RESEARCH REPORT
SWINE HEALTH

Title: Is humoral immunity defective in PCV-2 infected piglets?- NPB #07-201

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Date Submitted: 7/29/09

INDUSTRY SUMMARY

Porcine multisystemic wasting syndrome (PMWS) emerged in the mid and late 1990s in herds infected with porcine circovirus type-2 (PCV-2). A growing database of field observations and experimental animal infections considers PCV-2 to be either the cause of PMWS or to acts in concert with other factors to cause disease (Allen & Ellis, 2000). PMWS is responsible for significant economic losses in the US as well as around the world (Merial, 2004). There are no specific treatments for the disease although several vaccines have appeared. The complexity of PMWS may be related to the immunoregulatory properties of the virus. In an effort to address the immunological aspects of PCV-2 induced PMWS that might determine future vaccine strategies, we undertook a comparative study using 64 isolator piglets infected with either PCV-2, PRRSV or swine influenza (SIV); all are respiratory pathogens and the preliminary studies that were confirmed in this study indicated that germfree isolator piglets could resolve SIV infections in ~21 days. Therefore we wanted to identify parameters of immunity that correlated with this successful outcome while identifying immunological features that might explain why PCV-2 and PRRSV infected piglets were unable to clear the infection. It was thought that the optimal vaccine for both of these persistent infections should be designed to overcome the weaknesses in the immune response when compared to SIV.

This "3-virus" comparative study clearly showed that neither PRRSV nor PCV-2 infected piglets generated activated cytotoxic or helper T cells at the site of infection during the course of the study. The former cell type destroys virus-infected cells while the latter cell types stimulate the production of viral neutralizing (VN) antibodies. PCV-2, piglets did respond with normal production of immunoglobulins which argues against a direct inhibitor of overall B cell development. However and unlike SIV-infected piglets, PCV-2 infections did not promote antibody responses to irrelevant antigens (used as a test of function of the antibody system) and showed depressed development of helper T cells. Therefore our studies confirmed some aspects of the "immune suppressive" theory relating to PCV-2. However we were unable to pinpoint the cause of the depressed antibody response. Since PCV-2 infected piglets were unable to "mature their immune system" and to make robust antibody responses, we hypothesize that PCV-2 lacks the adjuvant effect of SIV and that future vaccine strategies should focus on resolving this apparent problem since the virus does not appear to cause any obvious immune dysregulation. One approach is to use polyvalent vaccines, perhaps including SIV or bacterial adjuvants, such as probiotics, to simultaneously stimulate the development of cytotoxic T cells and activated helper T cells.

The major feature of PRRSV infection was the extreme polyclonal and non-specific B cells activation that drove B cells to end-stage plasma cells while simultaneously depleting the population of activated B cells that could potentially respond to the virus. Perhaps a feedback loop from these cells also inhibited development of cytotoxic T cells. It seems that PRRSV vaccine development should focus on recombinant and modified vaccines that reduce B cell immune dysregulation while still promoting an immune response.

We believe the isolator piglet model can serve as an important test bed in developing and improving vaccines for PCV-2 because: (1) animals are especially sensitive to pathogens and (2) all piglets start with the same environmental background (unlike conventional piglets).

SCIENTIFIC ABSTRACT

A total of 64 germfree (GF) isolator piglets were studied in three different experiments that compared the effect of infection with PCV-2, SIV and PRRSV on: (1) the ability of piglets to resolve the viral infection, (2) changes in total IgG, IgM and IgG levels in blood and BAL, (3) phenotypic differences in blood NK, T and B cells and at the site of infection, (4) the ability to make antibody responses to the virus, (5) the ability to respond to irrelevant thymus dependent (TD) and thymus independent (TI-2) immunogens and (6) the clonotypic (spectratypic) pattern of B cells in the target tissues.

Our studies reveal that while GF piglets inoculated with SIV can resolve the infection in ~ 21 days, PCV-2 and PRRSV remain persistent infections. All SIV infected piglets had antibodies to the virus as did piglets infected with PRRSV but few PCV-2 infected piglets made anti-viral antibodies. In the case of PRRSV, the anti-viral antibodies may be simply the result of polyclonal B cell activation and hypergammaglobulinemia since there is no difference between immunized and PRRSV-infected piglets in their ability to make antibodies to an irrelevant control antigen, e.g. TNP. This would agree with our previous studies (Lemke et al 2004; Butler et al 2007; 2008). Most notable was the inability of either PCV-2 or PRRSV infected piglets to develop activated cytotoxic or helper T cells at the site of infection; perhaps this is the reason that the viral infections persist. Furthermore in PRRS, B cells are driven to end stage plasma cells which may interfere with their role as APCs and thus the generation of cytotoxic T cells and antigen driven anti-viral antibodies. PCV-2 infected piglets demonstrated a gradual increase in serum Ig levels of all isotypes similar to SIV-infected piglets indicating that PCV-2 does not suppress total B cell activity. However, PCV-2 infected piglets failed to develop antibodies to irrelevant, define immunogens as well as the virus which is consistent with their lack of activated helper T cells. It is our opinion that the defect in PCV-2 infections may be related to the lack of a strong adjuvant effect of the infection. This would suggest further studies using polyvalent vaccines or vaccines given together with strong adjuvants.

INTRODUCTION

Experimental PCV-2 infections in conventional, CDCD and gnotobiotic pigs have shown that PCV-2 is associated with granulomatous lesions of the respiratory tract and gut, enteritis, nephritis, depositions of protein in the kidney and generalized "wasting" of unthrifty pigs (Allan & Ellis, 2000). All lymphocytes, especially activated memory T cells, are decreased (Nielsen et al., 2003). While some have indicated that PMWS is associated with apoptosis, newer data suggest that the loss or collapse of T- and B-cell populations is more likely due to decreased cell proliferation (Chianni et al., 2003; Mandriola et al., 2004). Over 400 articles on PCV-2 and PMWS have been published but only a minority deal with the immune response and these collectively suggest: (a) lymphocyte proliferation is suppressed, (b) PCV-2 targets macrophages and dendritic cells which may impair their ability as APCs, and (c) PMWS is a disease caused by active immunosuppression or alternatively by failure of infection to stimulate an immune response. However, experiments designed to test these ideas are conspicuously lacking. Our proposal was to experimentally test whether PCV-2 interferes with: (a) B cell development, (b) immunoresponsiveness to irrelevant, defined immunogens and the virus and (c) development of protective immunity to SIV.

OBJECTIVES

1. In a comparative study of there different viruses, to test whether infection with PCV-2 uniquely interferes or suppresses humoral immune responses in GF isolator piglets

2. Determine whether PCV-2 infection can suppress an established immune response

3. Determine if PCV-2 infections can impair induction of adaptive immunity in GF piglets by studying the response to defined, model immunogens.

4. Determine whether PCV-2 infections can impair protective immunity to SIV.

MATERIALS AND METHODS

1. Experimental design: Table 1 describes the treatment of the 64 GF isolator piglets that were treated according to the generic experimental design shown in Figure 1.

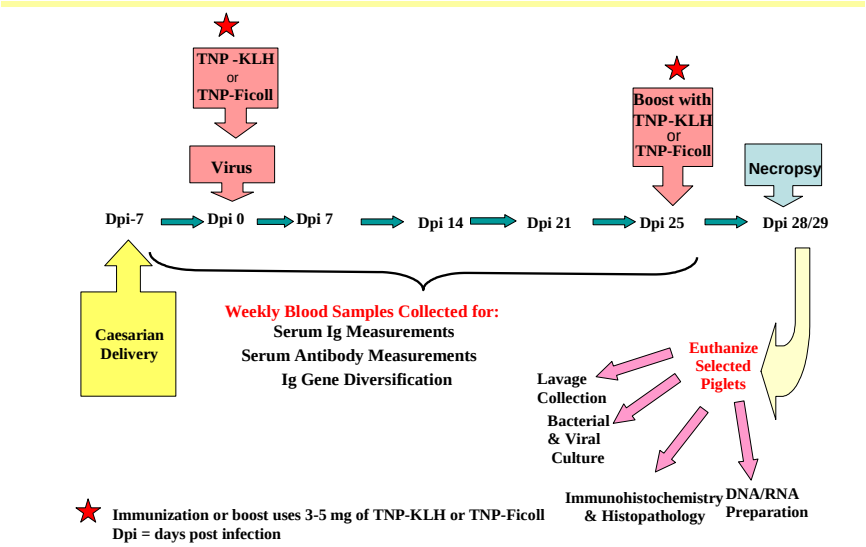


Figure 1. Generic experimental design for isolator piglet studies

Table 1
Animal Groups Studies

Animal Group	Nr	Virus	Immunogen	Expt Nr
90, 100, 103	12	SIV	none	475, 482,491
91 & 104	8	SIV	TNP-Ficoll	475 & 491
92 & 96	8	none	TNP-Ficoll, TNP-KLH	475
93 & 105	8	SIV	TNP-KLH	475 & 491
97 + 102	8	PCV-2	none	482 & 491
98 + 106	8	PCV-2	TNP-KLH	482 & 491
99 + 107	8	PCV-2	TNP-Ficoll	482 & 491
101	4	PRRSV *	none	482

* Our analysis includes data from earlier studies with PRRSV (Lemke, 2006)

2. Source and treatment of animals. All piglets were Caesarian-derived and reared in germfree isolators as previously described (Minatis and Jol___, Lemke et al 2004; reviewed in Butler et al 2009). All procedures followed approved IACAC protocols. Nine pregnant sows were purchased from a herd free of PRRSV and clinical reproductive disease to provide the 64 piglets used in the research. Periodic blood samples were drawn for measurement of Ig levels, anti-TNP, anti-viral activity and to test for viremia.

3. Specific in vitro tests

(a) Immunoglobulin (Ig) and antibodies. Igs of the major classes (IgM, IgG and IgA) antibodies to FLU and TNP and their specific activity were determined as previously described (Butler et al., 2002).

(b) Phenotypic analysis of T- and B-cells. Blood leucocytes were analyzed by four-color flow cytometry (FCM) to measure the relationship of T- and B-cell subsets with special reference to ratios and activation markers like CD8αα, CD25 MHC II on T-cells and CD2(+), IgM and CD21 on B cells

(c) Virus-specific antibodies. Commercially available ELISA tests (IDEXX Laboratories) were used to detect PRRSV and SIV-specific antibodies. PCV-2 antibodies were detected following the methods of Nawagitgul et al 2002.

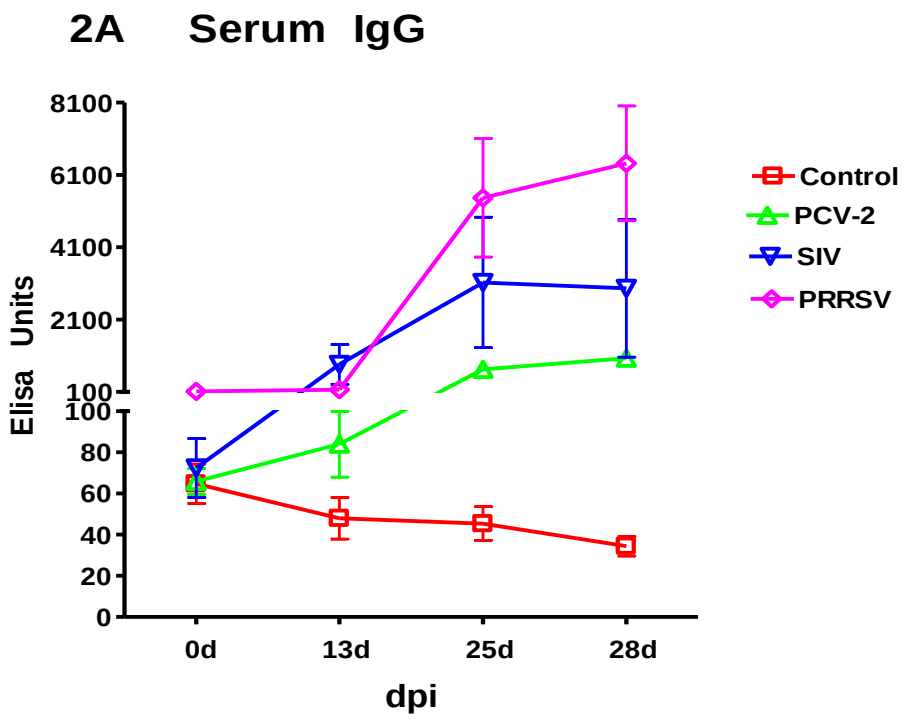
(d) Presence of virus Detection of PCV-2, PRRSV and SIV nucleic acid have been previously described (Opriessing et al 2003, Umthun et al 1999, Spackman and Suarez 2008).

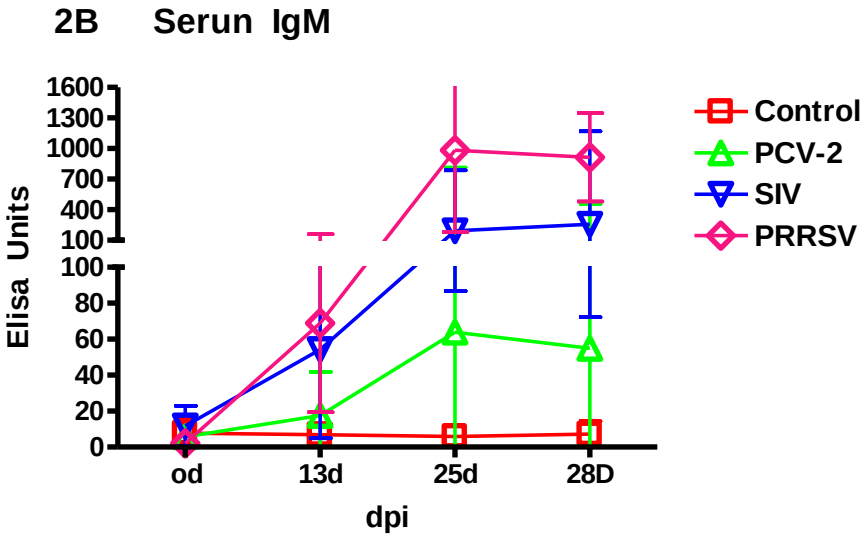
(e) Immune response to TD and TI-2 immunogens. TNP-KLH (TD) and TNP-Ficoll (TI-2) were prepared as previously described (Butler et al 2002). Animals in appropriate groups (Table 1) were immunized with 3-5 mg of the TD or TI-2 immunogens and on dpi 25, were boosted with an equal amount (Figure 1). Anti-TNP responses (IgG and IgM) were measured using TNP-albumin as the solid phase antigen (Butler et al 2005). Activity was expressed as ELISA Units/ml using the serum of a hyperimmunized pig (B1-5) as a reference standard that was assigned a certain number of ELISA Units of IgM and IgG activity.

RESULTS

Objective 1: **Does PCV-2 infection uniquely interfere with humoral immunity?**

Figure 2 shows the levels of IgG, IgM and IgA at four time points in the study. PCV-2 infected piglets have a normal increase in IgG and IgM although levels are lower that those for SIV-infected piglets. Interestingly IgA levels exceed those of IgA levels in SIV-infected animals and equal those in PRRSV-infected piglets in which Ig levels are thought to be the result of polyclonal B cell activation (Lemke et al 2004; Butler et al 2008).Thus PCV-2 does not inhibit normal B cell development and differentiation.





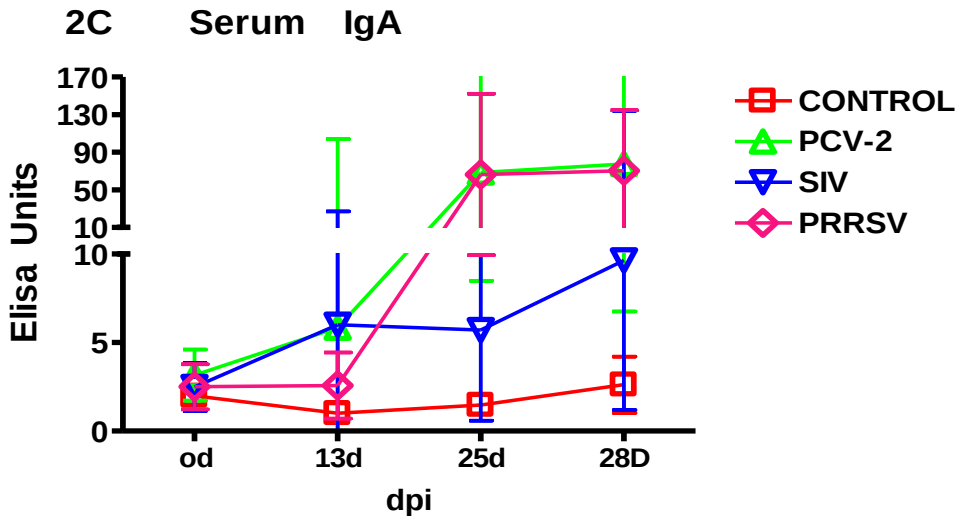


Figure 2. IgG, IgM and IgA serum concentrations in PCV-2, SIV and PRRSV-infected piglets

Objective 2. **Can PCV-2 infection interfere or suppress an established immune response?**
Time and increasing animal costs of maintenance did not allow this experiment to be done.

Objective 3. **Can PCV-2 suppress the induction of specific humoral immune responses?**
Figures 3 shows that in contrast to SIV-infected piglets, PCV-2 animals appear unable to make IgG or IgM antibodies to TNP. This may be explained by data in Figure 4 showing that they lack activated helper T cells even at the site of infection. Despite their failure to respond to irrelevant TD and TI-2 immunogens, some animals (2/8) did have antibodies to PCV-2 but all animals failed to resolve the infection (Fig. 5). At the time of necropsy, lymphocytes in the mesenteric and tracheal-bronchial lymph nodes (MLN; TBLN) bronchioalveolar lavage (BAL), spleen and blood were examined using 10 phenotypic markers. Except for PRRSV-infected animals that develop severe hypergammaglobulinemia and polyclonal B cell expansion (Figure 7; Lemke et al 2004; Butler et al 2007; 2008) all major differences were confined to BAL cells and TBLN at the site of the infections. The overall results are summarized in Figure 6.

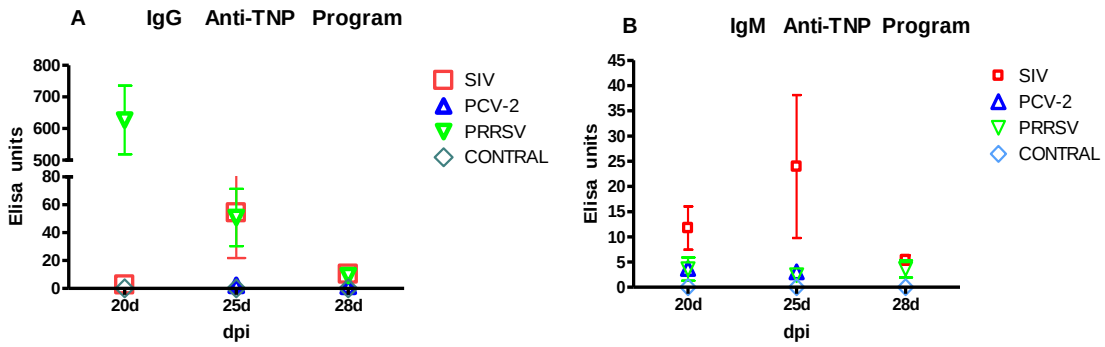


Figure 3. Failure of PCV-2 infected piglets to make immune responses to TD and TI-2 immunogens. IgG anti-TNP in PRRSV-infected piglets is probably non-specific due to polyclonal activation of the pre-immune repertoire. dpi=days post inoculation

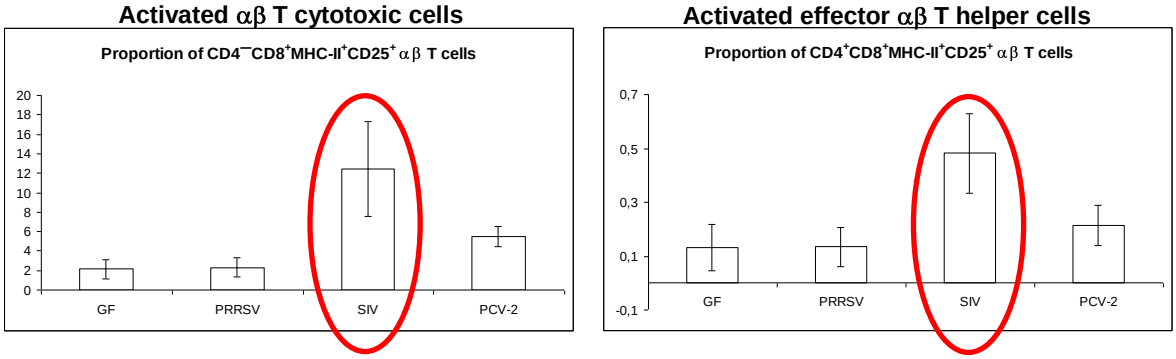


Figure 4. PCV-2 and PRRSV infected piglets fail to generate activated cytotoxic or helper T cells. Encircled bars denote significantly higher proportions of cells.

Group & Virus	PCR		ELISA		TTV1		TTV2	
	d 0	Necr	d 0	Necr	d 0	Necr	d 0	Necr
PRRSV	ND	15/15	0/15	12/15	ND	ND	ND	ND
SIV	0/12	0/12	0/12	12/12	0/12	0/12	?/12	0/12
PCV-2	0/12	12/12	--	2/12	0/12	0/12	?/12	0/12

ND= Test not done

Figure 5. PRRSV and PCV-2 infected isolator piglets fail to resolve the viral infection. Results include those from previous PRRS studies (Lemke, 2006). Necr= determined at necropsy at dpi 28.

	PRRSV	SIV	PCV-2
BAL			
Effector B cells	++++	+	+
Cytotoxic T cells	-	+++	-
NK cells	+	+	+
Helper T cells	-	+	-
	PATHOGENIC *many B cells producing Ab without any activation* *No cytototoxic T cells* *No activated helper T cells*	*NORMAL* *B cell response* *many cytotoxic T cells activated helper cells*	*SUPRESSIVE?* *B cell response?* *absence of cytotoxic T cells* *absence of helper T cells*

Figure 6. Summary of major difference in lymphocytes distribution.

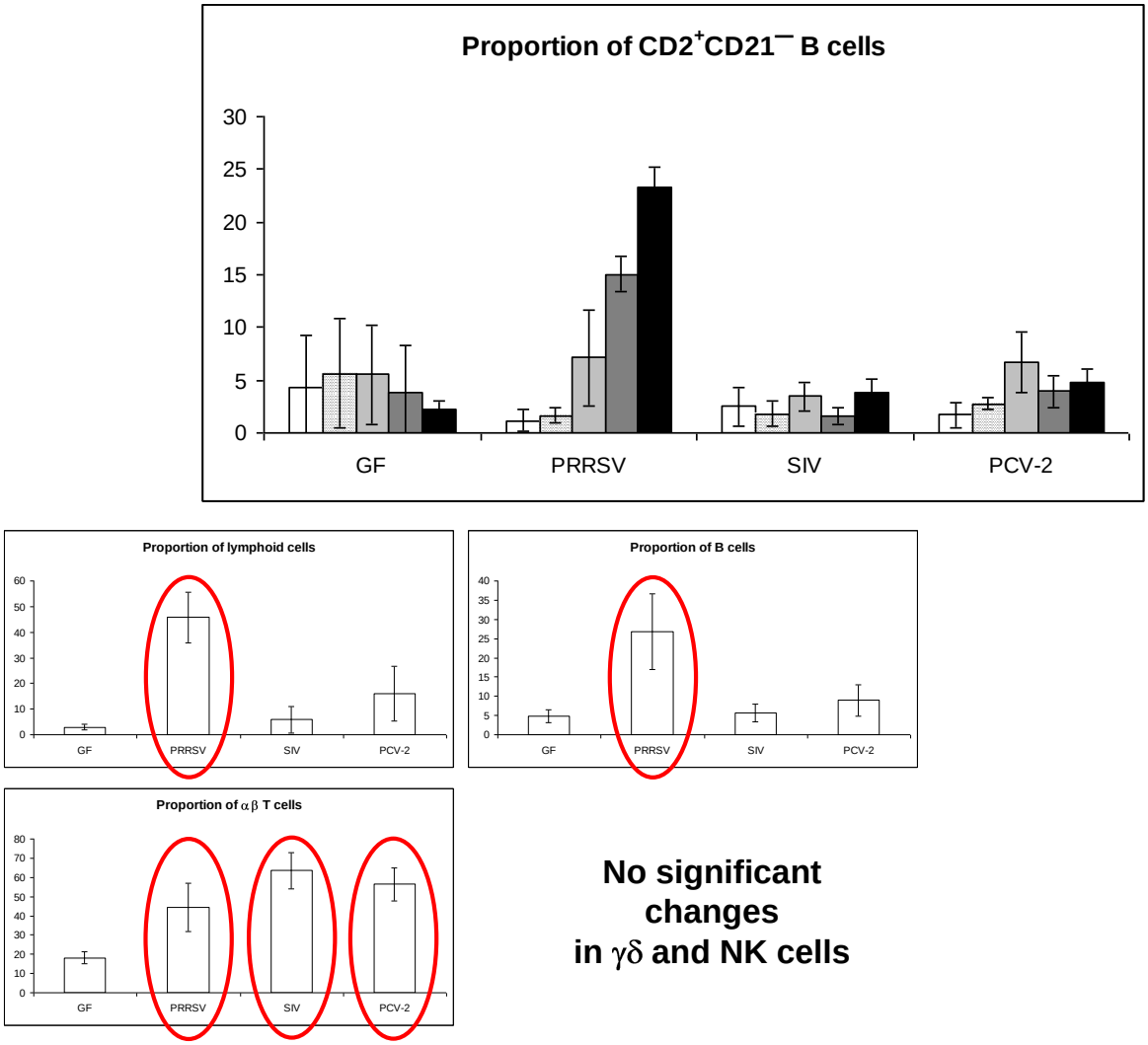


Figure 7. B cells are elevated in the BAL (below) and blood (above) in PRRSV-infected piglets. Total a/b T cell proportions are elevated in the BAL of all virus-infected piglets. Encircled bars indicate that the proportion of cells is significantly higher than in the GF control groups or other virus-infected piglets.

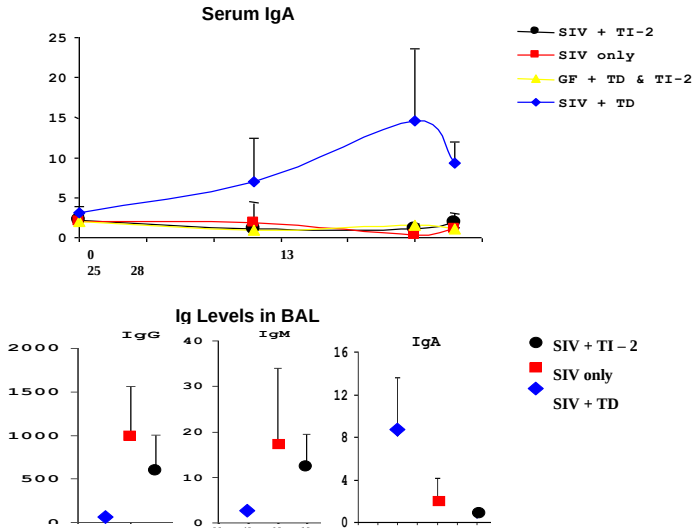


Figure 8. The concomitant administration of a TD immunogen and SIV elevates IgA levels in blood and BAL. Concentration in μg/ml. Data for GF piglets are not given for BAL.

Objective 4 **Can PVC-2 infection impair protection to SIV?**

The protracted time course of study, which required a no cost extension, only permitted us to address this objective in preliminary studies. In those studies, PCV-2 infected piglets were unable to resolve SIV infections in the normal 21 days. However, this study needs to be repeated with larger numbers. The response to PCV-2, i.e. the polyvalent vaccine concept, could be tested.

DISCUSSION

The 64 animal study was conducted in three settings; Expt 475 (April-May 2008); Expt 482 (Nov-Dec 2008); and Expt491 (April-May 2009; see Table 1). In addition, a series of preliminary studies were conducted in late 2007 and early 2008. The work was protracted because of administrative and bureaucratic problems at the NADC that were outside the control of the investigators. They have now been resolved but the delay required a no–cost extension, the deletion of objective 2 and the confinement of experiments addressing objective 4 only to preliminary studies.

We made several changes to the proposed work. Namely we considerably expanded the flow cytometric studies of lymphocytes and abandoned the use of quantitative PCR since we lost confidence with the method because of unreliability and failure to support data obtained using accepted but older and more labor intensive methods. As often the case, new technology is not always the best to resolve approach to a problem, just because it saves on labor.

The animal studies established several important issues as regards the humoral immune competence of PCV-2. The study also enhanced our understanding of PRRSV and SIV infections. This advancement is discussed below.

Figure 5 supports our use of SIV as a model of a viral disease that can be resolved by isolator piglets. Our results demonstrate that viral infection can substitute for colonization of the GI tract as a stimulus for development of a functional immune system. In addition to resolving the infection, all animals made anti-SIV antibodies (Figure 5) and especially anti-TNP responses when TNP is delivered as a TD immunogen (Fig.3). Of considerable interest was the effect of administration of a TD immunogen in SIV infected piglets on serum and BAL IgA levels (Fig. 8). IgA in the respiratory tract is generally associated with protective anti-viral immunity. This outcome contrasts with result obtained with PCV-2 infected animals that failed to resolve the infection and only a few made antibodies to the virus (Fig. 5) while all failed to respond to either TD or TI-2 irrelevant immunogens (Figure 3). These results are consistent with their lack of activated cytotoxic T cells and helper T cells at the site of infection (Fig.4). Nevertheless, PCV-2 infected piglets had a normal rise in serum Ig levels of all isotypes (Fig. 2) indicating there was no block in the pathway of B cells to plasma cell development.

However the data support the conclusion that there is a defect in development of specific antibody responses which may explains why so few made anti-viral responses and thus failed to resolve the viral infection.

A surprising and unexplained outcome of our studies is the elevated IgA levels in serum of PCV-2-infected piglets. This suggests that PCV-2 target the mucosal immune system. Unfortunately we have not yet measured the IgA response to either TNP or PCV-2 in these animals.

While PRRSV was not the focus of this NPB report, its use as second control virus providing additional data. First of all the severe hypergammaglobulinemia produced (Fig. 2; 10-fold increase compared to SIV or PCV-2) and the increase in B cells in BAL and blood (Fig. 7) support our previously published findings (Lemke et al 2004; Butler et al 2007;2008). Including PRRSV in the study also confirmed that like PCV-2, infection with PRRSV is not resolved in GF isolator piglets in the time course of the study (Fig. 5) and like PCV-2, there is no generation of activated cytotoxic or helper T cells (Fig. 4). Since PRRSV was not the focus of this study, further discussion will be confined to an oral presentation in Madison and manuscripts in preparation (see publications).

The overall goal of the research was to identify any defects in the immune responsiveness to PCV-2 that could form the basis for improving vaccines or management practices for the industry. While there are now PCV-2 vaccines, we believe these could be improved by research in the following areas.

Adjuvant Research. Our studies make it clear that SIV supplies its own adjuvant effect since infected animals make antibodies to the virus, antibodies to irrelevant immunogens and resolve the infection. A most interesting observation was the effect on the combination of a TD antigen with an SIV infection. This resulted in a highly significant elevation of serum and BAL IgA (Fig. 8) in animals that have activated cytotoxic and helper T cell at the site of infection (Fig. 4). Therefore one approach to improving vaccines is to determine what adjuvant features of SIV are missing in PCV-2. It seems possible that the addition of certain viral or bacterial PAMPs to PCV-2 vaccines could do what SIV infection does alone (Fig. 5). An important experiment but one that time did not permit us to complete (Objective 4) was to determine if SIV infection could serve as an adjuvant for a PCV-2 response. This would open the path to polyvalent vaccines that are sometimes used for the same reason in infant vaccination protocols.

In vitro Research. A comparison of SIV verus PCV-2 on lymphocytes and accessory cells (e.g. dendritic cells) *in vitro* should reveal difference in cytokine profiles and cell surface phenotype makers that might explain how resolution of SIV differs from the lack of resolution of PCV-2 (perhaps also PRRSV).

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Publications & Reports

[Submission of manuscripts has been delayed until data from all three experiments (Expt 475, 482 and 491) have been analyzed and data compiled. Most recent data were those provided in the presentation at the IVVDC in Madison]

Butler, J.E., C. Lemke, XiuZhu Sun, M. Sinkora, A. Vincent, Z. Bergman and K.M. Lager 2009 A comparative study of PRRSV, PCV-2 and SIV infections in germfree isolator piglets. Int'l Veterinary Vaccine Conf., Madison, WI 19-23 July

Sinkora, M., J. Sinkorova, K.M. Lager and J.E. Butler. The persistence of PCV-2 and PRRSV infections is correlated with the absence of activated cytotoxic and helper T cells at the site of infection. (In preparation).

Butler, J.E., Z. Bergman, A. Vincent and K.M. Lager. The adjuvant effect of an irrelevant thymus dependent immunogen on IgA levels in blood and BAL of SIV-infected piglets. (Brief report in preparation)

Butler, J.E., XiuZhu Sun, A. Vincent, K.M. Lager and M. Sinkora. The effect of PCV-2, PRRSV and SIV infections of germfree piglets on serum and local Ig levels, antibody responses to virus and irrelevant model immunogens

Butler, J.E., N. Wertz, D. Dieringer, D. Francis and K.M. Lager. The comparative effect of bacterial colonization and viral infection on IgG3 transcription and clonotype. (Manuscript in preparation).