RESEARCHREPORT
SWINE HEALTH

Title: Immunomodulation of humoral immunity in PRRSV-infected piglets – **NPB#03-118**

Investigator: John E. Butler, Ph.D.

Institution: The University of Iowa

Date Received: July 7, 2004

Abstract: The employment of the isolator piglet model allows the direct effect of environmental factors, including infections agents, on host physiology to be identified. This NPB-supported research shows that PRRSV induces immunodysregulation that results in autoimmunity which in turn causes kidney pathology and damages the vascular endothelial. PRRSV causes polyclonal activation of the pre-immune B cell repertoire in the absence of specific antigens. Since this repertoire also encodes autoantibodies that are deleted in normal development, PRRSV somehow interferes with the normal development of self-tolerancce. The immunopathology we have described in a recent article in the *Journal of Immunology* (Lemke et al., 2004) is not unique to isolator piglets but occurs in conventional neonates as well. The immune dysregulation we describe does not impair the piglet's ability to make specific antibodies to foreign antigens, including to PRRVs, especially during the primary response. Whether it effects secondary response is less certain.

The mechanism responsible for the dysregulation we describe remains unknown. While certain factors released from PRRSV-infected macrophages may directly stimulate B cells, we provide preliminary evidence that certain T-cell clones are selectively expanded in PRRSV infections but not in controls. It seems therefore important to determine the cause of the immunopathology (immunodysregulation) as part of any program that attempts to eradicate PRRSV as a major threat to pork production in the world.

Introduction: Immunological studies of PRRSV infections is in its infancy and much of the available information is contradictory or based on non-parallel investigations. We have recently shown that autoimmunity is one of the direct effects of PRRSV infections on the host immune system (Lemke et al., 2004). This is characterized by lymph node hyperplasia, polyclonal B cell activation and kidney pathology due to deposition of immune complexes and to autoantibodies directed to vascular endothelia and basement membrane. Hence, it is conceivable that such immunopathology could impair the host's immune system. This NPC-funded study was designed to address certain aspects of that question. It would seem important for the industry to know whether the ability to elicit protective immunity with any PRRSV vaccine would be impaired because of the virus-induced immunopathology.

Objectives: The research had three objectives, one of which was modified and a third replaced since it became redundant after our initial published work (Lemke et al., 2004). First we wished to determine if humoral immunity is impaired in animals infected by wild-type PRRSV. Our second objective was to determine whether colonization of the GI tract was required for piglets to mount a protective immune response to PRRSV. This was modified during the course of study because of the difficulty of showing whether immunity was really "protective" when using the isolator system. Third, we proposed to develop a reliable solid-phase immunoassay that overcomes/circumvents the non-specific ELISA problems resulting from arterivirus-induced polyclonal B cell activation. This objective was eventually considered of low priority and of little value to the industry once we had determined that PRRSV induced an autoimmune syndrome that could readily explain non-specific ELISA problems by the promiscuis behavior of antibodies of the pre-immune repertoire.

Material and Methods:

A. Animal studies. Isolator piglets were obtained and maintained in units at the National Animal Disease Center (NADC) using previously-described methods. Various animal groups were studied including: (a) groups that were colonized with a benign *E. coli* (G58-1) and inoculated with virus-free SHAM media (SCol), (b) colonized piglets given wild-type PRRSV (NADC - 8; PCol) and (c) piglets inoculated with wild-type PRRSV but maintained germfree (PGF). Animals in these three groups were then immunized with two major types of irrelevant antigens, i.e. a thymus-dependent (TD) antigen called FLU-KLH (fluorescein conjugated to keyhole limpet hemocyanin) and a type 2 thymus-independent antigen (TI-2) trinitrophenol-Ficoll (TNP-Ficoll). This design was used to test the piglets immunocompetence by immunization on week one whereas the week four booster tests for a secondary immune response.

In a second series of studies (not initially proposed) piglets were not immunized with the TD and TI-2 antigens in an effort to determine if PRRSV-induced polyclonal activation would yield the same response to the irrelevant antigens even though these antigens had never been encountered by the piglets. This question was not originally proposed since the work of Lemke et al. had not yet been published.

Finally, we conducted pilot studies with normal conventional piglets and with isolator piglets given maternal IgG for reasons discussed below.

B. Sample collection and analyses. Weekly blood samples were collected to recover both plasma and leucocytes; the latter were used to recover RNA for both B- and T-cells for subsequent transcriptional analyses.

Plasma samples were analyzed for total IgG, IgM and IgA and for IgG, IgA and IgM antibody activity to FLU (fluorescein) and TNP (trinitrophenol). Results are expressed as ELISA Units of activity (EUs) or as specific activity computed as EU/μg of the isotype being measured. The details of these methods have been previously published (Butler and Hamilton, 1991; Butler et al., 2000a; 2002).

RNA recovered from the leucocyte fraction of blood was used to prepare cDNA encoding rearranged V_H genes expressed with the various isotypes of porcine antibodies and those encoding expressed variable TCRβ and TCRδ genes. The former was used to test for polyclonal B cell activation and the latter to determine if PRRSV causes stimulation of certain T-cell clones in the manner of an infectious agent or in the manner of a T-cell superantigen. One of the submethods involved is called spectratyping. This can indicate whether certain clones of T- and B-cells have been expanded in the host because of an environmental event, e.g. viral infection. The details of these methods have been previously published (Butler et al., 2000b; 2001; Lemke et al., 2004; Holtmeier et al., 2004).

Figure 1

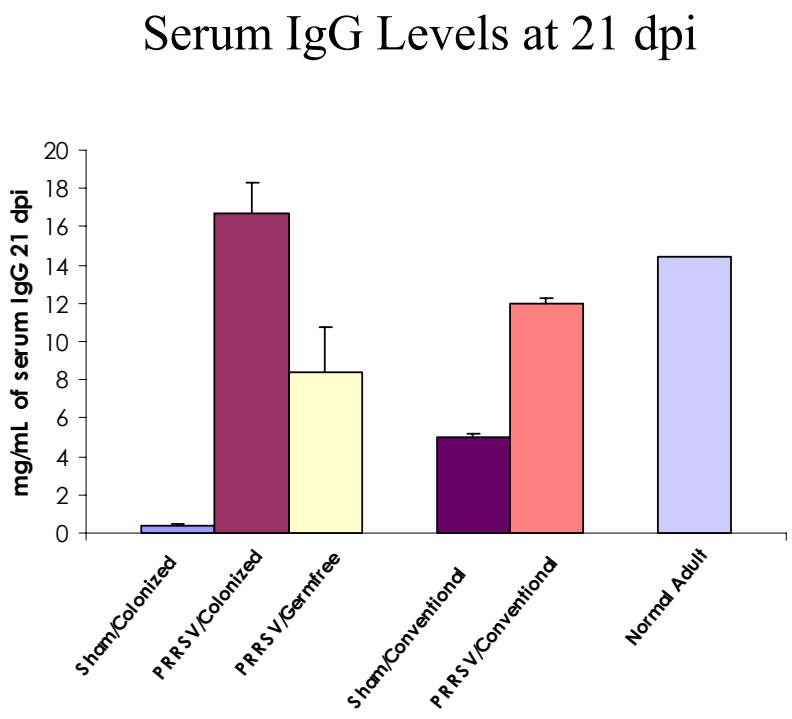


Figure 1. Serum IgG levels in isolator and conventional piglets experimental infected with wild type PRRSV NADC 8 and their controls. The three groups of animals (four/group) on the left were reared in isolators. Two groups of conventional piglets (four/group) are shown to the right. On the extreme right is the mean IgG content of normal adult swine serum. Data are means ± Sx. IgG levels are 10-fold higher in

PRRSV isolator piglets and >2-fold higher in conventional animals than corresponding controls. Since isolator piglets are colostrum-derived, it is quite remarkable that serum level equals that of adults 21 days post infection (dpi).

Figure 2

Serum IgM Levels at 21 dpi

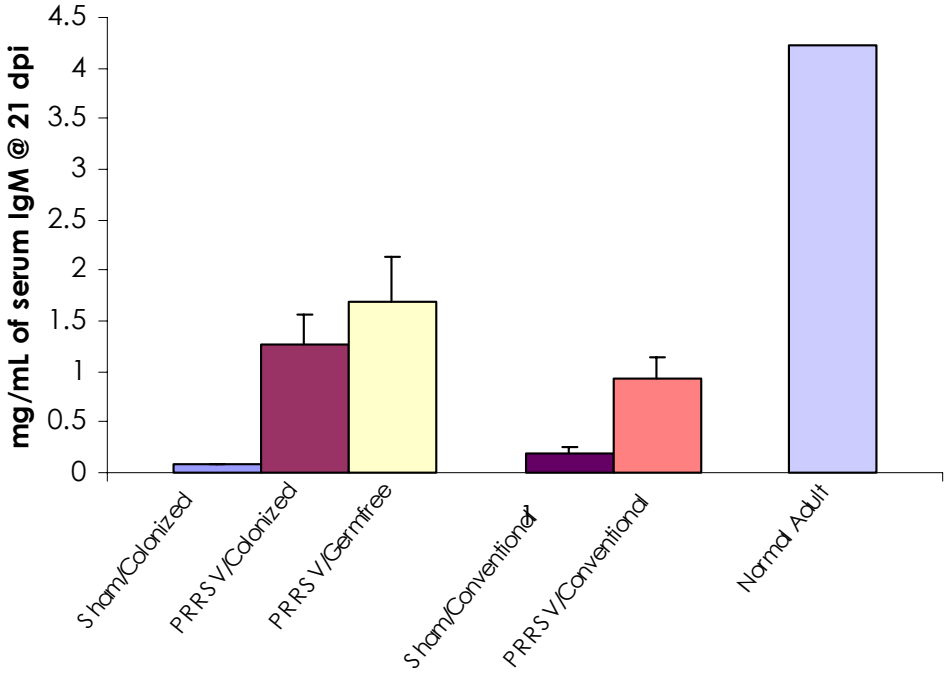


Figure 2. Serum IgM levels in PRRSV-infected isolator and conventional piglets and their corresponding controls. Legend is the same as for Figure 1. Noteworthy is that IgM levels are 5-fold higher in PRRSV-infected conventional piglets. Levels never achieve adult levels in PRRSV-infected isolator or conventional piglets.

Figure 3

Serum IgA Levels at 21 dpi

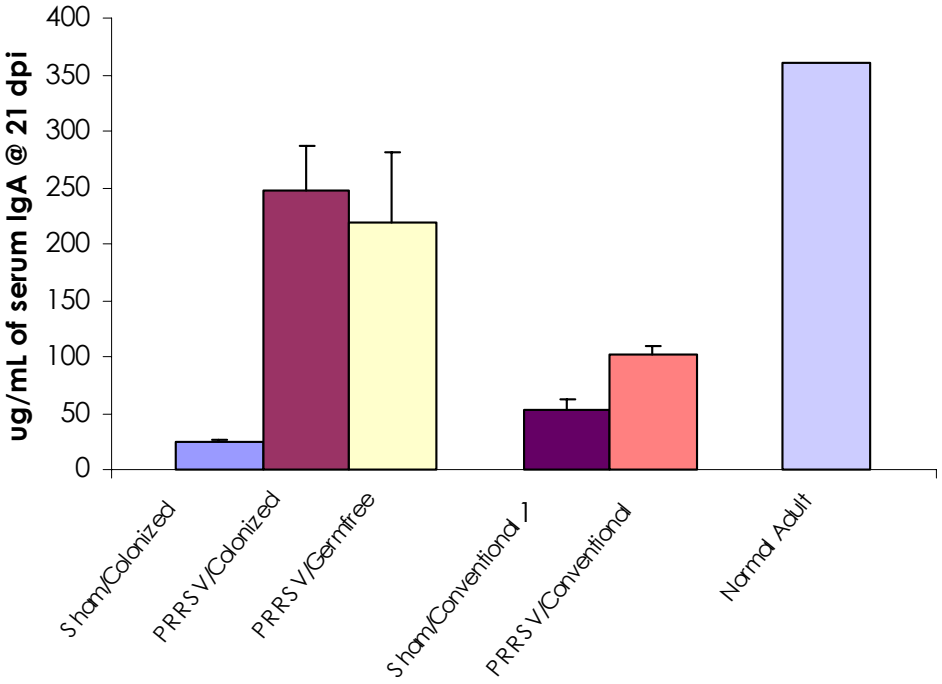


Figure 3. Serum IgA levels in PRRSV-infected isolator and conventional piglets and their corresponding controls. Legend is the same as Figure 1. IgA levels in infected isolator piglets approach adult levels but not in infected conventional animals.

Results:

A. PRRSV infection produces an autoimmune pathology in isolator piglets. The initial experimental design revealed an unexpected result: massive lymph node hyperplasia, 10-100-fold elevations of serum Ig levels (Figs. 1-3), high molecular IgG-containing aggregates, glomerular deposition of immune complexes and autoantibodies to vascular endothelia, basement membrane, dsDNA and Golgi antigen. Since this work was published in detail in the *Journal of Immunology* (Lemke et al., 2004) most of these findings will not be presented here. Rather, the abstract of the published work is inserted below.

Abstract of Lemke et al. 2004. Journal of Immunology 172:1916: Amidst growing evidence that numerous viral infections can produce immunopathology, including non-specific polyclonal lymphocyte activation, the need to test the direct impact of an infecting virus on the immune system of the host is crucial. This can best be tested in the isolator piglet model in which maternal and other extrinsic influences can be excluded. Therefore, neonatal isolator piglets were colonized with a benign *E. coli*, or kept germ-free, and then inoculated with wild-type porcine reproductive and respiratory syndrome virus (PRRSV) or sham media. Two weeks after inoculation, serum IgM, IgG and IgA levels were 30-50, 20-80 and 10-20 fold higher respectively, in

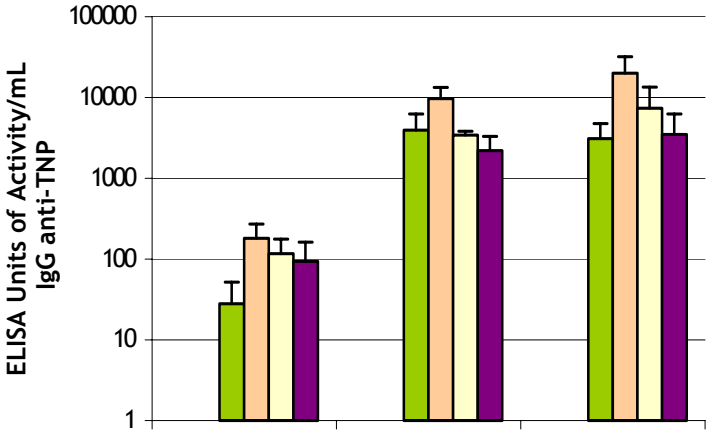
animals receiving virus versus sham controls, although <1% was virus-specific. PRRSV-infected piglets also had bronchial tree-associated lymph nodes and submandibular lymph nodes that were 5-10 times larger than colonized, sham-inoculated animals. Size exclusion FPLC revealed that PRRSV-infected sera contained high molecular weight fractions that contained IgG, suggesting the presence of immune complexes. Lesions, inflammatory cell infiltration, glomerular deposits of IgG, IgM and IgA and antibodies of all three isotypes to basement membrane and vascular endothelium were observed in the kidneys of PRRSV-infected piglets. Furthermore, autoantibodies specific for Golgi antigens and dsDNA could be detected 3-4 weeks after viral inoculation. These data demonstrate that PRRSV induces B-cell hyperplasia in isolator piglets that leads to immunologic injury and suggests that the isolator piglet model could serve as a useful model to determine the mechanisms of virus-induced immunopathology in this species.

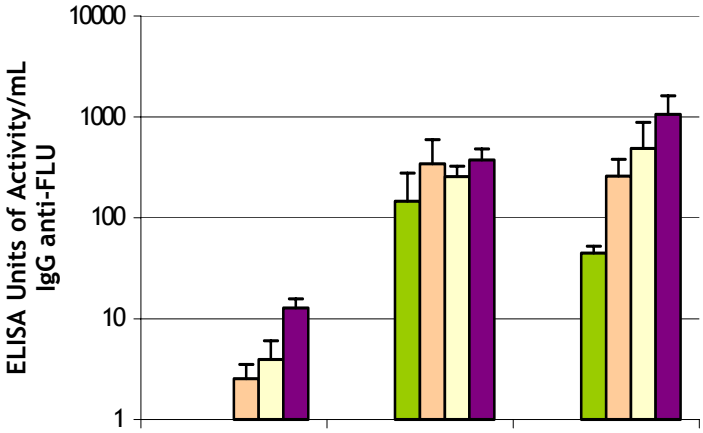
B. PRRSV-induced polyclonal activation is not an artifact of using the isolator piglet model. Critics of PRRSV research proposals submitted by us have questioned whether the work of Lemke et al. (2004) was an artifact of using isolator piglets. Such criticism persisted even after the study was published in the *Journal of Immunology* probably because this journal is not on their reading list. Since we also wondered why others had overlooked this obvious effect, we repeated the same study in conventionally-reared piglets, i.e. half were PRRSV inoculated and half were controls. Figures 1-3 summarize the results in terms of serum Ig levels. These show that as in isolator piglets, Ig levels of all isotypes are elevated after PRRSV infection. Of course Ig levels are much higher in conventional controls than isolator controls as a result of Igs acquired by passive immunity since these animals were not colostrum-deprived. Furthermore, virally-infected conventional piglets had kidney lesion and antibodies to Golgi antigens but no antibodies to dsDNA. Thus, our published observation on PRRSV-induced autoimmunity in isolator piglets is not an artifact, but also occur in conventionally-reared neonatal piglets although the autoimmune phenomenon appears to be less pronounced.

C. The primary response to irrelevant TD and TI-2 antigens **is not** impaired in PRRSV-infected piglets. Figure 4 summarizes the immune response of SHAM versus PRRSV-infected piglets in terms of their humoral immunocompetence. Results are expressed in terms of the absolute IgG titer of serum antibodies to the FLU epitope (top left), TNP epitope (top right) or the specific activity of the response to both epitopes (corresponding bottom panels). The term "Specific Activity" (bottom panels) expresses the response relative to the amount of IgG present. In other words, it indicates the proportion of the total IgG that is epitope-specific.

Figure 4 shows various features of the antibody response of PRRSV-infected piglets to irrelevant antigens. Incidentally, irrelevant antigens are used because of the ambiguity in the PRRSV literature caused by studying the immunocompetence of PRRSV-infected piglets to another pathogen. Such experimental designs cannot control for the synergistic influence of the second pathogen on the immune response rendering interpretation difficult. The major outcome of the studies summarized in Figure 4 are listed below.

(a) The absolute response to FLU and TNP are 10-100-fold higher in PRRSV-infected piglets. Readers should be cautioned that anti-FLU responses cannot



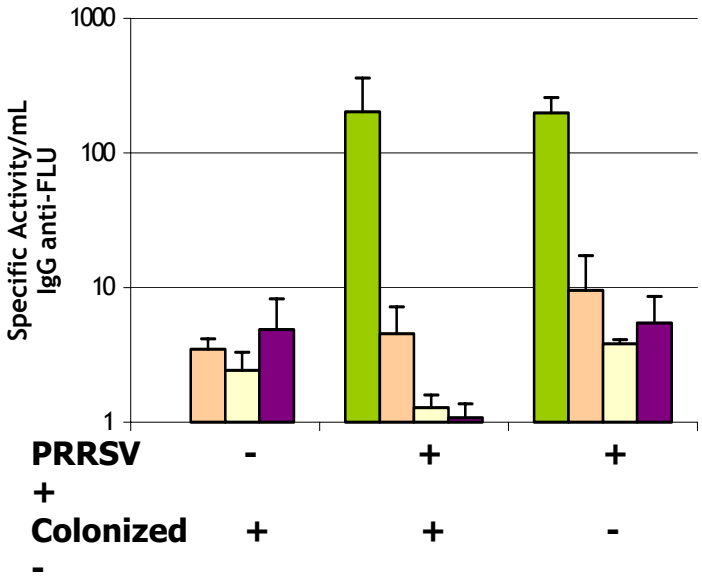


be compared to anti-TNP responses in absolute EUs, since each is determined against a separate standard.

(b) Colonization has no effect on the anti-FLU or anti-TNP response since PRRSV-infected germfree animals and those colonized with *E. coli*, give similar results.

(c) The specific activity of the anti-TNP or anti-FLU response is 10-fold higher on day 14 in PRRSV-infected than in controls. Thereafter, specific activity progressively drops in PRRSV piglets but not in controls.

(d) Evidence for a slight secondary response is seen in the control (Sham inoculated) piglets to the TD antigen but not in either PRRSV-infected groups, regardless of the antigen type.



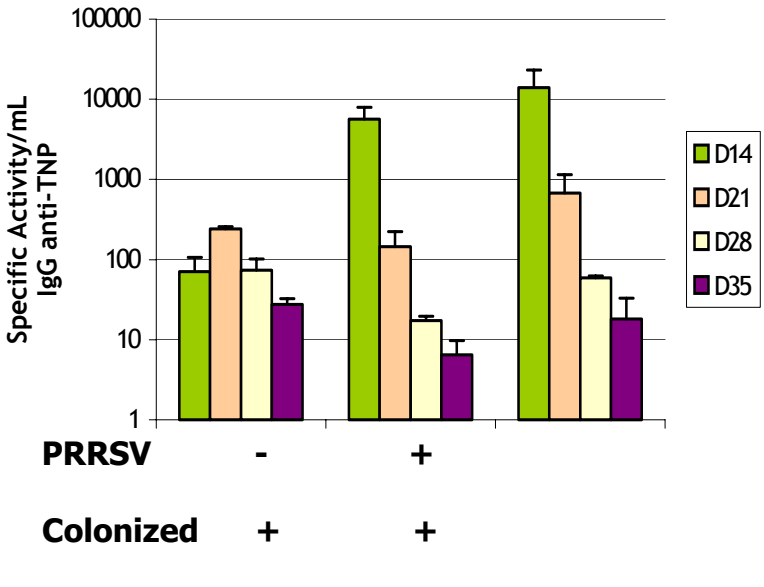


Figure 4. The IgG immune response of PRRSV-infected isolator piglets to fluorescein (FLU; upper left) and trinitrophenol (TNP; upper right). The former was delivered on a thymus-dependent carrier while TNP was delivered on a type 2 thymus-independent carrier. Three groups of piglets (four/group) were used and the treatment groups are indicated below each panel of the graph. Data are presented as raw antibody titer (TOP; ELISA Units/ml) and as Specific Activity (ELISA Units/μg of IgG; Bottom panels).

D. PRRSV-infected piglets show a pronounced response to epitopes they have never encountered. Our studies showing PRRSV induced autoimmunity suggest that B cells of the pre-immune repertoire that are polyclonally activated produce autoantibodies because the pre-immune B cell repertoire is driven to plasma cell differentiation before neonatal self-tolerance develops. It is well known that B cells of the pre-immune repertoire encode receptors recognizing autoantigens (and many other things). Thus we hypothesized that PRRSV-infected animals would produce antibodies to FLU and TNP even if they had never encountered these antigens since they are probably recognized by broadly specific, promiscuous B cells of the pre-immune repertoire. Figure 5 tests this hypothesis and shows that infection with PRRSV yields 10-100 fold increases in specific activity to TNP compared to non-infected controls. Thus, antibodies in arterivirus-infected mice to plastic (Cafruny et al., 1986) and self antigens (Lemke et al., 2004) probably result from the same type of non-antigen specific stimulation of the porcine pre-immune B cell repertoire.

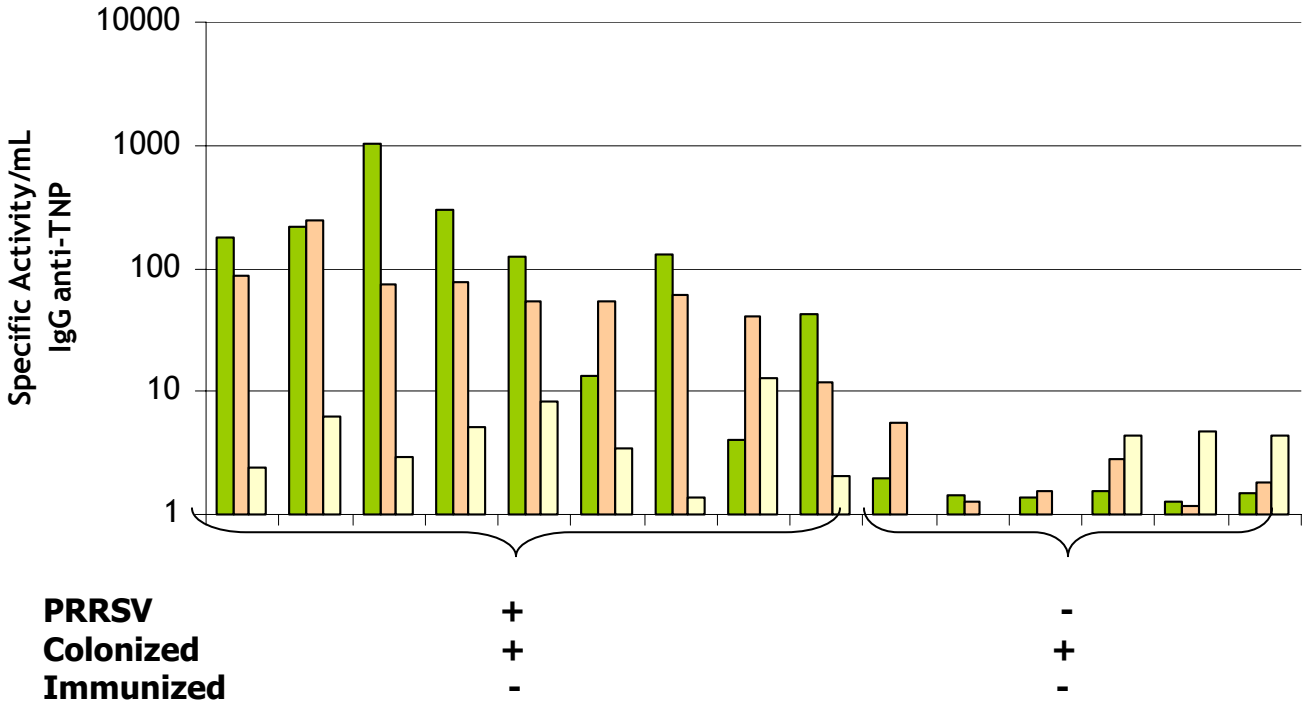


Figure 5. The immune response of non-immunized PRRSV-infected isolator piglets to TNP. Treatment of the various groups is designated with a plus (+) or minus (-). Data are expressed as specific activity on a log scale showing a 10-100 fold increase in PRRSV-infected animals.

E. PRRSV infection selectively expands certain TCRβ bearing T cells but not those bearing TCRδ. Collaborative studies with Wolfgang Holtmeier (Wolfgang Von Goethe University, Frankfurt) indicated that PRRSV-infected piglets showed no expansion of a particular TCRδ T-cell population (data not shown). However, this was not true for TCRβ-bearing T cells. Figure 6 shows the TCRβ spectratype for T-cells recovered from 15-PRRSV-infected animals as well as those from germfree piglets and PRRSV-free piglets colonized with *E. coli*. In general, germfree and colonized piglets reveal a Gaussian spectratype indicating there has been no selection of any T-cell clone. By contrast certain T-cell clones have been selected in all PRRSV-infected piglets. To be sure, each piglet is different but since pigs are an outbred species, these individual differences may reflect their MHC heterozygousity or that each animal responds to slightly different T-cell epitopes. Important is that no such selection is seen in either control group. We interpret this finding to mean that T-cells may be the initial target of PRRSV, not B-cells and that the polyclonal activation of B cells is the indirect result of stimulating selective T-cell clones. T-cell superantigens have been described for many viruses and our data makes this possibility more plausible than direct action on B cells.

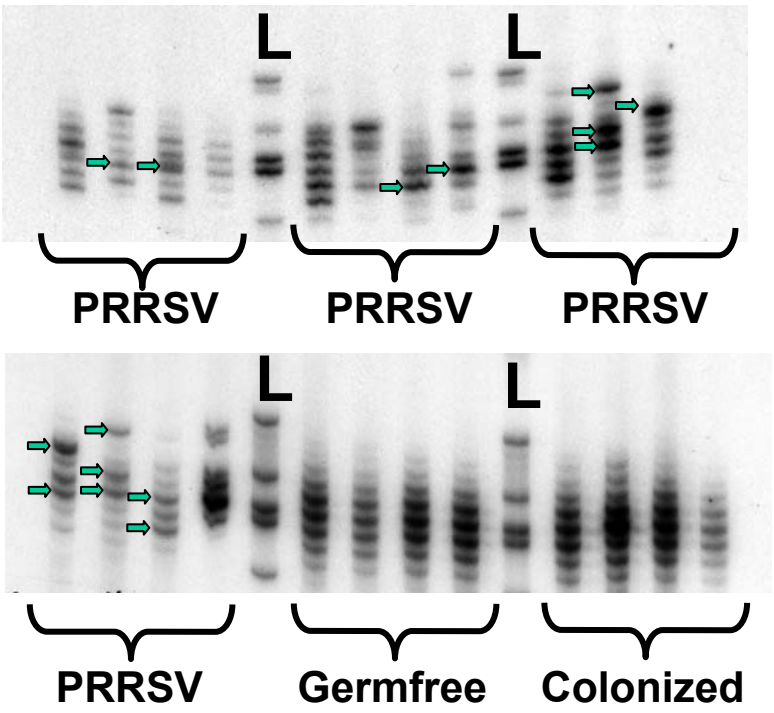


Figure 6. Spectratypic analysis of peripheral blood T cells in 15 PRRSV- infected piglets and age-matched germfree and E.coli colonized controls. L= CDR3 length ladder of decreasing nt length: 48,45,36,30,27 and 18. It should be noted that although all infected piglets give different patterns, ***all*** show selective expansion of certain T cells clones. This is not seen in uninfected piglets that instead give a Gaussian distribution of length indicating lack of selection. Arrows call attention to selected clones in PRRSV-infected piglets.

Discussion: This NPB-funded project unambiguously demonstrates the immune dysregulatory effect of PRRSV on the host immune. The B cell polyclonal activation, which also causes the lymph node hyperplasia, causes B cells of the pre-immune repertoire to differentiate to plasma cells, releasing autoantibodies (Lemke et al., 2004) and those recognizing other antigen never encountered by the infected animals (Fig. 5). These antibodies form immune complexes choking the glomerulus and causing endothelial damage throughout the body. Thus, PRRSV is a classical example of autoimmune disease like Lupus and rheumatoid arthritis in humans. While some critics believe our study is contrived because it was performed in isolator piglets, we show here that many of the same features occur in conventionally-reared newborns infected with PRRSV (Figs. 1-3). What remains to be determined is the long-term effect of this immune dysregulation. Is this transient autoimmunity that later wanes as the virus is cleared or are there long-lasting consequences? What should be of more immediate practical concern to the veterinary field is that the autoimmune crisis very likely increases the susceptibility of infected animals to opportunistic infection. In fact, we have even observed septicemia to commencal *E. coli* in PRRSV-infected piglets (Lager, Francis & Butler, unpublished).

Our studies also show that at least the initial (primary) immune response is not impaired although it is unclear as to whether secondary humoral responses are impaired (Fig. 4). The precipitous drop in specific activity to TNP or FLU after the initial response on day 14 (Fig. 4, Bottom) is not due to a lowering of the anti-TNP and anti-FLU titer (Fig. 4, Top) but due to the tremendous increase in serum IgG resulting from PRRSV-induced polyclonal activation. The simple explanation for the initial high specific activity is that TNP- and FLU-specific B-cell initially receive two signals while B-cells lacking receptors for TNP and FLU receive only one.

It is important to emphasize that irrelevant antigens were used to study PRRSV-induced impairment of the immune response because unlike using a second pathogen (e.g., another virus) they are non-infectious and produce no impairment (or augmentation) of their own. This is not true when secondary pathogens are used to examine the issue of immune competence and synergy between the two infections can cause its own immunopathology.

While there is no doubt that PRRSV causes polyclonal B cell activation and consequently autoimmunity, the mechanism remains unknown. Arguments that a glycoprotein from LDV is responsible for such a phenomenon in mice (Plagemann et al., 2001), are unconvincing. Here we show a definitive and striking effect on T-cells (Fig. 6). These data show that certain clones are selected for expansion that are not seen in germfree controls or those colonized with *E. coli*. It remains to be determined whether these stimulated clones represent T-cells bearing TCRs encoded by genes for certain TCRβ gene families. If so, PRRSV may exert its effect by acting as a T-cell superantigen.

The benefit to pork producers from knowledge about the pathology caused by a certain infectious agent follows the same logic as in human medical research. If we know what the negative pathological effects of PRRSV are, we can begin to develop measure to combat them. In a simple example, why not develop an attenuated virus that stimulates the development of protective immunity without causing the immunopathology seen with the wild-type virus?

Lay Interpretation: PRRS is caused by a virus that causes the host's immune system to malfunction much in the same manner in which infectious agents can trigger the onset of autoimmune syndromes like Lupus or rheumatoid arthritis in humans. The damage caused by such a syndrome effects kidney function and damages vascular and epithelial integrity. Both may explain the greater susceptibility of PRRSV-infected piglets to opportunistic pathogens of swine and the malaise and morbidity of infected neonatal piglets. It is generally true that if the pathology caused by an infectious agent is understood, the development of counter measures and the development of effective vaccines will rest on firmer scientific principles and less on non-directed trial and error vaccination schemes.

References:

Cafruny, W. A., D. P. Heerath, M. J. Jaqua and G. W. Plagemann. 1986. Immunoglobulins that bind uncoated ELISA plate surfaces: Appearance in mice during infection with lactate-dehydrogenase elevating virus and human anti-nuclear antibody positive sear. J. Med. Virology 19:175-186.

Butler, J. E., and R. G. Hamilton. 1991. Quantitation of specific antibodies: Methods of expression, standards, solid-phase considerations and specific applications. In J. E. Butler [Ed.], Immunochemistry of Solid-phase Immunoassay. Chapter 9. C.R.C. Press. pp. 173-198.

Butler, J. E., J. Sun, P. Weber and D. Francis. 2000. Antibody repertoire development in fetal and neonatal piglets. III. Colonization of the gastrointestinal tracts results in preferential diversification of the pre-immune mucosal B-cell repertoire. . Immunology (British) 100:119-130.

Butler, J. E., P. Weber, M. Sinkora, J. Sun, S. J. Ford and R. Christenson. 2000. Antibody repertoire development in fetal and neonatal piglets. II. Characterization of heavy chain CDR3 diversity in the developing fetus. J. Immunol. 165:6999-7011.

Butler, J.E., J. Sun. P. Weber, S.P. Ford, Z. Rehakova, J. Sinkora and K. Lager. 2001 Antibody repertoire development in fetal and neonatal piglets. IV. Switch recombination, primary in fetal thymus occurs independent of environmental antigen and is only weakly associated with repertoire diversification. J. Immunol. 167:3239-3249.

Butler, J. E., P. Weber, M. Sinkora, D. Baker, A. Schoenherr, B. Mayer and D. Francis. 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. J. Immunol. 169:6822-6830.

Plagemann, P. G., Q. A. Jones and W. A. Cafruny. 2001. Polyclonal activation of B cells by lactate dehydrogenase-elevating virus is mediated by N-glycans on the short ectodomain of the primary envelope glycoprotein. Adv. Exp. Med. Biol. 494:375-384.

Lemke, C. D., J. S. Haynes, R. Spaete, D. Adolphson, A Vorwald, K. Lager and J. E. Butler. 2003. Lymphoid hyperplasia resulting in immune dysregulation is caused by PRRSV infection in pigs. J. Immunol. 172:1916-1925.

Holtmeier, W., W. Geisel, K. Bernert, J. E. Butler, M. Sinkora, J. Sinkora and W. F. Caspary. 2004. Prenatal development of the porcine TCRδ repertoire: Dominant expression of an invariant T cell receptor δ chain. Eur. J. Immunol. 34:1941-1949.