Porcine Circovirus Associated Disease: Modulation of the Host Immune Response to PCV2 and PRRSV by Regulatory T Cells

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Abstract

Porcine circovirus associated disease (PCVAD) is currently one of the most economically important diseases facing the global swine industry. Porcine circovirus type 2 (PCV2) is the primary and essential causative agent of PCVAD, but development of clinical disease typically requires co-infection with other swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV). The specific mechanisms of co-infection that lead to clinical disease are not fully understood, but immune modulation by the co-infecting viruses is thought to play a critical role. The ability of dendritic cells (DC) infected with PRRSV, PCV2 or both to induce regulatory T cells (T_{regs}) was evaluated *in vitro*. DCs infected with PCV2 significantly increased $CD4^+CD25^+FoxP3^+$ T_{regs} ($p<0.05$) and DCs co-infected with PRRSV and PCV2 induced significantly higher numbers of T_{regs} than with PCV2 alone ($p<0.05$). This T_{reg} induction was found to be dependent on TGF-β and not IL-10. Further investigation of the *in vivo* swine immune response to acute co-infection with PCV2 and PRRSV failed to detect activation of T_{regs} in peripheral blood mononuclear cells (PBMCs) or bronchoalveolar lavage samples. The T_{reg} response to *in vitro* and *in vivo* PRRSV challenge in pigs persistently infected with PCV2 or vaccinated against PCV2 was evaluated. There was no significant difference in T_{regs} in PBMCs among chronically PCV2-infected, vaccinated PCV2 challenged or negative control pigs. However, following *in vitro* infection of monocyte-derived dendritic cells with PCV2, PRRSV, or both viruses, co-cultured lymphocytes from chronically infected and PCV2 vaccinated pigs

had significantly ($p<0.05$) decreased T_{reg} expression in the virus infected groups compared to the negative controls. In separate experiments, pigs vaccinated against PCV2 and subsequently challenged with an attenuated PRRSV strain and its pathogenic parental strain developed increased $CD4^{+}CD25^{+}FoxP3^{+}$ T_{regs} ($p<0.05$) in PBMC samples compared to uninfected controls, and this correlated with increased suppressor activity and IL-10 expression. The findings from these studies indicate that the interaction of PCV2 and PRRSV in swine modulates the host immune response mediated in part through the activity of T_{regs}. However, the extent to which T_{regs} orchestrate a dysregulated immune response in the pathogenesis of PCVAD *in vivo* remains to be determined.

Dedication

This work is dedicated with love and gratitude to my wife, Julie Tucker Cecere. I am forever grateful for your unwavering encouragement and support.

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at 0, 7, 14, and 21 dpi with an attenuated DS722 PRRSV or the parental VR2385 strain. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.

Attributions

Chapter 3: Co-infection of porcine dendritic cells with porcine circovirus type 2a (PCV2a) and genotype II porcine reproductive and respiratory syndrome virus (PRRSV) induces $CD4^{+}CD25^{+}FoxP3^{+}$ T cells *in vitro* and Chapter 4: Acute co-infection with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) does not induce regulatory T cells *in vivo*

Xiang- Jin Meng, MD, PhD: Dr. Meng provided guidance on study design, sampling strategy and manuscript preparation.

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Chapter 5: Immune response to PCV2 and PRRSV challenge in pigs persistently infected with PCV2 or vaccinated against PCV2

Xiang- Jin Meng, MD, PhD: Dr. Meng provided guidance on study design and data analysis.

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Chapter 1: Introduction

Porcine circovirus type 2 (PCV2) is universally recognized as the primary and necessary cause of a collection of clinical syndromes termed porcine circovirus associated disease (PCVAD) in North America and porcine circovirus diseases (PCVD) in Europe. Since the initial descriptions of a systemic wasting syndrome in post-weaning pigs in Canada, PCV2 has rapidly emerged as one of the most economically significant pathogens, along with porcine reproductive and respiratory syndrome virus (PRRSV), currently affecting the global swine population (Meng, 2012). PCV2 is currently endemic worldwide and PCVAD is a cause of severe production losses in every major swine producing country (Segales et al., 2005). Morbidity and mortality rates on affected farms are reported to be as high as 30% and 20% respectively (Segales and Domingo, 2002).

Although PCV2 is the primary etiological agent of PCVAD, infection with PCV2 alone rarely produces the full spectrum of clinical disease; rather, subclinical infection is the most common outcome in pigs solely infected with PCV2. Field cases of PCVAD typically reflect polymicrobial disease involving co-infection of PCV2 with other viral or bacterial swine pathogens. Similarly, experimental reproduction of PCVAD occurs most effectively and efficiently following co-infection with PCV2 and a range of other swine pathogens including porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, swine influenza virus, *Mycoplasma hyopneumoniae* and Torque teno sus virus (TTSuV) (Opriessnig and Halbur, 2012). The most common co-infecting pathogen identified in naturally occurring cases of PCVAD is PRRSV (Drolet et al., 2003; Grau-Roma and Segales, 2007; Pogranichniy et al., 2002). The exact mechanisms explaining this phenomenon of co-infection potentiating clinical PCVAD are not entirely clear, but modulation of the host immune system is considered a

key event in the pathogenesis of the disease (Gillespie et al., 2009; Krakowka et al., 2001). In fact, PCVAD is considered an immunosuppressive disease.

One specific mechanism viral pathogens have adapted to modulate the host immune response in favor of viral persistence is the induction of regulatory T cells (T_{regs}). T_{regs} serve several important functions in the immune system through maintenance of immunological self-tolerance and immune homeostasis, and they are therefore necessary for orchestrating a balanced immune response to pathogens (Chen et al., 2003). However, they can also be inappropriately induced by viruses in order to swing the balance of the immune response in favor of maintaining viral infection (Belkaid, 2007). PRRSV is known to induce T_{regs}, and this feature is thought to contribute to the immunosuppressive features of PRRSV infection. However, no available research addresses the role of T_{regs} in the immunopathogenesis of PCVAD. Since co-infection with PRRSV and PCV2 is common and is often associated with the development of PCVAD, the work in this dissertation is aimed at investigating the role of T_{regs} in modulating the swine immune response to co-infection with PCV2 and PRRSV.

In the first study, the relative contributions of PCV2, PRRSV, or both viruses to the induction of T_{regs} were assessed *in vitro*. These findings were correlated with cytokine expression of IL-10 and TGF-β to address the mechanism of T_{reg} induction. The second study was aimed at characterizing the *in vivo* T_{reg} response to infection with PCV2, PRRSV or both viruses. Thirty 4-week-old pigs were randomly divided into four groups: negative controls, PCV2 challenged, PRRSV challenged, and pigs challenged with both viruses. Percentages of $CD4^+CD25^+FoxP3^+$ T_{regs} among groups were compared in peripheral blood mononuclear cells and bronchoalveolar lavage samples and correlated with circulating viral load and lesion severity.

The objective of the third study was to determine if PCV2 infection or vaccination prior to challenge with porcine reproductive and respiratory syndrome virus (PRRSV) is associated with immunosuppression mediated by regulatory T cells (T_{reg}). In one set of experiments T_{reg} induction was evaluated *in vivo* and *in vitro* in pigs chronically infected with PCV2 or challenged with PCV2 following vaccination with a live or inactivated PCV2 vaccine. This was correlated with cell suppressor activity and cytokine expression. In the second set of experiments, the T_{reg} response in PCV2-vaccinated pigs challenged with an attenuated chimeric PRRSV or its virulent parent strain was characterized and associated with suppressor activity and cytokine profiles.

The advent of commercially available inactivated and subunit vaccines against PCV2 has proven highly effective at diminishing the burden of PCV2 replication and shedding, decreasing mortality, and enhancing growth parameters in commercial swine (Beach and Meng, 2012). However, vaccination against PCV2 does not prevent infection nor does it completely ameliorate viral shedding in affected herds. Subclinical PCV2 infection prior to vaccination with a modified-live PRRSV vaccine impairs the development of protective immunity against PRRSV (Opriessnig et al., 2006c). Therefore, unraveling the immunopathogenesis of PCVAD is particularly important for improving future vaccine efficacy for PCV2 and PRRSV.

Chapter 2: Literature Review

Introduction

Early descriptions of a systemic wasting disease affecting post-weaning pigs surfaced in Canada in the early 1990s (Clark, 1996; Harding, 1996) and this syndrome was later associated with a novel circovirus, termed porcine circovirus type 2 (PCV2) in 1998 (Allan et al., 1998a; Allan et al., 1998b; Ellis et al., 1998). Since then, PCV2 has rapidly emerged as one of the most economically significant pathogens currently affecting the global swine population and a growing list of disease conditions has been linked to PCV2 infection (Meng, 2012). Perhaps the most convincing argument for PCV2's tremendous impact on the swine industry is provided by the current widespread use of PCV2 vaccines, with an estimated 95-98% of market weight pigs in the United States receiving vaccines against PCV2. This has resulted in a substantial reduction in economic loss and clinical disease due to porcine circovirus associated diseases (PCVAD) (Opriessnig and Halbur, 2012). Despite this improved outcome in commercial swine production resulting from the availability of effective vaccines, significant gaps still exist in our understanding of the pathogenesis of PCVAD.

Taxonomy and Genomic organization

PCV2, along with its close relative PCV1, belong to the genus *Circovirus* in the family *Circoviridae* (Todd, 2005). Porcine circoviruses are among the smallest autonomously replicating viruses affecting animals and are single stranded, ambisense, circular, nonenveloped DNA viruses (Tischer et al., 1982; Tischer et al., 1974). Porcine circovirus type 1 was discovered

prior to PCV2 in the early 1970s as a contaminant of the porcine kidney cell line PK-15 and, despite its recent notoriety as a contaminant of the pediatric rotavirus vaccine Rotarix®, is non-pathogenic (Baylis et al., 2011; Tischer et al., 1982; Tischer et al., 1974).

PCV2 can be divided into 3 main genotypes based on sequence analysis: PCV2a (genotype 2), PCV2b (genotype 1) and PCV2c. PCV2a and PCV2b isolates have been identified worldwide and are capable of inducing clinical disease, while PCV2c has been documented in archived samples from Denmark (Dupont et al., 2008; Segales et al., 2008). Although PCV2a was originally the most prevalent genotype in North America, a major global shift occurred beginning in 2003 in Europe and spread to North America and Asia in 2005. This shift resulted in PCV2b predominating worldwide (Cheung et al., 2007; Dupont et al., 2008; Rose et al., 2012). A study that examined 97 cases of PCVAD using genotype-specific PCR demonstrated that PCV2a and PCV2b were present concurrently in 25% of cases (Hesse et al., 2008). Further sequence analysis revealed that PCV2a/PCV2b recombination can occur in the field in pigs naturally infected with multiple genotypes (Hesse et al., 2008).

PCVs contain two main open reading frames (ORFs) encoded in an antisense direction. ORF1 encodes two proteins involved in viral replication: Rep and Rep'. ORF2 encodes the capsid protein, which contains the immunodominant antigenic epitopes (Cheung, 2003a, b; Lekcharoensuk et al., 2004; Nawagitgul et al., 2000). An ORF3 protein has been described and is reportedly involved in PCV2-mediated apoptosis (Liu et al., 2006; Liu et al., 2005), but other independent laboratories have been unable to verify these findings (Juhan et al., 2010).

Virus life cycle and transmission

Prenatal and postnatal pigs are susceptible to PCV2 infection, but the outcome varies depending on the age at infection (Segales et al., 2005). Generalized infection of early embryos

culminates in embryonic death and uterine resorption with sows returning to estrus (Mateusen et al., 2007; Mateusen et al., 2004). Slightly later in development, in fetuses infected between 40-70 days of gestation, viral tropism is aimed at cardiomyocytes, hepatocytes and monocyte/macrophage lineage cells (Nauwynck et al., 2012; Saha et al., 2010; Sanchez et al., 2003; Sanchez et al., 2001). At this stage, the heart is particularly susceptible to viral replication, which can result in heart failure and subsequent fetal death and mummification (Pensaert et al., 2004). Cardiac tropism is lost postnatally and instead the virus primarily targets cells of monocyte/macrophage and lymphocyte origin (Lefebvre et al., 2008; Sanchez et al., 2004), although replication in epithelial/endothelial cells and fibrocytes has been reported (Steiner et al., 2008). Attachment of PCV2 to host target cells is mediated by glycosaminoglycan receptors, specifically heparin sulfate and chondroitin sulfate b (Misinzo et al., 2006). Uptake of virus into monocytic cells occurs via clathrin-mediated endocytosis and subsequent release from the endosome is dependent on an acidic environment (Misinzo et al., 2005). In contrast, PCV2 disassembly in epithelial cells occurs optimally at neutral pH and in monocyte or bone marrow derived dendritic cells there is no evidence of viral uncoating (Misinzo et al., 2008; Vincent et al., 2003). Following uncoating, viral replication is dependent on host cell enzymes expressed during the S-phase of the cell cycle due to the paucity of proteins expressed by PCV2 (Tischer et al., 1987). Replication of the viral genome occurs via a rolling-circle replication mechanism (Cheung, 2012).

Successful transmission of PCV2 is reliant upon multiple factors, including host susceptibility, virus characteristics that affect infectivity, and adequate exposure (Rose et al., 2012). PCV2 is present in high concentrations in nasal and fecal excretions and for extended periods in serum (up to 120 days post-inoculation) following infection (Hemann et al., 2012;

Opriessnig et al., 2007; Rose et al., 2012). Thus transmission occurs primarily via oronasal contact with infected feces and urine or direct contact with infected pigs (Gillespie et al., 2009; Patterson et al., 2011). Vertical transmission has been demonstrated resulting from transplacental infection following experimental intra-nasal infection of sows prior to farrowing (Ha et al., 2008; Park et al., 2005). PCV2 is also shed in semen of naturally and experimentally infected boars, and has been detected as early as five days post-inoculation (Kim et al., 2001; Larochelle et al., 2000; Madson et al., 2008; McIntosh et al., 2006). However, there is some speculation as to whether sufficiently high levels of PCV2 exist in semen from naturally infected boars to significantly contribute to fetal infections (Rose et al., 2012).

Clinical diseases associated with PCV2

The earliest descriptions of disease associated with PCV2 encompassed a postweaning multisystemic wasting syndrome and was initially referred to in the literature as PMWS. However, PCV2 was later determined to be highly prevalent among swine herds and the presence of PCV2 did not always equate to disease; therefore a case definition of PMWS was proposed that included the following criteria: 1. presence of typical clinical signs including wasting, respiratory disease and icterus; 2. identification of classical microscopic lesions in lymphoid organs including lymphoid depletion with histiocytic replacement ± intracytoplasmic basophilic botryoid inclusion bodies, and 3. demonstration of PCV2 antigen or nucleic acid within microscopic lesions (Opriessnig et al., 2007). It later became apparent that a growing number of disease manifestations were associated with PCV2 infection and, in an effort to consolidate the divergent abbreviations, diseases related to PCV2 infection are now referred to as porcine circovirus disease (PCVD) in Europe and porcine circovirus-associated disease

(PCVAD) in North America (Opriessnig et al., 2007). The salient features and updated terminology, as summarized by Segales (Segales, 2012) will be briefly discussed.

Serological studies indicate that PCV2 is omnipresent worldwide. However, disease resulting from PCV2 infection is far less prevalent (Segales et al., 2005). Therefore, the most common outcome of PCV2 exposure is subclinical infection. Despite the fact that pigs do not exhibit signs of clinical disease, subclinical PCV2 infection has been shown to have an adverse effect on the development of protective immunity induced by a PRRSV vaccine and PCV2 vaccination improves production parameters in subclinically infected pigs (Opriessnig et al., 2006c; Segales, 2012).

The most commonly encountered and best described syndrome associated with PCV2 is PCV2 systemic disease (previously termed PMWS). Typical clinical and pathological findings include weight loss (wasting), respiratory distress, diarrhea, icterus, and widespread lymph node enlargement characterized by lymphoid depletion and histiocytic replacement (Clark, 1996; Gillespie et al., 2009; Harding, 1996). Morbidity reportedly ranges from 4-30% while mortality can be 4-20% (Segales and Domingo, 2002). PCV2 lung disease presents clinically as respiratory distress and dyspnea and pulmonary lesions range from lymphohistiocytic to granulomatous interstitial or broncho-interstitial pneumonia with occasional bronchiolitis. PCV2 enteric disease is characterized by diarrhea due to granulomatous enteritis with depletion of Peyer's patches (Opriessnig et al., 2011b). Lesions of the lung and enteric forms overlap with those seen in the systemic disease, but the absence of lesions in lymph nodes excludes these subtypes from a diagnosis of PCV2 systemic disease (Segales, 2012). PCV2 reproductive disease culminates in reproductive failure associated with late-term abortions, still-births and mummification (Madson et al., 2009). Porcine dermatitis and nephropathy syndrome (PDNS) is an infrequent disease

associated with PCV2 infection and encompasses systemic necrotizing vasculitis and glomerulonephritis which produce irregular red-purple cutaneous macules and papules most often distributed on the hind limbs and perineal region and swollen kidneys with cortical petechia (Rosell et al., 2000; Segales et al., 1998).

Recently a novel disease syndrome associated with PCV2, termed acute pulmonary edema (APE), has been linked to herds previously vaccinated for PCV2 (Cino-Ozuna et al., 2011). Unlike other chronic progressive PCVADs, APE is characterized by peracute onset of respiratory distress and death in nursery or young finisher pigs, and often pigs are found dead with no premonitory signs. Lesions in affected pigs included pleural effusion, diffuse pulmonary edema most prominent in the intralobular septa, diffuse lymphohistiocytic interstitial pneumonia, and fibrinoid necrosis of blood vessel walls. Large quantities of PCV2 DNA were detected in affected tissues by PCR and viral antigen was identified within intravascular mononuclear cells and vascular endothelial cells. Development of APE was not linked to co-infection with other swine pathogens. Cino-Ozuna et al. proposed a mechanism for this disease wherein PCV2 infection is established in young pigs prior to vaccination and viral replication in mononuclear and vascular endothelial cells is enhanced in pigs lacking protective levels of maternal antibody. The resulting endothelial damage and cytokine release from monocytes contribute to loss of blood vessel wall integrity and contribute to acute pulmonary edema (Cino-Ozuna et al., 2011).

Role of co-infecting pathogens in PCVAD

One of the first reports of experimental reproduction of PCVAD described the development of lesions typical of field cases of systemic PCVAD (then termed PMWS) following inoculation of 3-day-old gnotobiotic pigs with tissue homogenates or passaged cell culture material from PMWS-affected piglets (Ellis et al., 1999). The investigators were able to

demonstrate seroconversion to PCV as well as immunohistochemical and molecular evidence of PCV in tissues bearing lesions. However, porcine parvovirus (PPV) was unexpectedly detected in affected tissue by virus isolation, polymerase chain reaction and immunohistochemistry. Upon further investigation, the authors concluded that PPV was likely present in low quantities in the lymphoid tissue used as a PCV source and was infective and replicated in susceptible gnotobiotic pigs. This study provided early evidence that co-infection with additional pathogens is important in the full expression of clinical PCVAD. Multiple subsequent studies have demonstrated that co-infection with PCV2 and PPV produces more severe clinical disease and lesions than infection with PCV2 alone and this phenomenon appears to be independent of the order of infection (Allan et al., 1999; Kennedy et al., 2000; Kim et al., 2003; Kim et al., 2006; Krakowka et al., 2000; Opriessnig et al., 2004a). There is evidence for the interaction of PCV2 and PPV in cases of naturally acquired PCVAD. Tissues from field cases of systemic PCVAD were examined for evidence of PCV2 and PPV via immunohistochemistry and PCR. Both viruses were detected in 12/69 cases examined (Ellis et al., 2000).

While it is now generally accepted that PCV2 is the primary etiological agent in the development of the aforementioned range of clinical syndromes encompassed by PCVAD, experimental reproduction of PCVAD occurs most effectively and efficiently following co-infection with PCV2 and a range of other swine pathogens (Opriessnig and Halbur, 2012). Arguably the most common co-infecting pathogen identified in naturally occurring cases of PCVAD is porcine reproductive and respiratory syndrome virus (PRRSV) (Drolet et al., 2003; Grau-Roma and Segales, 2007; Pogranichniy et al., 2002). PRRSV is a single-stranded, positive sense RNA virus in the family *Arteriviridae* of the order *Nidovirales* (Meulenberg et al., 1993, 1994). Pigs co-infected with PRRSV and PCV2 exhibit enhanced PCV2 replication compared to

pigs infected with PCV2 alone, increased severity of lesions and clinical disease, and prolonged shedding of PCV2 DNA in oronasal and fecal excretions (Harms et al., 2001; Sinha et al., 2011). Although acute PRRSV infection at the time of PCV2 vaccination does not impair PCV2 vaccine efficacy (Sinha et al., 2010), subclinical infection with PCV2 prior to vaccination with a modified-live PRRSV vaccine impaired the development of protective immunity against PRRSV (Opriessnig et al., 2006c). Additional swine viral pathogens that have been linked to co-infection with PCV2 include the recently identified Torque teno sus virus, porcine epidemic diarrhea virus and swine influenza virus (Aramouni et al., 2011; Dorr et al., 2007; Ellis et al., 2008; Jung et al., 2006a; Jung et al., 2006b; Kekarainen et al., 2006).

Development of PCVAD has been associated with co-infection by bacterial pathogens as well as viral pathogens. PCVAD has been experimentally reproduced following co-infection of pigs with PCV2 and *Mycoplasma hyopneumoniae*. In these studies, *M. hyopneumoniae* increased the severity of PCV2-associated lung and lymphoid lesions and this was associated with an increased amount of PCV2 DNA and prolonged viral shedding (Opriessnig et al., 2011c; Opriessnig et al., 2004b). In natural cases of PCVAD, a seropositive response to *M. hyopneumoniae* was associated with increased disease severity (Alarcon et al., 2011).

The mechanisms explaining this phenomenon of co-infection potentiating PCV2 replication and clinical PCVAD are not entirely clear (Opriessnig and Halbur, 2012). Some investigators have suggested that non-specific immune stimulation may provide the necessary co-stimulation to increase PCV2 replication, thereby enhancing clinical PCVAD, based on the finding that pigs treated with keyhole limpet hemocyanin in incomplete Freund's adjuvant and PCV2 developed moderate to severe PMWS (Krakowka et al., 2001). By this line of reasoning, co-infection with other swine pathogens may provide the necessary immune stimulation to

augment PCV2 replication, thereby recapitulating PCVAD. However, there is conflicting evidence in the literature whether immune stimulation alone may provide the necessary co-stimulation for the expression of clinical PCVAD (Haruna et al., 2006; Ladekjaer-Mikkelsen et al., 2002). Others have suggested that pathogens such as PRRSV may favor the persistence of PCV2 by suppressing or modulating the host immune response to co-infecting pathogens (Jung et al., 2009; Renukaradhya et al., 2010).

Porcine immune response to PCV2

The outcome of infection with PCV2 is in large part dictated by viral interaction with the host immune system. This is due, in part, to the fact that PCV2 directly infects multiple immune cells, including lymphocytes and cells of monocytic lineage. Early in the course of PCV2 exposure, the innate immune response to PCV2 is vital in orchestrating subsequent events in determining the outcome of infection (Darwich and Mateu, 2012). Despite the classic microscopic lesions of PCV2-associated intracytoplasmic botryoid inclusion bodies in histiocytes from pigs with PMWS, multiple studies provide evidence that cells of the monocyte/macrophage lineage and dendritic cells (DC) are not efficient targets for PCV2 replication (Perez-Martin et al., 2007; Steiner et al., 2008; Vincent et al., 2005; Vincent et al., 2003). Specifically, in monocyte and bone marrow-derived DCs there is no evidence of viral replication and PCV2 has been shown to persist in DCs without inducing apoptosis, modulating cell surface molecules or losing viral infectivity (Vincent et al., 2003). In addition to representing an effective means of immune evasion, these features may indicate that DCs serve as an efficient vehicle for viral transport due to their migratory capacity. Using an in situ hybridization probe specific for cells supporting active PCV2 replication, investigators demonstrated that tissues

from PMWS-affected pigs exhibited scarce viral replication in macrophages (Perez-Martin et al., 2007). A study describing the ultrastructural characteristics of lymph nodes from pigs with clinical PMWS identified PCV2 particles exclusively in histiocytes. Localization experiments using confocal microscopic and double immunolabeling transmission electron microscopy identified PCV2 antigen colocalized with mitrochondria, suggesting these organelles may play an important role in viral replication (Rodriguez-Carino et al., 2010). The limited or absent replication activity of PCV2 in macrophages and dendritic cells, respectively, has been suggested as evidence that the presence of cytoplasmic viral antigen may be reflective of phagocytic/endocytic activity rather than true 'infection' (Darwich and Mateu, 2012; Kekarainen et al., 2010; Steiner et al., 2008; Vincent et al., 2003).

Although exposure to PCV2 does not cause any identifiable functional change in myeloid DCs (mDC), PCV2 has been shown to modulate plasmacytoid DC (pDC) function. Specifically, pDCs exhibited impaired mDC costimulatory function following exposure to PCV2, and this was the result of PCV2-induced inhibition of IFN-α and TNF-α induction upon stimulation with CpG oligodeoxyribonucleotides (Vincent et al., 2005). Vincent and colleagues further demonstrated that this feature was mediated by viral DNA and induced a broad inhibition of pDC function including response to TLR-7 and TLR-9 agonists. Collectively these features were suggested as a novel modulation of the immune response to danger signals that could render the host more susceptible to secondary or concomitant microbial infections (Vincent et al., 2007).

Multiple investigators have provided evidence that IL-10 expression is elevated in peripheral blood mononuclear cells (PBMC) and tissue from PCVAD affected pigs. Kekarainen and colleagues reported that PCV2, but not PCV1, induced IL-10 secretion by monocytes. Furthermore, PCV2 inhibited IFN-γ, IFN-α and IL-12 secretion upon recall stimulation of

PBMCs (Kekarainen et al., 2008). The authors concluded that this may contribute to the suppression of a Th1 response by PCV2, thus contributing to the development of co-infections. Doster *et al.* reported increased IL-10 expression in mandibular lymph node, spleen and tonsil from PMWS-affected pigs. This IL-10 expression was predominantly associated with T cells, rather than B cells or macrophages, and was primarily expressed in bystander cells rather than PCV2-infected cells, suggesting a paracrine mechanism for cytokine expression (Doster et al., 2010). Darwich and colleagues further showed that pigs with subclinical PCV2 infection developed a transient virus-specific IL-10 response during the viremic phase of infection (Darwich et al., 2008). IL-10 exerts suppressor effects on a wide range of lymphocyte populations; is a known inhibitor of inflammatory cytokines, including IL-1β, IL-6, TNF and IL-12; and antagonizes the function of antigen presenting cells such as immature DCs. IL-10 treated DCs are known to induce regulatory T cells while producing antigen-specific anergy in CD4+ and CD8+ T lymphocytes (Enk, 2005).

In addition to modulating IL-10 expression, PCV2 has the ability to induce IL-1β and IL-8 while suppressing the release of IL-4 and IL-2 (Darwich et al., 2003). Shi et. al. examined the cytokine mRNA expression profiles of PBMCs from pigs experimentally co-infected with PCV2 and PRRSV. Following stimulation with concanavalin A, PBMCs from PRRSV/PCV2 co-infected pigs had reduced IL-2, IL-4, IL-6, IL-12p40 and IFN-γ but increased TNF compared to pigs infected with either virus alone. The authors concluded that these results suggest co-infection synergistically suppresses Th1 and Th2 responses, thus compromising cell-mediated and humoral immune responses and rendering pigs more susceptible to severe disease (Shi KC, 2010).

In cases of natural PCV2 infection, cell-mediated immunity and a strong neutralizing antibody response are important in mediating viral clearance (Darwich and Mateu, 2012). Neutralizing antibodies (NA) against PCV2 are known to develop beginning approximately four weeks post-inoculation (Pogranichnyy et al., 2000). Other investigators have shown that the adaptive immune response to PCV2 infection correlates with viral replication and disease severity. In pigs infected with PCV2, those with high NA and IFN-γ response exhibited lower viral replication compared to pigs with a weak NA and IFN-γ response. Furthermore, high NA titers were correlated with decreased clinical disease (Meerts et al., 2006; Meerts et al., 2005).

Several studies have investigated the antigenic determinants within the ORF2 encoded capsid protein. In one study, monoclonal antibodies against the capsid protein were used to map antigenic sites by analyzing their interaction with PCV1/PCV2-ORF2 chimeric infectious PCV DNA clones (Lekcharoensuk et al., 2004). The results indicated that the capsid protein immunodominant epitopes are likely located within amino acid residues 47-84, 165-200, and 230-233. In another study, PEPSCAN analysis of overlapping PCV2 peptide fragments identified four immunoreactive PCV2 ORF2 residues (Mahe et al., 2000). Overall, four PCV2 capsid protein epitopes have been identified (Trible and Rowland, 2012). An additional study was aimed at identifying capsid protein regions preferentially recognized by sera from PCV2 experimentally infected, vaccinated, and clinically diseased pigs (Trible et al., 2011). Vaccinated pigs preferentially recognized only the largest polypeptide fragment spanning amino acids 141-200. However, antibodies from experimentally infected and PDNS-affected pigs were directed against a small immunodominant capside protein oligopeptide spanning amino acids 169-180. The authors proposed that this epitope may function as a decoy epitope, subverting the humoral immune response away from viral clearance, as supported by the observation that antibodies

from vaccinated pigs had an approximately 4-fold increase in neutralizing activity compared to antibodies from infected pigs (Trible et al., 2011; Trible and Rowland, 2012). Further evidence for qualitative and quantitative differences in the antibody response to PCV2 vaccination compared to infection was recently reported by Trible et al. Vaccination with a PCV2 capsid protein induced a strong neutralizing antibody response, whereas PCV2 infection resulted in high levels of non-neutralizing antibody directed at the C-terminal region of the capsid protein. (Trible et al., 2012).

Regulatory T cells in the pathogenesis of viral infections

The past two decades have witnessed an explosion in research related to regulatory T cells (T_{reg}). A PubMed search with the keywords "regulatory T cell" returned 34,834 hits as of April 26, 2012. These cells represent an important arm of the immune system and function in the maintenance of immunological self-tolerance and immune homeostasis (Chen et al., 2003). In a seminal paper by Sakaguchi and colleagues, $CD4^+$ lymphocytes depleted of $CD25^+$ cells were inoculated into BALB/c athymic nude mice. The recipient mice spontaneously developed multiple autoimmune diseases and graft-vs-host wasting disease. However, the onset of these diseases could be prevented by reconstitution of the $CD4^+CD25^+$ cells, but not $CD8^+$ cells, in a dose dependent manner (Sakaguchi et al., 1995). These data and numerous ensuing studies provide evidence that $CD4^+CD25^+$ T_{regs} downregulate the immune response to self and non-self antigens in an antigen non-specific manner, thus contributing to the maintenance of self-tolerance. Thus far T_{regs} are best characterized in mice and humans where they represent approximately 5-10% of peripheral $CD4^+$ lymphocytes and less than 1% of $CD8^+$ cells. Two origins for T_{regs} have been identified: 1. natural T_{regs} (nT_{regs}) are generated in the thymus, have a

$CD4^+CD25^{high}$ phenotype and express the transcription factor FoxP3 (Fontenot et al., 2003; Itoh et al., 1999); 2. inducible T_{regs} (iT_{regs}, sometimes referred to as adaptive) are induced in the periphery from naïve $CD25^-$ T cells under subimmunogenic antigen presentation, chronic inflammation and during normal homeostasis of the gastrointestinal tract (Curotto de Lafaille and Lafaille, 2009; Mills, 2004). Findings on the similarities and differences between nT_{regs} and iT_{regs} have recently been reviewed and the authors suggested that nT_{reg} cells play an essential role in preventing autoimmunity whereas iT_{regs} primarily function in decreasing chronic inflammation, suppressing the immune response to allergens and maintaining a non-inflammatory environment in the gut (Curotto de Lafaille and Lafaille, 2009).

Expression of the transcription factor FoxP3 (forkhead box P3) is a defining characteristic of T_{regs}. Recognition of the importance of FoxP3 in immune regulatory function accompanied the discovery that FoxP3 deficiency was the underlying defect in a lymphoproliferative and multiorgan autoimmune disease in scurfy mice and human patients with immunodysregulation polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome (Feuerer et al., 2009). However, some subtypes of iT_{regs} exhibiting suppressor activity do not express FoxP3, including Tr1 cells, characterized by IL-10 secretion, and Th3 cells, characterized by TGF-β secretion (Bilate and Lafaille, 2012). $FoxP3^+$ T_{regs} recognize antigen via the αβ T-cell receptor and have a broad TCR repertoire.

T_{reg} differentiation is closely tied to that of Th17 T cells, another $CD4^+$ subtype characterized in part by the production of IL-17. Stimulation of the TCR of a naïve $CD4^+$ T cell in the presence of TGF-β and IL-6 induces the transcription factor RORγt and thus the differentiation of Th17 cells. However, in the absence of the inflammatory cytokine IL-6, TGF-β induced FoxP3 expression inhibits RORγt, thus leading to the differentiation of T_{reg} cells

(Ziegler and Buckner, 2009). The initial discovery that TGF-β was needed for differentiation of T_{reg} and Th17 cells suggested that they were somehow related. TGF-β is capable of inducing the signature transcription factors of T_{reg} and Th17 cells (FoxP3 and RORγt respectively). However, FoxP3 has been shown to associate with and inhibit RORγt as a transcriptional activator, thus resulting in the T_{reg} phenotype. In the presence of IL-6, this inhibition is reversed and RORγt function is restored leading to Th17 differentiation (Ziegler and Buckner, 2009). Of interest is the premise that commitment to the Th17 or T_{reg} phenotype resulting in a terminally differentiated T cell does not depict the whole story. There is some evidence that a transient state exists whereby the cell exhibits both T_{reg} and Th17 phenotypes. And, so-called terminally differentiated T_{regs} can revert to a Th17 phenotype (Tartar et al., 2009). Thus crosstalk between T_{reg} and Th17 signals may lead to plasticity in immune function.

Porcine T_{regs}

Kaser and colleagues were the first to identify and describe $FoxP3^+$ T_{regs} in pigs. They initially demonstrated, using RT-PCR and Southern Blot hybridization, that FoxP3 was selectively expressed on $CD4^+CD25^+$ T cells, similar to the phenotype of murine T_{regs} (Kaser et al., 2008b). These cells exhibited heterogeneous expression of CD45RC, CD8α, and MHC-II; produced IL-10; and demonstrated suppressor function when co-cultured with $CD4^+CD25^-$ T cells. With the aid of an anti-mouse/rat FoxP3 antibody with proven reactivity against porcine FoxP3, T_{regs} were identified in blood, spleen, lymph nodes, and thymus (Kaser et al., 2008a; Kaser et al., 2011a). The majority of T_{regs} were $CD4^+CD8^+$, but a minority of $FoxP3^+$ cells were identified in the $CD4^+CD8^-$ and $CD4^-CD8^+$ subpopulations. Investigators have demonstrated that, similar to human and murine T_{regs}, porcine T_{regs} utilize three main mechanisms of

suppression: cell-cell contact dependent suppression, the production of soluble factors such as IL-10, and competition for growth factors such as IL-2 (Kaser et al., 2011b). In this study the authors further describe the ability of porcine T_{regs} to suppress proliferation of T-helper cells, cytotoxic T lymphocytes, and TCR-γδ T cells, suggesting that T_{regs} play a role in regulating the adaptive and innate immune system of swine.

T_{regs} in Nidovirales infection

The role of T_{regs} in the pathogenesis of numerous viral infections has been reviewed elsewhere (Belkaid, 2007; Belkaid and Rouse, 2005; Belkaid and Tarbell, 2009; Billerbeck and Thimme, 2008; Keynan et al., 2008; Li et al., 2008; Rouse et al., 2006). However, the following is a review of the literature concerning the interaction of T_{regs} and viruses of the order Nidovirales, as recently summarized by Cecere *et al.* (Cecere et al., 2012b).

Porcine reproductive and respiratory syndrome virus

The ability of PRRSV to subvert the host immune system into mounting a weak and delayed humoral and cell-mediated immune response and the fact that infection in young pigs can be extremely persistent have been suggested as indirect evidence for the activity of T_{regs} in the pathogenesis of PRRSV infection (Darwich et al., 2010). Consequently, recent research efforts have investigated the role of virus-induced T_{regs} in PRRSV-induced disease.

Wongyanin and colleagues demonstrated that peripheral blood mononuclear cells (PBMCs) cultured with American genotype PRRSV *in vitro* induced virus-specific $CD4^+CD25^+FoxP3^+$ T_{regs} and the addition of monocyte-derived dendritic cells (MoDC) to the cell culture enhanced T_{reg} induction (Wongyanin et al., 2010). Not only was there a significant

increase in the numbers of $CD4^+CD25^+FoxP3^+$ cells, but these PRRSV-specific T_{regs} exhibited suppressive activity when co-cultured with PHA-stimulated autologous peripheral leukocytes. The authors reported in this study that PBMCs collected from PRRSV-infected pigs 10 days post-inoculation exhibited significantly higher numbers of $CD4^+CD25^+FoxP3^+$ lymphocytes when cultured in the presence of PRRSV compared to mock-infected cell lysate or PBMCs alone.

Silva-Campa *et al.* reported that PRRSV-infected MoDCs significantly increased $CD25^+FoxP3^+$ T_{regs} with suppressor activity following co-culture with autologous lymphocytes (Silva-Campa et al., 2009). This effect required the presence of viable PRRSV, because virus inactivation or treatment with IFN-α reversed T_{reg} induction. In this study T_{reg} induction was dependent on TGF-β and not IL-10. This same group reported that while American genotype PRRSV strains were capable of inducing T_{reg} and upregulating TGF-β production (Silva-Campa et al., 2009), DCs infected with European genotype PRRSV induced neither TGF-β nor T_{regs} (Silva-Campa et al., 2010).

Further supporting the role of T_{regs} in the pathogenesis of PRRSV infection, LeRoith and colleagues reported that an attenuated modified live PRRSV vaccine (MLV) and its pathogenic parent strain induced T_{regs} in pigs naturally infected with *Mycoplasma hyopneumoniae* (LeRoith et al., 2011). A significant increase in $CD4^+CD25^+FoxP3^+$ cells in PBMCs from PRRSV-challenged and vaccinated pigs was seen by day 10 post-inoculation, consistent with previous reports (Wongyanin et al., 2010), and this correlated with increased susceptibility to *M. hyopneumoniae*-induced respiratory disease. The findings from this study suggest that mutations in the PRRSV vaccine strain that attenuate the virus do not decrease its ability to induce T_{regs}. The authors concluded that T_{reg} induction in pigs following vaccination with a MLV may bias

towards IL-10 instead of IFN-γ production, thus contributing to the lack of heterologous protection in current PRRSV vaccines (Thacker et al., 2000). This illustrates the potential for improving next-generation PRRSV vaccines by altering T_{reg}-stimulating epitopes in the vaccine strain, which would elicit a strong virus-specific IFN-γ response and aid in protection against heterologous strains.

In order to assess the potential benefit of using a mucosal rather than parenteral immunization approach, Dwivedi *et al.* investigated the ability of an intranasally delivered PRRS-MLV augmented with a potent *Mycobacterium tuberculosis* whole cell lysate adjuvant to induce cross protective immunity against a heterologous PRRSV strain. Vaccinated pigs had a reduced frequency of T_{regs} in respiratory mucosal and systemic sites compared to unvaccinated pigs, and this correlated with decreased secretion of immunosuppressive cytokines IL-10 and TGF-β, diminished lung pathology, and increased PRRSV neutralizing antibody titers and IFN-γ secretion (Dwivedi et al., 2011a; Dwivedi et al., 2011b). These findings suggest that the route of immunization and adjuvant-mediated immunomodulation may influence T_{reg} dynamics, thereby facilitating or negating efficient viral clearance. This same group recently reported on pigs that were maintained on a commercial farm and experimentally infected with PRRSV. At two days post-infection, infected pigs had an increased frequency of circulating $CD4^+CD25^+FoxP3^+$ T_{regs}, reduced frequency of $CD4^-CD8^+$ and $CD4^+CD8^+$ T cells, and enhanced IL-10, IL-4 and IL-12 secretion (Dwivedi et al., 2012).

The contribution of PRRSV nucleocapsid protein (N) to T_{reg} induction was recently investigated. Wongyanin *et al.* reported that N-protein induced IL-10 producing cells and $CD4^+CD25^+FoxP3^+$ T_{regs} in a DC *in vitro* system (Wongyanin et al., 2012). In this study, T_{reg}

induction was found to be dependent, at least in part, on IL-10, as neutralization of IL-10 by anti-IL-10 antibody drastically reduced the PRRSV-induced T_{regs}.

Lactate dehydrogenase elevating virus

Lactate dehydrogenase-elevating virus (LDV) has been described as an "ideal" persistent virus since it is associated with life-long viral infection in mice in the absence of clinical disease while escaping the host immune response (Plagemann et al., 1995; Rowson and Mahy, 1985). Cytotoxicity is limited to resident tissue macrophages, in which viral replication is maintained. Lifelong immunotolerance is maintained and has been shown to be due in part to continuous generation of LDV antigens in the thymus (Rowland et al., 1994). In addition, LDV infection and replication within macrophages appears to be robustly resistant to typical antiviral immune responses including interferon-α/β, antiviral antibodies and virus-specific cytotoxic T lymphocytes (Plagemann et al., 1995). The antigen presenting ability of spleen, lymph node, and peritoneal macrophages from LDV-infected mice was shown to be diminished as measured *in vitro* by reactivation of memory T cells (Isakov et al., 1982). The LDV-mediated impairment of antigen presentation was not due to diminished uptake of antigen by macrophages in these experiments and LDV-infected peritoneal macrophages were not immunosuppressive in cell culture. Rather, the authors concluded that the decrease in antigen presenting ability of LDV-infected macrophages was related to reduced expression of Ia antigen or virus-mediated elimination of Ia-positive macrophages from the peritoneum (Isakov et al., 1982). Collectively these features suggest a role for T_{regs} in the pathogenesis of LDV infection.

Inada and colleagues demonstrated that mice challenged with heat-inactivated or killed LDV induced a strong virus-specific delayed-type hypersensitivity (DTH) reaction, whereas a

delayed hypersensitivity reaction was undetectable in young (1-3 month-old) mice challenged with live virus (Inada and Mims, 1986). In these experiments pretreatment with cyclophosphamide partially restored the DTH response, which was attributed to cyclophosphamide mediated elimination of suppressor T cells (Askenase et al., 1975; Mitsuoka et al., 1976). Live virus also elicited a DTH response in old mice (> 8 months) without cyclophosphamide treatment, which is not surprising in light of the fact that older mice have reduced suppressor T cell activity. The cumulative findings from this study suggest that inoculation with live LDV induces suppressor T cells, and that this dampens the virus-specific delayed hypersensitivity response (Inada and Mims, 1986).

Further supporting the immunosuppressive ability of LDV, Robertson *et al.* reported that co-infection with LDV and Friend virus (FV) delayed the FV specific CD8+ T cell response and that this resulted in an increase in duration and severity of the acute phase of FV infection (Robertson et al., 2008). The suppressed FV-specific CD8+ T cell response occurred in mice acutely co-infected with LDV and FV, as well as in mice inoculated with LDV 8 weeks prior to FV infection. In addition, mice infected with LDV exhibited significant regulatory T cell-mediated suppression of IFN-γ production by FV-specific CD8+ T cells that peaked at day 3 post-infection and was diminished by day 7 post-infection. However, failure of FV/LDV co-infected mice to mount a strong CD8+ T cell response was not attributed solely to T_{reg}-mediated suppression, because neither depletion of CD4+ cells nor pre-treatment with anti-CD25 antibody restored the normal CD8+ response (Robertson et al., 2008).

Coronaviruses

Much attention on the interaction of T_{regs} and viral pathogens has focused on chronic disease, demonstrating the ability of T_{regs} to delay or prevent viral clearance thus enabling persistent infection (Belkaid, 2007; Li et al., 2008). From the point of view of the host, virus-induced T_{regs} represent a detrimental factor in the context of chronic or persistent infection. However, coronavirus infection in the central nervous system of mice highlights a beneficial role of T_{regs} in reducing bystander damage related to immunopathology in the context of acute infection. Viral infection of the central nervous system exemplifies the necessity of a delicately balanced and finely orchestrated immune response. While a rapid and strong pro-inflammatory response will aid in viral clearance, host tissue destruction secondary to immunopathology can be a deleterious side effect. An appropriate anti-inflammatory response is thus necessary for minimizing collateral damage while still enabling clearance of the invading pathogen.

Mice infected with the neurovirulent strain of mouse hepatitis virus JHM (JHMV) develop a rapidly progressive, fatal disease that has been shown to be mediated, in part, by CD4 T cells. This was evidenced by the fact that infection with a recombinant JHMV strain containing a single mutation in an immunodominant CD4 T cell epitope (rJ.M_{Y135Q}) resulted in nonlethal mild encephalitis, and the decrease in mortality correlated with decreased numbers of virus-specific $CD4^+$ T cells in the brain (Anghelina et al., 2006). These findings were further investigated and significantly higher levels of the pro-inflammatory cytokines/chemokines IL-6, CCL2, CCL5 and IFN-γ were detected in the brains of mice infected with the non-mutated recombinant JHMV strain (rJ) compared to rJ.M_{Y135Q}-infected mice (Anghelina et al., 2009). CD4 T_{regs} were critical in ameliorating disease because greater numbers of T_{regs} were detected in the brains of rJ.M_{Y135Q}-infected mice compared to rJ-infected mice, their depletion in rJ.M_{Y135Q}-

infected mice increased morbidity and mortality, and adoptive transfer of T_{regs} into rJ-infected mice increased survival from 0% to 50% (Anghelina et al., 2009). The authors concluded that in the setting of acute encephalitis, T_{regs} may aid in limiting immunopathology, thus decreasing clinical disease without delaying viral clearance.

In contrast to the neurovirulent JHMV strain, mice infected with an attenuated JHMV variant (J2.2-V-1) develop chronic demyelinating encephalomyelitis and the demyelination is largely due to immunopathology associated with viral clearance (Bergmann et al., 2004; Wang et al., 1990; Wu et al., 2000). Trandem *et al.* demonstrated that adoptive transfer of T_{regs} ameliorated clinical disease and demyelination in J2.2-V-1-infected mice, but did not delay viral clearance in immunocompetent C57BL/6 mice (Trandem et al., 2010). The authors provided evidence that this improved clinical outcome was due in part to T_{regs} functioning in the draining cervical lymph nodes to suppress T cell proliferation, dendritic cell activation and expression of pro-inflammatory mediators. These findings further support the notion that T_{regs} play an important role in limiting neuropathology associated with mouse hepatitis virus (MHV) infection while still allowing for viral clearance.

Identification and characterization of pathogen-specific epitopes targeted by T_{regs} has received considerable interest due to possible therapeutic interventions aimed at diminishing inflammation-induced tissue damage in a pathogen-specific fashion. Zhao and colleagues identified T_{regs} that specifically recognize two mouse hepatitis virus-specific epitopes using the neurotropic rJ2.2 strain of mouse hepatitis virus, which is known to cause mild acute encephalitis and chronic demyelination (Fleming et al., 1986; Zhao et al., 2011). These virus-specific T_{regs} were present in the virus-infected central nervous system and, based on concurrent detection with virus-specific effector CD4 T cells and identification within the naïve T cell precursor pools in

the spleen and lymph nodes, are presumed to arise from the natural T_{reg} pool. In addition, the virus-specific T_{regs} expressed IL-10 and IFN-γ upon stimulation with viral peptide and suppressed proliferation of cognate-epitope specific effector CD4 T cells. While T_{regs} are known to play a critical role in reducing immunopathology and clinical disease in rJ2.2-infected mice, the results from this study suggest that virus-specific T_{regs} may be significantly more potent in diminishing immunopathology associated with encephalomyelitis compared to adoptive transfer of natural T_{regs}, particularly during acute infection when maximum viral antigen is present (Bergmann et al., 2006; Trandem et al., 2010; Zhao et al., 2011).

In contrast to the protective effect of T_{regs} in the pathogenesis of neurotropic mouse hepatitis virus infection, Shalev and colleagues demonstrated that T_{regs} contributed to more severe fulminant viral hepatitis in susceptible mice infected with murine hepatitis virus strain 3. This phenomenon was mediated in part by increased T_{reg} expression of the immunosuppressive cytokine fibrinogen-like protein 2 (Shalev et al., 2009). These findings suggest that T_{reg} activation following infection with mouse hepatitis virus may culminate in different outcomes depending on the anatomic location of disease, such that they are paradoxically beneficial to the host in limiting CNS disease but harmful in potentiating fulminant hepatitis.

The function of T_{regs} in coronavirus-infected mice highlights the potential dichotomy in protective versus harmful outcome, depending on the anatomic location of disease. Along this line of reasoning, one might argue that the perspective of T_{regs} protecting the host or enhancing disease may depend on which outcome is more deleterious, viral persistence or immunopathology associated with viral clearance.

Chapter 3: Co-infection of porcine dendritic cells with porcine circovirus type 2a (PCV2a) and genotype II porcine reproductive and respiratory syndrome virus (PRRSV) induces $CD4^+CD25^+FoxP3^+$ T cells in vitro

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Abstract

Porcine circovirus associated disease (PCVAD) is currently one of the most economically important diseases in the global swine industry. Porcine circovirus type 2 (PCV2) is the primary causative agent, however co-infection with other swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) is often required to induce the full spectrum of clinical PCVAD. While the specific mechanisms of viral co-infection that lead to clinical disease are not fully understood, immune modulation by the co-infecting viruses likely plays a critical role. We evaluated the ability of dendritic cells (DC) infected with PRRSV, PCV2, or both to induce regulatory T cells (T_{regs}) *in vitro*. DCs infected with PCV2 significantly increased $CD4^+CD25^+FoxP3^+$ T_{regs} ($p<0.05$) and DCs co-infected with PRRSV and PCV2 induced significantly higher numbers of T_{regs} than with PCV2 alone ($p<0.05$). Cytokine analysis indicated that the induction of T_{regs} by co-infected DCs may be dependent on TGF-β and not IL-10. Our data support the immunomodulatory role of PCV2/PRRSV co-infection in the pathogenesis of PCVAD, specifically via T_{reg}-mediated immunosuppression.

Introduction

Porcine circovirus associated disease (PCVAD), commonly known as porcine circovirus disease (PCVD) in Europe, is arguably the most economically significant disease facing the global swine industry today. This disease has devastated every major swine producing country in the world and has culminated in losses of up to twenty dollars per pig in the United States swine population (Gillespie et al., 2009). While PCVAD encompasses a spectrum of clinical syndromes, all are unified by the presence of porcine circovirus type 2 (PCV2) as the primary causative agent. PCV2 is a small, non-enveloped, single-stranded, circular DNA virus belonging to the family *Circoviridae* (Todd, 2005). PCVAD requires the presence of PCV2, but PCV2 infection alone rarely produces the full spectrum or severity of clinical disease. Other co-infecting pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, swine influenza virus, *Mycoplasma hyopneumoniae,* and Torque teno sus virus (TTSuV) augment the severity of clinical disease and result in increased PCV2 viral load in infected pigs (Opriessnig and Halbur, 2012). The exact mechanisms by which viral or bacterial co-infection with PCV2 potentiates clinical PCVAD are unknown, but modulation of the host immune system is likely a key event in the pathogenesis of this disease (Gillespie et al., 2009; Krakowka et al., 2001).

Virus-mediated induction of regulatory T cells (T_{regs}) is one specific mechanism of modulating the host immune response in favor of maintaining viral infection (Belkaid, 2007). T_{regs} are broadly divided into natural T_{regs}, originating from the thymus, and inducible (adaptive) T_{regs}, derived outside the thymus from naïve $CD4^+$ T cells (Askenasy et al., 2008). Although the phenotype of T_{regs} is variable, $CD4^+CD25^+FoxP3^+$ cells exhibiting suppressor activity by a variety of mechanisms have been identified in pigs (Kaser et al., 2008a, b; Kaser et al., 2011b).

By altering the host immune response to viral infection, T_{regs} contribute to persistent infection of many viruses including Friend virus, herpes simplex virus, hepatitis C virus, hepatitis B virus, human immunodeficiency virus, feline immunodeficiency virus, simian immunodeficiency virus, cytomegalovirus, and Epstein-Barr virus (Belkaid, 2007; Li et al., 2008; Rouse et al., 2006).

Recently, PRRSV has been shown to induce T_{regs} both *in vitro* and *in vivo* (LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010), although to our knowledge, T_{reg} induction by PCV2 has not been described. PRRSV-mediated T_{reg} induction appears to vary depending on the virus genotype (Silva-Campa et al., 2010). Since co-infection with PCV2 and PRRSV is very common in the swine population, and since pigs co-infected with PCV2 and PRRSV have reduced IFN-γ and increased IL-10 expression in peripheral blood mononuclear cells (PBMC) (Shi KC, 2010), we hypothesize that co-infection should induce higher numbers of T_{regs} *in vitro*.

Materials and methods

Viruses

PRRSV isolate ATCC VR2385 (Meng et al., 1994) and PCV2a isolate ISU-40895 (Fenaux et al., 2000) were used in this study. PRRSV VR2385 was propagated in confluent monolayers of MARC-145 cells. The viral titer was determined by an immunofluorescent assay (IFA) with an anti-PRRSV N antibody (SDOW17) and quantified in fluorescent focus-forming units (FFU) as described previously (Fang et al., 2006). Infectious virus stocks were generated for PCV2a by transfection of PK-15 cells in T25 flasks with Lipofectamine LTX (Invitrogen) using a dimerized infectious DNA clone as described previously (Fenaux et al., 2002; Fenaux et

al., 2003). The 50% tissue culture infective dose ($TCID_{50}$) per ml was calculated according to the method of Reed & Muench (Reed and Muench, 1938).

Isolation of PBMCs and generation of monocyte-derived dendritic cells

Peripheral blood samples from five PRRSV- and PCV2-free pigs were collected into heparinized syringes, diluted 1:2 with sterile phosphate buffered saline (PBS), overlaid on Ficoll-Paque™ (GE Healthcare, Piscataway, NJ) and PBMCs were collected as previously described (Silva-Campa et al., 2009). Porcine monocyte-derived dendritic cells (DC) were generated as previously described for pig 1 (Wang et al., 2007). Briefly, CD14-positive monocytes were purified from PBMCs by immunomagnetic labeling of cells using mouse anti-swine CD14 monoclonal antibody (R&D Systems, Minneapolis, MN) and goat-anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA). The CD14-positive monocytes were cultured for 5 days in complete medium supplemented with 20ng/ml recombinant porcine IL-4 and 20ng/ml recombinant porcine GM-CSF (Cell Sciences, Canton, MA). Due to low DC yields by this method, an alternative technique for generating DCs was employed for pigs 2-5 as previously reported (Carrasco et al., 2001; Flores-Mendoza et al., 2008). Freshly isolated PBMCs were seeded in T75 tissue culture flasks and incubated overnight in complete medium at 37 °C with 5% CO_2 to allow monocytes to adhere. Non-adherent cells were removed and frozen in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO). Adherent cells were cultured at 37 °C with 5% CO_2 in complete medium supplemented with 20ng/ml recombinant porcine IL-4 and 20ng/ml recombinant porcine GM-CSF. After 5 days, DCs were harvested using Cellstripper™, an enzyme-free cell dissociation medium (Cellgro, Manassas, VA). DC differentiation from both

methods was confirmed by typical veiled morphology and phenotyping with monoclonal antibodies specific for MHC I, MHC II, CD 172 (SWC3), CD 14, and CD 1 (Wang et al., 2007).

Infection of DCs by PRRSV and PCV2

DCs were inoculated with PRRSV at a multiplicity of infection (m.o.i.) of 0.1 as previously described (Silva-Campa et al., 2009), and with PCV2a at a m.o.i. of 0.01 (Vincent et al., 2003), or with both viruses, and incubated for 1 hour at 37 °C. Cells were then washed twice with complete medium and seeded into 96-well tissue culture plates at $5x10^4$ cells per well. After 24 hours of incubation at 37 °C with 5% CO_2, approximately $5x10^5$ lymphocytes (CD14-negative cell fraction for pig 1, non-adherent cells from an overnight culture of PBMCs for pigs 2-5) were added and co-cultured for 3 days. DC-lymphocyte co-cultures were performed in triplicate for each treatment group per pig.

Immunofluorescence assay (IFA) to determine PCV2 and PRRSV infectivity

DCs were inoculated with PCV2, PRRSV, or both as described above and infected cells were seeded in 35mm glass bottom dishes (MatTek Corporation, Ashland, MA). DCs were incubated for 36 hours at 37 °C with 5% CO_2, and the cells were then fixed with 80% acetone. After washing with PBS buffer, cells were sequentially incubated with an anti-PRRSV N monoclonal antibody SDOW17 and swine anti-PCV2 polyclonal serum followed by FITC-conjugated goat anti-swine antibody (KPL, Gaithersburg, MD) and Alexa-fluor647 conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA). The cells were then washed with PBS, covered with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a Nikon TE2000-E confocal microscope.

Flow cytometry

Following 3 days of co-culture with virus-infected or uninfected control DCs, lymphocytes were evaluated for their expression of CD4, CD25, and FoxP3 using flow cytometry. Briefly, cells were sequentially stained with mouse anti-porcine CD4 (VMRD, Pullman, WA), goat anti-mouse IgG2b:Alexa-fluor647 (Invitrogen, Carlsbad, CA), mouse anti-porcine CD25 (AbD Serotec, Raleigh, NC), and goat anti-mouse IgG:FITC (AbD Serotec, Raleigh, NC). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). Flow cytometric analysis was conducted on the lymphocytes using a FACSCalibur cytometer (Becton-Dickinson Biosciences, San Jose, CA) and analyzed using FlowJo 7.6.3 software. Dendritic cells were excluded based on forward and side scatter and 10,000 events (lymphocytes) were examined per replicate sample.

ELISA

Cell culture supernatants were collected following three-day co-culture of lymphocytes and virus-infected DCs. Levels of secreted IL-10 and TGF-β were quantified using commercial ELISA kits according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA). Differences among treatment groups were determined by Tukey-Kramer or paired *t*-test. Data analysis was

performed using JMP 8.0 (SAS Institute Inc. Cary, NC). Differences were considered to be statistically significant where $p<0.05$.

Results

Detection of viral antigen in DCs inoculated with PCV2 and PRRSV

An IFA assay was employed to confirm PRRSV and PCV2 infectivity of porcine DCs. PCV2 antigen was visualized as diffuse cytoplasmic staining with no viral antigen visible in the nucleus (Figure 3.1A and 3.1D). Punctate cytoplasmic and nuclear staining were observed using a PRRSV N-protein-specific monoclonal antibody (Figure 3.1F and 3.1H). Positive staining for PCV2 and PRRSV was visualized in DCs from both single virus and co-infected groups. In the co-infected group, no DCs exhibiting concurrent staining for both viruses were seen.

DCs infected with PRRSV, PCV2, or both viruses induce $CD4^+CD25^+FoxP3^+$ T_{regs}

Following three days co-culture with virus-infected DCs, lymphocytes were evaluated for CD4, CD25, and FoxP3 expression via tri-color flow cytometry. Following exposure to PCV2-infected DCs, $CD4^+CD25^+FoxP3^+$ T_{regs} were significantly increased in two pigs compared to uninfected controls (Figure 2B). PRRSV-infected DCs induced a significant increase in T_{regs} compared to uninfected controls in only one pig. DCs co-infected with PRRSV and PCV2 significantly induced T_{regs} compared to the uninfected controls in all pigs (Figure 3.2A and 3.2B). In pig 4, one-way analysis of variance revealed no statistically significant difference among treatment groups ($p<0.05$), which is likely due to high variance in the control triplicate samples. However, when the PRRSV/PCV2 co-infected group was compared directly to the uninfected control group using a paired *t*-test, the mean percentage of $CD4^+CD25^+FoxP3^+$ T_{regs} in

the co-infected group was significantly higher than that in the uninfected control group ($p=0.015$). Furthermore, PRRSV/PCV2 co-infected DCs induced significantly higher $CD4^+CD25^+FoxP3^+$ T_{reg} percentages than either virus alone in three of five pigs (Figure 3.2B).

TGF-β is up-regulated following co-infection with PCV2 and PRRSV

IL-10 and TGF-β in cell culture supernatant from co-cultured DCs and lymphocytes were quantified using commercial ELISA kits. Compared to infection with either PRRSV or PCV2 alone, co-culture of lymphocytes with PRRSV/PCV2 co-infected DCs induced significantly ($p<0.05$) higher levels of TGF-β (Figure 3.3A). Due to high levels of latent TGF-β in the cell culture media resulting from the addition of FBS, there was variability in TGF-β among pigs resulting from different lots of FBS used between experiments. Therefore, the quantity of TGF-β in cell-culture supernatant from the virus-infected groups was standardized against the uninfected control group for each pig and reported as relative levels. No significant difference in IL-10 levels among uninfected control or treatment groups was seen (Figure 3.3B)

Discussion

Our results further confirm a previous report that following *in vitro* PCV2 infection of DCs, PCV2 antigen was detectable only in the cytoplasm but not in the nucleus (Vincent et al., 2003). This indicates that PCV2 persists in DCs although reportedly there is no evidence of viral replication, transmission of virus to activated syngeneic T lymphocytes or cell death (Steiner et al., 2008; Vincent et al., 2005; Vincent et al., 2003). Similarly, PRRSV antigen was detected following *in vitro* infection of DCs as previously described (Wang et al., 2007). In the present study no DCs were visible that were concurrently expressing PCV2 and PRRSV antigen.

However, given the low m.o.i. of PCV2 this does not completely preclude the possibility that PCV2 and PRRSV can co-infect the same DC.

Modulation of the immune system is considered to play a critical role in the pathogenesis of PCVAD (Gillespie et al., 2009; Opriessnig et al., 2007). In addition, co-infection with PCV2 and PRRSV is one of the major contributors to development of clinical PCVAD (Opriessnig et al., 2007). However, the specific means by which PRRSV/PCV2 co-infection modulates the immune response in PCVAD is currently unknown. One potential mechanism is virus-mediated induction of T_{regs}. It has been reported that pigs co-infected with PCV2 and PRRSV have more severe lymphoid depletion and enhanced PCV2 replication and tissue distribution (Allan and Ellis, 2000; Harms et al., 2001; Rovira et al., 2002). While protective immunity against PCV2 is associated with neutralizing antibody and IFN-γ production (Meerts et al., 2005), T_{regs} decrease the IFN-γ response, block migration and proliferation of effector T cells, and inhibit IL-2 production (Askenasy et al., 2008). Therefore, PCV2/PRRSV-mediated activation of T_{regs}, as demonstrated in this study, may dampen the immune responses to PCV2, resulting in increased viral replication and clinical disease. In support of this model, PRRSV/PCV2 co-infection has been shown to upregulate IL-10 expression while suppressing IL-2, IL-4, IL-6, IL-12p40 and IFN-γ (Shi KC, 2010).

In the present study, 3-day co-culture of lymphocytes with virus-infected DCs was chosen, as opposed to a 5-day co-culture as previously described (Silva-Campa et al., 2009). This was due to morphologic evidence of cytolysis of DCs beginning approximately 5 days post-inoculation with PRRSV strain VR-2385. DCs did not exhibit morphological evidence of necrosis or apoptosis at the time lymphocytes were harvested for flow cytometry (following 3-day co-culture), but cell viability assays were not performed. Therefore, PRRSV mediated

cytopathic effects on DCs and the abbreviated co-culture time could potentially have impacted the results seen in this study.

PRRSV has been shown to induce IL-10 and it is thought that the potent immunosuppressive properties of this cytokine significantly contribute to modulation of the host immune system in the pathogenesis of PRRSV infection (Charerntantanakul et al., 2006; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003). However, a highly virulent PRRSV strain did not induce IL-10 *in vitro* or *in vivo*, and there is evidence that the ability of PRRSV to induce IL-10 varies depending on the strain (Darwich et al., 2011; Gimeno et al., 2011; Subramaniam et al., 2011). There are conflicting data in the literature regarding the relationship between PRRSV-mediated IL-10 production and T_{reg} induction. In one study PRRSV-mediated induction of T_{regs} was shown to be dependent on TGF-β, but not IL-10 (Silva-Campa et al., 2009). However, in a more recent study DCs pulsed with PRRSV N protein induced IL-10 producing cells and T_{regs}, suggesting a correlation between IL-10 production and development of PRRSV-induced T_{regs} (Wongyanin et al., 2012). In the present study co-infection of DCs with PCV2 and PRRSV was associated with an up-regulation of TGF-β compared to infection with either virus alone, but no increase in IL-10 was seen following infection with either virus alone or co-infection with both viruses compared to uninfected controls. These results are similar to previous reports of PRRSV-mediated T_{reg} induction (Silva-Campa et al., 2009). Because cytokine levels were quantified in the cell culture supernatant, the increase in TGF-β could reflect production by DCs or T lymphocytes. Individual pig TGF-β levels in the PRRSV/PCV2 co-infected groups did not directly correlate with the magnitude of $CD4^+CD25^+FoxP3^+$ T_{reg} induction (data not shown), which further suggests that TGF-β production is not solely attributable to T_{regs}. In addition, DCs were not treated with lipopolysaccharide (LPS) or an unrelated virus (ie. influenza virus or

transmissible gastroenteritis coronavirus). Therefore, it is uncertain whether the observed increase in T_{regs} occurs in a virus-specific manner or reflects a more general homeostatic response to inflammation.

Although induction of porcine T_{regs} by PRRSV has previously been reported *in vitro* and *in vivo* (LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010), the evidence for PRRSV induction of T_{regs} is inconsistent and some have speculated that this may be due to variation in viral genotype, since T_{reg} induction was seen with North American but not European PRRSV strains (Silva-Campa et al., 2010; Silva-Campa et al., 2009). In the present study, there was considerable individual variation among pigs in the T_{reg} response to infection with PRRSV, PCV2, or both viruses, even though the same genotype II North American PRRSV and PCV2a strains were used in all replicates. This may be due in part to genetic variation among pigs contributing to relative resistance or susceptibility of the host immune response, as has been described for PRRSV infection (Lunney and Chen, 2010). Although the results from this study suggest that T_{reg} induction may play a role in modulating the immune response to co-infection with PCV2 and PRRSV *in vitro*, additional studies investigating the T_{reg} response to PRRSV/PCV2 co-infection *in vivo* are needed to address the biological significance of virus-mediated T_{reg} induction in the pathogenesis of PCVAD.

Figures

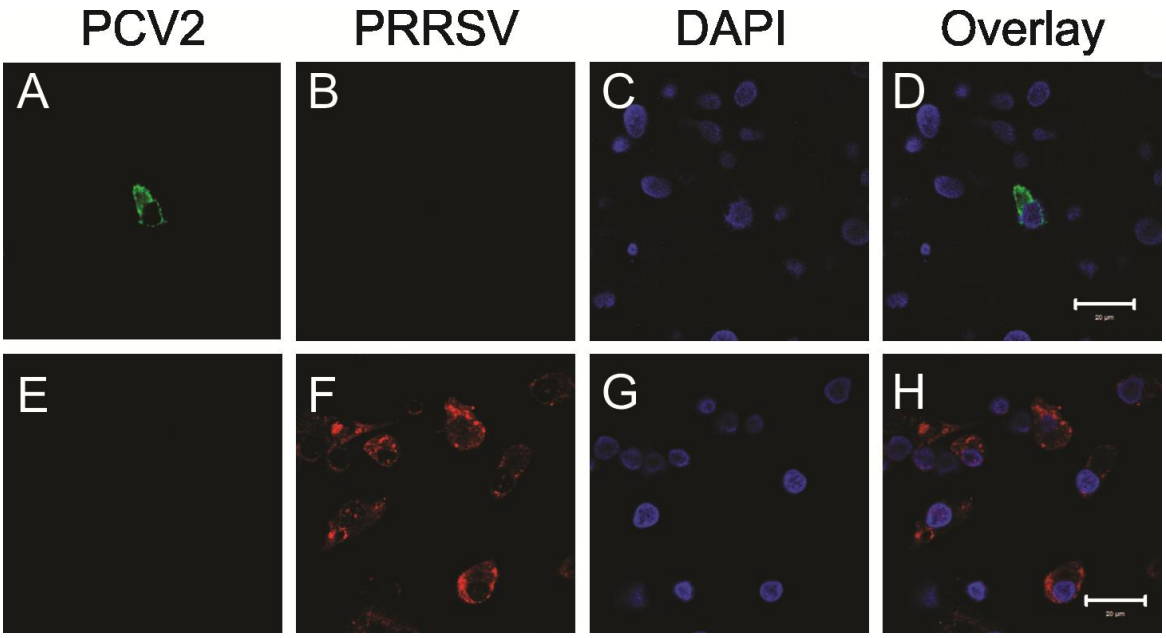


Figure 3.1. Porcine monocyte-derived dendritic cells 36 hours post-inoculation with PCV2 and PRRSV. DCs were immunostained with anti-PCV2 polyclonal antibody (green) and diffuse cytoplasmic immunoreactivity was observed (A, D). DCs were stained with a PRRSV N-protein-specific monoclonal antibody (red) and punctate cytoplasmic and nuclear immunoreactivity were seen (F, H). Bar represents 20µm.

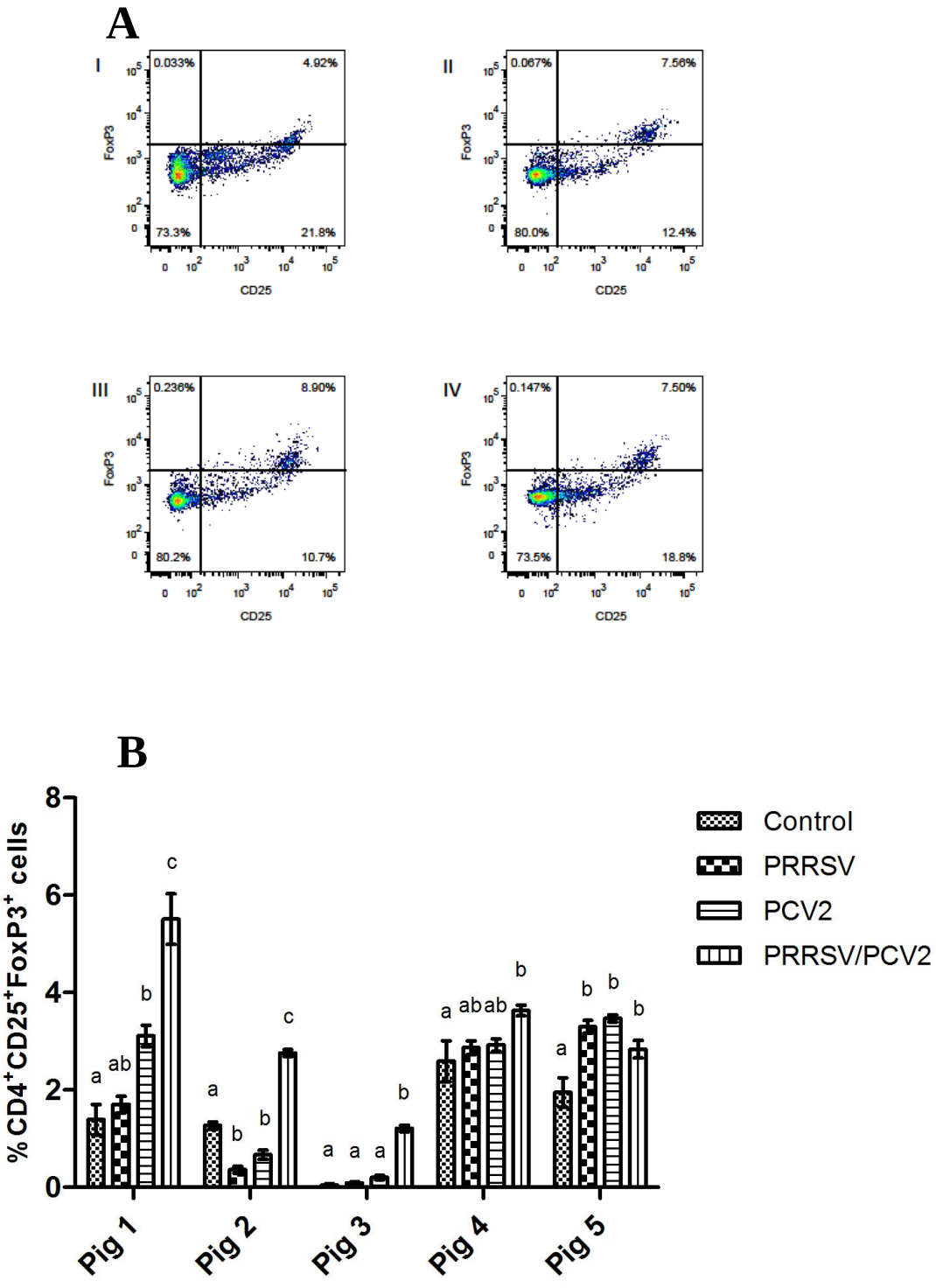
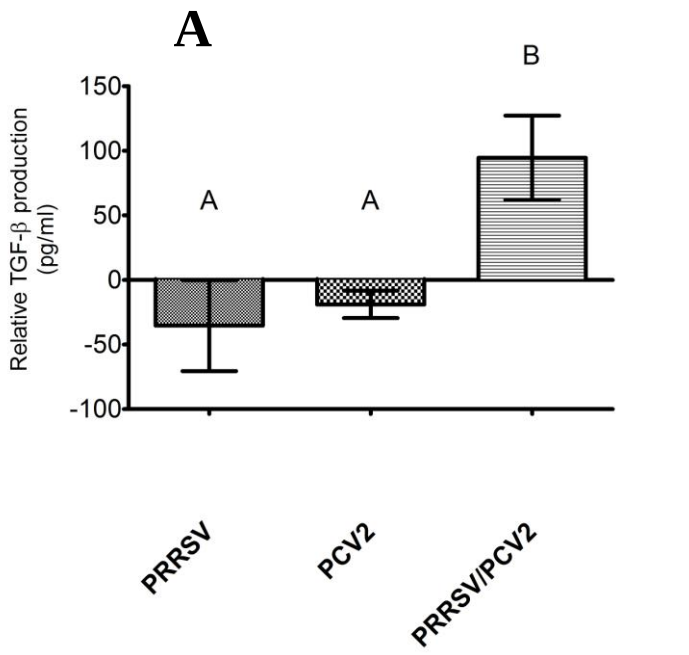

A
I
0.033%
4.92%
73.3%
21.8%
II
0.067%
7.56%
80.0%
12.4%
III
0.236%
8.90%
80.2%
10.7%
IV
0.147%
7.50%
73.5%
18.8%
FoxP3
CD25
B
% CD4+CD25+FoxP3+ cells
Control
PRRSV
PCV2
PRRSV/PCV2
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5

Figure 3.2. **(**A) Representative flow cytometry profile of lymphocytes following 3-day co-culture with virus infected DCs. CD4-gated lymphocytes expressing $CD25^+$ and $FoxP3^+$ are shown. I) uninfected control DCs; II) PRRSV-infected DCs; III) PCV2-infected DCs; and IV) PRRSV/PCV2 co-infected DCs. (B) Mean $CD4^+CD25^+FoxP3^+$ T cells ± standard error of the mean as a percentage of lymphocytes co-cultured with DCs infected with PRRSV, PCV2 or both viruses. Data represents three replicates per group from five independent experiments (n=5 pigs). Data not connected by the same letter are significantly different, $p<0.05$.


A
B
150
100
50
0
-50
-100
Relative TGF-β production (pg/ml)
A
A
PRRSV
PCV2
PRRSV/PCV2

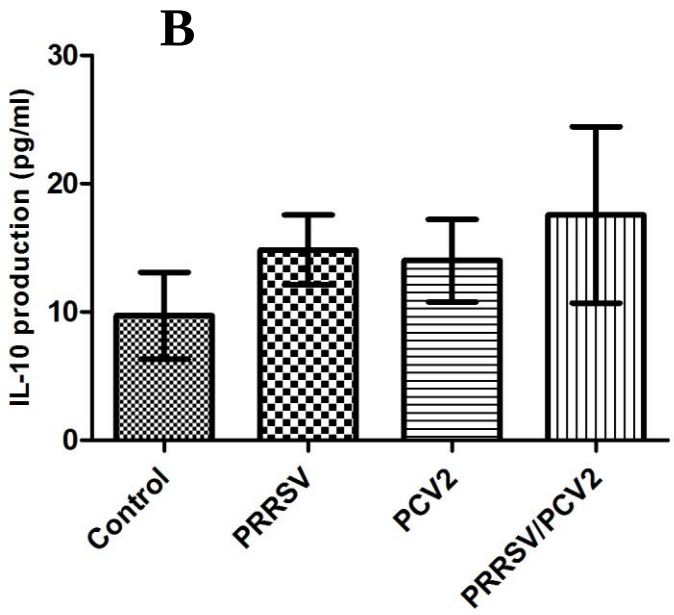

B
30
20
10
0
IL-10 production (pg/ml)
Control
PRRSV
PCV2
PRRSV/PCV2

Figure 3.3. Cytokine levels of TGF-β (A) and IL-10 (B) from cell culture supernatants following 3-day co-culture of lymphocytes with virus-infected DCs were quantified by ELISA. The data is from five independent experiments (n=5 pigs). TGF-β levels are expressed relative to the uninfected control group for each experiment. Data not connected by the same letter are significantly different, $p<0.05$.

Chapter 4: Infection with porcine circovirus type 2 (PCV2) or co-infection with PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) suppresses regulatory T cell induction during the subacute and chronic stages of infection

Abstract

The objectives of this study were to investigate regulatory T cell (T_{reg}) induction *in vivo* and *in vitro* in pigs infected with PCV2, PRRSV, or co-infected with both viruses. In the first experiment, we characterized the *in vivo* T_{reg} response to infection with PCV2, PRRSV, or both viruses and correlated this with viral load and lesion severity. Thirty, 4-week-old pigs were randomly divided into four groups: negative controls (n=6), porcine circovirus 2 (PCV2) infected (n=8), porcine reproductive and respiratory syndrome virus (PRRSV) infected (n=8), and pigs infected with both viruses (n=8). All animals were bled weekly through 14 days post-inoculation (dpi) and complete necropsies were performed on 14 dpi. There was no significant ($p<0.05$) difference among groups in $CD4^+CD25^+FoxP3^+$ T_{regs} in peripheral blood mononuclear cell (PBMC) samples at 7 dpi, but T_{regs} were significantly lower than the control group at 14 dpi. There was no significant ($p<0.05$) difference in suppressor activity in PBMC samples among groups. Similarly, no significant ($p<0.05$) difference in $CD4^+CD25^+FoxP3^+$-T_{regs} among groups was detected in bronchoalveolar lavage samples collected at necropsy. Pigs co-infected with PRRSV and PCV2 had significantly higher microscopic lung lesion scores than control pigs, but there was no difference in serum PCV2 viral load between PCV2-infected and PRRSV/PCV2 co-infected pigs. In the second experiment, we evaluated T_{reg} induction *in vivo* and *in vitro* in pigs chronically infected with PCV2 or challenged with PCV2 following vaccination with a live or inactivated PCV2 vaccine. There was no significant difference in T_{regs} in peripheral blood

mononuclear cells in the negative control group, PCV2 vaccinated pigs, or chronically infected pigs. However, following *in vitro* infection of monocyte-derived dendritic cells with PCV2, PRRSV, or both viruses, co-cultured lymphocytes from the chronically infected and live vaccinated pigs had significantly ($p<0.05$) decreased T_{reg} expression in the virus infected groups compared to the negative controls. These results suggest that T_{reg} activation may be suppressed during the subacute or chronic stage of infection with PCV2 or PCV2 and PRRSV. This may potentiate immune activation and enhance PCV2 viral replication, thus enhancing porcine circovirus associated disease (PCVAD).

Introduction

Porcine circovirus type 2 (PCV2) is a small, single-stranded, circular DNA virus of the genus *Circovirus* in the family *Circoviridae* (Todd, 2005). Porcine circovirus type 1 (PCV1) was discovered prior to PCV2 in the early 1970s as a contaminant of the porcine kidney cell line PK-15 and, despite its recent notoriety as a contaminant of the pediatric rotavirus vaccine Rotarix®, is non-pathogenic (Baylis et al., 2011; Tischer et al., 1982; Tischer et al., 1974). PCV2, in contrast, is universally accepted as the primary causative agent of a spectrum of clinical syndromes collectively termed porcine circovirus associated disease (PCVAD). Infection with PCV2 alone has been shown to produce severe disease in cesarean-derived, colostrum-deprived (CD/CD) pigs, but development of the full spectrum of clinical PCVAD is uncommon in conventional pigs infected solely with PCV2 (Allan et al., 2003; Allan et al., 2004; Bolin et al., 2001; Harms et al., 2001; Kennedy et al., 2000). Rather, development of severe PCVAD typically requires the presence of additional co-infecting pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, swine influenza virus, *Mycoplasma hyopneumoniae*, and Torque teno sus virus (TTSuV) (Allan et al., 1999; Allan et al., 2000b; Ellis et al., 2008; Kennedy et al., 2000; Krakowka et al., 2000; Opriessnig et al., 2004a; Opriessnig et al., 2004b; Pogranichniy et al., 2002). One mechanism contributing to this phenomenon is modulation of the host immune response to viral infection (Gillespie et al., 2009). There is conflicting evidence in the literature whether immune stimulation alone (rather than co-infection) may provide the necessary co-stimulation for expression of clinical PCVAD (Grasland et al., 2005; Haruna et al., 2006; Krakowka et al., 2007; Krakowka et al., 2001; Ladekjaer-Mikkelsen et al., 2002).

Recently, regulatory T cells (T_{reg}) have been implicated in altering the host immune response to infection, thus contributing to persistent infection of a growing list of viruses including Friend virus, herpes simplex virus, hepatitis C virus, hepatitis B virus, human immunodeficiency virus, feline immunodeficiency virus, simian immunodeficiency virus, cytomegalovirus, influenza virus, and Epstein-Barr virus (Aandahl et al., 2004; Belkaid, 2007; Belkaid and Rouse, 2005; Boettler et al., 2005; Dittmer et al., 2004; Estes et al., 2006; Fogle et al., 2010a; Fogle et al., 2010b; Karlsson et al., 2007; Marshall et al., 2003; Mexas et al., 2008; Pereira et al., 2007; Robertson et al., 2006; Suvas et al., 2003; Yang et al., 2007). T_{regs} are an important component of the normal immune system repertoire and play a role in preventing autoimmune disease, maintaining a controlled inflammatory response to infection and decreasing chronic inflammation (Kaser et al., 2011a). They are broadly divided into natural T_{regs}, originating from the thymus, and inducible (adaptive) T_{regs}, derived outside the thymus from naïve $CD4^+$ T cells (Askenasy et al., 2008). $CD4^+CD25^+FoxP3^+$ regulatory T cells exhibiting suppressor activity have been identified in pigs and have recently been implicated in the pathogenesis of PRRSV infection (Kaser et al., 2008a, b; Kaser et al., 2011b; LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010). Infection with PRRSV alone is associated with a delayed and weak neutralizing antibody and cell-mediated immune response and is known to produce persistent infection in pigs (Darwich et al., 2010; Lunney et al., 2010). Similarly, persistent PCV2 infection has been documented and piglets infected with PCV2 generated a delayed neutralizing antibody and IFN-γ response, which are important mediators of viral clearance (Fort et al., 2009). We have previously demonstrated that dendritic cells inoculated with PCV2 induce T_{regs} *in vitro*, and this effect is enhanced following co-infection with PRRSV and PCV2 (Cecere et al., 2012a). Based on these findings, the

objectives of the present study were twofold: 1.) to characterize the *in vivo* T_{reg} response to infection with PCV2, PRRSV, or both viruses and correlate this with viral load and lesion severity; 2.) to determine if persistent PCV2 infection or PCV2 vaccination are associated with immunosuppression, thus influencing the severity of ensuing clinical disease.

Experiment 1

Materials and Methods

Animal procedure and sample collections

Animal experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Thirty, four-week-old, crossbred pigs were obtained from a commercial herd known to be free of PCV2 and PRRSV by routine serological testing (Blacksburg, VA). All pigs were immunized with a *Mycoplasma hyopneumoniae* commercial vaccine at one week of age, followed by a booster at weaning at three weeks of age. Pigs were infected with the virulent strain of PRRSV VR-2385 (n=8), PCV2a (n=8), PRRSV and PCV2a (n=8), or saline (n=6). Blood samples were collected into uncoated and heparinized blood tubes (BD Biosciences) and PAXgene™ Blood RNA tubes (PreAnalytiX, Qiagen) on 0 (prior to inoculation), 7, and 14 dpi. Coagulated blood samples were centrifuged at 3000 x g, 23 °C for 10 minutes and the serum was aliquoted and stored at -80 °C. Serum samples were tested for the presence of PCV2 DNA and PAXgene™ blood samples were tested for the presence of PRRSV RNA. Peripheral blood mononuclear cells (PBMCs) were collected from heparinized blood by density gradient centrifugation. All pigs were humanely euthanized at 14 dpi and complete

necropsies were performed. Bronchoalveolar lavage cells (BALC) were collected at necropsy as previously described (Carter and Curiel, 2005).

Animal Housing

The pigs were randomly assigned to one of four groups and were housed in four separate rooms, approximately 80 ft^2 each. Each room was remotely monitored for temperature and humidity and was equipped with nipple drinkers. Pigs were fed free choice with a commercial pelleted feed.

Viruses

PRRSV isolate ATCC VR2385 (Meng et al., 1994) and PCV2a isolate ISU-40895 (Fenaux et al., 2000) were used in this study. The PRRSV isolate VR2385 is a highly pneumovirulent strain isolated from a pig in the United States in 1994 (Meng et al., 1994) and has been studied extensively. PRRSV VR2385 was propagated in confluent monolayers of MARC-145 cells. The viral titer was determined by an immunofluorescent assay (IFA) with an anti-PRRSV N antibody (SDOW17) and quantified in fluorescent focus-forming units (FFU) as described previously (Fang et al., 2006). The virus inocula was adjusted to a final concentration of 10^5 50% tissue culture infective dose ($TCID_{50}$)/2 ml, and pigs were given 2 ml of inocula intranasally by slowly dripping into each nostril.

PCV2a ISU-40895 was recovered from a pig with PCVAD in an Iowa farm in 1998 (Fenaux et al., 2000) and has been used extensively in PCV2 pathogenicity studies (Beach et al., 2010; Fenaux et al., 2002; Fenaux et al., 2004; Fenaux et al., 2003; Opriessnig et al., 2006a; Opriessnig et al., 2004a; Opriessnig et al., 2006b; Opriessnig et al., 2004b; Opriessnig et al.,

2003). Infectious virus stocks were generated for PCV2a by transfection of PK-15 cells in T25 flasks with Lipofectamine LTX (Invitrogen) using a dimerized infectious DNA clone as described previously (Fenaux et al., 2002; Fenaux et al., 2003). The titration of these infectious virus stocks by IFA was performed in 48-well plates (BD-Falcon) as described previously (Fenaux et al., 2002). The $TCID_{50}$ per ml was calculated according to the method of Reed & Muench (Reed and Muench, 1938) and a final infectious titer of $10^{4.5}$ $TCID_{50}$ per ml was used. Pigs were given 1 ml of virus inoculum by intramuscular injection in the right neck area.

Quantification of viral load by PCR

Serum viral load of PCV2 was quantified using a quantitative real-time PCR (qPCR) reaction as previously described (Beach et al., 2010). Total serum DNA was extracted using a QIAamp DNA minikit (Qiagen Inc.). A previously published qPCR assay that amplifies a conserved region of the PCV2 replicase gene and allows accurate quantification of PCV2a and PCV2b (McIntosh et al., 2009) was modified for use with Sensimix SYBR and fluorescein kit (Quantace). Each 25 µl reaction contained 200 nM of each primer (PCV2-83F: 5'-AAAAGCAAATGGGCTGCTAA-3'; PCV2-83R: 5'-TGGTAACCATCCCACCACTT-3'), 200 µM dNTP, 5mM $MgCl_2$, and 5 µl DNA extract. Triplicate reactions for each sample were run in an iQ5 qPCR thermocycler (BioRad) using the following program (95 °C 10min; 35 cycles of 95 °C 15 s, 60 °C 15s, 72 °C 15 s). Melt curve analysis was performed immediately following each run. Viral DNA genomes were quantified against duplicate standards of a PCV2b infectious DNA clone (Beach et al., 2010).

PRRSV RNA load in peripheral blood was quantified using a quantitative real-time PCR reaction as previously described (Ni, 2012). Briefly, peripheral blood RNA was collected from

PAXgene™ Blood RNA samples processed according to the manufacturer's instructions. RNA samples were treated with Ambion® DNA-*free*™ DNase Treatment & Removal Reagents (Invitrogen) according to the manufacturer's instructions and used to synthesize cDNA (Tetro cDNA synthesis kit, Bioline). The qPCR assay was conducted in a CFX96 real-time PCR system (Bio-Rad) using the following program (95 °C 10min; 40 cycles of 95 °C 5s, 58 °C 5s). Melt curve analysis was performed immediately following each run. Each 20µl reaction contained 10µl SsoFast EvaGreen Supermix (Bio-Rad), 0.5µl of each primer (10 µM), 5 µl of the template cDNA, and 4 µl of nuclease-free water. The primer sequences were designed based on the conserved region of the PRRSV nucleocapsid gene and the sequences are as follows: forward primer: 5'-TTAAATATGCCAAATAACAACGG-3'; reverse primer: 5'-TGCCTCTGGACTGGTT-3'.

Necropsy and histopathology

All pigs were humanely euthanized on 14 dpi by intravenous injection of pentobarbital sodium (Fatal Plus®, Vortech Pharmaceuticals, Ltd., Dearborn, MI) and complete necropsy examinations were performed. The extent of macroscopic lung lesions (from 0-100%) was subjectively scored as previously described (Halbur et al., 1995) and lymph nodes were subjectively scored from 0 (normal) to enlarged 3 times normal size (Opriessnig et al., 2004b). Samples of lungs; inguinal, tracheobronchial, hilar, and mesenteric lymph nodes; tonsil; spleen; and thymus were collected and fixed in 10% neutral buffered formalin. Tissues were then routinely processed for histological examination, embedded in paraffin, and stained with hematoxylin and eosin. Lung sections were scored by a board-certified veterinary pathologist (TC) blinded to the treatment groups and assigned a score for severity of pneumonia (0–6) as

previously described (Opriessnig et al., 2004b). Lungs were evaluated for presence and severity of type 2 pneumocyte hypertrophy and hyperplasia, alveolar septal infiltration with inflammatory cells, peribronchial lymphoid hyperplasia, amount of alveolar exudate, and amount of inflammation in the lamina propria of bronchi and bronchioles, and assigned a score ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse). Lymphoid tissues, including tonsil, spleen, and lymph nodes, were evaluated for the presence of lymphoid depletion and histiocytic replacement, as described previously (Opriessnig et al., 2004b).

Flow cytometry

Whole blood was collected into heparinized syringes, diluted 1:2 with sterile PBS, overlaid on Ficoll-Paque^{TM} (GE Healthcare, Piscataway, NJ), and centrifuged at 400 x g at 18 °C for 35 minutes. PBMCs were collected from the buffy coat into ice-cold complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, washed two additional times and resuspended in complete medium. PBMCs were then frozen in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. PBMC samples from 0, 7, and 14 dpi and BALC samples from 14 dpi were simultaneously thawed and evaluated for expression of CD4, CD25, and FoxP3 using flow cytometry. Briefly, cells were sequentially stained with mouse anti-porcine CD4 (VMRD, Pullman, WA), goat anti-mouse IgG2b:Alexa-fluor647 (Invitrogen, Carlsbad, CA), mouse anti-porcine CD25 (AbD Serotec, Raleigh, NC), and goat anti-mouse IgG:FITC (AbD Serotec, Raleigh, NC). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit at 4 °C for 12 hours followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine

FoxP3 for 30 minutes at 4 °C (eBioscience Inc., San Diego, CA). Flow cytometric analysis was conducted using a FACSCalibur cytometer (Becton-Dickinson Biosciences, San Jose, CA) and analyzed using FlowJo 7.6.3 software.

Cell proliferation assay

Cell suppression activity of T_{regs} was tested indirectly by evaluating proliferation of phytohemagglutinin (PHA) treated PBMCs. Cell proliferation was measured using the fluorescent dye carboxifluorescein succinimidyl ester (CFSE; Molecular Probes). Frozen PBMCs, as described above, were thawed and viable cells were counted by diluting the PBMC suspension 1:2 in trypan blue (Invitrogen, Carlsbad, CA). Ten µL of the PBMC/trypan blue suspension was transferred to a hemocytometer slide and viable cells were counted. PBMCs were then diluted to $5x10^6$ viable cells per ml in PBS/0.1% bovine serum albumin and stained with 5 µM CFSE for 10 min at 37° C in the dark. Cells were then quenched by addition of 5 volumes of ice-cold media and incubated on ice for 5 minutes. The cells were centrifuged at 300 × g for 10 min at 7° C and washed once with ice-cold media. Cells were then seeded in a 96-well tissue culture plate at $5x10^5$ cells per well with complete media containing 10 µg/ml PHA and cultured at 37° C, 5% CO_2 for 72 hours. CFSE staining was then assessed using flow cytometry with 488 nm excitation. Samples from each pig were run in duplicate and mean values for each treatment group were calculated. Results are expressed as mean proliferation index ± standard error of the mean. Proliferation index is defined as the average number of divisions that responding cells underwent.

Statistical analysis

Data analysis was performed using JMP 9.0.0 (SAS Institute Inc. Cary, NC) and GraphPad Prism version 5.04 (GraphPad Software Inc. La Jolla, CA). Differences among treatment groups were determined using one-way analysis of variance (ANOVA). If differences in group means were found, then Tukey-Kramer was used for each pair-wise comparison. Real-time PCR results were $\log_{10}$ transformed prior to statistical analysis as previously described (Hemann et al., 2012). Histopathology data were assessed using non-parametric Kruskal-Wallis ANOVA and differences among groups were measured using Dunn's multiple comparison test. Differences were considered to be statistically significant where $p<0.05$.

Results

Clinical observations

One pig in the PRRSV/PCV2 co-infection group developed bilateral subcutaneous abscesses at the base of the ear pinnae containing caseonecrotic exudate at 4 dpi. The pig was treated with 2 ml procaine penicillin G once daily for four days. By 7 dpi the pig had developed septic arthritis and was humanely euthanized. Samples were not collected for bacterial culture. One pig in the PCV2 group developed a draining abscess on the right carpus at 7 dpi, was treated with 2 ml procaine penicillin G once daily for five days and subsequently recovered. This pig was retained in the study. One pig in the PRRSV/PCV2 co-infection group became mildly dyspneic and depressed from 10 dpi to the end of the study.

Serum/blood viral nucleic acid detection

Blood samples from all pigs enrolled in the study were negative for PCV2 DNA and PRRSV RNA on day 0 as detected by PCR, defined by no signal detection in 40 cycles. All

negative control and PRRSV-inoculated pigs remained negative for PCV2 DNA for the duration of the study. PCV2 viremia was detected in all pigs infected with PCV2 or co-infected with PRRSV and PCV2 at days 7 and 14 (Figure 4.1). No significant difference ($p<0.05$) in serum viral load was detected between the two groups at either time point. All negative control and PCV2-inoculated pigs remained negative for PRRSV RNA throughout the duration of the study. On 7 dpi, 7/8 PRRSV-infected and 3/7 co-infected pigs were viremic, but no significant ($p<0.05$) difference in viral load was detected (Figure 4.2). On 14 dpi, 8/8 PRRSV-infected pigs and 5/7 co-infected pigs were viremic. PRRSV viral load was significantly ($p<0.001$) higher in pigs infected with PRRSV alone compared to co-infected pigs on 14 dpi.

$CD4^+CD25^+FoxP3^+$ T_{reg} Expression

In order to examine changes in circulating T_{reg} levels over time, PBMCs collected at 0, 7, and 14 dpi were evaluated for CD4, CD25, and FoxP3 expression via flow cytometry. The overall percentage of $CD4^+CD25^+FoxP3^+$ PBMCs in all groups decreased from 0 to 7 dpi (Figure 4.3A). No significant difference in T_{reg} expression was seen among groups at 0 or 7 dpi. However, at 14 dpi $CD4^+CD25^+FoxP3^+$ T_{regs} were significantly decreased in the PRRSV/PCV2 co-infected group compared to the negative control group ($p=0.03$). Similarly, T_{reg} expression was assessed in BALC samples collected at necropsy on 14 dpi. No significant difference in mean $CD4^+CD25^+FoxP3^+$ expression was found among groups. However, there was a trend ($p=0.077$) towards lower T_{reg} expression in the PRRSV/PCV2 co-infected group compared to the PRRSV infected group (Figure 4.3B).

T_{reg} suppressor activity in PBMCs

PBMCs collected from 0, 7, and 14 dpi were cultured *in vitro* and stimulated with the T cell mitogen PHA to determine if phenotypic T_{reg} expression correlated with functional suppressor activity. No significant difference in proliferation index was observed among groups at any of the time points in the study (Figure 4.4).

Macroscopic and microscopic lung lesions

At necropsy, none of the pigs had diffuse interstitial pneumonia. Several pigs from each group had random, small (less than 10% of affected lobe) patchy areas of atalectasis. One pig from the PRRSV/PCV2 co-infected group had bilateral consolidation of the cranioventral lung lobes that was more severe on the right (affecting approximately 50% of the right cranial lobe). Microscopically this lesion contained regionally extensive neutrophilic bronchointerstitial pneumonia with mild type II pneumocyte hyperplasia, suggestive of a secondary bacterial infection (Figure 4.5D). Three additional pigs in the PRRSV/PCV2 co-infected group had mild multifocal peribronchiolar and interstitial lymphohistiocytic infiltration. Two pigs in the PRRSV group had mild multifocal lymphohistiocytic interstitial pneumonia (Figure 4.5B). None of the pigs in the control or PCV2 group had microscopic evidence of interstitial pneumonia (Table 4.1, Figure 4.5A,C). Only the PRRSV/PCV2 co-infected group had significantly higher microscopic lung lesion scores than the control group (Table 4.1). There was no evidence of lymphoid depletion or histiocytic infiltration in spleen, tonsil, or lymph nodes in any of the pigs from all groups.

Experiment 2

Materials and methods

Experimental design

The experimental design for this study has been previously described (Hemann et al., 2012) and is summarized in Table 4.2. The protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. Briefly, twelve 6-week-old pigs obtained from a PCV2- and PRRSV-negative herd (determined by routine serology) were randomly divided into four groups. At day 0 the pigs were vaccinated with a commercial inactivated PCV1-2 vaccine (group 2, n=3), vaccinated with an experimental live-attenuated PCV1-2 chimeric vaccine (group 3, n=3), or remained unvaccinated (groups 1 and 4, n=6). On day 28 post-vaccination, 9 of 12 pigs (groups 2-4) were challenged with PCV2b. All pigs were bled weekly until necropsy at day 175 post-vaccination.

Vaccination, virus challenge, and sample collection

Pigs in group 2 were vaccinated with 2 ml of Suvaxyn® PCV One Dose™ (now reformulated and known as "Fostera™ PCV" from Pfizer Animal Health Inc.) based on a chimeric PCV1-2a, and pigs in group 3 were vaccinated with 2 ml of an experimental live-attenuated vaccine based on PCV1-2b (Beach et al., 2010). Vaccination was done by intramuscular injection in the right neck. On day 28 post-vaccination, pigs in group 4 (positive control) and vaccinated pigs (groups 2 and 3) were challenged with 2 ml (intranasally) and 1 ml (intramuscularly) of PCV2b virus stock. PCV2b isolate NC-16845 was used and propagated in PK-15 cells to an infectious titer of $10^{4.5}$ 50% tissue culture infective does ($TCID_{50}$) per ml

(Hemann et al., 2012). Weekly blood samples were collected from day 0 until necropsy at day 175 post-vaccination and serum samples were tested for the presence of PCV2 DNA, chimeric PCV1-2 vaccine virus DNA, and anti-PCV2 antibodies as previously described (Hemann et al., 2012). For the present study, heparinized whole blood samples were collected from all pigs on days 98 and 126 post-vaccination (dpv).

Isolation of PBMCs

Heparinized whole blood was diluted 1:2 with sterile PBS, overlaid on Ficoll-Paque™ (GE Healthcare, Piscataway, NJ) and peripheral blood mononuclear cells (PBMC) were collected as previously described (Silva-Campa et al., 2009). PBMCs from 98 dpv (day 70 post-challenge, dpc) were used to generate monocyte-derived dendritic cells for *in vitro* PCV2/PRRSV co-infection challenge and PBMCs from 126 dpv (98 dpc) were analyzed for CD4, CD25, and FoxP3 expression via flow cytometry and for cell suppression activity.

Generation of monocyte-derived dendritic cells (DCs)

Blood samples from 1 pig in each treatment group collected on 98 dpv (70 dpc) were used for the *in vitro* DC assay (due to adequate sample volume). Porcine DCs were generated as previously described (Carrasco et al., 2001; Flores-Mendoza et al., 2008). Briefly, freshly isolated PBMCs were seeded into T75 tissue culture flasks and incubated overnight in complete medium at 37 °C with 5% CO_2 to allow monocytes to adhere. Non-adherent cells were removed and frozen in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO). Adherent cells were cultured at 37 °C with 5% CO_2 in complete medium supplemented with 20ng/ml recombinant porcine IL-4 and 20 ng/ml recombinant porcine GM-CSF. After 5 days, DCs were

harvested using Cellstripper^{TM}, an enzyme-free cell dissociation medium (Cellgro, Manassas, VA). DC differentiation was confirmed by typical veiled morphology.

Infection of DCs by PRRSV and PCV2

DCs were inoculated with PRRSV at a multiplicity of infection (m.o.i.) of 0.1 as previously described (Silva-Campa et al., 2009), and with PCV2a at a m.o.i. of 0.01 (Vincent et al., 2003), or with both viruses and incubated for 1 hour at 37 °C. Cells were then washed twice with complete medium and seeded into 96-well tissue culture plates at $5x10^4$ cells per well. After 24 hours of incubation at 37 °C in 5% CO_2, approximately $5x10^5$ lymphocytes (non-adherent cells from an overnight culture of PBMCs) were added and co-cultured for 3 days. DC-lymphocyte co-cultures were performed in triplicate for each treatment group per pig.

Flow cytometry

Lymphocytes collected following 3-day co-culture with DCs (as described above) and PBMCs from heparinized blood samples collected on 126 dpv (98 dpc) were evaluated for their expression of CD4, CD25, and FoxP3 using flow cytometry. Briefly, cells were sequentially stained with mouse anti-porcine CD4 (VMRD, Pullman, WA), goat anti-mouse IgG2b:Alexa-fluor647 (Invitrogen, Carlsbad, CA), mouse anti-porcine CD25 (AbD Serotec, Raleigh, NC), and goat anti-mouse IgG:FITC (AbD Serotec, Raleigh, NC). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). Flow cytometric analysis was conducted on the lymphocytes using a FACSCalibur cytometer (Becton-

Dickinson Biosciences, San Jose, CA) and analyzed using FlowJo 7.6.3 software. Dendritic cells were excluded based on forward and side scatter.

Cytokine analysis

Plasma from blood samples collected on 126 dpv (98 dpc) was harvested following centrifugation at 1200 x g, (23 °C) for 10 minutes and frozen at -80 °C. Plasma was later thawed and levels of IL-10 and TGF-β were quantified using commercial ELISA kits according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Lymphocyte proliferation

Differences in cell suppression activity among treatment groups were tested indirectly by evaluating proliferation of PBMCs from whole blood collected on 126 dpv (98 dpc) as previously described (Mullarky et al., 2009). Briefly, 2×10^5 cells/well were placed into a 96 well round bottom plate (Fisher Scientific Company) and measured in triplicate after stimulation with 5 μg/ml of concanavalin A (ConA) or 10 μg/ml of phytohemagglutinin (PHA). Background proliferation was determined with PBMC cultured in complete medium. After incubating for 90 h at 37 °C in 5% CO_2, 20 μl cell proliferation reagent (CellTiter 96-Aqueous®, Promega,Madison, WI) was added to each well, the plates incubated (37°C and 5% CO_2) for an additional 4 hours, and then read at 490 nm with a microtiter well plate reader (Tecan Microplate Reader, Tecan Group Ltd. Mannedorf, Switzerland).

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA). Differences among treatment groups were determined by Tukey-Kramer or Dunnett's test. Data analysis was performed using JMP 9.0.0 (SAS Institute Inc. Cary, NC). Differences were considered to be statistically significant where $p<0.05$.

Results

PCV2 viremia

All negative control pigs remained negative for PCV2 DNA throughout the study (Hemann et al., 2012). PCV2b-inoculated animals (positive controls) became PCV2 viremic 35 dpv (7 dpc) and remained viremic until 147 dpv (119 dpc). Positive control animals had significantly ($p<0.05$) higher PCV2 serum concentrations than all other groups (Hemann et al., 2012).

Circulating T_{regs}, PBMC suppressor activity, and plasma cytokine levels

In order to determine whether persistent PCV2 infection or PCV2 challenge following vaccination induces changes in peripheral T_{regs}, PBMCs collected on 126 dpv (98 dpc) were evaluated for expression of CD4, CD25, and FoxP3 via flow cytometry. No significant ($p<0.05$) differences among treatment groups and negative control pigs were seen in mean $CD4^{+}CD25^{+}FoxP3^{+}$ T_{reg} expression (Figure 4.6). PBMC proliferation in response to stimulation with PHA was decreased ($p<0.05$) in the two vaccine groups and in the unvaccinated PCV2b infected group compared to unvaccinated/unchallenged (negative) controls (Figure 4.7B). A similar trend was seen in response to stimulation with ConA, but the differences among groups were not statistically significant (Figure 4.7A). Mean plasma levels of IL-10 and TGF-β were

slightly higher in the unvaccinated PCV2b infected group compared to the vaccinated and negative control groups, but the difference was not statistically ($p<0.05$) significant (Figure 4.8).

In vitro PCV2 and PRRSV challenge

Following three days co-culture with virus-infected DCs generated from each treatment group, autologous lymphocytes were evaluated for expression of CD4, CD25, and FoxP3 via flow cytometry. In samples collected from pigs vaccinated with a live-attenuated PCV1-2 vaccine or unvaccinated and challenged with PCV2b, the mean percentage of $CD4^{+}CD25^{+}FoxP3^{+}$ T_{regs} was significantly ($p<0.05$) reduced following co-culture with DCs inoculated with PCV2, PRRSV, or both compared to uninfected control DCs (Figure 4.9C and 4.9D). No significant difference among groups was seen for samples collected from unvaccinated/unchallenged control pigs or pigs vaccinated with a commercial inactivated PCV2 vaccine (Figure 4.9A and 4.9B).

Discussion

PRRSV is widely accepted as a common and important contributor to the pathogenesis of PCVAD. Multiple studies provide evidence that concurrent infection with PRRSV and PCV2 results in higher levels of PCV2 viremia and viral load in tissue, and that this is associated with increased severity of PCVAD-related lesions (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002). In addition to augmenting PCV2 replication and disease severity, concurrent PRRSV infection has been shown to prolong the presence of PCV2 DNA in serum and increase the amount of PCV2 in oral, nasal, and fecal excretions (Sinha et al., 2011). The mechanisms explaining this phenomenon are unknown, but the interaction of PRRSV and PCV2 is thought to

alter the host immune response, thus interfering with PCV2 clearance and favoring viral persistence (Opriessnig and Halbur, 2012). PRRSV is known to induce T_{regs} *in vitro* and *in vivo*, and this immune-modulating ability of the virus is thought to contribute to viral persistence (LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010).

Based on our previous findings that PCV2 induces T_{regs} *in vitro*, and that this effect is enhanced by co-infection with PRRSV and PCV2, we hypothesized that co-infection should induce greater numbers of T_{regs} *in vivo* than infection with PCV2 alone. This would further dampen the host immune response to PCV2, resulting in enhanced PCV2 replication and thus more severe PCVAD. However, the results from this study indicated there was no increase in $CD4^+CD25^+FoxP3^+$ T_{reg} expression in PBMCs at 7 after infection with PCV2, PRRSV, or both viruses. This correlated with no difference in cell suppressor activity among groups at all time points. Surprisingly, at 14 dpi, T_{regs} were decreased in the co-infected group compared to the control group. A similar trend in decreased T_{regs} in the co-infected group compared to controls was observed in BALC samples collected at 14 dpi, although this was not significantly different ($p<0.05$).

Previous experiments have demonstrated that PRRSV-mediated T_{reg} induction occurs between 10-14 dpi (LeRoith et al., 2011; Wongyanin et al., 2010). Therefore, the time frame for this study was chosen in order to capture the T_{reg} response in the lung (BALC) and in circulation (PBMC). Although no evidence of virus-mediated T_{reg} induction was seen in the present study, several factors may have contributed to this negative finding. In cases of PCVAD, lymphoid organs are one of the primary sites for PCV2 replication. If T_{reg} induction does occur in response to co-infection with PRRSV and PCV2, this phenomenon may be a localized immunomodulatory

effect and could be confined to lymphoid organs. This hypothesis is supported by the fact that *in vitro* T_{reg} induction by PRRSV/PCV2 co-infection was dependent on virus-infected dendritic cells (DC). Modulation of DCs by microbial pathogens, including viruses, is known to cause DC-mediated induction of T_{regs} (Belkaid, 2007). Other investigators have shown that *in vitro* induction of PRRSV-specific T_{reg} is dependent on DCs, and a recent study reported that PRRSV nucleocapsid (N) protein alone did not induce T_{regs}, but the addition of N protein-pulsed DCs to the culture system resulted in T_{reg} induction (Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010). Following antigenic stimulation and activation, DCs migrate to regional lymphoid tissue to present antigen, therefore DC-mediated induction of virus-specific T_{regs} may be limited to this microenvironment and T_{reg} induction would not be reflected in PBMC or BALC samples.

No gross or microscopic lesions of PCVAD were observed in this study. This is not surprising, given the short duration of the study. Classic lesions of PCVAD, including lymphoid depletion with histiocytic replacement and accumulation of intra-histiocytic botryoid inclusion bodies are reported to be most severe beginning at 21 dpi, but other investigators have seen gross lesions of pulmonary congestion and consolidation by as early as 14 dpi (Opriessnig and Halbur, 2012). The absence of severe pneumonia resulting from PRRSV infection is somewhat surprising, given that a virulent PRRSV strain (VR-2385) was used. However, lesions of interstitial pneumonia were not present at 14 dpi in previous experiments using this strain of PRRSV (LeRoith et al., 2011). One factor potentially contributing to the lack of severe pneumonia in the present study is the finding that not all pigs inoculated with PRRSV developed detectable viremia, and that, paradoxically, pigs co-infected with PRRSV and PCV2 had lower PRRSV viral loads in peripheral blood than pigs exposed to PRRSV alone at 14 dpi. Other

investigators have reported that concurrent infection with PCV2 and PRRSV does not alter replication of PRRSV compared to pigs singularly infected with PRRSV (Allan et al., 2000a).

Another potential confounding factor is that, in the present study, PCV2 was inoculated intramuscularly in order to produce consistent levels of viremia in all treated pigs. However, experimental reproduction of PCVAD using the PRRSV/PCV2 co-infection model is believed to be best achieved using a combination of intranasal and intramuscular inoculation of PCV2 (Tanja Opriessnig, personal communication). This most closely approximates the natural oro-nasal transmission of PCV2 and culminates in higher levels of PRRSV and PCV2 in the respiratory tract. Therefore, although all pigs in the PCV2 and PRRSV/PCV2 co-infected group developed PCV2 viremia, high levels of PRRSV and PCV2 may not have been present simultaneously in the respiratory tract. This could explain, in part, the relatively mild gross and microscopic lung lesions and lack of T_{reg} induction in the present study.

In the second experiment, no increase in circulating T_{regs} was detected in PBMCs from persistently PCV2-infected pigs despite measurable viremia at the time of blood collection. Similarly, T_{regs} were not increased in pigs challenged with PCV2 following vaccination with a commercial inactivated or live-attenuated PCV2 vaccine at 126 dpv (98 dpc). This is not surprising, given the chronicity of PCV2 infection and duration of time after vaccination. Experimental evidence from *in vivo* PRRSV infection studies indicates that T_{reg} induction is a transient event that occurs early in the course of infection, beginning approximately 10 dpi (LeRoith et al., 2011; Wongyanin et al., 2010).

Cell proliferation of PBMCs from persistently PCV2-infected pigs and pigs vaccinated with both the commercial inactivated and live-attenuated PCV2 vaccine was significantly lower than unvaccinated/unchallenged negative controls following stimulation with PHA. A similar

trend was observed upon stimulation with ConA but the difference was not statistically significant, which may be attributable in part to the fact that PHA is a more potent T cell mitogen in pigs than ConA (Vogel et al., 1981). This effect is likely unrelated to T_{reg} function, since no difference in circulating T_{regs} among groups was detected. Rather, the suppressed T cell proliferation may be due in part to vaccine and/or virus-dependent modulation of T cell subsets, such as induction of T cell anergy (LaSalle and Hafler, 1994). If T_{reg} function is partly responsible for this suppressed T cell proliferation, then the magnitude of the T_{reg} suppressor activity may differ between groups independent of the absolute number of T_{regs}, as others have demonstrated (Paula et al., 2011).

Although no statistically significant difference was detected in plasma IL-10 or TGF-β levels among treatment groups, the mildly increased levels of IL-10 in the unvaccinated, persistently PCV2-infected pigs correlates with previous reports of PCV2-mediated IL-10 upregulation (Darwich and Mateu, 2012). Of interest in this study was the observation that T_{reg} activation *in vitro* was significantly reduced in both vaccine groups and in unvaccinated/ persistently PCV2-infected pigs compared to controls following co-culture of lymphocytes with DCs exposed to PRRSV, PCV2, or both viruses. This parallels the results observed in the first experiment, in which T_{regs} were significantly reduced in the PCV2/PRRSV co-infected group at 14 dpi. Some investigators have suggested that activation of the host immune system is a pivotal event in the pathogenesis of PCVAD and may contribute to enhanced PCV2 replication (Krakowka et al., 2001). If this is true, then suppression of T_{reg} induction in persistently PCV2-infected or PCV2-vaccinated pigs, as observed in the present study, may play a critical role in potentiating this phenomenon. However, the strength of this conclusion should be interpreted

with caution, given the limitations of this study, which included a small sample size and results from a single time-point in a prolonged time-course of infection.

In conclusion, the results from this study indicate that T_{regs} were not induced in PBMC or BALC samples during the acute stage of infection with PCV2, PRRSV or both viruses. Whether this reflects a true absence of PRRSV/PCV2-mediated T_{reg} induction *in vivo*, or whether T_{reg} induction occurs in a site specific fashion (i.e. lymphoid tissue) warrants further studies. However, suppression of T_{reg} activation in the subacute or chronic phases of infection with PCV2 or PCV2 and PRRSV, as seen in these studies, may potentiate systemic inflammation and enhance PCV2 replication in the pathogenesis of PCVAD.

Figures and Tables

Table 4.1

Incidence and severity of microscopic lung lesions

Treatment group	Number of pigs with lung lesions	Lung lesion score
Control	0	0 ± 0
PRRSV	2/8	0.25 ± 0.16
PCV2	0/8	0 ± 0
PRRSV/PCV2	4/7	1.14 ± 0.67*

*Significantly different from the control ($p<0.05$)

Table 4.2

PCV2 challenge study following vaccination with an inactivated or live-attenuated vaccine

Group	No of pigs	Vaccination at day 0	Challenge virus at 28 days post-vaccination*
1	3	Unvaccinated	Unchallenged
2	3	Commercial inactivated PCV2 vaccine	PCV2b
3	3	Experimental live-attenuated PCV2 vaccine	PCV2b
4	3	Unvaccinated	PCV2b

*The challenge virus dose for PCV2b is $3 \times 10^{4.5}$ $TCID_{50}$/pig.

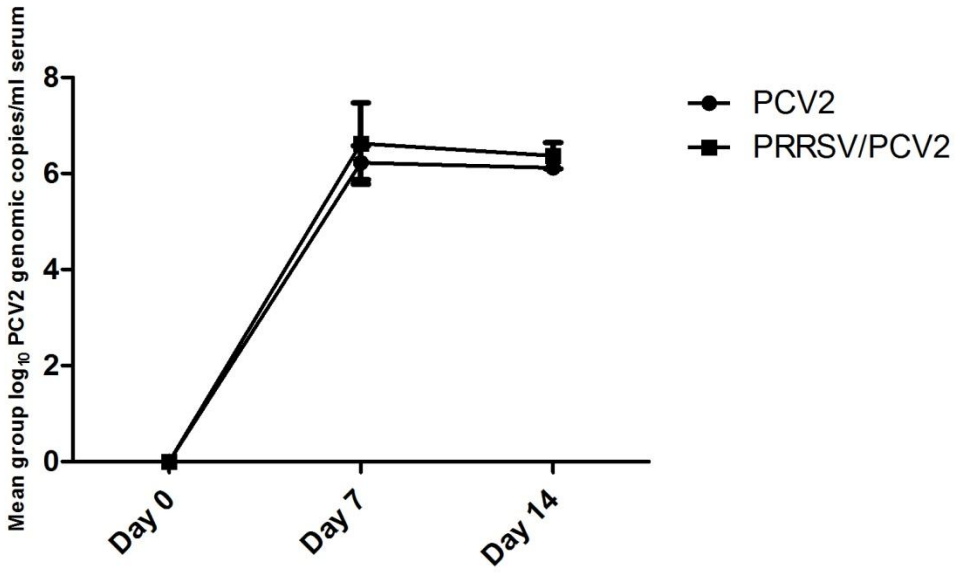


Figure 4.1. PCV2 DNA concentration in serum samples presented as mean group log_{10} PCV2 DNA copy number ± SEM. All pigs in the PCV2 (n=8) and PRRSV/PCV2 (n=7) infected groups were viremic on 7 and 14 dpi. No significant difference in PCV2 DNA copy number was observed between groups ($p<0.05$).

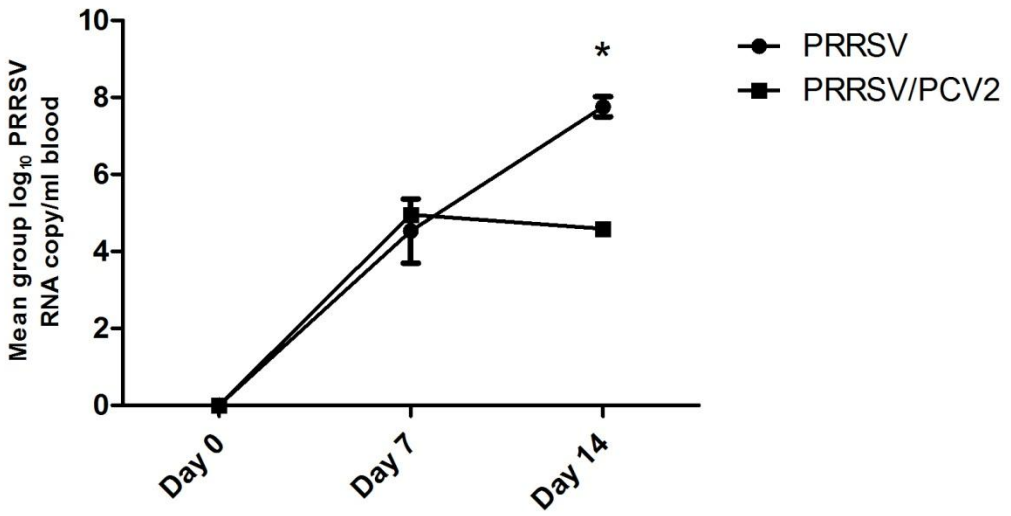


Figure 4.2. The amount of PRRSV RNA in peripheral blood samples presented as mean group $\log_{10}$ PRRSV RNA copy number ± SEM. For the PRRSV group, 7/8 pigs were viremic on 7 dpi and 8/8 pigs were viremic on 14 dpi. For the PRRSV/PCV2 co-infected group 3/7 pigs were viremic on 7 dpi and 5/7 pigs were viremic on 14 dpi. * denotes a significant difference between groups ($p<0.001$).

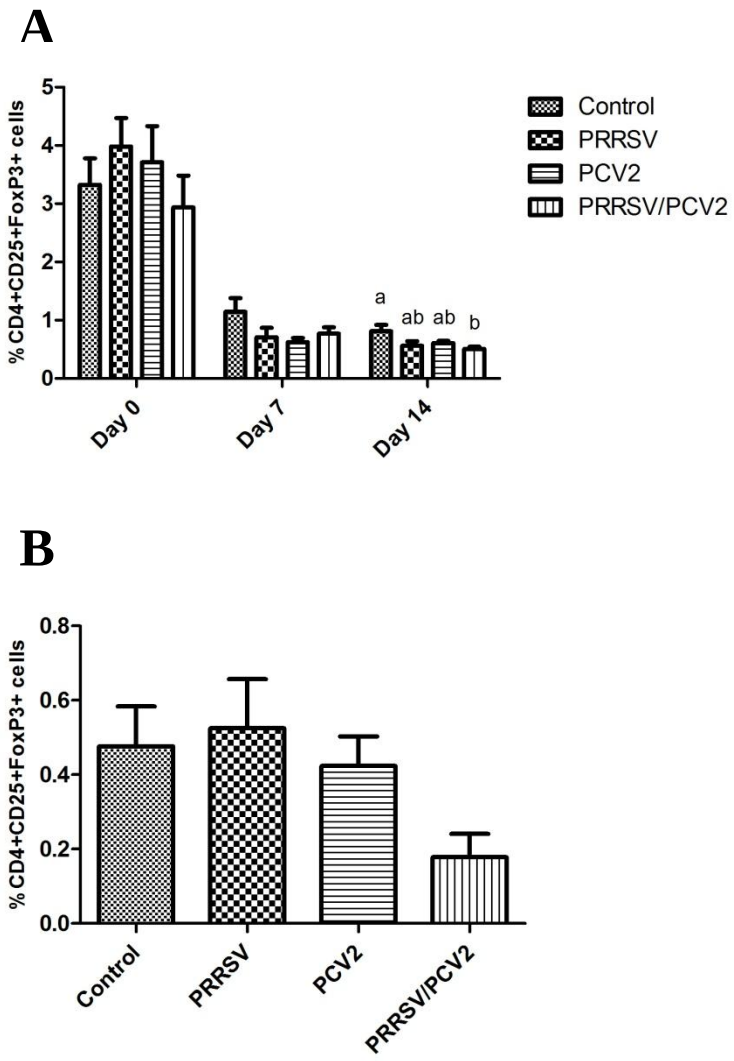


Figure 4.3. Regulatory T cell expression as measured by flow cytometry. Mean $CD4^{+}CD25^{+}FoxP3^{+}$ cells are expressed as a percentage of PBMCs (A) or BALCs (B). Error bars denote standard error of the mean. Data not connected by the same letter are significantly different ($p<0.05$).

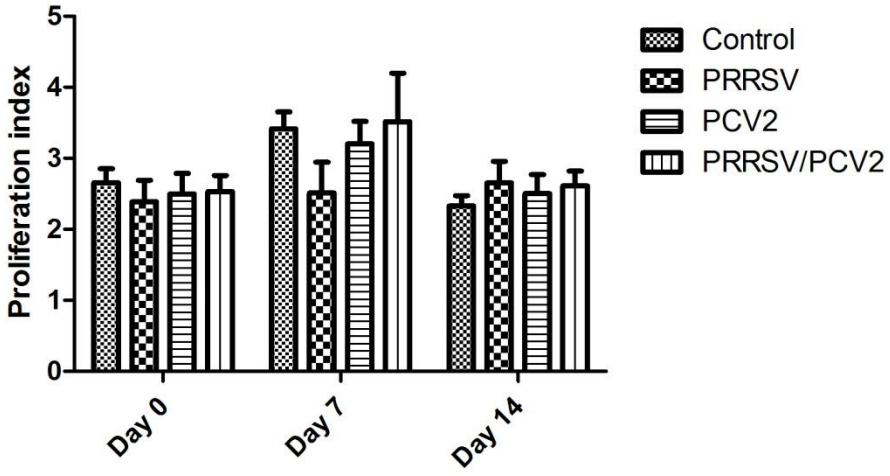


Figure 4.4. Suppressor activity of T_{regs} was assessed indirectly by determining the reduction in proliferation of CFSE-labeled PHA-stimulated PBMCs collected from 0, 7, and 14 dpi. Data are presented as the mean proliferation index (defined as the average number of divisions that responding cells underwent) ± SEM.

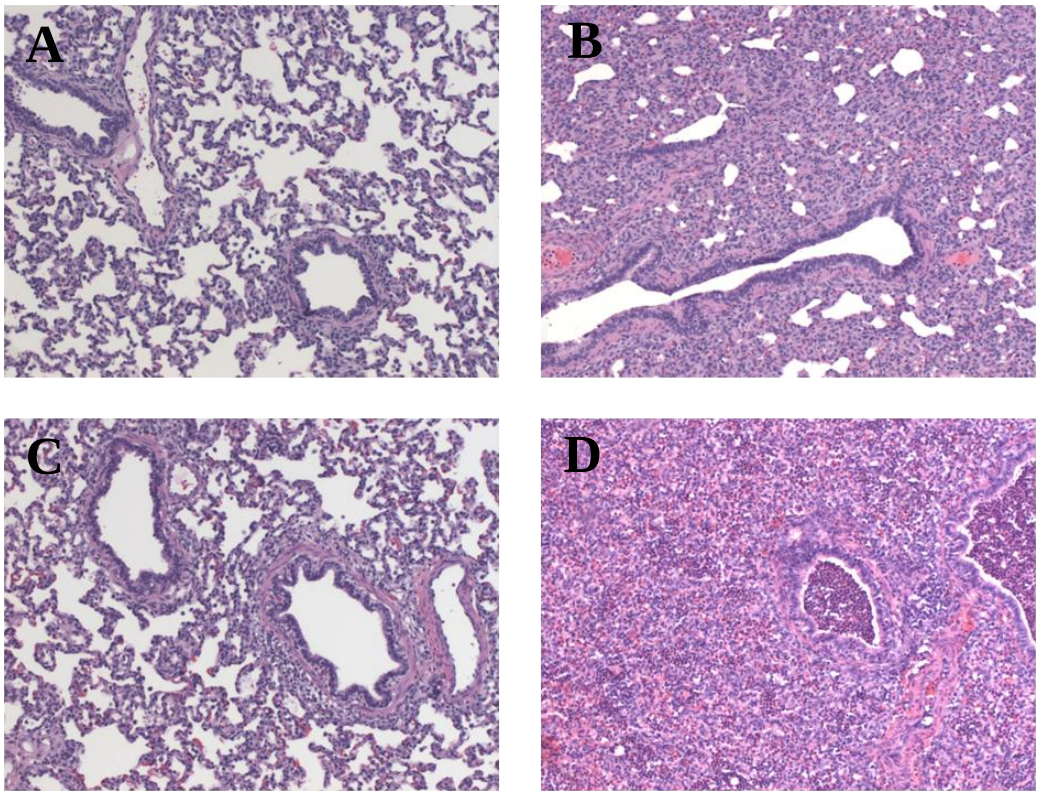


Figure 4.5. Lung collected at necropsy 14 dpi from pigs infected with (A) Saline (negative control). Normal. Hematoxylin and eosin (HE). (B) PRRSV. Alveolar septa are mildly expanded by infiltrates of lymphocytes and histiocytes. HE. (C) PCV2. No evidence of interstitial pneumonia is present. HE. (D) PRRSV/PCV2. Bronchiolar lumens are filled with neutrophils and necrotic cellular debris. Surrounding alveoli are filled with neutrophils and macrophages and alveolar septa are moderately expanded by similar inflammatory infiltrates. HE.

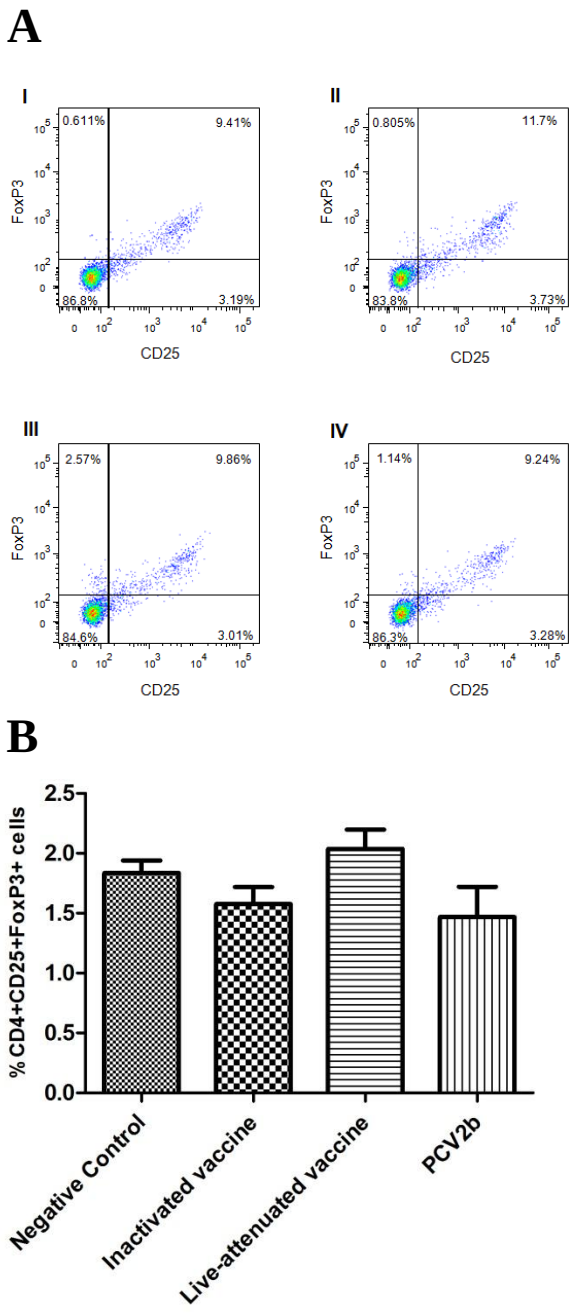


Figure 4.6. Regulatory T cell expression in PBMCs collected on 126 dpv. (A) Representative flow cytometry profiles of CD4-gated lymphocytes expressing $CD25^{+}$ and $FoxP3^{+}$ are shown. I) uninfected, unchallenged group (negative control); II) commercial inactivated PCV2 vaccine group; III) live-attenuated chimeric PCV2 vaccine group; and IV) unvaccinated PCV2b

challenge group (positive control). (B) Mean $CD4^+CD25^+FoxP3^+$ T cells (±SEM) as a percentage of PBMCs (n=3 pigs per group). No statistically significant difference in $CD4^+CD25^+FoxP3^+$ Tr_{egs} was detected among groups ($p<0.05$).

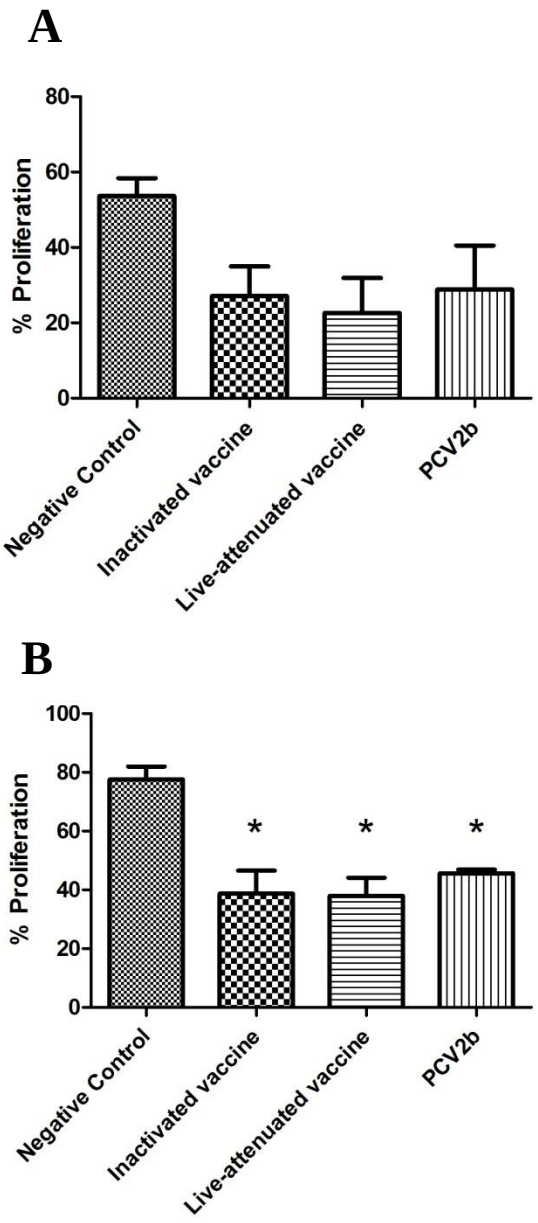


Figure 4.7. PBMC proliferation after stimulation with (A) ConA or (B) PHA for 90 hours (n=3 pigs per group). Values are mean percent proliferation compared to unstimulated controls, and error bars denote standard error of the mean. Significant differences from the negative control group are denoted by * ($p<0.05$). PBMC samples are from 126 dpv (98 dpc).

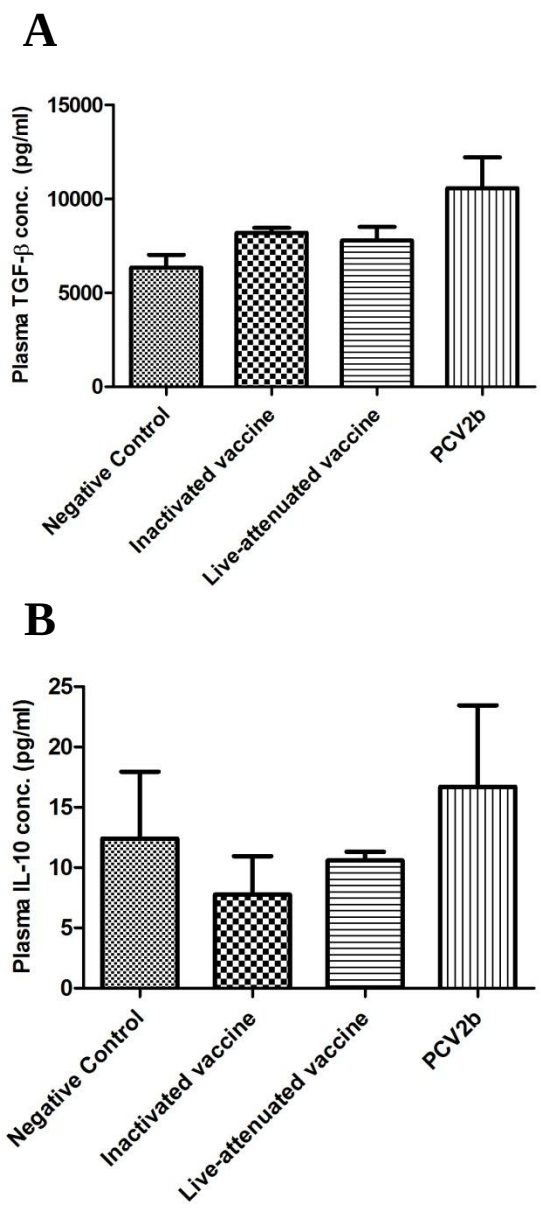
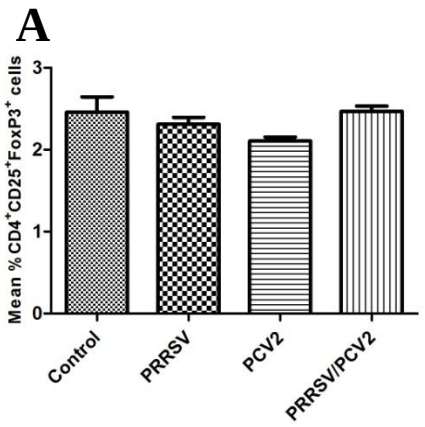
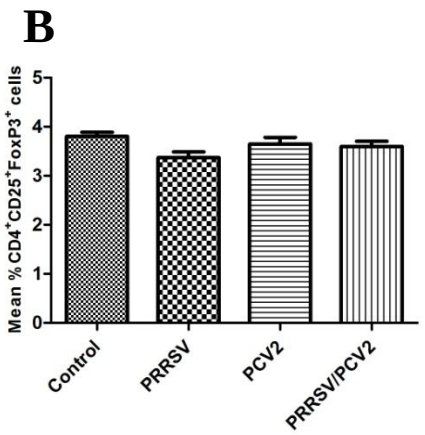


Figure 4.8. Cytokine analysis from blood samples collected on 126 dpv (98 dpc). Data are presented as mean plasma concentration of TGF-β (A) and IL-10 (B) ± SEM (n=3 pigs per groups). No significant ($p<0.05$) differences were detected among treatment groups.


A
Mean % CD4+CD25+FoxP3+ cells
0
1
2
3
Control
PRRSV
PCV2
PRRSV/PCV2


B
Mean % CD4+CD25+FoxP3+ cells
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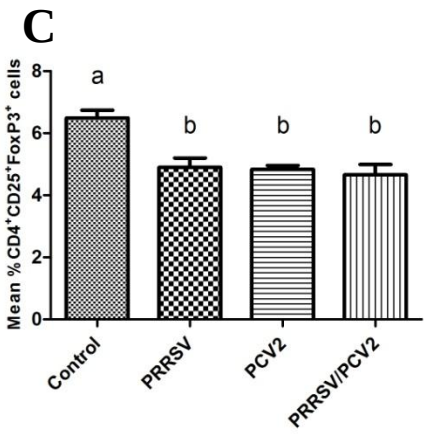
1
2
3
4
5
Control
PRRSV
PCV2
PRRSV/PCV2


C
Mean % CD4+CD25+FoxP3+ cells
0
2
4
6
8
a
b
b
b
Control
PRRSV
PCV2
PRRSV/PCV2

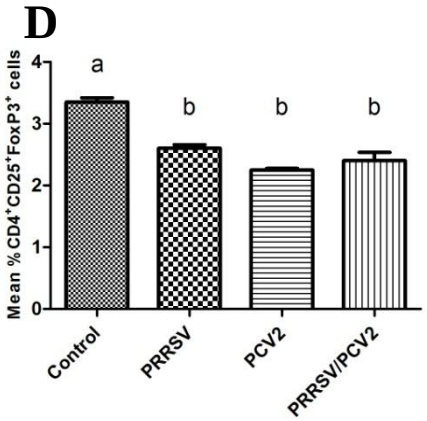

D
Mean % CD4+CD25+FoxP3+ cells
0
1
2
3
4
a
b
b
b
Control
PRRSV
PCV2
PRRSV/PCV2

Figure 4.9. Mean $CD4^+CD25^+FoxP3^+$ T cells as a percentage of lymphocytes following *in vitro* co-culture with uninfected control or virus infected DCs. Samples were collected on 98 dpv (70 dpc) from (A) unvaccinated unchallenged negative control pigs, (B) pigs vaccinated with a commercial inactivated PCV2 vaccine and challenged with PCV2b, (C) pigs vaccinated with a live-attenuated PCV2 vaccine and challenged with PCV2b, and (D) pigs unvaccinated and challenged with PCV2b. Data represent three replicates per group. Error bars denote standard error of the mean. Data not connected by the same letter are significantly different ($p<0.05$).

Chapter 5: Regulatory T cell response to PRRSV infection in pigs vaccinated against PCV2

Abstract

The objective of this study was to characterize the regulatory T cell (T_{reg}) response in pigs vaccinated against porcine circovirus type 2 (PCV2) following infection with porcine reproductive and respiratory syndrome virus (PRRSV). The T_{reg} response in PCV2-vaccinated pigs challenged with an attenuated PRRSV and its virulent parent strain was quantified using flow cytometry and associated with suppressor activity and cytokine profiles. Both the attenuated and virulent PRRSV strains induced significantly ($p<0.05$) higher $CD4^+CD25^+FoxP3^+$ T_{regs} in peripheral blood mononuclear cells (PBMC) and this correlated with decreased PBMC proliferation following mitogenic stimulation. IL-10 levels in serum were significantly ($p<0.05$) higher in pigs challenged with both strains compared to uninfected controls, and pigs inoculated with the attenuated PRRSV strain had increased IL-10 mRNA expression in tracheobronchial lymph node samples compared to controls. The results from this study indicate that an attenuated PRRSV strain and its virulent parent strain induce T_{regs} *in vivo* in pigs vaccinated with a commercial killed PCV2 vaccine.

Introduction

Porcine circovirus type 2 (PCV2) is a small single-stranded, ambisense, circular, nonenveloped DNA virus belonging to the genus *Circovirus* in the family *Circoviridae* (Todd, 2005). Since its initial discovery in 1998, PCV2 has rapidly emerged as one of the most economically significant pathogens currently affecting the global swine population and a growing list of disease conditions has been linked to PCV2 infection (Meng, 2012). The most commonly encountered and best described syndrome associated with PCV2 is PCV2 systemic disease (previously termed post-weaning multisystemic wasting disease or PMWS). Typical clinical and pathological findings include weight loss (wasting), respiratory distress, diarrhea, icterus, and widespread lymph node enlargement characterized by lymphoid depletion and histiocytic replacement (Clark, 1996; Gillespie et al., 2009; Harding, 1996). This disease is a cause of considerable morbidity and mortality, with reports ranging from 4-30% and 4-20% respectively (Segales and Domingo, 2002). While it is now generally accepted that PCV2 is the primary etiological agent in the development of PCVAD, experimental reproduction of PCVAD occurs most effectively and efficiently following co-infection with PCV2 and a range of other swine pathogens (Opriessnig and Halbur, 2012).

Arguably the most common co-infecting pathogen identified in naturally occurring cases of PCVAD is porcine reproductive and respiratory syndrome virus (PRRSV) (Drolet et al., 2003; Grau-Roma and Segales, 2007; Pogranichniy et al., 2002). PRRSV is a single-stranded, positive-sense RNA virus in the family *Arteriviridae* of the order *Nidovirales* (Meulenberg et al., 1993, 1994). Pigs co-infected with PRRSV and PCV2 exhibit enhanced PCV2 replication compared to pigs infected with PCV2 alone, increased severity of lesions and clinical disease, and prolonged shedding of PCV2 DNA in oronasal and fecal excretions (Harms et al., 2001; Sinha et al., 2011).

The mechanisms explaining this phenomenon of PRRSV co-infection potentiating PCV2 replication and clinical PCVAD are not entirely clear, but some investigators have suggested that pathogens such as PRRSV may favor the persistence of PCV2 by suppressing or modulating the host immune response to co-infecting pathogens (Jung et al., 2009; Renukaradhya et al., 2010) (Opriessnig and Halbur, 2012).

One of the proposed mechanisms by which PRRSV modulates the host immune system is via virus-mediated induction of regulatory T cells (T_{regs}). These T cells function in the maintenance of immunological self-tolerance and immune homeostasis and are thus a necessary component in mounting a balanced immune response (Chen et al., 2003). However, they can also be inappropriately induced by viruses in order to swing the balance of the immune response in favor of maintaining viral infection and persistence (Belkaid, 2007). PRRSV alone has been shown to induce T_{regs} *in vitro* and *in vivo* (LeRoith et al., 2011; Silva-Campa et al., 2009; Wongyanin et al., 2012; Wongyanin et al., 2010). We have previously demonstrated that dendritic cells (DC) inoculated with PCV2 alone induce $CD4^{+}CD25^{+}FoxP3^{+}$ T_{regs} *in vitro* and that this effect is enhanced following co-infection with PRRSV and PCV2 (Cecere et al., 2012a). However, following co-infection with PRRSV and PCV2, no T_{reg} induction was detected in peripheral blood mononuclear cells (PBMC) or bronchoalveolar lavage samples during the acute stage of the infection. We have also demonstrated that induction of T_{regs} *in vitro* following infection with PRRSV, PCV2 or both viruses is suppressed in pigs persistently infected with PCV2 or vaccinated with a live-attenuated PCV2 vaccine, but not in pigs vaccinated with an inactivated PCV2 vaccine.

The sequence of infection with PCV2 and PRRSV and the temporal relationship of co-infection likely contribute to the subsequent development and severity of PCVAD (Opriessnig et

al., 2011a; Opriessnig and Halbur, 2012). The objective of this study was to determine if PCV2 vaccination prior to infection with PRRSV alters the ability of PRRSV to induce T_{regs} *in vivo*, thus influencing the severity of ensuing clinical disease.

Materials and Methods

Experimental design and sample collection

The experimental design for this study has been described elsewhere (Ni, 2012) and was approved by the Virginia Tech and Iowa State University Institutional Animal Care and Use Committees. A subset of animals was sampled for the present study. Briefly, thirty, 3-week-old, specific pathogen-free pigs randomly divided into 3 groups of 10 pigs each and were inoculated with virus as shown in Table 5.1 (groups 1, 3, and 5). All pigs were bled at 0 (pre-inoculation), 7, 14, and 21 days post-inoculation (dpi) and coagulated and heparinized blood samples were collected into Vacutainer blood tubes (BD Biosciences, San Diego, CA). An additional pathogenicity study was conducted to compare the difference in virulence between the chimeric (DS722) and parental (VR 2385) virus. Eighteen 3-week-old SPF pigs were divided into 3 groups of 6 each and inoculated with virus as shown in Table 5.1 (groups 2, 4, and 6). All six pigs from each group were necropsied at 14 dpi. At necropsy, lung tissues were collected from each pig for histological examination and quantification of viral RNA loads. Lung, spleen, and tracheobronchial lymph nodes were collected for quantitation of cytokine mRNA expression via real-time PCR and phenotypic characterization via flow cytometry (spleen and lymph node). All pigs were vaccinated at 4 days of age with 1ml of a killed baculovirus vector PCV2 vaccine (Circumvent™ PCV, Intervet).

Viruses

The PRRSV isolate VR2385 is a highly pneumovirulent strain isolated from a pig in the United States in 1994 (Meng et al., 1994) and has been studied extensively. A chimeric PRRSV strain, DS722, was generated by molecular breeding of 7 distinct strains of PRRSV as previously described (Ni, 2012). DNA shuffling of the viral envelope gene GP5 resulted in a clone (DS722) that was inserted into the backbone of a DNA-launched infectious clone of PRRSV VR2385 (Ni et al., 2011). This chimeric virus was infectious but attenuated *in vitro* and *in vivo* (Ni, 2012).

Isolation of PBMCs, splenocytes, and lymphocytes

Heparinized whole blood collected on 0, 7, 14, and 21 dpi was diluted 1:2 with sterile PBS, overlaid on Ficoll-Paque™ (GE Healthcare, Piscataway, NJ) and peripheral blood mononuclear cells (PBMC) were collected as previously described (Silva-Campa et al., 2009). Spleen and tracheobronchial tissue samples were collected from all pigs at necropsy on 14 dpi and immediately submerged in ice-cold complete medium. After all necropsies were complete, spleen and lymph node samples were processed into single cell suspensions using a wire mesh as previously described (Levy et al., 1998). Cell suspensions were washed three times in complete medium and centrifuged at 300 x g at 4 °C for 10 minutes. Cells were then resuspended in fixation buffer (BD Biosciences, San Diego, CA), incubated on ice for 15 minutes, re-centrifuged, suspended in PBS with 1% BSA, and stored on ice until flow cytometry staining.

Flow cytometry

Flow cytometric analysis was carried out on blood collected at 0, 7, 14, and 21 dpi according to the technique of Gilbertie et al. (Gilbertie, 2010). Briefly, whole blood (3 ml)

collected into heparinized Vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was added to 12 ml ACK Lysis Buffer (8.3 g NH4Cl, 1.0 g KHCO3, 0.0327 g EDTA in 1L deionized water pH 7.2-7.4; Sigma-Aldrich, St. Louis, MO) and gently mixed by manual rotation for 3 minutes. Tubes were centrifuged at 300 x g at 4 °C for 5 minutes. Supernatant was discarded and 1 ml of PBS+1% bovine serum albumin (BSA) was added to the remaining pellet. The pellet was washed three times with 1 ml of PBS with 1% BSA and finally resuspended with 1 ml of PBS+1%BSA. Cells were sequentially stained with mouse anti-porcine CD4 (VMRD, Pullman, WA), goat anti-mouse IgG2b:Alexa-fluor647 (Invitrogen, Carlsbad, CA), mouse anti-porcine CD25 (AbD Serotec, Raleigh, NC), goat anti-mouse IgG:FITC (AbD Serotec, Raleigh, NC), and SPRD conjugated anti-CD8α (Fisher Scientific, clone 76-2-11). For intracellular staining, cells were permeabilized with a FoxP3 permeabilization/fixation buffer kit followed by staining with anti-mouse/rat FoxP3:PE that reacts with porcine FoxP3 (eBioscience Inc., San Diego, CA). Flow cytometric analysis was conducted using a FACSCalibur cytometer (Becton-Dickinson Biosciences, San Jose, CA) and analyzed using FlowJo 7.6.3 software. PBMCs were gated based on forward and side scatter. Splenocytes and lymphocytes collected at necropsy on 14 dpi were similarly stained and analyzed.

Serum cytokine analysis

Serum from blood samples collected on 0, 7, 14 and 21 dpi was harvested following centrifugation at 1200 x g, (23 °C) for 10 minutes and frozen at -80 °C. Serum was later thawed and levels of IL-10 and TGF-β were quantified using commercial ELISA kits according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

Analysis of cytokine mRNA expression

Spleen, lymph node, and lung were used for relative quantification of IL-10, TGF-β, IFN-γ, GAPDH, and β-actin mRNA by reverse transcription and real-time PCR. Briefly, spleen, lung, and tracheobronchial lymph node samples collected at necropsy on 14 dpi were immediately submerged in 10 volumes of RNAlater (Qiagen), stored at 7 °C for 48 hours, and then transferred to a -80 °C freezer. Tissue samples were later thawed and 100 mg of each tissue from each pig was homogenized in TriReagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA samples were treated with Ambion® DNA-*free*™ DNase Treatment & Removal Reagents (Invitrogen) according to the manufacturer's instructions and used to synthesize cDNA (Tetro cDNA synthesis kit, Bioline). From each reverse transcription reaction, 2.5 µl of the product were used to assess relative quantities of each specific mRNA of interest in separate reactions in triplicate wells. The SYBR Green PCR master mix Sensimix (Bioline) was used for all reactions according to the manufacturer's guidelines. All reactions were carried out using an iQ5Cycler PCR machine (BioRad). Quantification was carried out using the delta delta CT method (Winer et al., 1999) using GAPDH mRNA as the housekeeping gene for TGF-β and β-actin as the housekeeping gene for IL-10 and IFN-γ. The following programs were used for the GAPDH/ TGF-β samples (95 °C 10min; 40 cycles of 95 °C 15 s, 62.5 °C 30s, 72 °C 15 s) and β-actin/IL-10/ IFN-γ samples (95 °C 3min; 40 cycles of 95 °C 15 s, 65.5 °C 15s, 72 °C 15 s). Melt curve analysis was performed immediately following each run. The same calibrator sample, composed of pooled mRNA samples from all pigs in all treatment groups, was used to compare relative values within and between reactions (96-well plates). Sequences of primers for the detection of cytokines as well as for GAPDH and β-actin and accession numbers of target gene sequences are listed in Table 5.2.

Lymphocyte proliferation

Differences in cell suppression activity among treatment groups were tested indirectly by evaluating proliferation of PBMCs from whole blood collected on 7, 14, and 21 dpi as previously described (Mullarky et al., 2009). Briefly, 2×10^5 cells/well were placed into a 96-well round-bottom plate (Fisher Scientific Company) and measured in triplicate after stimulation with 10 μg/ml of PHA. Background proliferation was determined with PBMC cultured in complete medium. After incubating for 90 h at 37 °C in 5% CO_2, 20 μl cell proliferation reagent (CellTiter 96-Aqueous®, Promega,Madison, WI) was added to each well, the plates were incubated (37 °C in 5% CO_2) for an additional 4 hours, and then read at 490 nm with a microtiter well plate reader (Tecan Microplate Reader, Tecan Group Ltd. Mannedorf, Switzerland).

Statistical Analysis

Data were analyzed using a two-tail Student's t test to evaluate differences between treatment groups. Data analysis was performed using JMP 9.0.0 (SAS Institute Inc. Cary, NC). Differences were considered to be statistically significant where $p<0.05$.

Results

Peripheral and tissue T_{reg} expression

$CD4^+CD25^+FoxP3^+$ T_{regs} were significantly ($p<0.05$) increased in PBMCs from pigs infected with the DC722 PRRSV chimera compared to negative controls at 7 dpi (Figure 5.1A). $CD4^+CD25^+FoxP3^+$ T_{regs} were significantly ($p<0.05$) increased in PBMCs from pigs infected with the parental VR2385 PRRSV strain compared to negative controls at 21 dpi (Figure 5.1A).

No significant differences among treatment groups were observed at any of the time points for $CD8^+CD25^+FoxP3^+$ PBMCs (Figure 5.1B). The majority of $CD8^+$ cells expressing CD25 were also $CD4^+$ (Figure 5.2). Single cell suspensions from spleen and tracheobronchial tissue samples collected at necropsy 14 dpi did not retain cell surface staining via flow cytometry for CD4, CD8, or CD25. However, following cell permeabilization, FoxP3 staining was still present. The mean percentage of $FoxP3^+$ cells was higher in the DC722 PRRSV chimera group compared to VR2385-infected pigs or negative controls in both spleen and lymph node samples, but the difference was not statistically ($p<0.05$) significant (Figure 5.3).

Cell proliferation

PBMC proliferation in response to stimulation with PHA was significantly ($p<0.05$) reduced in pigs infected with the DC722 PRRSV chimera compared to negative controls at 7 dpi (Figure 5.4). Similarly, PBMC proliferation was significantly ($p<0.05$) reduced in pigs infected with the parental VR2385 PRRSV strain compared to negative controls at 7 and 21 dpi. No significant difference among treatment groups was observed at 14 dpi.

Cytokine analysis

The serum IL-10 concentration in DC722 chimera-infected pigs was significantly ($p<0.05$) higher than in negative control pigs at 7 dpi. Furthermore, the IL-10 levels in the VR2385 PRRSV-infected pigs were significantly higher than in the controls on 7 and 14 dpi (Figure 5.5A). IL-10 levels were below the limit of detection of the ELISA assay for all pigs at day 0. Similarly, all negative control pigs had undetectable levels of IL-10 at 21 dpi, but this was not significantly different from the virus-infected groups. The serum concentration of TGF-β

from DC722 chimera-infected pigs was significantly ($p<0.05$) lower than negative control pigs at 7 dpi, and no differences among treatment groups were detected on 0, 14 and 21 dpi (Figure 5.5B).

The relative tissue expression of IL-10, TGF-β, and IFN-γ mRNA in spleen, lung, and tracheobronchial lymph node on 14 dpi was quantified using real-time PCR. The tracheobronchial lymph node expression of IL-10 was slightly increased in DC722 chimera-infected pigs compared to controls and this was statistically ($p<0.05$) significant (Figure 5.6A). The mean IL-10 expression in the lungs of both virus-infected groups was lower than controls, but the difference was not statistically significant. Furthermore, no difference in splenic IL-10 expression was observed. No significant difference in TGF-β expression among groups was detected in any of the examined tissues (Figure 5.6B). Lung and lymph node IFN-γ mRNA expression were higher in both virus-infected groups compared to negative controls, but this difference was not statistically significant due to intragroup variation (Figure 5.6C).

Discussion

The objective of this study was to evaluate the T_{reg} response following infection with PRRSV in pigs vaccinated against PCV2. T_{regs} were quantified using flow cytometry following infection with an attenuated chimeric PRRSV strain (DC722) and its pathogenic parental strain (VR2385). This was correlated with the suppressor activity of PBMCs and the serum and tissue cytokine response to infection. In other experiments (Ni, 2012), the chimeric DS722 PRRSV strain was shown to replicate *in vivo* and produce detectable levels of viremia, although the serum viral load was significantly lower than VR2385 at 7 dpi. It was further demonstrated that this chimeric virus was attenuated in pigs, producing significantly lower gross and microscopic

lesion scores than its pathogenic parental strain, and conferred protective immunity against homologous and heterologous PRRSV challenge. Despite this attenuated phenotype, the chimeric DS722 strain induced significantly higher $CD4^+CD25^+FoxP3^+$ T_{regs} in PBMCs on 7 dpi compared to uninfected controls, and this correlated with a significant reduction in mitogen-induced PBMC proliferation indicating functional T_{reg} suppressor activity. PBMC proliferation was similarly suppressed in VR2385-infected pigs on 7 and 21 dpi, but the increase in $CD4^+CD25^+FoxP3^+$ T_{regs} was only significant on 21 dpi. A mild (though not statistically significant) increase in $FoxP3^+$ cells from both spleen and tracheobronchial lymph node cells in DS722-infected pigs was detected, further suggesting systemic activation of T_{regs}. These findings correlate with a previous report that, although attenuated, a PRRSV vaccine strain did not differ from the parent strain in its ability to activate T_{regs} (LeRoith et al., 2011). The PRRSV epitopes responsible for T_{reg} induction are currently unknown, but these results suggest that mutations in the vaccine strains that result in attenuation of the virus do not alter its ability to activate T_{regs}. These results have implications for future PRRSV vaccine development, in which it may be advantageous to modify T_{reg}-stimulating epitopes such that the attenuated vaccine strain still induces a robust cell-mediated immune response while avoiding the immunosuppressive consequences of T_{reg} activation.

No significant difference in $CD8^+CD25^+FoxP3^+$ PBMCs was detected among groups at any of the sampled time points. $CD8^+$ T_{regs} have been identified in pigs and in this study, as in previous reports, the majority of $CD8^+$ T cells expressing CD25 were also $CD4^+$ (Kaser et al., 2008b).

In the present study, serum IL-10 was increased in pigs infected with the DS722 chimera and its parental VR2385 strain, and IL-10 mRNA expression was increased in tracheobronchial

lymph nodes from the DS722 chimera group. These findings correlate with previous reports of PRRSV-mediated IL-10 induction. IL-10 is a pleiotropic cytokine with immunosuppressive properties that are thought to contribute to modulation of the host immune system in the pathogenesis of PRRSV infection (Charerntantanakul et al., 2006; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003). However, the host IL-10 response to PRRSV has been shown to vary considerably depending on the viral strain (Darwich et al., 2011; Gimeno et al., 2011; Subramaniam et al., 2011). There are conflicting data in the literature regarding the relationship between PRRSV-mediated IL-10 production and T_{reg} induction. In one study PRRSV mediated induction of T_{regs} was shown to be dependent on TGF-β, but not IL-10 (Silva-Campa et al., 2009), but in a more recent study the development of PRRSV-induced T_{regs} was associated with IL-10 production (Wongyanin et al., 2012). In the present study no difference was detected among groups in TGF-β tissue mRNA levels following PRRSV infection and the only significant difference in serum TGF-β levels was a decrease in the DS722 chimera group compared to controls at 7 dpi. Given that T_{regs} were increased in the chimera group at this time point, this finding may indicate that T_{reg} induction was dependent on IL-10 instead of TGF-β.

PRRSV infection is associated with a delayed and weak cell-mediated immune response, and development of virus-specific IFN-γ-secreting cells is typically delayed until 2-3 weeks post-inoculation. Even then, the levels of IFN-γ secretion are considerably lower than by other swine viral pathogens such as pseudorabies virus (Darwich et al., 2010). In the present study, although no statistically significant ($p<0.05$) differences were detected in IFN-γ mRNA expression in spleen, tracheobronchial lymph node, or lung at 14 dpi, lung IFN-γ expression in the DS722 chimera was higher than the negative controls and VR2385-infected group. This may correlate with the diminished severity of pulmonary lesions and attenuated phenotype of the chimera

strain, as the ability to induce a rapid IFN-γ response is an important component of viral clearance and heterologous protection by vaccination (Díaz I, 2006; Martelli P, 2009).

Based on the worldwide distribution and high prevalence of PCV2 (Ramamoorthy and Meng, 2009), we speculated that the reported inconsistency in the ability of PRRSV to induce T_{regs} may be due in part to the confounding effect of PCV2 circulating in pigs at the time of PRRSV challenge. We partially addressed this issue in the present experiment, in which pigs vaccinated with a commercial killed PCV2 vaccine (Circumvent™ PCV, Intervet) still demonstrated T_{reg} induction by an attenuated PRRSV strain and its virulent parent strain. This supports our previous findings, that vaccination with an inactive commercial PCV2 vaccine (Suvaxyn® PCV One Dose™) did not suppress the *in vitro* T_{reg} response to PRRSV. However, due to the experimental design, we were unable to control for this variable and compare differences in PRRSV-mediated T_{reg} induction related to presence or absence of PCV2 vaccination. The effects of persistent PCV2 infection or vaccination with a live-attenuated PCV2 vaccine on PRRSV-mediated T_{reg} induction *in vivo* remain to be determined.

Figures and Tables

Table 5.1

PRRSV infection and pathogenicity studies in pigs infected with the attenuated chimeric DS722 virus or with the parental VR2385 virus.

Group	Inoculation at day 0 (2×10^5 $TCID_{50}$/pig)	No. of pigs	Necropsy at 14 days post-inoculation
1	Parental VR2385	10	0
2	Parental VR2385	6	6
3	Attenuated chimera DS722	10	0
4	Attenuated chimera DS722	6	6
5	DMEM control	10	0
6	DMEM control	6	6

Table 5.2

Primer sequences (5'–3') used for cytokine mRNA detection.

Gene	Accession number	Forward primer	Reverse primer
TGF-β	AF461808	CTACTACGCCAAGGAGGTCAC	GCCCGAGAGAGCAATACAGG
IL-10	NM214123	CCGACTCAACGAAGAAGGCAC AG	CAGGCTGGTTGGGAAGTGGATG
IFN-γ	DQ839398	TTCAGAGCCAAATTGTCTCCTTC	AAGTCATTCAGTTTCCCAGAGC
GAPDH	DQ845173	CATCATCCCTGCTTCTACC	TGCTTCACCACCTTCTTG
β-actin	DQ845171	CTGCGGCATCCACGAAAC	TGTTGGCGTAGAGGTCCTTGC

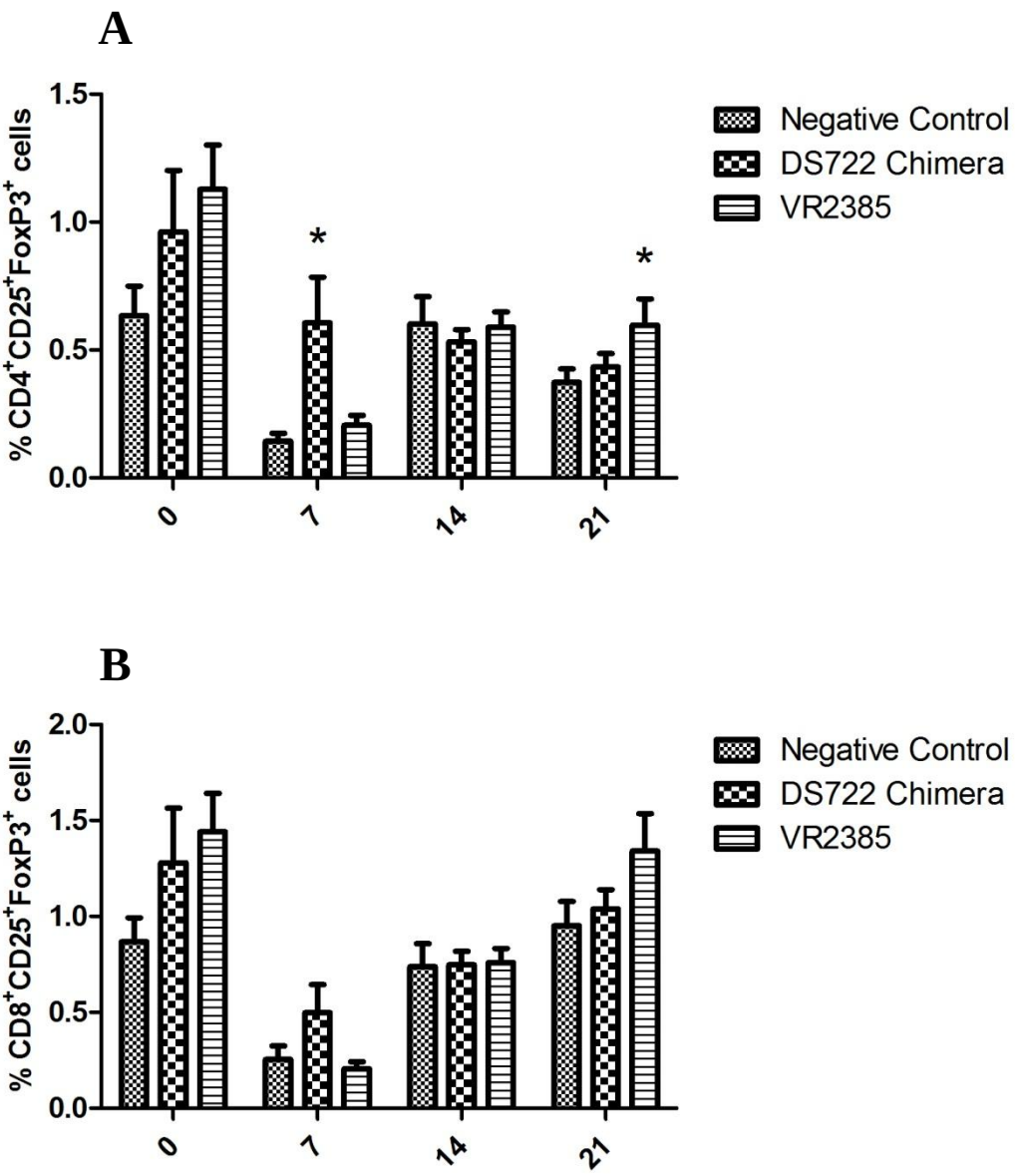


Figure 5.1. Regulatory T cell expression measured via four-color flow cytometry. Samples are PBMCs from pigs at 0, 7, 14, and 21 dpi with an attenuated DS722 PRRSV or the parental VR2385 strain. Mean $CD4^+CD25^+FoxP3^+$ (A) or $CD8^+CD25^+FoxP3^+$ (B) T cells are expressed as a percentage of PBMCs ± SEM (n=10 pigs per group). * denotes significant difference from the negative control group ($p<0.05$).

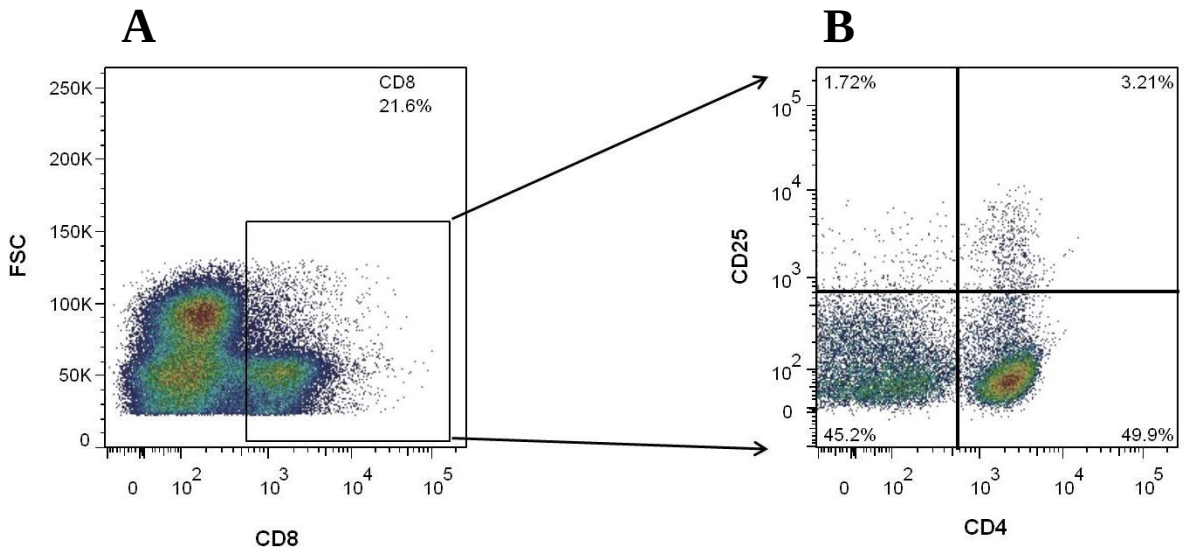


Figure 5.2. Representative CD4 and CD25 expression of $CD8^+$ subpopulations from PBMCs collected on 0 dpi from a pig in the DS722 attenuated PRRSV-infected group. (A) The pseudocolor plot shows expression of CD8 vs. forward scatter. $CD8^+$ gated cells are depicted in (B) expressing CD4 and CD25. The majority of CD25 expressing $CD8^+$ T cells are also $CD4^+$.

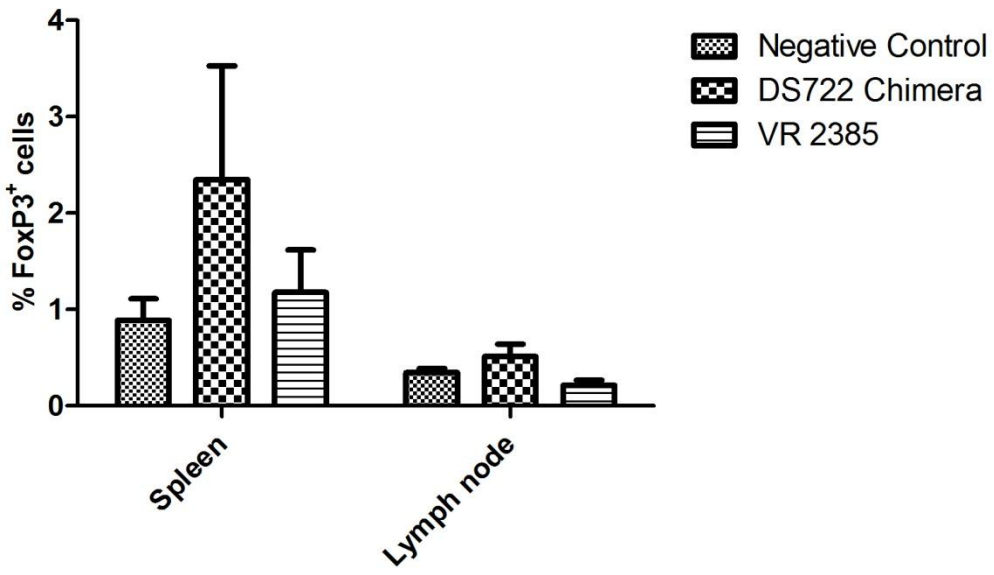


Figure 5.3. Cells expressing FoxP3 from spleen and tracheobronchial lymph node samples collected 14 days p.i. Single cell suspension were generated from tissue samples collected at necropsy and stained for FoxP3 expression via flow cytometry. Data are presented as mean percentage of $FoxP3^+$ cells ± SEM (n=6 pigs per group).

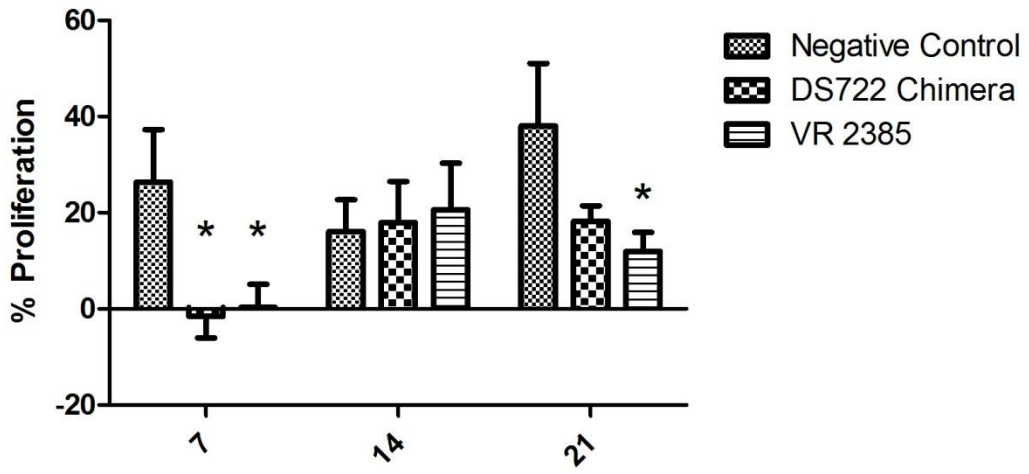


Figure 5.4. PBMC proliferation after stimulation with PHA for pigs at days 7, 14, and 21 post-challenge with a DS722 PRRSV chimera or the parental VR2385 strain. Values are mean percent proliferation compared to unstimulated controls and error bars denote standard error of the mean. Significant differences from the negative control group are denoted by * ($p<0.05$).

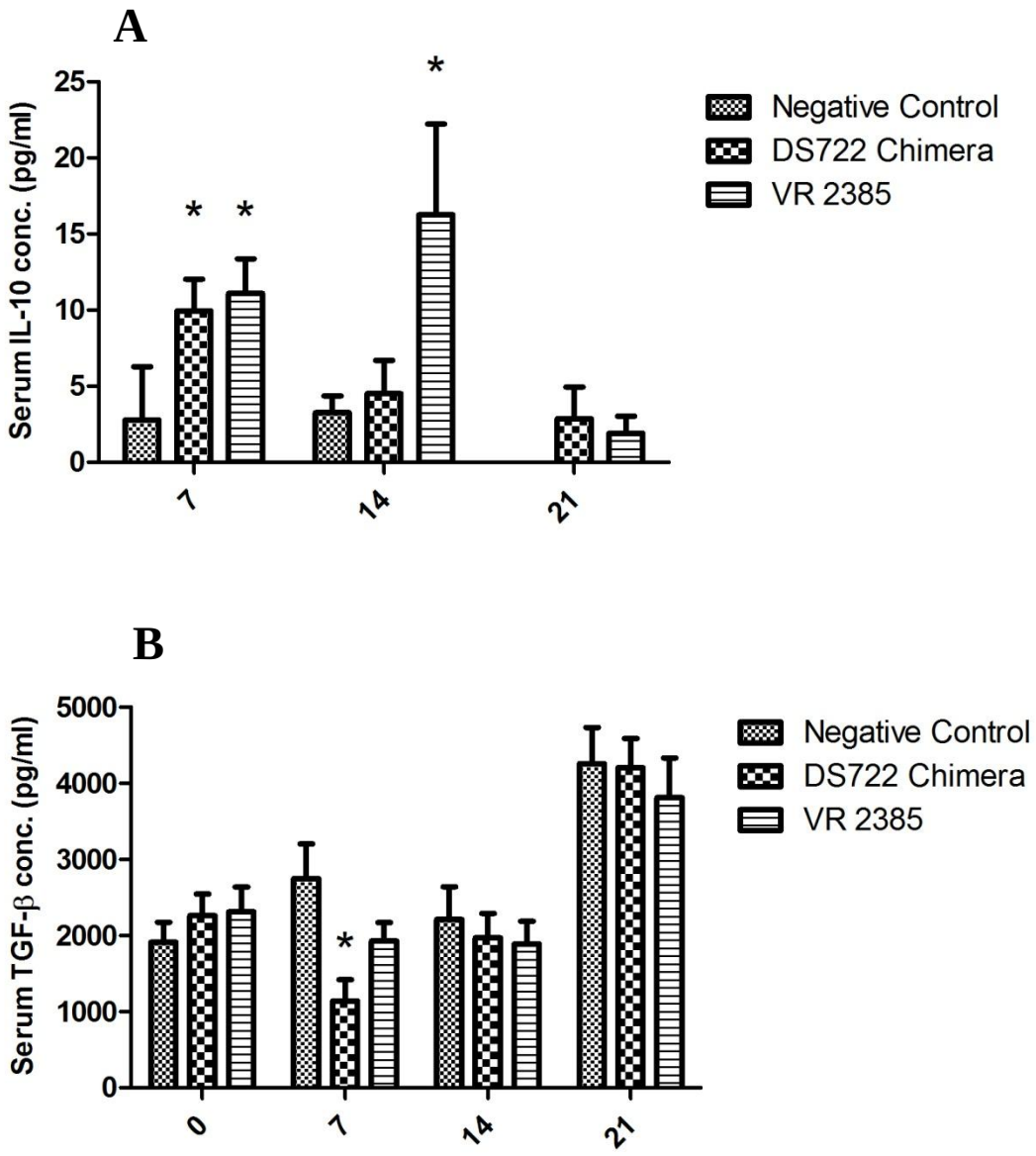


Figure 5.5. Cytokine analysis from pigs at days 0, 7, 14, and 21 post-challenge with a DS722 PRRSV chimera or the parental VR2385 strain. Data are presented as mean serum concentration of IL-10 (A) and TGF-β (B) ± SEM (n=10 pigs per groups). Significant differences from the negative control group are denoted by * ($p<0.05$).

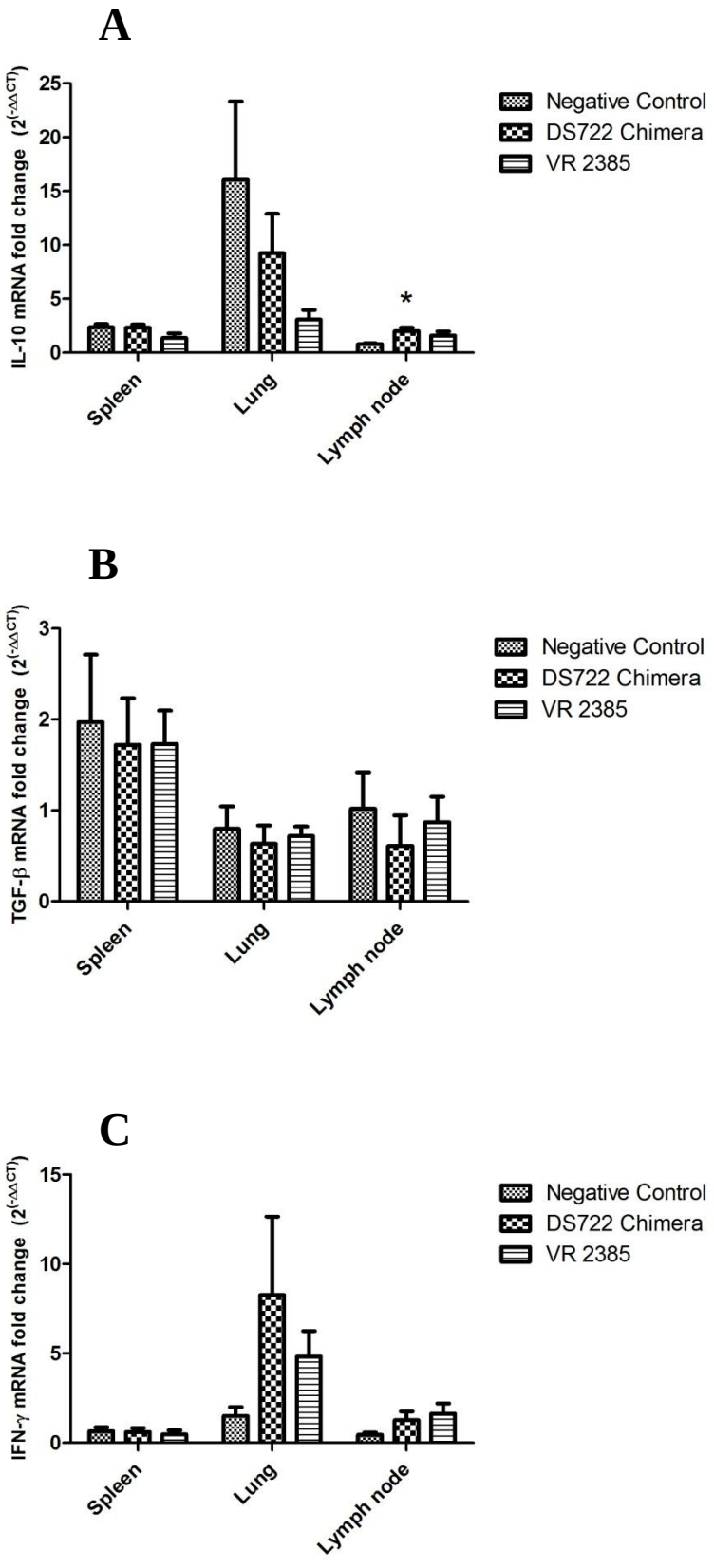

A
IL-10 mRNA fold change (2^(-ΔΔCT))
25
20
15
10
5
0
*
Spleen
Lung
Lymph node
Negative Control
DS722 Chimera
VR 2385
B
TGF-β mRNA fold change (2^(-ΔΔCT))
3
2
1
0
Spleen
Lung
Lymph node
Negative Control
DS722 Chimera
VR 2385
C
IFN-γ mRNA fold change (2^(-ΔΔCT))
15
10
5
0
Spleen
Lung
Lymph node
Negative Control
DS722 Chimera
VR 2385

Figure 5.6. Relative quantification of cytokine mRNA from pigs at day 14 post-challenge with a DS722 PRRSV chimera or the parental VR2385 strain. Relative levels of IL-10 (A), TGF-β (B) and IFN-γ (C) were measured in spleen, lung, and tracheobronchial lymph node samples collected at necropsy (n=6 pigs per group) using quantitative RT-PCR. Each bar represents the mean relative fold increase ± SEM using the delta delta CT method.

Chapter 6: Summary and conclusions

PRRSV-mediated induction of regulatory T cells (T_{regs}) has been identified as one mechanism involved in modulating the swine immune response to infection by the virus. It is thought that the broad suppressor activity of T_{regs} may contribute to a delayed and weak IFN-γ and neutralizing antibody response and potentiate chronic viral infection. Pigs infected with PCV2 display a similar immunosuppressive phenotype, including weak and delayed IFN-γ and neutralizing antibody responses in diseased pigs, as well as persistent infection associated with prolonged viremia and viral shedding. However, available research investigating the role of T_{regs} in mediating the host immune response in porcine circovirus associated disease (PCVAD) is lacking. Because co-infection of pigs with PCV2 and PRRSV is common and is often necessary for the development of severe clinical PCVAD, virus mediated induction of T_{regs} likely contributes to the immunopathogenesis of PCVAD. This dissertation provides the initial experimental observations of the *in vitro* and *in vivo* T_{reg} response to PCV2, PRRSV, or both viruses in vaccinated and unvaccinated pigs.

Because PCV2 and PRRSV are both known to target cells of monocytic lineage, including dendritic cells, the initial hypothesis was that clinical PCVAD resulted from a combination of PCV2-mediated maturation of dendritic cells (DC) and PRRSV-mediated activation of T_{regs}. Based on this hypothesis, the interaction of PCV2 with DCs might increase their susceptibility to PRRSV-mediated T_{reg} induction. The ensuing activation of T_{regs} would then suppress the cell-mediated and humoral immune response to PCV2, contributing to delayed viral clearance and increased replication. However, following *in vitro* experiments aimed at identifying the contribution of each virus to T_{reg} induction, alone or in combination, we

serendipitously discovered that DCs exposed to PCV2 alone were able to induce $CD4^+CD25^+FoxP3^+$ T_{regs}. PRRSV-infected DCs also induced T_{regs} and this effect was enhanced synergistically when the DCs were co-infected with both viruses. The induction of T_{regs} by PCV2/PRRSV co-infection in these experiments appears to be dependent on TGF-β and not IL-10, which is similar to *in vitro* PRRSV-mediated T_{reg} induction reported by other investigators. Because the cytokine levels were measured in cell culture supernatant, the cellular origin of TGF-β is uncertain. Although, given the short time frame of DC and lymphocyte co-culture, it is likely that TGF-β production is due in part to DC secretion, and is not solely attributable to production by activated T_{regs}. Although the response of dendritic cells to challenge with PRRSV or PCV2 is well documented, additional studies are needed to better characterize the phenotypic changes and cytokine response to co-infection with PCV2 and PRRSV. Co-localization studies using different multiplicities of infection are also needed to identify if DCs are simultaneously infected with PCV2 and PRRSV.

Because iT_{regs} are phenotypically indistinguishable from nT_{regs} once they are induced, the $CD4^+CD25^+FoxP3^+$ T_{reg} population examined in these experiments represents the combined contribution of iT_{regs} and nT_{regs}. Given the short time course of lymphocyte-DC co-culture in this experiment (72 hours), it is tempting to speculate that the increase in $CD4^+CD25^+FoxP3^+$ T_{regs} represents an expansion of the existing nT_{reg} population in response to viral challenge. However, investigators have previously demonstrated that human T_{regs}, when cultured *in vitro*, are anergic and thus unable to proliferate in response to activation (Baecher-Allan et al., 2001). Other studies showed that murine peripheral $CD4^+CD25^-$ naïve T cells were converted to $CD4^+CD25^+Foxp3^+$ T_{regs} following 3 day co-culture with antigen presenting cells in the presence of TGF-β and stimulation with anti-CD3 and anti-CD28 antibodies (Chen et al., 2003). Based on the up

regulation in TGF-β seen in the present study, it is possible that the virus-mediated increase in T_{regs} represents induction of iT_{regs} despite the short time course of lymphocyte co-culture with virus-infected DCs.

Based on the findings from this study, the revised hypothesis is that co-infection with PCV2 and PRRSV induces T_{regs}, and the resulting immunomodulation delays viral clearance, thus enhancing PCV2 proliferation and potentiating clinical PCVAD. To test this hypothesis, the *in vivo* T_{reg} response was investigated in pigs acutely infected with PCV2, PRRSV, or co-infected with both viruses. T_{reg} induction was not identified in peripheral blood mononuclear cells or bronchoalveolar lavage samples in pigs singly infected with either virus or co-infected with both viruses. These findings may reflect a true absence of T_{reg} induction due to co-infection with PRRSV and PCV2. However, multiple factors may have contributed to the lack of identifiable T_{reg} response in this study. Since the PRRSV strain used in this study has been shown to induce T_{regs} *in vivo* (LeRoith et al., 2011), the inability to detect T_{reg} induction may be a result of other contributing factors. The absence of severe disease following viral challenge observed in this experiment may correlate with a lack of T_{reg} induction *in vivo*. Alternatively, virus-mediated T_{reg} induction may occur in a transient, time-course specific manner and was not captured in this study or may be limited to lymphoid tissue rather than the pulmonary or systemic circulation.

In additional experiments, the *in vivo* and *in vitro* T_{reg} response to PRRSV or PCV2 was investigated in pigs persistently infected with or vaccinated against PCV2. A notable finding was that, following co-culture of lymphocytes with DCs exposed to PRRSV, PCV2, or both viruses, T_{reg} activation *in vitro* was significantly reduced in samples from pigs persistently infected with PCV2 or challenged with PCV2 following vaccination with a live-attenuated PCV2

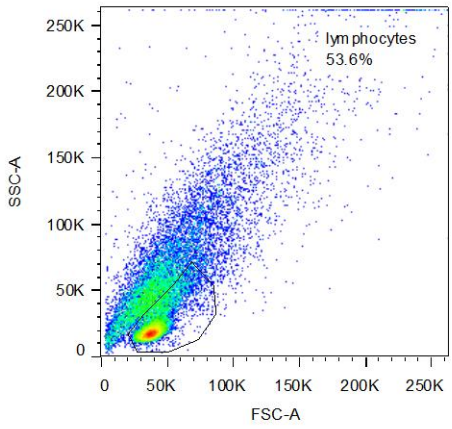
vaccine, as was seen in the co-infection group in the acute *in vivo* study (above). This seemingly paradoxical suppression of T_{reg} induction may potentiate immune activation, which has been suggested by some to be a pivotal event in the pathogenesis of PCVAD. In addition, it was demonstrated that PCV2-vaccinated pigs are susceptible to PRRSV-mediated T_{reg} induction *in vivo*, and this correlated with suppression of mitogen-induced PBMC proliferation and induction of the immunosuppressive cytokine IL-10. An attenuated vaccine candidate PRRSV strain was found to induce T_{regs} similar to its pathogenic parental strain, supporting previous findings that mutations in the vaccine strains that result in attenuation of the virus do not alter its ability to activate T_{regs}. These findings highlight the importance of future PRRSV vaccine development, in which it may be advantageous to modify T_{reg}-stimulating epitopes such that the attenuated vaccine strain still induces a robust cell-mediated immune response while avoiding the immunosuppressive consequences of T_{reg} activation.

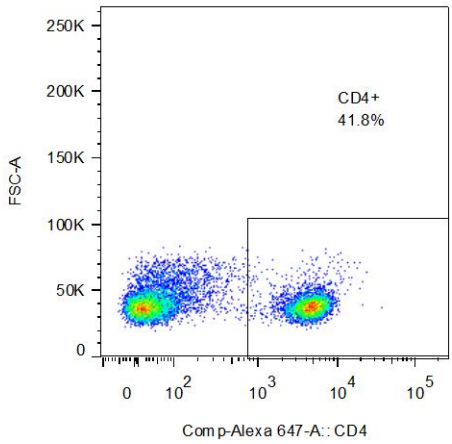
The work described in this dissertation suggests that PCV2 and PRRSV interacting in swine modulate the host immune response, in part, through the action of virus-induced regulatory T cells. Interpreting whether the action of T_{regs} confers a relative advantage to a viral pathogen in the context of infection is difficult, due in part to the fact that T_{reg} activation is a normal and expected consequence of a balanced immune response. T_{regs} are vital in returning the immune system to a quiescent state of homeostasis following an inflammatory response to a pathogenic microorganism. However, virus-mediated induction of T_{regs} prior to the onset of a strong cell-mediated and humoral immune response, as in the case of PRRSV infection, provides convincing evidence that the virus has subverted the host immune system in favor of viral persistence. Although it has been demonstrated that PCV2 alone can induce T_{regs} *in vitro* and that

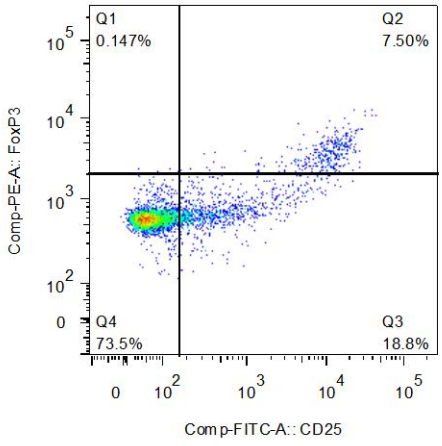
co-infection with PRRSV enhances this effect, it remains to be determined whether T_{regs} are induced *in vivo* in pigs infected with PCV2 and whether this enhances PCVAD.

Appendices

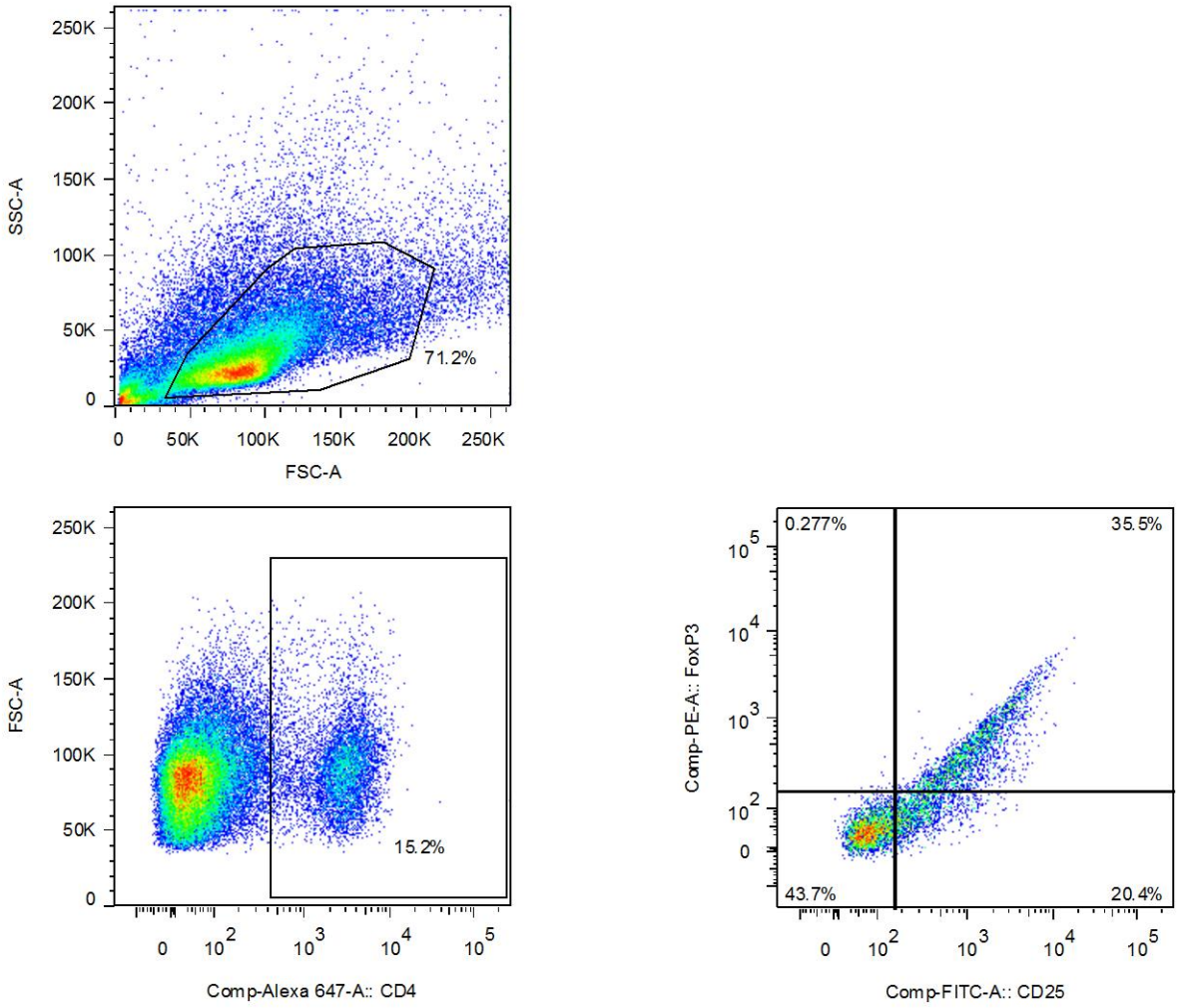
Appendix A: Representative flow cytometry profile and gating of lymphocytes following 3-day co-culture with DCs infected with PRRSV, PCV2, or both viruses. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.



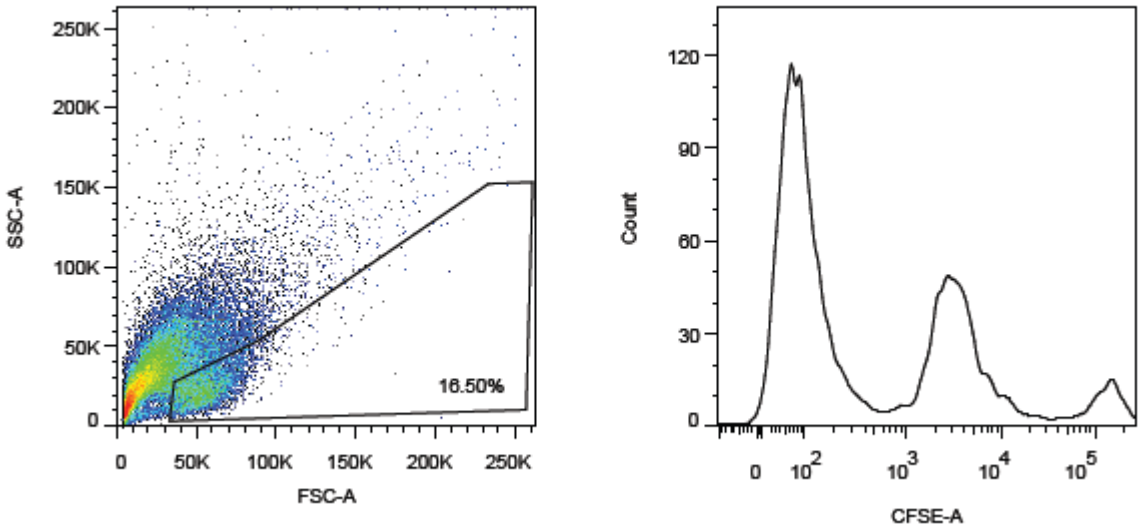




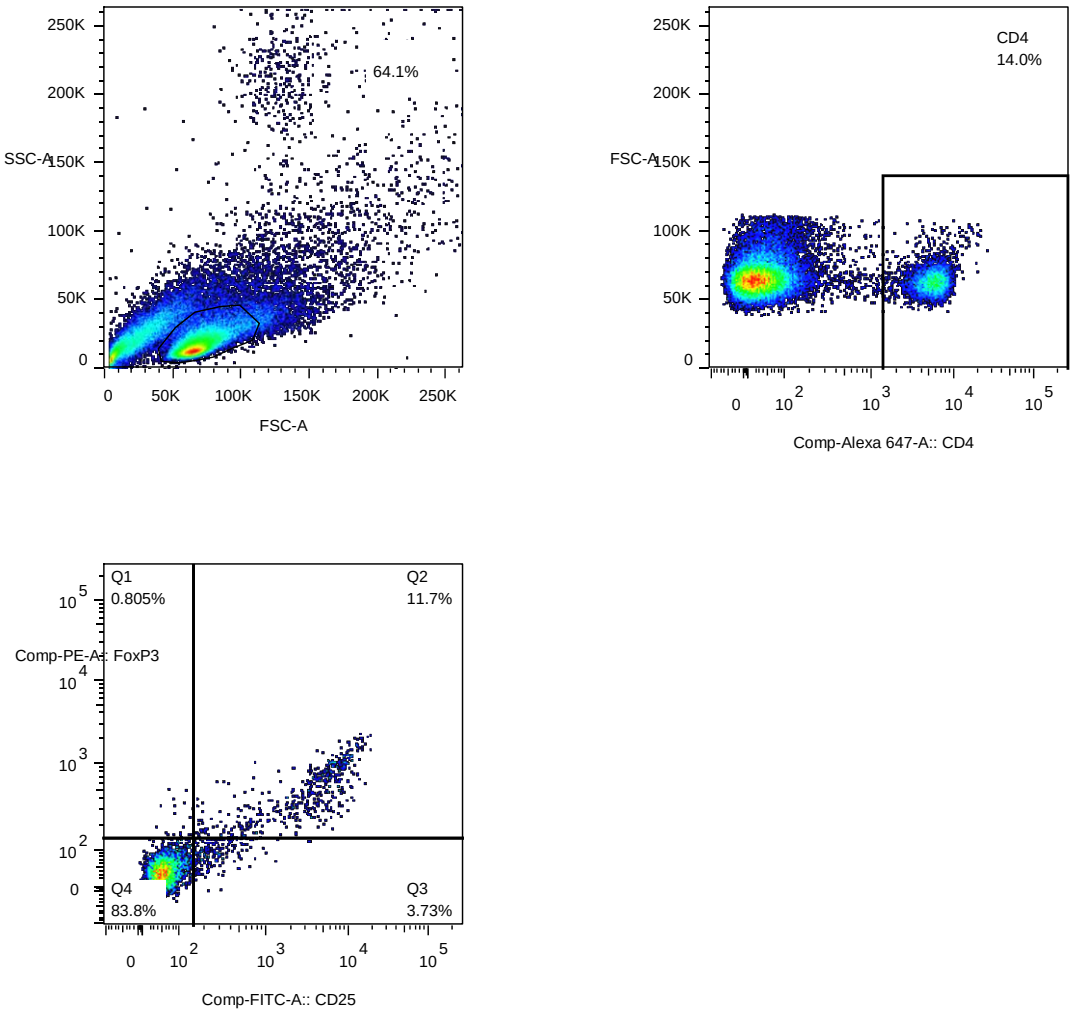
Appendix B: Representative flow cytometry profile and gating of PBMCs from pigs following infection with PRRSV, PCV2 or both viruses. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.



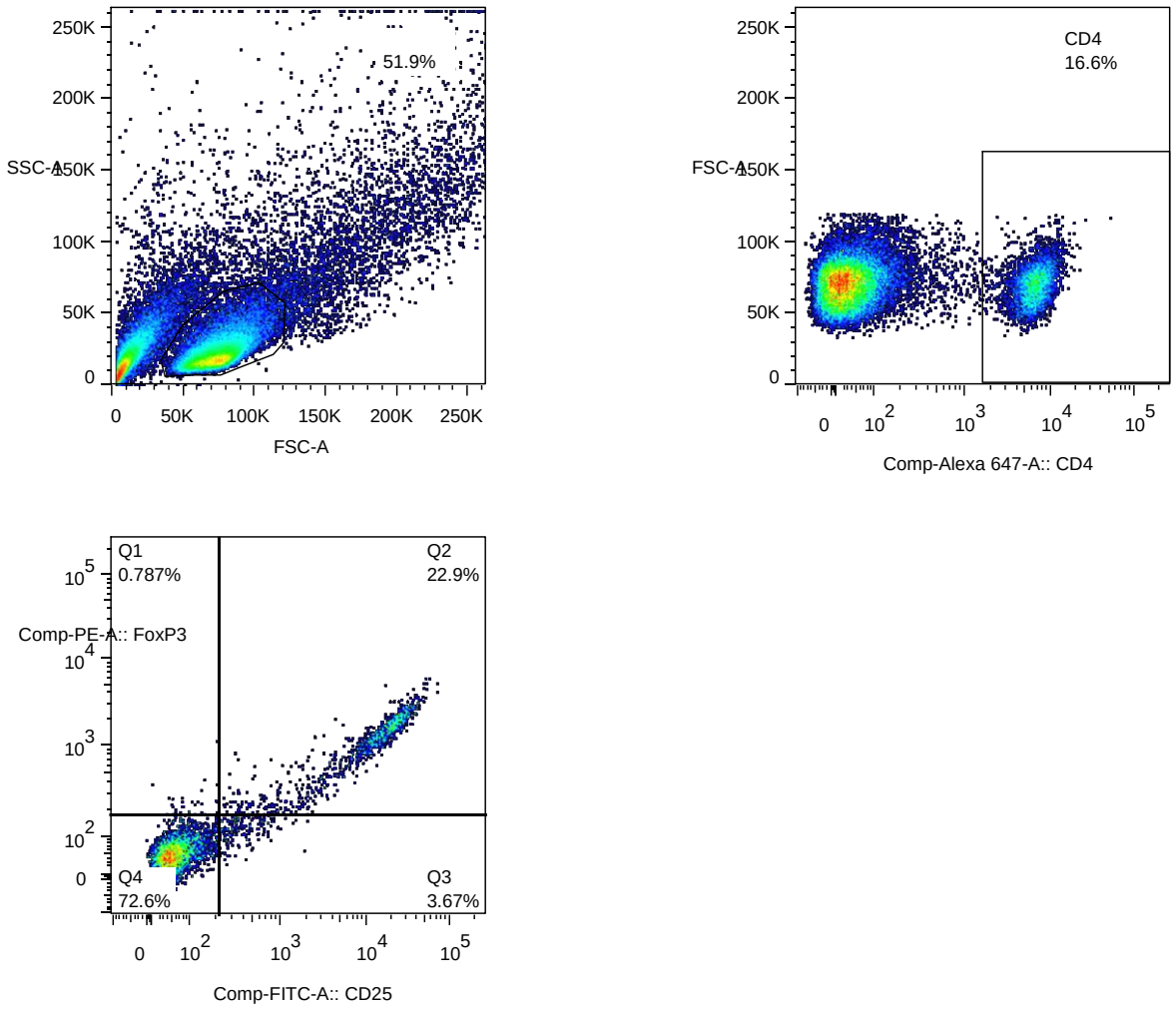
Appendix C: Representative flow cytometry profile and gating of PBMCs following CFSE staining and stimulation with 10 µg/ml PHA for 72 hours. PBMCs are from pigs co-infected with PRRSV and PCV2 14 dpi.



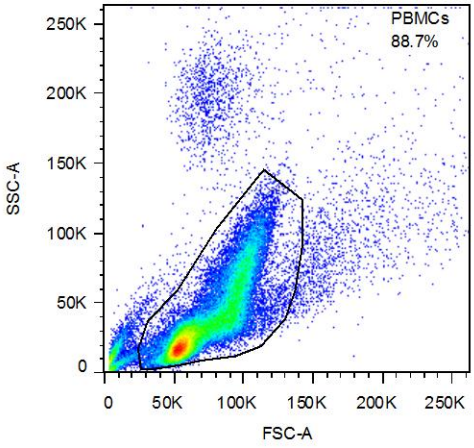
Appendix D: Representative flow cytometry profile and gating of PBMCs from pigs vaccinated with a commercial inactivated PCV1-2 vaccine, Suvaxyn® PCV One Dose™ (now reformulated and known as "Fostera™ PCV" from Pfizer Animal Health Inc.), and then challenged with PCV2b. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.

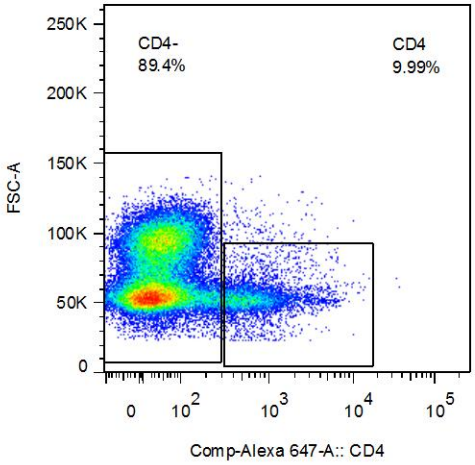


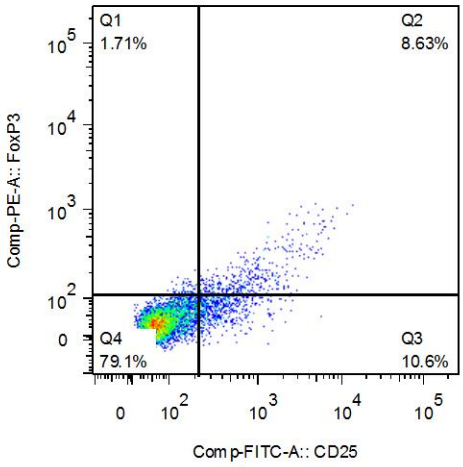
Appendix E: Representative flow cytometry profile and gating of lymphocytes following 3-day *in vitro* co-culture with DCs infected with PRRSV, PCV2 or both viruses. Samples were collected on 98 dpv (70 dpc) from: unvaccinated unchallenged negative control pigs, pigs vaccinated with a commercial inactivated PCV2 vaccine and challenged with PCV2b, pigs vaccinated with a live-attenuated PCV2 vaccine and challenged with PCV2b, and pigs unvaccinated and challenged with PCV2b. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.



Appendix F: Representative flow cytometry profile and gating of PBMCs from pigs at 0, 7, 14, and 21 dpi with an attenuated DS722 PRRSV or the parental VR2385 strain. Expression of FoxP3 and CD25 are shown for CD4-gated lymphocytes.







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