RESEARCH REPORT
SWINE HEALTH

Title: PCV2 replication and disease enhancement through lymphocyte stimulation – **NPB #06-095**

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Industry Summary:
Porcine circovirus type 2 (PCV2) causes a number of diseases including PMWS. The number of pigs that are infected with PCV2 that go on to clinical disease is extremely variable. The exact mechanism that causes PCV2 infection to result in disease is currently unknown. Currently, inducing clinical disease associated with PCV2 under laboratory conditions has been unreliable. Work in our laboratory had shown that the virus replicates in monocytes and replicating lymphocytes. In this study, we proposed to assess the replication of the virus in lymphocytes from tissue and blood from pigs concurrently infected with PCV2 and *Mycoplasma hyopneumoniae* (MHYO). We chose this model as we had previously observed an increase in the number of pigs exhibiting PCV2 associated disease when co-infected with MHYO in addition to PCV2. In this study, we studied the ability of lymphocytes to proliferate and the amount of virus produced in the presence of PCV2 with and without MHYO compared to non-infected pigs. Pigs from each group were sacrificed at 7, 14, and 21 days following PCV2 infection. While we confirmed that pigs in the appropriate groups were infected with either PCV2 and/or MHYO based on pneumonia, serology and the presence of virus, no clinical disease or symptoms occurred. The lymphoid depletion, which is a hallmark of PCV2 disease, was also extremely mild, although we demonstrated the presence of virus in the tissues. No alterations in the lymphocyte populations or their ability to proliferate and respond to antigen were observed in this study. This study did show that in the absence of clinical disease, PCV2 does not appear to suppress or modulate the immune system. More work needs to be done to identify why some pigs, such as in this study, remain healthy in the presence of MHYO and PCV2 and others go on to develop clinical disease that typically results in death.
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Scientific Abstract:
Porcine circovirus type 2 (PCV2) has been shown to induce a number of diseases including postweaning multisystemic wasting syndrome (PMWS). Many PCV2 infected pigs remain asymptomatic, while other pigs in the same herd and farm develop severe disease. The mechanism by which disease is triggered from infection is currently unknown. Based on earlier work that found proliferating lymphocytes had increased virus replication *in vitro*, we hypothesized that pigs with activated lymphocytes would result in an increase in the number of animals that developed clinical disease. A model using co-infection with *Mycoplasma hyopneumoniae* (MHYO) and PCV2, which had previously been shown to have an increased incidence of PCV2 disease, was used to evaluate the effect of dual infection on lymphocytes from infected pigs and their ability to respond to mitogens and MHYO antigens. Macroscopic and microscopic lesions, PCV2 viremia and antibody response, real-time PCR for MHYO and PCV2 DNA and a real-time RT-PCR that measures the PCV2 Cap as a measure of replication were assessed. No pigs demonstrated clinical disease throughout the trial. The pigs developed significant pneumonia consistent with MHYO infection, were viremic and developed serum antibodies to PCV2 indicating successful inoculations. However, microscopic evidence of PCV2 induced disease based on lymphoid depletion and clinical disease was lacking. Lymphocytes from peripheral blood, bronchial and inguinal lymph nodes and lungs were isolated, cultured with either mitogens or MHYO antigen and stained followed by assaying by flow cytometer for proliferation and apoptosis. Even though the pigs were infected with PCV2, in the absence of apparent disease, no alteration in lymphocyte populations, ability to be stimulated and proliferate or increased apoptosis was observed, demonstrating that the presence of PCV2 alone does not result in alteration or suppression of lymphocytes. More research is required to determine the mechanism by which PCV2 infection results in disease in pigs.

Introduction:

Porcine circovirus type 2 (PCV2) is a member of the *Circoviridae* family that causes PCV associated diseases (PCV-AD). Infection in pigs may result in a number of clinical syndromes including postweaning multisystemic wasting syndrome (PMWS). Microscopically, PCV2-induced lesions are characterized by changes in the lymphoid tissues including lymphocyte depletion of follicular and interfollicular areas with an accompanying infiltration by macrophages.[1] Consistently, PCV2 DNA and RNA are detected in various immune cells of PCV2-infected pigs including monocytes and lymphocytes. Studies have shown a strong correlation between the amount of PCV2 protein and/or genetic material detected in tissues and the severity of the lymphoid depletion.[2-4]

The etiology of PCV-AD and the mechanism behind its progression from PCV2 infection remains unclear. A number of investigators have hypothesized that progression of PCV2 infection to PCV-AD is through activation of the immune system.[5,6] Induction of PCV-AD has been difficult to achieve with disease occurring in only a very low percentage of pigs. Some of the co-factors used to induce PCV-AD have included other pathogens or immune activators such as vaccines or the use of non-specific mitogens.[7-9] A recent study demonstrated that infection with PCV2 and *Mycoplasma hyopneumoniae* (MHYO) increased the proportion of pigs that developed PCV-AD.[10] The interaction between these two pathogens provides a unique opportunity to investigate the mechanism behind the progression of PCV2 infection to disease.

We have demonstrated that PCV2 replicates in B and T lymphocytes in addition to monocytic cells.[11] One suggested mechanism for the lymphoid depletion observed with PCV2 infection is through apoptosis of virally infected lymphocytes. As the infected lymphocytes undergo apoptosis, macrophages take up PCV2 antigen resulting in depletion of lymphocytes and the presence of PCV2 antigen-filled macrophages in the lymphoid tissues of pigs with clinical disease consistent with PCV-AD. In an earlier study, PCV2 infection was shown to induce apoptosis in lymphocytes, followed by phagocytosis by macrophages resulting in lymphoid depletion.[12] However, another study contradicted these results by finding reduced levels of apoptosis in the B-cell areas of lymphoid tissues of PCV-AD-affected pigs compared to healthy non-PCV2 infected pigs.[13] Decreased cell proliferation and not increased apoptosis has also been suggested as an important factor in the

depletion of cells in lymphoid tissues from PCV-AD-affected pigs under field conditions.[14] Recent research in our laboratory found that PCV2 infection does not interfere with cell proliferation, and that without concurrent cellular stimulation apoptosis is actually *reduced* in peripheral blood mononuclear cells (PBMCs) in the presence of PCV2.[15] However, if PCV2-infected PBMCs were mitogenically stimulated then apoptosis increased significantly[15]. Any stimulation of the immune system, including vaccination or infection, induce lymphocytes to undergo proliferation thus potentially enhancing replication of PCV2 in lymphocytes as they divide as well as increasing apoptosis. A recent *in vitro* study in our laboratory demonstrated that PCV2 replication in peripheral blood monocytes (PBMCs) was significantly increased when stimulated with mitogens compared to unstimulated PBMCs.[16] In addition, it was found that the type of mitogen used to stimulate the lymphocytes affected the level of PCV2 replication. We demonstrated that stimulating lymphocytes with pokeweed mitogen (PWM) significantly increased viral replication and lymphocyte apoptosis compared to the mitogen, conconavlin A (ConA) which stimulates a different population of lymphocytes.[15] This suggests that the type of immune stimulation and perhaps more importantly the population of lymphocytes stimulated may impact viral replication and ultimately the severity of clinical disease observed with PCV2. MHYO has been reported to have mitogenic properties.[17]

Objectives:

The etiology and mechanism by which PCV2 infection progresses to PCV-AD remains unknown. **Our hypothesis for this study was that PCV2 replication is enhanced with the stimulation of specific populations of lymphocytes by co-infection with MHYO.** The effect of PCV2 on cell populations was evaluated using *in vitro* and *in vivo* studies and real-time RT-PCR assay to measure Cap mRNA of PCV2 to assess viral replication in lymphocytes. Concurrently, flow cytometry was performed to identify the specific populations of cells proliferating and undergoing apoptosis in response to stimulation. We had previously demonstrated that co-infection of pigs with MHYO and PCV2 resulted in an increased number of pigs exhibiting clinical disease consistent with PCV-AD. We used this model in the attempt to trigger the mechanisms that results in PCV2 infection progressing to disease. Our specific objectives for this study included:

1. Collect PBMCs from PCV2 negative pigs and measure *in vitro* cellular proliferation, viability and apoptosis as well as the level of PCV2 replication. Non-stimulated cells were compared to cells stimulated with PMW, ConA, or MHYO membrane preparations containing lipoproteins.
2. Lymphocytes from lymph nodes and PBMCs were collected from pigs infected with PCV2 with and without co-infection with MHYO. PCV2 replication and the specific lymphocyte populations undergoing proliferation or apoptosis/was determined and correlated to viral replication.

Materials & Methods:

Challenge preparations:

PCV2 isolate 40859 was propagated in PK-15 cells free of PCV as described previously.[18] The PK-15 cells were maintained in Eagle's minimum essential medium (MEM) and the 4th passage of the PCV2 in PK-15 cells were clarified by centrifugation. The titer for the virus was determined to be $10^{4.75}$ tissue culture infectious dose ($TCID_{50}$) / 100 µl as determined by titration on PK-15 cells using an immunofluorescence staining assay[18].

Mycoplasma hyopneumoniae (MHYO) strain 95MP1509 was grown in Friis media and membrane antigen was isolated using Triton X as described.[19]

Experimental design and sampling

All study procedures and animal care activities were conducted in accordance with the guidelines and under the approval of the Iowa State University (ISU) Institutional Committee on Animal Care and Use.

Sixty cross-bred pigs serologically negative for PCV2, MHYO, and PRRSV antibodies were randomly allotted to 4 groups with 15 pigs each. Throughout the study, pigs were housed in identical isolation rooms based on

their challenge status. Pigs were provided feed and water ad libitum. Pigs in Group 1 were not inoculated and served as negative controls. Pigs in Groups 2 and 4 pigs were inoculated intratracheally with 10 ml of MHYO strain 95MP1509 broth culture. Two weeks post MHYO inoculation, pigs in Group 3 and 4 were challenged with intranasally with PCV2 (Trial day 0). On trial days 7, 14, and 21, five pigs in each group were euthanized, and lung, lymph nodes, serum, bronchoaveolar lavage (BAL), and bronchial swabs were collected.

Table 1. Experimental design and necropsies

Trial Day*		0	7	14	21
Group 1	Negative	n=5	n=5	n=5	n=5
Group 2	MHYO†	n=5	n=5	n=5	n=5
Group 3	PCV2	n=5	n=5	n=5	n=5
Group 4	MHYO†+PCV 2	n=5	n=5	n=5	n=5

*Trial days indicate the days after PCV2 challenge.
Day 0 - bleeding;
Day 7 - bleeding and 1st necropsy;
Day 14 - bleeding, 2nd necropsy;
Day 21 - bleeding, 3rd necropsy.
†MHYO challenge was performed 14 days before PCV2 inoculation.

Necropsy:
Pigs were necropsied and lungs were scored for mycoplasmal pneumonia as previously described.[20] Macroscopic lesions associated with MHYO pneumonia, consisting of well demarcated dark-purplish areas of lung consolidation, were sketched onto a standard lung diagram. The proportion of lung surface with lesions was determined from the diagram using a Zeiss SEM-IPS image analyzing system as previously described.[20] Tissues for histopathological examinations were fixed in 10% formaldehyde in PBS. Tissues, BAL and serum samples for nucleic acid extractions were snap frozen on dry ice and preserved at -80 °C. Peripheral blood was collected by venipuncture for lymphocytes proliferation and apoptosis. Portions of lungs, bronchial lymph nodes and inguinal lymph nodes were placed in 10% formalin, processed and embedded in paraffin using an automated tissue processor. Microscopic examination was made for interstitial pneumonia, peribronchiolar lymphoid hyperplasia, bronchopneumonia, and lymphoid depletion as previously described.[10,19]

PCV2 and MHYO DNA and PCV2 transcript RNA extractions
Viral and mycoplasmal genomic DNA was extracted and purified from 200 µl of serum (for PCV2) or BAL fluid (for MHYO) by using QIAmp DNA Mini Kit following the steps as described previously.[21,22] DNA was eluted in 60 µl (for PCV2) or 100 µ (for MHYO) of elution buffer from the kit.

PCV2 RNA transcripts in tissues from 20 mg by using RNAqueous Kit from Ambion Company as described previously.[22] RNA was eluted in a total volume of 70 µl of elution buffer and was further treated with Turbo DNA-free reagent from Ambion Company to remove DNA contaminations. Final purified RNA was kept at -80 °C.

Real-time PCR assays of PCV2 and MHYO DNA and real-time RT-PCR assays of PCV2 genome transcript
Genomic DNA copy numbers of PCV2 in serum sample and MHYO in BAL were analyzed by real-time PCR assays with primers and probes using Taqman universal PCR master mix (Applied Biosystems) as described previously.[21] PCV2 ORF2 transcript RNA were analyzed by a previously described real-time RT-PCR method

with Taqman one-step universal RT-PCR master mix (Applied Biosystem). All real-time (RT-) PCR reactions were performed on Rotor-Gene RG-300 real-time PCR system (Corbett Research, Sydney, Australia).

Enzyme-linked immunosorbent assay (ELISA) detection of PCV2 antibody
Blood samples were collected on 0, 7, 14 and 21 DPI of PCV2, and tested for PCV2 antibodies using a PCV2 ORF2 ELISA protocol as described previously.[23] Briefly serum samples were diluted in 1:40 with 5% milk diluents (KPL, Gaitherburg, MD) and 100 µl diluted samples and controls was used in the ELISA assay. Plates were incubated at 37 °C for 30 min followed by incubation with peroxidase-labeled anti-pig IgG. The optical density of each well was read at 405 nm with a plate reader (Molecular Devices). Sample/positive (S/P) ratios were calculated. A S/P ratio of 0.20 was used as cut-off value.

Lymphocyte proliferation assay using CFSE staining and apoptosis assays
Peripheral blood mononuclear cells were isolated by centrifugation from blood collected through venipuncture in BD Vacutainer CPT tubes with heparin from pigs at 7, 14, and 21 days post PCV2 infection as described previously.[22] Mononuclear cells from bronchial lymph nodes were isolated as previously described.[23] Mononuclear cells were washed three times in PBS and red cells were removed. The mononuclear cells were either suspended in PBS or whole RPMI1640 medium to proceed for CFSE labeling or cell apoptosis assays.

Cell proliferation assays were performed with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA) stained PBMC as described previously. Briefly, 1×10^6 CFSE stained PBMC cells were added wells of 96-well plates. Cells in each well were left untreated, or stimulated with ConA at 5.0 µg/ml, pokeweed mitogen (PWM) at 10 µg/ml, or MHYO membrane antigen. The stimulated and unstimulated cells were incubated for 5 days, and then stained anti-CD4-Tricolor and anti-CD8-PE antibodies for T cells, or anti-CD19-PE antibody for B cells. Raw data of CFSE, tricolor and PE three color for T cells, or CFSE and PE two color for B cells were acquired from 10,000 events per sample. The raw data from flow cytometry were further analyzed with ModFit LT 3.0 to calculate proliferation indices for each mitogen or MHYO antigen stimulated cells as described previously.[15]

For the apoptosis assay, mononuclear cells from either peripheral blood or lymph nodes were suspended in whole RPMI1640 medium and seeded in flat-bottom 96-well plates with 1×10^6 cells per well in 200 µl medium as previously described.[23] The cells were left either unstimulated or stimulated with ConA (5 µg/ml) or PWM (10 µg/ml). After 5 days incubation, cells were stained with CD3-FITC antibody for T cells, or CD21-FITC for B cells, followed by Annexin V (AN)-PE (BD bioscience) and 7-amino-actinomycin D (7AAD) (BD Bioscience) staining followed by assaying in a flow cytometer. Raw data for FITC, PE, and 7AAD intensity were acquired for 10,000 events. FlowJo software was used to further analyze flow cytometry data. Apoptotic indices (AI) for stained T cells and B cells were calculated in the gated T or B cell subpopulations with the percentage of AN positive and 7AAD negative cells divided by the percentage of AN positive and 7AAD negative cells plus the percentage of AN negative and 7AAD negative cells).

Statistic analysis
Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. $P < 0.05$ was considered to be statistically significant for all test procedures. The correlation of the copy numbers of PCV2 Cap mRNA, stimulation index and
apoptotic index were evaluated by nonparametric correlations test, Spearman's Rho in Multivariate Methods using JMP5.1 (SAS Institute Inc., Cary, NC).

Results:

Clinical disease:

No clinical disease consistent with PCV-AD was observed in any of the pigs throughout the trial. By day 21 post infection, the pigs infected with PCV2 weighed more than either of the MHYO infected groups, but did not differ from the Negative control pigs. There was no difference in weights between the two MHYO infected groups.

Table 2. Body weight of pigs before and after challenge

Groups	D-35	D-15	D0	D7	D14	D21
Negative	11.0±1.8	26.2±3.7	47.7±5.0^{b}	57.5±6.2	68.5±5.0	79.2±4.8^{ab}
MHYO	11.3±1.4	28.7±2.4	48.4±4.2^{b}	59.5±4.8	66.6±4.7	71.8±5.9^{b}
PCV2	12.4±2.0	29.0±4.2	54.0±6.4^{a}	61.7±6.8	73.0±8.9	88.6±4.34^{a}
MHYO and PCV2	11.7±1.5	27.6±3.1	49.6±5.5^{ab}	58.5±6.3	66.7±8.6	70.6±12.1^{b}

Pig were weighed day -35, -15, 0, 7, 14, and 21 following PCV2 infection. Values presented are the means and standard deviations of body weight in pounds of pigs in each group.

Pathology and PCV2 IHC staining:

As can be observed in Table 3, pigs inoculated with MHYO had significant pneumonia at 7 DPI, which was 3 weeks post inoculation with MHYO. No differences in the severity of MHYO pneumonia was observed in pigs challenged with PCV2 in addition to MHYO. These results were confirmed by the microscopic scoring.

Table 3. Pathology scores of lungs

Days	Groups	% Pneumonia	IntPn	PBLH	Bronch
7 DPI	Negative	0.8^{3} ± 1.5^{C}	0.6§ ± 0.6^{b}	0.4 ± 0.9^{bc}	0.0 ± 0.0^{b}
	MHYO	18.4 ± 7.5^{a}	2.0 ± 1.0^{a}	1.8 ± 0.8^{a}	0.6 ± 0.6^{ab}
	PCV2	0.3 ± 0.4^{c}	1.0 ± 0.7^{ab}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}
	MHYO & PCV2	9.8 ± 7.6^{b}	2.0 ± 0.71^{a}	1.4 ± 1.3^{ab}	0.8 ± 0.8^{a}
14 DPI	Negative	0.3 ± 0.7^{b}	0.2 ± 0.5^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0a
	MHYO	14.3 ± 6.8^{a}	1.4 ± 0.6^{a}	1.8 ± 1.1^{a}	0.6 ± 1.3^{a}
	PCV2	0.0 ± 0.0^{b}	0.8 ± 0.8^{ab}	0.40 ± 0.6^{b}	0.0 ± 0.0^{a}
	MHYO & PCV2	21.6 ± 18.4^{a}	1.6 ± 0.6^{a}	2.6 ± 0.6^{a}	0.2 ± 0.45^{a}
21 DPI	Negative	0.3 ± 0.5^{b}	0.8 ± 0.5^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{a}
	MHYO	2.8 ± 2.0^{ab}	1.2 ± 0.8^{a}	1.8 ± 1.1^{a}	0.6 ± 0.9^{a}
	PCV2	1.2 ± 0.6^{b}	1.0 ± 0.7^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{a}
	MHYO & PCV2	6.7 ± 5.6^{a}	1.2 ± 0.5^{a}	1.4 ± 1.1^{ab}	0.2 ± 0.5^{a}

Pathology scores of lung tissues collected on day 7, 14, and 21 following PCV2 infection. Lung tissues were scored for percentage of pneumonia, the presence of interstitial pneumonia (InPn), perobronchiolar lymphoid hyperplasia (PBLH), and suppurative bronchopneumonia (Bronch). The values represent mean scores of each group on a scale of 0 to 3 with standard deviations The values presented are the means and standard deviations from each group. Different superscripts (a, b) within each column indicate significant difference of mean score within treatments for each day post-infection ($P < 0.05$).

Lymphoid tissues were scored for the presence of lymphoid depletion and histocytic replacement of follicles. No differences were observed in any of the groups at any date (data not shown). But PCV2 IHC staining of the lymphoid tissues and lungs did show the presence of PCV2 in these tissues (Table 3).

Table 4. IHC scores of PCV2 in lungs and lymphoid tissues

Groups	Neg	Mhyo	PCV2	Mhyo+PCV2
7dpi	0	0	0.4 ± 0.9	0
14dpi	0	0	1.2 ± 1.3	2.8 ± 2 .3
21dpi	0	0	0.6 ± 0.9	0.2 ± 0.

Numbers show number of cells staining positive/100 cells and standard deviation

PCV2 and MHYO DNA and Cap RNA, and antigen:
No differences were observed in the levels of MHYO DNA between any of the infected groups at any point in the study (Table 4).

Table 5. Quantitative real-time PCR of Mhyo in lung lavage

Groups	Neg*	Mhyo	PCV2	PCV2 and Mhyo
7dpi	ND	1.6 ± 1.6	ND	0.6 ± 0.8
14dpi	ND	2.3 ± 2.4	ND	1.0 ± 1.1
21dpi	ND	4.7 ± 4.2	ND	2.2 ±2.2

*ND means not done. Values show mean ng/µl of Mhyo genome DNA and SD in lung lavage fluid and standard deviation.

As shown in Tables 5, PCV2 DNA and replication were assessed using real-time PCR assays. PCV2 viremia was observed in the appropriate groups following inoculation with PCV2. However, no differences in the level of viremia were observed between the two groups, independent of MHYO infection status. Similarly, the presence or absence of MHYO made no difference in the replication of PCV2 in any of the tissues assessed using the quantitative real-time assay for the Cap mRNA (Table 7).

Table 6. Quantitative real-time PCR assays of PCV2 DNA in serum samples

Groups	D0	D7	D14	D21
Negative	ND*	0	0	0^{b}
MHYO	ND	0	0	0^{b}
PCV2	0.078 ±0.548	2.389 ± 1.114^{a}	4.142 ± 0.852^{a}	3.137 ± 0.972^{a}
MHYO and PCV2	0.168 ± 0.390	1.856 ± 1.251^{a}	3.981 ± 1.283^{a}	2.429 ± 1.240^{a}

*ND means not done. PCV2 DNA in serum samples collected on day 7, 14, and 21 following PCV2 infection was quantified by real-time PCR. Values presented are the means and standard deviations of the logarithmically transformed PCV2 DNA copy numbers per microliter sample. Different superscripts (a, b) within each column indicate significant difference of mean score within treatments for each day post-infection ($P < 0.05$).

Table 7. Quantitative real-time RT-PCR assays of PCV2 Capsid transcript RNA in tissues

Groups		BLN	ILN	LNG
7 DPI	PCV2	2.352±2.847	1.922±2.162	1.832±1.846
	MHYO and PCV2	2.890±2.191	2.165±1.452	2.148±2.754
14 DPI	PCV2	4.121±0.843	2.154±1.890	4.213±1.453
	MHYO and PCV2	4.898±1.034	4.132±1.026	4.209±1.693
21 DPI	PCV2	3.024±1.288	1.625±0.801	1.683±1.498
	MHYO and PCV2	2.815±0.434	1.070±1.637	2.191±1.593

Real-time RT-PCR were used to analyze PCV2 capsid transcript RNA in bronchial lymph nodes (BLN), inguinal lymph nodes (ILN) and lungs (LNG) in pigs infected with PCV2 only and dually infection pigs by PCV2 and MHYO on day 7, 14, and 21 following PCV2 infection. Values presented are the means and standard deviations of the logarithmically transformed copy numbers of PCV2 capsid transcript RNA. Statistical analysis showed there were no significant differences in the PCV2 RNA copy number between the PCV2 only infected and PCV2 and MHYO dually infected pigs.

PCV2 Serology

Pigs infected with PCV2 were seropositive by Day 21. The negative control and MHYO infected groups remained negative for PCV2 antibodies throughout the trial (data not shown).

Lymphocyte proliferation and apoptosis

Although there occasionally were significant differences observed in the proliferation and apoptosis of lymphocytes between groups, no biologically significant differences were observed. Figures 1 and 2 show the cell proliferation and apoptosis assay data at day 21 post PCV2 inoculation. We used several different statistical and analysis programs while evaluating the data, but no significant differences in the overall response by the lymphocytes was observed in any of the groups and date point post PCV2 inoculation.

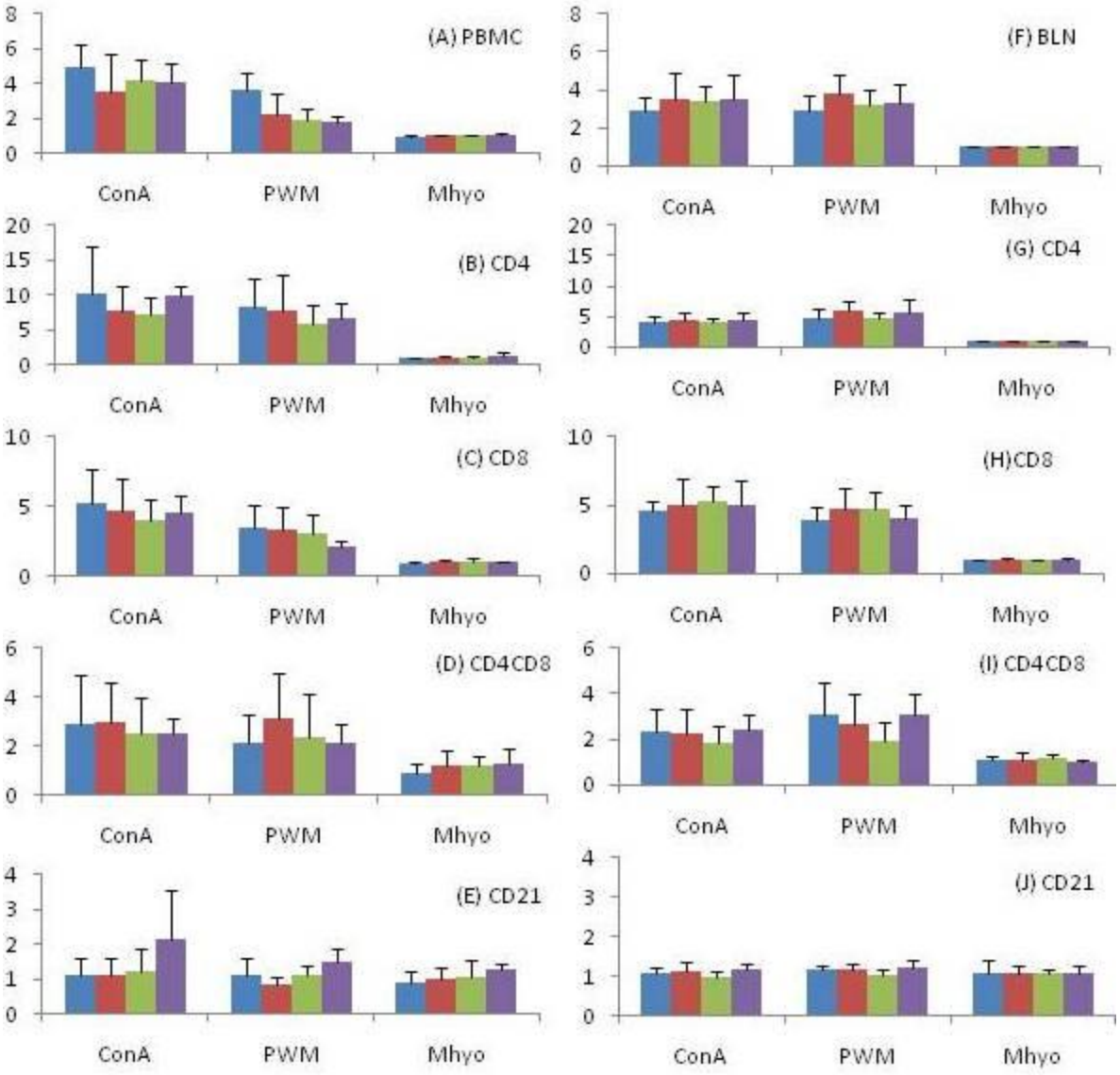

Fig. 1 Cell proliferation assays of PBMC (A-E) and bronchiolar lymph node cells (F-J) stimulated by ConA, PWM, and MHyo antigen on day 21 post PCV2 inoculation. Stimulation index of PBMC or bronchiolar lymph node cells, CD4, CD8, CD4CD8, and CD21 cells are shown on Y-axis. The four experimental groups of pigs are labeled as the following:

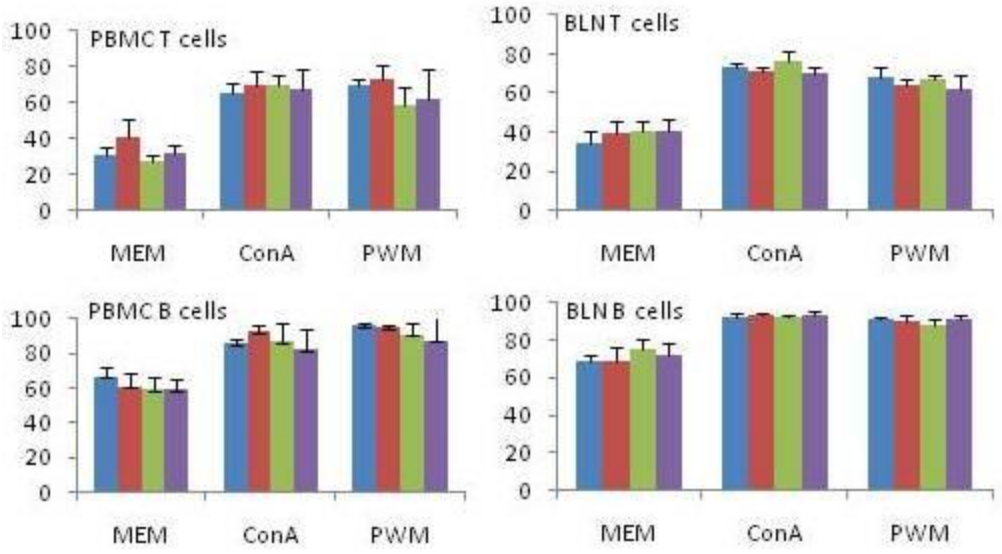

Fig. 2 Apoptosis assay of PBMC with no stimulation (MEM), ConA and PWM stimulation on day 21 post PCV2 inoculation. Apoptotic index of T (A) and B (B) cells is shown on the Y-axis. The four experimental groups of pigs are labeled as the following:

Discussion:

The goal of this study was to assess the impact that PCV-AD had on lymphocytes. Because it is difficult to reliably induce the progression of PCV2 infection to disease, we co-infected the pigs with MHYO. In past studies, this model increased the number of pigs that developed clinical disease associated with PCV2 infection. However, in those studies, all pigs did not develop disease. Unfortunately, in the study reported here, none of the pigs developed clinical disease associated with PCV2 infection. We confirmed that the pigs had been challenged successfully with MHYO, based on lesions and the presence of the organism by PCR. We also confirmed that the pigs were infected with PCV2 based on the presence of the virus in serum and tissues. Unfortunately, there was no significant lymphoid depletion or other microscopic evidence of disease, although by immunohistochemistry we did confirm that the virus was present in the tissues.

Several factors may have played a role in the lack of disease in our pigs, the challenge/inoculation times may not have been optimal or the pigs in the study did not have the trigger factors needed to result in the progression of PCV2 to disease. At this point, it is unknown why some pigs infected with PCV2 develop disease, while others do not.

This study did confirm that PCV2 infection alone is not enough to result in significant alteration in the ability of lymphocytes to proliferate. This would explain why pigs infected with the virus don't appear to have a high number of concurrent infections.

Due to the lack of clinical disease, we were not able to prove or disprove our hypothesis. Further work is needed to identify the triggering factors that cause PCV2 infection to develop into the lymphoid depletion and wasting associated with PCV-AD.

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