RESEARCH REPORT
SWINE HEALTH

Title: Antigen-specific T cell responses associated with PCVAD pathogenesis - **NPB # 10-012**

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Industry Summary: This project was intended to determine whether or not pig with PCVAD had T lymphocytes that responded to different epitopes of the PCV CP, when compared with vaccinated pigs or pigs that had been infected with PCV2 alone. The PCVAD model used was the dual-infection model with PRRSv and PCV2 as the pathogens. Work from Dr. Rowland's lab had shown that serum antibodies from infected pigs identified a region of the coat protein (CP) that was less readily recognized by antibodies from vaccinated pigs, and our previous studies had shown that PCV2 vaccinated pigs had T cells that recognized a specific pattern of epitopes from CP. Because there were differences in antibody epitopes, we reasoned that differences would also exist in T cell epitopes. Therefore, we hypothesized that infected pigs would recognize a different array of peptides from CP compared with vaccinated pigs, and that dual-infected (PCVAD) pig T cell epitopes would differ from those of PCV2-infected pigs. Experiments were performed using sets of 4 pigs, each one either either:1) untreated, 2) vaccinated with a commercially available vaccine, 3) infected with PCV2, or 4) infected with PCV2 and PRRSv. Blood was collected for serum at intervals throughout the experimental period, and PRRSv and PCV2 antibodies were evaluated. During active infection, CP polypeptide ELISA assays were run to evaluate antibody binding to either the full-length or truncated CP peptide. At necropsy, lymph nodes were taken for histology, and single cell suspensions were prepared for immunophenotyping by flow cytometry, and T cell proliferation and Interferon-gamma ELISPOT assays to detect responses to individual chemically synthesized CP 30-mer peptides. The T cell epitope results showed that different patterns of epitope recognition occurred among vaccinated, PCV2-infected and dual-infected pigs, with vaccinated pig responses resembling our previous study, PCV2 infected pig T cells recognizing primarily 2 peptides at the amino terminus of the protein, and dual-infected pig T cells primarily recognizing 5 peptides at the carboxy terminus of the CP protein. These data were consistent with our hypothesis. Additional support was obtained from the antibody assays, lymphoid cell measurements, immunophenotyping and histological examinations. These findings confirm that PCV2- and dual-infected pigs have T cells that respond to fewer peptides compared with vaccinated pigs and that the epitopes recognized by PCV2-infected pig T cells are in a different location on the protein compared with dual-infected pig T cells. These findings suggest that PCVAD represents an inability of the pig immune system to adequately control the virus, perhaps as a result of co-infection with PRRSv. Thus, prevention of infection by PRRSv likely represents an important control strategy for PCVAD.

Keywords: PCVAD, T lymphocytes, epitopes, coat protein (CP), lymph nodes, Interferon-gamma

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Abstract: The hypothesis for this project was that the T cell epitope response pattern of pigs infected with PCV2, or dual-infected with PCV2 and PRRSv, will differ from that of vaccinated pigs, with the differences associated with disease severity. Pigs were infected using a PCV2-PRRSv dual-infection model to induce PCVAD, and lymphoid cells, isolated from lymph nodes taken from those pigs at necropsy, were used to map T cell epitopes recognized by the pigs. The T cell epitope expression pattern was compared with the patterns from T lymphocytes from pigs infected with PCV2 alone, and from pigs vaccinated with a commercially available PCV2 vaccine. Untreated control pigs were included to confirm specificity of the responses. Supporting data included anti-PCV2 antibody titers during the infection period, CP B cell epitopes recognized by sera during infection, lymph node weights, lymph node histopathology and PCV2 antigen immunohistochemistry, and immunophenotyping of lymph node lymphoid cell populations. The results indicate that PCV2-infected and dual-infected pigs have T lymphocyte epitope recognition patterns that differ from vaccinated pigs and from each other, with dual-infected pig T cell epitopes clustering at the carboxy-terminus of the protein and PCV-2 infected pig T cell epitopes more prominent at the amino-terminus of the protein. There are no significant differences between the number of lymphoid cells per gram of lymph node tissue among any of the treatment groups. However, T lymphocytes tend to predominate over B cells and macrophages in control pig nodes and have low CD25 expression, compared with vaccinated pigs that have a larger proportion of B lymphocytes and/or follicular dendritic cells and substantial CD25 expression, and PCV2 single- and dual-infected pigs that have roughly equal proportions of T cells and B cells/follicular dendritic cells and somewhat less CD25 expression. Histopathology shows that dual-infected pigs have epithelioid clusters of macrophages in lymph node germinal centers and collections of macrophages positive for cytoplasmic PCV2 antigen, compared with the other treatments. Anti-PCV2 titers show that PCV2- and dual-infected pig titers rise late in the infection process and remain high while vaccinated pig titers rise early after vaccination and remain elevated. Control pigs do not have high titers of anti-PCV2 antibodies. B cell epitope data largely agree with previous studies.

There was one pig in the dual-infected group that differed substantially from other dual-infected pigs. This pig had anti-PCV2 titers that were relatively low early in infection and became negative by 40 days post infection. The T cell epitope expression pattern from this pig resembled the vaccinated pig pattern in that epitopes at both ends of the CP protein were recognized; however, the number of epitopes recognized was limited compared with the vaccinated pigs. Histologically, lymph nodes from this atypical dual-infected pig did not have epithelioid macrophages and contained rare cells expressing PCV2 antigen in the nucleus, rather than the cytoplasm.

Introduction: the hypothesis for this project is that pigs infected with both PCV2 and PRRSv will have T lymphocytes that recognize different epitopes from the capsid protein (CP) of PCV2 compared with PCV2 infected and vaccinated pigs. Confirmation of this hypothesis adds to the knowledge of the immunology and pathogenesis of PCVAD, and represents important information about differential immune responses between infected and protected pigs.

Objectives: The objective is to determine whether or not the T cell epitope response pattern of pigs infected with PCV2, or dual-infected with PCV2 and PRRSv, differs from that of vaccinated pigs.

Materials and Methods:

Our overall approach was to infect pigs using a PCV2-PRRSV dual-infection model to induce PCVAD, and to use lymphoid cells isolated from lymph nodes taken from those pigs at necropsy to map T cell epitopes that are recognized by sick pigs. The T cell epitopes recognized by PCVAD pigs were compared with those recognized by T lymphocytes taken from pigs infected with PCV2 alone and from CP vaccinated pigs. Negative control pigs were used to confirm specificity of the T cell responses. Additional supporting data was gathered, including lymph node histopathology, immunophenotype of lymph node lymphoid cell populations, and B cell epitopes recognized by sera taken from the pigs.

Pigs were arbitrarily assigned to one of four treatment groups (see Table 1). A total of 28 pigs were used for the experiment. For vaccination, two doses of CP vaccine were delivered three weeks apart in the neck muscle. Negative control pigs received saline at the same intervals. Two weeks after the last vaccination, the remaining pigs were infected and blood was taken for serum at intervals over the course of the study. Sera were tested for the presence of PCV2 and PRRSV by standard PCR and serological assays performed in the Kansas State Veterinary Diagnostic Laboratory (KSVDL), and in collaboration with Dr. Rowland. At the end of the experiment, pigs were necropsied, and blood was collected for serum to map B cell epitopes to CP using an ELISA developed in Dr .Rowland's laboratory. Lymph nodes were collected for histopathology and evaluated by Dr. Nietfeld at the diagnostic laboratory, and lymphoid cell suspensions were generated in order to perform immunophenotyping to characterize T cell, B cell, and monocytic cell populations, and to perform functional assays to characterize T cell responses to CP peptides (IFN-γ ELISPOT and flow cytometric proliferation).

Table 1. Experimental infection model treatments

			Treatment		
Grp	Description	n	Vaccine	PCV2	PRRSV
1	Control	7	-	-	-
2	Vaccine	7	+	-	-
3	PCV2	7	-	+	-
4	PCV2/PRRSV	7	-	+	+

Methods

CP polypeptide and oligopeptide ELISA. ELISAs using CP polypeptide and oligopeptides are in use in Dr. Rowland's laboratory. Briefly, Each CP polypeptide or BSA-conjugated CP oligopeptide is diluted in 0.05 M carbonate binding buffer (pH 9.6) and 100 µL added to each well of an ELISA plate (Costar). Commercially prepared 20-mer oligopeptides (21st Century Biochemicals) are conjugated to BSA by the manufacturer. For the purpose of conjugation to BSA, a cysteine was added to the N or C-terminal end of the peptide. Antigen-coated ELISA plates were incubated overnight at 4^0C. Wells are washed with 0.01% Tween 20 in PBS (PBST) then blocked at room temp with 5% goat serum. Dilutions of pig sera are prepared in PBS with 5% goat serum (PBS-GS) and incubated at room temp. After washing with PBST, bound porcine antibody is detected using peroxidase-labeled goat anti-swine antibody (Accurate Chemical & Scientific Corp.) diluted in PBS-GS and incubated at room temperature. After extensive washing with PBST, peroxidase activity is detected using an ABTS chromogenic substrate kit (KPL) and absorbance read at 405 nm.

Preparation of lymph node cells. Peripheral lymph nodes were collected and a sample was stored in 10% buffered formalin for subsequent sectioning and evaluation of structure and cellularity. The remaining nodes were placed into sterile, cold PBS containing antibiotics for transport to the laboratory. Fat and stroma were dissected away, and the nodes were blotted dry and weighed on sterile gauze pads. The nodes were minced to approximately 1 mm pieces in PBS containing 20% ACD. Leukocyte rich supernatants were collected, the remaining pieces were pressed through wire mesh screens, and the resulting suspensions added to the supernatants. The tubes were placed on ice 30' to allow small pieces to settle out, and then the suspensions were collected, centrifuged and resuspended in PBS/ACD. The suspensions were underlayered with Ficoll-Hypaque and centrifuged. The interface cell layers containing lymphoid cells were collected, washed, counted and used in the assays. Viabilities were checked by Trypan blue dye exclusion.

Immunophenotyping of lymphoid cells from lymph nodes. Monoclonal antibodies (mAb) to T lymphocyte subsets (CD4, CD8, WC1), B lymphocytes (CD79, CD21), macrophage subsets (SWC3, CD163), and the IL-2 receptor (CD25, used as an indicator of activation/regulation) were used for immunophenotyping of lymph node cell suspensions. The method is described in Wyatt et al (1994). Briefly, cells diluted into buffer containing fetal bovine serum and azide were incubated in wells of 96-well V-bottom plates, each containing a single antibody. After incubation on ice for 30 minutes, followed by washing, the cells were

incubated in the secondary detection antibody for 30 minutes on ice. After additional washing, the cells were resuspended in formaldehyde-containing buffer and stored at 4^0C until analyzed by flow cytometry. The detection antibodies were FITC-labeled (FL-1) goat anti-isotype-specific antibodies.

In vitro cultures of lymphoid cells from lymph nodes. Cells were cultured at 2 X 10^6 cells/well in RPMI-1640 containing 10% fetal bovine serum, HEPES, 2-mercaptoethanol, and antibiotics in 24-well culture plates. Cultures were incubated with no stimulation, or with chemically synthesized peptides that overlapped by 10 amino acids each and that were based on the amino acid sequence of the ORF2 capsid protein of PCV2. Culture controls included: a) Con A (positive control) and, b) unstimulated cells.

ELISPOT assay for IFN-γ secretion from lymphoid cells. The ELISPOT assay was performed on cultures incubated on Immulon plates coated with anti-porcine IFN-γ following the method published by Meier et al. The antibody reagents are commercially available and the assay is routinely used in our laboratories.

T lymphocyte proliferation. For proliferation studies, MLN cell suspensions were labeled with PKH67, a green fluorescing molecule (Fl-1) that inserts into cell membranes and remains in the membranes. As the cell divides, the amount of label in the membranes is decreased by half, so that cells that have proliferated are less fluorescent compared with cells that have not responded (the protocol we use was originally provided by Dr. M. McCaw, N.C. State [deceased]). Controls included: a) Con A (positive control) and, b) no stimulation. After culture, the cells were collected, and aliquots are surface labeled with mAb to CD4. Secondary, RPE-labeled anti-mouse antibody (Fl-2) was added to detect bound mAb. All of the reagents are commercially available. The labeled cells were subjected to dual fluorescence flow cytometric analyses to determine the proportion of $CD4^+$ T cells that proliferated to each peptide.

Results:

Anti-PCV2 titers during infection. Figure 1 contains representative titers from control, vaccinated, PCV2 infected, and dual-infected pigs. Figure 1A shows that antibodies to PCV2 from vaccinated pigs rose early after vaccination, by 27 days post inoculation, and remained high through the period. In contrast, anti-PCV2 titers from single- and dual-infected rose later in infection. Control pigs tended to remain antibody negative. The exception was the atypical dual-infected pig (Figure 1B) in which antibody titers were low early in the infection, and turned negative at approximately 42 days post infection.

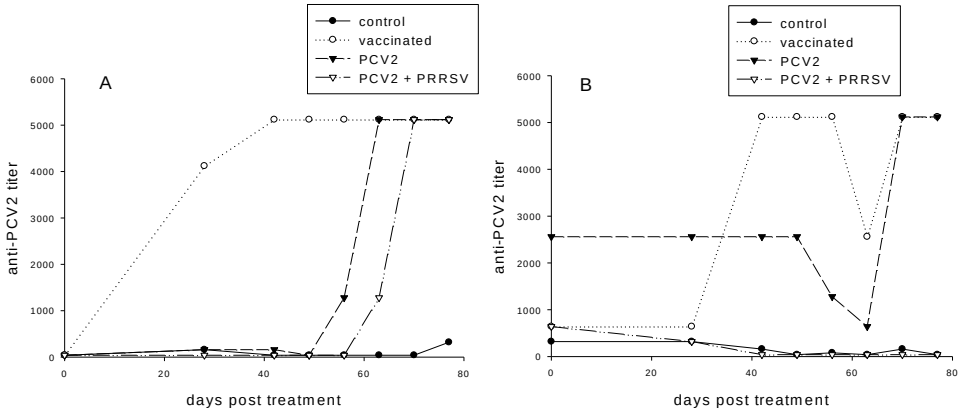

Figure 1. Anti-PCV2 titers with time after inoculation of pigs with commercial PCV2 vaccine, PCV2 virus, or PCV2 and PRRSv. Panel A is a representative set of sera from the majority of pigs in the study. Panel B shows the anti-PCV2 titer pattern of the dual-infected pig with the atypical disease presentation.

Antibody epitopes of infected/vaccinated pigs. Sera from the groups of pigs were assayed against the large polypeptide (amino acids 43-233) and a smaller peptide (amino acids 169-180) from the PCV2 coat protein. Figure 2 contains a representative set of sera from the study pigs. Pigs tended to have likely maternal

antibodies to PCV2 upon arrival at the facility. Thus, both the large and the small peptides were recognized by sera from those pigs. During active infection, vaccinated and PCV2 infected pigs tended to have antibodies predominantly directed against the large polypeptide, while the dual-infected pigs tended to have antibodies that recognized both the large and the smaller peptides to roughly equal degrees. Serum from the atypical pig had a recognition pattern similar to the vaccinated and PCV2 infected pigs (not shown).

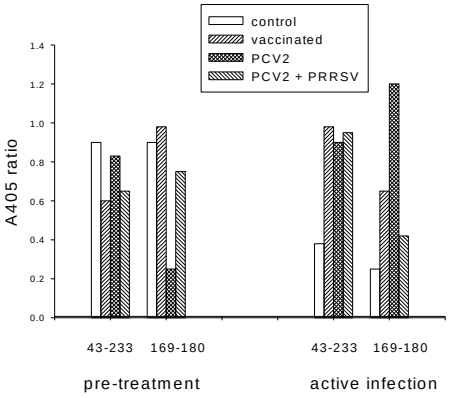

Figure 2. Representative anti-PCV2 titers to the large and smaller peptides from CP.

Histopathology and PCV2 antigen in lymph nodes. At necropsy, lymph nodes from control pigs were relatively quiescent, while those from vaccinated and PCV2-infected pigs showed substantial numbers of germinal centers. No PCV2 antigen was detected in sections from those nodes (sections not shown). However, the dual-infected pigs had clusters of epithelioid macrophages and macrophages with cytoplasmic PCV2 antigen. Figure 3 shows a representative sample of dual-infected pig nodes. Figure 3A shows the pattern seen with the majority of dual-infected pigs. In contrast, Figure 3B shows the pattern seen in the node of the atypical dual-infected pig.

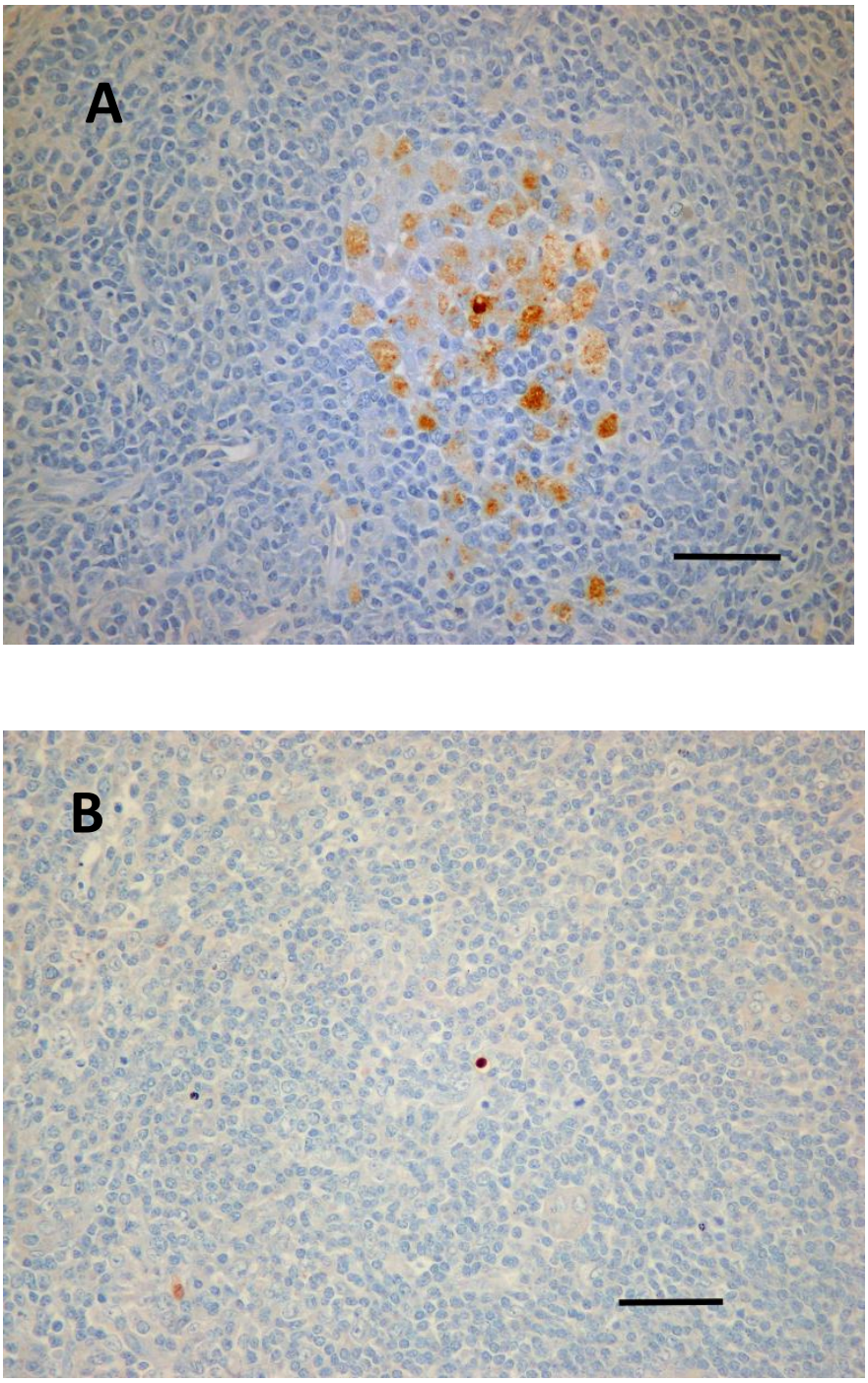

Figure 3. Representative immunohistochemistry/histopathology of nodes from dual-infected pigs. Panel A depicts the pattern seen with the majority of dual-infected pigs. Panel B shows the pattern seen with the atypical dual-infected pig. Bars are 50 μm.

Lymphoid cell recoveries and immunophenotypes from study pigs.

Figure 4 depicts the number of lymphoid cells/gm of tissue (Panel A) and the immunophenotype of those cells (Panel B). There was a trend toward a decreased number of lymphoid calls/gm of tissue in the control pigs, which is consistent with relative quiescence in those animals. There were substantially increased numbers of lymphoid cells/gm of tissue in the vaccinated, PCV2 infected, and dual-infected pig nodes, consistent with

lymphoid cell activity, germinal centers and macrophage function in those animals. However, the differences were not statistically significant (P=0.191 by Kruskal-Wallis ANOVA), likely because of variability among individual pigs in response to inoculation. The immunophenotype data showed that T lymphocytes predominate over B cells and macrophages in control pig nodes and CD25 expression was low, consistent with lack of immune activity. Vaccinated pigs had a larger proportion of B lymphocytes and/or follicular dendritic cells and substantial CD25 expression, and PCV2 single- and dual-infected pigs had roughly equal proportions of T cells and B cells/follicular dendritic cells and somewhat less CD25 expression, consistent with activation and an immune response.

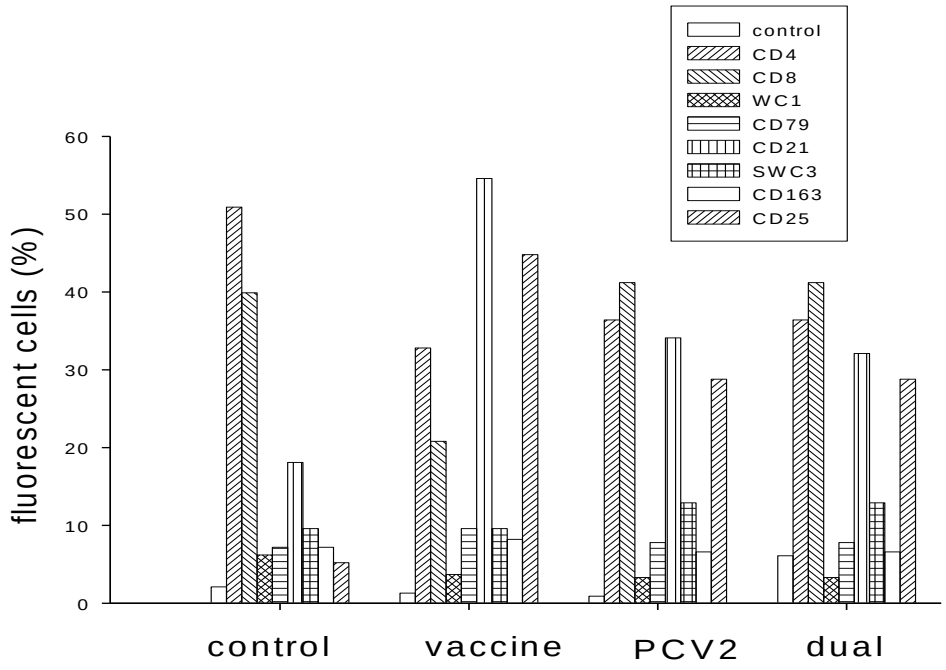

Figure 4. Immunophenotype of lymphoid cells from control, vaccinated, PCV2 infected, and dual-infected pigs. Each bar represents the relative percentage of fluorescent cells in a representative pig set.

T cell epitope patterns from study pigs to CP peptides. Figure 5 shows the patterns of T cell epitope expression in lymphoid cells from vaccinated, PCV2 infected, and dual infected pigs. The data represent Interferon (IFN)-γ secretion by lymphocytes after culture with individual peptides synthesized based on the amino acid sequence of PCV2 CP. Control pig expression was subtracted from the inoculated pigs within each group. Figure 5A depicts the response pattern from vaccinated pig T lymphocytes. Similar to our previous vaccination study (not shown), the pattern shows responses to epitopes from the amino-terminal end (P1, P2, and P4), and the carboxy-terminal end (P6-P10), but not to P5 or the final carboxy-terminal P11. The response pattern from PCV2-infected pigs, depicted n Figure 5B, differs from the vaccinates in that the predominant reactivity is at the amino-terminal end (P1 and P2), with relatively little reactivity at the carboxy-terminal end. The response pattern for the dual-infected pigs (Figure 5C) differs from both of the other treatments in that the main responses are to epitopes at the carboxy-terminal end (P6-P10). In Figure 5D is the atypical dual-infected pig T cell epitope response. It resembles the vaccinates in that peptides at both ends of the protein induce a response, but there are fewer total epitopes recognized compared with the vaccinates, and fewer.

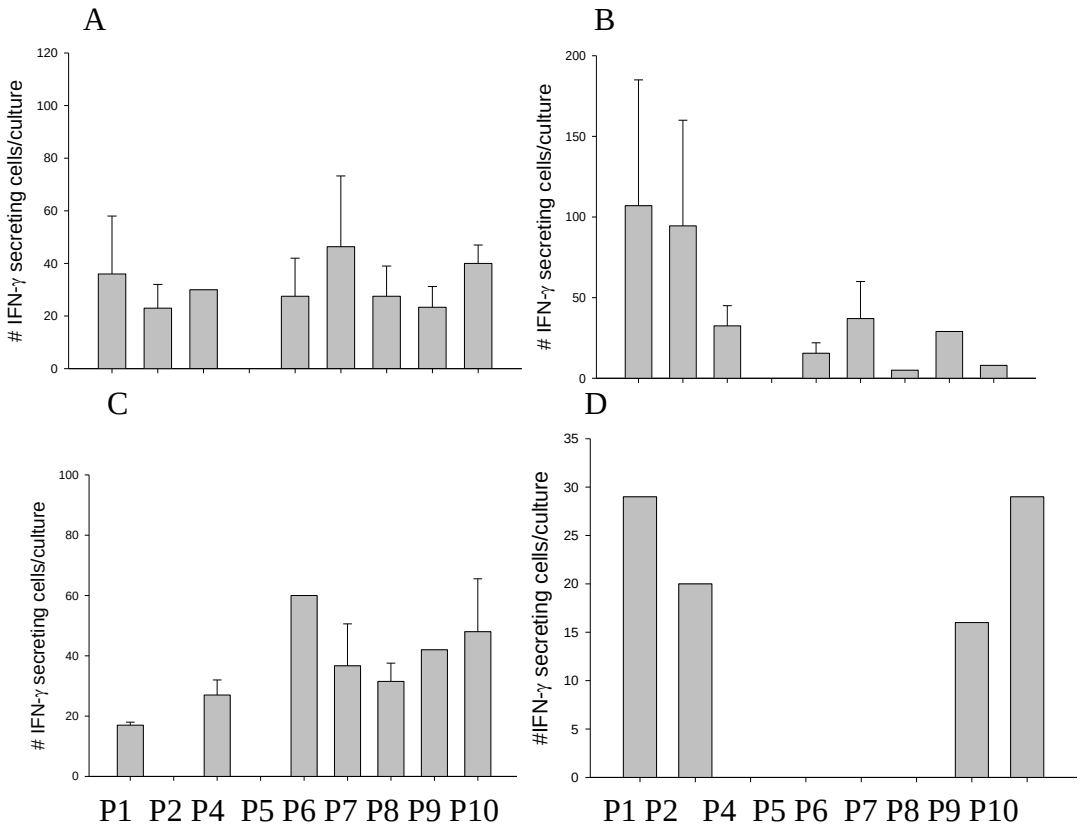

Figure 5. Representations of the T cell epitope response patterns from the pig treatment groups. Panel A vaccinated, Panel B PCV2 infected, Panel C majority of dual-infected pigs, Panel D atypical dual-infected pig.

T lymphocytes responded by secreting IFN-γ, compared with the other dual-infected pigs. The proliferation assay results were similar, with CD4+ T cells proliferating in response to the epitopes (not shown).

Discussion: The results confirm our hypothesis that dual-infected pig T lymphocytes respond to T cell epitopes from PCV2 CP in a different pattern compared with vaccinated or PCV2 infected pigs. Interestingly, the PCV2 infected pigs had a different T cell epitope response pattern from the vaccinated pigs as well. The supporting data are consistent with anti-PCV2 antibody titers rising at expected times after vaccination or infection, and are supported by previous studies on B cell antibody epitopes from Dr. Rowland's laboratory. The differing immunophenotype patterns are consistent with active lymph nodes in infected and vaccinated pigs and relatively quiescent lymph nodes in controls. The histopathology data indicate that dual-infected pig nodes retain substantial amounts of PCV2 antigen in cytoplasm, suggesting that PCV2 infection continues longer in those animals compared with the other inoculated groups. Together, these data indicate that dual-infected pigs have an abnormal immune response to PCV2 possibly as a result of co-infection by PRRSv with subsequent suppression or decoying of the immune response away from the PCV2 virus. This research is potentially important to pork producers because it dissects the differences in immune responsiveness that occur in PCVAD, laying the groundwork for potential treatments or vaccines that can control this disease.