complement factors C1q and C3, and increased numbers of CD8+ lymphocytes [65].

Multiple cofactors associated with the presence of PCV2 have been postulated as causative agents of PDNS. In naturally infected animals, PCV2 and PRRSV, have been detected and isolated from affected tissues [67]. Numerous other pathogens such as Staphylococcus hyicus, Actinobacillus pleuropneumoniae, Pseudomonas spp, and Pasteurella multocida [68, 69] have been isolated from tissues with PDNS concomitant with the presence of PCV2. However, the exact role of PCV2 in the development of PDNS is still under debate. Recent studies demonstrate that lesions resembling PDNS can be reproduced with PRRSV and Torque Teno Virus (TTV) without the presence of PCV2 [70].

**Respiratory porcine circovirus associated disease (R-PCVAD)**

Porcine respiratory diseases complex (PRDC) is a multifactorial entity that affects pigs from 8 to 22-weeks of age [14]. Numerous agents have been proposed as causative agents, such as PCV2, swine influenza virus (SIV), PRRSV, pseudorabies virus (PRV), porcine respiratory coronavirus (PRCV), Mycoplasma hyopneumoniae, Pasteurella multocida, Streptococcus suis, and Actinobacillus pleuropneumoniae [71]. However, it is considered that PCV2 may play an
important role not only as a primary pathogen but also predisposing to secondary infections [72]. Clinical signs are characterized by dyspnea, cough, lethargy, anorexia and fever. Common gross lesions are characterized by interstitial pneumonia with interlobular septal edema and major airway edema. The presence of these gross changes can be seen either in R-PCVAD or S-PCVAD, an indicative of an overlap between these two PCV2 presentations [15]. Differentiation between these two clinical presentations is based on histological findings. Characteristic lesions are histiocytic or granulomatous bronchointerstitial pneumonia, peribronchiolar fibroplasia and necrotizing bronchiolitis [14, 72] without the pathognomonic lymphoid lesions observed in S-PCVAD. PCV2 can be detected in interstitial macrophages and bronchiolar lamina propria by IHC and HIS. PCV2 has also been linked with a more severe form of necrotizing interstitial pneumonia (PNP) in association with PRRSV, PRV and less frequently SIV [46, 73]. Histological lesions observed in PNP are characterized by large numbers of coagulates of necrotic cells with acidophilic and granular debris within alveolar spaces and marked hypertrophy and proliferation of type 2 pneumocytes with varying degrees of lymphohistiocytic interstitial inflammation [46]. Although numerous reports suggest the possibility of an overlap between S-PCVAD and R-PCVAD [14, 44, 71], recent studies strongly indicate these are not two separate entities and PCV2 mainly contributes to PRDC in relation to PCV2-SD occurrence [74].

**Enteric porcine circovirus associated disease (E-PCVAD)**

Pigs 16- to 22-weeks old can present with clinical diarrhea caused by PCV2 infection of the intestinal tract. Clinical signs are non-specific and can be confounded with other enteric diseases affecting growers and finisher pigs. Diarrhea is yellow and can progress to dark-red, and is associated with growth retardation and wasting. Morbidity varies from 10-20% and mortality is 50-60% [75]. E-PCVAD case definition is still controversial; however, gastrointestinal lesions
have to be associated with the presence of PCV2 and no other S-PCVAD characteristic lesions and no PCV2 has to be detected [15, 76]. The diagnosis of E-PCVAD is appropriate when (1) there is clinical diarrhea, (2) the hallmark microscopic lesions are present in Peyer’s patches but not in other lymphoid tissues, and (3) PCV2 DNA or antigen can be demonstrated within lesions [75]. Gross lesions are characterized by enlarged mesenteric lymph nodes and thickness of the intestinal mucosa. Histological lesions in E-PCVAD are granulomatous inflammation of the Peyer’s patches characterized by lymphoid depletion and replacement of follicle architecture by macrophages, histiocytes and occasional multinucleated giant cells. The lamina propria and submucosa can also be diffusely infiltrated by macrophages and multinucleated giant cells [77]. Large, multiple, basophilic or amphophilic, grape-like intracytoplasmic inclusion bodies are often seen in the cytoplasm of histiocytes and multinucleated giant cells [47, 75]. PCV2 antigen can be detected in histiocytes on Peyer’s patches, submucosa and crypt epithelium by IHC and HIS [14, 78]. Numerous concurrent infections have been observed concomitant with PCV2 infection. The histological and clinical features of the coinfection between PCV2 and Lawsonia intracellularis have been widely studied [78-80]. Other pathogens such as Salmonella typhimurium have also been observed in field and experimental studies associated with PCV2 [47, 79].

Reproductive failure porcine circovirus associated disease (RF-PCVAD)

Reproductive failure associated with PCV2 was first reported in Canada in 1999 [81]. Since the initial description, naturally occurring reproductive failures associated with PCV2 have been widely reported [82-86]. Moreover, experimental studies have also linked PCV2 to reproductive failures [87-89]. Although the first reports of S-PCVAD from Canada are dated back in 1991, retrospective studies failed to demonstrate the presence of PCV2 in fetus tissues in cases of abortion from the period 1955-1998 [82]. Clinical signs of RF-PCVAD are abortions associated
with increased rates of mummified, macerated, stillborn and weak-born piglets [81, 85]. However, these clinical presentations can also be the result of numerous infectious and no infectious causes. Infectious agents including PRRSV, porcine parvovirus (PPV), porcine pseudorabies virus (PRV), and porcine enterovirus can cause almost identical clinical presentations. Non-infectious causes such as mycotoxins, environmental changes or nutritional imbalances might also result in reproductive failures. Coinfection with other pathogens might occur, and numerous pathogens such as PPV[85, 90, 91], PRRSV [83, 91], PCV1 [90], have also been reported in association with PCV2; however the significance of these findings is still unknown. PCV2 does not affect specific gestation periods, and virus antigen has been detected in early gestation associated with embryonic death, irregular returns to estrus [84, 87], mid gestation associated with mummified fetuses and abortions [84], and late gestation associated with mummies, stillborn, weak-born piglets, and delay in farrowing [85, 89, 92]. Histologic lesions are normally observed in the fetus heart, characterized by lymphocytic infiltration and occasional fibrosis. PCV2 antigen can be detected in the myocardium but can also be observed in other tissues such as liver, kidney and lung [81, 82]. Numerous studies have attempted to demonstrate a relation between different PCV2 strain and reproductive failures. Experimental infection of fetuses by intrauterine inoculation [93] or artificial insemination with PCV2 contaminated semen [48, 94] isolated from cases of RF-PCVAD, S-PCVAD, and PDNS, showed no differences in virus replication, tissue tropism and clinical outcome. Boars can be infected with PCV2 with subsequent viral seeding in semen [95-97]. These studies concluded that semen could be an important source of viral dissemination.
PCV2 Pathogenesis and host immune response

Innate immune response

During the early infection, PCV2 has a close interaction with monocytes, macrophages and dendritic cells (DCs) [62]. Probably the immune modulation observed during the course of the disease is the result of an early interaction of PCV2 with antigen presenting cells (APCs). It has been demonstrated that PCV2 can persist in DCs without evidence of virus replication, loss of infectivity or changes in cell viability. PCV2 antigen can persist for several days post-infection (dpi) in DCs leading to the conclusion that PVC2 uses DCs infection as a mechanism for viral spread and transmission [98], and the presence of viral particles in those cells is the result of phagocytic or endocytic activity [99, 100]. Although, there are reports of lymphocytes-carrying PCV2, this has been considered a transient effect due to DCs-lymphocyte aberrant cross talk induced by PCV2-DCs association [101]. No impairment of humoral and cytotoxic response [102-104] has been observed in clinically-affected pigs, leading to the conclusion that infection of DCs does not affect immune regulation of lymphocytes by DC cells.

Internalization without infectivity has also been observed between PCV2-pulmonary-alveolar-macrophages (PAMs) and PCV2-monocyte–derived macrophages (MdM) [105]. However, the production of different cytokines in these cells is affected by PCV2 infection. The expression of IL-8 and TNF-α in PAMs, is up-regulated after PCV2 infection [106] in association with the increase of mRNA expression of macrophage-derived neutrophil chemotactic factors-II (AMCF-II), granulocyte colony-stimulating factor (G-CSF), and monocyte chemotactic protein-1 (MCP-1) [106, 107]. In splenic lymphocytes, PCV2 infection decrease IL-4 and IL-2 levels [108] and increases IL-12 [109]. There is also downregulation of IFN-γ, IL-2, IL-4 and IL-12 expression in secondary lymphoid tissue [110]. PCV2 infection of PBMC is also responsible for modulating
pro inflammatory cytokines. In PMBC isolated from PCV2-infected animals, IFN-γ and IL-2 production is impaired [111]. Upregulation of proinflammatory cytokines IL-1β and IL-8 has been observed in PBMC from PCV2-infected pigs [110]. In conclusion, PCV2 can interact with APCs and modulate the immune response without active cellular replication.

In addition, PCV2 infection modulates cytokine profiles, which is an important part of the pathogenesis and development of clinical disease. The increase of IL-10 is a common finding in tissues and PBMC of S-PCVAD affected animals [108, 110-113]. Overexpression of IL-10 mRNA in multiple lymphoid tissues and cytokine production are important in T-cell rich areas and occasionally in B-cell and macrophages [113]. Interestingly, this IL-10 stimulation cannot be achieved with Cap or Rep proteins but rather the whole virus is necessary [112]. In vivo studies have demonstrated that IL-10 is transiently increased in serum that is correlated with the viremic phase observed in subclinically infected animals [114].

Adaptive immune response

Cell-mediated immune response

Studies of cell-mediated immune responses in PCV2 subclinically-affected animals are scarce. IFN-γ gamma secreting cells (IFN-γ-SC) are the major focus of study in the role of PCV2 and cellular immune response in subclinically-infected animals. Studies in cesarean-derived and colostrum-deprived pigs (CD-CD) showed that after peak viremia, coincident with the decreasing serum viral load, there is an increase of PCV2-specific IFN-γ-SC [115]. These findings support the notion that viral clearance is also due to the contribution of specific IFN-γ-SC in addition to virus neutralization [116]. In addition, CD4+ and CD8+ lymphocytes seem to play a role in the presence of specific IFN-γ-SC. Depletion of these T-lymphocytes subsets impairs specific IFN-γ-
SC [103]. Lymphocytic depletion and histiocytic infiltration are the main cellular response observed in clinically affected animals.

The role of the cell-mediated immune response during clinical presentation is still unclear; however, the lymphocyte impairment observed during clinical presentation suggests its contribution in the immunity against PCV2. High PCV2 replication viral load leads to impairment of neutralizing and T-cell response [102, 104, 117]. The upregulation of IFN-γ-mRNA observed in PBMC in clinically affected pigs also suggests the importance of IFN-γ-SC. Studies of the cell-mediated immune response after vaccination showed that the main cellular component is associated with specific IFN-γ-SC induction in response to the Cap protein of PCV2 [118, 119].

**Humoral response**

**Immunosuppression induced by PCV2**

It is known that PCV2 affects lymphoid tissues, causing lymphoid depletion followed by granulomatous inflammation with a subsequent immunosuppression during the course of the clinical disease [52, 58, 120]. These histological changes are normally associated with high amounts of PCV2 nucleic acid in affected lymphoid tissues. Lymphoid depletion affects a wide variety of lymphoid cells such as B cells, NK cells, γδ T cells, CD4+ and CD8+ T lymphocytes, and interfollicular dendritic cells (DCs) [60, 121]. Although, B and T lymphocytes are the most severely-affected cells in lymph nodes; PCV2 nucleic acid and antigen have been found infrequently in lymphocytes [122], instead resident macrophages and APC’s showed intranuclear and intracytoplasmic PCV2 signals [123]. However, studies demonstrate that PCV2 can be internalized without evidence of replication in DCs, macrophages and monocytes. The same studies also showed that PCV2 remains infectious and does not induce cell death [98, 105]. Virus replication and cell death were not observed in co-cultured DCs and lymphocytes infected with
PCV2. Therefore, it has been postulated that APC can play a role in viral immune evasion [98]. Immune suppression has also been associated with the presence of lymphopenia in clinically S-PCVAD animals, characterized by depletion of CD8^+ and CD4^+ CD8^+ lymphocyte subsets [60]. The effect of PCV2 in lymphocyte populations is still unknown, and several theories have been proposed such as reduced production in bone marrow, reduced proliferation in secondary lymphoid tissues and induction of apoptosis [14, 61, 124]. Based on in vitro data and supported by a mouse model, PCV2 ORF3 has been proposed to be responsible for lymphoid apoptosis [26, 27]. Upregulation of several key factors in the induction of apoptosis has been linked to PCV2 infection. In vitro experiments in PK-15 cells showed an increase on porcine p53 expression and resulting in apoptosis [27]. PCV2-ORF3 has also been associated with induction of NF-κβ in PK-15 cells and lymphocytes leading to apoptosis [29], and upregulation of Fas/Fas ligand activity in a PCV2-PRRSV coinfection model. However, the role of apoptosis induced by PCV2 is also still debatable, studies showed that apoptotic rate is inversely correlated with the amount of PCV2 in lymphoid tissues [124]. Experimentally infected pigs with ORF3 deficient virus did not show differences in lymphoid histological lesions compared to piglets infected with PCV2 wild-type [28]. Finally, there is a report that compares lymphoid turnover rate against the apoptotic rate in PCV2 infected animals, which concludes that lymphoid depletion likely occurs due to detriments in cellular proliferation but not apoptosis [125]. PCV2 can also interfere with lymphocyte B growth factor IL-4, and T cell and macrophage activator IL-2 [108]. This might interfere with lymphocyte proliferation and interferon activity, increasing pro-inflammatory cytokines (IL-1 and IL-8) [126]. Another factor that supports the immune suppression produced by PCV2 is the fact that cytokine production is impaired during the acute infection. PCV2 cannot replicate in DCs [98]; nevertheless, it has been demonstrated that viral infection can affect functionality of plasmacytoid dendritic cells
(pDCs) [127]. The presence of viral DNA, but not viral replication, is sufficient to impair the induction of TNF-α, IFN-α, IL-12 and IL-6 [126] in pDCs. In addition to impairment of cytokines during PCV2 infection, immunosuppression is supported by the fact that IL-10 is up-regulated in PBMCs, which is responsible for Th1 suppression [113]. In a coinfection model, DCs were infected with PCV2 or dually infected with PCV2 and PRRSV. The results showed an increase in T-reg activation, and its induction is likely due to IFN-β production but not IL-10 [128]. All these data lead to the notion of the immune deficiency induced by PCV-2 infection in cases of S-PCVAD.

Although, available information supports the role of PCV2 in immunosuppression in S-PCVAD; it is also known that the presence of PCV2 is necessary but not sufficient for the development of S-PCVAD. Multiple attempts to reproduce PCV2 clinical disease with PCV2 infection alone has failed. Therefore it is possible that PCV2 needs a cofactor that helps to develop clinical presentation. Originally immunosuppression was proposed; however steroid-induced immunosuppression has not been a successful model to reproduce clinical disease. Field studies demonstrate there was a high correlation with PMWS clinical presentation and the presence of PCV2 and multiple coinfections such *Mycoplasma hyopneumoniae, Pasteurella multocida, Lawsonia intracellularis, Salmonella spp. and PRRSV, PPV, SIV PSR PEDV, TTV*. Experimentally coinfections with multiple bacteria and viruses have been used to reproduce clinical disease. Probably the most efficient and widely used coinfection model is PCV2-PPV. The importance of this model relies not on immunosuppression as was originally stated but rather immunomodulation [55, 129, 130]. PCV2-*Mycoplasma hyopneumoniae* is another experimental coinfection model. However, *Mycoplasma hyopneumoniae* adjuvant is likely the key factor inducing immunomodulation and enhancing PMWS manifestation. Interaction amongst multiple
pathogens naturally occurs on pig farms; and amongst those the most devastating clinical presentation is observed during PCV2 and PRRSV infection causing severe respiratory disease with a high mortality rate.

**Vaccines**

Currently the vaccines available on the market are subunit-vaccines, inactivated virus vaccines based on the PCV2a or chimeric PCV1-2a genotype. Inactivated PCV2a virus vaccine was the first type of vaccines commercially available [131]. This vaccine provides protection in grower animals by passive antibody transfer, when pregnant sows are vaccinated 3-4 weeks before farrowing. In order to provide a good level of passive immunity, gilts require two doses of vaccine. Inactivated PCV2a vaccines also confer protection to grower animals, reducing clinical signs, ADWG and feed conversion, when the vaccine is administered to animals older than three weeks of age [132]. Recent studies comparing inactivated PCV2a vaccine, and baculovirus-expressed PCV2a cap protein vaccine demonstrated that inactivated virus vaccine confers better passive immunity [133].

Baculovirus has been successfully used to express the PCV2a capsid protein as a subunit vaccine and to induce protection against PCV2a [134]. The subunit vaccine can induce immunity without the risk of adverse reactions that might be observed with inactivated vaccines. Although, researchers have tried numerous attempts with subunit vaccines in breeding stock, they is currently only indicated as a single dose in weaned piglets [133]. In a clinical trial performed in a large-scale PMWS-positive farm, production parameters such as morbidity, mortality rate, ADG, and feed conversion were improved with a single dose administration of this vaccine. In the same trial, there was also a reduction in viral load and virus shedding in infected animals. These effects were
correlated with activation of humoral and cell-mediated immune responses in vaccinated animals [135]. Subunit vaccines carrying PCV2a cap protein were able to confer humoral protection against multiple strains from different geographical regions [136]. With the appearance an sudden increment in prevalence of mPCV2b [137] there was concerns over efficacy of current PCV2a-based vaccine. In order to produce a broad protective vaccine, the subunit vaccine produced using the alphavirus replicon (RP) expression system has been evaluated. Previous study successfully demonstrate the used of the alphavirus vaccine vector platform to induce immunity against African swine fever virus, and swine influenza classical [138, 139]. Studies comparing the efficacy of PCV2a-based vaccine and experimental alphavirus mPCV2b-based vaccine sowed that both vaccines are capable of reduce the viremia in pig experimentally infected with mPCV2b [137]. Moreover the survival rate in animals vaccinated with PCV2a-based vaccine and experimental alphavirus mPCV2b-based vaccine was 85.7% and 57.1% respectively compared with 28.6% of positive controls [137].

The first USDA-fully-licensed vaccine available on the market was a PCV1-2a chimeric inactivated virus. In this vaccine, the cap region of PCV2a was cloned into a PCV1 backbone. Thus, animals are exposed to a whole virus but only the cap belongs to a pathogenic strain. Experimentally, it has been demonstrated that the PCV1-2a chimeric vaccine can reduce histological lesions and clinical signs associated with PMWS in the presence of maternal antibodies [140]. The reduction of viremia and clinical signs observed in experimentally infected animals is attributed to the level of neutralizing antibodies induced by the chimeric PCV1-2a vaccine [141]. A newly reformulated chimeric PCV1-2a inactivated virus vaccine successfully induces neutralizing antibodies, and also stimulates IFN-γ-SCs, improving the cell-mediated immune response [142]. In a clinical trial in PMWS-positive farms, there was dramatic
improvement of clinical signs, mortality, ADWG, antibodies against PCV2, virus load and PMWS-associated lymphoid microscopic lesions [141]. No maternal antibody interference was observed, and reduction of clinical signs associated with coinfection was also improved with a single dose of vaccine to weaned piglets [143]. Recently, the administration of live chimeric virus demonstrates an ability to induce protective immunity against PCV2a in experimentally infected pigs [144].

Despite the already efficacious and successful vaccines available on the market, there have been numerous attempts to generate new vaccine systems for PCV2 protection. Bacterial vectors, expressing Cap protein, such as E. coli [145], Lactococcus lactis [146] or yeast such as Saccharomyces cerevisiae [147] have been investigated. Numerous viral vectors, such as recombinant adenovirus [148] and recombinant pseudorabies virus [149], both expressing the cap protein of PCV2, demonstrated the ability to induce specific antibodies. Unfortunately, most of these systems have only been tested in mice and there are no data to support the functionality of these systems in pigs. An infectious molecular DNA clone capable of reproducing clinical signs, lesions and an immune response similar to those observed with natural infection, leads to the hypothesis that DNA vectors could be used to induce immunity in pigs. Different inoculation routes have been evaluated, such as intrahepatic, intra-lymph node and intramuscular, and developed lesions similar to natural infection [150]. Thus, DNA plasmids containing two identical copies of the chimeric PCV1-2a ligated in tandem have been used as a delivery system to induce immunity. Virulence was attenuated and good levels of neutralizing antibodies were observed in this experimental trial [151]. This type of vaccine has the advantage of easy plasmid storage and manipulation.
The most promising technology is a modified live-attenuated vaccine, constructed with a PCV2a cap region inserted in a PCV1 backbone [144]. These live-attenuated vaccines have overcome the potential problem of reversion to a wild type, as opposed to most of the traditional attenuated vaccines. This construct proves to be genetically stable after several passages in vitro and in vivo, and is capable of inducing humoral and cellular immunity as well. Animals challenged with PCV2a and PCV2b showed the same level of protection, preventing clinical signs, and viral shedding, after vaccination with this live attenuated vaccine. Experimental vaccines based on the PCV2b capsid inserted in PCV1 backbone have also been shown to confer good protection in animals infected with PCV2b, or animals dually infected with PCV2b-a strains [152, 153]. In this experimental trial, the vaccine demonstrates the ability to improve the level of antibodies and decrease viral shedding, not only in animals infected with PCV2b but also in those dually infected with PCV2a and PCV2b [154].

Traditionally, the most prevalent PCV2 strains have been PCV2a and PCV2b. Farms have been affected by PCV2a, but during the last decade there has been a genetic shift and PCV2b is now the most prevalent subtype [155]. Despite the fact that there is no data supporting that PCV2b is more pathogenic than PCV2a, the emergence of this strain has been associated with more severe clinical cases [156]. The existence of a PCV2c has been documented in archived tissues in Denmark [35]. Moreover new genotypes [37, 38] are being described and demonstrate that they can co-exist even within the same farm [157]. Finally, a description of a PCV1-2a natural chimeric virus has been described in Canada [158]. Although there is information that PCV1-2 chimeric vaccines, either containing PCV2a or the PCV2b cap region, can confer heterologous protection, there are no reports regarding vaccine protection against some of the newly emerged strains such as PCV2d or PCV2e. Based on this new global genetic map, where multiple strains have emerged
and displaced the traditional strain, and the fact that some of these strains can coexist within the same farms and reduce the effectiveness of homologous vaccines, there is a necessity for a broader vaccine that can provide protection against multiple PCV2 strains.

**PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS**

**General history, clinical presentation and epidemiology**

Porcine reproductive and respiratory syndrome (PRRS) first emerged in the late 80’s as a mysterious disease progressing simultaneously in Europe and North America [159-161]. PRRS virus (PRRSV) was identified for the first time in Europe in 1991 and designated as Lelystad virus (LV) [162], which is considered the European prototype of PRRSV. Subsequently the virus was identified in the United States [160]. Clinical description of cases in Europe and North America followed the same pattern, characterized by increases in mortality during early nursery associated with pneumonia and increases in reproductive failures in pregnant sows and gilts, including mummified, stillborn, and aborted fetuses [163, 164]. Genetic analyses of both viruses showed high genetic divergence despite similar clinical presentations in Europe and North America. PRRSV is currently classified into two distinct genotypes, type 1 (European) and type 2 (North American); and type 2 is further subdivided into at least 9 distinct genetic lineages [165-167]. Both types share approximately 60% of nucleotide sequence, but they are antigenically different [168-171]. Moreover, within the same genotype, high genetic variability has been reported [171, 172]. It is estimated that the PRRSV mutation rate is approximately $10^{-2}$/site/year, which is higher than any of the other known RNA viruses ($10^{-3}$ to $10^{-5}$/site/year) [173]. Due to reversion to virulence of the available live-attenuated vaccine, the type 2 U.S. strain is now present in Europe [174]. Previously the LV strain was only confined to Europe; however importation of pigs from Europe
to Canada allowed widespread dissemination of the European type 1 strain in Canada [175].

PRRSV is considered endemic in most swine producing-countries which might allow PRRSV to evolve into new virus variants. Despite all the efforts during the last 20 years to control this disease, there is not yet a universal vaccine that can protect against the diversified field strains. Vaccination commonly confers good protection against homologous strains; however, the heterogeneity of strains present in the field is detrimental for the development of efficient broadly-protective vaccines [176, 177]. The extensive heterogenic nature of PRRSV presents challenges for the efficacy of current commercial vaccines which are all based on a single virus strain isolated many years ago, and consequently the current available vaccines confer only a limited level of cross-protection against heterologous PRRSV strains [178-180]. PRRS is still clinically present and is considered one of the most economically devastating diseases affecting the swine industry.

**Taxonomy and genomic organization**

PRRSV is a single-stranded, positive-sense RNA virus of approximately 15 Kb that belongs to the family *Arteriviridae* (genus *Arterivirus*) [181], with another three members of this family: lactate dehydrogenase-elevating virus (LDV) (mice), equine arteritis virus (EAV) (horses and donkeys), and simian hemorrhagic fever virus (SHFV) (monkeys). The genome consists of nine open reading frames (ORFs): ORFs 1a, 1b, 2a, 2b, 3, 4, 5, 6, and 7. The 5’ three-quarters of the genome consists of two slightly overlapping ORFs, 1a and 1b, which are translated directly from the genome-sense RNA.

PRRSV has adapted a discontinuous transcription strategy to synthesize a nested set of subgenomic mRNAs (sg mRNAs) which possess the same 5’-UTRs and 3’-UTRs with the genomic RNAs [182]. Replicase ORF1a and ORF1b, via ribosomal frameshift-mediated translational
reprogramming, generate nonstructural proteins (nsps) that direct viral genome replication and sg mRNA synthesis [183]. The structural protein-encoding region produces glycoprotein GP2a (encoded by ORF2a), the envelope proteins E (ORF2b), GP3 (ORF3), GP4 (ORF4), GP5 (ORF5), membrane protein M (ORF6), nucleocapsid protein N (ORF7) and recently identified small hydrophobic protein ORF5a (ORF5a) [166, 184-186]. These structural proteins are critically important for the viral replication life cycle and for inducing protective immunity.

GP5, the major envelope glycoprotein, contains 3-4 N-linked glycosylation sites and a neutralizing epitope which may induce protective immunity [187-189]. In viral particles, GP5 forms heterodimers with the M protein by interacting with cellular heparin sulfate involving virus entry into cells [190]. GP5 is also the most variable structural protein showing only about 90% nucleotide sequence identity among type 2 PRRSV strains and about 50% identity between type 1 and type 2 PRRSV [191-193]. The GP5 has been extensively studied as a target for PRRSV vaccine development. Several minor envelope proteins (GP2a, GP3, GP4 and E) form different oligomeric complexes containing abundant N-linked glycosylation sites and induce neutralizing antibodies [194-196]. Recent studies have shown that the minor envelope proteins play an important role in determining the cell tropism by interacting with the cellular receptor CD163 [197, 198].

**PRRSV pathogenesis**

Viruses of the family *Arteriviridae* possess certain common characteristics related to viral pathogenicity. These viruses are characterized by chronic or persistent infection, and cytopathic effect in macrophages causing severe disease. PRRSV specifically, is host and tissue specific restricted to swine cells of the monocyte lineage including pulmonary perivascular macrophage,
subset of macrophages of lymph node, and spleen, and intravascular macrophages of the umbilical
cord and placenta [199-201]. Although PRRSV is considered to cause a chronic infection, one of
the major clinical manifestations develops acutely after infection in naïve pigs. Respiratory disease
is the result of close viral interaction with pulmonary macrophages resulting in cytokine storm
with consequent pulmonary damage [202]. The chronic or persistent stage is characterized by
lower viral replication in just a few organs. After entering the respiratory system, the virus is
carried to the local lymph nodes and tonsils by local macrophages and dendritic cells resulting in
severe viremia [164]. Viral titers in serum increase and reach a maximal concentration
approximately 7 to 14 days post-infection (dpi) followed by a sharp decrease with a complete
resolution approximately four to six weeks post-infection [203-205]. Virus replication can be
detected in tissue twelve hours after infection. The highest viral replication has been observed in
pulmonary macrophages, retropharyngeal lymph nodes, bronchial lymph nodes and thoracic aortic
lymph nodes [200].

Clinical manifestations of reproductive failure vary by gestational age of affected fetuses
at the time of infection. PRRSV infection can cause mummified and weak-born piglets, elevated
pre-weaning mortality and late-term abortion [206]. PRRSV infection during the onset of the
gestation does not affect fertilization or conception rates [207, 208]. Infection during the first and
second trimester might result in a low number of infected fetuses or high number of still-births
[209, 210]. PRRSV can cross the placental barrier more specifically during the third trimester of
gestation [206, 211]. Systemic clinical signs in sows and gilts vary from none to mild anorexia,
lethargy and fever [164, 212]. In boars, the virus can be found in testis and semen with consequent
temporary decrease of semen quality and viral shedding in semen [213]. Systemic signs are not
evident, but can occasionally be associated with mild fever, anorexia and libido reduction [214].
**Immunology**

In order to understand the immune modulation exerted by PRRSV, it is necessary to review the major factors that play a role in the immune response against PRRSV. Probably the most important factors that define protective immunity against PRRSV are the levels of virus neutralizing antibodies and presence of IFN-γ production.

During the acute infection phase, antibodies are produced rapidly against viral infection. Specific antibodies (IgM) against PRRSV can be seen approximately 14 dpi and last approximately 5 weeks, whereas the peak IgG concentration is approximately 21 dpi and last for 3-4 weeks [215-217]. However, early IgG antibody production does not correlate with production of neutralizing antibodies. NA antibodies against the major structural component, GP5, do not appear in circulation until 28 dpi. Other studies also proved that early immune response included antibodies against the N and M proteins, and also nsps proteins more specifically nsps 1 and 2 [217-219]. The early presence of no-specific antibodies seems to play a major role in viral infection. Non-neutralizing antibodies can serve as a Trojan horse providing a coating that enhance viral internalization and replication in alveolar macrophages [220]. The epitopes responsible for inducing antibodies associated with enhancement in internalization are located in the N protein and GP5 [205, 221, 222]. The importance of NA antibodies has been proved in numerous experiments where administration of serum from convalescent animals, has been shown to block placental viral passage, prevent reproductive failures, and prevent clinical signs and viremia in growers pigs [223, 224].

The host cell-mediated immune response is the other component of the immune response modulated by PRRSV. Numerous studies demonstrate that T-cell response appears approximately 4 weeks after infection and is directed against GP5, M and N proteins and is concomitant with the
appearance of NA antibodies [225]. Additional studies demonstrated that concentration of interferon-\(\gamma\) producing lymphocytes is low during acute PRRSV infection. Thus, lymphocyte response during PRRSV infection is considered weak, transient and variable amongst strains. Vaccination with a MLV showed there is an increase in interferon secreting cells with an increase of CD4\(^+\)-CD8\(^+\) CD4\(^-\)-CD8\(^+\) lymphocytes in PBMC [216]. Interferons play an important role during viral infection, PRRSV is sensitive to type I interferon and the virus has the ability to down-regulate its expression in the respiratory system. The mechanism by which PRRSV regulates interferon-\(\alpha\) production is unknown, but has a detrimental effect in recruitment of the interferon secreting cells and could also impair the Th1 immune response. Although there were multiple attempts to correlate the presence of T-reg cells with immune modulation induced by PRRSV, the available data is still contradictory.

PRRSV has also been proven to regulate the expression of certain cytokines. The data available regarding some of the proinflammatory cytokines such as TNF-\(\alpha\), IL-1 and IL-8 is controversial. In \(\textit{vitro}\) studies showed that TNF-\(\alpha\) mRNA and protein expression in pulmonary alveolar macrophages are down-regulated [226, 227], while \(\textit{in vitro}\) and \(\textit{in vivo}\) studies indicate that PRRSV upregulates the expression of TNF-\(\alpha\) mRNA [228-231]. These differences suggest a differential expression of nsps2 due to genomic changes and deletions [232]. Most studies report that PRRSV induce IL-6 and IL-8 \(\textit{in vivo}\), and IL-10 mRNA expression is also increased in PBMC [231, 233, 234]. In addition, IL-8 and IL-1\(\beta\) were reported to have significant correlation with viral load levels, which indicate that these two cytokines are linked to PRRSV clearance [235]. PRRSV infection can also play a role in the host immune modulation of regulatory T cells. Experimentally, T-reg cells co-cultured with PRRSV-infected monocytes derived dendritic cells (MDDC) up-regulate both IL-10 and TGF\(\beta\) mRNA [236].
Vaccines

Heterologous protection is the major problem in the development of a universal vaccine against PRRSV. Two major genotypes of PRRSV are currently circulating worldwide. These two types, type 1 (European) and type 2 (North American) can cause the same clinical disease but are antigenically different sharing only approximately 50-60% nucleotide sequence identity [176]. Like other RNA viruses, PRRSV is prone to genetic drift caused by inaccurate RNA replication. It has been demonstrated that multiple strains and quasispecies can circulate within the same farm and infect the same animal [237]. The coexistence of both types 1 and 2 strains in some countries create a risk of intertypic recombination [238]. Due to immune evasion and genetic diversity of PRRSV, the current vaccines can only confer partial protection. Currently there are two types of commercial vaccines, modified live-attenuated vaccines (MLV) and inactivated killed vaccines (KV) [176, 177, 239-245].

**Modified live-attenuated virus (MLV) vaccines**

MLV have only relative successes due to the viral heterogeneity in the field. Although MLV vaccine can confer clinical protection especially against homologous or closely-related strains; immunogenicity studies demonstrated that humoral and cellular responses are weak and antibodies appear approximately two weeks after vaccination. Moreover, antibodies are targeted against the N protein which does not induce neutralizing activity [246, 247]. Amongst other problems observed with MLV, viral shedding and reversion to a wild-type have been observed experimentally [248] as well in the field [249]. It has been demonstrated that PRRSV MLVs can be shed in placenta, and semen with potential reversion to wild type [174, 240, 242, 245]. PRRSV MLVs have been shown to reduce the virus replication in pigs and the severity and duration of disease after challenge with a homologous strain, but it does not have a strong impact in vaccinated
pigs upon challenge with a virulent heterologous strain [176]. Although, heterologous MLV does not confer full protection, pigs vaccinated with heterologous-MLV showed significant reduction in severity of clinical signs, duration and severity of infection and more efficient cell-mediated immune response compared with unvaccinated pigs [250]. Vaccination with a MLV homologous strain has demonstrated reduction in virus shedding in semen compared with heterologous strains [251]. However, boar vaccination with MLV did not show reduction in viral shedding and tissue viral load upon challenge with heterologous strains [252].

**Inactivated virus vaccine or killed virus vaccine (KV)**

Inactivated vaccines are not licensed in the United States, therefore there is no abundant information to measure the real benefit of these vaccines in field conditions. Few reports from the experience gathered in Europe, described that killed vaccines elicit a weak cell-mediated immunity (CMI). When killed vaccine is used in infected animals, it reportedly enhances CMI and humoral response making this a good therapeutic vaccine [253]. Inactivated vaccines have the advantage that they can be easily customized and generated against specific and multiple strains circulating in the field [254]. Despite these benefits, KV does not overcome the problem of cross protection against heterologous strains observed with MLV, and does not reduce virus replication and viral shedding [216, 245]. Immunogenicity studies demonstrate that KV does not induce NA and does not elicit CMI response determined by lymphocyte proliferation and IFNγ levels [255, 256]. KV has been shown to improve sows reproductive performance (i.e. reduction of premature farrowing, abortions and increase of farrowing rate) and litter characteristics (i.e. increase of the number of live born and weaned pigs and decrease of stillborn, mummified, weak and splay-legged piglets) [257]. KV does not improve viremia detected in boars or viral load in semen [251].
Experimental vaccines

Numerous other systems to express PRRSV proteins, and induce heterologous and long-lasting protection are still under investigation. Viral vector vaccines have been used to express immunogenic PRRSV proteins. Baculovirus expression system has been used to co express GP5 and PCV2 cap, His tagged-GP3, and recombinant GP3 GP5 and N proteins [258-261]. Replication-deficient virus has been used to express PRRSV viral proteins. Combined or individually, PRRSV GP5 and M protein have been expressed in vaccinia virus Ankara, and in vivo studies in mice demonstrate that combined recombinant vaccines can induce humoral and cellular response [262]. Fowl pox virus expressing GP3 and GP5 associated with IL-18 genes induce specific PRRSV antibodies, as well as VN antibodies and T cell response in pigs [263]. Adenoviral vectors has been used to express GP5, GP3, N and M proteins conferring humoral response in pigs [264]. Other studies using the same viral vector but expressing GP3-GP5, GP4-GP5 and GP3-GP4-GP5 demonstrated that, in mice, this chimeric virus confers better levels of VN and strong lymphocyte proliferation response compared with GP5, GP3, N and M proteins [265]. However, these systems have limited duration and cannot confer heterologous protection. Genetically engineered plants are able to express immunogenic proteins of PRRSV. Recombinant tobacco [266, 267], banana [268], potato [269] and corn [270] demonstrate that they can induce humoral and cellular immune response when they are feed to pigs. However, these delivery systems present the same problem observed with baculovirus regarding immunity duration and effectiveness.

DNA vectors carrying different PRRSV proteins have been developed. PRRSV ORFs have been cloned individually in DNA vectors. ORF7 induced specific PRRSV antibodies, ORF1 and ORF4 induce specific antibodies against nsp51 and GP4, and NA were detected in pigs inoculated with plasmid containing GP5 [271]. Immune modulators such as IL-2 and IL-4 has been included
in PRRSV DNA vaccines containing ORF5 [272]. These DNA vaccines showed to induce higher levels of humoral and cellular immune responses.

**INTERACTION OF PCV2 AND PRRSV**

It has been demonstrated that most of the clinical features of PCVAD cases are exacerbated in animals co-infected with PRRSV compared with those suffering PCV2 infection alone [273]. In addition, field surveys in the Netherlands showed that 83% of animals with PCVAD clinical signs were dually infected with PCV2 and PRRSV, while 35% of animals in a control group without PCVAD signs were also dually infected [274]. Amongst the most prevalent viral pathogens that affect pigs, the combination of PCV2 and PRRSV have been shown to be the most prevalent in nursery pigs [275, 276]. However, this dual infection appears not to be the most frequent in suckling and fattening pigs [275]. Several studies demonstrated the impact of PCV2, PRRSV or combination of both in porcine necrotizing pneumonia (PNP). One study in Canada demonstrated that PRRSV infection and PCV2-PRRSV coinfection can cause lesions characterized as PNP in suckling pigs [46]. In another study, the prevalence of PCV2 is similar (39.1%) to the PCV2-PRRSV coinfection (40.5%) in animals with lesions characteristic of PNP [73]. In a prevalence study of pathogens associated with PNP in Italy, singular infection of PCV2 and PRRSV was approximately 14.3%, while animals showing PCV2-PRRSV coinfection was 28% [277]. Therefore, the data indicate that there is not a unique pattern of infection causing PNP lesions; moreover, other pathogens such as SIV, AVD have also been associated with the presence of PNP lesions in early nursery and suckling pigs. Boars can be naturally co-infected with PCV2 and PRRSV without evident clinical manifestation. Serological and virological evidences showed a high prevalence of PRRSV and PCV2. Moreover, there was a high sequence identity between viruses detected in serum and semen [278].
**In vivo experiments**

In order to evaluate the importance of PCV2 and PRRSV virus coinfection, numerous animal models have been developed. Concurrent PCV2-PRRSV infection in colostrum-deprived piglets and five weeks-old weaned conventional pigs showed that the prevalence of PCV2 antigen is higher in those animals co-infected, compared with groups singly infected with PCV2. This led to the conclusion that PCV2-PRRSV dual infection exacerbates PCV2 replication [279, 280]. Dual infection in CD-CD piglets showed that the severity of PRRSV interstitial pneumonia is exacerbated in PCV2-PRRSV co-infected pigs. However lymphoid depletion, granulomatous inflammation and necrotizing hepatitis is due to the sole effect of PCV2 in dually infected animals [281]. Numerous studies attempt to demonstrate if different PRRSV or PCV2 subtype have a greater impact in severity of clinical presentation. Six week-old pigs dually infected with different combination of PCV2a and PCV2b or PRRSV type 1 or type2 showed significant differences in virulence and pathogenicity of type 1 and type 2 PRRSV, but no significant differences in virulence and pathogenicity of PCV2a and PCV2b related to the production of PCV2-associated lesions [282]. Studies using different type 2 PRRSV strains, emphasize the differences observed in replication amongst PRRSV strains *in vivo* but no difference was observed in PCV2 clinical signs regardless the PCV2 subtype used [283]. The synergistic effect of PCV2 and PRRSV was attributed to a specific infection order in nursery pigs infected with PCV2 and the new high pathogenic-PRRSV strain (HP-PRRSV). Thus, viral replication was enhanced and more-severe clinical signs and lesions were observed in pigs infected with HP-PRRSV followed by PCV2 [284].
**In vitro experiments**

Coinfection studies with PCV2 and PRRSV have been scarce due to the lack of stable cell lines that allows coinfection. Few experiments tried to phenotype the cytopathic effect and immune modulation in coinfection of pulmonary alveolar macrophages (PAM). It has been described that PCV2 does not induce cytopathic effect (CPE) in PAM while PRRSV infection can cause severe CPE. However, when PAMs were coinfected, CPE was significantly reduced. There was also a high level of cell death and apoptosis in PRRSV infected cells compared with singular PCV2 infection. Nevertheless, coinfected cells showed lower apoptotic and cell death rate. These results lead to the theory that PCV2 reduces PRRSV infectivity and PRRSV associated-CPE. In the same experiment, the levels of TNFα in singular PCV2 infection were significantly higher compared with those of PRRSV infected group [285]. PCV2-ORF3 has also been associated with induction of NF-κβ in PK-15 and lymphocytes leading to apoptosis [29], and upregulation of Fas/Fas ligand activity in a PCV2-PRRSV coinfection model [286]. In a coinfection model, dendritic cells were infected with PCV2 or dually infected with PCV2 and PRRSV. The results showed an increase in T-reg activation, and its induction is likely due to INF-β production but not IL-10 [128].

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CHAPTER 2

Evaluation of the use of non-pathogenic porcine circovirus type 1 as a vaccine delivery virus vector to express antigenic epitopes of porcine reproductive and respiratory syndrome virus

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ABSTRACT

We previously demonstrated that the C-terminus of the capsid gene of porcine circovirus type 2 (PCV2) is an immune reactive epitope displayed on the surface of virions. Insertion of foreign epitope tags in the C-terminus produced infectious virions that elicited humoral immune responses against both PCV2 capsid and the inserted epitope tags, whereas mutation in the N terminus impaired viral replication. Since the non-pathogenic porcine circovirus type 1 (PCV1) shares similar genomic organization and significant sequence identity with pathogenic PCV2, in this study we evaluated whether PCV1 can serve as a vaccine delivery virus vector. Four different antigenic determinants of porcine reproductive and respiratory syndrome virus (PRRSV) were inserted in the C-terminus of the PCV1 capsid gene, the infectivity and immunogenicity of the resulting viruses are determined. We showed that an insertion of 12 (PRRSV-GP2 epitope II, PRRSV-GP3 epitope I, and PRRSV-GP5 epitope I), and 14 (PRRSV-GP5 epitope IV) amino acid residues did not affect PCV1 replication. We successfully rescued and characterized four chimeric PCV1 viruses expressing PRRSV linear antigenic determinant (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We demonstrated that all chimeric viruses were stable and infectious in vitro and three chimeric viruses were infectious in vivo. An immunogenicity study in pigs revealed that PCV1-PRRSV_EPI chimeric viruses elicited neutralizing antibodies against PRRSV VR2385. The results have important implications for further evaluating PCV1 as a potential vaccine delivery vector.
INTRODUCTION

Porcine circoviruses (PCV) belong to the genus *Circovirus* of the family *Circoviridae* [287]. The viral genome is packaged in an icosahedral capsid approximately 17 nm in diameter, and PCV is the smallest virus infecting mammals. Two types of PCV, PCV1 and PCV2, have been identified thus far. PCV1 was first described in 1974 as a contaminant of the porcine kidney cell line, PK-15, and is non-pathogenic in pigs [1]. PCV2 is pathogenic and causes an economically-important porcine circovirus-associated diseases (PCVAD) in swine worldwide [12, 57, 288]. Both PCV1 and PCV2 are non-enveloped, single-stranded circular DNA molecules of 1,759 (PCV1) and 1,768 (PCV2) kb in size [287].

The non-pathogenic PCV1 shares similar genomic organization with the PCVAD-associated PCV2 [75], which is characterized by 11 potential open reading frames (ORFs) with predicted protein sizes ranging from 2 to 36 kDa [24]. However, thus far only two major ORFs are believed to be essential for completing the basic functions of the virus: ORF1 encodes the replicase (Rep) (314 aa) and the truncated, spliced Rep’ (178 aa), whereas the ORF2 encodes the immunogenic capsid protein (233 aa). Sequence analyses revealed that PCV1 shares a 76% nucleotide sequence identity with its pathogenic counterpart PCV2. The ORF1-encoded replicase protein has approximately 80% amino acid sequence identity between the two viruses, whereas the ORF2 capsid protein has about 60% amino acid sequence identity [156, 289]. The ORF1 and ORF2 genes are oriented in opposite directions, resulting in an ambisense orientation. Between the 5’ end of ORF1 and ORF2, there exists an intergenic region that contains the origin of virus replication characterized by a stem-loop structure [25].

Since the initial identification of PCV2 [12], several genotypes have now been described [37, 38] and demonstrated to co-exist in pigs [22, 157]. For a single-stranded DNA virus, PCV2
has been shown to have the highest DNA mutation rate that is comparable to single-stranded RNA viruses [43]. In contrast, the non-pathogenic PCV1 has been demonstrated to have a low mutation rate and low genetic diversity worldwide [290, 291]. While PCV2 is highly prevalent in most swine-producing countries and is associated with clinical PCVAD, PCV1 is non-pathogenic and has a low prevalence in swine herds [2-8, 292, 293]. A recent survey demonstrated that, while PCV2 DNA and PCV2-specific antibodies are present in more than 80% of the samples evaluated, the molecular and serological prevalence of PCV1 is less than 2.4% [294].

We previously demonstrated that a genetically modified infectious PCV2 can tolerate up to a 27 aa insertion in the C-terminus of the ORF2 capsid gene [295]. We showed that insertion of single, dimeric, and trimeric hemagglutinin (HA) tags, a GLu-GLu epitope tag of a mouse polyomavirus, and the KT3 epitope tag of the simian virus 40 in the C-terminus of PCV2 capsid gene resulted in infectious chimeric viruses that induce both PCV2-neutralizing antibodies and anti-epitope tag antibodies [295]. Another study reported that insertion of a VP1 epitope region (aa 141–160, LTNVRGDLQVLAQKAARPLP) of the foot and mouth disease virus (FMDV) in PCV2 produced infectious virus in vitro and in a mouse model, and the PCV2-FMDV chimera elicited dual immunity against PCV2 and FMDV [296].

Because of the low prevalence of PCV1 in swine herds, the non-pathogenic nature, the low mutation rate, and the systemic tropisms of PCV1 for multiple tissues and organs, it is logical to explore the potential use of PCV1 as a vaccine delivery virus vector. Therefore, in this study, as a proof-of-principle, we evaluated whether PCV1 can express known antigenic determinants of porcine reproductive and respiratory syndrome virus (PRRSV), an economically-important swine pathogen. Generation of chimeric viruses containing neutralizing antigenic epitopes of PRRSV in the backbone of the non-pathogenic PCV1 could potentially elicit protective immunity against
PRRSV with the benefit of a live virus-vectored vaccine, but without the risk of pathogenicity or reversion to virulence often associated with the traditional modified live-attenuated vaccines.

MATERIALS AND METHODS

Construction of chimeric PCV1-PRRSV<sub>EPI</sub> (epitope) infectious clones:

Four different known antigenic epitopes derived from PRRSV strain VR2385, including GP2 epitope II (aa 40–51, ASPSHVGWWSFA), GP3 epitope I (aa 61–72, QAAAEAYEPGRS), GP5 epitope I (aa 35–46, SSSNLQLIYNLT), and GP5 epitope IV (aa 187–200, TPVTRVSAEQWGRP), were each cloned individually in frame into the C-terminus of the PCV1 capsid gene (GenBank accession number GU799575). Chimeric viruses were constructed by overlapping extension and fusion PCR following a method previously described [295]. Briefly, a pCR2.1-PCV1 infectious clone plasmid containing the full-length PCV1 genome was used as the template to generate two amplicons of 200 bp and 1,800 bp with complementary overhangs containing individual PRRSV antigenic epitope sequences (Table 1). A second round of fusion PCR was performed to assemble the previously synthesized amplicons. The PCR product was digested with KpnI (New England Biolabs) and inserted into the pCR2.1TOPO vector (Invitrogen) (Fig. 1). Recombinant plasmids containing the insert were transformed into the alpha-select strain of E. coli (Bioline). Positive clones were selected via blue-white screening, and insertion of each specific PRRSV epitope was confirmed by DNA sequencing. The viral genomic DNA was excised from the plasmid by enzymatic digestion with KpnI and concatemerization was carried out through a ligation reaction with T4 DNA ligase (Invitrogen), overnight, at room temperature. The infectious chimeric virus was generated by transfection of the concatemerized genomic DNA into the PCV1-free PK15 cells at 30%–40% confluency with lipofectamine ltx (Invitrogen). After 72
h post-transfection, the infectious virus was harvested by three cycles of freezing and thawing of the cells.

**In vitro infectivity, epitope expression, and titration of chimeric PCV1-PRRSV\textsubscript{EPI} viruses:**

PCV1-free PK-15 cells were seeded at a concentration of $2 \times 10^5$ cells/well in a 48-well plate. After reaching approximately 40%–50% confluency, cells were washed once with Hank’s Balanced Salt Solution (Gibco). The cells were then incubated with 100 μL of 1:10 serial dilution of the virus stock for 1 h at 37°C in 5% CO₂, after which the cells were washed once with 200 μL of minimum essential media (MEM) (Gibco). Infection was carried out in 300 μL MEM supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimycotic (Fisher) at 37°C in 5% CO₂. After 72 h post-infection, the cells were fixed with 80% acetone and the infectivity was assessed by an immunofluorescence assay (IFA).

Briefly, infected cells were incubated with 100 μL of mouse anti-PCV1-Cap monoclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (KPL, Kirkegaard & Perry Laboratories, Inc.). Expression of PRRSV-specific antigenic epitopes was also confirmed by IFA using custom polyclonal rabbit antibodies (Biomatik) against each synthetic PRRSV epitope followed by secondary goat anti-rabbit IgG (DyLight 550). Cells positive for both PCV1 Cap and PRRSV epitopes were visualized using a Zeiss LSM 880 confocal microscope (Zeiss, Pleasanton, CA) with a 40X objective, using the argon 488 and helium-neon 594 lasers. Serial ten-fold dilutions of the virus stock were performed in order to determine the 50% tissue culture infectious dose (TCID\textsubscript{50}) of the virus stocks according to the method described by of Reed and Muench [297].
In vivo characterization of the infectivity and immunogenicity of four PCV1-PRRSV\textsubscript{EPI} chimeric viruses

Experimental design for the animal study

A total of 21 specific-pathogen-free (SPF) pigs were randomly assigned into seven groups of three pigs each, including two positive control groups (PCV1 and PRRSV), a negative control (MEM-treated group), and four groups for each of the PCV1-PRRSV\textsubscript{EPI} chimeric viruses. Pigs in each of the PCV1-PRRSV\textsubscript{EPI} chimeric virus groups and the PCV1-positive control group were intramuscularly inoculated with 5 mL (4.64 × 10\textsuperscript{2} TCDI\textsubscript{50}/mL) of the respective viruses. Pigs in the PRRSV VR2385-positive control group were each inoculated with 5 mL (2 × 10\textsuperscript{5} TCDI\textsubscript{50}/mL) of PRRSV VR2385. Serum samples were collected from each pig prior to inoculation and weekly thereafter for a period of 7 weeks.

Quantification of viral DNA loads in sera and lung tissues

The viral DNA was extracted from serum samples at 0, 7, 14, 21, 28, 35, and 42 days post-inoculation (dpi) and from tissues (lung and tracheobronchial lymph node) at 42 dpi, using Ambion MagMAX-96 Viral DNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The DNA standards used for the qPCR were virus stock used for inoculation as well as plasmids containing the full-length PCV1 infectious clone. In order to rule out potential cross-contamination, DNA extracted from PCV2a and PCV2b virus stocks, and empty pCR2.1TOPO vector (Invitrogen), were included as the negative control. The PCV1 DNA copy numbers in sera or tissues were quantified by a TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Corp.) according to the manufacturer’s protocol. The PCR primers and probes (PCV1 P9/PCV1 P10/PCV1 probe) (Table 1) used in the qPCR assay were designed to target a specific amplicon.
of 97 bp in ORF2. The qPCR assay was conducted using the ABI 7500 (RT) PCR system (Life Technologies Corp). The PCR conditions included denaturation at 95°C for 20 s, annealing at 95°C for 3 s, amplification at 60°C for 30 s, and a final extension at 72°C for 5 min, with a total number of 40 cycles. Each reaction was performed in triplicate.

**Serological evaluation of anti-PCV1 antibodies and anti-PRRSV\textsubscript{EPI} antibodies:**

Specific antibodies against PCV1-Cap were tested by an indirect immunofluorescence assay (IIFA). For the IIFA, the PCV1-free PK-15 cells were inoculated with 100 μL of PCV1 and incubated for 72 h at 37°C, and fixed in 80% acetone. Sera from pigs in each of the chimeric PCV1-PRRSV\textsubscript{EPI}-infected groups as well as from pigs in both the positive and negative control groups were serially diluted and incubated for 1 h at 37°C. Cells were washed three times with PBS, followed by addition of 100 μL (1:100) of fluorescent-labeled secondary anti-swine IgG antibody (KPL, Kirkegaard & Perry Laboratories, Inc.). The cells were then washed again, and positive cells were detected using a fluorescence microscope within the excitation and emission spectrum peak wavelengths of approximately 495 and 519 nm, respectively. The virus titer was defined as the highest positive dilution and expressed as a mean geometric titer. The anti-PRRSV N antibody response was also evaluated using the IDEXX HerdCheck X3 ELISA kit according to the manufacturer’s instructions.

Four different PRRSV KLH-conjugated synthetic antigenic peptides (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP) were used as the antigen for the four PRRSV peptide-based antibody ELISAs. Each vial of lyophilized peptide (5 mg) was resuspended in 1 mL of UltraPure\textsuperscript{TM} Distilled Water (Gibco\textsuperscript{®}, Life Technologies) to a
final stock concentration of 5 mg/mL, aliquotted, and stored at –80°C. Following titration and optimal dilution, 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated with 100 μL per well of each peptide at a concentration of 5 μg/mL in phosphate-buffered saline (PBS) at pH 7.4 (Gibco®, Life Technologies) and incubated at 4°C overnight. After incubation, plates were washed five times, blocked with 300 μL per well of a solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc.), and incubated at 25°C for 2 h. Plates were then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs. The ELISA conditions, including coating and blocking, buffers, sample and conjugate dilutions, and incubation conditions (time and temperature), were identical for the four different peptide-based ELISAs. Serum samples were diluted at 1:50, after which plates were loaded with 100 μL of the diluted sample per well. Plates were incubated at 37°C for 1 h and washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 μL of peroxidase-conjugated goat anti-pig IgG (Fc) antibody (Bethyl Laboratories Inc.), diluted at 1:15,000, were added to each well and the plates were incubated at 37°C for 1 h. After a washing step, the reaction was visualized by adding 100 μL of tetramethylbenzidine-hydrogen peroxide (Dako North America, Inc.) substrate solution to each well. After 10 min incubation at room temperature, the reaction was stopped by the addition of 50 μL of a stop solution (1 M sulfuric acid) to each well. Reactions were measured according to the optical density at 450 nm using an ELISA plate reader (Biotek® Instruments Inc.) operated with commercial software (GEN5TM, Biotek® Instruments Inc.).

Serum virus neutralization assay to evaluate the neutralizing activity against PRRSV

VR2385:

The neutralizing antibody titers against PRRSV VR2385 were determined by a serum virus neutralization assay essentially as previously described [298]. Briefly, two-fold diluted serum
samples collected at 28, 35, and 42 dpi from each pig were mixed with an equal volume of the PRRSV VR2385 virus at an infectious titer of $2 \times 10^3$ TCID$_{50}$/mL and incubated at 37°C for 1 h. The mixtures were then inoculated onto MARC-145 cells in 96-well plates and incubated for 1 h at 37°C. After washing with PBS, the cells were maintained in DMEM with 2% FBS. At approximately 20 hpi, the cells were assayed by IFA for virus infection. The neutralizing antibody titers were expressed as the highest dilution that showed a 90% or above reduction in the number of fluorescent foci compared to that of antisera from negative control pigs. Samples were evaluated in triplicate and three independent tests were performed for each serum sample.

**Statistical analysis**

The Student’s $t$-test (unpaired) was used to evaluate the differences ($P < 0.05$) between the samples in the two groups. Repeated measure two-way ANOVA with Tukey’s correction was calculated for multiple comparison. Statistical significance was set to alpha = 0.05. All analyses were performed using commercially available software GraphPad Prism® 6 (GraphPad Software, Inc, CA).

**RESULTS**

**Chimeric PCV1 viruses containing PRRSV VR2385 antigenic epitopes inserted in the C-terminus of the PCV1 capsid are infectious *in vitro*:**

Each of the chimeric PCV1-PRRS$_{EPI}$ clones was verified by full-length genomic sequencing for the presence in frame of each of the inserted PRRSV antigenic epitopes in the C-terminus of the PCV1 capsid gene. Transfection of each full-length chimeric virus DNA clone in PK-15 cells resulted in the production of infectious virions. Confocal microscopy revealed that each of the PCV1-PRRS$_{EPI}$ chimeric viruses expressed PCV1 Cap as well as the respective PRRSV
antigenic determinant (Fig. 2). Infected cells showed dual nuclear staining with both anti-PCV1 monoclonal antibodies and anti-PRRSV epitope peptides (GP2 II, GP3I, GP5I, and GP5IV) mono-specific antibodies. The stability of chimeric viruses was confirmed after five successful serial passages in PK-15 followed by dual IFA staining of the PCV1 capsid and respective PRRSV antigenic determinant, as well as by sequence confirmation of the chimeric viruses harvested after the five passages (data not shown).

**PCV1-PRRSV_{EPI} chimeric viruses are viremic and replicate in tissues of experimentally inoculated pigs:**

All serum samples, evaluated by TaqMan® qPCR for the presence of PCV1 prior to inoculation at day 0, were negative. Viremia was detected at as early as 7 dpi for PCV1-PRRSV_{EPI}GP3I and PCV1-PRRSV_{EPI}GP5IV viruses, and at 21 dpi for PCV1-PRRSV_{EPI}GP2II and PCV1-PRRSV_{EPI}GP5I viruses (Table 2). The frequency of pigs showing viremia in each group varied during the trial. The number of animals used and the frequency variability do not allow for robust statistical analysis; however, the average DNA viral loads in serum samples from each chimeric virus were within one log_{10} difference: 1.39 \times 10^5 \text{ genomic copies/mL} for PCV1-PRRSV_{EPI}GP2II; 1.87 \times 10^5 \text{ genomic copies/mL} for PCV1-PRRSV_{EPI}GP3I; 4.22 \times 10^5 \text{ genomic copies/mL} for PCV1-PRRSV_{EPI}GP5I; and 1.96 \times 10^5 \text{ genomic copies/mL} for PCV1-PRRSV_{EPI}GP5IV, and had at least two-log_{10} lower genomic copies/mL than the parental PCV1 (1.26 \times 10^7 \text{ genomic copies/mL}) (Fig. 3A). After 42 dpi, all animals were necropsied and no significant pathological lesions were observed. However, all infected groups had detectable viral DNA in the tracheobronchial lymph nodes and lungs (Table 2), indicating virus replication in tissues. No significant differences in viral genomic copy number/gram of tissue were observed.
between parental PCV1 and PCV1-PRRSV\textsubscript{EPI} chimeric viruses in tracheobronchial lymph nodes and lungs (Fig. 3B). No evidence of PCV1 replication was observed in the PRRSV VR2385 and MEM control groups.

**PCV1-PRRSV\textsubscript{EPI} chimeric viruses induce both PCV1-specific and PRRSV antigenic epitope-specific antibodies in pigs:**

Anti-PCV1 IgG antibodies were detected in the sera of the parental PCV1 control group as well as all the PCV1-PRRSV\textsubscript{EPI} chimeric viruses-inoculated groups. IgG anti-PCV1 antibodies were detected from 14 dpi in parental PCV1-infected pigs and remained seropositive at 42 dpi. Anti-PCV1 IgG antibodies were detected in PCV1-PRRSV\textsubscript{EPI}GP3I- and PCV1-PRRSV\textsubscript{EPI}GP5IV-infected groups at 14, 21, and 28 dpi, followed by a significant titer reduction compared to the parental PCV1 at 35 and 42 dpi. The remaining chimeric viruses-infected groups, PCV1-PRRSV\textsubscript{EPI}GP2II and PCV1-PRRSV\textsubscript{EPI}GP5I, showed a delayed seroconversion to anti-PCV1 IgG antibodies (35 dpi) and significantly lower levels of anti-PCV1 IgG antibodies, compared to the parental PCV1-infected group, at 35 and 42 dpi (Fig. 4A). Anti-PCV1 IgG antibodies were not detected in PRRSV or MEM control groups. As expected, anti-PRRSV N antibodies were only detected in the PRRSV 2385 infected group (Fig. 4B).

Antibody responses against the inserted PRRSV antigenic epitopes were detected by specific epitope-based ELISA. Antibody response against PRRSV-GP2 epitope II was not detected in pigs experimentally infected with the PCV1-PRRSV\textsubscript{EPI}GP2II chimeric virus (Fig. 5A). However, specific antibodies against PRRSV-GP3 epitope I were detected in the PCV1-PRRSV\textsubscript{EPI}GP3I chimeric virus group at 28 dpi and remained positive at 42 dpi (Fig. 5B). Specific antibodies against PRRSV-GP5 epitope I were detected at 21 dpi in the wild-type PRRSV.
VR2385-infected group, and at 35 dpi in the PCV1-PRRSV$_{EPI}$GP5I chimeric virus-infected group at 35 dpi, and remained positive at 42 dpi (Fig. 5C). The presence of anti-PRRSV-GP5 epitope IV antibodies was detected at 21 dpi in the PCV1-PRRSV$_{EPI}$GP5IV chimeric virus group and at 28 dpi in wild-type PRRSV VR2385 group, and remained at a high level in the PCV1-PRRSV$_{EPI}$GP5IV chimeric virus group at 35 and 42 dpi (Fig. 5D). The low number of animals per group, as well as individual variation amongst animals may play a role in the different levels of antibodies response observed amongst groups.

**PCV1-PRRSV$_{EPI}$ chimeric viruses-infected pigs develop neutralizing antibodies against the PRRSV VR2385:**

To investigate whether PCV1-PRRSV$_{EPI}$ chimeric viruses can induce neutralizing antibodies against PRRSV, a serum virus neutralization assay against PRRSV VR2385 strain was performed. Anti-PRRSV VR2385 neutralizing antibodies were detected in the PCV1-PRRSV$_{EPI}$GP3I, PCV1-PRRSV$_{EPI}$GP5I, and PCV1-PRRSV$_{EPI}$GP5IV chimeric viruses-infected groups at 28 dpi and remained detectable at 42 dpi. No statistical difference in neutralizing antibody titers were observed throughout the experiment between wild-type PRRSV VR2385 and PCV1-PRRSV$_{EPI}$GP3I, PCV1-PRRSV$_{EPI}$GP5I, and PCV1-PRRSV$_{EPI}$GP5IV, except for PCV1-PRRSV$_{EPI}$GP5I at 42 dpi (Fig. 6). PRRSV 2385 neutralizing antibodies were not detected in PCV1 or MEM control groups.

**DISCUSSION**

PCV1 is a non-pathogenic virus in pigs infecting multiple tissues and organs [7, 8, 292]. Early field studies reported a high serological prevalence of anti-PCV1 antibodies in the swine population [2, 5, 299], although no disease could be associated with the presence of this virus
either naturally or experimentally [4, 7]. However, more recent field studies have demonstrated that the serological prevalence of anti-PCV1 antibodies as well as virus circulation in the swine population are very low [294]. Sequence and phylogenetic analyses have also demonstrated a low mutation rate and low genetic diversity of the PCV1 strains worldwide [290]. Thus, the low prevalence of PCV1, lack of evidence of pathogenicity, low mutation rate, and systemic tropisms for multiple tissues and organs make PCV1 an attractive candidate for a potential live vaccine vector.

Previous studies have successfully used the non-pathogenic PCV1 as the genomic backbone for the development of PCV2 vaccines [144]. Cloning of PCV2 ORF2 capsid gene into the backbone of PCV1 proved to be viable \textit{in vivo} and conferred full protection against PCV2, whilst still retaining the non-pathogenic nature of PCV1 [144, 151]. Additionally, pigs experimentally infected with a PCV2-1 reciprocal chimeric virus, containing PCV1 ORF2 in a PCV2 backbone, showed specific anti-PCV1 IgG antibody response [151]. Herein in this study, we further demonstrated that insertion of known PRRSV antigenic determinants in the C-terminus of PCV1 ORF2 capsid gene produced infectious chimeric viruses and did not impair the humoral immune response against PCV1. Although different levels of anti-PCV1 IgG were detected, all PCV1-PRRSV\textsubscript{EPI} chimeric viruses were capable of induce anti-PCV1 IgG antibodies in infected SPF pigs.

The different levels of anti-PCV1 IgG antibodies might be associated with a different replication timeline and viral DNA load observed amongst the various PCV1-PRRSV\textsubscript{EPI} chimeric viruses compared with parental PCV1. The extension of the C-terminus of the PCV1 capsid gene through the addition of PRRSV antigenic epitopes might also affect the structural conformation and antibody induction capability of the PCV1 capsid. Indeed, previous studies demonstrated that
mutation of the last four amino acid residues of the PCV2 ORF2 (-PLKP) to three amino acid residues of the PCV1 ORF2 (-LNK) reduces viral antibody recognition [300]. Therefore, in the current study, anti-PCV1 IgG antibodies generated against PCV1-PRRSV\textsubscript{EPI} chimeric viruses may not completely bind to the full PCV1 Cap expressed by the parental PCV1 that was used for the IIFA serology test. Further studies will be necessary to demonstrate whether the insertion of a foreign amino acid sequence at the C-terminus may alter the conformation of the PCV1 capsid protein.

It has been previously demonstrated that the C-terminus of the PCV2 capsid is a type-specific immune reactive epitope that is displayed on the surface of the virion capsid [300, 301]. Moreover, following the insertion of epitope tags in the C-terminal region, infectious chimeric PCV2 was generated and shown to elicit dual immunity against both PCV2 capsid and the inserted foreign epitope tags, whereas mutation of the N-terminus of PCV2 capsid impaired viral replication [295]. Herein in the present study, we inserted four different known PRRSV antigenic epitopes into the C-terminus of the non-pathogenic PCV1 capsid gene, and demonstrated that the insertions did not significantly affect virus infectivity \textit{in vitro} or viral replication \textit{in vivo}. We previously showed that epitopes as large as 27 amino acids can be inserted in the PCV2 capsid gene without impairing viral viability [295]. In the present study, we demonstrated that insertions, varying from 12 aa for PRRSV-GP3 epitope I and PRRSV-GP5 epitope I, 12 aa for PRRSV-GP2 epitope II, and 14 aa for PRRSV-GP5 epitope IV, did not affect viral infectivity \textit{in vitro} or replication \textit{in vivo}. Future study is necessary to determine the tolerance of maximal length of amino acid insertion in PCV1 capsid without affecting the viability of the virus.

In the current study, PCV1 chimeric viruses expressing four known B-cell linear epitopes of PRRSV, previously demonstrated to be immunogenic against PRRSV [218, 302, 303], were
generated. It has been reported that GP5 plays a major role in PRRSV neutralization [302, 304, 305]. PRRSV-GP5 epitope IV is an important immunogenic epitope (P^{188}LTR (V/T) SAEQW^{197}) that has also been proved to be reactive with sera raised against European PRRSV strains. Despite a few amino acid changes, this epitope seems to be well conserved amongst type 2 PRRSV strains [219]. We showed in this study that PCV1-PRRSV_{EPI}GP5IV chimeric virus induced neutralizing antibody levels comparable to those induced by the PRRSV VR2385 virus. The PRRSV-GP5 epitope I neutralizing epitope, 37SHLQLIYNL, for the PRRSV VR2332 is located in the GP5 ectodomain sequence and is considered as the primary neutralizing epitope for the type 2 PRRSV isolates [302]. The PCV1-PRRSV_{EPI}GP5I chimeric virus in this study induced similar neutralizing antibody levels compared to those induced by PRRSV VR2385 virus. However, the neutralizing antibody titers induced by the PCV1-PRRSV_{EPI} viruses appeared to decline more rapidly compared to the PRRSV VR2385.

PRRSV ORF3 is considered as the second most variable PRRSV structural protein, with four consecutive peptides from aa 61–105, all of which are considered as important immunodominant domains of GP3 [218, 306]. The PCV1-PRRSV_{EPI}GP3I chimeric virus generated in this study contains the aa 61–72 (QAAAEAYEPGRS) and was shown to induce similar levels of neutralizing antibodies compared to the PRRSV VR2385. Despite the fact that PCV1-PRRSVEPIGP2II chimeric virus is infectious in vitro, no viremia and viral DNA were detected in tissues of inoculated pigs, probably due to the short duration of viremia not being detectable with the current sampling scheme. This explanation was also supported by the presence of anti-PCV1 IgG at 14 dpi, indicative of virus replication. Furthermore, the chimeric virus also failed to induce antibodies against PRRSV-GP2 epitope II. Therefore, we have successfully demonstrated that three of the four PCV1-PRRSV_{EPI} chimeric viruses generated in this study
induced PRRSV epitope-specific antibodies and neutralizing antibodies against PRRSV VR2385 at a level comparable to those induced by wild-type PRRSV VR2385.

In summary, we successfully generated and rescued four PCV1 chimeric viruses expressing different known PRRSV linear-B epitopes (GP2 epitope II: aa 40–51, ASPSHVGWWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We further showed that three of these chimeric viruses were infectious in vitro and in pigs, and genetically stable. Importantly, we found that three PCV1-PRRSV_EPI chimeric viruses elicited neutralizing antibodies against PRRSV VR2385. Therefore, the results from the present study provided a proof of concept for further exploring the use of the non-pathogenic PCV1 as a live virus vector for vaccine delivery.

ACKNOWLEDGEMENTS

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[41] Plagemann PGW, Rowland RRR, Faaberg KS. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch Virol. 2002;147:2327-47.

FIGURE LEGENDS

Fig. 1. A schematic diagram for the construction of the PCV1-PRRSV\textsubscript{EPI} chimeric DNA clones. The epitope insertion was accomplished by two rounds of overlapping extension PCR. The first amplicon of 200 bp containing an overhanging GPxx\textsubscript{EPI} region (xx denote different inserted epitopes, GP2II, G3I, GP5I and GP5IV) was generated with M13-F and GPxx\textsubscript{EPI}-R primers (black arrows). The second amplicon of 1778 bp containing a complementary GPxx\textsubscript{EPI} overhanging region was generated with GPxx\textsubscript{EPI}-F and M13-R primers (empty arrow heads). The full-length PCV1-PRRSV epitopes chimeric clones were assembled by a fusion PCR using previously generated amplicons as templates, and with M13-F and M13-R primers.

<table>
<thead>
<tr>
<th>GPXX (aa)</th>
<th>Sequence</th>
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<tr>
<td>GP2II (39-51)</td>
<td>ASPSHVGWWSFA</td>
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<tr>
<td>GP3I (61-72)</td>
<td>QAAAEAYEPEGRS</td>
</tr>
<tr>
<td>GP5I (35-46)</td>
<td>SSSNLQLIYNLTI</td>
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<tr>
<td>GP5IV (186-200)</td>
<td>TPVTRVSAEQWGRP</td>
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Fig. 1
Fig. 2. Confocal microscopy of PK-15 cells infected with parental PCV1 as well as with four different PCV1-PRRSV_{EPI} chimeric viruses. PCV1-PRRSV_{EPI} chimeric viruses (PCV1-PRRSV_{EPI}GP2II, PCV1-PRRSV_{EPI}GP3I, PCV1-PRRSV_{EPI}GP5I, and PCV1-PRRSV_{EPI}GP5IV) and parental PCV1 were assayed by dual immunofluorescence staining. Infected cells were dually labeled with a mixture of mouse anti-PCV1 capsid monoclonal antibody (1:1000) (Mab) and PRRSV epitope-specific polyclonal antibodies (1:500) (Pab). In order to determine cross reactivity, cells infected with each specific chimeric virus group were tested against each respective PRRSV-specific epitope antibody. After incubation with the primary antibody, a mixture of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:100) (KPL, Kirkegaard & Perry Laboratories, Inc.) and goat anti-rabbit IgG-DyLight (1:500) (Thermo Scientific) were added. Dually infected cells were visualized using a Zeiss LSM 880 confocal microscope (Zeiss, Pleasanton, CA) with a 40X objective, using the argon 488 and helium-neon 594 lasers.
Fig. 3. Detection and quantification of PCV1 viral DNA loads in serum, lymphoid tissues and lung samples in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-PRRSV<sub>Epi</sub> chimeric viruses. Pigs were experimentally infected with parental PCV1 as well as each of the four different PCV1-PRRSV<sub>Epi</sub> chimeric viruses (PCV1-PRRSV<sub>Epi</sub>GP2II, PCV1-PRRSV<sub>Epi</sub>GP3I, PCV1-PRRSV<sub>Epi</sub>GP5I, and PCV1-PRRSV<sub>Epi</sub>GP5IV). Determination of viral DNA loads in serum and tissues was performed using TaqMan® qPCR. The number of animals used and the frequency variability in each time point, do not allow for robust statistical analysis. (A) Group mean log viral genomic copies/ml of serum is plotted for each treatment group, and the error bars indicate standard errors. (B) Mean viral DNA loads in tracheobronchial lymph node and lung were determined for each treatment group. Mean log viral genomic copies/gram of tissue is plotted for each treatment group, and the error bars indicate standard errors.
Fig. 3
Fig. 4. Anti-PCV1 IgG antibodies and anti-PRRSV N antibodies in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-PRRSV_{EPI} chimeric viruses. Pigs were infected with parental PCV1 as well as with each of the four different PCV1-PRRSV_{EPI} chimeric viruses. (A) Anti-PCV1 IgG antibodies were detected by an indirect immunofluorescence assay (IIFA). PCV1-free PK15 cells were infected with 1 MOI of parental PCV1. Immunoreactivity against PCV1 was evaluated in serum samples generated in pigs infected by parental PCV1, PCV1-PRRSV_{EPI} chimeric viruses, PRRSV VR2385 and MEM control. Detectable anti-PCV1 IgG antibodies were seen as early as 14 dpi in parental PCV1, PCV1-PRRSV_{EPI}GP3I, and PCV1-PRRSV_{EPI}GP5IV, followed by PCV1-PRRSV_{EPI}GP2II, and PCV1-PRRSV_{EPI}GP5I at 21 dpi. Different letters denote statistical difference. (B) The anti-PRRSV N antibody titers at indicated time points were detected using the IDEXX HerdCheck X3 ELISA kit. The level of antibody was expressed as a sample/positive (S/P) value ratio. The dash line shows the cutoff threshold (S/P value ≥0.4). Each plot represents the mean value of 3 pigs per infected group at each time point. Statistical comparison was performed using repeated-measures analysis of variance, followed by Tukey’s post-hoc procedure for multiple comparisons. Statistical significance was set to alpha = 0.05.
Fig. 4

A

Geometric mean titer of positives (log2)

B

S/P Ratio

PCV1
PCV1-PRRSV_EPI_KP2II
PCV1-PRRSV_EPI_KP3I
PCV1-PRRSV_EPI_KP5I
PCV1-PRRSV_EPI_KP5IV
PRRSV-VR2385
MEM
Fig. 5. PRRSV antigenic epitope-specific ELISAs for detection of the inserted PRRSV epitope antibodies induced by PCV1-PRRSV<sub>EPI</sub> chimeric viruses. Specific-pathogen-free pigs were infected with parental PCV1, as well as with each of the four PCV1-PRRSV<sub>EPI</sub> chimeric viruses containing respective PRRSV antigenic epitopes. PRRSV epitope-specific antibody responses were tested in serum samples of pigs infected with PCV1-PRRSV<sub>EPI</sub>GP2II, PCV1-PRRSV<sub>EPI</sub>GP3I, PCV1-PRRSV<sub>EPI</sub>GP5I, and PCV1-PRRSV<sub>EPI</sub>GP5IV, parental PCV1, and PRRSV VR2385. All infected groups were tested individually against each epitope peptide. (A) PCV1-PRRSV<sub>EPI</sub>GP2II; (B) PCV1-PRRSV<sub>EPI</sub>GP3I; (C) PCV1-PRRSV<sub>EPI</sub>GP5I; and (D) PCV1-PRRSV<sub>EPI</sub>GP5IV. The average of three animals is plotted for each time point, and standard errors are indicated. Asterisks indicate significant differences on that day for each of the PCV1-PRRSV<sub>EPI</sub> chimeric viruses compared to PRRSV VR2385 infected group. The dotted horizontal line indicates the cutoff of each assay. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig. 5
Fig. 6. Kinetics of anti-PRRSV neutralizing antibody response in pigs experimentally infected with each of the four PCV1-PRRSVEPI chimeric viruses as well as with the PRRSV VR2385 virus. Neutralizing antibody (NA) titers induced against the PRRSV VR2385 by each of the four PCV1-PRRSVEPI chimeric viruses as well as by the parental PRRSV VR2385 virus were detected as early as 28 dpi. NA antibodies titers observed in the PCV1-PRRSVEPI GP3I, PCV1-PRRSVEPI GP5I, and PCV1-PRRSVEPI GP5IV groups against PRRSV VR2385 were comparable to those observed in the PRRSV VR2385-infected group at 28 and 35 dpi. At 42 dpi, the NA antibodies titers in PCV1-PRRSVEPI GP5IV group were significantly lower than those observed in the PRRSV VR2385-infected group. The NA titers against parental strain PRRSV VR2385 and each of the PCV1-PRRSVEPI chimeric viruses were expressed as the highest dilution (2n) that showed a 90% or above reduction in the number of fluorescent foci compared to that of serum from negative control group. The NA titers against PCV1-PRRSVEPI GP2 II was not shown because of undetectable NA titer. Three independent experiments were performed for each test, and the error bars indicate standard errors. The P value shows whether one chimeric virus group had significant differences in NA titers compared to the parental PRRSV VR2385 group.
Fig. 6.
### Table 1. Primer sequences used in the construction and detection of the PCV1-PRRSV<sub>EPI</sub> chimeric viruses

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<td>CGATGTTGAATCTGAGGTGG</td>
</tr>
<tr>
<td>PCV1</td>
<td>P10</td>
<td>R581-602</td>
<td>AGAAAGCGGAATGAGATA</td>
</tr>
<tr>
<td>PCV1</td>
<td>Probe</td>
<td>R528-553</td>
<td>ACCGACTGGGCGTCGAGTATCC</td>
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Table 2. Detection of viremia and virus replication in tissues of pigs infected by parental PCV1, and each of the four PCV1-PRRSV<sub>EPI</sub> chimeric viruses

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of pigs with detectable viremia and viral genome in tissues (positive/total no. of pigs)</th>
<th>dpi</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42</th>
<th>Total</th>
<th>Lung</th>
<th>TBLN</th>
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<tr>
<td>PCV1-PRRSV&lt;sub&gt;EPI&lt;/sub&gt; GP2II</td>
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<tr>
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<tr>
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dpi: days post infection, TBLN tracheobronchial lymph nodes
CHAPTER 3

Expression of antigenic epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) in a modified live-attenuated porcine circovirus type 2 (PCV2) vaccine virus (PCV1-2a) as a potential bivalent vaccine against both PCV2 and PRRSV


Virus Research (2015) 154–164
**ABSTRACT**

Co-infection of pigs in the field with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) is common and poses a major concern in effective control of PCV2 and PRRSV. We previously demonstrated that insertion of foreign epitope tags in the C-terminus of PCV2 ORF2 produced infectious virions that elicited humoral immune responses against both PCV2 capsid and inserted epitope tags. In this study, we aimed to determine whether the non-pathogenic chimeric virus PCV1-2a, which is the basis for the licensed PCV2 vaccine Fostera™ PCV, can express PRRSV antigenic epitopes, thus generating dual immunity as a potential bivalent vaccine against both PCV2 and PPRSV. Four different linear B-cell antigenic epitopes of PRRSV were inserted into the C-terminus of the capsid gene of the PCV1-2a vaccine virus. We showed that insertion of 12 (PRRSV-GP2 epitope II, PRRSV-GP3 epitope I, and PRRSV-GP5 epitope I), and 14 (PRRSV-GP5 epitope IV) amino acid residues did not impair the replication of the resulting PCV1-2a-PRRSVEPI chimeric viruses *in vitro*. The four chimeric PCV1-2a viruses expressing PRRSV B-cell linear epitopes were successfully rescued and characterized. An immunogenicity study in pigs revealed that two of the four chimeric viruses, PCV1-2a-PRRSVEPIEPIGP3IG and PCV1-2a-PRRSVEPIEPIGP5IV, elicited neutralizing antibodies against PRRSV VR2385 as well as PCV2 (strains PCV2a, PCV2b, and mPCV2b). The results have important implications for exploring the potential use of PCV1-2a vaccine virus as a live virus vector to develop bivalent MLVs against both PCV2 and PRRSV.
INTRODUCTION

Porcine circovirus type 2 (PCV2) is the causative agent of porcine circovirus-associated disease (PCVAD) [57, 75, 307]. Among the most common clinical presentations associated with PCVAD is post-weaning multisystemic wasting syndrome (PMWS) [308]. PCV2 is also considered to be a part of the porcine respiratory disease complex (PRDC) [309]. Numerous reports have shown the importance of PCV2 in the development of PCVAD, however PCVAD has rarely been reproduced experimentally by PCV2 infection alone [294, 307]. Nevertheless, clinical PCVAD has been experimentally reproduced in PCV2 co-infection models with porcine parvovirus infection [57, 308, 310], Mycoplasma hyopneumoniae [311, 312], or porcine reproductive and respiratory syndrome virus (PRRSV) [279, 313].

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRSV [170, 171]. The major clinical manifestations of PRRS are reproductive failures, which are characterized by abortions and high percentages of mummified fetuses [314]. Additional clinical manifestations of PRRS include respiratory problems in nursery and growing-finishing pigs, growth reduction, poor performance, and high mortality rate [315, 316]. Both PRRSV and PCV2 have caused devastating diseases in swine industry worldwide, resulting in immense economic losses. Co-infection of pigs with PCV2 and PRRSV in the field are common in swine production worldwide. The prevalence of natural PRRSV and PCV2 co-infection in swine herds varies from 20% in Canada, 48% in Spain and 60% in the United States [280, 282, 317-319]. Therefore, prevention and control of PRRSV and PCV2 co-infection have been a priority for the global swine industry.

These two economically-important swine diseases are caused by completely unrelated viruses. PCV2 belongs to the family Circoviridae [16], which is a non-enveloped, single-stranded circular DNA virus of approximately 1.7 kb in genome size with 11 predicted potential open reading frames.
ORFs [24]. PCV2b is currently the predominant subtype infecting pigs worldwide, although PCV2a subtype is also prevalent in swine herds [156, 320, 321]. The two major ORFs of PCV2 are ORF1 which encodes the full-length replicase (Rep) of 314 amino acids (aa) and a truncated, spliced Rep’ protein (178 aa), and ORF2 which encodes the viral capsid protein (233 aa). PRRSV is a single-stranded, positive-sense RNA virus of approximately 15 kb in genome size that belongs to the family *Arteriviridae*. The genome of PRRSV consists of nine ORFs encoding nonstructural proteins (ORFs 1a, 1b) and structural proteins (ORFs 2a, 2b, 3, 4, 5, 6, and 7). There exist two distinct genotypes of PRRSV: North American (type 2) and European (type 1) [168-171]. The two genotypes share approximately 60% nucleotide sequence identity and are antigenically different [305, 322-324].

Both PRRSV and PCV2 are capable of tolerating mutations and insertion of foreign nucleic acids sequences without affecting their infectivity *in vivo* [295, 296, 325]. PCV2 can tolerate an insertion of up to 27 aa residues of foreign antigenic epitopes into the C-terminus of the ORF2 without impairing its infectivity *in vivo* [295]. More recently, insertion of a foot and mouth disease virus (FMDV) VP1 epitope in PCV2 resulted in infectious viruses that elicited dual immunity against PCV2 and FMDV [296].

Several commercial vaccines against PCV2 are currently available, including inactivated whole virus, inactivated chimeric PCV1-2a virus, and subunit vaccines [326]. Currently, a licensed modified live-attenuated vaccine (MLV) against PCV2 is still lacking. It has been shown that the replication factors of the non-pathogenic PCV1 and the PCVAD-associated PCV2 are interchangeable [327, 328]. A candidate MLV against PCV2 has been developed using a chimeric virus approach, in which the ORF2 capsid gene of pathogenic PCV2 was inserted into the backbone of the non-pathogenic PCV1. The resulting chimeric PCV1-2a virus is non-pathogenic.
but elicits protective immunity against PCV2 in pigs [144] and was the basis for the first USDA-fully licensed vaccine against PCV2. Similarly, another chimeric virus PCV1-2b, which the ORF2 capsid gene of PCV2b subtype was inserted in the backbone of PCV1, has been demonstrated to confer cross-protection against different PCV2 subtypes [153]. Due to PRRSV immune evasion and high genetic diversity among PRRSV strains, the current PRRSV vaccines only confer protection against closely-related strains but not against heterologous strains. Currently there are two type of commercial vaccine available, MLV and inactivated killed vaccines (KV) [243, 329].

Since PCV2 and PRRSV co-infections are very common in swine herds worldwide, it would be of great interest to develop a bivalent MLV vaccine that can protect pigs against both viruses. Therefore, it is logical to explore the potential use of the non-pathogenic chimeric PCV1-2a vaccine virus, which is the basis of the current commercial killed vaccine Fostera™ PCV, as a live virus vector to express PRRSV antigenic epitopes for use as a potential PCV2-PRRSV bivalent MLV vaccine.

**MATERIAL AND METHODS**

**Construction of chimeric PCV1-2a-PRRSVEPI (epitope) infectious DNA clones**

Four different antigenic epitopes of PRRSV strain VR2385, including GP2 epitope II (aa 39–51, ASPSHVGWWSFA), GP3 epitope I (aa 61–72, QAAAEAYEPGRS), GP5 epitope I (aa 35–46, SSSNLQLIYNL), and GP5 epitope IV (aa 186–200, TPVTRVSAEQWGRP), were cloned individually in frame into the C-terminus of the ORF2 capsid gene of the chimeric PCV1-2a vaccine virus previously developed in our laboratory [144]. Chimeras were constructed by overlapping extension PCR following a method described previously [295]. Briefly, two amplicons of 212 bp and 1800 bp with an overhanging complementary region containing different PRRSV antigenic epitopes (Table 1) and a SacII restriction site were constructed by overlapping
extension PCR using the PCV1-2a infectious clone plasmid pBSK-PCV1-2a [144] as the template. A second round of fusion PCR was used to assemble the two amplicons. The PCR product was digested with SacII (New England Biolabs) and ligated into pBluescript II SK (+) (pBSK+) (Stratagene) to generate each of the chimeric PCV1-2a-PRRSV_EPI DNA clones (Fig. 1). Recombinant plasmids containing the respective insert were transformed into the alpha-select strain of *Escherichia coli* (Bioline). Positive clones were selected via blue-white screening and the insertion of each specific PRRSV antigenic epitope was confirmed by sequencing. Chimeric PCV1-2a-PRRSV_EPI viruses were rescued by transfection of concatemerized genomic DNA of each chimera into the PCV1-free PK-15 cells as previously described [151]. Infectious viruses were harvested by freezing and thawing the cell cultures three times and were stored at -80 °C for further use.

**Infectivity, PRRSV antigenic epitope expression, and comparative growth kinetics of chimeric PCV1-2a-PRRSV_EPI viruses**

To determine the infectivity of the PCV1-2a-PRRSV_EPI chimeric viruses, PK-15 cells were seeded at a concentration of 2 × 10^5 cells/well in 48 well plates and infected at 40-50% confluency with 100 µl of 1:10 serial dilutions of each virus stock. After 72 h, the cells were fixed, and virus infectivity was detected by immunofluorescence assay (IFA) using 1:1000 dilution of a mouse anti-PCV2-Cap specific monoclonal antibody (mAb) (RTI, Brookings, SD), followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG-FITC (KPL, Kirkegaard & Perry Laboratories, Inc.). The positive fluorescence signals were detected using a fluorescent microscope. Expression of the PRRSV-specific antigenic epitopes was detected by IFA using custom polyclonal antibodies (Biomatik) against each of the PRRSV synthetic antigenic epitope, followed by a secondary goat anti-rabbit IgG (DyLight 550; emission spectrum peak wavelength
of approximately 576 ± 4 nm; Thermo Scientific). Serial ten-fold dilutions of each virus stock were performed in order to determine the tissue culture infectious dose 50 (TCID$_{50}$) according to the method described by Reed and Muench [297].

To compare the replication and growth kinetics of the PCV1-2a-PRRSV$_{EPI}$ chimeric viruses, and its parental PCV1-2a vaccine virus, six well plates were seeded with PK15 cells and infected at a multiplicity of infection (MOI) of 0.1, at a 40-50% confluency. Infection was carried out for 96 h, and the cells were collected every 12 h by three cycles of freezing and thawing. At different time points, the viral genomic copy numbers in infected cells were quantified by a TaqMan-based real time PCR. Briefly, viral DNA was extracted using the ZR Viral DNA Kit™ according to the manufacture’s protocol (Zymo Research Corporation). The DNA genomic copy numbers of each virus were quantified by a PCV1-2a TaqMan-based real-time PCR with primers and probes targeting the intergenic region of the PCV1-2a virus genome (Table 1). The real-time PCR was performed using a Bio Rad CFX96 detection system (Bio Rad) under the following conditions: an activation step at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds, 55-60 °C for 60 seconds, and 72 °C for 60 seconds. A ten-fold serially diluted pBSK-PCV1-2a DNA plasmid containing the full-length sequence of the PCV1-2a virus was used to construct the standard curve. Each reaction was performed in triplicate.

**In vivo characterization of infectivity and immunogenicity of four PCV1-2a-PRRSV$_{EPI}$ chimeric viruses**

**Experimental design for the pig infection study**

To determine whether the chimeric viruses can infect pigs and induce PRRSV epitope-specific antibodies, a total of 21 specific-pathogen-free (SPF) pigs were randomly assigned into 7 groups with three pigs per group, including a PCV1-2a and a PRRSV VR2385 positive control.
groups, a negative control group, and four groups with each of the PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses that were confirmed to be viable \textit{in vitro}. The pigs were each inoculated intramuscularly with 5 mL of $4.64 \times 10^2 \text{TCDI}_{50}/\text{mL}$ of each of the four PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses and of the PCV1-2a positive control as well as each with 5 mL of $2 \times 10^5 \text{TCDI}_{50}/\text{mL}$ of PRRSV VR2385 in the PRRSV positive control group. The negative control group was inoculated intramuscularly with 5 mL of sterile MEM cell culture media. Serum samples were collected from all pigs prior to inoculation and weekly thereafter for a period of 8 weeks. All animals were euthanized at 56 days post-inoculation (dpi), and samples of lung and tracheobronchial lymph node tissues were collected during necropsy.

\textbf{Quantification of viral DNA loads in sera and lung tissues}

Viral DNAs were extracted from serum samples collected at 0, 7, 14, 21, 28, 35, 42, 49, and 56 dpi as well as from lung and tracheobronchial lymph node tissues at 56 dpi, using the Ambion MagMAX-96 Viral DNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. The DNA standard used for the qPCR was derived from the virus stock used for inoculation as well as from the infectious clone plasmid containing the full-length PCV1-2a genome. To evaluate and rule out potential cross-contamination, PCV1 DNA was also extracted from the virus stock generated in our laboratory, and an empty pBSK+ vector (Stratagene) was included as a negative control as well. The PCV1-2a DNA copy numbers in sera or tissues were quantified using a TaqMan\textsuperscript{®} Fast Virus 1-Step Master Mix (Life Technologies Corp.) according to the manufacturer’s protocol. The PCR primers and probes (PCV1-2a cap P11/PCV1-2a Cap P12/PCV1-2a probe 2) (Table 1) used in the qPCR assay were designed to target the specific amplicon of 95 bp in ORF2. The qPCR assay was conducted using the ABI 7500 (RT) PCR system (Life Technologies Corp). The PCR conditions included denaturation at 95°C for 20 s, annealing
at 95°C for 3 s, amplification at 60°C for 30 s, and a final extension at 72°C for 5 min, with a total number of 40 cycles. Each reaction was performed in triplicate.

**Detection of anti-PCV1-2a antibodies and anti-PRRSV_{EPI} epitope antibodies by ELISA**

All serum samples were tested for the presence of anti-PCV2 IgG antibodies using an PCV2 ORF2-based ELISA [330]. Samples were considered positive if the calculated sample-to-positive (S/P) ratio was equal to 0.2 or greater. The anti-PRRSV N antibody response was evaluated using the HerdCheck X3 ELISA kit according to the manufacturer’s protocol. Antibody levels were expressed as a sample/positive (S/P) value ratio with a cutoff threshold of S/P value ≥0.4.

Four different PRRSV KLH-conjugated synthetic antigenic epitope peptides (GP2 epitope II: aa 39–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNLT; and GP5 epitope IV: aa 186–200, TPVTRVSAEQWGRP) were synthesized and used to standardize four different PRRSV antigenic epitope peptide-based ELISAs. Each vial of lyophilized peptide (5 mg) was resuspended in 1 mL of UltraPure™ Distilled Water (Gibco®, Life Technologies) to a final stock concentration of 5 mg/mL, aliquoted and stored at -80 °C. Following titration and optimal dilution, 96-well microtitration plates (Nunc, Thermo Fisher Scientific) were manually coated with 100 μL per well of each peptide at a concentration of 5 μg/mL in phosphate-buffered saline (PBS) at pH 7.4 (Gibco®, Life Technologies) and incubated at 4 °C overnight. After incubation, the plates were washed five times, blocked with 300 μL per well of a solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc.), and incubated at 25 °C for 2 h. The plates were then dried at 37 °C for 4 h and stored at 4 °C in sealed bags containing desiccant packs. The ELISA conditions, including coating and blocking, buffers, sample and conjugate dilutions, and incubation conditions (time and temperature), were
identical for the four peptide-based ELISAs. Antibodies were detected in 100 µL of a 1:50 serum dilution per well. The plates were incubated at 37 °C for 1 h and washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 µL of peroxidase-conjugated goat anti-pig IgG (Fc) antibody diluted at 1:15,000 (Bethyl Laboratories Inc.) was added to each well, and the plates were incubated at 37 °C for 1 h. After the washing step, the reaction was visualized by adding 100 µL of tetramethylbenzidine-hydrogen peroxide (Dako North America, Inc.) substrate solution to each well. After incubation for 10 min at room temperature, the reaction was stopped by adding 50 µL of a stop solution (1 M sulfuric acid) to each well. The reactions were measured according to the optical density at 450 nm using an ELISA plate reader (Biotek® Instruments Inc.) operated with commercial software (GEN5TM, Biotek® Instruments Inc.).

Detection of neutralizing activity against PCV2 and PRRSV using serum virus neutralization assays

Neutralizing antibody titers against PCV2 were tested with a serum virus neutralization assay (SVN) using serum samples collected at 28, 35, 42, 49, and 56 dpi, essentially as previously described [295]. Briefly, serum samples were first heat-inactivated for 1 h at 56 ºC, and subsequently serial 2-fold dilutions of each serum sample were incubated with equal volumes of PCV2a, PCV2b and mPCV2b at an infectious titer of 5 × 10^3 TCID_{50}/mL for 1 h at 37 ºC. The virus–serum mixtures were then dispensed in 96-well plates containing PK-15 cells at 50-60% confluency and incubated for 1 h at 37 ºC. Infected cells were maintained in 150 µL of complete MEM media at 37 ºC and 5% CO_2. After 72 hours post-infection (hpi), the cells were assayed by IFA with anti-PCV2-Cap specific mAb (RTI, Brookings, SD), followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG-FITC (KPL, Kirkegaard & Perry Laboratories, Inc.) as previously described.
For testing the neutralizing antibody titers against PRRSV, a SVN test was conducted as previously described [298]. Briefly, two-fold diluted serum samples collected at 28, 35, 42, 49, and 56 dpi from each pig were mixed with an equal volume of PRRSV VR2385 virus at an infectious titer of $2 \times 10^3$ TCID$_{50}$/mL and incubated at 37°C for 1 h. The mixtures were then dispensed into MARC-145 cells in 96-well plates and incubated for 1 h at 37 °C. After washing with PBS, the cells were maintained in DMEM with 2% fetal bovine serum (FBS). At approximately 20 hpi, the cells were assayed by IFA with anti-PRRSV N mAb SDOW17 (Rural Technologies, Inc.) followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG-FITC (KPL, Kirkegaard & Perry Laboratories, Inc.) for evidence of virus infection. The neutralizing antibody titers were expressed as the highest dilution that showed a 90% or above reduction in the number of fluorescent foci compared to that of serum samples from negative control pigs. Samples were evaluated in triplicate and three independent tests were performed for each serum sample.

**Statistical analysis**

A repeated measure two-way ANOVA with Tukey’s correction was calculated for multiple comparison. The statistical significance was set at alpha = 0.05. All analyses were performed using a commercially available software GraphPad Prism® 6 (GraphPad Software).

**RESULTS**

**Insertion of PRRSV antigenic epitopes at the C-terminus of ORF2 capsid gene of the chimeric PCV1-2a vaccine virus does not impair virus infectivity in vitro**

The four chimeric PCV1-2a-PRRSV$_{EPI}$ clones were fully sequenced and confirmed to contain each of the respective PRRSV antigenic epitopes in frame within the C-terminus of the ORF2 gene of the PCV1-2a vaccine virus. After five consecutive passages in PK-15 cells, the
viability and genetic stability of each of the four PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses was confirmed by dual IFA staining against both PCV2 Cap and PRRSV-specific epitopes, as well as by sequencing the full-length genome of each PCV1-2a-PRRSV<sub>EPI</sub> chimeric virus (data not shown). Confocal microscopy showed that each PCV1-2a-PRRSV<sub>EPI</sub> chimeric virus co-expressed both PCV2 Cap and respective PRRSV antigenic epitope in the nucleus of infected PK-15 cells (Fig. 2). The genomic copy numbers of the PCV1-2a-PRRSV<sub>EPI</sub>GP3I chimeric virus were significantly higher at 48 and 72 hpi, and of PCV1-2a-PRRSV<sub>EPI</sub>GP3I chimeric virus were significantly higher at 60 and 84 dpi when compared to that of the PCV1-2a vaccine virus in infected cells. However, there was no significant difference in the genomic copy numbers between PCV1-2a and PCV1-2a-PRRSV<sub>EPI</sub> chimera at 96 hpi (Fig. 3).

**PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses expressing PRRSV Gp3I and Gp5IV epitopes produce viremia and replicate in tissues of inoculated pigs**

All serum samples collected prior to inoculation at day 0 were tested negative for PCV1-2a DNA by qPCR. All pigs in the PCV1-2a-PRRSV<sub>EPI</sub>GP3I and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV chimeric virus groups as well as in the PCV1-2a virus group developed viremia. Viral DNA was detected at 7 dpi in all positive groups. Although all pigs in these three groups were positive, the frequency of positive animals observed at different time points varied throughout the trial. The number of animals used and the variability in frequency did not allow for a robust statistical analysis. However, the average genome copy number in serum from each chimeric virus was within one log difference (PCV1-2a-PRRSV<sub>EPI</sub>GP3I, $3.08 \times 10^4$ genomic copies/mL; and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV, $1.51 \times 10^4$ genomic copies/mL) and had at least two-fold lower genomic copies/mL than that of the PCV1-2a vaccine virus (PCV1-2a, $5.74 \times 10^6$ genomic copies/mL) (Fig. 4A). Surprisingly, viral DNAs from pigs inoculated with two of the chimeric viruses, PCV1-2a-
PRRSV\textsubscript{EPI}GP2II and PCV1-2a-PRRSV\textsubscript{EPI}GP5I, were undetectable in serum or tissues, even though these two chimeras were tested viable \textit{in vitro}.

No significant gross lesion was observed in the PCV1-2a-PRRSV\textsubscript{EPI} chimeric virus-infected groups or in the PCV1-2a, PRRSV VR2385, or MEM control groups at 56 dpi. All animals inoculated with PCV1-2a-PRRSV\textsubscript{EPI}GP3I, PCV1-2a-PRRSV\textsubscript{EPI}GP5IV, and PCV1-2a had detectable viral DNA in lung and tracheobronchial lymph nodes, suggestive of virus replication in tissues (Table 2). No significant difference in the genomic copy number/gram of tissues was observed between PCV1-2a and PCV1-PRRSV\textsubscript{EPI}GP3I, or between PCV1-2a and PCV1-PRRSV\textsubscript{EPI}GP5IV (\textbf{Fig. 4B}). No PCV1-2a viral DNA was detected in the PRRSV VR2385 and MEM control groups.

\textbf{PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses induce both anti-PCV2 and anti-PRRSV epitope-specific antibodies in pigs}

Anti-PCV2 IgG antibodies were detected in the sera of the PCV1-2a group, as well as in the PCV1-2a-PRRSV\textsubscript{EPI}GP3I, and PCV1-2a-PRRSV\textsubscript{EPI}GP5IV chimeric virus groups. Significant levels of IgG anti-PCV2 antibodies were detected between 28 and 35 dpi in PCV1-2a and PCV1-2a-PRRSV\textsubscript{EPI}GP3I groups, and between 35 and 42 dpi in the PCV1-2a-PRRSV\textsubscript{EPI}GP5IV group, all remained seropositive at the end of the study at 56 dpi (\textbf{Fig. 5A}). There was no detectable level of anti-PCV2 IgG antibodies in the PCV1-2a-PRRSV\textsubscript{EPI}GP2II and PCV1-2a-PRRSV\textsubscript{EPI}GP5I chimeric virus groups or in the PRRSV and MEM control groups. As expected, anti-PRRSV N antibodies were detected only in the PRRSV VR2385-infected group (\textbf{Fig. 5B}).

Antibody responses against the inserted PRRSV antigenic epitopes were detected by using PRRSV epitope-specific ELISAs. There were no detectable antibodies against PRRSV-GP2 epitope II or PRRSV-GP5 epitope I in pigs inoculated with the PCV1-PRRSV\textsubscript{EPI}GP2II or PCV1-
PRRSV<sub>EPI</sub>GP5I chimeric viruses (Fig. 5A, 5C). However, antibodies against PRRSV-GP3 epitope I were detected in the PCV1-2a-PRRSV<sub>EPI</sub>GP3I chimeric virus group between 28 and 35 dpi, and remained positive at 56 dpi (Fig. 5B). Antibodies specific against PRRSV-GP5 epitope IV was detected at 14 dpi in the wild-type PRRSV VR2385 infected group, and at 35 dpi in the PCV1-2a-PRRSV<sub>EPI</sub>GP5IV chimeric virus group, and they remained seropositive at 56 dpi (Fig. 5D).

**PCV1-2a-PRRSV<sub>EPI</sub> chimeric virus-infected pigs develop neutralizing antibodies against both PCV2 and PRRSV**

Neutralizing antibodies (NA) against PCV2a were detected in pigs infected with PCV1-2a as well as with the two chimeric viruses PCV1-2a-PRRSV<sub>EPI</sub>GP3I and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV at 42 dpi and remained detectable at 56 dpi. There was no difference in NA antibody levels among these three infected groups at different time points except, at 56 dpi, when there was a significantly higher level of NA in the PCV1-2a group compared to that of the PCV1-2a-PRRSV<sub>EPI</sub> chimeric virus groups (Fig. 7A). NA antibodies against PCV2b were also detected in both PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV chimeric virus groups at 49 and 56 dpi, and the anti-PCV2b NA levels were higher in PCV1-2a-infected pigs than that in the PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV infected-groups (Fig. 7B). Similar levels of anti-mPCV2 NA were detected in PCV1-2a as well as in PCV1-2a-PRRSV<sub>EPI</sub>GP3I and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV infected groups at 42 and 49 dpi (Fig. 7C).

A SVN assay against PRRSV VR2385 was performed in order to determine whether the PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses induce neutralizing antibodies against PRRSV. Anti-PRRSV neutralizing antibodies were detected in the PCV1-2a-PRRSV<sub>EPI</sub>GP3I and the PCV1-2a-PRRSV<sub>EPI</sub>GP5IV chimeric virus groups at 28-56 dpi. No statistical difference in the neutralizing antibody titers was observed at 28, 35, and 42 dpi between PRRSV VR2385 wild-type, PCV1-2a-
PRRSV\textsubscript{EPI}GP3I, or PCV1-PRRSV\textsubscript{EPI}GP5IV (Fig. 7D). As expected, anti-PRRSV neutralizing antibodies were not detected in the PCV1-2a and MEM control groups.

**DISCUSSION**

PCV2 and PRRSV are two of the most economically-important global swine pathogens causing tremendous economic losses in the swine industry worldwide. Field epidemiological data revealed that PCV2 and PRRSV co-infection in the field is common in PCVAD outbreaks [280, 282, 317-319]. The clinical importance of these two viruses, and the high prevalence of co-infection in the field demand a PCV2-PRSSV bivalent vaccine that can prevent both virus infections.

Previously, we demonstrated that ORF1 can be interchangeable between PCV2 and PCV1 without impairing virus infectivity [151], and that chimeric PCV1-2 viruses with PCV2 capsid gene in the backbone of PCV1 are non-pathogenic but elicit protective immunity against the PCV2 [144]. We also showed that the chimeric PCV1-2a vaccine virus can tolerate insertion of up to 27 aa foreign antigenic epitopes (HA, Glu and KT3 epitope tags) without affecting virus infectivity [295]. Therefore, in this study, we evaluated whether the chimeric PCV1-2a vaccine virus can be used as a live virus vector to express PRRSV antigenic epitopes as a potential bivalent MLV vaccine. We demonstrated that the viability of the PCV1-2a vaccine virus was not impaired \textit{in vitro} by the insertion of four different PRRSV VR2385 epitopes, respectively, in the C-terminus of the capsid gene of the PCV1-2 vaccine virus. Although there were some differences in virus replication during the time course of the \textit{in vitro} study, at 96 hpi there was no difference in the genomic copy numbers between PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses and PCV1-2a virus. A subsequent pig infection study demonstrated that only two of the four chimeric viruses, PCV1-2a-PRRSV\textsubscript{EPI}GP3I and PCV1-2a-PRRSV\textsubscript{EPI}GP5IV, were capable of replicating in pigs and eliciting
dual humoral immune responses against both PCV2 and PRRSV *in vivo*. Although the genomic copy numbers in sera was slightly lower in pigs infected with the two PCV1-2a-PRRSV_{EPI} chimeric viruses compared to that of the PCV1-2a virus, there was no difference in the levels of anti-PCV2 antibody or early PCV2 neutralizing antibody levels. Therefore, it appears that the expression of capsid protein of PCV2 in PCV1-2a vaccine virus was not impaired by the insertion in-frame of PRRSV antigenic epitopes. Although the PCV1-2a-PRRSV_{EPI}GP2II and PCV1-2a-PRRSV_{EPI}GP5I chimeras were shown to be viable *in vitro*, evidence of virus replication was not detected in inoculated pigs as there was no viremia or detectable viral DNA in tissues. It is possible that the lack of viral replication *in vivo* may be associated with a short duration of viremia that is undetectable with the sampling scheme used in this study, a short or insufficient antigen exposure to induce sufficient immune response or the inability of the two chimeric viruses to effectively infect pigs.

It has been demonstrated that the presence of neutralizing antibodies is inversely correlated with PCVAD clinical signs [117]. With the emergence of PCV2 mutants such as mPCV2 in the field [40] and the association of PCV2 cases with vaccine failures [331], it would be interesting to evaluate whether PCV1-2 MLV vaccine could protect against known PCV2 subtypes such as PCV2a, PCV2b and mPCV2. It has been reported that pathogenicity did not differ among PCV2a, PCV2b, and the newly described mPCV2 strain [41], and the current PCV2a-based vaccines protect against PCV2b and the new mutant strain mPCV2 [332]. In this study, we demonstrated that the PCV1-2a chimeric virus induced neutralizing antibodies against PCV2a, PCV2b, and mPCV2. The PCV1-2a-PRRSV_{EPI} chimeric viruses induced comparable levels of neutralizing antibodies against all three PCV2 strains (PCV2a, 2b, and mPCV2) evaluated in this study. Thus,
it appears that insertion of PRRSV antigenic epitopes in the C-terminus of the ORF2 did not impair the ability of the PCV1-2a vaccine virus to induce neutralizing antibodies against PCV2 strains.

In the present study, we constructed four PCV1-2a chimeric viruses expressing B-cell linear epitopes of PRRSV, which were previously demonstrated to be immunogenic against PRRSV [218, 302, 303, 333, 334]. It has been reported that anti-GP5 plays an important role in PRRSV neutralization [302, 304, 305]. PRRSV-GP5 epitope IV is an important immunogenic epitope (P^{188}_{\text{LTR}} (V/T) SAEQW^{197}) that has also been shown to be reactive with antisera raised against type 1 European PRRSV strains. This epitope seems to be well conserved amongst type 2 strains with only a few aa differences [219]. We demonstrated in this study that PCV1-2a-PRRSV_{EPI}GP5IV chimeric virus induced anti-PRRSV neutralizing antibody at a level that is comparable with the wild-type PRRSV VR2385. The ORF3 of PRRSV is considered the second most variable PRRSV protein with four consecutive peptides spanning aa 61–105, which are considered as an important immunodominant domains of GP3 [218, 306]. The PCV1-2a-PRRSV_{EPI}GP3I chimeric virus contains aa 61–72 (QAAAEAYEPGRS) and it was shown to induce similar levels of anti-PRRSV neutralizing antibodies when compared to the wild-type PRRSV VR2385.

In summary, we successfully generated four PCV1-2a chimeric viruses expressing PRRSV linear-B cell epitopes (GP2 epitope II: aa 40–51, ASPSHVGWWSFA; GP3 epitope I: aa 61–72, QAAAEAYEPGRS; GP5 epitope I: aa 35–46, SSSNLQLIYNL; and GP5 epitope IV: aa 187–200, TPVTRVSAEQWGRP). We further demonstrated that two of the chimeric viruses, PCV1-2a-PRRSV_{EPI}GP3I and PCV1-2a-PRRSV_{EPI}GP5IV, were infectious in pigs. Importantly, these two PCV1-2a-PRRSV_{EPI} chimeric viruses elicited neutralizing antibodies against both PCV2 (PCV2a, PCV2b, mPCV2) and PRRSV VR2385. Thus, the present study provides a proof of
concept for the potential use of the PCV1-2a vaccine virus as a live vaccine virus vector to develop candidate bivalent MLV vaccine against both PCV2 and PRRSV. Future challenge and efficacy studies are necessary to evaluate the degree of protection in a PCV2-PRRSV co-infection model.

ACKNOWLEDGEMENTS

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(PCV2) Cloned into the Genomic Backbone of the Nonpathogenic PCV1 Induces Protective Immunity against PCV2 Infection in Pigs. J. Virol. 78(12), 6297-6303.


FIGURE LEGENDS

Fig. 1. A schematic diagram for the construction of the PCV1-2a-PRRSV<sub>EPI</sub> chimeric DNA clones. The epitope insertion was accomplished by two round of overlapping extension PCR. The first amplicon of 200 bp containing an overhanging GPxx<sub>EPI</sub> region (xx denote different inserted epitopes, GP2II, G3I, GP5I and GP5IV) was generated with M13-F and GPxx<sub>EPI</sub>-R primers (black arrows). The second amplicon of 1778 bp containing a complementary GPxx<sub>EPI</sub> overhanging region was generated with GPxx<sub>EPI</sub>-F and M13-R primers (empty arrow heads). The full-length PCV1-2a-PRRSV<sub>EPI</sub> chimeric clones were assembled by a fusion PCR using previously generated amplicons as templates, and with M13-F and M13-R primers.

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Fig. 1.
Fig. 2. Confocal microscopy of PK-15 cells infected with wild-type PCV1-2a vaccine virus as well as with four different PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses. PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses (PCV1-2a-PRRSV\textsubscript{EPI}GP2II, PCV1-2a-PRRSV\textsubscript{EPI}GP3I, PCV1-2a-PRRSV\textsubscript{EPI}GP5I, and PCV1-2a-PRRSV\textsubscript{EPI}GP5IV) and PCV1-2a vaccine viruses were assayed by dual immunofluorescence assay (IFA) staining. Infected cells were dually labeled with a mixture of mouse anti-PCV2 capsid monoclonal antibody (1:1000) (Mab) and anti-PRRSV epitope peptide-specific mono-specific polyclonal antibodies (1:500) (Pab). In order to determine cross-reactivity, cells infected with each chimeric virus were tested against each respective anti-PRRSV epitope-specific peptide antibody. After incubation with the primary antibody, a mixture of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:100) (KPL, Kirkegaard & Perry Laboratories, Inc.) and goat anti-rabbit IgG-DyLighttm (1:500) (Thermo Scientific) were added. Infected cells were visualized using a Nikon TE2000-E confocal microscope at 488 nm (525/50 emission filter) to detect the PCV2 capsid, and at 647 nm (710/50 emission filter) to detect the PRRSV antigenic epitope.
Fig. 2.
Fig. 3. Replication kinetics of PCV1-2a and four PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses \textit{in vitro}. The replication kinetics of PCV1-2a and PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses were compared by quantifying the viral DNA loads during a period of 96 h. The PK-15 cells were infected with PCV1-2a vaccine virus as well as with the four PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses at 0.1 MOI. Genomic copy numbers for PCV1-2a, PCV1-2a-PRRSV\textsubscript{EPI}GP2II, PCV1-2a-PRRSV\textsubscript{EPI}GP3I, PCV1-2a-PRRSV\textsubscript{EPI}GP5I, and PCV1-2a-PRRSV\textsubscript{EPI}GP5IV in cell cultures at each time point were determined by real-time PCR based on 95 nt amplicon in the intergenic region of the PCV1-2a genome. Statistical comparison was performed using repeated-measures analysis of variance, followed by Dunnett's multiple comparisons test. Statistical significance was set to alpha = 0.05. The asterisks (*) indicate a significant difference between the PCV1-2a and chimera PCV1-2a-PRRSV\textsubscript{EPI} chimeric viruses. Statistical significance were observed between PCV1-2a-PRRSV\textsubscript{EPI}GP5IV and PCV1-2a at 48 hpi (p=0.0023) and 72 hpi (p< 0.0001), and PCV1-2a-PRRSV\textsubscript{EPI}GP3I and PCV1-2a at 60 hpi (p=0.0126) and 84 hpi (p= 0.0049).
Fig. 4. Detection and quantification of PCV2 viral DNA loads in serum, lymphoid tissues and lung samples in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses. Pigs were experimentally infected with PCV1-2a vaccine virus as well as each of the four different PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses (PCV1-2a-PRRSV<sub>EPI</sub>GP2II, PCV1-2a-PRRSV<sub>EPI</sub>GP3I, PCV1-2a-PRRSV<sub>EPI</sub>GP5I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV). (A) Determination of viral DNA loads in serum was performed using TaqMan® qPCR. (B) Determination of viral DNA loads in tracheobronchial lymph node and lung tissues were performed using TaqMan® qPCR. The number of animals used and the frequency variability in each time point, do not allow for robust statistical analysis; mean viral DNA loads in tracheobronchial lymph node and lung were determined for each treatment group. Mean log viral genomic copies/gram of tissue is plotted for each treatment group, and the error bars indicate standard errors.
Fig. 4.
Fig. 5. Anti-PCV2 IgG antibodies and anti-PRRSV N antibodies in specific-pathogen-free (SPF) pigs experimentally infected with PCV1-2a-PRRSV_{EPI} chimeric viruses. Pigs were infected with PCV1-2a vaccine virus as well as with each of the four different PCV1-2a-PRRSV_{EPI} chimeric viruses. (A) Anti-PCV2 IgG antibodies were detected by an ELISA. Immunoreactivity against PCV2 was evaluated in serum samples generated in pigs infected by PCV1-2a vaccine virus, PCV1-2a-PRRSV_{EPI} chimeric viruses, PRRSV VR2385, and MEM control. Detectable anti-PCV2 IgG antibodies were seen as early as 28 dpi in PCV1-2a group, and 35 dpi in PCV1-2a-PRRSV_{EPI}GP3I, and PCV1-2a-PRRSV_{EPI}GP5IV groups. (B) The anti-PRRSV N antibody titers at indicated time points were detected using the IDEXX HerdCheck X3 ELISA kit. The level of antibody was expressed as a sample/positive (S/P) value ratio. The dash line shows the cutoff threshold (S/P value $\geq 0.4$). Each plot represents the mean value of 3 pigs per infected group at each time point. Statistical comparison was performed using repeated-measures analysis of variance, followed by Tukey’s post-hoc procedure for multiple comparisons. Statistical significance was set to alpha = 0.05.
Fig. 5.
Fig. 6. PRRSV epitope peptide-specific ELISAs for detection of anti-PRRSV epitope antibodies induced by PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses. Specific-pathogen-free pigs were infected with PCV1-2a vaccine virus or with each of the four PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses expressing respective PRRSV antigenic epitopes. PRRSV epitope-specific antibody responses were tested in serum samples of pigs infected with PCV1-2a-PRRSV<sub>EPI</sub>GP2II, PCV1-2a-PRRSV<sub>EPI</sub>GP3I, PCV1-2a-PRRSV<sub>EPI</sub>GP5I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV, PCV1-2a, and PRRSV VR2385. All infected groups were tested individually against each epitope peptide. (A) PCV1-2a-PRRSV<sub>EPI</sub>GP2II; (B) PCV1-2a-PRRSV<sub>EPI</sub>GP3I; (C) PCV1-2a-PRRSV<sub>EPI</sub>GP5I; and (D) PCV1-2a-PRRSV<sub>EPI</sub>GP5IV. The average of three animals is plotted for each time point, and standard errors are indicated. Asterisks indicate significant difference on that day for each of the PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses compared to PRRSV VR2385 infected group. The dotted horizontal line indicates the cutoff of each assay. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig. 6.
Fig. 7. Kinetics of anti-PCV2 and anti-PRRSV neutralizing antibody responses in pigs experimentally infected with each of the four PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses as well as with the PRRSV VR2385 virus. (A) Neutralizing antibody (NA) titers induced against PCV2a were detected as early as 42 dpi in PCV1-2a and PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV groups. (B) Detection of NA against PCV2b were observed at 28 dpi in PCV1-2a and remained detectable at 56 dpi. NA induced by PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV were detectable at 49 and 56 dpi, although the NA titers were significantly lower than that induced by PCV1-2a vaccine virus. (C) The NA titers against mutant PCV2 mPCV2b were detected as early as 42 dpi in PCV1-2a and PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV. There was no difference in NA antibodies tires at 42 and 49 dpi, although at 56 dpi, the NA titer induced by the PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses were significantly lower than that induced by PCV1-2a. Three independent experiments were performed for each test, and the error bars indicate standard errors. The asterisks (*) indicate a significant difference between the PCV1-2a and chimera PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses. The NA titers against each PCV2 strain and each of the PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses were expressed as the highest dilution (2n) that showed a 90% or above reduction in the number of cells compared to that of serum from negative control group. (D) Neutralizing antibody (NA) titers against PRRSV VR2385 by PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses as well as by the PRRSV VR2385 virus were detected as early as 28 dpi. Anti-PRRSV VR2385 NA titers in the PCV1-2a-PRRSV<sub>EPI</sub>GP3I, and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV groups were comparable to those in the PRRSV VR2385-infected group at 28, 35 and 42 dpi. At 49 and 56 dpi, the NA titers in PCV1-2a-PRRSV<sub>EPI</sub>GP3I and PCV1-2a-PRRSV<sub>EPI</sub>GP5IV groups were significantly lower than those observed in the PRRSV VR2385-infected group. The NA titers against PCV1-2a-PRRSV<sub>EPI</sub> GP2 II and PCV1-2a-PRRSV<sub>EPI</sub>GP5I
were not shown because of undetectable NA titers. Three independent experiments were performed for each test, and the error bars indicate standard errors. The asterisks (*) indicate a significant difference between the PRRSV VR2385 and the PCV1-2a-PRRSV_{EPI} chimeric viruses.
Fig. 7.
### Table 1. Primer sequences used for construction and identification of the PCV1-2a-PRRSV<sub>EPI</sub> chimeric viruses

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Table 2. Detection of viremia and virus replication in tissues of pigs infected by parental PCV1, and each of the four PCV1-PRRSV<sub>EPI</sub> chimeric viruses

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dpi: days post infection, TBLN tracheobronchial lymph nodes
CHAPTER 4

Modulation of proinflammatory cytokines in monocyte-derived dendritic cells (MoDCs) by porcine reproductive and respiratory syndrome virus (PRRSV) through interaction with the porcine intercellular-adhesion-molecule-3-grabbing non-integrin (pDC-SIGN)


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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes arguably the most economically-important global swine disease which is characterized by reproductive failure and abortion in sows, and respiratory diseases with a high mortality rate in nursery pigs. One of the key mechanisms that PRRSV induces immune suppression is the modulation of numerous interleukins, such as type I interferons (IFN-I), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-12 in infected pigs. Antigen-presenting cells (APCs) are the first line of defense, and play an important role in innate immune regulation during early stages of immune responses to PRRSV infection. Among the APCs, pulmonary alveolar macrophages (PAMs), pulmonary interstitial macrophages (PIMs), and dendritic cells (DCs) are the main targets for PRRSV replication. The role of PRRSV-DCs interaction in PRRSV infection is largely unknown, and current research focuses on the production and regulation of interferons and specific inflammatory cytokines in DCs that may play a role in immune modulation after infection. In this study, we evaluated the immunomodulation of MoDCs through the pDC-SIGN receptor, which interacts with PRRSV, by blocking the pDC-SIGN receptor with recombinant hICAM-3-Fc or anti-pDC-SIGN mAb. We demonstrated that recombinant hICAM-3-Fc enhances the mRNA expression levels of proinflammatory cytokines. We also showed that anti-pDC-SIGN mAb inhibits the mRNA expression of TNF-α and IL-1α but enhances the expression of IL-12 induced by PRRSV in MoDCs. The results help understand the mechanisms of immunomodulation by PRRSV.

Keywords: dendritic cell (DC); dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN); intercellular-adhesion-molecule-3 (ICAM-3).
INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) causes one of the most economically-important diseases in the swine industry worldwide [335, 336]. The disease is characterized by reproductive failure and abortion in sows, as well as respiratory diseases with a high mortality rate in nursery pigs [170, 315]. PRRSV is a single-stranded, positive-sense RNA virus of approximately 15 kb. The virus belongs to the family of Arteriviridae, consists of two genotypes: the North American (type 2) and the European (type 1). The two genotypes share approximately 60% nucleotide sequence identity but are antigenically different [168-170, 337]. Although commercial vaccines against PRRSV are available, the disease is still clinically present. The extensive genetic diversity observed among field strains, the continuous evolution of PRRSV strains, and the virus evasion of host immune system [172, 338, 339] hamper the development of more effective vaccines.

Host inmate immune responses play an important role during early stages of virus infection and in the development of the clinical disease. Antigen-presenting cells (APCs) are the first line of defense, and play an important role in innate immune regulation during early stages of immune responses to PRRSV infection [340]. Among the APCs, pulmonary alveolar macrophages (PAMs), pulmonary interstitial macrophages (PIMs), and dendritic cells (DCs) are the main targets for PRRSV replication [204]. PRRSV can replicate and establish a productive infection in pulmonary DCs, monocyte-derived-DCs (MoDCs), and bone marrow-derived-DCs [204, 341, 342], but plasmacytoid DCs remain unresponsive after infection in vitro [343]. One of the key mechanisms that PRRSV induces immune suppression is the modulation of numerous interleukins, such as type I interferons (IFN-I), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6 and IL-12 in infected pigs [285, 344, 345]. Innate immune modulation has been associated with the functional
modulation of natural killer (NK) cells, which is driven by increased plasmatic levels of IL-4, IL-10, and IL-12 [346] and is also associated with an incremental increase of IL-1β, IL-6, and TNF-α levels via the primary positive modulation of IL-10 after PRRSV infection [347].

Dendritic cells (DC) possess a large repertoire of pathogen recognition receptors (PRRs), including Toll-like receptors and C-type lectin receptors (CLR) that recognize the molecular patterns expressed by pathogens such as PRRSV [348]. Dendritic cell-specific intercellular-adhesion-molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD 209) is a type II transmembrane protein that mediates DC differentiation, migration, and pathogen internalization and plays an important role in the immune regulation of DCs [349, 350]. DC-SIGN contains a carbohydrate-specific domain (CRD), a neck region composed of seven and half repeats containing 23 amino acid residues, a transmembrane region, and a cytoplasmic tail [351]. Signaling through the DC–SIGN receptor includes down-stream mediators from the Raf-1 pathway that leads to the production of proinflammatory cytokines IL-12, IL-6, TNF-α, and immunosuppressive cytokine IL-10. Certain pathogens can modulate T helper type 1 (Th1) polarization, Th2 response, and/or the induction of regulatory T cells by DC-SIGN binding. It has been demonstrated that mannose-rich pathogens enhance the expression of IL-10, IL12, and IL-6 and that fucose-expressing pathogens enhance the expression of IL-10 but down-regulate the expressions of IL-12 and IL-6 [352].

The role of PRRSV-DC interaction during PRRSV infection is largely unknown, and current research focuses on the production and regulation of interferons and specific inflammatory cytokines in DCs, which may play a role in immune modulation after infection. Plasmacytoid DCs are considered to be key effectors that express high levels of IFN-I in the early stages of antiviral innate immunity [353]. In vitro experiments with porcine pDCs exposed to PRRSV demonstrated
an up-regulation of CD80/86 and an increased secretion of IL-10 [107, 341], but no effect was observed on IFN-α, IL-6, IL-8, IL-12, IFN-γ, or TNF-α secretion [343]. Also, in vitro cultured MoDCs infected with PRRSV showed an increase in IL-10, IL-12 and TNF-α production [354]. Whether PRRSV-DCs interaction occurs through specific receptor(s) is unknown, however, previous studies have shown that PRRSV interacts with porcine DC-SIGN (pDC-SIGN), possibly through certain N-glycans in structural proteins, and enhances PRRSV transmission in vitro [355].

In this present study, we evaluated the immunomodulation of MoDCs through the pDC-SIGN receptor, which interacts with PRRSV, by blocking the pDC-SIGN receptor with recombinant hICAM-3-Fc or anti-pDC-SIGN mAb. We demonstrated that recombinant hICAM-3-Fc enhances the mRNA expression levels of proinflammatory cytokines and that anti-pDC-SIGN mAb inhibits the mRNA expression of TNF-α and IL-1α and enhances the expression of IL-12 induced by PRRSV in MoDCs.
MATERIALS AND METHODS

Preparation of monocyte–derived dendritic cells (MoDCs)

MoDCs were generated essentially as previously described [356, 357]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from blood by density-gradient centrifugation at 400 x g for 40 min at room temperature over Ficoll-paque PREMIUM (1.077 g/liter) (GE Healthcare). The PBMCs were washed twice with PBS at 250 x g for 10 min at room temperature. After washing, the cells were suspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. Monocytes were harvested by plastic adhesion overnight. After overnight incubation, cells were washed vigorously with PBS and cultured in IMDM supplemented with 10% FBS, 20 ng/ml of recombinant porcine granulocyte macrophage colony-stimulating factor (GM-CSF) (Gibco), and 20 ng/ml of recombinant porcine IL-4 (Invitrogen) for 5 days. On day 3, half of the cell culture supernatant was replaced with fresh medium supplemented with the aforementioned cytokines. On day 5, MoDCs were harvested with cell dissociation buffer (Invitrogen) and scraped off the plate gently. The cells were washed with IMDM once, counted after tryphan blue staining, and plated with fresh IMDM supplemented with 10% FBS.

Flow cytometry analysis of surface markers

The effect of PRRSV infection on the maturation status of MoDCs in the presence or absence of the pDC-SIGN receptor blockade due to competitive hICAM-3 interaction was analyzed by flow cytometry analysis of the surface expressions of SLA class I, SLA class II, and CD80/86. As a positive control, cells were treated with lipopolysaccharide (LPS) (1µg/ml). MoDCs infected with 1 MOI of PRRSV strain VR2385 with and without hICAM-3 treatment, as well as sham-inoculated and LPS controls, were incubated for 18 h at 37°C in 5% CO₂.
washed with flow cytometry (FCM) buffer (calcium- and magnesium-free PBS pH 7.4, 2% FBS, and 0.1% sodium azide). Viable cells were counted after staining with trypan blue (0.4%) and resuspended in FCM buffer at a concentration of 1 x 10^6 cells/mL. Treated cells (100 µl) were incubated with primary antibody mouse anti-MHC-II or anti-MHCI and CD80/86 (VMRD, Inc) at a concentration of 15µg/mL for 45 min. Cells were washed twice with 1 mL of FCM buffer by centrifugation at 250 x g for 5 min and incubated with goat anti-mouse IgG conjugated with Alexafluor 488 (Invitrogen) at a dilution of 1:200 for 30 min in the dark. Subsequently, cells were washed twice with 1 mL of FCM buffer via centrifugation for 5 min. Finally, cells were resuspended in FCM buffer, and the data were acquired with a FACScan flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Blocking pDC-SIGN-PRRSV interactions in MoDCs through recombinant hICAM-3-Fc or anti-pDC-SIGN mAb

A total of 2 x 10^5 MoDCs were suspended in 100 µl of IMDM that was supplemented with 10% v/v FBS and incubated for 30 min at 4ºC with 1 µg of hICAM3-Fc (R&D systems). Cells were washed twice with 500 µl of IMDM by centrifugation at 250 x g for 10 min at 4ºC. After washing, one group was suspended in 100 µl of IMDM (ICAM-3/M), and another group was infected with 1 MOI of a PRRSV strain VR2385 for 2 h at 4ºC (ICAM-3/V). The negative control group was treated only with IMDM (M/M), and the positive control groups were infected with the same dose and strain of PRRSV without hICAM-3 treatment (V/M).

For the anti-pDC-SIGN-specific blockage assay, a total of 2 x 10^5 MoDCs were suspended in 100 µl of IMDM supplemented with 10% v/v FBS and incubated for 30 min at 4ºC with various concentrations of an anti-pDC-SIGN mAb [358] targeted to the carbohydrate-recognition domain (CRD) domain of pDC-SIGN. Cells were washed twice with 100 µl of IMDM by centrifugation.
at 250 x g for 10 min at 4°C. After washing, one group was suspended in 100 µl of IMDM (mAb/M), and a second group was infected with 1 MOI of PRRSV strain VR2385 for 2h at 4°C (mAb/V). A negative control group was treated with IMDM (M/M), and a positive control group was infected with the same dose and virus strain but without the DC-SIGN-mAb treatment (M/V). After treatments, the cells were plated in a 24-well plate, and one well per treatment was collected every 6 h for a period of 24 h post-infection. Cell culture supernatants were aliquoted and stored at -80°C for further evaluation of viral release and interleukin secretion. Cells were scraped off the well and resuspended in 300 µl of Trizol (Invitrogen) for further mRNA quantification by quantitative PCR. Total RNAs were also extracted from the harvested MoDCs via an RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. RNAs were reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad) and random primers. The following cycling conditions were used to synthesize the cDNAs: 1 cycle at 25°C for 5min, 1 cycle at 42°C for 30 min, and 1 cycle at 85°C for 5 min.

qRT-PCR and cytokine ELISA to quantify the mRNA expression and secretion levels of cytokines

The relative quantification of gene expressions of TNF-α, IL-12p35, IL-1α, IL-1β, and IL-6 was performed with a Bio Rad CFX96 detection system (Bio Rad, USA). Reactions were performed in duplicate, with cyclophilin A (CYP A) being used as the internal control. All primers were synthesized (Integrated DNA Technologies, Inc) to target amplicons from 160 to 190 nt based on the cDNA sequence of each gene of interest from the NCBI database (Table 1). The mRNA expression levels were quantified according to the ∆∆Ct method [359]. A 15µl reaction mixture, containing 12.5 ng of cDNA, 7.5 µl of SYBR Green PCR Master Mix (Bioline’s Sensimix SYBR & Fluorescein Kit), and 500 nM of each primer, was assembled. The following thermal cycle
conditions were used: an initial activation step at 95°C for 10 minutes, followed by a 3-step PCR program of 95°C for 15 seconds, 55-60°C for 60 seconds, and 72°C for 60 seconds for 40 cycles. A dissociation curve was obtained for each quantitative PCR run to assess the specificity of the PCR run.

The cytokine secretion levels in the cell culture supernatants were measured using commercially available multiplex ELISA MILLIPLEX MAP Porcine Cytokine and Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (PCYTMAG-23), which was customized for the detection of TNF-α, IL-12p35, IL-1α, IL-1β, and IL-6 (EMD, Millipore). The reporter fluorescence of the beads was determined by using a dual-laser Bio-Rad Bio-Plex 200 instrument with Bio-Plex Manager software Version 6.0 (Bio-Rad) and expressed as the median fluorescent intensity (MFI) of ≥50 microspheres per set per well.

Virus titration in the supernatant was measured by immunofluorescence assay (IFA) using anti-PRRSV N-specific antibody (SDOW17) (RTI, Brookings, SD), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (KPL, Kirkegaard & Perry Laboratories, Inc.), and detected with a fluorescence microscope within excitation and emission spectrum peak wavelengths of approximately 495 nm and 519 nm, respectively. The tissue culture infectivity dose 50 (TCID$_{50}$) was calculated using the Reed and Muench method [297].

**Statistical analysis**

The significance between treatment groups was determined via two-way ANOVA with a Tukey HSD post-hoc test using Graph pad Prism v5 and Microsoft Excel. A $p$-value of less than 0.05 was considered to be statistically significant.
RESULTS

pDC infectivity, activation and maturation are not affected by DC-SIGN blockage via hICAM-3

We first investigated whether the blockage of pDC-SIGN with hICAM-3 affects PRRSV-VR2385 infectivity or the activation and maturation of pDCs. It has been reported that pDC-SIGN is not an entry receptor for PRRSV into pDCs, but it does enhance PRRSV transmission in vitro [355]. Dendritic cell differentiation is characterized by morphological changes characterized by the presence of large cytoplasmic projections of irregular shape with pseudopodia and the expressions of SLA class I, SLA class II, and the CD80/86 co-stimulatory molecules [354]. In this study, we did not observe morphological difference between hICAM-3-blocked cells and mock-treated cells at 24 hours post-infection (hpi) with PRRSV VR2385 (Fig. 1A, 1B). Both groups, ICAM-3/V and M/V, infected with 1 MOI of PRRSV-VR2385 showed marked cytopathic effects (CPE) at 24 hpi. Morphological changes observed in infected cells were characterized by cell rounding, cytoplasmic swelling, and the loss of the characteristic cytoplasmic dendritic projection as compared to the mock-infected cells (Fig. 1B).

The infectivity of PRRSV VR2385 was not affected, as there was no difference in TCDI_{50} viral titers in the culture supernatant between M/V and ICAM-3/V (Fig. 1C). In this study, we found that PRRSV infection reduced significantly the expression of SLA class I and co-stimulatory CD80 molecules (Fig. 1D), as was previously reported [354]. There was not a significant reduction of SLA class II in M/V and ICAM-3/V compared to the LPS control; however PRRSV had a detrimental effect in SLA class II molecule expression. Therefore, the results indicate that the blockage of pDC-SIGN with hICAM-3 does not affect PRRSV CPE formation or infectivity but down-regulates the expression of SLA class I (but not class II) CD 80 expression.
In vitro blockage of pDC-SIGN with human hICAM-3 up-regulates proinflammatory mRNA cytokine expression but does not affect protein secretion in the supernatant of MoDC infected by PRRSV

Numerous studies have demonstrated that PRRSV has an effect on proinflammatory cytokine regulation in MoDC [343]. However, the specific target receptor of this proinflammatory cytokine induction is not understood. To our knowledge, there is no information regarding the role of pDC-SIGN as a mediator in proinflammatory modulatory effects during PRRSV infection. Previous studies have demonstrated that DC-SIGN enhances PRRSV transmission in trans and enhances lymphocyte infection [355]. Here in this study, we measured the effect of pDC-SIGN blockage with hICAM-3 on proinflammatory cytokine gene expression and protein production in MoDCs infected with PRRSV.

No differences in TNF-α, IL-12p35, IL-1α IL-1β, or IL-6 mRNA expression levels were observed at 6, 12, 18 and 24 hpi between the MEM-PRRSV (M/V) and hICAM-3-PRRSV (ICAM-3/V) treatments. Although all genes under study showed almost a two-log increase during the time course of 24 h as compared to the mock-treated cells (M/M), there was no significant difference.

The effect of the blockage of pDC-SIGN with hCIAM-3 was observed at 24 hpi. All proinflammatory cytokines examined in this study, except for IL-1α, showed a marked mRNA up-regulation in ICAM-3/V as compared to M/M at 24 hpi. The up-regulatory effect of PRRSV on the mRNA expression of TNFα, IL-12p35, IL-1β, and IL-6 was enhanced in cells pretreated with hCIAM-3 (Fig. 2 A-E).

The cytokine fluorescence intensities were evaluated in MoDC supernatants at 6, 12, 18, and 24 hpi. The results obtained for M/V, ICAM-3/V, and mock-infected cells used as a control
(M/M) were compared. There was no significant difference in TNF-α, IL-12p35, IL-1α IL-1β, and IL-6 cytokine protein levels between the M/V, ICAM-3/V, and M/M groups throughout the experiment. The intensities of TNF-α and IL-12p35 did not show a significant difference among the various treatments and also remained constant during the four time points evaluated in this experiment (Fig. 3 A, B). Although there was not a significant difference among treatments at any time point, the IL-1α, IL-1β, and IL-6 proteins showed similar secretion patterns, which were characterized by a slight, constant, measurable increase in protein concentration over the four time points evaluated (Fig. 3 C, D, and E).

In vitro blockage of pDC-SIGN with anti-pDC-SIGN mAb down-regulates proinflammatory mRNA cytokine expression but does not affect proinflammatory cytokine levels in MoDC culture supernatants

In order to evaluate the specific effect of PRRSV proinflammatory cytokine gene regulation through the pDC-SING receptor, MoDCs were treated with anti-pDC-SIGN mAb prior to PRRSV infection (mAb/V). No statistical differences in proinflammatory mRNA cytokine modulation were observed between PRRSV-infected MoDCs (M/V) and MoDCs pretreated with anti-pDC-SIGN mAb treatment (mAb/V) at 6, 12, and 18 hpi. At 24 hpi, there was a significant down-regulation of TNFα and IL-1α and up-regulation of IL-12 (Fig. 4 A-E).

The effect of PRSSV cytokine production after DC-SIGN blockage with anti-pDC-SIGN was evaluated in MoDCs supernatants at 6, 12, 18, and 24 hpi. No significant difference in TNF-α, IL-12p35, IL-1α IL-1β, and IL-6 cytokine protein levels were observed between the M/V, mAb/V, and M/M groups throughout the experiment. Although no significant differences were observed, there is a trend of a lower concentration for all cytokines evaluated at 24 hpi in mAb/V-pretreated cells as compared to the M/V-positive group (Fig. 5 A-E).
DISCUSSION

Evasion of host immune response via immunosuppression and establishment of a persistent infection is one of the mechanisms of PRRSV pathogenesis. The key mechanism that PRRSV induces immunosuppression is probably through the modulation of numerous interleukins such as IFN-γ, TNF-α, IL-1, IL-6, and IL-12p35 in infected pigs [285, 344, 345]. In this study, we evaluated the role of pDC-SIGN in cytokine gene expression and protein production in MoDCs experimentally infected with PRRSV in an in vitro model. Our data suggest that anti-pDC-SIGN mAb induces the down-regulation of the gene expression of multiple cytokines but does not affect cytokine protein production.

Productive infection of porcine MoDCs by PRRSV has previously been reported [342]. PRRSV has a specific cell tropism, but few receptors have been identified in PRRVS susceptible cell lines. CD163 and sialodhesin (SN) are responsible for virus entry and infectivity. However, heparin sulfate, CD151, and vimentin have also been identified as potential PRRSV receptors due to their involvement in virus attachment, internalization, or uncoating [360]. C-type lectin receptors (CLRs) are expressed on the surfaces of DCs and are associated with capturing pathogen-derived glycosylated proteins and internalizing them for efficient antigen presentation [349]. Although it has been postulated that pDC-SIGN is not an entry receptor for PRRSV in pDCs, it does enhance viral transmission in vitro [355]. In the present study, we demonstrated that blockage of the DC-SIGN receptor by hICAM-3 did not affect virus intake or infectivity. Therefore, the results from this study indicated that pDC-SIGN does not play a role in virus entry or infectivity.

DCs activation is characterized by morphological changes and the expression of SLA-I, SLA-II, and CD80/86 co-stimulatory molecules [354]. It has previously been reported that PRRSV infection induces the down-regulation of SLA-II and CD80/86 in mature DCs [341]. Other studies
have found that the PRRSV (strain CNV-3) down-regulates SLA-I and SLA-II expression among infected MoDCs [354]. In addition to the SLA-I and SLA-II down-regulation observed due to PRRSV infection in DCs, the CD14 and CD11b/c have also been down-regulated by PRRSV infection [342]. No differences in terms of morphological changes, such as the presence of large cytoplasmic projections, irregular cellular shapes and the presence of pseudopodia, which are associated with DCs activation, were observed between PRRSV-infected MoDCs with and without h-ICAM-3 treatment and mock-treated cells after PRRSV infection. The results from this study demonstrated that pDC-SIGN blockage in MoDCs with hICAM-3 did not affect the regulatory effect of PRRSV on SLA-I, SLA-II, or co-stimulatory molecule CD80, suggesting that pDC-SIGN does not play an exclusive role in PRRSV’s activation and maturation activities of MoDCs.

DCs are a pivotal component of the immune system and are crucial in the determination of the class of the adaptive immune response. In addition to adhesive and Ag-recognition properties, the engagement of DC-SIGN with DCs results in the activation of signal transduction pathways that can cause an extensive modulation of immune responses [361]. The adaptive immune response by DCs is controlled by TLRs (Toll-like receptors), and it has been demonstrated that DC-SIGN can differentially modulate the fate of the adaptive immune response through a series of intermediate pathways (the serine/threonine kinase Raf-1 a pathway) or the interaction-activation of various LTR receptors [352, 362].

Infection with PRRSV can induce weak TNF-α production both in vivo and in vitro [226, 363]. Moreover, it has been demonstrated that during the early stages of PRRSV infection, TNF-α mRNA expression levels do not differ between PRRSV-infected and mock-infected MoDCs and that PRRSV may induce a late, observable up-regulation between 12 and 24 hpi [364]. Although, in this study, no significant difference between anti-pDC-SIGN mAb and hICAM-3 with mock-
treated cells was found, we did observed a moderate increase in gene expression level during the time course of the experiment. However, at 24 hpi, MoDCs pretreated with hICAM-3 and infected with PRRSV showed a significant and delayed up-regulatory effect on TNF-α mRNA expression as compared to PRRSV-infected MoDCs. Interestingly, a significant down-regulatory effect was observed in cells treated with specific DC-SIGN anti-pDC-SIGN mAb after PRRSV infection at 24 hpi. These results may indicate that anti-pDC-SIGN mAb can block DC-SIGN more specifically than hICAM-3. Thus, pDC-SIGN mAb treatment may abrogate PRRSV’s delayed up-regulatory effect on TNF-α mRNA expression. It has been postulated that PRRVS affects a post-transcriptional mechanism in order to suppress TNF-α production at the translation or secretion levels [343, 364, 365]. No differences were observed in protein production between treatments and between time points evaluated in this study. This data is consistent with previous studies in which PRRSV failed to induce TNF-α protein secretion. In the present study, protein concentrations remain at the basal level throughout the study.

Numerous interleukins, including IL-1, IL-6, and TNF-α, are important mediators of the local inflammatory response and are considered responsible for the clinical signs of disease, such as fever, depression, anorexia, and respiratory distress [366]. Previous studies demonstrate that PRRSV can induce IL-1 and IL-6 in bone-marrow-derived dendritic cells [234]. However, in vivo studies have shown that IL1-β and IL-6 were not elevated by 10 dpi, which is coincident with the presence of acute clinical signs and pulmonary lesions, but these interleukins were elevated during the persistent stage at 28 dpi [345]. In this present study, the treatment of MoDCs with hICAM-3 increased IL-1α, IL-1β, and IL-6 gene expressions, but no differences in protein production were observed. Interestingly, there was a reduction in IL-1 and IL-6 gene expressions in MoDCs treated with anti-pDC-SIGN mAb, although there was no statistical difference. DCs can produce IL-12,
which is coded by two independent genes, IL-12p35 and IL-12p40. IL-12 production is associated with the differentiation of naïve T cells into Th1 cells. Previous studies have demonstrated that the production of IL-12 increases in DCs and pulmonary alveolar macrophages infected with PRRSV [345, 354]. Mature DCs resist the suppression of IL10 but synthesize high levels of IL-12, which enhances both innate (natural killer) and acquired (B and T cell) immunity. Interestingly, in this study, we found that the mRNA levels of IL-12p35 were upregulated in both DC-SIGN blockage treatments. These results may suggest that PRRSV’s up-regulatory effect on IL-12p35 is not achieved through DC-SIGN or could be the result of multiple pathways induced by PRRSV. Recently, it has been demonstrated that certain combinations of TLR act synergistically to induce and release IL-12 in DCs [367]. In this present study, we measured only the levels of IL12p35 but not those of IL12p40. Interestingly, it has been reported that IL12p35 can couple with IL12p40 and form the heterodimer IL-12p70, which has a proinflammatory effect [368]. However, this effect has not been demonstrated for IL12p35, and more research regarding the role of this molecule in PRRSV infection is warranted.

The absence of differences in cytokine production among the treatments and time points evaluated with this experimental design is in agreement with previous reports [369]. \textit{In vivo} experiments have shown a low level of expression of pro-inflammatory cytokines, both at the mRNA and protein levels, in pigs infected with PRRSV-1 and PRRSV-2 [347]. It has been proposed that the lack of substantial changes in serum concentrations of pro-inflammatory cytokines may reflect a strategy by which PRRSV evades the host immune response. Therefore, samples of blood and/or serum do not necessarily reflect the events occurring in the lungs of PRRSV-infected animals at any given time [369]. In this present study, the differential genetic modulation observed among various cytokines may be the result of the differential or incompletely
specific modulation of DC-SIGN through hICAM-3 or anti-pDC-SIGN mAb. Previously, it has been demonstrated that hICAM-3 causes an incomplete inhibition of DC-SIGN, resulting in a failure to inhibit HIV transmission through DC-SIGN [370]. In addition, the role of TLRs was not explored in this study, and it might be interesting to evaluate the additive result of TLRs in the activation and modulation of various cytokines. Due to the complex interaction between DC-SIGN-TLRs and their cross-talk through various intermediate pathways, more studies will be necessary to dissect the role of DC-SIGN in PRRSV cytokine modulation.

In summary, we demonstrate that blocking pDC-SIGN with specific anti-pDC-SIGN mAb abrogates PRRSV’s modulatory effect on a group of proinflammatory effects. Although the immune responses against PRRSV infection are regulated by a complex balance of negative and positive signals and are further complicated by the interactions between numerous receptors, here in this study, we provide data to support a role of pDC-SIGN in the immunomodulation caused by PRRSV in pDCs.

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FIGURE LEGENDS

Fig. 1: Effect of PRRSV infection after pDC-SIGN blockage with hICAM-3 on MoDCs infectivity, activation and maturation. (A) MoDCs were treated with hICAM-3 and infected with 1 MOI of PRRSV strain VR2385 (ICAM-3/V) or mock treated with IMDM and infected with the same viral strain and infectious dose (M/V). Negative controls consist of MoDCs treated with IMDM (M/M) and hICAM-3 and IMDM (ICAM-3/M). Cell viability and morphological changes were evaluated every 6 h for a period of 24 h. No morphological difference was observed in MoDCs ICAM3/V and M/V treatments. Infected cells showed a severe rounding and cytoplasmic swelling with partial loss of cytoplasmic projection 6 hpi. (B) Both groups, ICAM3/V and M/V, showed a marked cytopathic effects (CPE) at 24 hpi. (C) Viral titration was carried out by serial dilutions of MoDCs supernatant and infection of MARC-145 cells at 90% confluency. Virus infectivity was detected by immunofluorescence assay (IFA) using 1:1000 dilution of anti-PRRSV N monoclonal antibody SDOW17. The infectivity of PRRSV VR2385 was not affected by hICAM-3 treatment. There were no differences in viral TCDI50 recovered between M/V and ICAM3/V supernatant. (D) Flow cytometry analysis of MoDCs was performed by incubating 1 million cells with 1µg of indicated antibodies followed by appropriate FITC-conjugated secondary antibodies. Staining with isotype control or secondary antibody only served as background controls. Ten thousand events were acquired after excluding dead cells through DAPI staining. PRRSV infection reduced significantly the expression of SLA-I and co-stimulatory CD80 molecules. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig. 1.

A

B

C

D

Mean Fluorescence Intensity

\( \text{MFI} = \log^{10} \text{ICID}_{10} \text{ml} \)

\( p = 0.01 \)

\( p = 0.02 \)

\( p = 0.001 \)

\( p = 0.001 \)

SLA II

SLA I

CD80
Fig. 2: Regulatory effect of proinflammatory cytokines mRNA expression in MoDCs induced by PRRSV infection after pDC-SIGN blockage with hICAM-3. (A-E) Relative abundance of cytokine (TNF-α, IL-12p35, IL1-α, IL-β and IL-6) mRNA expressions in MoDCs at 6, 12, 18 and 24 hpi. MoDCs were treated with hICAM-3 and infected with 1MOI of PRRSV VR2385 (ICAM3/V) or mock treated with IMDM and infected with the same viral strain and infectious dose (M/V). Negative controls consist of MoDCs treated with IMDM (M/M) and hICAM-3 and IMDM (ICAM-3/M). The cytokine mRNA expression levels were determined by quantitative real-time RT-PCR. Data are expressed as fold increase in gene expression relative to mock-infected cells. Three independent experiments were performed for each test, and the error bars indicate standard errors. Significant difference between ICAM-3/V, M/V and M/M are expressed with their p-values. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig. 2
Fig 3: Cytokine production of MoDCs infected with PRRSV after pDC-SIGN blockage with hICAM-3. (A-E) Relative abundance of cytokine (TNF-α, IL-12p35, IL1-α, IL-β and IL-6) protein production in the supernatant of MoDCs at 6, 12, 18 and 24 hpi. MoDCs were treated with hICAM-3 and infected with 1 MOI of PRRSV VR2385 (ICAM3/V) or mock treated with IMDM and infected with the same viral strain and infectious dose (M/V). Negative controls include MoDCs treated with IMDM (M/M) and hICAM-3 and IMDM (ICAM-3/M). The cytokine protein secretion levels in the cell culture supernatants were measured using the commercial multiplex ELISA MILLIPLEX MAP Porcine Cytokine and Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (PCYTMAG-23), which was customized for the detection of TNF-α, IL-12p35, IL-1α, IL-1β, and IL-6 (EMD, Millipore). The reporter fluorescence of the beads was determined by using a dual-laser Bio-Rad Bio-Plex 200 instrument with Bio-Plex Manager software Version 6.0 (Bio-Rad) and expressed as the median fluorescent intensity (MFI) of ≥50 microspheres per set per well. Data are expressed as fold increase in gene fluoresce intensity relative to mock-infected cells. Three independent experiments were performed for each test, and the error bars indicate standard errors. Significant difference between ICAM-3/V, M/V and M/M are expressed with their $p$-values. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig 3
Fig. 4: Regulatory effect of proinflammatory cytokine mRNA expressions in MoDCs induced by PRRSV infection after pDC-SIGN blockage with anti-pDC-SIGN mAb. (A-E) Relative abundance of cytokine (TNF-α, IL-12p35, IL1-α, IL-β and IL-6) mRNA expressions in MoDCs at 6, 12, 18 and 24 hpi. MoDCs were treated with anti-pDC-SIGN mAb and infected with 1MOI of PRRSV VR2385 (mAb/V) or mock treated with IMDM and infected with the same viral strain and infectious dose (M/V). Negative controls include MoDCs treated with IMDM (M/M) and anti-pDC-SIGN mAb and IMDM (mAb/M). The cytokine mRNA expression levels were determined by quantitative real-time RT-PCR. Data are expressed as fold increase in gene expression relative to mock-infected cells. Three independent experiments were performed for each test, and the error bars indicate standard errors. Significant difference between mAb/V, M/V and M/M are expressed with their p values. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig 4. Relative increment normalized to mock control (log_{10})
Fig 5: Cytokine production of MoDCs infected with PRRSV after pDC-SIGN blockage with anti-pDC-SIGN mAb. (A-E) Relative abundance of cytokine (TNF-α, IL-12p35, IL1-α, IL-β and IL-6) protein production in the supernatant of MoDCs at 6, 12, 18 and 24 hpi. MoDCs were treated with anti-pDC-SIGN mAb and infected with 1MOI of PRRSV VR2385 (mAb/V) or mock treated with IMDM and infected with the same viral strain and infectious dose (M/V). Negative controls consist of MoDCs treated with IMDM (M/M) and anti-pDC-SIGN mAb and IMDM (mAb/M). The cytokine secretion levels in the cell culture supernatants were measured using a commercially available multiplex ELISA MILLIPLEX MAP Porcine Cytokine and Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (PCYTMAG-23), which was customized for the detection of TNF-α, IL-12p35, IL-1α, IL-1β, and IL-6 (EMD, Millipore). The reporter fluorescence of the beads was determined by using a dual-laser Bio-Rad Bio-Plex 200 instrument with Bio-Plex Manager software Version 6.0 (Bio-Rad) and expressed as the median fluorescent intensity (MFI) of ≥50 microspheres per set per well. Data are expressed as fold increase in gene fluorescence intensity relative to mock-infected cells. Three independent experiments were performed for each test, and the error bars indicate standard errors. Significant difference between mAb/V, M/V and M/M are expressed with their p values. Statistical comparison was performed using two-way ANOVA followed Tukey’s correction for multiple comparison. Statistical significance was set to alpha = 0.05.
Fig 5.
### TABLES

**Table 1. Primer sequences for real-time polymerase chain reaction analysis of cytokine genes expression**

<table>
<thead>
<tr>
<th></th>
<th>Primer sequence (5’-3’)</th>
<th>Anti-sense</th>
<th>Amplicon</th>
<th>Accession number</th>
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<tr>
<td>TNFα</td>
<td>CCTACTGCACTTCGAGGTTATC</td>
<td>GCATACCCACTCTGCCATT</td>
<td>177 bp</td>
<td>JF831365.1</td>
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<tr>
<td>IL-6</td>
<td>CAAGGAGGTACTGGCAGAAA</td>
<td>CAGCCTCGACATTCCCTTAT</td>
<td>185 bp</td>
<td>JQ839263.1</td>
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<tr>
<td>IL-1α</td>
<td>ACCCGACTGTTTGAGTG</td>
<td>CCTGCCTTTGGCAATAAAC</td>
<td>178 bp</td>
<td>NM_214029.1</td>
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<tr>
<td>IL-1β</td>
<td>GAAGCATCCAGCTGCAAATC</td>
<td>GACGATGGGCTTTCTTCAA</td>
<td>167 bp</td>
<td>NM_001005149.1</td>
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<tr>
<td>IL-12p35</td>
<td>CAGGCCCAGGAATGTCTC</td>
<td>CGTGGCTAGTTCAAGTGGA</td>
<td>188 bp</td>
<td>NM_213993.1</td>
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<tr>
<td>CYPA</td>
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<td>CACATGTTTGCCATCCAACC</td>
<td>189 bp</td>
<td>JX523418.1</td>
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</tbody>
</table>

IL, interleukin; TNF, tumor necrosis factor; CYPA, cyclophilin A.
CHAPTER 5

Final conclusions

Both viruses, PCV2 and PRRSV, individually or during co-infection, have caused devastating diseases in the swine industry worldwide. Although advances in vaccine development have produced commercial vaccines against both viruses, clinical diseases associated with single and co-infection are still present. The heterogeneity of PRRSV field strains and the emergence of new variants of PCV2 make necessary a constant review of the epidemiology and virology features related to immunogenicity and current approaches for vaccine strategies against these two agents.

Here in this PhD dissertation research we evaluated the ability of PCV viruses as expression vectors of foreign antigenic epitopes and their capability to induce immunogenicity in vivo against the inserted epitopes. Based on previous information where PCV1-2 showed to be an effective vector of foreign epitope tags, we engineered the non-pathogenic PCV1 and the vaccine strain PCV1-2a in order to express B-linear epitopes of PRRSV. Additionally, we evaluated the role of PRRSV-pDC-SIGN receptor interaction in modulation of proinflammatory cytokines during PRRSV infection in MoDCs, in order to develop novel approaches for PRRSV vaccine development.

We successfully generated and rescued four PCV1 chimeric viruses expressing different PRRSV linear B-cell epitopes (GP2 epitope II, GP3 epitope I, GP5 epitope I, and GP5 epitope IV). We further demonstrated that three of these chimeric viruses were infectious in pigs. Importantly, we found that the PCV1-PRRSV_{EPI} chimeric viruses elicited neutralizing antibodies against PRRSV VR2385. Therefore, the results of this study provided the bases for further exploring the use of the non-pathogenic PCV1 as a live virus vector for vaccine delivery.
For the chimeric PCV1-2a viruses we were able to express four PRRSV B-cell linear epitopes \textit{in vitro} (GP2 epitope II, GP3 epitope I, GP5 epitope I, and GP5 epitope IV). Immunogenicity studies in pigs revealed that two of the four chimeric viruses, elicited neutralizing antibodies against PRRSV VR2385 as well as against heterologous strains of PCV2 (strains PCV2a, PCV2b, and mPCV2b). The results have important implications for exploring the potential use of PCV1-2a vaccine virus as a live virus vector to develop bivalent MLVs against both PCV2 and PRRSV.

Additionally, we demonstrated that recombinant hICAM-3-Fc enhances the mRNA expression levels of proinflammatory cytokines, and blocking pDC-SIGN with specific anti-pDC-SIGN mAb selectively regulates PRRSV’s modulatory effect on a group of proinflammatory cytokines. Although the immune responses against PRRSV infection are regulated by a complex balance of negative and positive signals and are further complicated by the interactions between numerous receptors, here in this study, we provide data to support a role of pDC-SIGN in the immunomodulation caused by PRRSV in pDCs.

In conclusion, we demonstrated that PCV can be used as a delivery vector of small foreign antigenic epitopes without affecting infectivity \textit{in vitro} and \textit{in vivo}. Both PCV1 and PCV1-2a backbones were able to not only induce PRRSV epitopes specific antibodies but also their own PCV Cap-specific antibodies. Most importantly, all PRRSV epitope antibodies induced either by PCV1 or PCV1-2a backbone had neutralizing activities against the PRRSV strain used in this study. Future challenge and efficacy studies are necessary to evaluate the degree of protection in a PRRSV or PCV2-PRRSV co-infection model. Additionally, we concluded that PRRSV-pDC-SIGNs interaction plays a role in modulatory effect in proinflammatory cytokines in MoDCs.
Further exploration of this specific host-pathogen interaction would be important in order to develop a potential more effective subunit vaccine or a therapeutic agent against PRRSV infection.