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Interaction of Porcine circovirus 2 with the Swine Immune System

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Interaction of Porcine circovirus 2 with the Swine Immune System

by

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Abstract

Porcine circovirus 2 (PCV2) is a virus with a single-stranded, DNA circular genome that is ubiquitous in pig populations worldwide. PCV2 is the causative agent of the post-weaning multisystemic wasting syndrome (PMWS), a multifactorial disease that affects six to twelve-week-old pigs, and which is characterized by weight loss and immunosuppression. PCV2 vaccination has diminished the presentation of PMWS in the field, although PCV2 infection is not prevented.

PCV2 infects lymphocytes and it depends on the host enzymes to replicate. Enhanced PCV2 infection rates are associated with increased mitotic activity, although the effect of PCV2 replication on lymphoid cell function is unknown. The main goal of this thesis was to understand the interactions of PCV2 with the swine immune system, by determining the effect of PCV2 on the antibody response in pigs under field conditions, and studying the impact of the primary PCV2 infection on lymphocyte activation, proliferation, and viability. As shown in Chapter 2, a PCV2 persistent infection was detected in farmed pigs of all age groups. Furthermore, a great variability in their neutralizing antibody titers was observed, regardless of their vaccination status. Chapter 3 provides details about the establishment of an *in vitro* cell model to study the effect of primary PCV2 infection on the immune cells, by using snatched-farrowed, porcine colostrum deprived (SF-pCD) PCV2-free pigs as blood donors of PCV2-naïve peripheral blood mononuclear cells (PBMCs). These cells were exposed to the polyclonal mitogens ionomycin/PMA and PCV2 infection. As shown in chapters 4 & 5, enhanced PCV2 infection rates in ionomycin/PMA-stimulated PBMCs, decreased proliferation in PCV2 infected cells, and high bystander cell death rates in PCV2-exposed PBMCs were observed. This thesis contributes to the current knowledge on PCV2 immunology by bringing insight into factors that contribute to

viral pathogenesis and immune modulation and emphasizes the need of developing new vaccines that prevent PCV2 infection.

Preface

The following manuscript was published using data that is being presented in this thesis. Cristina Solis Worsfold was involved in study design, data collection and analysis, result interpretation and manuscript writing with guidance from her supervisor and other collaborators. All authors contributed important intellectual content and provided critical review of the paper. Written permission for reproduction of the article in its entirety for this thesis has been obtained from the publisher and all co-authors.

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Dedication

To my parents.

Thank you for your unconditional love and guidance.

“No te rindas que la vida es eso,
continuar el viaje,
perseguir tus sueños,
destrabar el tiempo,
correr los escombros,
y destapar el cielo.”
Mario Benedetti

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
7-AAD	7-Aminoactinomycin D
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ACC	Animal Care Committee
ACK	Ammonium-Chloride-Potassium
APS	Ammonium persulfate
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BSL-2	Biosafety level-2
Ca^{2+}	Calcium
CAP	PCV2 Capsid protein
CD	Cluster of differentiation
CH	Constant heavy
CME	Clathrin-mediated endocytosis
CTL	Cytotoxic T lymphocyte
Con A	Concanavalin A
ddH20	Double distilled water
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GC	Germinal center
GD	Gestation day
GP4	Glycoprotein 4
HCl	Hydrogen chloride
HEV	High endothelial venules
HRP	Horseradish peroxidase
IFN-γ	Interferon gamma
IFN-γ-SC	IFN-γ-secreting cells
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IPEC-J2	Intestinal porcine epithelial cell line
IPP	Ileal Payer's Patches
IQR	Inter-quartile range
IV	Intravenous
kg	Kilograms
LMER	Liner-mixed effects model
M	Molar
MDA	Maternally-derived antibodies

MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute
mL	Millilitre
mRNA	Messenger ribonucleic acid
NAb	Neutralizing antibody
NFAT	nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLS	Nuclear localization signal
NPTr	Newborn pig trachea epithelial
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PB	Permeabilization buffer
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PCV1	Porcine circovirus 1
PCV2	Porcine circovirus 2
PCVAD	Porcine circovirus 2-associated diseases
PDNS	Porcine dermatitis and nephropathy syndrome
PE	Phycoerythrin
PED	Porcine epidemic diarrhea

PI	Propidium iodide
PIn	Proliferation index
PK-15	Porcine epithelial kidney cells
PMA	Phorbol myristate acetate
PMWS	Post-weaning multisystemic wasting syndrome
P.O.	Per os, orally
PPV	Porcine parvovirus
PRRSV	Porcine reproductive and respiratory syndrome virus
PVDF	Polyvinylidene fluoride
PWM	Pokeweed mitogen
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
REML	Restricted maximum likelihood
RPMI	Roswell Park Memorial Institute
SF-pCD	Snatch-farrow, porcine colostrum deprived
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
ST	Swine testis cells
$TCID_{50}$	Tissue culture infectious dose 50%
TCR	T cell receptor

TEMED	Tetramethylethylenediamine
Th	T helper
TNF-α	Tumor necrosis factor-alpha
VNA	Virus neutralization assay
VR1BL	Porcine fetal retina cells
VSRS	Veterinary Sciences Research Station

Chapter One: Introduction

1.1 Canadian pork industry

Pork is the most consumed meat worldwide, followed by chicken and beef[1]. In Canada, pork production is the fourth largest agricultural industry after canola, dairy products, and beef, with revenues of more than 4 billion dollars in 2015[2]. Quebec is the biggest producer of pork nationwide, followed by Manitoba, Ontario, and Alberta[3], and over 50% of the pork produced is exported to the United States, Japan, and Europe[4].

Over the last 10 years, the Canadian pork industry has gone through major challenges due to an international drop in hog market prices and a significant increase of feed costs[5]. In 2011, the number of pig farms in Canada was reduced by 36%, which resulted in a decrease of 15.7% in pork production[5]. These changes have led to specialization of farms and breeding facilities to assure increased meat quality and quantity with reduced costs per pig.

Industrialization of pig farms has also led to an increase in biosecurity measures to reduce the introduction and circulation of production-limiting and/or zoonotic infectious agents in swine herds[6]. Segregation of farms, control and disinfection of materials that enter or exit farms, and all-in/all-out production systems minimize the risks of disease transmission. Nonetheless, the constant stress factors and high animal density (Figure 1.1A.) make the intensive pig production systems ideal for emergence of new pathogens[6]. One example is the outbreak caused by the Porcine epidemic diarrhea (PED) virus in the United States in 2013, which was estimated to kill up to 7 million newborn piglets[7]. Consequently, continuous research and vaccine development are necessary to prevent or mitigate the effect of infectious diseases in swine production.

1.2 Porcine circovirus 2

Porcine circovirus 2 (PCV2) was identified in 1998 as an emergent pathogenic virus that causes clinical diseases in swine[8]. Economic losses due to weight loss and mortality in pigs during the PCV2 epidemic reached $130 million dollars per year in England alone[9], making it one of the most important pathogens to affect swine industry in the last 20 years. Currently, PCV2 is enzootic in herds worldwide[10] and causes subclinical infections in pigs. Understanding the mechanisms of viral infection and pathogen-host interactions are essential to recognise the consequences that PCV2 infection could have on swine production[9].

1.2.1 *Classification and genomic organization of Porcine circoviruses*

The *Circoviridae* family consists of a group of circular DNA-genome viruses that mainly infect birds and mammals[11]. The family is divided into two genera: *Cyclovirus* and *Circovirus.* The genus *Circovirus* consists of 22 virus species, including *Porcine circovirus 1* (PCV1, type species) and PCV2[12]. PCV1 was first described in 1982 as a contaminant of the porcine kidney cell line PK-15[13], and was later identified as a non-pathogenic virus that infects pigs. PCV1 and PCV2 share 77% sequence identity and do not have antibody crossreactivity[14]. A new virus, *Porcine circovirus 3,* has recently been proposed to cause dermatitis, and reproductive and renal failure in sows but its significance in disease progression still needs to be confirmed[15].

PCV2 is a small, non-enveloped virus of 17nm diameter, with a 1.767 kb single-stranded circular DNA genome[16](Figure 1-2). Four open reading frames (ORF) encode the five major proteins of PCV2: ORF-1 is a 945 base pair (bp) gene on the positive strand that encodes the replication-assisting proteins Rep (35kDa) and Rep' (28.5kDa)[17], ORF-2 is a 702bp gene on the negative strand that encodes the structural and immunogenic capsid (CAP) protein (28kDa)[18],

and the ORF-3 (315bp) and ORF-4 (180bp) genes, embedded in ORF-1 in the antisense strand, encoding the ORF-3 (11.9kDa) and ORF-4 (6.5kDa) proteins, which are associated with PCV2 pathogenesis[19,20].

1.2.2 *PCV2 entry and viral replication*

PCV2 infects epithelial cells of the respiratory, reproductive, and digestive tract, as well as cells of the immune system[21,22]. PCV2 has been found in secretions (e.g. colostrum, milk, and semen)[23,24], and excretions (e.g. saliva, feces, and urine)[25,26] of pigs, contributing importantly to the horizontal transmission of PCV2 within the herd and perinatal infection of piglets[27]. Vertical transmission of PCV2 and its consequences for farmed pigs is still controversial, although PCV2 infection has been associated with reproductive failure in breeding sows[28]. Experimental intrauterine exposure of fetuses to PCV2 after gestation day (GD) 70 demonstrated viral infection in hepatocytes, cardiomyocytes, and cells of the monocyte lineage without causing abortions[29].

PCV2 attaches to glycosaminoglycans (GAGs), like heparan sulphate and chondroitin sulphate B in the cell membrane[30]. In monocytic cell lines, PCV2 is internalized by clathrin-mediated endocytosis and subsequently released into the cytoplasm after endosome acidification[31]. Interestingly, in epithelial cell lines, the use of endosome acidification inhibitors increased PCV2 infection rates, so the mechanism behind viral endosome release is not known[32]; nonetheless, the use of protease inhibitors completely block PCV2 infection so it is hypothesized that a serine protease is involved in endosome viral release[22,33]. This is continued by the transport of PCV2 to the nucleus by means of the nuclear localization signal (NLS) sequence of the CAP protein and its association with the cell's microtubules[34].

ORF-1, encoding the non-structural viral proteins Rep and Rep', is transcribed using the host's polymerase, followed by the replication of the viral genome[35]; Rep and Rep' are the only viral proteins necessary for viral DNA replication[36].

To start DNA replication, the single-stranded DNA genome of PCV2 is converted to a double-stranded intermediate using the host's enzymes[35]. The origin of replication of PCV2 is found in a non-coding region between the CAP and Rep gene, forming a stem-loop with a sequence that is conserved between circoviruses (5'-AAGTATTAC-3'), with hexamer repeats on the 3' side (H1 and H2)[35](Figure 1-2). The newly synthetized Rep and Rep' proteins are imported from the cytoplasm and bind to the origin of replication, destabilizing and cleaving the ds-DNA, generating a 3'-OH end that is used to start the genome replication via a rolling circle melting-pot mechanism; since Rep and Rep' have no polymerase activity, it is believed that PCV2 uses the host's polymerase to replicate[37]. After genome replication, the virus assembles to a particle using the newly produced CAP protein, and exits the cells using unknown mechanisms, although it is hypothesized that cell death induction is required for this step[35].

1.2.3 *Post-weaning multisystemic wasting syndrome (PMWS)*

PCV2 infection has been implicated with several clinical syndromes that have collectively been named PCV2-associated diseases (PCVAD)[38]. These include PCV2-associated enteritis[39], PCV2-associated reproductive disease[40], the porcine respiratory disease complex[41], porcine dermatitis and nephropathy syndrome (PDNS)[42], and the post-weaning multisystemic wasting syndrome (PMWS)[8]. PCV2 is the causative agent of PMWS, and it is the main disease discussed through-out this thesis due to its effects on the immune system.

PMWS was first described in Canada in 1996[43], causing mortality rates of up to 30% in nursery piglets of 6 to 12 weeks of age[44]. The clinical signs associated with PMWS include diarrhea, weight loss, enlarged lymph nodes, and general immunosuppression[45](Figure 1-1B). No specific treatment is available against the disease; consequently, wasting animals in a herd are euthanized, resulting in an important economic loss[9].

PCV2 infection is a necessary factor for PMWS development, but due to the ubiquitous nature of PCV2, antigen and antibody presence in pigs is not enough to diagnose PMWS[38]. Diagnostic criteria to identify a PMWS case includes the presence of clinical signs[38], microscopic lesions in lymph nodes (e.g. severe lymphocyte depletion and granulomatous infiltration with presence of high amounts of PCV2-CAP protein, determined by immunohistochemistry[46]; Figure 1-1C. and 1-1D.), and high amounts of PCV2 genome copies ($>10^6$) in affected tissues determined by quantitative polymerase chain reaction (qPCR)[38].

Analysis of archive samples of tissues demonstrated that PCV2 strains were circulating in pig herds since the 1950s without causing an apparent clinical disease[47]. Phylogenetic studies have been conducted using strains isolated worldwide to determine if specific PCV2 genotypes or mutations were responsible for the PCV2 disease outbreak reported in the late 1990s[48]. Yet, it was concluded that PCV2 isolates have more than 96% sequence identity, and no specific geographical strain or amino-acid sequence has been correlated with increased virulence[48].

Four PCV2 genotypes were established based on differences in the ORF-2 nucleotide sites: PCV2a, PCV2b, PCV2c, and PCV2d, with recombinant strains classified as intermediate clades[49]; a recombinant PCV1-2a genotype was also described in Canada in 2007, possibly due

to a vaccine escape strain[50]. Until the early 1990s, most strains were classified as PCV2a with low genetic diversity, and they were used for vaccine development[10]. A genotype shift was observed with the emergence of PCV2b and PCV2d in the early 2000s[10], and in Canada, the emergence of PCV2b was related to a significant increase in PMWS cases[51]. PCV2d strains represent approximately 60% of the strains isolated in China and 37% of the strains from the United States[49]. PCV2c includes strains isolated in Denmark only and are considered extinct[52].

1.2.4 *Pathogenesis of PMWS*

Pigs are infected with PCV2 by oronasal route and the virus initially replicates in epithelial cells of the respiratory and digestive system[53]. Macrophages and dendritic cells phagocytose infected or dying cells and aid in virus dissemination towards bronchial, inguinal and mesenterial lymph nodes[54,55]. Once inside the lymph node, PCV2 replicates in lymphocytes, induces apoptosis, and causes a mild depletion of B and T cells in the tissue approximately one week after infection[21]. In this stage, a low level of infiltration of monocytes and macrophages in the lymph nodes can also be observed[56,57].

If the virus continues to replicate for two to three weeks after infection, sustained lymphocyte apoptosis can lead to a substantial loss in architecture of the lymph node[21]. In secondary lymphoid tissues of diseased animals, there is an absence of B cells in the follicles and germinal centers, undefined distribution of T cells in parafollicular areas, and infiltration of histiocytes[55,57](Figure 1-1C). Advanced stages of disease are characterized by high antigen concentration in tissues and serum[21] and PMWS clinical signs[57].

As PCV2-load in tissues and blood increases and disease progresses, a depletion of circulating peripheral blood mononuclear cells (PBMCs) is observed[58], with decreased IgM+ B cells, CD4+ and CD8+ T cells, and increased circulation of monocytes in PMWS animals[59,60]. This reduction was also observed in experimentally PCV2-infected germ-free pigs, with a severe lymphocyte depletion at day 21 post-infection; the subpopulations affected were mostly CD21+ B cells and CD4+ effector/memory cells[61].

A major issue in the PCV2 field is that clinical signs of PMWS cannot be reproduced experimentally, and similar clinical disease is only observed if the pigs are co-infected with other pathogens or if the immune system is stimulated[62]. Co-infection of pigs with PCV2 and Porcine reproductive and respiratory syndrome virus (PPRSV)[63] or Porcine parvovirus (PPV)[64] increased PCV2 replication and lesions in lymphoid tissues[65]. Clinical signs were also observed after PCV2-subclinically infected pigs were vaccinated with Freund's incomplete adjuvant and keyhole limpet hemocyanin[62], and enhancement of PCV2 infection rates has been reported after treatment of PBMCs with mitogen *in vitro*[66], indicating that external factors might be needed for disease development[62].

The lack of a proper animal model to study the molecular mechanisms of disease made researchers turn to tissue culture models to understand PCV2 infection[67]. The commercially available porcine epithelial kidney cells (PK-15 cells) and swine testis cells (ST cells) have been used extensively for *in vitro* studies with the disadvantage of low infectivity rates[68]. Enhancement of viral replication in these cells has been achieved by exposing PCV2 infected PK-15 to interferon gamma (IFN-γ)[69], glucosamine or Concanavalin A (Con A)[68].

PCV2 infection has also been observed in porcine fetal retina cells (VR1BL)[67], newborn pig trachea epithelial cell line (NPTr)[70], and intestinal porcine epithelial cell line (IPEC-J2)[71]. In primary cell cultures, PCV2 infection has been demonstrated in hepatic cells and PBMCs[72].

1.2.5 *Vaccination and immune response against PCV2*

1.2.5.1 Immune response after vaccination

The goal of vaccination is to elicit long-lasting B and T cell immune responses that protect from infection or disease caused by a specific pathogen[73]. In the case of inactivated or sub-unit vaccines, the presence of pathogen-associated molecular patters (PAMPs) in the vaccine, assisted by adjuvants, initiate an innate immune system response by macrophages and DCs, which process and present the antigens to T cells in the local draining lymph nodes[73].

In the lymph node, processed peptides are presented to naive CD8+ and CD4+ T cells via major histocompatibility complex (MHC) -I and MHC-II, respectively[74]. Activated T helper (Th) or cytotoxic T lymphocytes (CTLs) are then able to perform their effector responses as direct killing of infected cells in the case of CTLs, and secretion of lymphocyte-proliferative cytokines, like IL-2 and IL-4, in the case of Th cells[74]. After the initial expansion phase, the majority of effector T cells die, but a small percentage differentiate into memory T cells that circulate for long periods of time and are able to respond rapidly after a secondary infection[74].

In the case of naïve B cells, their receptor recognizes and binds to the antigen protein of the vaccine[74]. With the aid of Th cells, B cells are activated and driven into proliferation in lymph nodes, forming germinal centers (GCs), and differentiating into antibody-secreting plasma cells and memory B cells[74]. In the GCs, B cells undergo two major events: somatic

hypermutation and class switch recombination, that increases the binding capacity of B cell receptors[75]. Somatic hypermutation is a process that alters the sequence of the B cell receptor variable region, and results in the production of high affinity antibodies by plasma cells, including neutralizing antibodies (NAbs)[75]. The proliferation of B cells in GC results also in class-switch recombination, where the immunoglobulin (Ig)-M switches to IgG, IgA, or IgE[74]. Antigen-specific plasma cells do not survive long periods of time, but a fraction of memory B cells migrates to the bone marrow where they initiate a rapid immune response after a secondary infection[74].

NAbs are secreted by plasma cells of the germinal centers, and are usually measured late in the primary immune response, 10 to 14 days after initial contact by the antigen[75]. NAbs have different mechanisms for virus neutralization including inhibition of virus binding to cell receptors, interference of viral attachment and entry to cells, and inhibition of genome uncoating in endosomes[76,77]. Because of this important biological function, the induction of high titers of NAbs, and long lasting immune memory cells, are some of the preferred outcomes of vaccination[78].

1.2.5.2 PCV2 vaccine evaluation

The use of PCV2 vaccines significantly diminished the cases of PMWS worldwide[79]. Vaccines contain the PCV2a-CAP protein expressed as a virus-like particles using a baculovirus system, an inactivated PCV2a virus, or an inactivated chimeric virus containing the CAP protein of PCV2a in the backbone of the non-pathogenic, PCV1[79]. One vaccine has been licensed to be used at three days of age[80], but pigs are usually vaccinated at approximately three weeks of age

when they are weaned-off maternal milk[81]; all vaccines are administered intramuscularly. The characteristics of the available commercial vaccines are described in table 1-1.

Efficacy of PCV2 vaccines has been evaluated using clinical and immunological parameters. PCV2 vaccinated pigs present an increase in average daily weight gain[82], diminished PCV2-associated tissue lesions[73], reduced PCV2 shedding in fecal and oral discharges[83], and reduced circulating PCV2 in serum, compared to non-vaccinated animals[81,84].

The immunological parameters used to determine PCV2 vaccine efficacy are induction of NAbs[85,86] and IFN-γ-secreting cells (IFN-γ-SC)[81,87]. The presence of NAbs correlates with reduction in circulating PCV2[88] and protection against the clinical disease[89]. Experimental trials have demonstrated vaccine efficacy by induction of NAbs 20 days after vaccination[87], clearance of PCV2 viral load in serum after vaccination and viral challenge[90, 86], and increased number of IFN-γ-SC three weeks after vaccination that correlate with reduction of viremia[81]. Under field conditions, similar levels of NAs are not reached before four weeks post-vaccination and require a virus challenge as a booster to increase NAb titers[84]. In addition, PCV2 vaccines do not prevent PCV2 infection since pigs still have high levels of PCV2 viral load in serum, regardless of vaccination[84].

Maternally-derived antibodies could also influence the response of pigs to PCV2 vaccination[90,91], since farmed pigs receive different levels of antibodies against PCV2 in colostrum and milk[23]. Nonetheless, studies have determined that under field conditions, the presence of these antibodies does not affect the response to vaccines unless they are present in

very high levels[92,93], so the factors that interfere with vaccine efficacy in the field should be studied further.

1.3 PCV2 interaction with the immune system

PCV2 infection is closely associated with the immune system and infection could result in modulation of the immune system, affecting the way the animal reacts towards pathogens[94, 95].

PCV2-CAP protein has been detected in the cytoplasm of monocytes, macrophages[96] and dendritic cells (DCs)[97] after *in vitro* exposure to PCV2. Still, these cells do not seem to support virus replication, and could act as a reservoir that aids in virus dissemination[96]. A virus kinetic study demonstrated that PCV2 persists as an infectious virus in monocyte-derived and bone marrow-derived DCs for several days without replicating or causing apoptosis, opposed to what is observed in lymphocytes[97]. Similarly, alveolar macrophages that phagocytize PCV2 do not support viral replication, but demonstrated a diminished capability of antigen presentation, reduction in production of reactive oxygen species, and increased production of tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) *in vitro*[96].

High PCV2 loads in tissue were found to correlate with a generalized lymphocyte depletion in PMWS pigs[57]. Apoptosis induction is proposed to be the cause of lymphocyte depletion in diseased animals, but the mechanism is still unclear and a topic of controversy in the PCV2 field[94]. Early studies by Shibahara *et al*. (2000) described that PCV2 replication was the direct cause of apoptosis and lymphocyte depletion[55].

At least three proteins encoded by PCV2 have been shown to induce apoptosis and cell death *in vitro*: ORF-3, ORF-4, and CAP. The accumulation of CAP protein in the nucleus and

cytoplasm of PK-15 was cytotoxic, and a high percentage of non-infected cells died by bystander effect in cell culture.[98] ORF-3 increases expression of p53[99], activating caspases 8 and 3[100] and inducing apoptosis *in vitro*[101]. Similarly ORF-4 was shown work in conjunction with ORF-3 to regulate cell apoptosis.[20]

Depletion of lymphocytes could also occur as an indirect effect of PCV2 infection[94,95]. A cytokine imbalance has been described in PMWS cases, including increased levels of IL-10 mRNA expression in atrophic thymus cases, and decreased IL-2 and IL-4 mRNA in lymph nodes[95]. In the same study, authors reported an increase in IL-8 secretion that could induce macrophage infiltration in tissues[95]. In addition, PBMCs isolated from PMWS-affected animals produce lower anti-inflammatory cytokines (e.g. IFN-γ) after mitogen stimulation[102], demonstrating a diminished immune response in diseased animals that could lead to low proliferation of cells, and subsequent cell death.

1.4 Distinct characteristics of the swine immune system

The swine immune system has unique characteristics that distinguish them from human and mice. The understanding of these differences is important since they could affect the way pigs react to pathogens; this section explains some of these differences.

Gestation in swine is 114 days long, and the type of placenta is epitheliochorial, meaning that there are six layers separating fetus from sow; this results in an absence of maternal antibody transfer before birth[103]. As in other mammals, swine hematopoiesis starts in the yolk sac at GD 20, continued by the fetal liver after GD30, and in the bone marrow after GD40[104]. After GD40, IgM+ B cells are found in blood and spleen, and the thymus is populated by two

waves of progenitors, first from the liver, and then from the bone marrow[104]. The bone marrow expands the populations of B and T cells present in the periphery, and from GD65-100 is where the majority of B cells are produced. By GD70, the adaptive immune response of the pig is active, but it is still not known if the bone marrow continues lymphopoietic activity in adult swine[104].

The thymus is colonized with T-cell precursors after they migrate from bone marrow to thymus at GD40[105]. As all other vertebrates, swine have two types of T cell receptors (TCR): αβ and γδ[105]. TCR-γδ T cells can represent more than half of the periphery T cells in young pigs, but the population decreases rapidly with age[105]. TCR-γδ T cells are usually CD4-, but can be CD8 positive or negative[105]. Their function is still unknown, but expression of MHC-II leads to believe they could be antigen presenting cells with cytolytic activity[105].

Mature TCR-αβ T cells are exported from the thymus as CD4-CD8+ αβ cytotoxic T cells, or CD4+CD8- αβ T helper cells[105]. Once in blood, encounter with antigens leads to activation of helper T cells, becoming double positive $CD4^+$ $CD8\alpha^+$ T cells, a type of memory T helper cell with expression of MHC-II and no cytolytic activity[105]. Because their conversion is dependent on antigen exposure, pigs are born with few double positive cells but can represent up to 60% in adults[105].

Swine genome encodes constant heavy (C_H) genes for five antibody isotypes: IgG, IgM, IgE, IgA, and IgD, and they are all transcribed before birth[106]. Seven IgG subclasses have been identified (IgG1, $IgG2^a$, $IgG2^b$, IgG3, IgG4, IgG5, and IgG6), but the function of each subclass is unknown. Like in other species, the concentration of IgG in blood is 10 times higher than IgM

and IgA, and 80% of the mucosal secretions and milk are IgA[106]. An important consideration is that in late gestation, the sow's blood IgG is transferred to the epithelial cells of the mammary gland[106]. This process makes IgG whole molecules available in colostrum for the newborn piglet to absorb using specialized columnar enterocytes, before "gut closure", which happens 24h after birth[106]. After this period, the sow's mammary glands secrete mainly IgA in milk[106]. Swine also has a lower IgA blood concentration compared to humans, since one-third is derived from the intestinal mucosa; this contrasts to humans where the majority of IgA is derived from the bone marrow[106]. In addition, a population of B cells exists in the medulla (IgG and IgA expressing cells), cortex, and in the interstitial region (no expression of Ig) of the thymus[105]. They develop independently of other B cells and their function is unknown[104].

Like in humans, pig light chains are equally identified as kappa or lambda, differing from mice that use 95% kappa light chains[106]. Two families of V_K have been identified (IGKV1 and 2), with approximately 60 and 30 genes on each family respectively, and six J_K segments; the number of $V_\lambda J_\lambda$ genes have not been identified[106].

An important difference of the swine B cell development compared to human and mice is the restriction of heavy chain V-D-J recombination genes[103]. In swine, heavy chain rearrangements are first detected at GD20 in the yolk sac[103], but pre-immune diversity is built using only two D_H genes, one J_H gene, and 4 V_H genes belonging to only one family (V_H3)[104]. This makes junctional diversity, and not combinatorial diversity, the main pathway in heavy chain diversity in pigs[103].

Diversification of B cells was also thought to occur in the ileal Payer's Patches (IPP), an organ that appears late in gestation in the ileal-cecal junction of the small intestine of artiodactyls and involutes a few months after birth[107]. In lambs, it was demonstrated that the IPP are an important site for B cell development and diversification, and the same was expected for swine. Recently, a study demonstrated that IPP are not necessary for B cell lymphogenesis since removal of the organ did not lead to decreased circulating B cells, and the phenotype of the IPP B cells did not resemble the B cells of the bone marrow, demonstrating that IPP might be a secondary lymphoid organ in the swine[108].

1.4.1 *Peripheral blood mononuclear cells (PBMCs) distribution in swine*

Swine have higher circulating PBMCs compared to other species due to a particularity in their lymphatic circulation[107]. In pigs, the immigration of cells into the lymph nodes occurs by the afferent lymph vessels, but the lymphocytes exit through the high endothelial venules (HEV), resulting in higher numbers of circulating mononuclear cells in blood (approximately $6.5 \pm 2.7 \times 10^6$/ mL)[107]. Another particularity in swine is that they have an "inverted lymph node structure", meaning pigs lack a medullary area, with mostly cortical and paracortical areas[107]. The distribution of the different types of peripheral blood lymphocytes in swine are described in table 1-2.

1.5 Thesis aim and overview

The overall aim of this thesis was to investigate the interaction of PCV2 with the porcine immune system, specifically the humoral immune response in farmed animals, and the effects of PCV2 infection on lymphocyte activation, proliferation, and cell death.

This project has three main components divided in four data chapters. The first part of this thesis (Chapter 2) describes the humoral immune response of PCV2 vaccinated and non-vaccinated farmed pigs of different age groups, and aids to understand the types of antibodies induced in farmed pigs after vaccination and the PCV2 viral load in each production stage. The second part (Chapter 3) describes the establishment of a PCV2-free pig cohort to be used as a source for PCV2-free mononuclear cells to study PCV2 pathogenesis. The third part of this thesis addresses PCV2 infection of PBMCs, the specific immune cell subsets that are susceptible to PCV2 infection *in vitro*, and cell death induction (Chapter 4). In addition, Chapter 5 describes how PCV2 infection modulates PBMC activation and proliferation *in vitro.* Finally, Chapter 6 contains the general discussion and concluding remarks about the study.

This study contributes significantly to the current knowledge on PCV2 immunology and the mechanisms behind viral infection in lymphocytes. In addition, it brings insight into factors that contribute to viral pathogenesis and describes humoral responses against PCV2 vaccines and virus-host interactions in farmed pigs.

Table 1-1 Characteristics of current PCV2 vaccines.

Company	Name	Antigen	Age	Vaccination schedule	Availability
Merial	Circovac®	Inactivated PCV2a virus	Sows/gilts	One injection, followed by a second injection 3w later at least 2w before farrowing.	Worldwide except USA
			Pigs	Single dose, 3w of age	
Boehringer Ingelheim	Ingelvac CircoFLEX®	PCV2a CAP protein	Pigs	Single dose, 2w of age	Worldwide
MSD/Merck Animal health	Porcilis® PCV	PCV2a CAP protein	Pigs	Single dose, 3w of age	Europe, Asia, and South Am.
	Circumvent™ PCV	PCV2a CAP protein	Pigs	Single dose, 3w of age	North and Central Am.
	Circumvent™ G2 PCV	PCV2a CAP protein	Pigs	Single dose, 3 w of age Two dose, 3 days of age and 3w of age	Canada and USA
Zoetis	Suvaxyn® PCV	Inactivated recombinant PCV1 with PCV2a CAP	Pigs	Single dose, 3 w of age	Europe and Asia
	Fostera™ PCV	Inactivated recombinant PCV1 with PCV2a CAP	Pigs	Single dose, 3 w of age Two dose, 3w of age, and 2-3w later	North America, South Africa, and Asia

Source: Segales, 2015, modified[109]

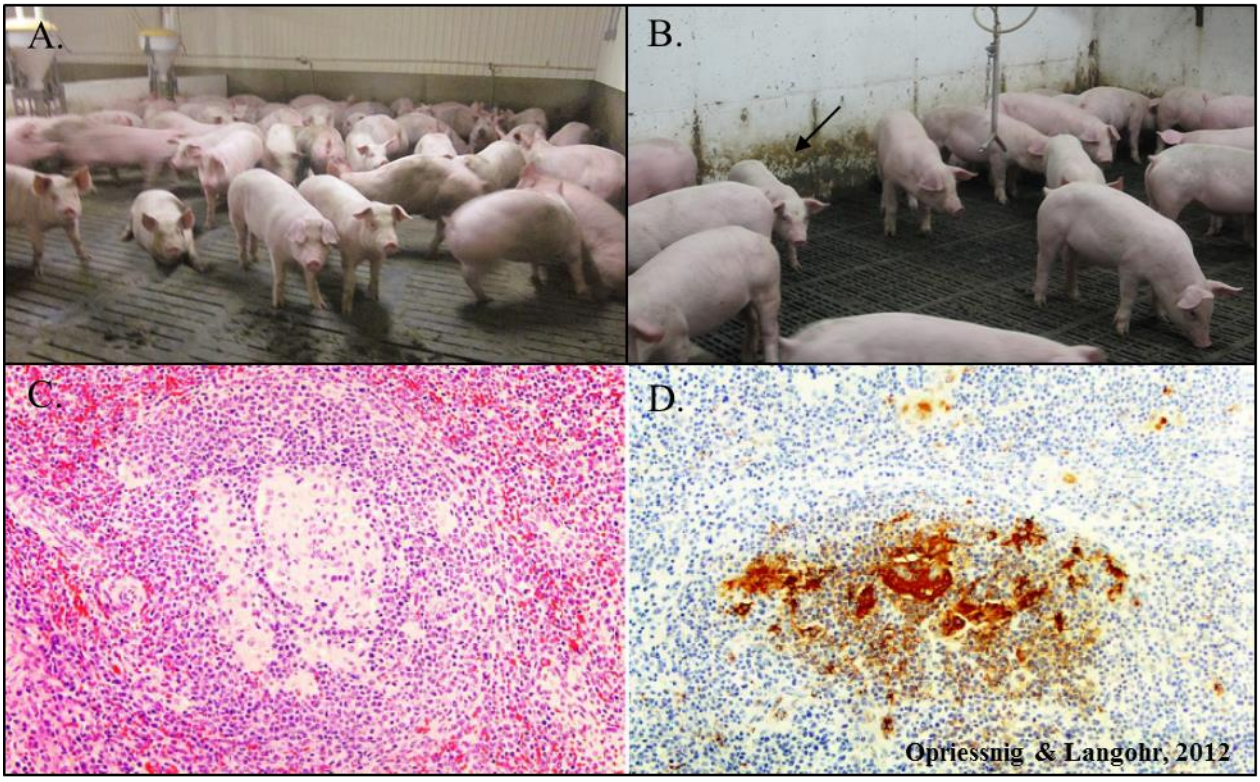
Table 1-2 Distribution of swine lymphocyte subsets present in blood, phenotype, and frequency within peripheral blood lymphocytes (PBL).

Lymphocyte subset	Phenotype	Frequency within PBL
Cytotoxic T lymphocytes	$CD3^+$ $CD8\alpha^+$ $CD8\beta^+$	8-21%
T-helper cells	$CD3^+$ $CD4^+$ $CD8\alpha^-$ $CD3^+$ $CD4^+$ $CD8\alpha^+$	19-60%
TCR-γδ T cells	$CD3^+$ TCR-$\gamma\delta^+CD8^{+/-}$	5 -50%*
Natural Killers (NK)	$Perforin^+$ $CD2^+$ $CD3^-$ $CD4^-$ $CD5^-$ $CD6^-$ $CD8\alpha^+$	2-10%*
NKT cells	$Perforin^+$ $CD3^+$ $CD6^-$ $CD11\beta^+$ $CD16^+$	0.5-3%
T reg	$CD4^+$ $CD25^{High}$ $FoxP3^+$	1-3%
Naïve mature B cells	CD2+ CD21+ sIg+	15-25%

*age dependent

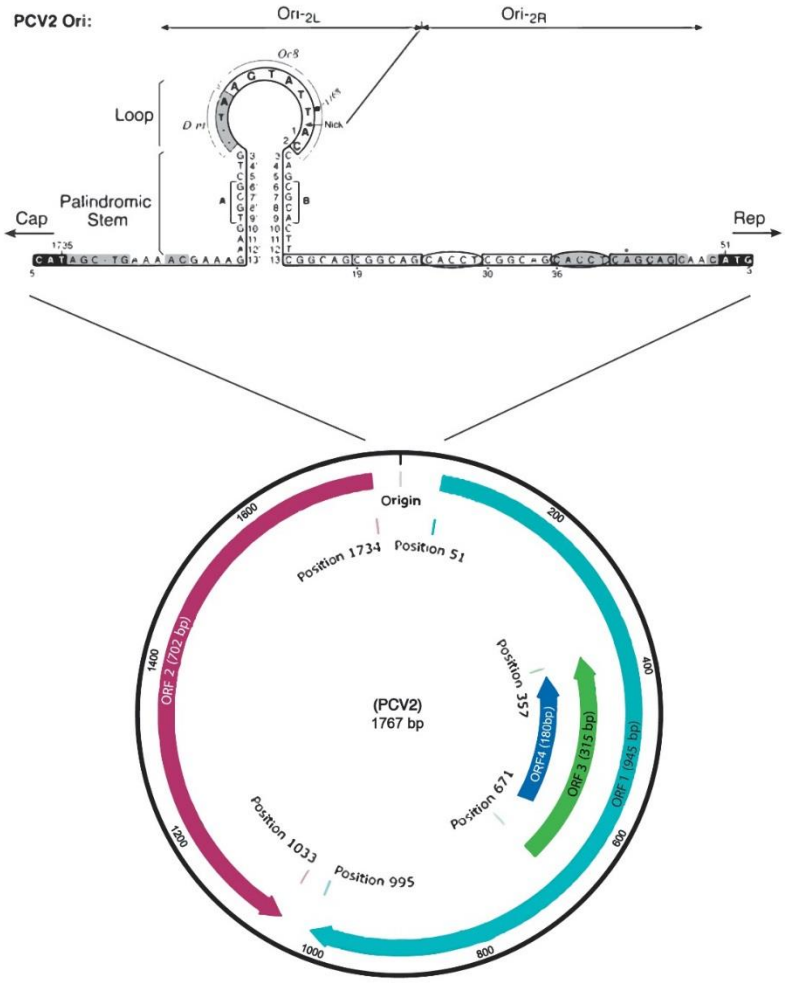
Source: Gerner *et al.*, 2009[105] & Sinkora *et al.*, 2011[108], modified.

Figure 1-1 Post-weaning multisystemic wasting syndrome (PMWS) clinical signs and microscopic tissue lesions. 1-1A. Representation of animal density in a pig herd visited in rural Alberta. 1-1B. PMWS case depicted by an arrow. The pig is smaller than his age-matched pen mates. 1-1C. Lymph node of a pig experimentally-infected with PCV2. Histiocytic infiltration of follicle and lymphocyte depletion. 1-1D. Immunohistochemical staining of PCV2 antigen in the same lymph node.



Source: A & B: Czub laboratory library; C & D: Opriessnig & Langohr, 2012 ©SAGE Publishing[46]

Figure 1-2 Schematic representation of the PCV2 single stranded, circular genome, with a detailed view of the origin of DNA replication (Ori). Color arrows represent the size and position of the four major open reading frames (ORF) of the viral genome: ORF-1, ORF-2, ORF-3, and ORF-4.



Source: Cheung, A. *et al*. 2007 ©ELSEVIER[110] & Zhai, S.L *et al*. 2014 © Bio Med Central[111], modified

Chapter Two: Assessment of neutralizing and non-neutralizing antibody responses against Porcine circovirus 2 in vaccinated and non-vaccinated farmed pigs

2.1 Introduction

Since the 1990s, PCV2 has been recognized as one of the most significant pathogens in pig production globally[112,113]. PCV2 is the smallest autonomously replicating virus known. It consists of a circular ssDNA genome with three major ORFs and one structural protein, the CAP protein[35]. PCV2 is aetiologically involved in several syndromes affecting pigs, together known as PCVADs. Of these, PMWS of 6–12-week-old piglets is the most prevalent and devastating disease[114]. PCVADs are characterized by PCV2-induced functional and structural defects of the immune system[115]. In non-vaccinated pig herds of modern production, the morbidity and mortality of PMWS can reach over 20%[116].

Vaccination with commercially available PCV2 vaccines leads to enhanced performance of vaccinated pigs in pig production. This is reflected by reduced mortality, stronger weight gain and shortened time periods to market[82,117]. Experimental vaccination studies have demonstrated that the levels of PCV2 genome copies in serum after PCV2 challenge are significantly lower in vaccinated than in non-vaccinated animals[86,118,119]. This correlates with elevated PCV2-NAbs in serum[88], suggesting that these antibodies prevent higher levels of circulating PCV2 genomes. As high PCV2 load is an essential component for clinical disease[120,121], it has been suggested that the levels of PCV2-NAbs are an indicator of protection against PCVAD[88,122].

The immunological response to PCV2 under production conditions is less clear: whilst there is little doubt about the effects of vaccination on curtailing PCVAD, PCV2 viremia is neither consistently prevented nor reduced by vaccinating farmed pigs[123]. This study serves to

enlighten the role of PCV2 neutralizing and non-neutralizing anti-PCV2 antibodies of pigs from modern pig production facilities.

2.2 Material and methods

2.2.1 *Serum sample collection*

Samples belonging to 160 pigs from 13 pig farms in Alberta were examined. Eighty animals had been vaccinated using different types of commercial PCV2 vaccines following the instructions of the vaccine manufacturers. Serum samples were further classified into four groups according to the age the donor pig belonged to at the time point of sampling (suckling pigs: 0–21 days of age; weaned/nursery pigs: 21–84 days of age; finisher/grower pigs: 84–180 days of age; sows/boars: more than 180 days of age).

2.2.2 *PCV2 real-time quantitative polymerase chain reaction (qPCR)*

Levels of PCV2 genome copies in serum were determined with real-time qPCR. DNA extraction from 100 µL serum was performed using a Mag-Bind Viral DNA/RNA kit (Omega Biotek, catalog # M6246-03) following the manufacturer's instructions. The master mix was prepared using PCV2 primers (forward: 5'-CTGACTGTGGTTCGCTTGAT-3'; reverse: 5'-GTTACCGCTGGAGAAGGAAA-3'), a PCV2 Taq-Man probe(5'-ATGTAAACTACTCCTCCCGCCATACAATC-FAM-3'), nuclease-free water (Ambion), 2X buffer and 25X enzyme (Ambion AgPath-ID One-Step reverse transcription-PCR). A standard curve was constructed by log10 dilutions of a plasmid construct of PCV2. The qPCR program used was 45°C for 10 min, 95°C for 10 min, and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds (Bio-Rad CFX96 Real-time PCR). Results were log10 transformed and reported as PCV2 DNA copy number/mL. Based on the limit of detection of the qPCR assay, a sample was

considered positive when the result was equal to or higher than 10^4 PCV2 DNA copies/mL; only positive samples were considered for statistical analysis of the qPCR assay.

2.2.3 *PCV2 viral stock production*

PCV2 viral stock production was performed using reverse genetics. Two copies of a PCV2b genome (strain 05-32650, Accession # EF394779) were cloned into plasmid pJ201; a tandem was used to generate a clone that could amplify the complete PCV2 genome[124]. The plasmid was amplified in transformed *Escherichia coli (E. coli)* and PCV2 DNA extraction was performed using the QIAGEN Plasmid Maxi Kit protocol (catalog# 12163). PCV2 DNA was transfected into PK-15 cells using Lipofectamine 2000 (Invitrogen, catalog #52887) and OptiMEM (Gibco, catalog #31985), and infectious virus was collected in the supernatant after five days of incubation at 37°C. PK-15 cells were infected with the transfection supernatant, and incubated for 7 days, when the supernatant was collected, freeze/thawed three times to release intracellular infectious viral particles, sonicated and clarified at 400 x g for 5 minutes. Infectivity of the virus stock was determined using the Reed-Muench titration, and is defined as the dose that will infect 50% of the wells exposed to the virus (50% tissue culture infectious dose 50/mL, TCID50)[125]. The PCV2 virus stock titer was calculated as $6.3x10^4$ TCID50/mL, and PCV2 infection on PK-15 cells can be observed in Figure 2-1.

2.2.4 *Virus neutralization assay*

A virus neutralization assay (VNA) was performed to determine the PCV2-NAb titers in serum[88]. Serum was thawed and heat inactivated for one hour at 56°C, and threefold serially diluted in minimum essential media (MEM, Gibco® catalog #11095-080, supplemented with 5% fetal bovine serum, 1% non-essential amino acids, 100Upenicillin/mL, 100μg streptomycin/mL,

and 1% sodium pyruvate 100nM) in a 96-well skirted plate (VWR, catalog# 82006-704). Fifty µL of the PCV2 stock (containing $3.1x10^3$ TCID50/50µL) were added to each well for a final volume of 100µL, and incubated for one hour at 37°C. Subsequently, the samples were transferred to a 96-well culture plate (VWR, catalog #29442-058), containing PK-15 cells (50% confluency, 10,000 cells/well seeded 24h before the assay), and incubated at 37°C for 90 minutes. The wells were then washed once with 1X sterile phosphate buffer solution (PBS) and 100µL of fresh media was added to each well, followed by incubation at 37°C for 48h, before fixing and indirect staining against PCV2-CAP.

All samples were tested once on a plate and the duplicate was performed the next day under the same conditions. One serum sample known to be positive in the assay was used as a serum positive control on each plate, and a serum belonging to a newborn pig that did not consume colostrum at birth, was used as a serum negative control on each plate. Two wells were used as a virus positive control (50µl PCV2 stock in 50µL of MEM) and two wells as a serum/virus negative control, adding 100µL of MEM to the cells (Appendix 2.5.1 shows an example of a VNA plate).

2.2.5 *Indirect staining against PCV2 capsid*

To determine PCV2 infection on PK-15 cells, an intracellular, indirect staining was performed against the CAP protein. Media was discarded from the plate and cells were washed once with 1X PBS, followed by fixing by addition of 50µL/well of 4% paraformaldehyde, and incubation at room temperature (RT) for 30 minutes. Subsequently, cells were washed 3X with 100µL of 1X-PBS, and 50µL of permeabilization buffer (PB, 1X PBS - 0.1% saponin - 0.1% bovine serum albumin (BSA, Sigma-Aldrich, catalog #05470)) was added to each well, and incubated at RT for 30 minutes. After the buffer was discarded, 40µL of the primary antibody

(rabbit anti-CAP) diluted 1:400 in PB was added to each well and incubated for 90 minutes at 37°C. Subsequently, the plate was washed 3X with 100µL 1X PBS - 0.05% TWEEN® 20 (Sigma-Aldrich, catalog #P9416), and 50 µL of a 1:400 dilution in PB of the secondary antibody (goat anti-rabbit Alexa Fluor 568; Life Technologies) was added to each well and incubated for 60 minutes at 37°C. The plate was washed 3X with 100µL 1X PBS-0.05%TWEEN®, followed by the addition of 50µL of 4'6-Diamidino-2-Phenylindole (DAPI, 300nM, Life Technologies) on each well and incubated for 10 minutes at RT. Subsequently, the wells were washed 3X with 100µL of 1X PBS, and 100µL of 1X PBS were added on each well before analysis.

2.2.6 *Image acquisition and neutralizing antibody titer calculation*

The NAb titer of the serum is defined as the dilution where the sample is able to neutralize 50% of the virus infected cells compared to a virus positive control well. Images of each well were acquired to determine the percentage of infected cells/per well, and the NAb titer was calculated using a non-linear regression.

The automated microscope INCell Analyzer 2000 (GE Healthcare) was used for image acquisition of each well. Three different fluorescent channels were defined based on the staining performed to the cells: brightfield, DAPI for nucleus, and Cy3 for anti-PCV2-CAP staining (Figure 2-2A). Nine images per well were acquired from randomly-chosen 10X fields of view.

Images were analyzed using the "Multi Target Analysis" of the INCell Analyzer 1000 Workstation (GE Healthcare). The software localizes the cells in the images acquired previously and analyzes the staining of each individual cell. A "top hat" selection localizes the cell nucleus

based area (40.11μm) and DAPI staining. Subsequently, it localizes the cytoplasm following a "collar selection" which considers a diameter of 5μm around the nucleus.

The Cy3 fluorescence (indicating the PCV2 CAP+ cells) was scanned in the nucleus and in the cytoplasm of the cells. The intensity threshold of the Cy3 fluorescence was set on each plate based on the intensity of background of the PCV2-uninfected negative control cells. If the Cy3 intensity was detected to be above background in the nucleus and/or cytoplasm, the cell was considered positive (Figure 2-2B); details on validation of this analysis can be found in Appendix 2.5.4.

The percentage of positive cells is used to determine the reduction in infectivity on each well using the following formula:

$$\frac{(\%\ \text{total infected cells in control well} - \%\ \text{of infected cells in sample})}{\text{total infected cells in control well}} * 100$$

Sigma plot version 12.5 was used to perform a logistic four parameter non-linear regression calculation using the percentage of reduction in infectivity (Appendix 2.5.2). The formula used was:

$$y = y0 + (a / 1+ (x/x0)b)$$

where y is the percentage reduction in infectivity, x is the dilution of the sera, a is the max (y), b is the slope, and x0 is the point of inflection considered as the NAb50 titer of the sample. A macro was developed to analyze the samples in a quick manner and is found in Appendix 2.5.3.

2.2.7 *Indirect ELISA using a linear peptide of the PCV2 capsid*

An indirect ELISA was performed as described previously to measure the levels of antibodies against a conserved linear peptide of PCV2-CAP (169-STIDYFQPNNKR-180), an epitope that has been suggested to 'divert' the humoral immune response away from generating NAb[126]. The peptide was prepared by a commercial company (Peptide 2.0 Inc.), and was conjugated to previously activated BSA (using Sulfo-SMCC, 5mg/ml of ddH_20; Sigma-Aldrich) for a final concentration of 1mg/mL.

The peptide was diluted to a concentration of 1.5µg/mL in carbonate buffer and 100µL (150ng/well) were used to coat each well of a half 96-well ELISA plate (Thermo Fisher, catalog #15041) overnight at 4°C; the other half of the plate was coated with 1X PBS-BSA 0.1%. The serum sample was diluted 1:50 in 1X PBS-BSA 3% and pre-adsorbed to BSA by incubating overnight at 4°C.

Coated wells were washed 3X using 1X PBS-Tween 0.1% (PBST), and 100µL of blocking buffer (5% skim milk – 1X PBS – Tween 0.1%) was added to each well and incubated for 1h, RT. After blocking, 50µL of the diluted sample was added to the peptide and BSA coated wells, and incubated for 3 hours at 37°C. Subsequently, wells were washed 3X with PBST, and 50µL of a 1:2000 dilution of rabbit anti-pig HRP (Sigma, cat# A05678) were added to each well, followed by incubation for one hour at 37°C. Wells were washed 3X with PBST, and 100µL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were added and incubated for 30min at 37°C. Lecture of absorbance was done using the BIO-Rad iMark™ spectrophotometer with a 415nm filter.

All samples were tested twice on the same plate. One serum sample, known to be positive in the assay, was used as a serum positive control on each plate, and a serum from a newborn pig that did not consume colostrum at birth was used as a serum negative control on each plate. Two wells were used as a conjugate control.

To avoid considering the unspecific binding towards BSA of each sample, the absorbance in the BSA well was subtracted to the absorbance in the peptide well. This followed the normalization of the samples by calculation of the sample to positive (s:p) values using the following formula:

$$s:p = \frac{\text{(Absorbance of serum positive control) – (Absorbance of serum negative control)}}{\text{(Absorbance of sample) – (Absorbance of serum negative control)}}$$

2.2.8 *Statistical analysis*

Statistical analyses of data were performed on duplicate test results for each sample and dilution using SPSS v.17.0 software. The Kolmogorov–Smirnov test was performed to test the normality of distribution. If the distribution was normal (VNA results), an independent samples Student's t-test was performed to analyse the differences between PCV2-vaccinated and non-vaccinated animals of the same age group. One-way ANOVA was performed to analyse the variation of results between different ages but identical vaccination status groups (vaccinated/non-vaccinated), followed by post-hoc analysis using Tukey's HSD test with Bonferroni adjustment. If the distribution of data was not normal (qPCR and ELISA results), Mann–Whitney U tests were performed to analyze the differences between results of PCV2 vaccinated and non-vaccinated animals of the same age group. A Kruskal–Wallis H test was

performed to analyze the results between different ages but identical vaccination groups, followed by post-hoc analysis using Dunn's multiple comparison test with Bonferroni adjustment. Spearman's test was performed to test correlation between NAb titers and s:p ratio values of the ELISA. A p value < 0.05 was considered significant.

2.3 Results

2.3.1 *PCV2 genome copies in serum*

To reveal the effect of vaccination on the levels of circulating PCV2, a qPCR was used to determine the number of viral genome copies in serum, and they were compared between and within groups of pigs. Fifty-two animals from the vaccinated group (n=52/78), and forty-one of the non-vaccinated group (n=41/77) were negative for PCV2 genome copies in serum (Figure 2-3); two samples from the vaccinated sow/boars group, and three samples of the non-vaccinated suckling age group could not be used in this test due to insufficient amount of sera**.**

The PCV2 genome copies/mL were calculated and compared within age groups of vaccinated and non-vaccinated animals using a Mann–Whitney U test, and between age groups of the same vaccination group using a Kruskal–Wallis H test, followed by Dunn's multiple comparison test with Bonferroni adjustment. Positive results for the qPCR are shown as PCV2 genome copies Log 10/mL, and significance is shown as an asterisk (Figure 2-3).

No significant difference in the levels of PCV2 genome load was measured when comparing vaccinated with non-vaccinated pigs of the four age groups ($p>0.05$). A significant difference was observed between the distribution of PCV2 genome copies between age groups of vaccinated pigs (mean rank = 13.04, $p<0.01$). Pairwise comparisons demonstrated that

nursery/weaned vaccinated animals had significantly higher genome copy numbers compared with grower/finisher (mean rank = 9.10, $p<0.01$) and sow/boar groups (mean rank = 10.77, $p<0.05$; Figure 2-3). Similarly, non-vaccinated pigs had a significant difference between PCV2 genome copies between age groups. (mean rank = 16.29, $p<0.001$). Pairwise comparisons demonstrated that the sow/boar group had significantly lower levels of PCV2 genomes compared with weaned/nursery (mean rank = 20.12, $p<0.01$) and grower/finisher group (mean rank = 19.05, $p<0.01$; Figure 2-3).

These results demonstrate that vaccination had no effect on reducing the amount of PCV2 genome copies in any age group, and that older animals have lower PCV2 genome copies in serum, compared to younger animals.

2.3.2 *PCV2 neutralizing antibody titers*

To determine if vaccination induced the production of neutralizing antibodies, the titers of PCV2-NAbs were determined for each serum sample and they were compared within and between groups of pigs. Only two animals (n=2/80) from the vaccinated group and six (n=6/80) from the non-vaccinated group were negative to the assay (Figure 2-4).

The titers were calculated and compared within age groups using a Student's t-test, and between age groups using a one-way ANOVA, with Tukey's HSD test with Bonferroni adjustment. Positive results for assay are shown as NA titer 50 log 2 and significance is shown as an asterisk (Figure 2-4). A higher NAb titer mean was measured in vaccinated animals of 21-84 days of age (5.28 ± 2.84SD), compared to non-vaccinated animals of the same age group (3.53±2.41), with a significant difference of 1.75 ((95% CI 0.06 – 3.43), $t=2.10$, $p<0.05$; Figure

2-4); no significant difference was measured between vaccinated and non-vaccinated animals of other age groups.

A significant difference in NAb titers was measured between different age groups of vaccinated pigs ($F=13.92$, $p<0.001$). Pairwise comparisons demonstrated a significant increase in NAb titers in vaccinated animals of the grower/finisher compared to suckling (4.28, 95% CI (2.34 - 6.22), $p<0.001$), and weaned/nursery group (3.57, 95% CI (1.63 - 5.50), $p<0.001$). Similarly, non-vaccinated animals also demonstrated a difference of NAb titers between age groups ($F=7.07$, $p<0.001$). Pairwise comparisons demonstrated a significant increase in titers of vaccinated sow/boars compared to the suckling group (2.71, 95% CI (0.77 - 4.65), $p<0.01$) (Figure 2-4). In addition, non-vaccinated animals of the weaned/nursery group had significantly lower NAb titers than grower/finisher (-3.39, 95% CI (-5.83 - 0.96), $p<0.01$), and sow/boar groups (-3.62, 95% CI (-5.83 - -1.41), $p<0.001$) (Figure 2-4).

These results demonstrate an effect of vaccination on the production of PCV2-NAbs in the nursery pigs only. Also, a higher NAb titer was observed in older pigs, compared to younger animals, but these levels were independent from vaccination.

2.3.3 *Indirect ELISA using a linear peptide of the PCV2 capsid*

An indirect ELISA was used to demonstrate the presence of antibodies directed against the 169-180 epitope of the PCV2-CAP. Ten animals from the vaccinated group (n=10/80), and nine of the non-vaccinated group (n=9/80) were negative to this test (Figure 2-5). The s:p values were calculated and compared within age groups of vaccinated and non-vaccinated animals using a Mann–Whitney U test, and between age groups of the same vaccination group using a

Kruskal–Wallis H test, followed by Dunn's multiple comparison test with Bonferroni adjustment; significance is shown as an asterisk (Figure 2-5).

A significantly higher antibody level against aa 169–180 was found to be present in non-vaccinated grower/finisher group (mean rank =24.42), compared to vaccinated animals of the same age group (mean rank =16.58, U=278.5, $p<0.05$). Similarly, a significantly higher antibody level was measured in the non-vaccinated sow/boar group (mean rank =33.08), compared to their vaccinated counter parts (mean rank =15.02, U=529.5, $p<0.001$); no significant difference was measured between vaccinated and non-vaccinated animals of other age groups.

A significant difference was found in the distribution of antibodies towards the linear epitope 169-180 between different age groups in vaccinated pigs (mean rank = 36.72, $p<0.05$). Pairwise comparisons demonstrated significantly lower antibody levels in the vaccinated suckling group compared to the grower/finisher (mean rank = -33.43, $p<0.001$), and sow/boar group (mean rank = -40.33, $p<0.001$). Similarly, non-vaccinated pigs also presented a significant difference in the distribution of antibodies between age groups (mean rank = 39.35, $p<0.05$). Pairwise comparisons demonstrated that the vaccinated sows/boar group also had lower antibody levels compared to the weaned/nursery group (mean rank = -24.47, $p<0.01$) (Figure 2-5). In non-vaccinated pigs, antibody levels in the suckling group were significantly lower than the grower/finisher group (mean rank = -45.68, $p<0.001$), and sow/boar group (mean rank = - 47.53, $p<0.001$). Also, the antibody levels in the non-vaccinated weaned/nursery group were significantly lower than the grower/finisher group (mean rank = -23.03, $p<0.05$), and sow/boar group (mean rank = - 24.88, $p<0.001$) (Figure 2-5). These results demonstrate an effect of age, and not vaccination, on the induction of antibodies against the linear epitope 169-180.

A Spearman's test was performed to test if a correlation existed between neutralizing and non-neutralizing antibodies. Results demonstrate a positive, significant correlation between the titers of neutralizing and (non-neutralizing) antibodies against aa 169–180 ($r = 0.42$; $p<0.001$; Figure 2-6), demonstrating an association between both types of antibodies.

2.4 Discussion

This study aimed to determine the role of PCV2 vaccines on inducing neutralizing and non-neutralizing anti-PCV2 antibodies and their correlation with PCV2 load in serum of pigs from modern production facilities.

PCV2 genome copies were measured to determine the effect of vaccination on reducing viremia. Interestingly, no significant difference in the PCV2 genome load was found when comparing PCV2 vaccinated with non-vaccinated pigs. Thus, the protective effects of PCV2 vaccination were not reflected by reduced PCV2 serum load under field conditions[123], contrasting the results of previous studies[119] and demonstrating persistence of PCV2 infection in vaccinated pigs.

From all age groups, weaned/nursery pigs are at highest risk for developing PCVAD[114], and in the current study, this group presented the highest levels of PCV2 genome copies in sera, compared to older animals (sow/boars). Declining maternal antibodies, in addition to other typical age- and production-related stress factors[114] could contribute to a weak adaptive immune response in the weaned/nursery pigs, resulting in elevated PCV2 levels in blood. A different response was observed in older pigs, where a permanent exposure to PCV2 through the

environment could enhance secondary memory responses able to decrease circulating viral loads[127].

As vaccinated pigs are significantly protected from developing PCVAD[82,117,128], and as the levels of PCV2-NAbs were found to correlate inversely with viremia in experimental[89,129] but not field studies[88], the levels of PCV2-NAbs were determined in the cohort. A newly established VNA allowed to calculate PCV2 NAb titers based on analyses of more than 5000 cells and a minimum of 500 infectious foci (cells) per serum dilution (Appendix 2.6.5). Images of PCV2-infected cells were captured using an INCell 2000 analyzer high-content screening system (GE Healthcare) and analyzed with the INCell Analyzer 1000 Workstation (GE Healthcare). This method provides greater accuracy over traditional immunofluorescence-based VNA due to increased sampling, objective determination of positive foci, and standardization of image exposure capture settings[130].

The virus neutralization assay results demonstrate that vaccinated animals belonging to the high-risk group (weaned/nursery pigs) represented the only age group that had significantly higher PCV2-NAbs compared with the non-vaccinated counterparts. This result indicates that vaccination had a positive impact on PCV2-NAb induction in the group of pigs with the highest risk for developing PCVAD, although it is not known if the titers measured have clinical relevance with regard to protection against disease[89]. As the titers of PCV2-NAbs in vaccinated pigs were not significantly higher than those of non-vaccinated pigs in the other age groups, it is speculated that under field conditions, PCV2-NAbs need assistance from other host defence mechanisms to successfully prevent PCVAD and reduce the amount of infectious circulating virus and PCV2-infected cells[17].

As most of the older animals had substantial titers of PCV2-NAbs, it appears likely that sows transfer some of these antibodies to piglets via colostrum and milk[23]. This would explain the presence of PCV2-NAbs and the low PCV2 viral load of the suckling piglets[90]. It is, however, striking how long it takes for most pigs in production facilities to generate significantly higher titers of PCV2-NAbs, assuming that newborn piglets are first exposed to PCV2 around the time of parturition or shortly after[123]. This seroconversion time period appears to be much longer than for pigs used in experimental PCV2 vaccine studies[81,84], or pigs exposed to PPV[131] or influenza virus[132]. As pigs are capable of generating substantial titers of PCV2-NAbs within 2–5 weeks under experimental conditions[84], the lengthened time period needed to produce substantial titers of PCV2-NAbs by farmed pigs must thus be due to an environmental and not an intrinsic factor like host or viral genetics.

It has been suggested that non-NAbs directed against the linear PCV2 epitope represented by aa 169–180 of the CAP protein 'divert' the humoral immune response away from generating NAbs[126]. However, in the current study, a positive correlation was found between the titers of neutralizing and (non-neutralizing) antibodies against the CAP 169–180 epitope, contrasting the suggested inverse correlation, and demonstrating an association between these antibody types[126].

As vaccination alone did not lead to an increased level of non-neutralizing aa 169–180-specific antibodies in all the age groups, this immunogenic peptide must have originated mainly during PCV2 replication and not from the vaccine[122]. The release of this PCV2 peptide from infected cells could have happened in the process of PCV2-induced cell killing or as a consequence of an attack of CD8+T-lymphocytes on PCV2-infected cells. In either case, a

higher antibody titer against this peptide probably reflects a more intense interaction between PCV2 and the immune system of non-vaccinated animals than occurs in vaccinated pigs[133].

As protection from PCVAD is induced by PCV2 vaccination[82,117,128], it is plausible that, under field conditions, PCV2-specific T-cell immunity is most likely an essential and substantial component of protection from PCVAD[17, 134]. The stronger antibody response against aa 169–180 in older non-vaccinated pigs was probably due to repeated or persisting exposure of this linear epitope to antigen-presenting cells and T-lymphocytes. It thus appears that antibodies against aa 169–180 may serve as an indirect marker for the extent of PCV2 replication in tissues, although further studies would need to be carried out to test this possibility.

The current study is not the first to demonstrate the absence of efficacy of PCV2 vaccination in terms of reducing PCV2 serum load in farmed pigs[84], and results are in contrast to those from experimental PCV2 vaccine studies[135]. This discrepancy could be due to lower infectious 'pressure', i.e. less infectivity and/or limited exposure to PCV2 in experimental setups than exists in pig production[136]. It is also likely that farmed pigs experience a higher level of immunosuppression at the time around weaning than experimental pigs do[137]. It is proposed that subclinical infection with PCV2 – which may affect most piglets perinatally[123] – hampers a timely and strong development of PCV2-NAbs.

Based on the results of the current study, it is proposed that a subclinical infection with PCV2 before or shortly after weaning could play a major role for compromising functions of the immune system, although clinical outcomes seem to occur later. In conclusion, subclinical

infection with PCV2 deserves more attention and research, together with efforts to improve current PCV2 vaccines.

Figure 2-1 Microscopy images (10X, INCell Analyzer 2000) of PK-15 cells after mock or PCV2 infection after 48h of incubation. Indirect-immunofluorescence assay was performed using the antibodies rabbit anti-PCV2 CAP, followed by goat anti-rabbit IgG Alexa Fluor 568 (AF-568) and nuclear counterstain (DAPI).

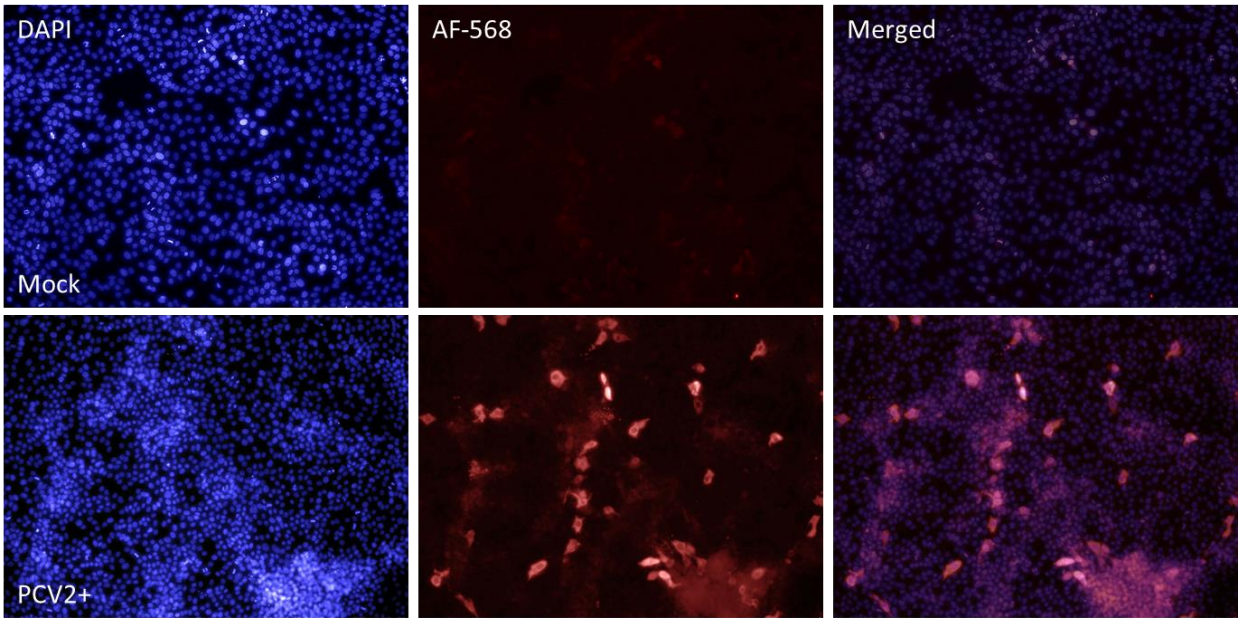
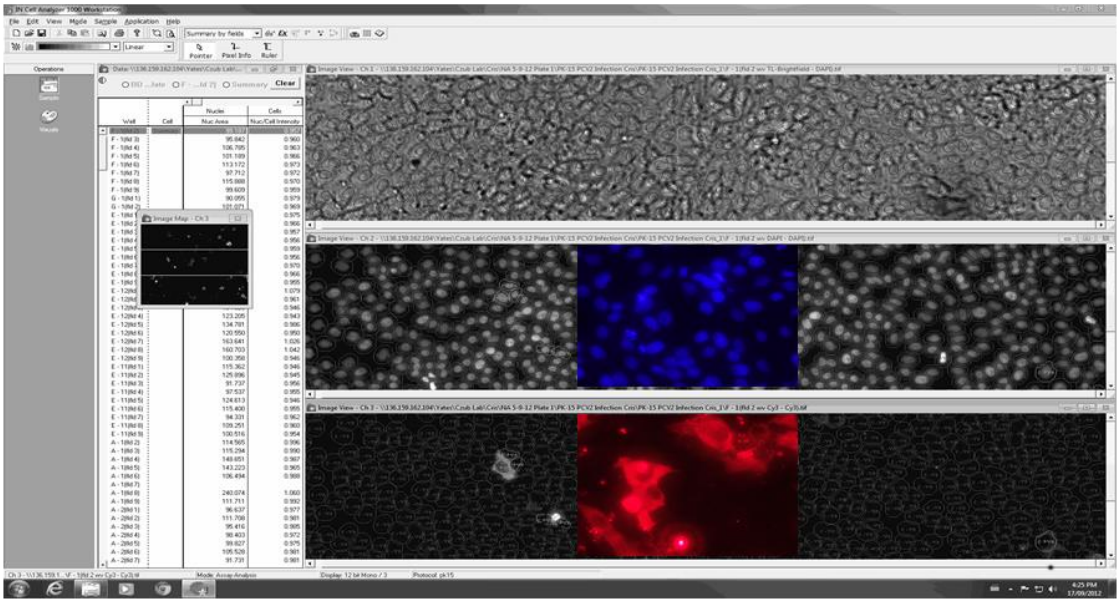


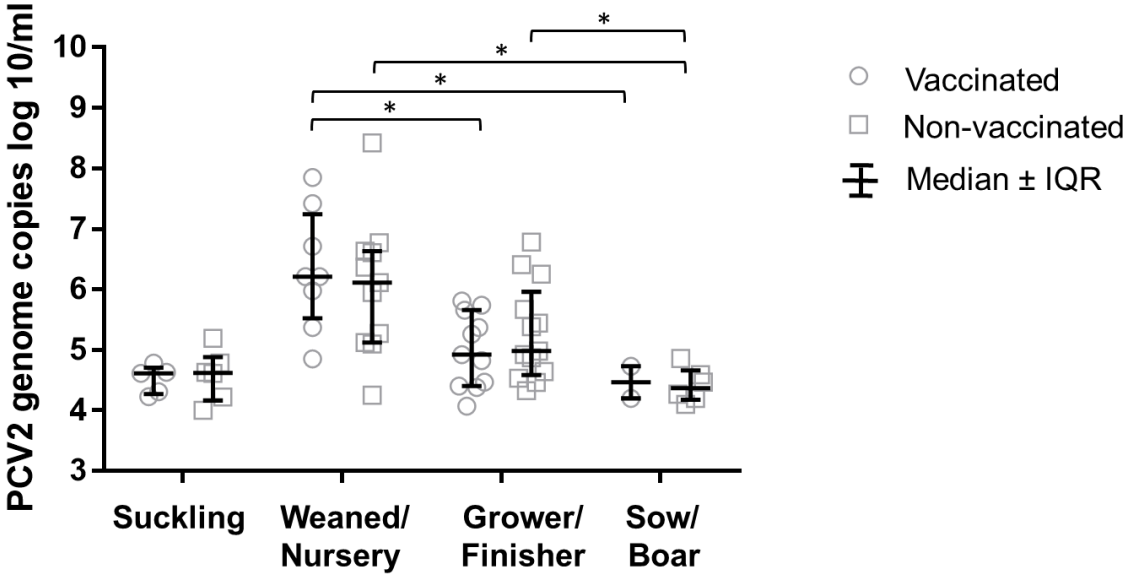
Figure 2-2 Example of the analysis performed by the INCell Analyzer 2000 (GE Healthcare). **Figure 2-2A** Screenshot of the analysis software INCell Analyzer 2000: Brightfield, DAPI, and Cy3. **Figure 2-2B** PCV2 CAP + cells using the INCell Analysis software: The nucleus is localized by DAPI, then the nucleus and cytoplasm are scanned for Cy3 signal over background, indicating PCV2 CAP+ cells. Red circles indicate positive cells.

A.



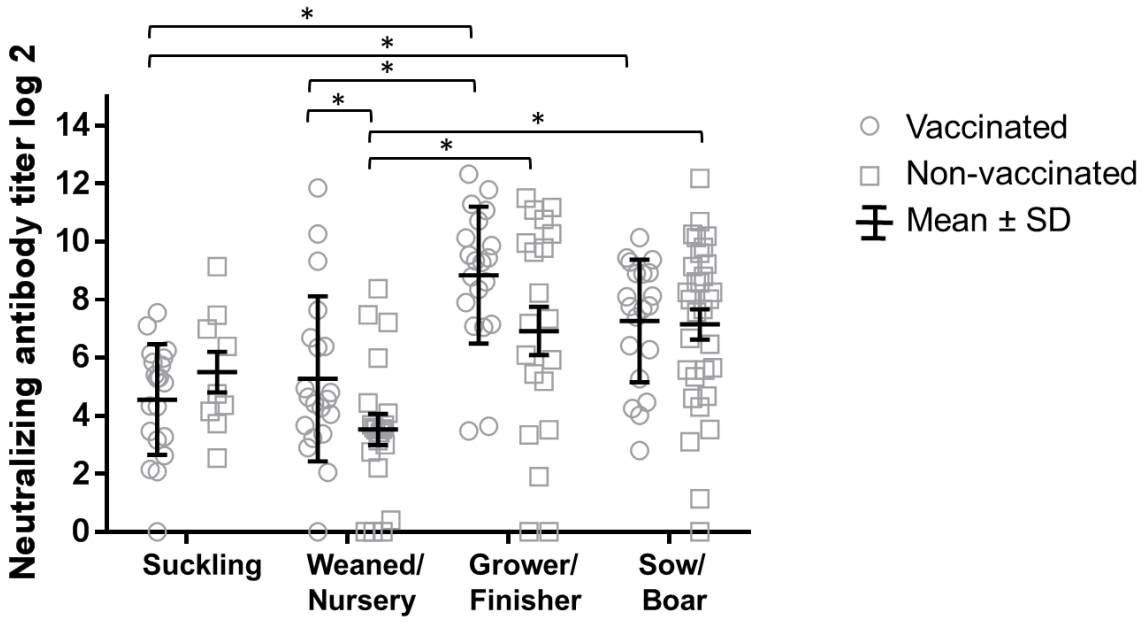
B.

Figure 2-3 Levels of PCV2 genome copies in sera from qPCR-positive pigs grouped into four age categories and further divided into vaccinated and non-vaccinated animals. The results of individual samples are depicted as open symbols; the median interquartile range (IQR) are denoted in the graph. Based on statistical analysis, groups of animals that showed a significant difference are indicated by an asterisk in the graph ($p<0.05$). The prevalence of qPCR-positive animals is demonstrated in the table below the graph.



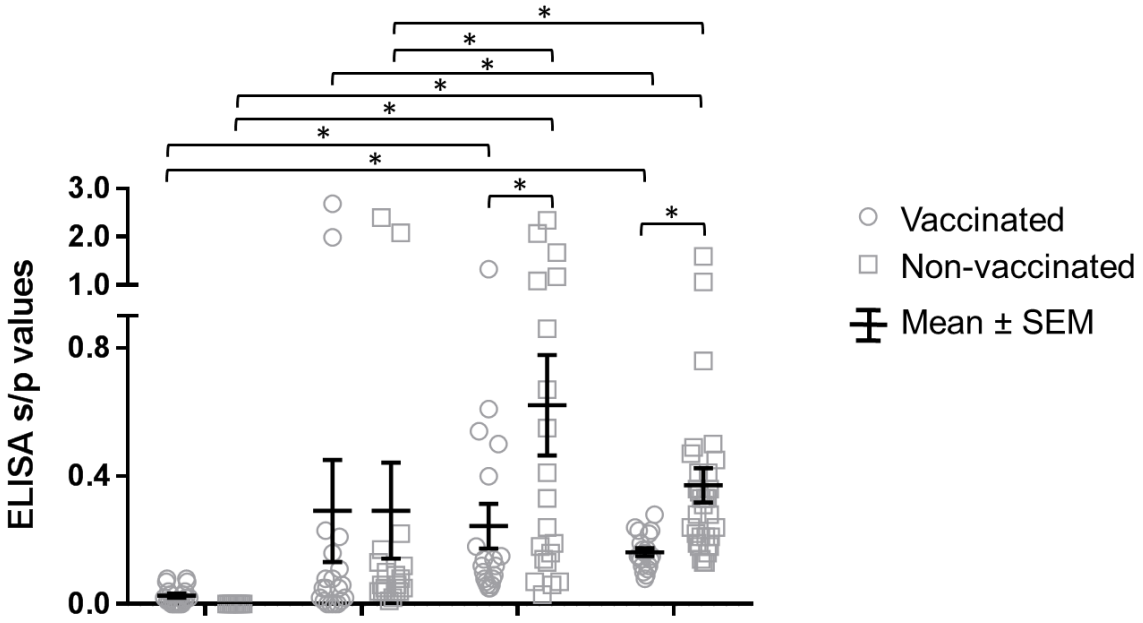
PCV2+	V	NV	V	NV	V	NV	V	NV
n/total	5/20	6/6	8/20	11/20	11/20	13/20	2/18	6/31
%	25	100	40	55	55	65	11	19

Figure 2-4 Titers of PCV2-neutralizing antibodies in sera from pigs grouped into four age categories and further divided into vaccinated and non-vaccinated animals. The results of individual samples are depicted as open symbols; mean ± SD are denoted on the graph. Based on statistical analysis, groups of animals that showed a significant difference ($p<0.05$) are indicated by an asterisk on the graph. The prevalence of VNA-positive animals is shown in the table below the graph.



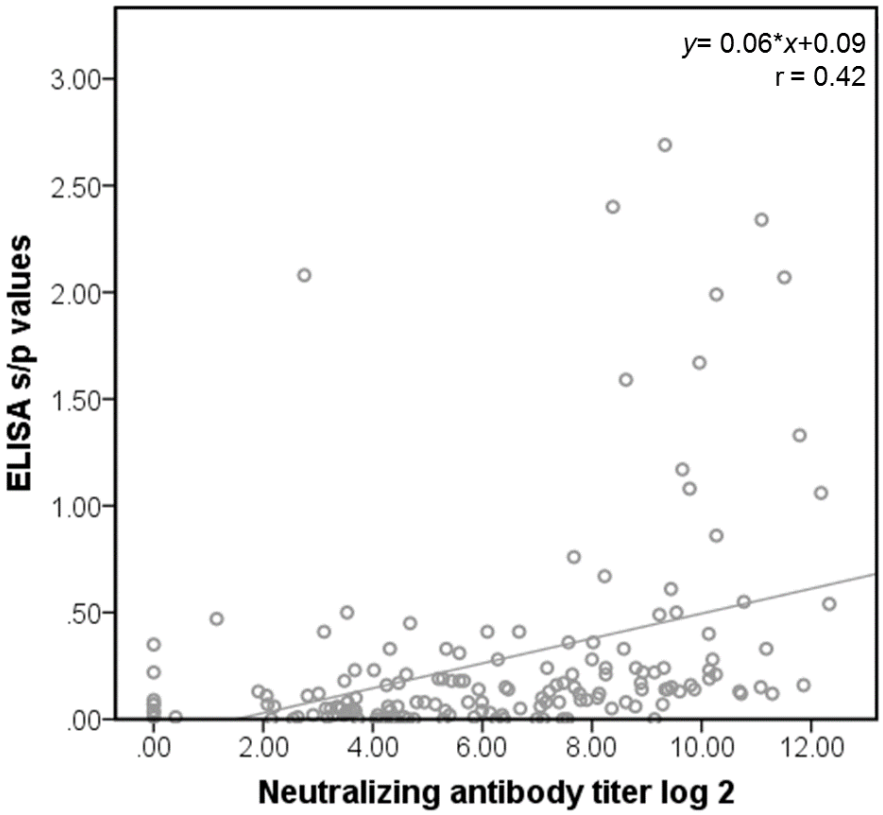
VNA+	V	NV	V	NV	V	NV	V	NV
n/total	19/20	9/9	19/20	17/20	20/20	18/20	20/20	30/31
%	95	100	95	85	100	90	100	96

Figure 2-5 Levels of antibodies directed against PCV-CAP in sera from pigs grouped into four age categories and further divided into vaccinated and non-vaccinated animals. The results of individual samples are depicted as open symbols; mean ± SEM are denoted in the graph. Based on statistical analysis, groups of animals that showed a significant difference ($p<0.05$) are indicated by an asterisk in the graph. The prevalence of ELISA-positive animals is shown in the table below the graph.



ELISA+	V	NV	V	NV	V	NV	V	NV
n/total	14/20	0/9	16/20	20/20	20/20	20/20	20/20	31/31
%	70	0	80	100	100	100	100	100

Figure 2-6 Correlation between neutralizing antibody titers and s : p values of the anti-CAP peptide aa 169–180 ELISA. The results of individual samples are depicted as open symbols. A linear regression was calculated and is represented as a line.



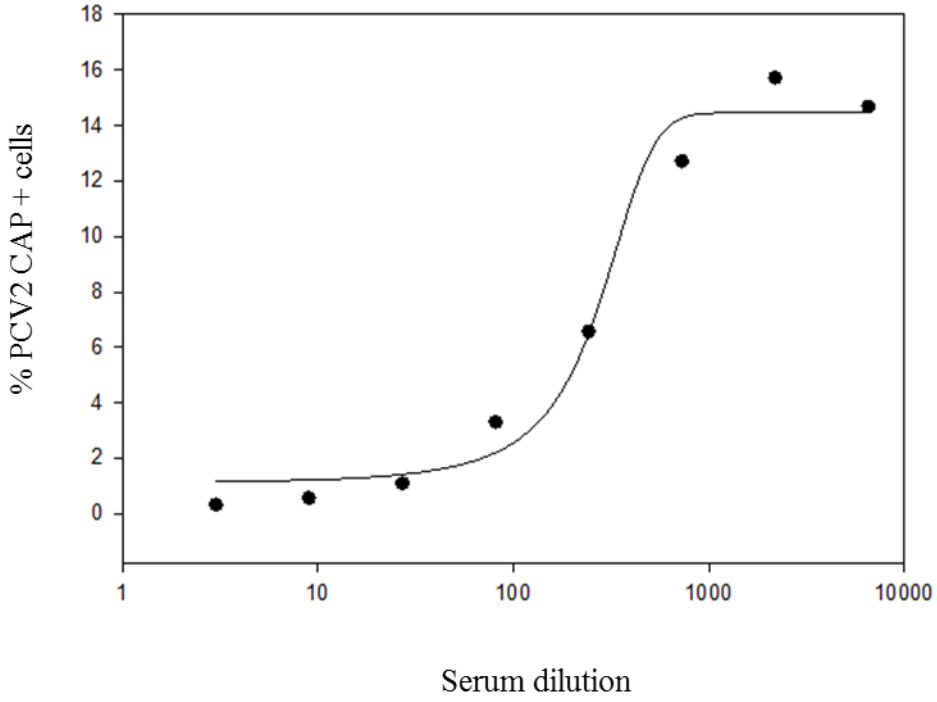
2.5 Appendix

2.5.1 *Example of a VNA plate set-up.*

	1	2	3	4	5	6	7	8	9	10	11	12
	S 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	SC+	
A	1:3										1:3	Mock
B	1:9										1:9	Mock
C	1:27										1:27	Mock
D	1:81										1:81	Mock
E	1:243										SC- 1:3	PCV2
F	1:729										1:9	PCV2
G	1:2187										1:27	PCV2
H	1:6561										1:81	PCV2

SC+: Positive control serum; SC-: Negative control serum

2.5.2 ***Example of a non-linear regression curve to calculate the 50% reduction in infectivity using Sigma Plot version 12.5.***



*In this example, the r^2=0.98 and the VN50 of this sample was calculated to be 264.7.

2.5.3 *Sigma Plot macro used to calculate the NAb titer*

```
Option Explicit
Function FlagOn(flag As Long)
FlagOn = flag Or FLAG_SET_BIT ' Use to set option flag bits on, leaving others unchanged
End Function
Function FlagOff(flag As Long)
FlagOff = flag Or FLAG_CLEAR_BIT ' Use to set option flag bits off, leaving others unchanged
End Function
Sub Main
Dim I As Integer
For I = 1 To 20
ActiveDocument.CurrentDataItem.Open
ActiveDocument.NotebookItems.Add(CT_GRAPHICPAGE)
Dim ColumnsPerPlot()
ReDim ColumnsPerPlot(2, 1)
ColumnsPerPlot(0, 0) = 11
ColumnsPerPlot(1, 0) = 0
ColumnsPerPlot(2, 0) = 31999999
ColumnsPerPlot(0, 1) = 0
ColumnsPerPlot(1, 1) = 0
ColumnsPerPlot(2, 1) = 31999999
Dim PlotColumnCountArray()
ReDim PlotColumnCountArray(0)
PlotColumnCountArray(0) = 2
ActiveDocument.CurrentPageItem.CreateWizardGraph("Scatter Plot", "Simple Scatter", "XY Pair",
ColumnsPerPlot, PlotColumnCountArray, "Worksheet Columns", "Standard Deviation", "Degrees",
0.000000, 360.000000, , "Standard Deviation", True)
```

```
ActiveDocument.CurrentPageItem.Select(False, -1269, -1742, -1269, -1742)
ActiveDocument.CurrentPageItem.Select(False, -1885, 1695, -1885, 1695)
ActiveDocument.CurrentPageItem.SetCurrentObjectAttribute(GPM_SETAXISATTR,SAA_TYPE, 2)
Dim CurItem As Object
Set CurItem = ActiveDocument.CurrentItem
Dim ActiveDoc As Object
Set ActiveDoc = ActiveDocument
Notebooks.Open("C:\Users\Prionlab\Desktop\STANDARD.JFL", ".JFL")
Dim FitFile As Object
Set FitFile = Notebooks("C:\Users\Prionlab\Desktop\STANDARD.JFL")
ActiveDoc.Activate
CurItem.IsCurrentItem = True
Dim FitObject As Object
Set FitObject = Notebooks("C:\Users\Prionlab\Desktop\STANDARD.JFL").NotebookItems("Logistic, 3
Parameter ")
FitObject.Open
FitObject.DatasetType = CF_XYPAIR
FitObject.Variable("x") = "col(161)"
FitObject.Variable("y") = "col(" + CStr(I) + ")"
FitObject.Run
ActiveDocument.NotebookItems("Data 1").Open
ActiveDocument.CurrentDataItem.Open
ActiveDocument.CurrentDataItem.Open
ActiveDocument.CurrentDataItem.Open
FitObject.OutputReport = True
FitObject.OutputEquation = False
FitObject.ResidualsColumn = -2
```

```
FitObject.PredictedColumn = -2
FitObject.ParametersColumn = -2
FitObject.OutputGraph = True
FitObject.OutputAddPlot = True
FitObject.ConfidenceBands = False
FitObject.ExtendFitToAxes = False
FitObject.AddEquationToTitle = False
FitObject.AddPlotGraphIndex = 0
FitObject.XColumn = -1
FitObject.YColumn = -1
FitObject.ZColumn = -2
FitObject.Finish
FitFile.Close(False)
Set FitFile = Nothing
ActiveDocument.CurrentPageItem.Select(False, -1269, -1742, -1269, -1742)
ActiveDocument.CurrentPageItem.Select(False, -1885, 1695, -1885, 1695)
ActiveDocument.CurrentPageItem.SetCurrentObjectAttribute(GPM_SETAXISATTR,SAA_TYPE, 2)
Set FitFile = Nothing
Next I
End Sub
```

2.5.4 *Validation of the VNA analysis: Manual vs automated analysis*

A VNA was performed using control samples and the plate was analyzed following the manual count protocol described by Fort *et al.* (2007)[88], and compared to the results of the automated analysis.

The plate reading in the manual count was performed using an inverted microscope at 40X. Three random fields of view were selected and the positive cells were counted. Afterwards, the percentage of neutralization was calculated following the formula:

[1-(mean # positive cells of the two replicas of each serum dilution/ mean # positive cells in negative control)]*100.

The same plate was run in the automated microscope INCell Analyzer 2000 following the protocol described in this chapter. The results for both counts were plotted and the linear regression between both counts was calculated as r^2= 0.97 (Figure 2.5.4).

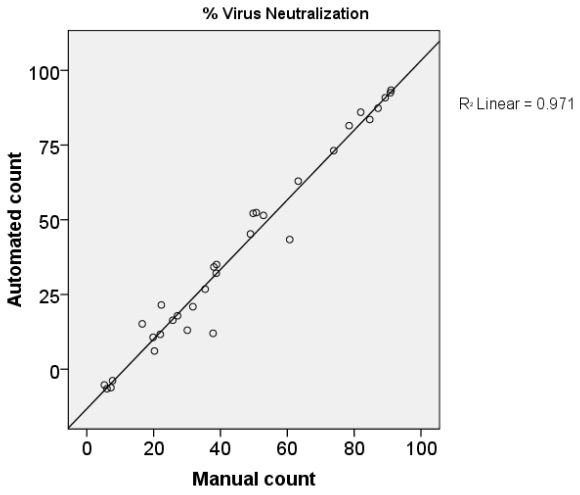


Figure 2.5.4 Automated and manual count comparison of the VNA assay.

2.5.5 *Example of raw data from the INCell Analyzer 1000 Workstation*

2.5.5A. Total cell count and PCV2 infection rates (percentage and numbers) in the mock control wells of the 16 plates used in the study including the first and second runs, with duplicates.

Plate ID	Run 1 Total Cell Count	Run 2 Total Cell Count	Run 1 Total PCV2 (%)	Run 2 Total PCV2 (%)	Run 1 Total PCV2 (n)	Run 2 Total PCV2 (n)
1	10620 10464	4183 7834	0.04 0.01	0.10 0.01	4 1	4 1
2	4914 9107	5565 3233	0.18 0.00	0.04 0.43	9 0	2 14
3	3476 2121	2639 2363	0.14 2.08	0.04 0.00	5 44	1 0
4	3567 2611	2688 1867	0.20 0.08	0.34 0.16	7 2	9 3
5	2498 4463	4533 4806	0.04 0.02	0.31 0.10	1 1	14 5
6	5944 3581	4933 7685	0.03 0.92	0.06 0.00	2 33	3 0
7	10035 10848	4918 2334	0.05 0.07	0.04 0.13	5 7	2 3
8	11904 11141	2885 4009	0.06 0.24	0.10 0.05	7 27	3 2
9	7725 7757	10852 10275	0.18 0.05	0.28 0.53	14 4	30 54
10	8267 7726	6635 3137	0.05 0.02	0.29 0.77	4 2	19 24
11	9086 7567	11202 11284	0.99 0.17	0.07 0.02	90 13	8 2
12	8385 6269	11862 11100	0.72 0.16	0.06 0.05	60 10	7 5
13	5800 7618	13110 13119	0.21 0.28	0.12 0.10	12 21	16 13
14	3316 1679	13655 5520	0.85 0.36	0.27 0.69	28 6	37 38
15	11809 10627	11202 11549	0.17 0.55	0.37 0.11	20 58	41 13
16	8193 6572	4354 3268	0.24 0.00	0.14 0.21	562 20	6 7
Total	7031.10	6831.22	0.29	0.19	33.72	12.06

2.5.5B. Total cell count and PCV2 infection rates (percentage and numbers) in the PCV2+ control wells of the 16 plates used in the study including the first and second runs, with duplicates.

Plate ID	Run 1 Total Cell Count	Run 2 Total Cell Count	Run 1 Total PCV2 (%)	Run 2 Total PCV2 (%)	Run 1 Total PCV2 (n)	Run 2 Total PCV2 (n)
1	3661	2828	21.36	13.51	782	382
	4126	2588	18.57	16.07	766	416
2	3553	1781	8.78	15.61	312	278
	4622	2705	10.21	10.24	472	277
3	2295	2688	12.55	7.03	288	189
	2024	3300	12.11	8.52	245	281
4	2032	1585	17.37	13.19	353	209
	2209	1360	17.97	13.24	397	180
5	1642	3151	16.14	13.58	265	428
	1854	9421	15.32	12.14	284	1144
6	3122	3119	19.92	11.48	622	358
	4623	5072	16.42	11.38	759	577
7	8361	4752	10.24	12.98	856	617
	7181	6135	12.91	10.71	927	657
8	8214	2804	14.04	13.73	1153	385
	8792	3393	11.07	13.23	973	449
9	5741	7648	13.20	12.27	758	938
	4641	6640	12.58	13.57	584	901
10	5788	3053	15.48	11.76	711	359
	7663	6000	15.74	8.93	911	536
11	5714	10162	10.94	9.75	625	991
	6191	9155	11.16	9.95	691	911
12	5661	8932	16.23	8.32	919	743
	6246	9651	15.53	7.24	970	699
13	4547	11182	12.25	7.83	557	876
	3957	10465	16.45	7.83	651	819
14	1620	10953	14.44	9.78	234	1071
	1413	10954	16.77	7.99	237	875
15	7314	9888	11.74	9.44	859	933
	6620	8834	11.03	10.37	730	916
16	5828	2162	11.67	11.66	606	252
	4789	2380	11.74	12.69	680	302
Total	4751.38	5773.16	14.12	11.13	630.53	592.16

Chapter Three: Establishment of a PCV2-free swine cohort

3.1 Introduction

PCV2 is the causative agent of the PWMS, a disease characterized by severe lymphocyte depletion and immunosuppression[60]. Even though PCV2 infects and replicates in PBMCs[138], not all infected pigs develop clinical disease, and the mechanisms behind PCV2 pathogenesis and the effects of viral infection on lymphocyte function are unknown[139]. Currently, no animal model is available to reproduce the whole spectrum of PMWS, therefore, an *in vitro* system using PCV2-free primary swine PBMCs is ideal to understand host-pathogen interactions[72].

Swine research has increased in the last three decades as a result of biomedical and infectious disease studies[105]. Outbred, conventional pigs are suitable for research due to the availability of herds worldwide, large litter sizes, standardized breeding procedures, and strict biosafety protocols in farms[107,140]. However, the widespread presence of exogenous pathogens, like PCV2, that cause perinatal infections and are ubiquitous in swine herds has raised concerns regarding their use for *in vivo* and *in vitro* studies[141]. Several models have been developed to raise PCV2-free pigs[70, 142, 143, 144], yet the most common methodologies used are gnotobiotic piglets[142], and snatch-farrow, porcine colostrum deprived (SF-pCD) pigs[143,144].

Gnotobiotic piglets are germ-free animals raised in controlled, sterile environments[142]. Pregnant sows are usually obtained from a high-health farm, and transported to a clean facility one week before the farrowing date[103]. The gravid uterus is removed at approximately day 113 of gestation, and piglets are immediately introduced into sterile chambers that isolate them from environmental pathogens[103]; pigs are fed sterile milk replacements and managed using sterile tecniques[142]. Gnotobiotic pigs are ideal to study the development of the swine immune system

without gut colonization[103]; however, due to their size, pigs can only be kept in the isolators for approximately one month[142]. In addition, this method requires specialized facilities and trained personnel to raise the piglets[142,103]

SF-pCD piglets are born naturally in the farm, but additional contact with the environment is controlled by snatching them immediately after farrowing, avoiding colostrum intake and possible contact with pathogens[144]. The piglets are then transported to a research facility, reared in a bio-containment, non-sterile environment, and fed bovine colostrum[144]. This method allows pathogenesis studies without the interaction of maternal antibodies and pathogens, and reduces costs significantly compared to gnotobiotic piglets, since a caesarian section and isolators are not required[144].

This study aimed to determine whether farmed-raised pigs have a persistent PCV2 infection, and to raise PCV2-free pigs using a SF-pCD method. The establishment of a PCV2-free pig cohort would allow pathogenesis studies to be conducted without the use of tissues and cells derived from PCV2-persistently infected pigs[141].

3.2 Material and methods

3.2.1 *Determination of a persistent PCV2 infection in swine PBMCs*

To determine if farmed-raised pigs carried a persistent PCV2 infection, PBMCs were isolated from seven Landrace-Large white cross pigs. The animals were born in a commercial herd but were transported at three months of age to a biosafety level 2 (BSL-2) room of the Veterinary Sciences Research Station (VSRS) of the University of Calgary; none of the pigs presented PCV2-associated clinical signs while housed in the facility.

Thirteen whole blood samples were collected by jugular venipuncture and collected on Ethylenediaminetetraacetic acid (EDTA) tubes in a period of 6 months. PBMCs were isolated using Ficoll gradient, and re-suspended to a concentration of $2x10^6$ cells/ml of Roswell Park Memorial Institute (RPMI) medium (see Chapter 4 for details on cell isolation). DNA was extracted from 200µL of PBMC suspension and a qPCR was used to determine the PCV2 genome copy number present in the cells (see details below).

PCR results demonstrated that 3 out of 13 PBMC samples were positive to PCV2 DNA copies (>$4.0X10^4$ PCV2-DNA copies/mL of isolated PBMCs). In addition, the PCV2-CAP protein was determined in the samples using flow cytometry (results not shown). Due to the possibility of a persistent PCV2 infection in PBMCs isolated from farmed animals, it was decided to establish a PCV2-free swine model to use as whole blood source for future experiments.

3.2.2 *SF-pCD animal model*

A SF-pCD method was used to raise PCV2-free pigs, by snatching the piglets from the sows immediately after birth and transporting them to a BSL-2 controlled environment[144]; the animal protocol was approved by the Animal Care Committee of the University of Calgary (ACC-15-0036).

3.2.2.1 Sows and snatching of piglets

A commercial breeding facility with Large White/Landrace cross sows was chosen and approved by the University of Calgary. Two visits were performed prior to the expected

farrowing date to inspect the facilities and to determine possible routes to minimize pathogen exposure for the newborn piglets.

At the collection day, three clinically-healthy sows were chosen randomly as they started parturition, at approximately 115 days of gestation. When parturition signs were observed, the peri vulvar area was washed and cleaned with chlorhexidine 2% and water. Once the farrowing started, a disposable pad was placed around the area to reduce the risk of environmental contamination.

Newborn piglets were snatched from the sows before they touched the floor, or equipment of the farm. The piglet was cleaned with a new towel (Figure 3-1A), the umbilical cord was immediately clamped, cut, and cleaned with povidone-iodine (7.5%, Betadine®), and the pig was placed in a new plastic box. It was then transported to a previously disinfected, heated van that was waiting outside the farrowing facility. In the van, the animals were cleaned, dried, and placed in a new plastic box, containing a heat pad and a high-efficiency particulate air (HEPA) filter on the lid; a maximum of four piglets were placed in each box. To minimize exposure of piglets to pathogens, the person in the van did not enter the facilities, and wore a face mask and gloves while manipulating the animal (Figure 3-1B).

The piglets were bottle-fed with bovine colostrum once an hour (see section 3.2.2.2 for details; Figure 3-1B), and were kept in a warm environment inside the box (approximately 30°C). After the last pig had been snatched, cleaned, and fed, they were transported to the VSRS of the University of Calgary.

3.2.2.2 Rearing of snatch-farrowed piglets and sampling pre-weaning

Piglets were moved to a previously disinfected, BSL-2 room with positive pressure ventilation; the temperature of the room was set to 30°C, and was recorded four times a day. Piglets were identified using a livestock marker, and placed inside 3 pig containment pens (67 inches wide x 42 inches deep x 34 inches high; Penner Services, catalog ID: KPD), with 2-3 animals per unit. A heat lamp was placed on top of the unit, at a height determined by the pigs' behaviour (comfortably lying on their sides, underneath the heated space). Each pen had a liquid feeder per pig (Miller Manufacturing, USA, catalog #BPW4), and metal chains and rubber toys for enrichment.

The animals were fed a liquid diet prepared fresh inside the room, consisting of dried bovine colostrum powder with 20% (first 72h only), or 18% bovine IgG (The Saskatoon Colostrum Company Ltd.), spray-dried whole egg powder with polyclonal antibodies against *E. coli* (2g/pig/day, Hyper Egg K88, J. H. Hare & Associates Ltd.), and an oral iron supplement (250mg/kg, 75mg/mL, Ferodan®), re-suspended in 37°C water[144]; details on the volume of colostrum fed per pig per day are shown in Table 3-1, and the protocol followed to prepare the liquid diet mix is shown in Appendix 3.5.1. The piglets were fed hourly for the first 6h, every 2h from 6 to 24h of age, every 4h from 24h to 5 days of age, and 4 times per day thereafter until they were 21 days of age (8am, 12pm, 4pm, 10pm).

The piglets were bottle-fed initially (Figure 3-1C), but learned to drink colostrum from the liquid feeder after the first 48-72h. Fresh water was left inside the feeders in between feeding times. On day 17, the piglets were introduced to commercial starter feed pellets inside the

colostrum (100gr/pen/feeding, Masterfeeds, Vigor™ Pre-starter 2, non-medicated (plain) crumbles, catalog #305207), until weaned-off milk, on day 22 of age.

A clinical score sheet was filled once a day per animal, to assess the animal's behaviour inside the unit (e.g. breathing, alertness), and when handled (e.g. body condition score, hydration; Appendix 3.5.2). Since an increase on daily weight gain has been considered as a parameter for good health in piglets[144], body weight was measured once a day using a scale. The average weekly weight gain of the pigs was calculated using the following formula: (weight at day 7 – weight at day 1)/ 7), and then averaged between individuals.

Feces were observed daily for any signs of diarrhea, and rectal temperature was taken if the animal presented a decrease in body weight or any clinical signs. If the animal presented clinical signs, it was treated with anti-inflammatory (meloxicam, Metacam®, Boehringer, 1.5mg/mL, 0.4mg/kg, P.O., every 48h), and antibiotic drugs (ceftiofur hydrochloride, Excenel®, Zoetis, 50mg/ml, 3mg/kg IM, q.d for eight days).

A BSL-2 protocol was followed to enter the room at all times (Appendix 3.5.3). A separate room was used to change from street clothes into the study designated autoclaved scrubs, and foot covers were used to cover the shoes on the way to the room. Once inside the pig room, the shoes and foot covers were left in a designated "dirty area", and rubber boots were used to move around the "clean area" of the room. New gloves, mouth, and hair covers were used at all times (Figure 3-1C). In addition, each containment unit had an autoclaved surgical gown that was worn when cleaning and manipulating the animals of that pen, and gloves were changed in-between units. The floor of the room was disinfected daily using Virkon 1% for 10

minutes (Virkon Disinfectant Technologies), before washing thoroughly with warm water. Each pen was washed four times a day with soap and warm water, after each feeding (Appendix 3.5.4). In addition, no person in contact with other pigs in the last 24h was permitted inside the room.

An oral swab was taken from each animal on days 1, 8, 15, and 22, to test for PCV2 DNA copies in saliva using qPCR. Briefly, a new swab was placed inside the piglet's mouth for 15 seconds, then placed back into the sleeve. Individual swabs were placed in 1mL of sterile PBS, and stored at -20°C until analysis.

3.2.2.3 Post-weaning rearing

At 22 days of age, the pigs were weaned-off milk, and moved into a previously cleaned and disinfected nursery room; the animals were transported using a cart to avoid contact with the facility's floors. Piglets were swabbed, weighed, and ear-tagged, and a prophylactic anti-inflammatory (meloxicam, Metacam®, Boehringer, 1.5mg/mL, 0.4mg/kg, P.O.), and antibiotic treatment (ceftiofur hydrochloride, Excenel®, Zoetis, 50mg/ml, 3mg/kg IM, q.d for three days) was given, followed by daily monitoring for one week.

Animals were divided into two pens with elevated slatted flooring and concrete floor underneath. Each pen had a three space-feeder (Figure 3-1D), and an individual feeder on the side. Two nipple drinkers allowed water *ad libitum*, and the animals were monitored over the first days to assure they learned how to use them. The commercial starter pellets that had been introduced before weaning (Vigor™ Pre-starter 2, Masterfeeds) were fed three times a day for the first three days, followed by twice a day thereafter. At 10 weeks of age, the feed was

transitioned into grower pellets (Masterfeeds, Vigor™ 16% Hog grower, non-medicated pellets, catalog #308434) until the end of the study.

The RT was checked daily, initially set to 24°C, and decreased one degree weekly until the RT was 21°C. A heat lamp was placed in a corner of each pen for the first 10 days after weaning, until it was observed that the pigs were not using the heated space. Rubber mats were placed in front of the feeders, and on the corners of the pens. Plastic toys and metal chains were placed on each pen for enrichment (Figure 3-1F).

Since the pigs were placed in a new room, changes were applied to the BSL-2 protocol to enter the rooms (Appendix 3.5.5). Three different areas were designated: a “dirty area” where the staff changed from street clothes to autoclaved scrubs, followed by an “intermediate area” that was crossed using the area’s rubber boots, and a “clean area”, consisting of a sampling room, and a separate room where the pigs were housed. Once inside the “clean” rooms, the staff changed into boots and disposable coveralls to be used for cleaning and feeding for each pen.

At 16 weeks of age, the pigs walked on the concrete floor for 10 minutes daily, while the pen was being cleaned. The animals were also made to stand from 5 to 10 minutes on a foot bath containing an antiseptic solution (povidone-iodine 7.5%, Betadine®) to avoid presentation of foot lesions.

3.2.3 *Blood sampling and euthanasia*

Sampling was performed once a week after weaning, for a total of 13 weeks. Only half of the cohort was bled each week, assuring that each individual would be bled only once every

two weeks. The volume of blood sampled was never more than 0.05% of the animal's estimated body weight, and it was collected using EDTA and serum-separating tubes.

The anatomic site sampled was determined based on age:

3.2.3.1 Cranial vena cava: from 4 to 14 weeks of age (approximately 8-65kg of weight)

Pigs of less than 11 weeks of age were sedated inside the pen using azaperone (0.2mg/kg, IM), while pigs of 11 to 14 weeks of age were sedated using a combination of dexmedetomidine (0.01mg/kg, IM), azaperone (0.4mg/kg, IM), and alfaxalone (2mg/kg, IM). After the animal was sedated, it was carried to the sampling room where it was manually restrained on a procedure table. The pig was placed on it's back, with one person firmly holding down the back legs, and another person holding down the front legs. A third person hold the pig's head with one hand, and directed a 20-22gauge X 1.5-inch needle on a vacutainer holder towards the thoracic inlet area, aiming the needle towards the opposite scapula, in a centre of an area formed by the cranial aspect of the sternum, the shoulder joint, and the trachea. The needle was then inserted ¾ of it's full length, the vacutainer tube inserted into the needle, until the blood started to flow (Figure 3-1E). After sampling, the animals were taken back to the pen where they were monitored until they were able to walk and drink water correctly (approximately 1 hour).

3.2.3.2 Jugular venipuncture: from 15 to 22 weeks of age (65 to 90kg of weight approximately)

When pigs were 15 weeks of age or older, they were directed towards the sampling room using plastic herding boards, where they were restrained using a hog Iowa holder. The holder kept the pigs in standing position, with their neck extended. An 18-gauge X 1.5-inch needle was

directed into the pig's right jugular grove, two-thirds down the neck. Once inside the skin, the needle was connected into the vacutainer, and the blood started to flow.

3.2.3.3 Terminal bleeding and euthanasia

Once the humane or experimental endpoint was reached, the animal was sedated, anesthetised, and euthanized following humane guidelines (ACC-15-0036). Briefly, the pigs were pre-medicated using dexmedetomidine (0.01mg/kg, IM), azaperone (0.4mg/kg, IM), and alfaxalone (2mg/kg, IM); induction was performed using alfaxalone (1mg/kg, IV). Once the animal was confirmed to be under deep anesthesia, a cardiac puncture was performed using a 14-gauge, 3.5-inch needle, with a 20ml syringe to collect a maximum of 300ml of blood. Afterwards, the animal was euthanized with an overdose of sodium pentobarbital (20-25ml per animal).

3.2.4 *PCV2 genome testing*

A qPCR was performed to determine the PCV2 genome copies in blood and saliva. Briefly, 200µl of saliva suspension or 200µl of whole blood were used for DNA extraction, using the QIAamp DNA mini kit (QIAGEN Catalogue # 51306) following the manufacturer's instructions; PBS was used as a negative extraction control and a PBS spiked with 1µl of PCV2 10^8 plasmid was used as a positive extraction control. The master mix was prepared using 0.75µl of PCV2 primers at a final concentration of 300nM (Forward- CTG ACT GTG GTT CGC TTG AT, and Reverse- GTT ACC GCT GGA GAA GGA AA), 12.5µl of PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences Catalogue # 95056-02K), and 8.5µl of sterile PCR water. The standard curve was constructed by log 10 dilutions of a plasmid construct of PCV2. The qPCR program used was 95°C for 5 minutes, and 40 cycles of 95°C for 15 seconds and

60°C for 30 seconds (Bio Rad® CFX96 Real Time PCR). Based on the limit of detection of the qPCR assay, a sample was considered positive if the result was higher to 10^4 PCV2-DNA copies/ml blood or saliva; assay results are reported as log10 of PCV2-DNA copy number/ml blood or saliva.

3.2.5 *PCV2 antibody testing*

A Western Blot was performed to determine the presence of antibodies against PCV2 in serum of SF-pCD pigs, using purified PCV2 as antigen. Briefly, an sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, with a single sample well was prepared using a 12% resolving gel (ddH_20, 30% acrylamide/bis-acrylamide solution, 1.5M Tris/HCl, 10% SDS, 10% ammonium persulfate (APS), and Tetramethylethylenediamine (TEMED)), and a 4% stacking gel (ddH_20, 30% acrylamide/bis-acrylamide solution, 0.5M Tris/HCl, 10% SDS, 10% APS, and TEMED). Fifty µL of purified PCV2 and 50µL of lysis buffer were boiled for 10 minutes, mixed with 100µL of sample buffer, and loaded into the single sample well of the polymerized gel. A protein ladder (PageRuler Prestained Protein Ladder, Fermentas #SM0671), with a magic mark (MagicMark XP Western Standard Ladder, Invitrogen #P/N LC5602) were used as reference, and the samples were run in an electrophoresis system (Mini-protean Tetra cell, Bio Rad, catalog #1658005EDU) for 100 minutes, at 100V.

The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer method. Briefly, filter papers, PVDF membrane, and the gel, were equilibrated in semi-dry transfer buffer, and placed in layers in a semi-dry electrophoretic transfer cell (Trans-Blot®, Bio Rad, catalog #1703940), for 90 minutes at 25V. Subsequently, the membrane was blocked using 5% skim milk in 1X PBS-0.1% tween for 1h, RT.

The membrane was cut vertically into 20 strips of approximately 0.3cm, and each strip was used to test individual swine serum samples against PCV2 antibodies; sera positive and negative to PCV2 antibodies were used as controls. Each swine serum was diluted 1:100 in 5% skim milk in 1X PBS-0.1% tween, placed on individual chambers, and incubated with a membrane strip overnight, at 4°C. The strip was washed 3X with 1X PBS-0.1% Tween, followed by incubation with a 1:10000 dilution of the secondary antibody (rabbit anti pig-IgG-HRP, Sigma catalog #A5670), for 60 minutes, RT. The membrane was washed 3X with 1X PBS-0.1% tween, and incubated with the chemiluminescent substrate (Pierce™, ECL, Thermo Fischer, catalog #32106), for 3 minutes in the dark, RT; the membrane was analyzed using a VersaDoc 5000 Imaging System and Quantity One software.

3.2.6 *Statistical analysis*

A liner-mixed effects model (LMER) was used to examine the effects of time (as fixed effects) on body weight (predictor variable), using individual pigs as a random effect. A restricted maximum likelihood (REML) method was used for parameter estimation (see Chapter 4 for details on the model). The normality and equal variance assumptions were assessed to validate the fitness of each model; a $p<0.05$ was considered statistical significant. Analyses were performed using R version 3.3.1 (lme4 package, version 1.1.12)[145].

3.3 Results

3.3.1 *Health status of SF-pCD pigs*

Nine piglets were snatched from three sows; information on the sex, weight, and sow identification for individual pigs is displayed in Table 3-2. Pig #9 died during the first 24h and was not considered for the clinical status analysis.

An increase on weight gain was considered as a parameter for good health in the piglets[144], so the weight of the animals was taken daily for the first 22 days, and is displayed per individual pig in Figure 3-2. The average daily weight gain of the pigs was 113g ± 32 SD for the first week, 244g ± 44 SD for the second week, and 243g ± 57 SD for the third week. Time had a positive effect on the weight gain ($p<0.001$), and a significant variation of daily weight gain was observed between individual pigs ($p<0.001$).

The clinical signs observed throughout the rearing of the pigs, and the age of death or euthanasia are detailed in Table 3-2. Diarrhea, swollen joints, jaundice, and foot lesions were observed in different animals.

A black, pasty to watery diarrhea was observed in six out of the eight animals, during different days of the first 3 weeks of rearing (Table 3-2). Pigs #2, #6, and #8 presented diarrhea in the first 48h, and pig #5 on day six, but they remained alert, gaining weight, and eating correctly so treatment was not necessary at this time. In contrast, pigs #1 and #4 presented swollen leg joints and diarrhea after 48h and 96h, respectively, and pigs #2 and #8 presented weight loss, lethargy, and fever at day 18; in these cases, the animals were treated with antibiotic and anti-inflammatory drugs.

Jaundice was observed in pig #1 at six weeks of age, with an evident icterus of skin, mucous membranes, and serum (Table 3-2). A complete physical exam did not suggest any other pathology, and a complete blood count and chemistry was performed; the results suggested a liver lesion with increased alkaline phosphatase (918 U/L; ref. 118-395), total bilirubin (109.5 umol/L; ref. 0.0-17.1), and cholesterol (2.28 mmol/L; ref. 0.93-1.4) (Appendix 3.5.6). Since the

pig continued to eat and defecate normally, it was kept in the cohort until it died overnight, 9 days later. Necropsy findings determined that the cause of death was a hemorrhage due to a diffuse esophageal ulcer. It was also determined that the icterus was due to a chronic portal fibrosis with obstructive cholestasis (Appendix 3.5.7).

At 10 weeks of age, all seven animals presented some degree of lameness. Pig #3 was the most affected, with swollen hind limbs, hoof bruising, and white line separation; it was euthanized at 11 weeks of age after no improvement was observed with anti-inflammatory treatment and foot baths (Table 3-2). To diminish foot lesions in the remaining animals, exercise was encouraged on the concrete floor while the pen was being cleaned, anti-inflammatory treatment was provided when necessary, and the pigs were made to stand in foot baths once a day, until improvement was observed; the most affected pigs were #5 and #8, which presented mild foot lesions until the end of the study.

The sampling days, with the amount of blood sampled per animal is described on Table 3-3. No irregularities were observed during sedation, handling, blood sampling, or euthanasia of the pigs. Pigs #2 and 4 reached the experimental endpoint at 14 and 15 weeks of age, respectively. The remaining four animals were separated into two pens until the end of the study at 22 weeks of age.

3.3.2 *Determination of PCV2 status of pigs*

A qPCR was performed to determine the presence of PCV2-DNA in saliva or blood of pigs; results are shown in Figure 3-3. All animals were negative on the four swabs performed

pre-weaning (Figure 3-3A), and on the blood samples taken weekly until 22 weeks of age at the end of the study (Figure 3-3B).

A Western Blot was performed to determine if the pigs had antibodies against PCV2. All SF-pCD pigs raised were negative in this assay (Figure 3-4).

3.4 Discussion

PCV2 is a pathogen that infects pigs worldwide and is associated with the immune system of swine[60]. The mechanisms leading to PCV2-associated clinical disease are not well understood, and a reproducible *in vitro* system would aid to study PCV2 pathogenesis[72]. In this study, an SF-pCD PCV2-free cohort was established as a source of primary PBMC cells to study PCV2 infection.

Farmed pigs are commonly used to study PCV2 infection due to the availability of outbred pig herds and the practicality of studying PCV2 infection in a natural setting[140]. A disadvantage of this model is the possibility of a subclinical PCV2 infection in the pigs that could influence experimental results[72]. In this study, a persistent PCV2 infection was confirmed in farmed-raised pigs by the presence of viral DNA and CAP protein in the isolated PBMCs. Other studies have also shown a reactivation of a PCV2 infection in PBCMs after mitogen treatment and incubation for 72h[72]. Taken together, these results demonstrate the difficulties of using persistently-infected PBMCs for swine pathogen research, and the need of PCV2-free pigs for future experiments.

In the current study, eight pigs were successfully raised PCV2-free using a SF-pCD method[144]. The animals were bled regularly and they served as a source of primary PBMCs for

PCV2 infection studies for a total of 14 weeks. In addition, since the pigs were deprived of porcine colostrum, their PCV2-antibody free status allowed to use them in a PCV2-vaccination pilot study (results not shown), demonstrating the different uses of this model.

The PCV2-free status of the pigs was determined by the absence of PCV2 antigen and antibodies in whole blood and serum. Snatching the piglets from the sow immediately after birth and keeping the animals under controlled, biosafety level-2 (BSL-2) conditions for the remaining of the study prevented PCV2 infection due to contact with contaminated farm environment or maternal colostrum. A PCR also demonstrated that the pigs were negative for the Porcine Lymphotropic Herpesvirus 1 and 3 (Hunt, J. 2016, personal communication), a virus that infects newborn piglets[146], demonstrating that the biosafety and biosecurity measures taken throughout the study prevented these common peri-natal infections.

Daily monitoring of weight and clinical signs of the piglets was vital to initiate treatment promptly, avoiding mortalities throughout the first weeks of rearing[144]. Six out of the eight piglets presented a pasty, black diarrhea, starting 48h after farrowing accompanied by weight loss, fever, and lethargy. The absence of maternal antibodies, and the clean, but not sterile environment where the pigs are housed, makes gastrointestinal infections caused by *E. coli* common when rearing colostrum-deprived pigs[147,148]. Prophylactic treatment with antibiotics is not recommended to prevent diarrhea occurrence in colostrum-deprived pigs[147], therefore stringent cleaning of the units using disinfectants, and filtered water to prepare the liquid diet, could prevent cases of pathological diarrhea in the future.

All pigs presented lameness after 10 weeks of age, with hoof bruising, or white-line separation in severe cases. Foot lesions are common in farmed pigs, with a prevalence of 39.6% in swine of 4 to 14 weeks of age[149]. Treatment with anti-inflammatory drugs, as well as foot baths and additional rubber mats inside the pens significantly improved lesions in the animals[150]. Additionally, foot lesions can be prevented by moving the animals from mesh to concrete flooring before the finishing stage begins at 8 weeks of age[149].

In conclusion, it was possible to raise PCV2-free pigs using a SF-pCD method and preventing a PCV2 infection of the animals throughout the study. These animals can reliably be used as tissue and cell donors for *in vitro* PCV2 studies, avoiding the use of material derived from PCV2-persistently infected pigs.

Figure 3-1 Raising of snatch-farrow porcine-colostrum deprived piglets. **Figure 3-1A** Newborn piglets were snatched from the sows before they touched the floor or equipment of the farm. The piglet was cleaned with a new towel, the umbilical cord was immediately clamped and cleaned with povidone-iodine, and the pig was placed in a new plastic box. **Figure 3-1B** The piglets were bottle-fed with bovine colostrum once an hour, immediately after birth. **Figure 3-1C** Feeding of piglets in containment facility. A biosafety-level 2 protocol was followed to enter the room, and designated boots, coveralls, surgical gowns, face masks, gloves, and hair covers were used at all times. **Figure 3-1D** After weaning, individual feeders were installed on each pen to avoid competitions. **Figure 3-1E** Pigs of less than 14 weeks of age were manually restrained, and the cranial vena cava was used for blood collection. **Figure 3-1F** After weaning, pigs were placed in two pens with mesh elevated floors, rubber mats, and plastic toys for enrichment.

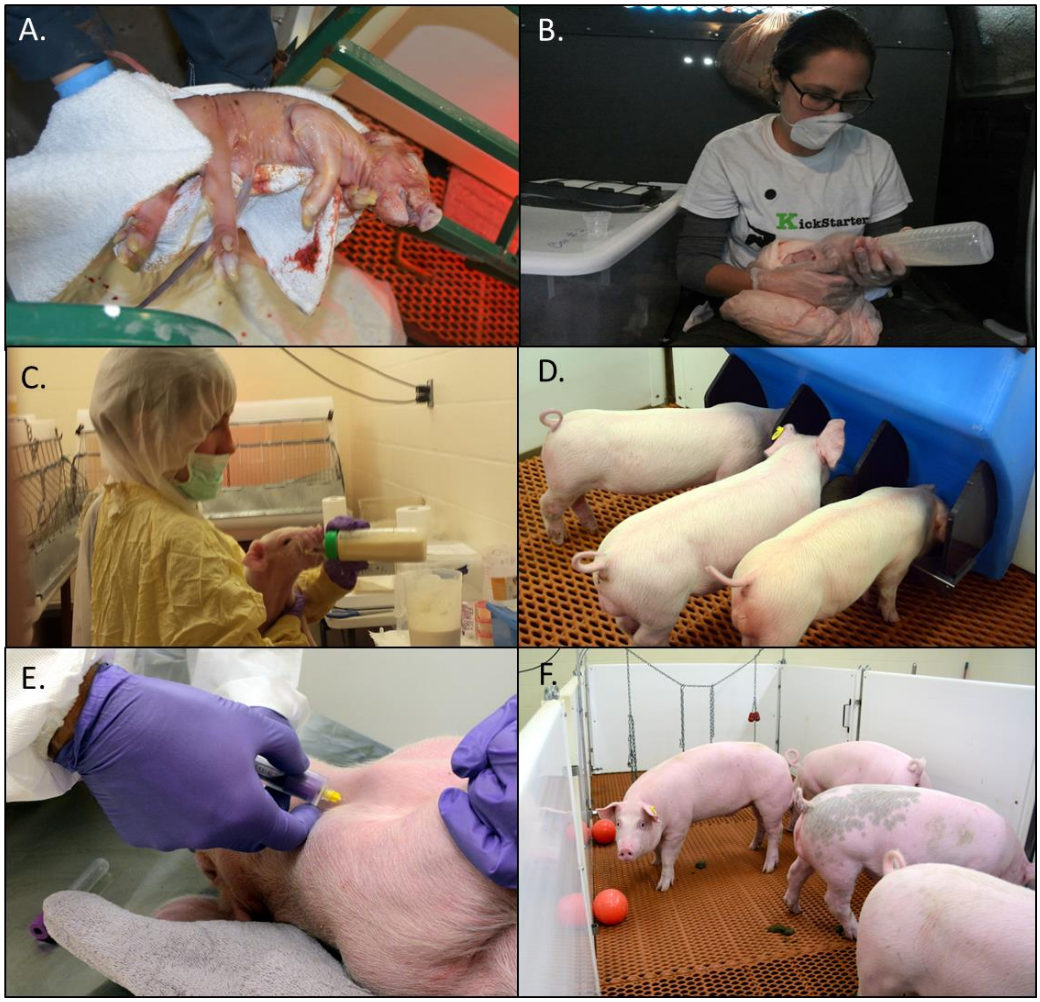


Table 3-1 Components and quantity of the liquid diet fed to the SF-pCD piglets from day 1 to 21. The volume displayed is the amount required daily (*)

Ingredients	Days 1-3					
	1 pig	X10 pigs*				
20% Colostrum (%, w/v)	20	1060gr				
Iron (mg/kg milk solids)	250	250mg				
IgY K88 (g/pig/day)	2	20				
Warm water (L/pig/d)	0.53	5.3 L				
	Days 4 – 9		**Days 10 - 14**		**Days 15 -21**	
	1 pig	X10 pigs*	1 pig	X10 pigs*	1 pig	X10 pigs*
18% Colostrum (%, w/v)	15	2.5 kg	15	3.7 kg	15	4.9kg
Iron (mg/kg milk solids)	250	625mg	250	925mg	250	1225mg
IgY K88 (g/pig/day)	2	20	2	20	2	20
Warm water (L/pig/d)	1.67	16.7 L	2.5	25 L	3.3	33 L

Source: Huang *et al.*, 2013 (modified)

Table 3-2 Sex, weight at day 2 (kg), weight at weaning (kg), sow ID for individual piglets, clinical signs (week of age), and week of age at death or euthanasia.

Pig ID	Sow	Sex	Weight day 2 (kg)	Weight at day 22 (kg)	Clinical signs (week of age)	Death or euthanasia (week of age)
1	1	M	2.3	5.7	Diarrhea (w1) Joint swelling (w1) Jaundice (w6)	8
2	1	F	1.8	5.6	Diarrhea (w1 and w3)	14
3	1	M	2.2	7.6	Lameness (w10)	11
4	1	M	2.0	7.1	Diarrhea (w1) Joint swelling (w1)	15
5	2	M	1.8	6.6	Diarrhea (w1) Lameness (w10)	22
6	3	M	1.8	7.2	Diarrhea (w1)	22
7	3	F	2.1	8.0	Lameness (w10)	22
8	3	M	1.6	6.3	Diarrhea (w1 and w3) Lameness (w10)	22
9	3	M	-	-	-	1

Figure 3-2 Daily weight (kg) of individual pigs on the first 22 days of age.

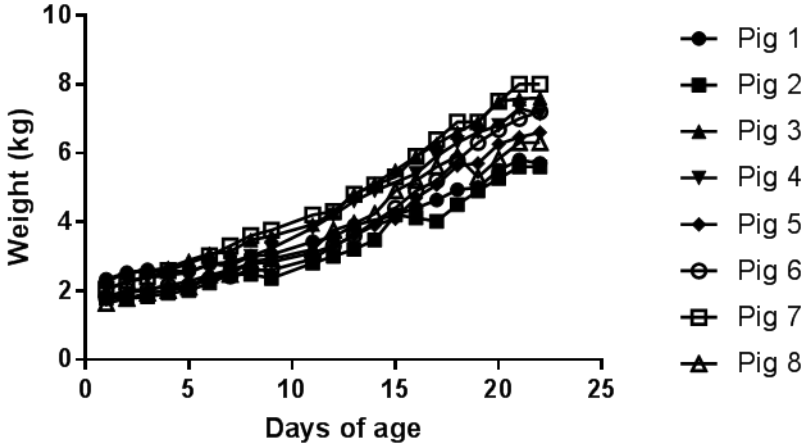


Table 3-3 Amount of whole blood (mL) sampled per animal per sampling day. Terminal bleeding of the animals is demonstrated by an asterisk (*).

Sampling week	Pig ID	mL whole blood
1	5	5
	6	8
	7	8
	8	3
2	1	5
	2	10
	3	10
	4	5
3	5	10
	6	10
	7	10
	8	5
4	2	16
	3	32
	4	26
5	5	48
	6	19.5
	7	54
	8	50
6	2	44
	3	44
	4	45
7	5	29
	6	47
	7	45
	8	46
8	2	18
	3	48
	4	39
	3	104*
9	5	49
	6	47
	7	44
	8	58
10	2	153*
	4	54
11	4	221*
	7	47
	8	46
12	5	74
	6	76
13	7	45
	8	25
14	5	91*
	6	113*
	7	80*
	8	110*

Figure 3-3 PCV2 DNA copies determined by qPCR in saliva and blood of SF-pCD pigs. **Figure 3-3A** PCV2 DNA copies/200µL of saliva (log 10), collected from oral swabs of individual pigs (1-8), once a week for the first month of rearing. Samples #9 and #10 represent the positive and negative controls for the assay, respectively. **Figure 3-3B** PCV2 DNA copies/mL of blood (log 10), per pig, per sampling week. Samples + and x represent the positive and negative controls for the assay, respectively.

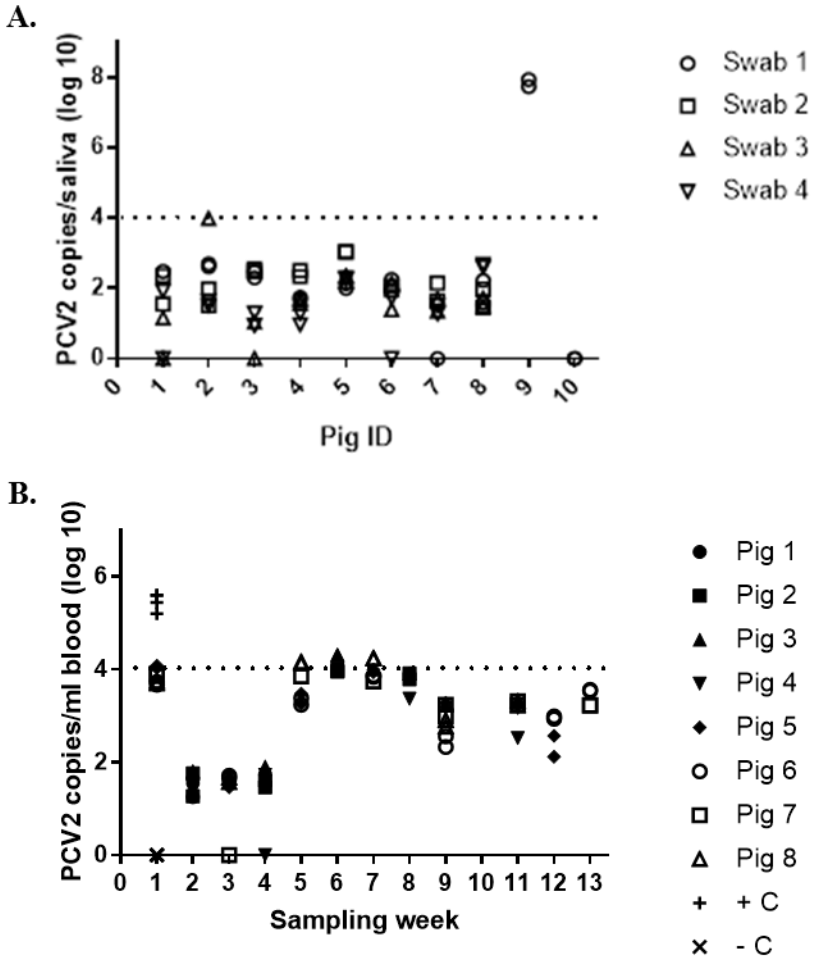
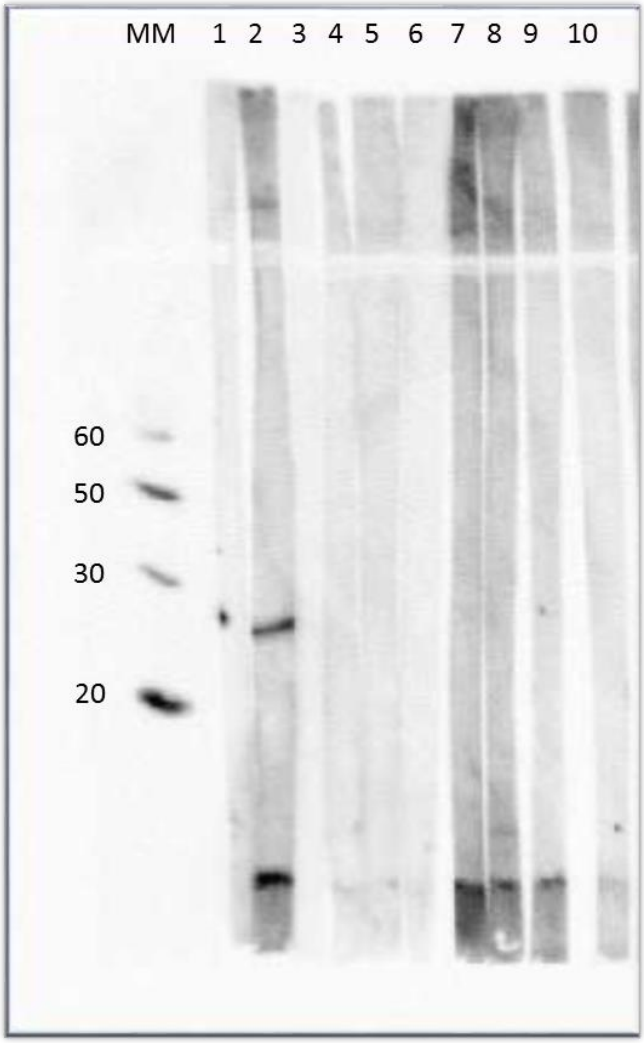


Figure 3-4 Western Blot to determine antibodies against PCV2-CAP protein (27kDa) in terminal bleed serum sample from SF-pCD pigs. Each strip represents a sample (1-10), with magic mark (MM) used as reference for protein weight (kDa). Samples ID are as follow: 1: Polyclonal rabbit anti-PCV2-CAP; 2: Positive control (ID: RD4); 3: Negative control (newborn piglet); 4: Pig #2; 5: Pig #3; 6: Pig #4, 7: Pig#5; 8: Pig #6; 9: Pig #7; 10: Pig#8.



3.5 Appendix

3.5.1 *Standard operating procedure (SOP) for liquid diet preparation*

Standard operating procedure (SOP) – Animal handling BSL-2

COLOSTRUM PREPARATION*

Project: Derivation and handling of snatch-farrowed, pig colostrum deprived piglets (SF-pCD).

AC15-0036, February - March, 2016

***Total feeding volume will vary depending on the date of the visit. The amount of colostrum to be prepared and fed/pig is available in a table inside the room.**

1. A powder mixture containing bovine colostrum and Hyper Egg Immune IgY will be available for each feeding and will be placed in a properly identified Ziploc bag on top of the table.

2. Add the appropriate amount of iron supplement (Ferodan) to the mixture following the proper amount/date shown in the table.

3. Warm-up 1.7L of water in the kettle. Do not let the water boil.

4. Mix hot and cold water in the pink bucket labeled “food” to reach the final volume required/day, reaching a final temperature of 37°C. Measure the temperature of the water using the green digital thermometer.

5. Mix the powder mixture containing the colostrum, IgY, and iron into the water. Mix thoroughly using the manual beater.

6. Distribute colostrum in all the feeders, offering the volume required per pig, following the table in the room. Do not overfeed.

7. Clean the feeders and the decks with soap and water.

3.5.2 *Clinical score sheet for pre-weaned piglets*

PRE-WEANED PIGLETS	Date and time										
Clinical observation											
Normal (N) or Absent (A)											
Undisturbed (20min)											
Skin/Ears/Tail											
Behaviour											
Breathing											
Movement											
Eating											
Drinking											
Alert/Sleeping											
Breathing											
Respiratory signs											
On Handling											
Alert											
Body condition											
Hydration											
Eyes											
Vocalisation											
Urine											
Feces											
Sampling site											
Body Weight											
Temperature (pig)											
Temperature											
(microenvironment)											
Comments											
Initials											

3.5.2 (cont.)

Signs	Clinical score			
	0	1	2	3
Alert/Sleeping	Normal	Dull or depressed	Little response to handling	Unconscious
Behaviour	Normal	Isolated, abnormal posture	Inactive, abnormal position (cont) aggressive to pen mates	Aggressive OR Moribund
Breathing	Normal	Rapid, shallow	Rapid, abdominal breathing	laboured, irregular, cyanotic
Body condition	Normal	Loss OR increased weight	Loss of body fat OR increase fat	Loss of muscle OR no detection of bones
Body weight	Normal	Reduced growth rate	Chronic weight loss	Stagnation of growth
Skin/Ears/Tail	Normal	Small wounds	Un-kept coat, wounds, mutilation	Major wounds, bleeding, mutilation
Drinking	Normal	Increased OR decreased in 24h	Increased OR decreased in 48h	Constantly drinking OR no drinking over 48h
Eating	Normal	Increased OR decreased over 24h	Increased OR decreased in 48h	Overeating OR inappetence over 48h
Eyes	Normal	Wetness OR dullness, abnormal movement for 24h	Discharge, abnormal movement for 48h	Eyelids matted, nystagmus
Feces	Normal	Moist feces OR excessively dry feces (no mucus)	Loose feces, soiled perineum, blood OR less feces	Continuous diarrhea, blood in feces OR constipation
Hydration	Normal	Weakness, dry snout for 24h	Weakness, dry snout for 48h	Incoordination, convulsions
Movement	Normal	Slight incoordination, lameness	Reluctance to move after rest	Limb dragging OR paralysis
Respiratory signs	None	Coughing or sneezing for 24h	Coughing, sneezing and discharge for 48h	Continuous discharge and coughing Difficulty breathing.
Sampling site	Normal	Mild lesions after sampling	Mild edema, hematoma formation	Excessive edema/hematoma formation
Temperature (pig)	Normal	>40°C over 12h	>40°C over 24h	>40°C after treatment
Temperature (env)	Normal	< or > 30°C		
Urine	Normal	Abnormal volume	Abnormal volume and hematuria	Incontinence, hematuria
Vocalisation	Normal	Excessive OR decreased vocalisation for 24h	Excessive OR decreased vocalisation for 48h	Continuous abnormal vocalisation
Other				

3.5.3 *SOP for handling of pre-weaned piglets*

Standard operating procedure (SOP) – Animal handling BSL-2

Project: Derivation and handling of snatch-farrowed, pig colostrum deprived piglets (SF-pCD).

AC15-0036, February, 2016

This room will be subjected to the following practices at all times:

Entering the room:

DO NOT ENTER THE ROOM IF YOU HAVE BEEN IN CONTACT WITH SWINE

WITHIN THE PAST 24 HOURS

1. Wear the study designated scrubs only – change in bathroom 124A (sheep area). Put in disposable hearing protection at this time as well.
2. Take one pair of the purple, disposable plastic boot covers from this room with you to put on immediately outside Room 123 before entering.

3. On the log sheet outside Room 123 before entering, record:
    a. - your name
    b. the entry time ("time-in")
    c. room temperature – from thermometer viewed through the door window.

Before entering room, look at pigs through the window to briefly observe behaviour. Do they look comfortable? Make note of any suspiciously acting piglets

Inside the room:

1. Enter the room and stay in "dirty area" (as marked by floor tape).
2. Put on hair net and face mask. Step out of your shoes and put on a disposable overall.
3. Carefully step into the boots located in the "clean area". Place latex/nitrile gloves on.
4. Observe the piglets before disturbing them and look for any abnormal behaviour. Check water remaining in bowls in pen and function of heat lamps.
5. Clean surface of table using 1% VirkonS solution.

6. Heat water and prepare mix of colostrum following instructions (See SOP). Place in feeders and assure pigs eat. Spot clean pig deck flooring using water.
7. **Researchers:** Weigh, take rectal temperature of piglets and record on clinical score sheet (once a day ONLY, record completion on exit sheet).
   Researchers: Deep cleaning of the piggy decks (soap and water) and the floors will be performed ONCE a day ONLY (record on exit sheet; See SOP).
8. Take feeders out of pens and wash them with water and soap. Place feeders in again filled with water.
9. **Researchers:** Prepare the powder mixture for next feeding, without adding water or iron.
10. Do a final walkthrough of the room to assure everything is in order.
11. Provide any necessary summary comments in the log book within the room

Exiting the room:

1. Step out of "clean" boots into "dirty area" in your sock feet leaving the boots in the clean area.
2. Take off face mask, and hair net and place in garbage container. Take off coverall and hang back up on wall hook if still visibly clean or discard in autoclave bag if soiled. Put on shoes with the shoe covers on and exit the room.
3. Once outside the room:
   a. - record your exit time ("time-out")
   b. remove shoe covers and carry back to garbage bin in bathroom 124A .
4. In room 124A:
   a. - Remove scrubs and, if clean, place in designated locker for reuse or, if dirty, place in laundry hamper.
   b. All scrubs must be laundered and autoclaved at least once per week.

3.5.4 *SOP to clean room and containment units of pre-weaned piglets*

Standard operating procedure (SOP) – Animal handling BSL-2

PIGGY DECK AND FLOOR CLEANING

Project: Derivation and handling of snatch-farrowed, pig colostrum-deprived piglets (SF-pCD).

AC15-0036, February - March, 2016

Piggy deck "deep" cleaning: ONCE A DAY

1. Place a big garden bag in metal bin. Tape the edges of the bag to the bin using duck tape.
2. Place the piglets in the metal bin.
3. Take feeders out and rinse with soap and water.
4. Clean the flooring of the piggy deck using soap and water. Scrub using a sponge. Discard sponge after using and rinse the flooring with water.
5. Lift the flooring and rinse bottom of deck using water. Lift a corner of the deck to help drain the water.
6. Dry the surface of the flooring with a paper towel.
7. Place water in feeders, place in deck and return the piglets to the deck.
8. Record cleaning on the sheet outside the room.

Floor cleaning: ONCE A DAY

1. Clean the metal bin with Virkon 1% and water.
2. All the boxes and material that is on the floor should be placed inside the metal bin while the floor is being cleaned.
3. Spray floor with warm water. Clean floor using dish soap and water. Scrub floor using broom, rinse with water.
4. Prepare 2L of 1% Virkon. Distribute through the floor and leave it to air dry.
5. Spray the "dirty" area of the entrance (signaled by tape) with Virkon. Leave the room.

Direction of cleaning: start from the far end of the room (near pen#3) and clean towards the "dirty area" at the entrance of the room.

3.5.5 *SOP to enter rooms of pigs (post-weaning)*

Standard operating procedure (SOP) – Animal handling BSL-2

Project: Derivation and handling of snatch-farrowed, pig colostrum deprived piglets (SF-pCD).

AC15-0036, March-May 2016

These rooms (131, 138, 138A) will be subjected to the following practices at all times:

Entering the room:

DO NOT ENTER THESE ROOMS IF YOU HAVE BEEN IN CONTACT WITH

SWINE WITHIN THE PAST 24 HOURS

1. On the log sheet outside Room 131 before entering, record:
   a. your name, b. the entry time ("time-in")

2. Enter "dirty area" in room 131 and change into the study designated scrubs.
3. Step into covered boots belonging to the "intermediate area" in the same room. Cross the room until you reach the second door leading to room 138.
4. Leave boots in "intermediate area" and step into "clean area" boots, which will be placed in a Virkon 1%-foot bath inside room 138. Rooms 138 and 138A are considered "clean areas" and can be accessed using these boots.
5. Place gloves, hair cover and mask if needed. Step into animal room.
6. Use designated corveralls and boots for each pen. Change gloves, coveralls, and boots if changing pens.
7. Step into each pen and place proper amount of dry pellets in the feeder, following schedule in Table 1.
8. Clean each pen with water and assure water nipples are working. Take off coverall to proceed to next pen.
9. Write the amount of food given to animals and any other comment in log book and notify the researchers if any abnormality is observed. Make a final walkthrough of the room.
10. Go back to room 138 and take off any PPE.
11. Take off boots and leave in foot bath in "clean area". Step into "intermediate area" boots in room 131 and walk towards "dirty area".
12. Leave the "intermediate area" boots and step into "dirty area".
13. Step out of room 131 and record your time out and room 138A temperature in the log sheet.

3.5.6 *Whole blood results, Pig#1*

03/28/2016 12:16:08 PM -0700 FAXCOM PAGE 1 OF 1

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ANTECH Acct No. 600157

Doctor GREG MUENLH

Accession No. CYAA02793571
Received 03/28/2016
Reported 03/28/2016 12:06 PM

Owner	Pet Name	Species	Breed	Sex	Pet Age	Chart#
CRISTINA SOLIS	PIG1	Porcine		M	6W	N

Superchem

Tests	Results	Ref. Range	Units
Total Protein	**46 (LOW)**	**78-89**	**g/L**
Albumin	27	19-34	g/L
Globulin	**19 (LOW)**	**52-64**	**g/L**
A/G Ratio	**1.4 (HIGH)**	**0.3-0.5**	**Ratio**
AST(SGOT)	**26 (LOW)**	**32-84**	**U/L**
ALT(SGPT)	40	31-58	U/L
Alk Phosphatase	**918 (HIGH)**	**118-395**	**U/L**
GGTP	145		U/L
Result Verified			
Billirubin,Total	**109.5 (HIGH)**	**0.0-17.1**	**umol/L**
BUN	**2.9 (LOW)**	**3.57-10.71**	**mmol/L**
Creatinine	**62 (LOW)**	**88-239**	**umol/L**
Bun/Creatinine Ratio	47		Ratio
Phosphorus	2.83	1.71-3.10	mmol/L
Glucose	5.4	4.7-8.3	mmol/L
Calcium	2.77	1.75-2.90	mmol/L
Magnesium	1.6		mmol/L
Sodium	147	135-150	mmol/L
Potassium	6.3	4.4-6.7	mmol/L
Sodium/Potassium Ratio		23	Ratio
Chloride	104	94-106	mmol/L
Cholesterol	**2.28 (HIGH)**	**0.93-1.40**	**mmol/L**
Triglycerides	0.49		mmol/L
Amylase	1094		U/L
PrecisionPSL™	11		U/L
CPK	259		U/L

Comment(s)
Icterus 1+. AST may be increased by 20%. See online reports for specific comments regarding this interference. No other significant analyte interference.

Complete Blood Count

Tests	Results	Ref. Range	Units
WBC	18.1	11.0-22.0	10^9/L
RBC	7.2	5.0-9.0	10^{12}/L
Hemoglobin	142.0	100-160	g/L
Hematocrit	48	30.0-50.0	%
MCV	68	50-68	fL
MCH	19.8	17-21	pg
MCHC	**294 (LOW)**	**300-340**	**g/L**
Platelet Count	462		10^9/L
Giant platelets present may indicate active thrombopoiesis.			
Platelet Estimate	Adequate		

Differential	**Absolute**	**%**	
Neutrophils	11.22	62	10^9/L
Bands	0.18	1	10^9/L
Lymphs	5.79	32	10^9/L
Monocytes	0.72	4	10^9/L
Eosinophils	0.18	1	10^9/L
Basophils	0.00	0	10^9/L

Poikilocytosis
Echinocytes Moderate
Keratocytes Slight
Polychromasia Moderate
Blood Parasites None Seen

3.5.7 *Final report of necropsy, Pig#1.*

UNIVERSITY OF CALGARY
FACULTY OF VETERINARY MEDICINE

Report
Accession No. 16-259

Pathologist: Dr. Jan Bystrom

Diagnostic Services Unit
11877 85th Street, NW
Calgary, AB T3R 1J3
Phone: (403) 220-2806
Fax: (403) 239-6984

Date Received: 04/04/16
Date Reported: 05/12/16

Referring Vet: Dr. Greg Muench
VSRS
11481 85 St NW
Calgary, AB T3R 1J3

Billing: 10 30001 VET001036

Clinic: Veterinary Science Research Station
Animal Name/ID: Pig #1
Species: Porcine
Breed:
Age: 6 Week
Sex: M
Owner: Czub, Markus

Specimens Received

Body

Case History

Brought in after snatch farrowing on farm and raised on commercial powdered bovine colostrum products in attempt to raise them free of PCV2. Weaned onto commercial piglet starter at 24 days of age.
This pig noted to have icteric serum on blood sample collected 2 weeks ago. On closer examination - clinically mildly icteric. Bloodwork done (see attached) - significant elevated bilirubin and alk phos and hypoprotenemic (slightly). Otherwise was eating and drinking normally with normal observed activity although he was not growing as quickly as his penmate (pretty subtley). Noted to be a bit lethargic last night and becoming somewhat pale. All other pigs are fine clinically with normal serum colouration on blood samples collected over past couple of weeks.

Diagnosis

FINAL DIAGNOSES:

1. BLOOD LOSS ANEMIA AND MELENA ASSOCIATED WITH SEVERE DIFFUSE PARS ESOPHAGEAL ULCERATION
2. MILD ICTERUS DUE TO MODERATE TO SEVERE, CHRONIC PORTAL FIBROSIS WITH OBSTRUCTIVE CHOLESTASIS
3. SPLEEN: MULTIFOCAL EXTRAMEDULLARY HEMATOPOIESIS

Comments

PRELIMINARY INTERPRETATION: April 4, 2016

This was a classical case of gastric pars esophageal ulceration in the pig with the most severe sequelae of complete diffuse ulceration, vascular leakage within the ulcer and death due to severe blood-loss anemia. This did not appear to be a sudden catastrophic bleed - there were no large frank blood clots in the stomach. Rather it is likely the pig was bleeding slowly for some time with semidigested blood in the stomach and evidence for melena seen throughout the tract and distally to the rectum. Histology is underway, results to follow.

FINAL INTERPRETATION: May 12, 2016

The mild icterus noted grossly in this pig is explained by a liver lesion. Portal tracts are fibrotic with mild associated inflammation. Pathogenesis of the icterus is obstructive cholestasis.

The underlying cause of the portal fibrosis is unknown. This may be a response to a drug or toxin excreted via the biliary tree. Chronic exposure to proinflammatory cytokines could also result in this lesion. The ulcer may have indirectly caused this lesion by leading to long term release of proinflammatory cytokines.

The spleen is attempting to respond to the anemia with extramedullary hematopoiesis noted.

Necropsy

Report Status

☐ Gross, pending results	☐ Gross and final
☐ Pathology completed. Pending ancillary tests.	☑ Final

GROSS NECROPSY FINDINGS: One white weaner pig submitted dead, LW x Landrace cross, born February 17, 2016, 16 kg., yellow ear tag #1. Pig was in good body condition with adequate but not abundant internal fat stores. Bloody fluid was seen at nostrils with blood beginning to dry on the nasal planum and along the right body wall, presumed to be down at death. Carcass was generally very pale with thin, watery blood throughout and a mildly icteric discoloration to mucous membranes and tissues generally. Parenchymatous tissues were very pale, particularly noted in the lungs and liver. Red-brown fluid material was seen coating the oral cavity and esophageal and tracheobronchial mucosal surfaces. Stomach contained copious red-brown fluid with suspended flecks of semidigested blood, with a "coffee ground" like appearance, and feed material. Pars esophagea of the stomach was diffusely and deeply ulcerated with a deep lip surrounding a central crater. Adherent hemorrhagic fibrin patches were seen multifocally throughout the ulcerated tissue, interpreted as sites of vascular rupture with attempts to close with fibrin clots. Ingesta through the small intestine was dark brown becoming more black and tarry in the spiral colon/distal colon/rectum, characteristic of melena, and with very dark, semipelleted feces seen terminally.

FROZEN: Bags: lung, kidney, spleen. Petri plates: lu/Li/ki/sp; thymus/mesLN (Petri plates released to graduate student).

GROSS MORPHOLOGICAL DIAGNOSES:

1. BLOOD LOSS ANEMIA ASSOCIATED WITH SEVERE DIFFUSE PARS ESOPHAGEAL ULCERATION

2. MELENA

3. MILD ICTERUS

HISTOLOGICAL FINDINGS: LIVER: Lobular pattern is accentuated by marked portal tract fibrosis which is separating lobules. Within fibrous tracts, bile ducts are difficult to find, appearing to be caught up in fibrous tissue, and perhaps mildly hyperplastic. Inflammatory infiltrate in fibrous tissue is light, made up of lymphocytes and occasional eosinophils. Deposits of bright yellow pigment are seen within bile ducts, ductules and canaliculi within lobules. Central veins are difficult to

identify. Hepatic cords are breaking apart to mild degree with moderate variation in hepatocellular size, cytoplasmic vacuolation in individual hepatocytes and fine eosinophilic granularity to hepatocellular cytoplasm. SPLEEN: Red pulp is devoid of normal red cell complement with prominent background stroma being visible. Numerous deposits of extramedullary hematopoiesis are seen including megakaryocytes. THYMUS: Many tingable body macrophages are seen throughout a robust cortical zone. STOMACH: There is abrupt transition from intact glandular mucosa to very deep ucleration with no visible squamous mucosa remaining. Ulcerated surface varies from heavy granulation and deeper organizing fibrous tissue to heavily coated by fibrinous and cellular debris, mostly degenerate neutrophils, admixed with semilaminated rafts of frank hemorrhage. Numerous deposits of plant debris and bacteria are seen throughout surface debris. LUNG, HEART, KIDNEY, ADRENAL GLAND, LYMPH NODES, BRAIN: NVL.

HISTOMORPHOLOGICAL DIAGNOSES:

1. LIVER: PORTAL FIBROSIS, MODERATE TO SEVERE, CHRONIC WITH OBSTRUCTIVE CHOLESTASIS

2. SPLEEN: MULTIFOCAL EXTRAMEDULLARY HEMATOPOIESIS

3. STOMACH: PARS ESOPHAGEAL ULCERATION, SEVERE, CHRONIC-ACTIVE WITH SURFACE FIBROSIS, GRANULATION TISSUE, FIBRINOCELLULAR DEBRIS, HEMORRHAGE AND INTRALESIONAL PLANT DEBRIS AND BACTERIA

05/13/16

Dr. Jan Bystrom, DVM, MSc, Veterinary Pathologist

End of Report

Chapter Four: PCV2 infection and cell death in PBMCs

4.1 Introduction

PCV2 is a small, non-enveloped virus that infects pigs worldwide[16]. PCV2 initially infects and replicates in epithelial cells of the respiratory and digestive system[53], and then disseminates towards regional lymph nodes[57]. Viral DNA can be found in both lymphocytes and macrophages[21,138], while PCV2 mRNA is mainly found in circulating and tissue lymphocytes[21], which suggests that lymphocytes are an important replication site[21].

PCV2 is the aetiological agent of PMWS, a disease that affects 6 to 12 week-old pigs[8] and is characterized by high viral loads in tissues and a severe generalized depletion of lymphocytes[57]. In naturally-occurring PMWS cases, the depletion of lymphocytes results in a loss of normal lymphoid follicle architecture, infiltration of histiocytes in lymphoid tissues, and immunosuppression[57,56]. Although the mechanisms behind cell depletion in PMWS are still unclear, it has been suggested that virus-induced cell death combined with a decrease in lymphocyte proliferation could play a role[55].

A challenge to study PMWS pathogenesis and cell depletion is the lack of a proper animal model. Even though PCV2 infection is ubiquitous among pigs and is necessary for disease, not all infected animals develop PMWS[151]. Experimentally PCV2-infected pigs only present PWMS-like clinical signs after being co-infected with a secondary pathogen (e.g. PRRSV)[152], or by activating their immune system using vaccines with keyhole limpet hemocyanin in Freund's incomplete adjuvant[153].

Following the clinical observation that activation of the immune system leads to increased PCV2 viral loads *in vivo*, higher PCV2 infection rates *in vitro* were achieved by activating PBMCs using mitogens[72]. Previous studies used Con A[72] or Pokeweed mitogen (PWM)[66] to activate lymphocytes before PCV2 infection, resulting in increased PCV2 replication and apoptosis rates after stimulation[62]. In these studies, the mitogens used usually targeted only one type of lymphocyte subpopulation, biasing the type of stimulation in cell cultures. In addition, reactivation of persistent PCV2 infections were observed if the PBMCs used were isolated from PCV2-carrier pigs[72].

To overcome these issues, the current study used PCV2-free pigs as PBMCs donors, exposed the cells to the polyclonal B and T cell-activating mitogens ionomycin and phorbol myristate acetate (PMA), and determined the effect of mitogen stimulation on PCV2 infection rates. In addition, the subsets of lymphocytes infected by PCV2 were determined, and the effect of PCV2 on PBMC apoptosis and viability was assessed.

4.2 Material and methods

4.2.1 *Experimental animals and whole blood sampling*

Seven Large White/Landrace cross pigs were raised PCV2-free using a snatch-farrow, porcine colostrum-deprived protocol[144]. The animals were housed in a BSL-2 facility of the University of Calgary with controlled air circulation. Half of the cohort was bled once a week, from 4 to 22 weeks of age (Chapter 3). The animal protocol was approved by the ACC of the University of Calgary (ACC-15-0036).

4.2.2 *PBMC isolation from whole blood*

Whole blood samples from individual pigs were diluted 1:1 in cold RPMI-1640 media with L-glutamine (Lonza®, #12-702F; supplemented with 5% fetal bovine serum (Gibco®, #16000044), 1% penicillin/streptomycin (Gibco®, #15240-062)). Subsequently, 13mL of Ficoll-Paque (GE Healthcare, #17-1440-03) were placed in a 50mL Falcon tube (VWR, #89004-364), and 18mL of the diluted blood were placed carefully on top to avoid mixing blood with the Ficoll-Paque. The sample was centrifuged at 400 x g for 30 min RT, with the brake turned off. Migration of cells during centrifugation results in erythrocytes and granulocytes to sediment at the bottom of the tube, and PBMCs to stay at the interface between the plasma and the Ficoll-Paque. The layer containing the PBMCs was collected carefully and placed in a 50mL tube, washed once with cold RPMI media, and centrifuged for 5 min at 4°C and 200 x g with maximum acceleration and deceleration. The supernatant was then discarded and the remaining erythrocytes were lysed adding 1-3ml of Ammonium-Chloride-Potassium (ACK, Gibco®, #A10492-01) for one minute. The PBMCs were washed with 20mL of sterile 1XPBS and centrifuged for 5min at 200 x g at 4°C. The cells were then re-suspended in 5mL of cold media and viable cells were counted using a blood counting chamber (Lumicyte, #090001), and Trypan Blue 0.4% (Gibco®, #15250-061).

4.2.3 *PCV2 viral stock*

PCV2 viral stock used was produced as described in Chapter 2. Infectivity of the virus stock was determined using the Reed-Muench titration, and was defined as the dose that infects 50% of the wells exposed to the virus (tissue culture infectious dose 50/mL, TCID50)[125]. The PCV2 viral stock titer was calculated as $6.3x10^4$ TCID50/mL.

4.2.4 *Stimulation of swine PBMCs and PCV2 infection*

PBMCs from each individual pig were re-suspended at a concentration of 2 x 10^6 PBMCs/mL RPMI 1640 media, and 100µL of cell suspension were added to each well of a U-shaped, 96-well plate. A solution containing ionomycin (1µg/mL, Calbiochem, catalog number 407953) and PMA (1ng/mL, Sigma, catalog number P8139) was added to the PBMCs using a volume of 100µL/well and incubated for 30 minutes at 37°C. The cells were centrifuged at 200 x g for 3 min, the supernatant was carefully discarded, and the cells were resuspended in 100µL of media. Subsequently, 100µL of the PCV2 viral stock (6.3X10^3 TCID50/100µL, MOI 0.01) was added to the cell suspension. The cells were incubated for 24, 48, 72 and 96 hours, centrifuged, and stained for membrane or viability markers. The cells were fixed with 2% formaldehyde for 20 minutes at RT, resuspended in 50µL of PB (1 X PBS, 0.1% BSA-0.1% saponin-PBS), and stained for intracellular viral proteins (see details below).

4.2.5 *Phenotypic analysis of CD3+ T-cell subsets*

The 96-well plates were centrifuged, supernatant was discarded, and 50µL of blocking buffer (1X PBS, 5% goat serum, 0.01% sodium azide) were added to the cells and incubated for 20 minutes at 4°C. A mouse anti-swine CD3-PE (IgG1, 0.1mg/mL, Southern Biotech, catalog #45-0-09) monoclonal antibody was used to identify swine T cells, by adding 1µL of a 1:3 dilution of the antibody directly to each well (final concentration: 0.03µg/50µL). The cells were incubated for 45 minutes at 4°C, washed once with washing buffer (2% BSA, 1X PBS), and then fixed with 2% formaldehyde for 20 minutes at RT. The antibody anti-mouse IgG1-PE (0.2 mg/mL, BD Pharmigen, catalog # 554680) was used as an isotype control at the same

concentration (0.03µg/50µL); details of the optimization protocol of these markers can be found in Appendix 4.5.1.

4.2.6 *Viability assay using the LIVE/DEAD® fixable stain*

Viability of PBMCs was assessed by the LIVE/DEAD® fixable Violet dead cell stain kit (Thermo Fisher, cat#L34955) following the manufacturer's instructions. The LIVE/DEAD stain binds to the amines present in the outer and inner cell membranes, and the cells with compromised membranes can be distinguished by having a higher fluorescence stain compared to live cells. The fluorescence is not affected by the downstream processing of cells[154], and it was used in conjunction with the anti-CAP antibody to determine the viability of PCV2-infected cells.

Plates were centrifuged, supernatant was removed carefully, and the cells were resuspended in 100µL of sterile PBS. The dye was diluted 1:25 in a sterile PBS and 1µL was added to each well at a final dilution of 1:2500. The treated cells were incubated for 10 minutes, washed once with PBS, fixed in 50µL of 2% formaldehyde for 20 minutes. After one wash with PBS, the cells were permeabilized and stained against intracellular viral proteins, and analyzed using flow cytometry (see details below).

4.2.7 *Apoptosis and viability stain using Annexin V and Propodium iodide*

The percentage of apoptotic cells was determined using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmigen, #556547), in combination with the propidium iodide (PI) as a viability stain. Annexin V binds to phosphatidylserine, a phospholipid exposed in the external cell membrane during apoptosis, and PI is an intercalating dye that binds to DNA in cells that have lost membrane integrity[155]. Together, these dyes can be used to determine the percentage of

apoptotic (Annexin V+/PI-) and live (Annexin V-/PI-) cells. Cells were immediately analyzed using flow cytometry since no downstream processing is possible after PI staining.

Briefly, the plates were centrifuged at 200 x g for 3 minutes, the supernatant was removed carefully, and the cells were washed once with 100µL of sterile PBS. The cells were resuspended in 100µL of Annexin binding buffer (100µL/100,000 cells), followed by the addition of 5µL of FITC-Annexin V and 5µL of PI to each well, and incubated in the dark for 15 minutes at RT. Subsequently, the cells were centrifuged, washed twice with 100µL Annexin binding buffer, and resuspended in 50µL of Annexin binding buffer. The cells were fixed with 50µL of 2% formaldehyde for 20 minutes in the dark, washed once with 1X PBS, re-suspended in 200µL of Annexin binding buffer, and analyzed immediately. The apoptosis-inducer etoposide (50mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich # E1383) was added to apoptosis-control wells (2µL/well) 24h before the end of the experiment.

The apoptotic index per sample was defined as the rate of apoptotic cells in the totality of live and apoptotic cells, and was calculated using the following formula:

(% Apoptotic cells) / (% Live cells + % Apoptotic cells)

4.2.8 *PCV2 capsid indirect immunofluorescent staining*

Fixed and permeabilized cells were incubated with 50µL of a 1:1000 dilution of rabbit-hyperimmune serum towards CAP (final dilution 1:2000), for one hour at 37°C. Subsequently, the cells were washed once with 1X PBS- BSA 1%, resuspended in 50µL of PB and incubated for 60 minutes at 37°C with 50µl of 0.31ng/mL of goat anti-rabbit Alexa Fluor 635 (1:13000 final dilution, 2mg/ml, Invitrogen®) in PB, washed once with 1X PBS- BSA 1% and resuspended in 200µL of PBS for flow cytometry analysis.

For light microscopy evaluation, the secondary antibody goat-anti rabbit IgG-FITC (BD Pharmigen, catalog #554020, 0.5mg/ml) was used in a 1:200 dilution, followed by one wash with 1X PBS-BSA 1%. Nuclear counterstain was achieved by adding 100µL of Hoechst (2µg/mL; Molecular probes, #H1399) for 10 minutes in the dark, followed by one wash with 1X PBS, and resuspension of cells in 200µL of PBS.

4.2.9 *Flow cytometry analysis*

Cells were re-suspended in 200µL of PBS and the complete volume was transferred from plates to individual 5mL round bottom tubes (Falcon, #352008). Samples were read by two different instruments, depending on the necessity of specific lasers due to fluorophore combinations. The Attune Flow cytometer (Thermo Fisher Scientific), was mainly used for apoptosis and phenotypic characterization using the Attune analysis software (Thermo Fisher Scientific); the LSR II™ (BD Bioscience) equipped with four lasers and using the DIVA software (BD Bioscience) was mainly used for the experiments of live/dead and CAP stain. Electronic compensation was set on each experiment to eliminate spectral overlaps between fluorophores. FlowJo version 10.0.08 was used for data analysis; debris and aggregates were eliminated after initial gating, followed by a specific analysis depending on the experiment. A detailed description of gating strategies for each experiment is explained in Appendix 4.5.2.

4.2.10 *Immunofluorescent microscope image acquisition*

Cells were transferred to a flat-bottom optical plate (Nunc®, #160376) and plate reading was performed using the automated inverted microscope IN Cell Analyzer 2000 (GE Healthcare®). Eight images per well were acquired using a 40X field of view; the microscope software randomly determined the area of the well where the images were acquired, and repeated

them on each well. Three different fluorescent channels were defined: brightfield (exposure 0.03s, offset 0.00), FITC (exposure 2.5s, offset 10.0), and DAPI (exposure 0.05s, offset 6.00); laser autofocus was set to 10%. Images of the DAPI and FITC channel were merged using the INCell Analyzer software (GE Healthcare).

4.2.11 *Statistical analysis*

Statistical analyses of data were performed using SPSS Statistics 22.0 software (IBM) and R version 3.3.1 (R Foundation for Statistical Computing). Outlier calculation, descriptive statistics, and paired-samples t-tests were performed using SPSS. Outliers were determined as any number outside the range: (Q1- k(Q3-Q1), Q3 + k(Q3-Q1)), with k=2.2, and were excluded from any further analysis.

A LMER was used to examine the effects of treatment and time (as fixed effects) on each of the predictor variables (e.g. PCV2 infection rates, percentage of dead cells, percentage of apoptotic cells), using individual pigs as a random effect. A REML method was used for parameter estimation. Pairwise comparisons within and between groups were analyzed using Tukey's post hoc test with Bonferroni method for p-value adjustment for multiple comparisons. The normality and equal variance assumptions were assessed to validate the fitness of each model. Analyses were performed using R; the 'lme4' package version 1.1.12 for the linear mixed effects models analysis, and 'lsmeans' package version 2.24 for multiple comparisons[145]. A $p<0.05$ was considered statistically significant. The reasoning for choosing this model and an example of the set-up can be found in appendix 4.5.2.

The pigs used for each experiment, as well as the amount of independent experiments performed per assay are detailed on each figure. Duplicates of samples used in the same experiment were analyzed individually and averaged to determine the final percentage.

4.3 Results

4.3.1 *Microscopic evaluation of PCV2-antigen on PBMCs*

To determine if swine PBMCs were infected with PCV2, the presence of the viral CAP antigen in the nucleus or cytoplasm of infected cells was analyzed using the automated microscope INCell Analyzer 2000. As illustrated in Figure 4-1, morphological differences between cells, as a diversity in nuclear size, and mitotic activity of cells were observed at all incubation time points, as well as nuclei shrinkage and fragmentation in dying cells.

After 24h of incubation, PCV2-CAP antigen was observed in the cytoplasm of mitogen-treated and non-treated cells, and in cell debris throughout the wells (Figure 4-1A). After 48h of incubation, some cells of the mitogen-treated control wells were organized in small groups, possibly arranging for cell division. In PCV2 treated cells, PCV2-CAP antigen was mainly observed in the cytoplasm (non-mitogen treated) and nucleus/cytoplasm (mitogen treated) of infected PBMCs; some PCV2-CAP positive cells displayed an enlarged nucleus in the mitogen treated wells (Figure 4-1B). After 72h and 96h, PCV2-CAP antigen was observed in both nucleus and cytoplasm of mitogen-treated cells (Figure 4-1C and 4-1D).

4.3.2 *PCV2 infection rates of PBMCs*

The rate of PCV2-CAP positive cells in mitogen-treated and non-treated PBMCs was determined using flow cytometry. The effect of treatment and incubation time on PCV2 infection

within and between groups was evaluated using an LMER model, with Tukey's post hoc test for pairwise comparisons (Figure 4-2, Table 4-1).

Treatment had a significant, positive effect on PCV2 infection rates in the PCV2-only (3.9% ± 0.5 SE, $p<0.001$), and PCV2-I/PMA (7.5% ± 0.6 SE, $p<0.001$) groups, compared to mock. Pairwise comparisons demonstrated that the infection rates in the PCV2 treated groups were significantly higher than background ($p<0.001$; Figure 4-2). In addition, PCV2-I/PMA treated group had higher infection rates than PCV2-only, at all incubation time points ($p<0.001$; Figure 4-2).

Incubation time had a negative effect on PCV2 infection rates at 96h (-1.9% ± 0.8 SE, $p<0.05$) compared to 24h. Pairwise comparisons demonstrated that infection rates were lower at 96h compared to 72h in PCV2 ($p=0.04$) and PCV2-I/PMA ($p=0.04$) treated groups (Figure 4-2). A borderline significant variation was observed between individual pigs ($p=0.051$).

These results demonstrate higher PCV2 infection rates in ionomycin/PMA treated PBMCs compared to non-treated cells at all incubation time points, and a decrease of PCV2 infection rates after 96h of incubation.

4.3.3 *CD3+ T cells in activated and infected PBMCs*

To determine if the number of T cells in culture was affected after mitogen treatment, the percentage of CD3+ T cells was observed after 48h of incubation using flow cytometry. The effect of mitogen treatment on the percentage of CD3+ T cells was determined using an LMER model, with Tukey's post hoc test for pairwise comparisons. Statistical analysis demonstrated no

effect of treatment or individual pigs on the percentage of CD3+ cells at 48h ($p>0.05$; Figure 4-3, Table 4-2).

To determine if T cells are infected with PCV2, the percentage of PCV2+/CD3+ T cells in culture was demonstrated using flow cytometry, and compared between mitogen treated and non-treated infected groups using a paired samples t-test. Results demonstrate a lower mean percentage of PCV2+/CD3+ T cells in the PCV2-Ionomycin/PMA group (32.5% ± 9.8 SD), compared to PCV2 only (61.6% ± 8.0 SD, $t=4.2$), but the p-value was just over the significance threshold ($p=0.051$; Figure 4-4).

These results demonstrate that the absolute number of T cells is not different between mitogen treated or non-treated cells after 48h of culture, but approximately twice as many T cells are infected in the PCV2-only treated group, compared to the mitogen treated group, although this difference is marginally significant.

4.3.4 *Cell death of activated and infected PBMCs using the LIVE/DEAD® fixable stain*

To determine the viability of PBMCs in mitogen treated and non-treated cells after different incubation time points, the percentage of dead cells after each treatment was determined using the LIVE/DEAD® fixable stain, and analyzed using flow cytometry. An LMER model was performed, with Tukey's post hoc test for pairwise comparisons (Figure 4-5; Table 4-3).

Treatment had a significant, positive effect on cell death in the Ionomycin/PMA (9.7 ± 2.6 SE, $p<0.001$), PCV2 (9.6% ± 2.5 SE, $p<0.001$), and PCV2-Ionomycin/PMA (24.2% ± 2.5 SE, $p<0.001$) groups, compared to mock. Incubation time had a negative effect on cell death at 72h (-

8.4% ± 2.9 SE, $p<0.005$) and 96h (-6.8% ± 3.4 SE, $p<0.05$) compared to 24h, and no significant variation was observed between individual pigs ($p=0.07$).

Pairwise comparisons demonstrated that the percentage of dead cells was significantly lower in the mock treated wells, compared to all other treatment groups at all incubation time points ($p<0.05$; Figure 4-5). Also, a higher cell death was measured in the PCV2-Ionomycin/PMA cells compared to all other treatment groups, at all incubation time points ($p<0.001$; Figure 4-5).

These results demonstrate that PBMC treatment with ionomycin/PMA and PCV2 separately increased cell death, but a significantly higher cell death is observed when both treatments (ionomycin/PMA and PCV2) were used on the cells.

4.3.5 *PCV2-CAP positive cell rate in live or dead cells using LIVE/DEAD® fixable stain*

To determine PCV2 infection rates in live or dead cells, the viability status (live or dead) of cells was determined using the LIVE/DEAD® fixable stain, and the PCV2 infected cells were identified using an antibody against the viral CAP. The effects of incubation time, treatment, and viability status on infection rates were determined using an LMER model, with Tukey's post hoc test for pairwise comparisons (Figure 4-6, Table 4-4).

Treatment affected the PCV2 infection rates in a significant, positive way in the PCV2 (10.0% ± 1.1SE, $p<0.001$) and PCV2-Ionomycin/PMA (10.3% ± 1.1 SE, $p<0.001$) groups, compared to Ionomycin/PMA. The viability status of cells (12.0% ± 0.9 SE, $p<0.001$), and incubation time also affected PCV2 infection rates in a significant, positive way at 48h (3.5% ±

1.4 SE, $p<0.05$) and 72h (3.9% ± 1.4 SE, $p<0.01$), compared to 24h. No significant variation was observed between individual pigs ($p=0.2$).

Pairwise comparisons demonstrated that the percentage of CAP positive staining in PCV2-Dead, and PCV2-I/PMA-Dead were significantly higher than the percentage of CAP positive cells in the PCV2-Live, and PCV2-I/PMA-Live treatment groups, at all incubation time points ($p<0.001$; Figure 4-6). The background controls had significantly lower CAP positive cells, than the PCV2-treated cells (dead), at all incubation time points (Figure 4-6). These results demonstrate higher PCV2 infection rate in dead cells, compared to the PCV2 infection rate in live cells, independently of mitogen treatment.

4.3.6 *Apoptosis in activated and infected cells*

To determine if apoptosis was the cause of the increased cell death observed in the experiments, the percentage of apoptotic (AnnexinV+/PI-) and live (Annexin V-/PI-) PBMCs after 72h of incubation were determined using Annexin V and PI, and the apoptotic index was compared between treatment groups. The effects of treatment on the apoptotic index were determined using an LMER model, with Tukey's post hoc test for pairwise comparisons (Figure 4-7, Table 4-5).

The apoptotic index was affected in a significant, positive way by treatment with PCV2 (0.34% ± 0.07SE, $p<0.001$), PCV2-Ionomycin/PMA (0.14% ± 0.07 SE, $p<0.05$), and etoposide (0.30% ± 0.07 SE, $p<0.001$), compared to mock. Pairwise comparisons demonstrated a significantly higher apoptotic index in the PCV2-only treated cells, compared to mock ($p<0.001$), Ionomycin/PMA ($p<0.001$), and PCV2-Ionomycin/PMA ($p<0.05$) treated groups

(Figure 4-7), and in etoposide treated cells compared to mock ($p<0.001$) and Ionomycin/PMA ($p<0.001$) treated groups (Figure 4-7). Results demonstrate that the apoptotic index increased after incubation with PCV2, independently of the mitogen treatment.

4.4 Discussion

PMWS is characterized by a general depletion of lymphocytes, resulting in immunosuppression and secondary infections[21]. In this study, we explored the effect of a polyclonal mitogen stimulation on PCV2 infection in PBMCs from PCV2-naive pigs, and evaluated the impact of the enhanced viral replication on cell viability.

Our results clearly indicate that PCV2 infection rate increases in PBMCs when exposed to the ionomycin/PMA. The highest rate of PCV2 infection (8%) was measured in PBMCs after 24h of incubation, which decreased after 96h of incubation as assessed by flow cytometry. The increased PCV2 infection rates in early incubation time points (24h) suggests that ionomycin/PMA treatment influences virus replication[156], so the increased rates at 24h do not seem to be due to a secondary infection in culture or increased cell division. Treatment of cells with mitogens could enhance PCV2 entry. An increase in heparan sulfate expression, a cell receptor for PCV2, was reported in endothelial cell lines after PMA treatment[157], and the expression should be assessed in PBMCs. Mitogen treatment could also stimulate viral protein expression, since Con A treatment resulted in increased PCV2-CAP mRNA in PBMCs, 18h post-infection[138]. Whether any of these mechanisms is used by PCV2 to increase infection rates in ionomycin/PMA treated PBMCs still needs to be elucidated.

In the current study, we compared the PCV2 infection rates of PBMCs stimulated with Con A, PWM, or Ionomycin/PMA, and incubated for 48h (Appendix 4.5.5). Our results confirmed that the highest infection rates were obtained by pre-treating PBMCs with Ionomycin/PMA (Appendix 4.5.5). Previous studies have reported PCV2 infection rates of less than 2% in PCV2-free PBMCs treated with Con A and PCV2 simultaneously[72], or with cells exposed to PCV2 first and then treated with Con A for 96h[158]. The difference between infection rates could be explained by the type of subpopulations stimulated by each mitogen, since both B and T cells are activated by ionomycin/PMA (Appendix 4.5.4)[159,160], while only T or B cells are stimulated by Con A or PWM, respectively[161].

Even with the enhanced infection rates after mitogen stimulation, our results also confirmed that PCV2 infection in PBMCs is characterized by low infection rates. This is similar to what is observed in the immortalized cell line PK-15, where only around 5-10% of cells are positive to PCV2 antigen[98], increasing to 15% if the cells are treated with IFN-γ[98], or D-glucosamine[68]. Low infection rates in cell lines also represent a constraint for virus stock production, since the low $TCID_{50}$ of the stock will result in low MOIs common for PCV2 *in vitro* experiments[158]. The reasons of low susceptibility for infection are not at all clear, but it could be due to an epigenetic phenomenon.

Exploring the PCV2 infectivity rate in lymphocyte subpopulations demonstrated that T cells represent the main target for PCV2 infection, with approximately 60% of PCV2+ being CD3+ T cells in the non-mitogen treated population. Our data are in line with Lefebvre *et al.* (2008), who reported PCV2 infection rates of up to 40% in CD4+ T cells, and 54% in CD8+ T cells[158]. Even though we were not able to determine the PCV2 infection rate in B cells because of

some technical difficulties with the anti-porcine B cell antibodies (results not shown), contrasting data has been reported about the infection rate in B cells. While Lefebvre *et al.* (2008) reported that only 11% of the IgM+ cells were positive for PCV2[158], Lin *et al.* (2008) reported that almost 50% of the IgM+ cells were PCV2 positive after Con A treatment[72]. Future studies should continue elucidating on the specific lymphocyte subsets infected by PCV2 *in vitro*.

Our data further indicate that the enhanced PCV2 replication in PBMCs after mitogen stimulation is correlated with an increase in cell death[66]. Cell death percentage of approximately 30% was observed in the PCV2-ionomycin/PMA treated wells as early as 24h post-infection. In addition, data indicates that PBMCs that expressed the PCV2 capsid died. Even though treatment with ionomycin can induce death in cultured cells[162], the expression of PCV2-CAP protein can also be associated to the high cell death in PBMCs, since CAP expression resulted in death of 40% of PK-15 epithelial cells[98]. However, these data do not exclude the possibility that expression of ORF-3 may contribute to cell death in PBMCs as well, since cell death was observed in 15% of PK-15 epithelial cells expressing ORF-3[101] and in 42.6% of ORF-3 transfected swine PBMCs[163].

PCV2 infection induces apoptosis of cells *in vivo*[164] and *in vitro*[101], so we tried to elucidate if apoptosis was the mechanism behind the high death rates in PBMCs. Apoptotic cells were observed after 72h of incubation in cells exposed to all treatment, but a higher apoptotic index was observed in the PCV2-only treated PBMCs (0.51) compared to the PCV2-Ionomycin/PMA treated cells (0.31). These results are in line with Yu *et al*. (2009), who reported cell death in approximately 50% of PBMCs and an apoptotic index of 0.4 after treatment with PWM and PCV2 exposure for 3 days[66].

Considering the low PCV2 infection rates (6-8%) and the high percentage of cell death (27%-34%) in the PCV2-Ionomycin/PMA treated PBMCs, we suggest the presence of a soluble factor in the culture media that causes bystander cell death. TNF-α is a pro-inflammatory cytokine mainly secreted by monocytes and activated macrophages, but can also be secreted by lymphocytes in response to infection[165]. TNF-α can act as a Fas ligand, inducing apoptosis on lymphocytes that express a Fas receptor[165]. Increased TNF-α secretion was measured in PBMCs isolated from PCV2 infected animals, after Con A activation[166]. Therefore, the high cell death observed in the PCV2-Ionomycin/PMA treatment groups could be due to the combined effect of PCV2 replication, and immunosuppressive and pro-apoptotic cytokine secretion.

In conclusion, this study has demonstrated primary PCV2 infection in swine PBMCs, with increased rates on mitogen-stimulated cells, possibly due to an effect on virus replication. In addition, apoptosis seems to be an important mechanism for cell death *in vitro*, induced directly by PCV2 infection or other soluble factors that could be present in the cell culture supernatant.

Figure 4-1 Microscopy images (40X, INCell Analyzer 2000) of swine PBMCs after mock, ionomycin/PMA, PCV2, or PCV2-Ionomycin/PMA treatment, and four incubation time points. Indirect-immunofluorescence assay was performed using rabbit anti-PCV2 CAP stain, followed by goat anti-rabbit IgG FITC, and nuclear counterstain (Hoechst).

Figure 4-1A Anti PCV2-CAP stain in PBMCs, after 24h of incubation

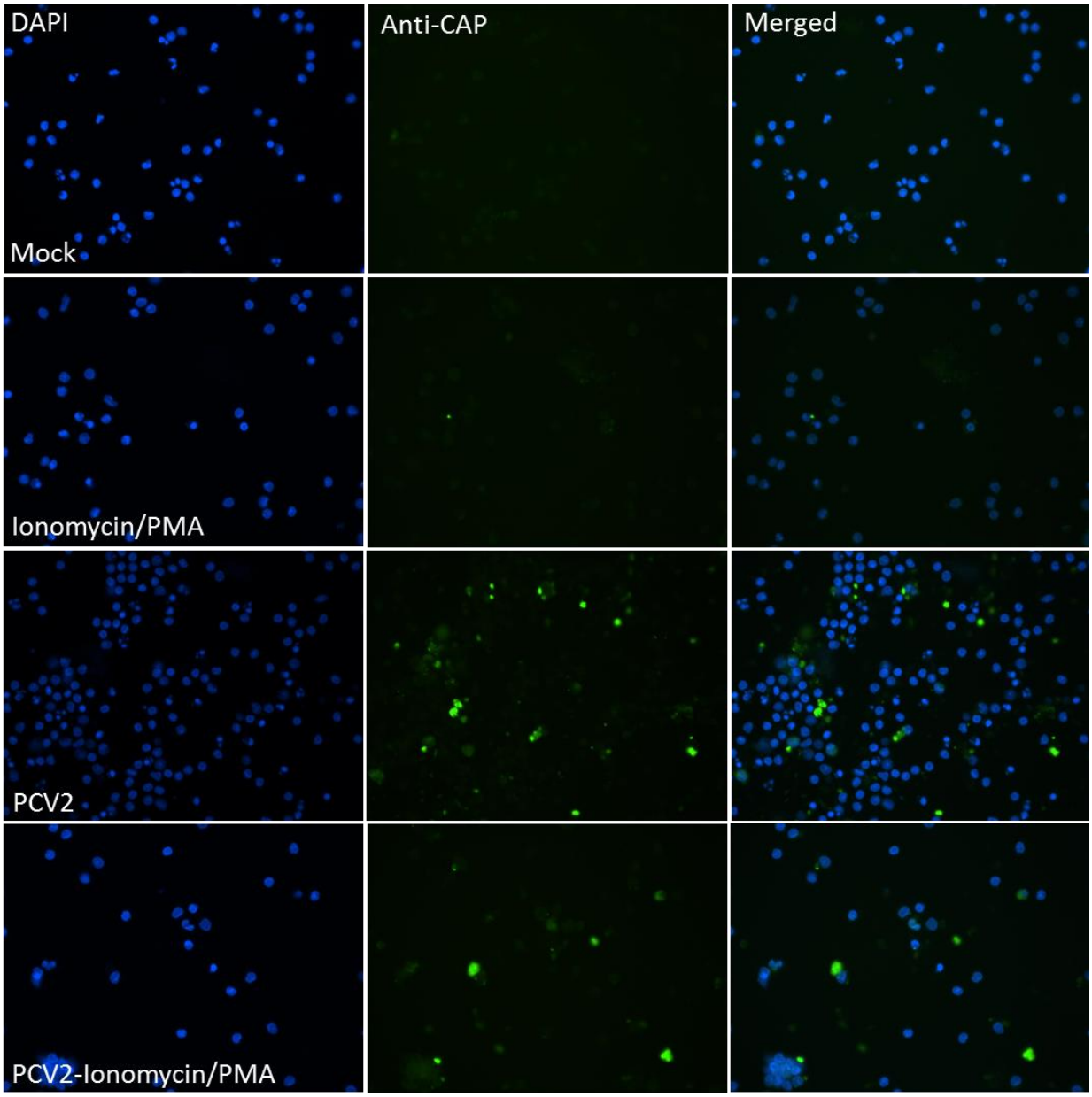


Figure 4-1B Anti PCV2-CAP stain in PBMCs, after 48h of incubation.

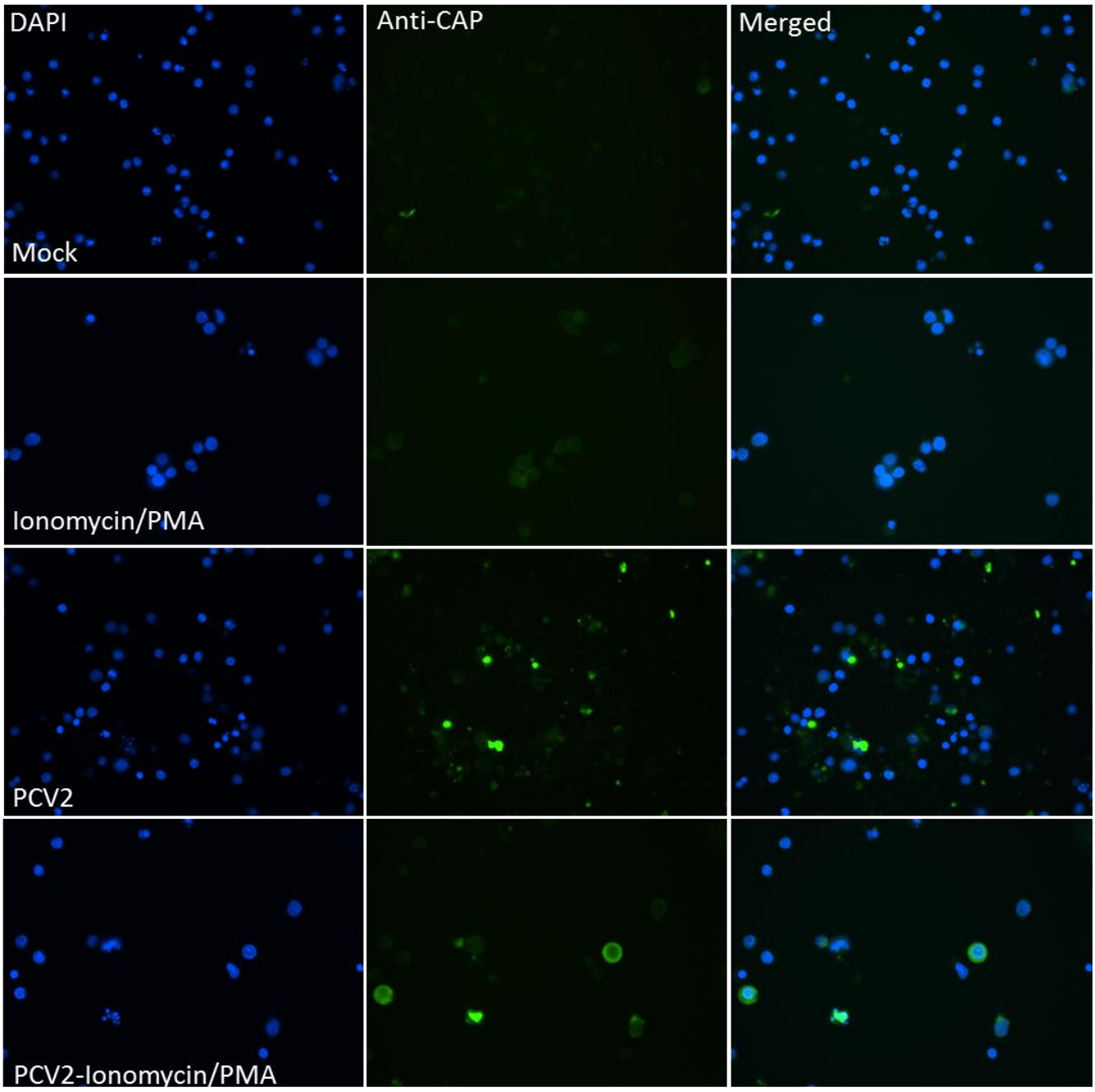


Figure 4-1C Anti PCV2-CAP stain in PBMCs, after 72h of incubation.

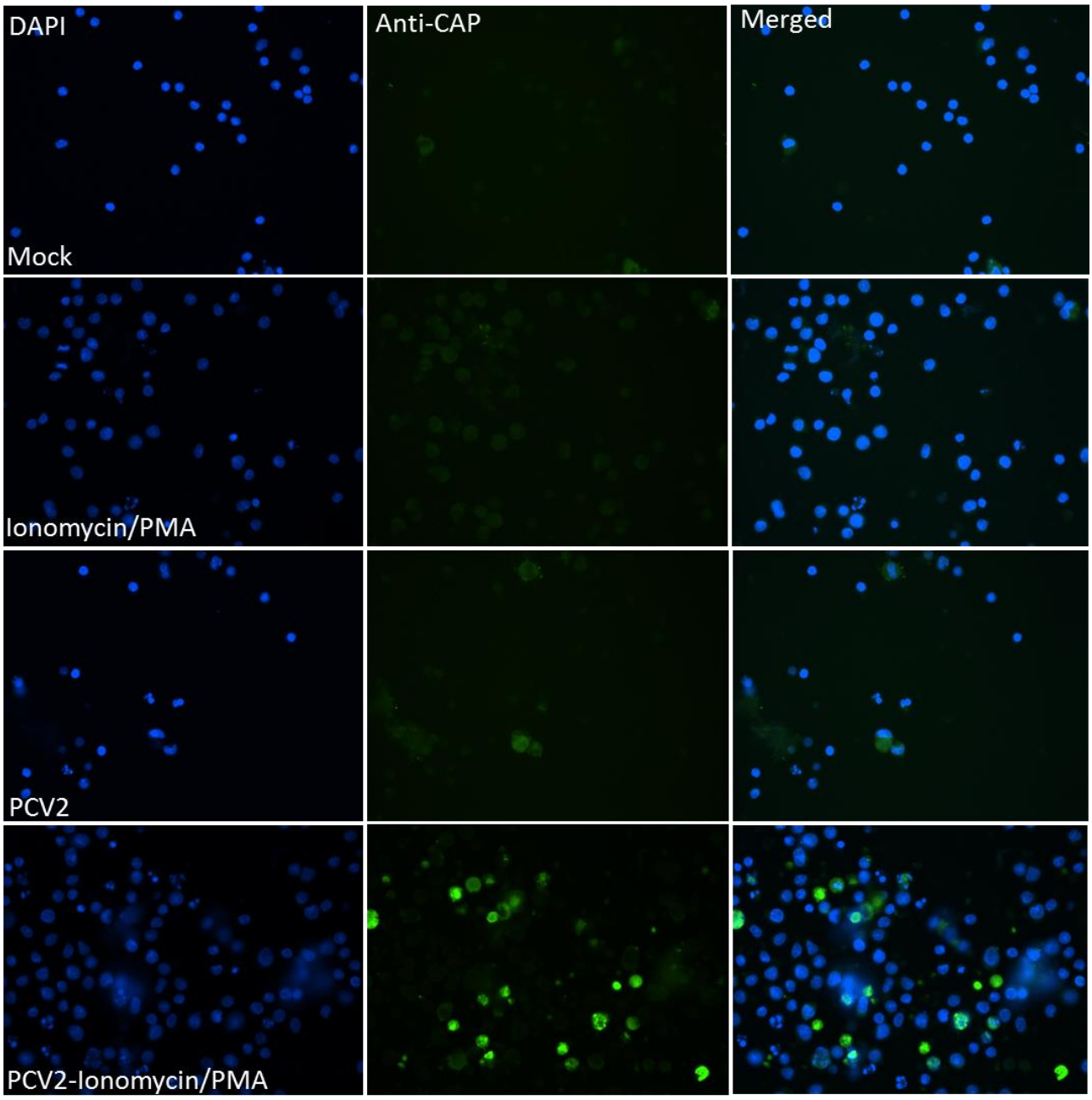


Figure 4-1D Anti PCV2-CAP stain in PBMCs, after 96h of incubation.

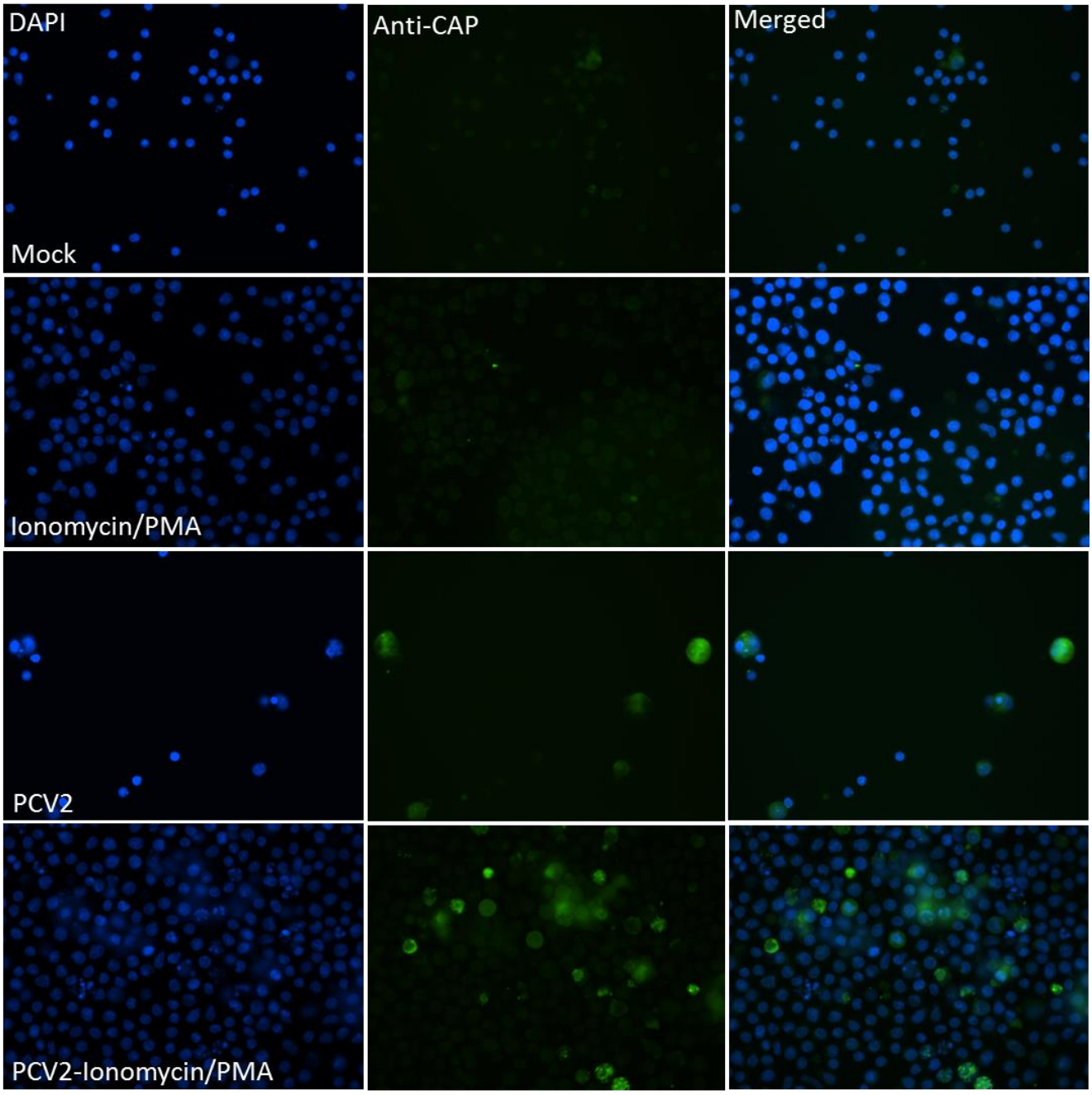


Figure 4-2 Percentage of PCV2-CAP positive PBMCs after four different treatments, and four incubation time points. Results represent the parameter estimates of the LMER model, of two (24h and 96h; pig #:4, 5, 6, 7), and four (48h, 72h; pig #: 2, 3, 4, 5, 6, 7) independent experiments. Error bars equal to mean ± CI, and significance is shown as asterisks (*=p<0.05; ***=p<0.001 demonstrates significance between all groups of the same time point).

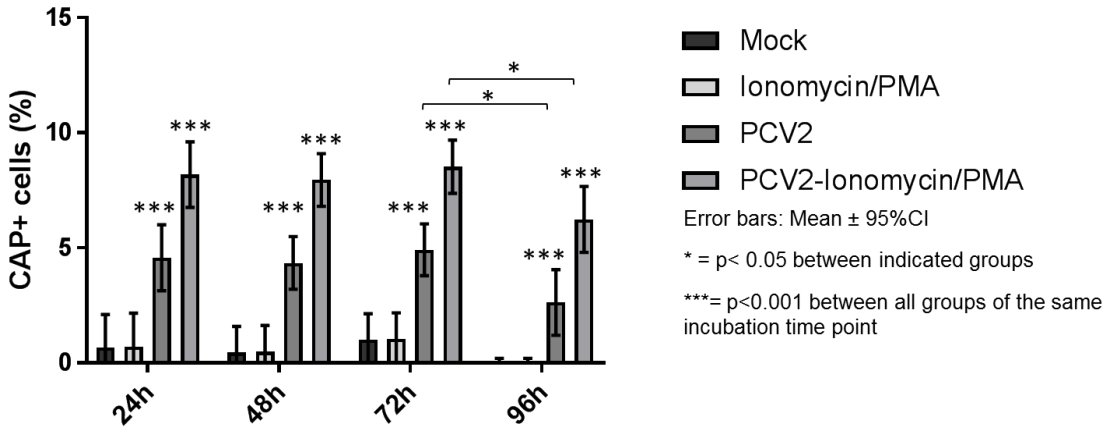


Table 4-1 Percentage of PCV2-CAP positive PBMCs after four treatments and four incubation time points. Distribution (mean ±SD) of raw data, and parameter estimates (95% CI) of the LMER model; negative estimates were truncated to 0.

		PCV2-CAP infection (%)	
	Time / Treatment	Mean ± SD	Estimate (95%CI)
24h	Mock	0.20 ± 0.17	0.68 (0 - 2.11)
	Ionomycin/PMA	0.21 ± 0.16	0.71 (0 - 2.17)
	PCV2	5.01 ± 2.94	4.58 (3.15 - 6.01)
	PCV2-Ionomycin/PMA	9.96 ± 3.31	8.18 (6.75 - 9.61)
48h	Mock	0.29 ± 0.25	0.45 (0 - 1.60)
	Ionomycin/PMA	0.65 ± 0.40	0.49 (0 - 1.63)
	PCV2	4.62 ± 3.04	4.35 (3.21 - 5.49)
	PCV2-Ionomycin/PMA	7.88 ± 3.19	7.96 (6.81 - 9.10)
72h	Mock	1.06 ± 0.90	1.02 (0 - 2.15)
	Ionomycin/PMA	0.89 ± 0.74	1.05 (0 - 2.18)
	PCV2	5.11 ± 3.70	4.92 (3.79 - 6.04)
	PCV2-Ionomycin/PMA	9.09 ± 2.36	8.52 (7.37 - 9.68)
96h	Mock	1.07 ± 0.68	0 (0 - 0.20)
	Ionomycin/PMA	0.31 ± 0.27	0 (0 - 0.20)
	PCV 2	2.21 ± 1.36	2.64 (1.21 - 4.06)
	PCV2-Ionomycin/PMA	4.61 ± 1.08	6.24 (4.81 - 7.67)

Figure 4-3 Percentage of CD3+ T cells in swine PBMCs, after mock, ionomycin/PMA, PCV2, and PCV2-Ionomycin/PMA, after 48h of incubation. Results are the average of two independent experiments (pig #: 1, 2, 3, 4, 7, 8). Error bars equal to mean ± SD.

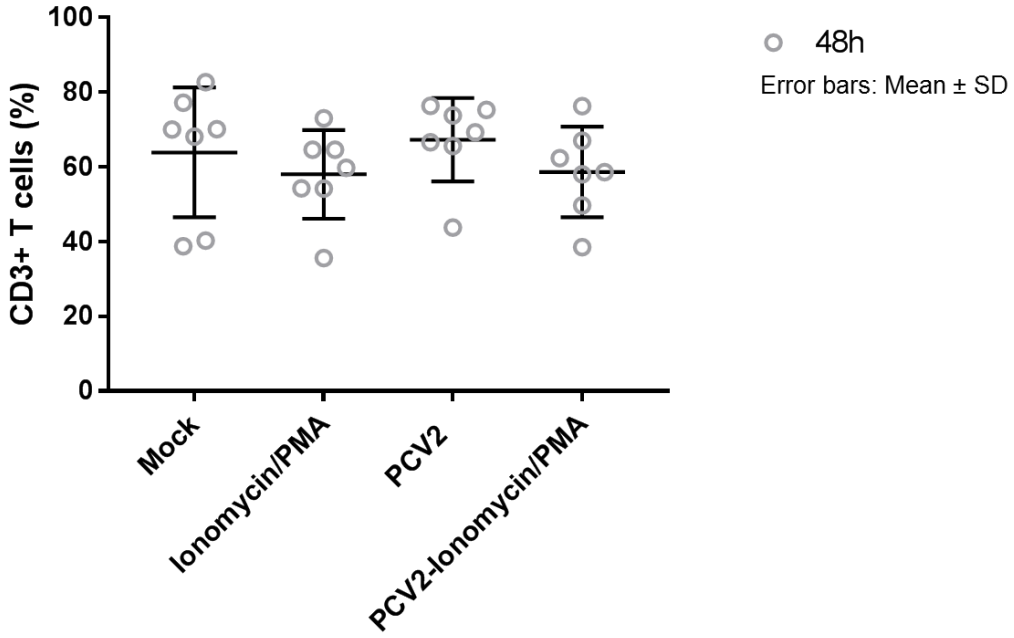


Table 4-2 Percentage of CD3+ T cells after four different treatments and 48h of incubation. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model.

	CD3 + T cells (%)	
Treatment	Mean ± SD	Estimate (95%CI)
Mock	63.89 ± 17.37	63.85 (53.36 - 74.35)
Ionomycin/PMA	58.01 ± 11.87	57.99 (47.49 - 68.48)
PCV2	67.26 ± 11.17	67.22 (56.73 - 77.72)
PCV2-Ionomycin/PMA	58.64 ± 12.12	58.61 (48.11 - 69.11)

Figure 4-4 Percentage of CD3+ T cells in PCV2+ and PCV2- cell populations, after 48h of incubation (mean ± SD).

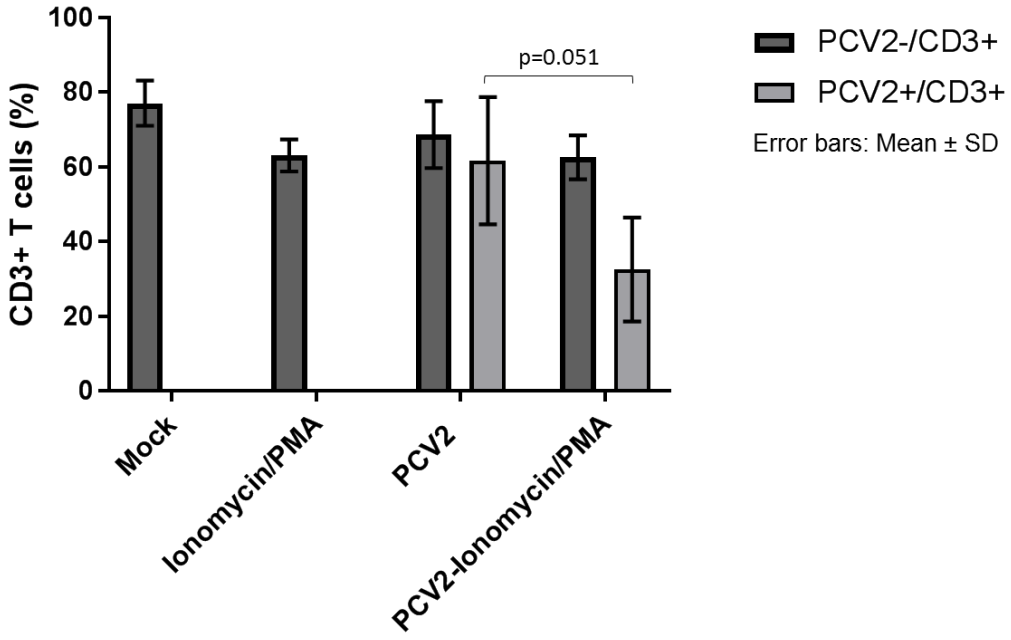


Figure 4-5 Percentage of dead PBMCs after four different treatments, and four incubation time points. Results represent the parameter estimates of the LMER model of two (24h and 96h; pig #: 4, 5, 6, 7), and four (48h, 72h; pig #: 2, 3, 4, 5, 6, 7) independent experiments. Error bars equal to mean ± 95%CI, and significance is shown as asterisks (***p<0.001 demonstrates significance between all groups of the same time point).

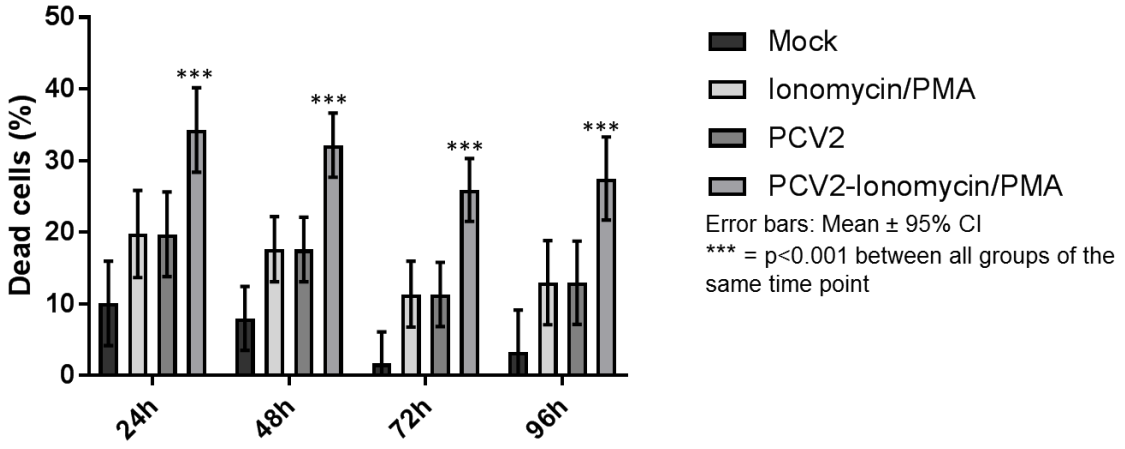


Table 4-3 Percentage of dead PBMCs after four treatments and incubation time points. Distribution (mean ± SD) of raw data, and the parameter estimates (95% CI) of the LMER model; negative parameter estimates were truncated to 0.

		Dead cells (%)	
Time / Treatment		Mean ± SD	Estimate (95%CI)
24h	Mock	4.95 ± 0.63	10.09 (4.20 - 15.99)
	Ionomycin/PMA	18.58 ± 5.67	19.76 (13.70 - 25.85)
	PCV2	15.86 ± 6.12	19.74 (13.83 - 25.65)
	PCV2-Ionomycin/PMA	43.2 ± 9.12	34.30 (28.40 - 40.20)
48h	Mock	5.82 ± 1.23	7.967 (3.50 - 12.44)
	Ionomycin/PMA	19.69 ± 6.71	17.63 (13.09 - 22.18)
	PCV2	15.18 ± 5.03	17.61 (13.11 - 22.11)
	PCV2-Ionomycin/PMA	34.52 ± 11.27	32.17 (27.70 - 36.65)
72h	Mock	4.66 ± 1.72	1.7 (0 - 6.10)
	Ionomycin/PMA	11.51 ± 3.89	11.37 (6.76 - 15.97)
	PCV2	11.20 ± 5.69	11.35 (6.86 - 15.83)
	PCV2-Ionomycin/PMA	22.73 ± 17.12	25.91 (21.51 - 30.31)
96h	Mock	4.71 ± 3.20	3.32 (0 - 9.13)
	Ionomycin/PMA	7.89 ± 6.28	12.98 (7.12 - 18.84)
	PCV2	22.49 ± 22.51	12.96 (7.14 - 18.78)
	PCV2-Ionomycin/PMA	20.54 ± 9.19	27.52 (21.71 - 33.33)

Figure 4-6 Percentage of PCV2-CAP positive PBMCs in live or dead cell populations. Results represent the parameter estimates of the LMER model of two (24h and 96h; pig #: 4, 5, 6, 7), and four (48h, 72h; pig #: 2, 3, 4, 5, 6, 7) independent experiments. Error bars equal to mean ± 95% CI, and significance is shown as asterisks (***p<0.001 demonstrates significance between groups of the same time point).

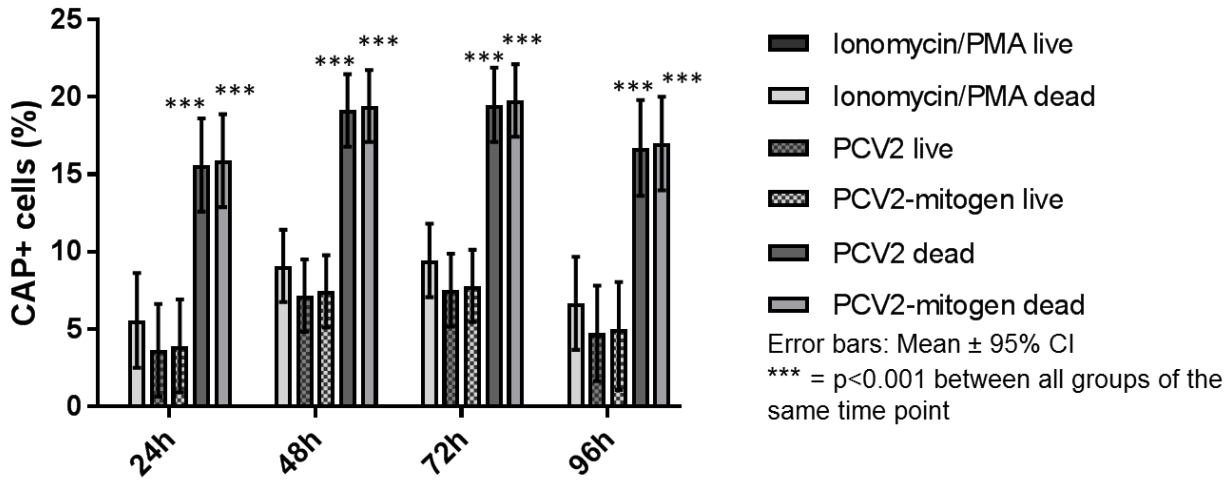


Table 4-4. Percentage of PCV2-CAP positive cells in live or dead cells after four incubation time points. Distribution (mean ± SD) of raw data, and parameter estimates of the LMER model (95% CI); negative estimates were truncated to 0.

		PCV2-CAP + cells (%)	
	Time / Treatment / Viability	Mean ± SD	Estimate (95%CI)
24h	Ionomycin/PMA (L)	0.08 ± 0.05	0 (0 - 3.32)
	Ionomycin/PMA (D)	1.21 ± 1.52	5.57 (2.50 - 8.63)
	PCV2 (L)	1.68 ± 0.90	3.646 (0.65 - 6.64)
	PCV2-Ionomycin/PMA (L)	4.00 ± 1.95	3.93 (0.92 - 6.93)
	PCV2 (D)	14.93 ± 5.95	15.60 (12.59 - 18.61)
	PCV2-Ionomycin/PMA (D)	17.60 ± 3.17	15.89 (12.89 - 18.88)
48h	Ionomycin/PMA (L)	1.61 ± 1.35	0 (0 - 0)
	Ionomycin/PMA (D)	4.22 ± 2.86	9.09 (6.76 - 11.42)
	PCV2 (L)	3.51 ± 2.27	7.17 (4.84 - 9.50)
	PCV2-Ionomycin/PMA (L)	7.38 ± 2.95	7.45 (5.12 - 9.78)
	PCV2 (D)	24.36 ± 6.63	19.13 (16.78 - 21.47)
	PCV2-Ionomycin/PMA (D)	18.13 ± 5.55	19.41 (17.09 - 21.73)
72h	Ionomycin/PMA (L)	1.04 ± 0.91	0 (0 - 0)
	Ionomycin/PMA (D)	4.11 ± 3.36	9.448 (7.07 - 11.83)
	PCV2 (L)	3.28 ± 2.72	7.53 (5.17 - 9.89)
	PCV2-Ionomycin/PMA (L)	7.24 ± 2.62	7.81 (5.47 - 10.14)
	PCV2 (D)	24.63 ± 9.82	19.49 (17.09 - 21.88)
	PCV2-Ionomycin/PMA (D)	22.20 ± 7.30	19.77 (17.42 - 22.11)
96h	Ionomycin/PMA (L)	0.24 ± 0.32	0 (0 - 0)
	Ionomycin/PMA (D)	4.15 ± 3.64	6.67 (3.67 - 9.67)
	PCV2 (L)	1.78 ± 1.32	4.745 (1.66 - 7.83)
	PCV2-Ionomycin/PMA (L)	2.59 ± 1.01	5.03 (1.10 - 8.06)
	PCV2 (D)	20.08 ± 12.33	16.7 (13.61 - 19.80)
	PCV2-Ionomycin/PMA (D)	18.21 ± 4.12	16.98 (13.96 - 20.00)

L= live, D= dead

Figure 4-7 Apoptotic index of PBMCs after five different treatments and 72h of incubation. Results represent the parameter estimates of the LMER model of three independent experiments (pig #: 2, 3, 4, 5, 6, 7). Error bars equal to mean ± 95% CI, and significance is shown as asterisks (***p<0.001, *p<0.05). Brackets demonstrate significance between the first group on the left of the graph.

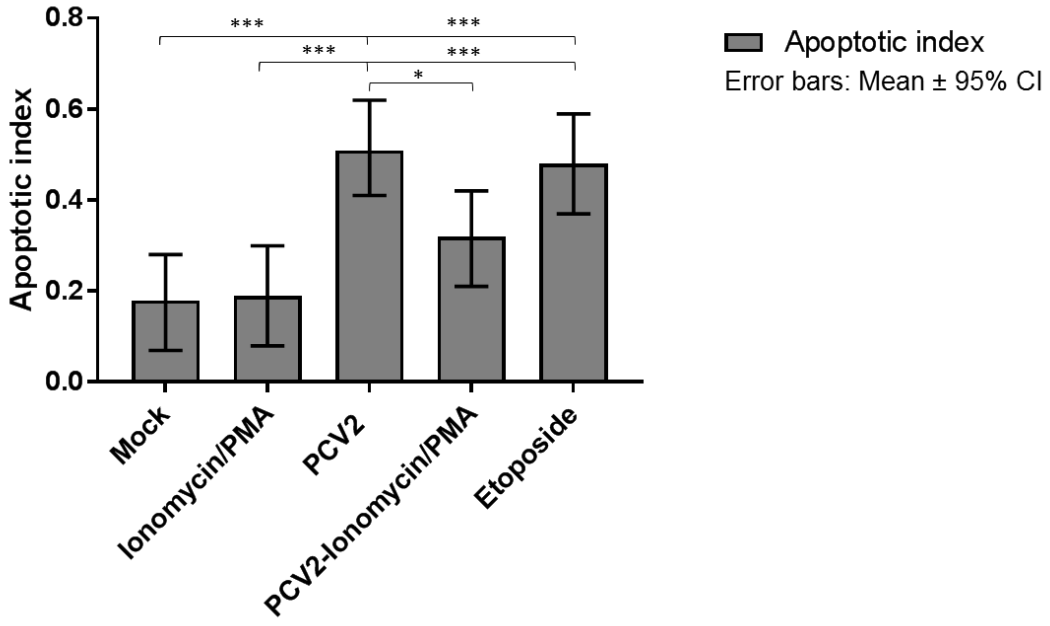
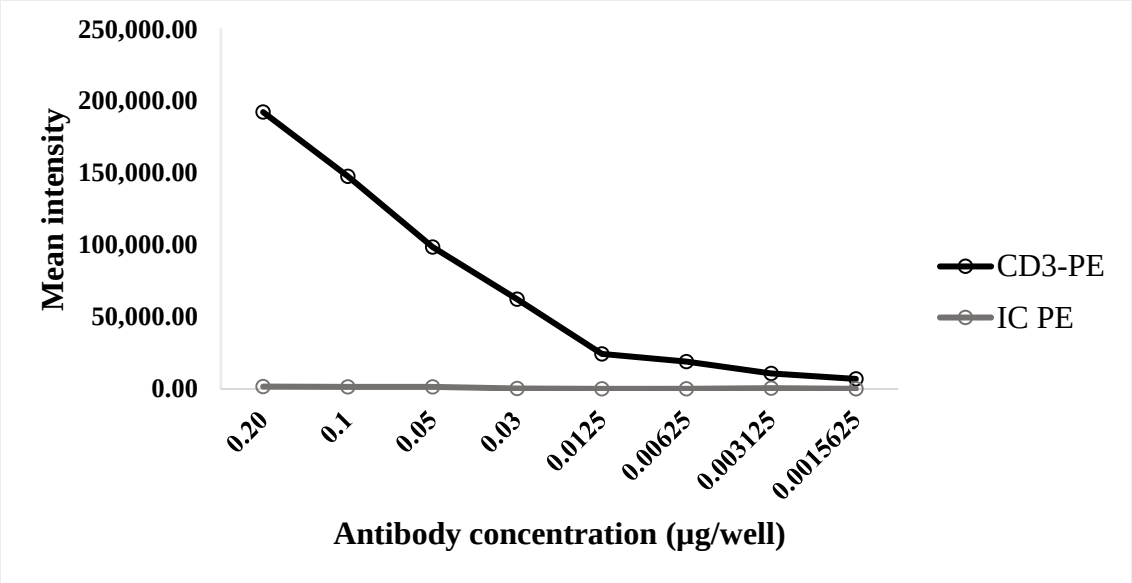


Table 4-5 Apoptotic index of PBMCs after four treatments, and 72h of incubation. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model

	Apoptotic index	
Treatment	Mean ± SD	Estimate (95%CI)
Mock	0.17 ± 0.05	0.18 (0.07 - 0.28)
Ionomycin/PMA	0.19 ± 0.10	0.19 (0.08 - 0.30)
PCV2	0.51 ± 0.18	0.51 (0.41 - 0.62)
PCV2-Ionomycin/PMA	0.31 ± 0.22	0.32 (0.21 - 0.42)
Etoposide	0.48 ± 0.28	0.48 (0.37 - 0.59)

4.5 Appendix

4.5.1 *Optimization of anti-swine CD3 membrane marker*



4.5.2 *Flow cytometry analysis examples*

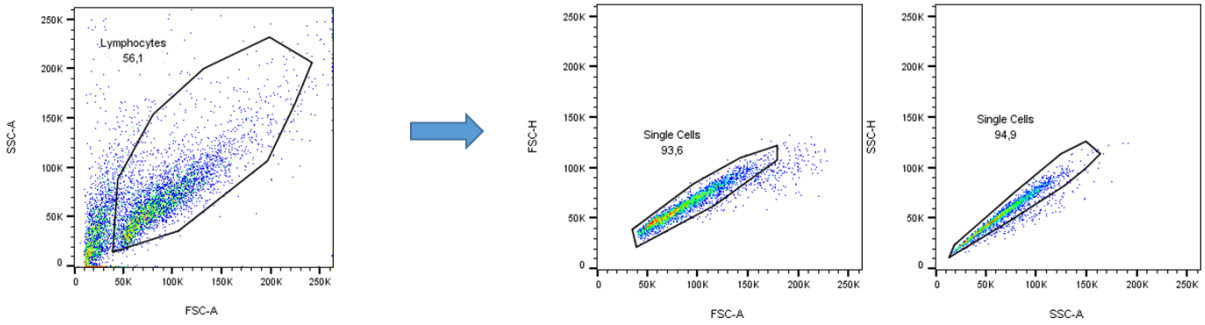
Continued is a detailed description of the analysis performed on each experiment using the Flowjo software version 10.0.08.

4.5.2.1 Gating strategy to determine PCV2-CAP+ cells using goat anti-rabbit Alexa Fluor 635 as a secondary antibody against a rabbit-anti CAP antibody

Cell debris was gated-out, followed by doublet exclusion in the FSC and SSC channels, and control samples were used to establish a two-parameter histogram, distinguishing PCV2 CAP + and populations in the red channel (APC-A in the x-axis of the graphs in Step 3).

Step 1. Gating-out cell debris

Step 2. Doublet exclusion (FSC and SSC)

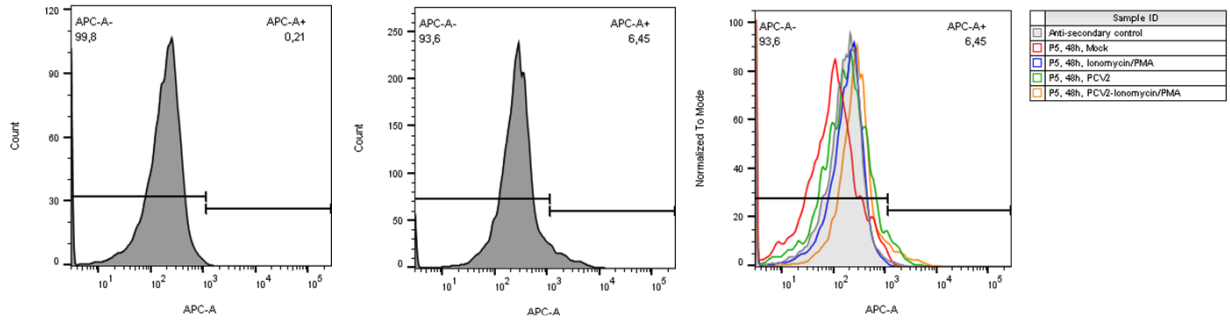


Step 3. Establishment of a two-parameter histogram to distinguish PCV2+ and PCV2- populations (based on control samples).

Ionomycin-PMA

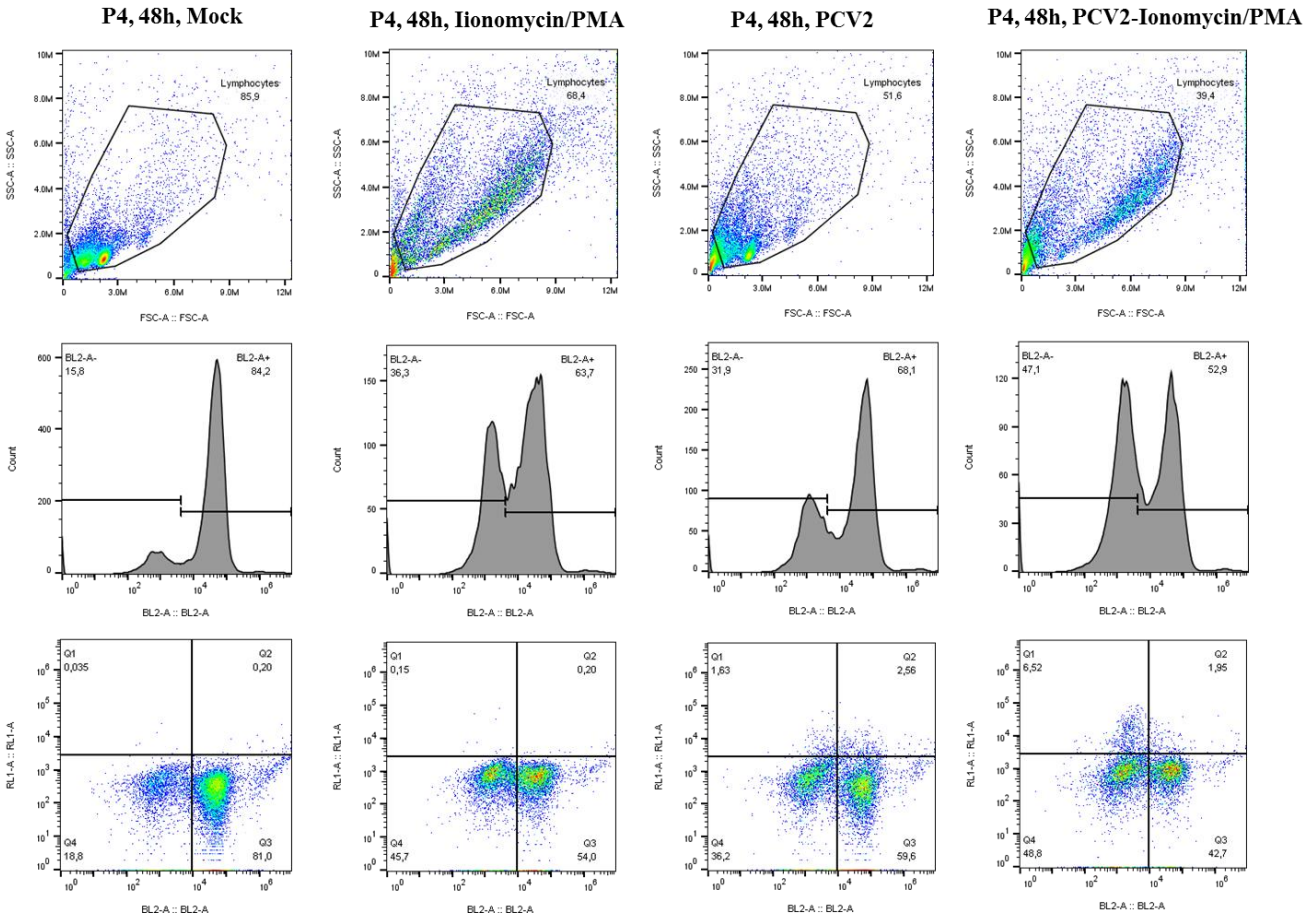
PCV2- Ionomycin-PMA

Overlay example: Pig 5, 48h



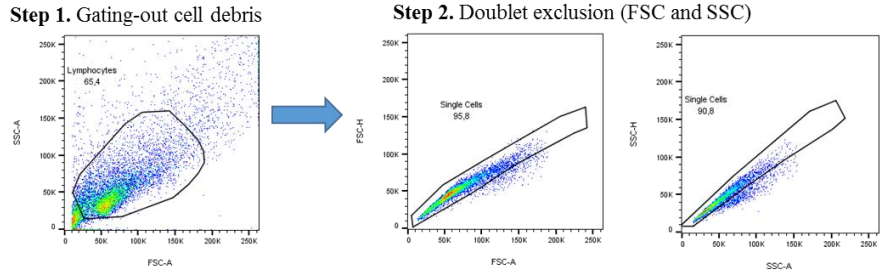
4.5.2.2 Gating strategy to determine the percentage of CD3+ T cells and the percentage of PCV2+ CD3+ T cells using the anti-swine CD3-PE antibody and the goat anti-rabbit Alexa Fluor 635 as a secondary antibody for the anti-CAP antibody

Cell debris was gated-out, followed by doublet exclusion in the FSC and SSC channels (not shown), and a two-parameter histogram to calculate the percentage of CD3+ T cells (BL-2-A+) and CD3- cells (BL-2A-). In the same population, a two axis dot plot was constructed using the PE (BL-2) and red (RL-1) channels, to determine the CD3+/PCV2+ and CD3+/PCV2- populations.

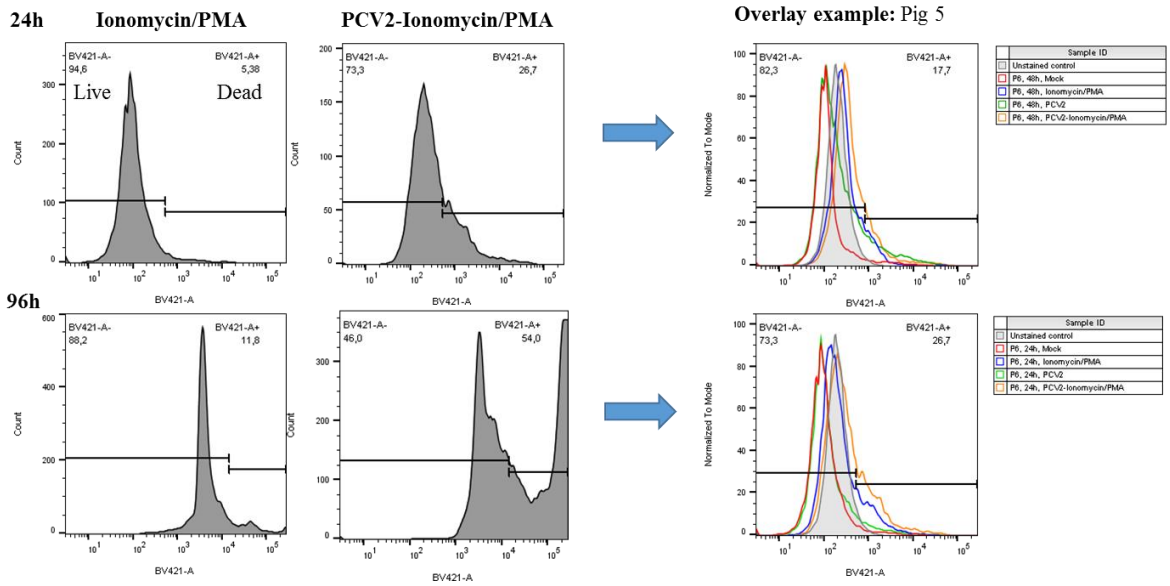


4.5.2.3 Gating strategy to determine the percentage of live or dead cells using LIVE/DEAD® fixable Violet dead cell stain kit

Cell debris was gated-out, followed by doublet exclusion in FSC and SSC channels. Control samples were used to establish a two-parameter histogram to distinguish live (Brilliant violet-421-) from dead (Brilliant violet-421+) cells. Gating was performed for each incubation time point, since we observed a shift to the right on negative populations over time.



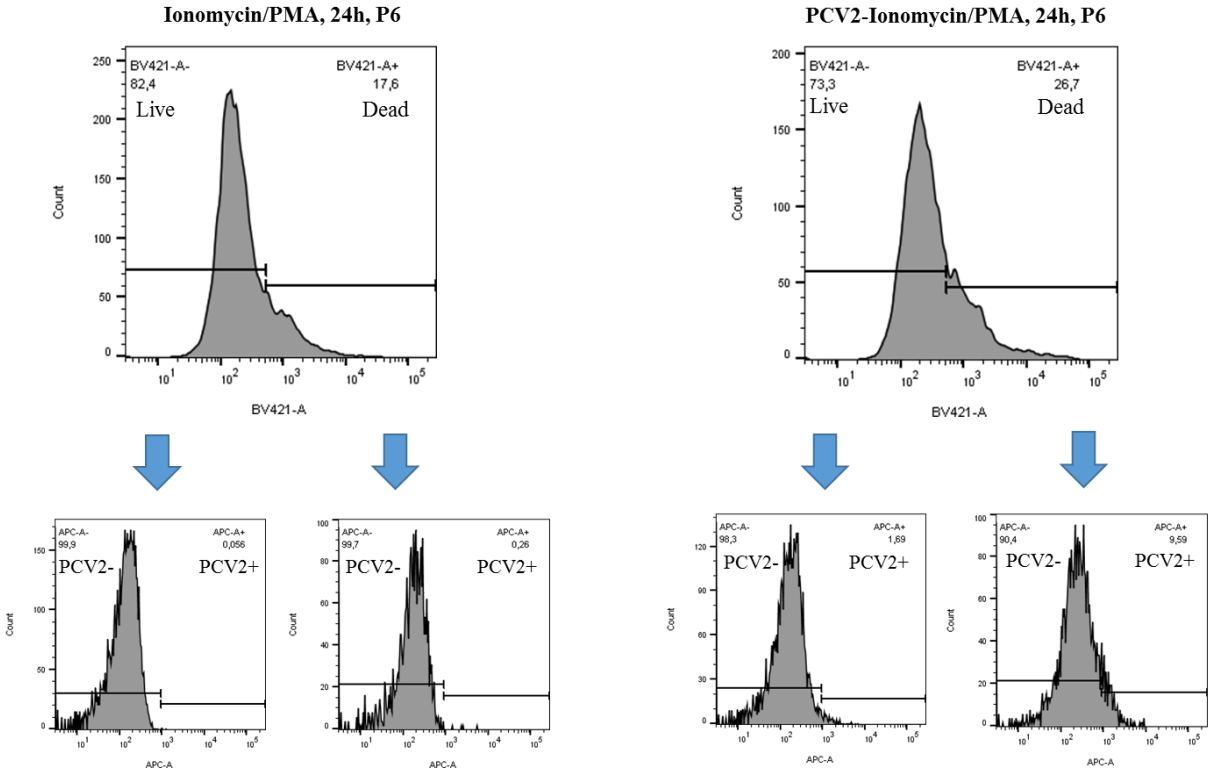
Step 3. Establishment of a two parameter histogram to distinguish live (BV421-) from dead cells (BV421+) Gating was performed for each incubation time point.



4.5.2.4 Gating strategy to determine the percentage of PCV2+ cells in live or dead cells

After the live-dead gating, a two-parameter histogram was performed to determine the percentage of PCV2-CAP positive cells in the live or dead populations.

Step 4: After the gating described to determine live or dead cells, the analysis was continued with PCV2 gating:



4.5.3 *Example of the script used for statistical analysis of PCV2 infection rates data using a linear mixed effect model (LMER) in R (version 3.3.1)*

A LMER is an extended linear regression model, and it is used to analyze categorical data with repeated measurements using the same statistical unit[167]. As in a regression model, the LMER assesses the relationship between independent variables to predict the value of a dependent variable, taking in account random components of the experiment that could influence the result[167]. This model has the advantage of taking the whole data set into account to calculate the estimates, instead of averaging each group separately[168]. This makes the model more robust because it is not affected by missing values or small sample sizes[168].

This model has two components: fixed effects and random effects[168]. In this example, the treatments (e.g. mock, mitogen, PCV2, PCV2+mitogen) and incubation times (e.g. 24h, 48h, 72h, and 96h) are independent variables (i.e. fixed effects) that are used to predict the dependent variable (e.g. percentage of CAP+ cells); the individual pigs that were used as blood donors for each experiment are used random effects (Step 1).

To calculate the estimates, the model uses a hierarchical system, where each group is compared to a reference[168]. In this example, the reference groups are Treatment A (i.e. mock) and Time 1 (i.e. 24h) (Step 1). After the LMER, pairwise comparisons (Step 2) and model validation (Step 3) followed.

Step 1: Linear mixed effects model (LMER) script:

Formula: capsid ~ Treatment + Time + (1 | Pig)
Data: CAP
fit <- lmer (capsid ~ Treatment + Time + (1|Pig), data=CAP, REML=TRUE)

Results: Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	0.679	0.712	48.250	0.953	0.3451
TreatmentB	0.031	0.552	98.430	0.058	0.9541
TreatmentC	3.897	0.547	98.350	7.122	1.78e-10 ***
TreatmentD	7.503	0.558	98.500	13.443	< 2e-16 ***
Time2	-0.225	0.637	102.650	-0.353	0.7247
Time3	0.342	0.632	102.120	0.541	0.5895
Time4	-1.941	0.755	98.520	-2.570	0.0117 *

Analysis of Random effects Table:

	Chi.sq	Chi.DF	p.value
Pig	3.87	1	0.051

Step 2: Tukey's post hoc test with Bonferroni method for p-value adjustment for multiple comparisons

Formula:
Lsmeans (fit, pairwise~Treatment+Time)

Results: Parameter estimates

Treat.	Time	lsmean	SE	df	lower.CL	upper.CL
A	1	0.679	0.712	48.25	-0.752	2.110
B	1	0.711	0.727	50.71	-0.748	2.170
C	1	4.577	0.710	47.87	3.148	6.005
D	1	8.182	0.712	48.93	6.751	9.614
A	2	0.453	0.557	26.86	-0.690	1.598
B	2	0.485	0.556	26.84	-0.656	1.628
C	2	4.351	0.554	26.42	3.2135	5.489
D	2	7.957	0.558	27.32	6.812	9.102
A	3	1.021	0.548	25.51	-0.107	2.149
B	3	1.053	0.548	25.49	-0.075	2.182
C	3	4.919	0.546	25.10	3.794	6.043
D	3	8.524	0.564	28.22	7.369	9.680
A	4	-1.262	0.726	50.88	-2.721	0.196
B	4	-1.230	0.712	48.30	-2.662	0.200
C	4	2.635	0.710	47.98	1.207	4.063
D	4	6.241	0.712	49.05	4.810	7.671

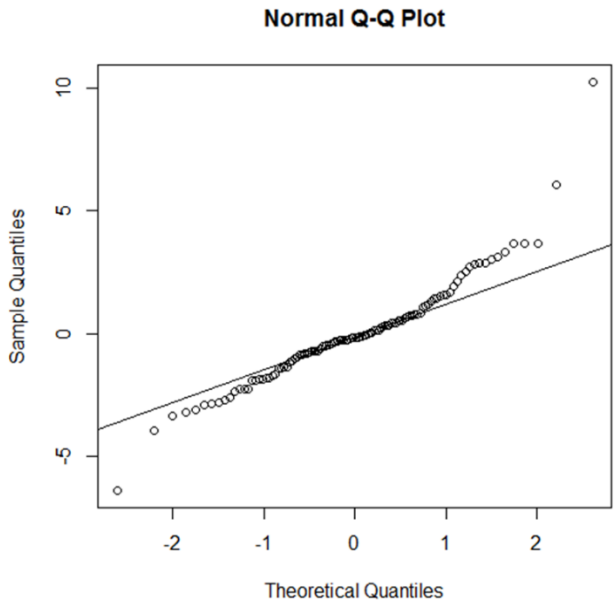
contrast	estimate	SE	df	t.ratio	p.value
A,1 - B,1	-0.03191879	0.5527453	98.43	-0.058	1.0000
A,1 - C,1	-3.89796951	0.5473494	98.35	-7.122	<.0001
A,1 - D,1	-7.50362409	0.5581631	98.50	-13.443	<.0001
A,1 - A,2	0.22530564	0.6378750	102.65	0.353	1.0000
A,1 - B,2	0.19338685	0.8306173	100.91	0.233	1.0000
A,1 - C,2	-3.67266387	0.8396949	100.93	-4.374	0.0030
A,1 - D,2	-7.27831846	0.8479279	101.19	-8.584	<.0001
A,1 - A,3	-0.34213743	0.6320466	102.12	-0.541	1.0000
A,1 - B,3	-0.37405621	0.8269052	100.63	-0.452	1.0000
A,1 - C,3	-4.24010693	0.8360015	100.64	-5.072	0.0002
A,1 - D,3	-7.84576152	0.8534693	100.94	-9.193	<.0001
A,1 - A,4	1.94166530	0.7556148	98.52	2.570	0.4315
A,1 - B,4	1.90974651	0.9133338	98.33	2.091	0.7661
A,1 - C,4	-1.95630420	0.9216089	98.38	-2.123	0.7461
A,1 - D,4	-5.56195879	0.9280478	98.42	-5.993	<.0001
B,1 - C,1	-3.86605072	0.5473338	98.34	-7.063	<.0001
B,1 - D,1	-7.47170530	0.5581256	98.48	-13.387	<.0001
B,1 - A,2	0.25722442	0.8572622	101.22	0.300	1.0000
B,1 - B,2	0.22530564	0.6378750	102.65	0.353	1.0000
B,1 - C,2	-3.64074508	0.8529696	101.09	-4.268	0.0043
B,1 - D,2	-7.24639967	0.8610614	101.33	-8.416	<.0001
B,1 - A,3	-0.31021864	0.8522020	100.73	-0.364	1.0000
B,1 - B,3	-0.34213743	0.6320466	102.12	-0.541	1.0000
B,1 - C,3	-4.20818815	0.8485986	100.69	-4.959	0.0003
B,1 - D,3	-7.81384273	0.8657980	100.97	-9.025	<.0001
B,1 - A,4	1.97358409	0.9585319	98.63	2.059	0.7856
B,1 - B,4	1.94166530	0.7556148	98.52	2.570	0.4315
B,1 - C,4	-1.92438542	0.9442713	98.53	-2.038	0.7979
B,1 - D,4	-5.53004000	0.9505437	98.57	-5.818	<.0001
C,1 - D,1	-3.60565459	0.5526030	98.39	-6.525	<.0001
C,1 - A,2	4.12327514	0.8413466	101.20	4.901	0.0004
C,1 - B,2	4.09135635	0.8278649	101.03	4.942	0.0003
C,1 - C,2	0.22530564	0.6378750	102.65	0.353	1.0000
C,1 - D,2	-3.38034895	0.8451001	101.30	-4.000	0.0109
C,1 - A,3	3.55583208	0.8362116	100.70	4.252	0.0046
C,1 - B,3	3.52391329	0.8234044	100.65	4.280	0.0042
C,1 - C,3	-0.34213743	0.6320466	102.12	-0.541	1.0000
C,1 - D,3	-3.94779201	0.8499468	100.95	-4.645	0.0011
C,1 - A,4	5.83963481	0.9443130	98.54	6.184	<.0001
C,1 - B,4	5.80771602	0.9216332	98.39	6.302	<.0001
C,1 - C,4	1.94166530	0.7556148	98.52	2.570	0.4315
C,1 - D,4	-1.66398928	0.9360977	98.47	-1.778	0.9184
D,1 - A,2	7.72892973	0.8472776	100.96	9.122	<.0001
D,1 - B,2	7.69701094	0.8338770	100.78	9.230	<.0001

D,1 - C,2	3.83096022	0.8428019	100.81	4.546	0.0016
D,1 - D,2	0.22530564	0.6378750	102.65	0.353	1.0000
D,1 - A,3	7.16148667	0.8328553	100.42	8.599	<.0001
D,1 - B,3	7.12956788	0.8199806	100.35	8.695	<.0001
D,1 - C,3	3.26351716	0.8290334	100.36	3.937	0.0136
D,1 - D,3	-0.34213743	0.6320466	102.12	-0.541	1.0000
D,1 - A,4	9.44528939	0.9506453	98.60	9.936	<.0001
D,1 - B,4	9.41337061	0.9281069	98.44	10.143	<.0001
D,1 - C,4	5.54731989	0.9361456	98.48	5.926	<.0001
D,1 - D,4	1.94166530	0.7556148	98.52	2.570	0.4315
A,2 - B,2	-0.03191879	0.5527453	98.43	-0.058	1.0000
A,2 - C,2	-3.89796951	0.5473494	98.35	-7.122	<.0001
A,2 - D,2	-7.50362409	0.5581631	98.50	-13.443	<.0001
A,2 - A,3	-0.56744306	0.4582389	99.04	-1.238	0.9970
A,2 - B,3	-0.59936185	0.7188595	98.90	-0.834	1.0000
A,2 - C,3	-4.46541257	0.7146935	98.82	-6.248	<.0001
A,2 - D,3	-8.07106715	0.7337293	99.00	-11.000	<.0001
A,2 - A,4	1.71635967	0.6359748	102.27	2.699	0.3461
A,2 - B,4	1.68444088	0.8307681	100.94	2.028	0.8040
A,2 - C,4	-2.18160984	0.8272009	100.90	-2.637	0.3857
A,2 - D,4	-5.78726443	0.8332057	100.65	-6.946	<.0001
B,2 - C,2	-3.86605072	0.5473338	98.34	-7.063	<.0001
B,2 - D,2	-7.47170530	0.5581256	98.48	-13.387	<.0001
B,2 - A,3	-0.53552427	0.7171204	98.46	-0.747	1.0000
B,2 - B,3	-0.56744306	0.4582389	99.04	-1.238	0.9970
B,2 - C,3	-4.43349378	0.7138075	98.60	-6.211	<.0001
B,2 - D,3	-8.03914837	0.7328494	98.78	-10.970	<.0001
B,2 - A,4	1.74827845	0.8542874	100.65	2.046	0.7930
B,2 - B,4	1.71635967	0.6359748	102.27	2.699	0.3461
B,2 - C,4	-2.14969105	0.8390828	100.75	-2.562	0.4366
B,2 - D,4	-5.75534564	0.8449886	100.50	-6.811	<.0001
C,2 - D,2	-3.60565459	0.5526030	98.39	-6.525	<.0001
C,2 - A,3	3.33052645	0.7129949	98.45	4.671	0.0010
C,2 - B,3	3.29860766	0.7138580	98.67	4.621	0.0012
C,2 - C,3	-0.56744306	0.4582389	99.04	-1.238	0.9970
C,2 - D,3	-4.17309765	0.7286770	98.77	-5.727	<.0001
C,2 - A,4	5.61432917	0.8507932	100.67	6.599	<.0001
C,2 - B,4	5.58241039	0.8390568	100.81	6.653	<.0001
C,2 - C,4	1.71635967	0.6359748	102.27	2.699	0.3461
C,2 - D,4	-1.88929492	0.8413380	100.51	-2.246	0.6633
D,2 - A,3	6.93618103	0.7104219	98.42	9.763	<.0001
D,2 - B,3	6.90426224	0.7112708	98.63	9.707	<.0001
D,2 - C,3	3.03821152	0.7069200	98.54	4.298	0.0039
D,2 - D,3	-0.56744306	0.4582389	99.04	-1.238	0.9970
D,2 - A,4	9.21998376	0.8589459	100.94	10.734	<.0001

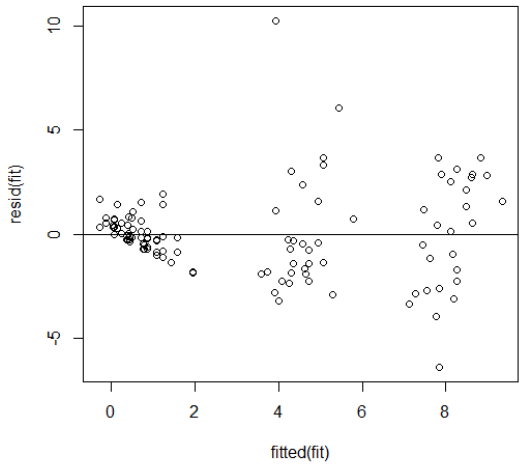
D,2 - B,4	9.18806497	0.8473079	101.08	10.844	<.0001
D,2 - C,4	5.32201425	0.8436933	101.04	6.308	<.0001
D,2 - D,4	1.71635967	0.6359748	102.27	2.699	0.3461
A,3 - B,3	-0.03191879	0.5527453	98.43	-0.058	1.0000
A,3 - C,3	-3.89796951	0.5473494	98.35	-7.122	<.0001
A,3 - D,3	-7.50362409	0.5581631	98.50	-13.443	<.0001
A,3 - A,4	2.28380273	0.6319220	102.05	3.614	0.0372
A,3 - B,4	2.25188394	0.8269150	100.64	2.723	0.3313
A,3 - C,4	-1.61416678	0.8233531	100.62	-1.960	0.8404
A,3 - D,4	-5.21982136	0.8199168	100.32	-6.366	<.0001
B,3 - C,3	-3.86605072	0.5473338	98.34	-7.063	<.0001
B,3 - D,3	-7.47170530	0.5581256	98.48	-13.387	<.0001
B,3 - A,4	2.31572152	0.8520077	100.62	2.718	0.3345
B,3 - B,4	2.28380273	0.6319220	102.05	3.614	0.0372
B,3 - C,4	-1.58224799	0.8360369	100.61	-1.893	0.8731
B,3 - D,4	-5.18790258	0.8326381	100.32	-6.231	<.0001
C,3 - D,3	-3.60565459	0.5526030	98.39	-6.525	<.0001
C,3 - A,4	6.18177224	0.8484829	100.62	7.286	<.0001
C,3 - B,4	6.14985345	0.8359676	100.62	7.357	<.0001
C,3 - C,4	2.28380273	0.6319220	102.05	3.614	0.0372
C,3 - D,4	-1.32185186	0.8289114	100.30	-1.595	0.9658
D,3 - A,4	9.78742682	0.8657247	100.92	11.305	<.0001
D,3 - B,4	9.75550803	0.8534478	100.93	11.431	<.0001
D,3 - C,4	5.88945731	0.8498806	100.91	6.930	<.0001
D,3 - D,4	2.28380273	0.6319220	102.05	3.614	0.0372
A,4 - B,4	-0.03191879	0.5527453	98.43	-0.058	1.0000
A,4 - C,4	-3.89796951	0.5473494	98.35	-7.122	<.0001
A,4 - D,4	-7.50362409	0.5581631	98.50	-13.443	<.0001
B,4 - C,4	-3.86605072	0.5473338	98.34	-7.063	<.0001
B,4 - D,4	-7.47170530	0.5581256	98.48	-13.387	<.0001
C,4 - D,4	-3.60565459	0.5526030	98.39	-6.525	<.0001

Step 3: Validating fitness of model

Normality assumption:
Formula:
qqnorm(residuals(fit))
qqline(residuals(fit))

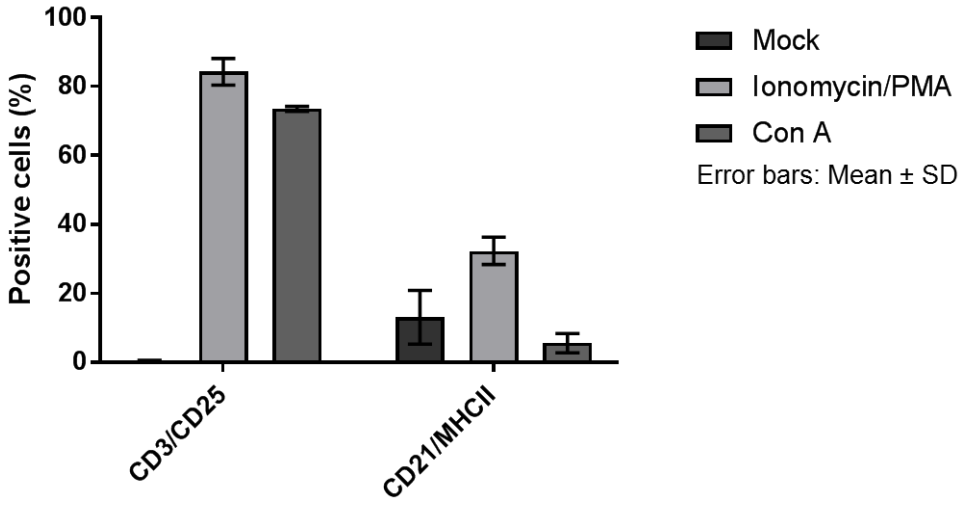


Equal variance assumption: residuals vs fitted data
Formula:
plot(resid(fit)~ fitted (fit))
abline(h=0)



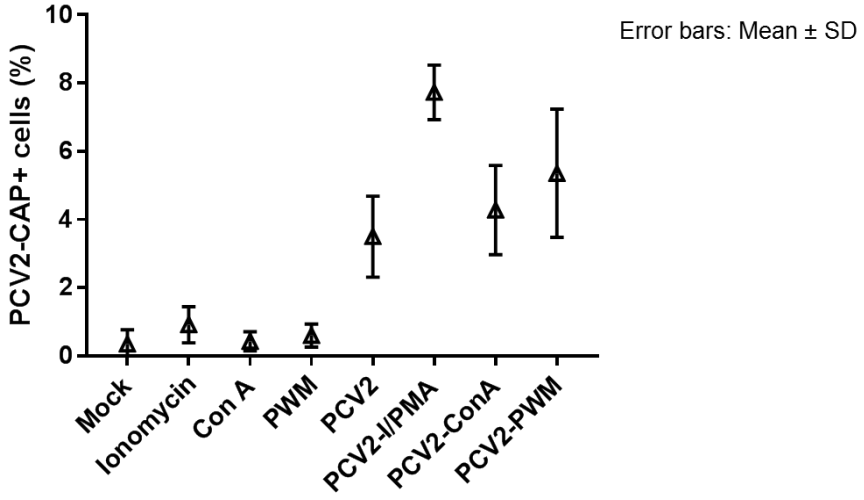
4.5.4 *Percentage of CD3+/CD25+ and CD21+/MHCII+ PBMCs after mock, Ionomycin/PMA (1µg/mL and 1ng/mL, respectively) and Con A (5µg/mL) treatment for 30 minutes, followed by incubation for 48h*

Graph represents the result of one experiment using three PCV2-free pigs (pig #: 4, 7, and 8).



4.5.5 *Percentage of PCV2 CAP+ PBMCs after treatment with Ionomycin/PMA (1µg/mL and 1ng/mL, respectively), Con A (5µg/mL), and PWM (5µg/mL) for 30 minutes, followed by incubation of cells with PCV2 for 48h*

Graph represents the result of two experiments, using PBMCs isolated from three PCV2-free pigs (pig#: 2, 3, 4).



Chapter Five: PCV2 infection and cell cycle dependency

5.1 Introduction

Porcine circovirus 2 (PCV2) is a non-enveloped virus, with a single-stranded, circular DNA genome of 1.7 kb[16]. It has been predicted that the PCV2 genome contains ORFs[169] encoding for five proteins: the replication assisting proteins Rep and Rep' encoded in the ORF-1[170], the pro-apoptotic proteins ORF-3[101] and ORF-4[171], and the capsid, the only structural protein of PCV2 encoded in the ORF-2[172]. PCV2 does not contain a viral polymerase, so the limited coding capacity of the viral genome and the lack of polymerase suggests that PCV2 depends on the host enzymes to replicate[173], and a cell cycle arrest in S phase would generate the ideal environment for PCV2-DNA synthesis and gene expression[174].

The cell cycle is divided into the interphase (G1, S, G2), and mitosis (M), and the quiescence phase G0. Each phase contains checkpoints with cyclins and cyclin dependent kinases (CDKs) that assure cell cycle continuation[175]. G1 phase is characterized by the expression of transcription factors that prepare the cell for DNA synthesis, and it is regulated by cyclins D and E[174]. S phase results in a new DNA synthesis, and it is regulated by the cyclin A/CDK2 complex [174]. The G2 phase prepares the cell for mitosis and has cyclin B/CDK1 as regulators, and is followed by mitosis[174].

A cell cycle dependency for PCV replication was first proposed by Tischer e*t al* (1987), who demonstrated increased PCV2-DNA synthesis during S phase in synchronized glucosamine-treated PK-15 cells[156]. A recent study demonstrated that PCV2 infection induces S phase arrest in PK-15 cells[176], with a downregulation of Cyclin A/CDK2 expression[176,173]. A G0/G1 arrest was also suggested due to the downregulation of Cyclins E and D in PCV2-infected PK-15

cells[177]. Whether the infection with PCV2 will exert a similar effect on PBMCs has not been explored yet.

Impaired cell proliferation[178] and downregulation of antigen presentation[179] were observed in lymph node sections of PMWS pigs, although the same phenomenon has not been reported *in vitro*[66]. These mechanisms could be associated with enhanced PCV2 replication and persistence in tissues of diseased pigs. The demonstration of the effect of PCV2 infection on cell proliferation *in-vitro* could elucidate the mechanism by which PCV2 regulates normal cell processes to increase viral replication[66].

In this study, PCV2-naïve PBMCs were stimulated with the polyclonal mitogens ionomycin/PMA, and exposed to PCV2. By pushing the cells into mitosis, the activation and proliferation activity of the PBMCs were analyzed, with measurements on cell cycle and DNA synthesis. This study aids in understanding the effect of PCV2 on activation and proliferation of PBMCs *in-vitro*, and to determine if a cell-cycle dependency is necessary for PCV2 infection in PBMCs.

5.2 Material and methods

5.2.1 *Cell source and PCV2 infection*

Whole blood source, isolation of PBMCs, Ionomycin/PMA stimulation, and PCV2 infection and anti-PCV2 CAP staining were performed following the methodology described in Chapter 4.

5.2.2 *Indirect immunostaining of the swine IL-2 receptor (CD25) and MHC-II*

After 48h of incubation, the plates were centrifuged, the supernatant was discarded, and 50µL of the blocking buffer (1X PBS, 5% goat serum, 0.01% sodium azide) were added to the cells for 20 minutes at 4°C. The primary antibody (mouse anti-swine CD25 antibody (IgG1 isotype, Serotec, catalog #MCA1736) or mouse anti-swine MHC-II (IgG2a isotype, WSU antibody centre, catalog #MSA3)) was added directly to the cells, and incubated for 45 minutes at 4°C. The cells were washed once with the washing buffer (2% BSA, 1X PBS), and a secondary antibody (goat anti-mouse IgG-FITC (BD Pharmigen, catalog #554001) or goat anti-mouse IgG2a-PE (Life technologies, catalog #P21139)) was added to the plates for 45 minutes at 4°C. The cells were washed once and resuspended in 2% formaldehyde for 20 minutes at RT.

5.2.3 *Total PBMC count*

To determine the total number of cells after each treatment, Hoechst stained PBMCs were transferred to a flat-bottom optical plate (Nunc®, #160376), and analyzed using the automated inverted microscope IN Cell Analyzer 2000 (GE Healthcare®). Eight images per well were acquired using a 10X field of view in the DAPI channel (exposure 0.05s, offset 6.00), and used to determine the absolute numbers of cells using the multi-target analysis of the INCell Analyzer 1000 Workstation (GE Healthcare). The total number of nuclei/well was determined based on the Hoechst stain intensity, and a "top hat" selection based on nuclear area (11μm^2).

5.2.4 *PBMC proliferation*

PBMC proliferation analysis was performed using the Cell Trace™ carboxyfluorescein succinimidyl ester (CFSE) kit (Thermo Fisher, cat# C34554) following the manufacturer's instructions. Briefly, PBMCs were resuspended in RPMI media to a concentration of 10^6

cells/mL in a 15mL Falcon tube, and 1µL/mL of the Cell Trace™ stock solution was added to the cells, and then incubated for 20 minutes at 37°C. Five times volume of the staining media was added to the cells, and incubated for 5 minutes at RT. The cells were pelleted at 200 x g for 3 minutes, re-suspended in warm media to a concentration of 2 x 10^6 PBMCs/mL media, and 100µL were placed on each well of a U-shaped 96-well plate, followed by the Ionomycin/PMA treatment for 30 minutes. The cells were washed, and PCV2 was added for 24h, 48h, and 72h. After the incubation, the plates were centrifuged, the supernatant was removed carefully, the cells were resuspended in 100µL of sterile PBS, and then stained using the LIVE/DEAD® fixable Violet dead cell stain kit (Thermo Fisher, cat#L34955) following the manufacturer's instructions (see Chapter 4 for details). After staining, the cells were fixed and stained against intracellular viral proteins (see Chapter 4), and analyzed using flow cytometry.

5.2.5 *Cell cycle analysis*

Cell cycle analysis was performed by detecting new DNA synthesis using the synthetic analog of thymidine, Bromodeoxyuridine (BrdU), and measuring DNA content of cells using the intercalating dye 7-Aminoactinomycin D (7-AAD). The analysis was performed using the FITC-BrdU flow kit (BD Pharmigen, cat #559619) following the manufacturer's instructions. Briefly, 3µL of BrdU stock (1mM BrdU in 1X DPBS) were added to the PBMC cultures, and incubated for 1h at 37°C. Subsequently, plates were centrifuged at 200g for 3 minutes, the supernatant was discarded, and the cells were fixed using 100µL of the BD Cryofix/Cytoperm Buffer for 30 minutes at RT. The cells were then washed using 100µL of the 1X BD Perm/Wash Buffer, centrifuged, and incubated with 100µL of the BD Cytoperm Permeabilization Buffer Plus for 10 minutes at 4°C. The cells were washed once, fixed again for 5 minutes at RT, and washed. The

incorporated BrdU was exposed by adding 100 µL of DNAse (300µg/mL) per well, incubated for 1h at 37°C, and washed once. Subsequently, the anti-BrdU antibody was diluted 1:50 in staining buffer and incubated with the cells for 20 minutes RT. The cells were washed once and stained for the intracellular PCV2-CAP protein, and DNA content using 20µL of the 7-AAD solution for 15 minutes. The cells were washed once, resuspended in 200µL of PBS, and analyzed using flow cytometry. Details on the optimization of the cell cycle protocol are found in Appendix 5.5.1.

5.2.6 *Flow cytometry analysis*

Cells were re-suspended in 200µL of PBS and the complete volume was transferred from the plates to individual 5mL round bottom tubes (Falcon, #352008). Samples were read in two different instruments, depending of the necessity of specific lasers due to fluorophore combinations. The Attune Flow cytometer (Thermo Fisher Scientific) was used to read the experiments with activation and cell cycle markers using the Attune analysis software (Thermo Fisher Scientific); the LSR II™ (BD Bioscience) equipped with four lasers and using the DIVA software (BD Bioscience) was used for the cell proliferation analysis. Electronic compensation was set on each experiment to eliminate spectral overlaps between fluorophores.

FlowJo version 10.0.08 was used for the activation membrane markers and BrdU data analysis. Debris and aggregates were eliminated after initial gating, followed by a specific analysis depending on the experiment. A detailed description of gating strategies for each experiment is explained in Appendix section 5.5.2 to 5.5.5 (MHC-II and CD25), and 5.5.6 (BrdU).

For the analysis of CD25 and MHCII positive cells, control samples were used to distinguish the percentage of membrane marker positive cells. Subsequently, the geometric mean fluorescence intensity (MFI) was determined for the MHC-II+ or CD25+ population. The intensity of the CD25 or MHCII positive cells at time 0 was transformed to a baseline value of 1, and was used as reference to normalize the fluorescence of all other treatments at 48h (Appendix section 5.5.2 to 5.5.5).

ModFit LT version 4.1 (Verity Software House) was used for proliferation and cell cycle analysis. For the cell proliferation analysis, a model was built using the cell track wizard, with adjustment of the parent population and determination of the cell generations by the software (see Appendix 5.5.7 to 5.5.9 for details on the analysis). The proliferation index (PIn) was computed by the software and is defined as "the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment"[180].

For the cell cycle analysis, the determination of the percentage of cells in each cell cycle phase was performed by the software using 7-AAD as reference for DNA content. The 2N peak of the G0/G1 was adjusted automatically by the program, and the G2/M phase was determined based on a G2/G1 ratio of 2 (4N); the S phase was determined as the area under the curve between G1 and G2 (see Appendix 5.5.10 and 5.5.11 for details on the analysis).

5.2.7 *Statistical analysis*

Statistical analyses of data were performed using SPSS Statistics 22.0 software (IBM) and R version 3.3.1 (R Foundation for Statistical Computing). Outlier calculation, descriptive statistics, and paired-samples t-tests were performed using SPSS. Outliers were determined as

any number outside the range: (Q1- k(Q3-Q1), Q3 + k(Q3-Q1)), with k=2.2, and were excluded from any further analysis.

A LMER was used to examine the effects of treatment and time (as fixed effects) on each of the predictor variables (e.g. CD25 and MHC-II upregulation, MFI of CD25 and MHC-II, total PBMC counts, PIn, cell cycle analysis), using individual pigs as a random effect. A REML method was used for parameter estimation. Pairwise comparisons within and between groups were analyzed using Tukey's post hoc test with Bonferroni method for p-value adjustment for multiple comparisons. The normality and equal variance assumptions were assessed to validate the fitness of each model. Analyses were performed using R; the 'lme4' package version 1.1.12 for the linear mixed effects models analysis, and 'lsmeans' package version 2.24 for multiple comparisons[145]. A $p<0.05$ was considered statistical significant.

5.3 Results

5.3.1 *IL-2 receptor (CD25) upregulation and MFI*

The expression of the alpha chain of the IL-2 receptor (CD25) was used to assess if the cells were activated by mitogen treatment at 48h of incubation. The differences in the percentage of CD25+ cells at 48h from 0h were calculated and compared using a one-sample t-test. The results showed that the percentage of positive cells at 48h were significantly higher to time 0 ($t=4.7$, $p< 0.001$; Figure 5-1).

An LMER model determined that treatment had a significant, positive effect on CD25 upregulation in the ionomycin/PMA (53.22% ± 2.5 SE, $p<0.001$), and PCV2-Ionomycin/PMA (67.5% ± 2.1 SE, $p<0.001$) groups, compared to mock. Pairwise comparisons demonstrated that

the percentage of CD25+ cells was significantly higher in the ionomycin/PMA treatment group as compared to mock and PCV2-only treated groups ($p<0.001$; Figure 5-1, Table 5-1). Also, the PCV2-ionomycin/PMA treated group had significantly higher percentage of CD25+ cells, compared to all other groups ($p<0.001$; Figure 5-1, Table 5-1). No significant variation was observed between individual pigs ($p=0.8$).

To determine the level of CD25 expression in the cells, the geometric MFI of CD25+ cells at 48h and 0h were calculated and compared using a one-sample t-test. The results showed that the MFI of CD25+ cells at 48h was significantly higher than at time 0 ($t=3.3$, $p<0.003$).

An LMER model demonstrated that treatment had a significant, positive effect on the MFI of CD25+ cells in the ionomycin/PMA (0.43 ± 0.09 SE, $p<0.001$) and PCV2-Ionomycin/PMA (0.45 ± 0.09 SE, $p<0.001$) groups, compared to mock. Pairwise comparisons demonstrated that the MFI of CD25+ cells of the mock group was significantly lower than the cells in the ionomycin/PMA group, and the PCV2-Ionomycin/PMA groups ($p<0.001$, Figure 5-2, Table 5-1). In addition, the MFI of the cells in the PCV2-Ionomycin/PMA group was also significantly higher than the MFI of the PCV2-only group ($p<0.05$, Figure 5-2, Table 5-1). No significant variation was observed between individual pigs ($p=0.1$).

These results demonstrate that the PBMCs were activated 48h after mitogen treatment, and a higher percentage of CD25+ cells was observed in the PCV2-Ionomycin/PMA treated cells.

5.3.2 *MHC-II upregulation and MFI*

The expression of MHC-II was also used as a marker to assess the activation status of PBMCs after treatment. To determine if MHC-II was upregulated after 48h of incubation, the differences between the percentage of MHC-II+ cells at 48h and 0h were calculated and compared using a one-sample t-test. The results showed that the percentage of MHC-II+ cells at 48h were significantly higher than at time 0 ($t=8.0$, $p< 0.001$).

An LMER model was performed to determine the effect of treatment on expression of MHC-II at 48h, with Tukey's post hoc test for pairwise comparisons. Treatment had a significant, positive effect in the ionomycin/PMA (24.8% ± 2.3SE, $p<0.001$), and PCV2-Ionomycin/PMA (20.8% ± 2.3 SE, $p<0.001$), compared to mock. Pairwise comparisons demonstrated that the percentage of MHC-II+ cells was significantly higher in the ionomycin/PMA treatment group, compared to mock, and PCV2 exposed groups ($p<0.001$; Figure 5-3, Table 5-2). Similarly, PCV2-ionomycin/PMA treated group had significantly higher percentage of MHC-II+ cells, compared to mock and PCV2 ($p<0.001$; Figure 5-3, Table 5-2). No significant variation was observed between individual pigs ($p=0.5$).

To determine if the level of MHC-II expression in the cells at 48h was different from 0h, the MFI differences were calculated, and compared using a one-sample t-test. The results showed that the MFI of MHC-II positive cells at 48h was significantly higher than time 0 ($t=5.1$, $p<0.000$).

An LMER demonstrated a significant, negative effect of treatment on the geometric MFI at 48h in the PCV2 (-0.6 ± 0.2 SE, $p<0.05$) and PCV2-Ionomycin/PMA (-0.6 ± 0.2 SE, $p<0.05$)

groups, compared to mock. Pairwise comparisons demonstrated that the MFI of Ionomycin-PMA was significantly higher than MFI of cells in the PCV2 ($p<0.01$) and PCV2-Ionomycin/PMA ($p<0.001$) treated groups (Figure 5-4, Table 5-2).

These results demonstrate that PBMCs were activated after mitogen treatment, and they demonstrate a decrease in MHC-II expression in the PCV2-exposed groups.

5.3.3 *CD25 and MHC-II in PCV2 infected cells*

To determine if PCV2 affected CD25 or MHC-II upregulation, the expression of CD25 or MHC-II was determined in CAP+ population and was compared to the expression in CAP- population using a paired-samples t-test.

A significantly higher expression of CD25 was observed in the CAP+ cells, compared to the CAP- cells of the PCV2-only group ($t=5.0$, $p=0.02$; Figure 5-5A). Likewise, a significantly higher percentage of MHC-II cells was observed in the CAP+ cells, compared to the CAP- cells of the PCV2-only ($t=3.3$, $p=0.02$) and PCV2-Ionomycin/PMA treated groups ($t=4.8$, $p=0.03$; Figure 5-5B). These results demonstrate a high PCV2 infection on activated cells.

5.3.4 *Total PBMC counts after PCV2 infection and mitogen treatment*

To determine the total number of PBMCs in the wells after each treatment and incubation time point, eight 10X images per well were acquired using the automatic microscope INCell Analyzer 2000. Total cells per well were determined based on nuclear staining, and averaged between wells and animals.

The effect of treatment and incubation time on cell count within and between groups was evaluated using an LMER model, with Tukey's post hoc test for pairwise comparisons (Figure 5-6 and Table 5-3). Treatment had no effect on cell count, but incubation time had a significant, positive effect on cell count after 96h (3057.08 ± 1479.61 SE, $p<0.005$), compared to 24h. Pairwise comparisons demonstrated a higher cell count in the ionomycin/PMA, PCV2, and PCV2-ionomycin/PMA treated groups at 96h, compared to mock and PCV2-only groups at 48h ($p<0.05$; Figure 5-6).

5.3.5 *Proliferation index (PIn)*

To determine if proliferation of cells was affected by activation and PCV2 exposure, the PIn of each treatment group was determined using flow cytometry. The effect of treatment and incubation time on proliferation within and between groups was evaluated using an LMER model, with Tukey's post hoc test for pairwise comparisons using the Bonferroni method for p-value adjustment (Figure 5-7, Table 5-4).

Treatment had a significant, positive effect on the PIn in the Ionomycin/PMA (0.81 ± 0.17SE, $p<0.001$), and PCV2-Ionomycin/PMA (1.12 ± 0.17SE, $p<0.001$) groups, compared to mock. Incubation time had no significant effect on the PIn, and a significant variation was observed between individual pigs ($p=0.04$).

Pairwise comparisons demonstrated a significantly higher PIn in the Ionomycin/PMA and PCV2-Ionomycin/PMA treated groups, compared to mock at all incubation time points ($p<0.001$; Figure 5-7). In addition, a higher PIn was observed between mock and Ionomycin-PMA ($p<0.001$), Ionomycin/PMA and PCV2 ($p<0.001$), and between PCV2 and PCV2-

Ionomycin/PMA ($p<0.001$) at 72h (Figure 5-7). Ionomycin-PMA, PCV2, and PCV2-Ionomycin/PMA had higher PIns at 72h compared to the same groups at 48h ($p<0.05$; Figure 5-7).

5.3.6 *Proliferation index in PCV2-CAP+ cells*

To determine if PBMC proliferation was affected by PCV2 infection, the PIn of CAP+ cells were compared to the PIn of CAP- cells of the PCV2-Ionomycin/PMA treatment group using an independent samples t-test.

Results demonstrate a significantly lower PIn in CAP+ cells compared to CAP- cells of the PCV2-Ionomycin/PMA group at 72h ($t=3.16$, $p<0.05$; Figure 5-8), but no significant difference at 48h (Figure 5-8).

5.3.7 *Cell cycle analysis on activated and infected PBMCs*

The percentage of cells in G0-G1, S, and G2-M were determined using DNA content and analyzed using flow cytometry. The effect of treatment and incubation time on the percentage of cells on each phase was analyzed using an LMER, with Tukey's post hoc test for pairwise comparisons.

Treatment had a negative effect on the percentage of cells in G0-G1 in the ionomycin/PMA (-18.44% ± 4.49 SE, $p<0.001$), and PCV2-Ionomycin/PMA treatment (-18.88% ± 4.54 SE, $p<0.001$) groups, compared to mock. Pairwise comparisons demonstrated a significantly lower percentage of cells in G0-G1 phase in the Ionomycin/PMA, and PCV2-Ionomycin/PMA treatment groups, compared to mock, at all incubation time points ($p<0.05$, Figure 5-9). Incubation time also had a negative effect on the percentage of cells in G0-G1 (-

26.19% ± 8.82 SE, $p<0.01$), compared to time 0, and no significant variation was observed between individual pigs ($p=0.09$).

Treatment had a positive effect on the percentage of cells on S phase in the Ionomycin/PMA (15.70% ± 4.63 SE, $p<0.01$) and PCV2-Ionomycin/PMA (18.84% ± 4.68 SE, $p<0.001$) groups, compared to mock. Pairwise comparisons demonstrated a higher percentage of cells in S phase in the PCV2-Ionomycin PMA treated group, compared to mock at all incubation time points ($p<0.05$, Figure 5-10). Incubation time had a positive effect on the percentage of cells in S phase (19.40% ± 9.05 SE, $p<0.01$), and no significant variation was observed between individual pigs ($p=0.6$).

Incubation time and treatment had no effect on the percentage of cells in G2-M phase (Figure 5-11).

5.3.8 *BrdU incorporation in activated PBMCs*

DNA synthesis after activation and PCV2 exposure was determined by the incorporation of BrdU in PBMCs. The effect of treatment and incubation time on the percentage of BrdU positive cells was determined using an LMER, with Tukey's post hoc test for pairwise comparisons.

Treatment had a positive effect on the percentage of BrdU+ cells in the ionomycin/PMA (15.39% ± 4.2SE, $p<0.001$), and PCV2-Ionomycin/PMA treatment (25.97% ± 4.2SE, p0.001) groups, compared to mock (Figure 5-12, Table 5-8). Pairwise comparisons demonstrated a higher percentage of BrdU positive cells in PCV2-Ionomycin/PMA treated groups at all incubation time points, compared to mock and PCV2-only ($p<0.001$; Figure 5-12). Incubation

time had a positive effect at 48h (8.98% ± 4.08SE, $p<0.05$) compared to 12h, while no variation was observed between individual pigs ($p=0.6$).

5.3.9 *BrdU incorporation and PCV2 infection*

To determine if there was a difference of DNA synthesis between infected and non-infected cells, the percentage of BrdU positive cells were compared between CAP+ and CAP- cells at 48h and 72h using a paired samples t-test (Figure 5-13).

A significantly lower incorporation of BrdU was observed in CAP+ cells compared to CAP- cells of the PCV2-only group at 48h (-9.4% ± 1.4SE, $t=-6.6$, $p<0.05$), and 72h (-8.6% ± 0.86, $t=-10.0$, $p<0.05$) (Figure 5-13). No significant difference was determined between the mean percentage of BrdU+ cells in the CAP+ and CAP- cells of the PCV2-Ionomycin/PMA treated cells.

5.4 Discussion

PCV2 has a very limited coding capacity that relies on the host enzymes for replication, and a cell cycle arrest in G0/G1 and S has been suggested for viral replication[156]. Clinical observations have demonstrated that an activation of the immune system leads to disease in pigs[62], suggesting that increased mitosis rates could aid in PCV2 pathogenesis. This study investigated the effect of PCV2 infection on activation and proliferation of mitogen-treated swine PBMCs *in vitro*.

Activation of swine PBMCs after ionomycin/PMA treatment was confirmed by the upregulation of the low affinity chain of the IL-2 receptor (IL-2Rα or CD25) and MHCII. CD25 is upregulated in the presence of co-stimulatory factors such as a soluble IL-2, which activates a

transduction signal that leads to naïve T cell proliferation[165]. CD25 is upregulated in activated B and T lymphocytes[181], and is expressed in regulatory T cells. In swine, MHC-II is expressed in antigen presenting cells as monocytes, macrophages, B cells, dendritic cells, and in CD8+ T cells and NK cells[182]. Our results showed that treatment with ionomycin/PMA increased the expression of CD25 and MHC-II in PBMCs, demonstrating activation of cells 48h after stimulation with the mitogens. For cells exposed to PCV2, an increased percentage of CD25+ PBMCs were measured in the PCV2-Ionomycin/PMA treated group, compared to controls. In addition, approximately 40-60% of PCV2-CAP positive cells in the PCV2-only and PCV2-Ionomycin/PMA treated groups presented an upregulation of CD25 or MHC-II, suggesting a preference of PCV2 for activated cells. These results are in line with Grierson *et al* (2009), who reported an increased expression of CD25 in circulating T cells of pigs with PMWS[183], supporting the hypothesis that an activation of the immune system is required for high PCV2 replication[62].

In this study, the effect of PCV2 infection on cell proliferation was assessed by measuring new DNA synthesis, cell cycle progression, and proliferation analysis. Our results demonstrate new DNA synthesis in cells of the PCV2-Ionomycin/PMA treated group, by the increase in BrdU incorporation starting after 12h of incubation. In addition, an increased percentage of cells in S phase was observed in the PCV2-Ionomycin/PMA treated group, compared to mock, starting after 24h of incubation.

An S phase dependency for PCV replication was first suggested by Tischer *et al* (1987), who reported that the quantity of viral DNA increased before the first round of mitosis in PK-15 cells[156]. A study demonstrated that the S phase arrest in PK-15 cells depends on Cyclin A

downregulation and p53 activation, resulting in increased PCV2 replication[176]. Although no significant increase in the percentage of cells in S phase was observed in the PCV2-Ionomycin/PMA treated cells compared to Ionomycin/PMA treated controls, results suggest that a stimulation of mitotic activity in the cells could aid PCV2 replication[176]. Future studies should assess if there is an S phase dependency for PCV2 infection in PBMCs by determining the cell cycle phases of PCV2-infected cells.

Regarding the mitotic activity of the cells, an increase in the proliferation index and the total cell count in non-infected cells treated with ionomycin/PMA was observed after 72h and 96h, respectively, similar to what has been reported in swine PBMCs after Con A and PWM stimulation[161]. In PCV2-exposed PBMCs, no difference was seen between the proliferation of PCV2-exposed cells and controls when the whole population of cells was analyzed. Interestingly, when PCV2-infected cells specifically were assessed, a lower PIn was observed in the CAP+ cells after 72h of incubation, suggesting that PCV2 infection decreases the mitotic rate of PBMCs. Our results also demonstrated a decrease in the MFI of MHCII+ cells of the PCV2-only and PCV2-Ionomycin/PMA treated groups, suggesting a dysregulation on antigen presentation on cells after exposure to PCV2[179].

PMWS is characterized by immunosuppression[184], and a disruption of MHC-II presentation by antigen presenting cells could decrease interactions with CD4+ T cells, resulting in impaired immune cell activation and proliferation[178]. A downregulation of MHC-II has been reported in lymph node tissues of PMWS-affected pigs with moderate lymphoid depletion, specifically in DCs and lymphocytes in follicular areas, and to a lesser extent in macrophages of the interfollicular area[179]. A decrease in cell proliferation was also reported in lymphoid tissues

of pig in early stages of PMWS[178], and an unresponsiveness to stimulation in PBMCs isolated from PMWS and PCV2 sub-clinically infected pigs[102], but this had not been demonstrated in *in vitro* experiments using PBMCs until now. The dysregulation in antigen presentation and cell proliferation could be caused by the secretion of immune regulating cytokines (e.g. IL-2, IL-6, IL-10)[102] into the supernatant, and their presence should be assessed in future studies.

In conclusion, the current study demonstrates possible mechanisms of immunomodulation used by PCV2 that could aid in viral persistence and increased replication in PBMCs. Downregulation of MHC-II expression, decreased cell proliferation, and a possible S phase arrest in swine PBMCs after mitogen exposure and PCV2 infection, demonstrates how PCV2 replication could affect normal cellular pathways for its own benefit.

Figure 5-1 Identification of swine CD25 receptor in PBMCs after four different treatments. Results are the average of two independent experiments, using five pigs (pig #: 2, 3, 4, 5, and 7). Error bars equal to mean ± SD, and significance is shown as asterisks (***p<0.001).

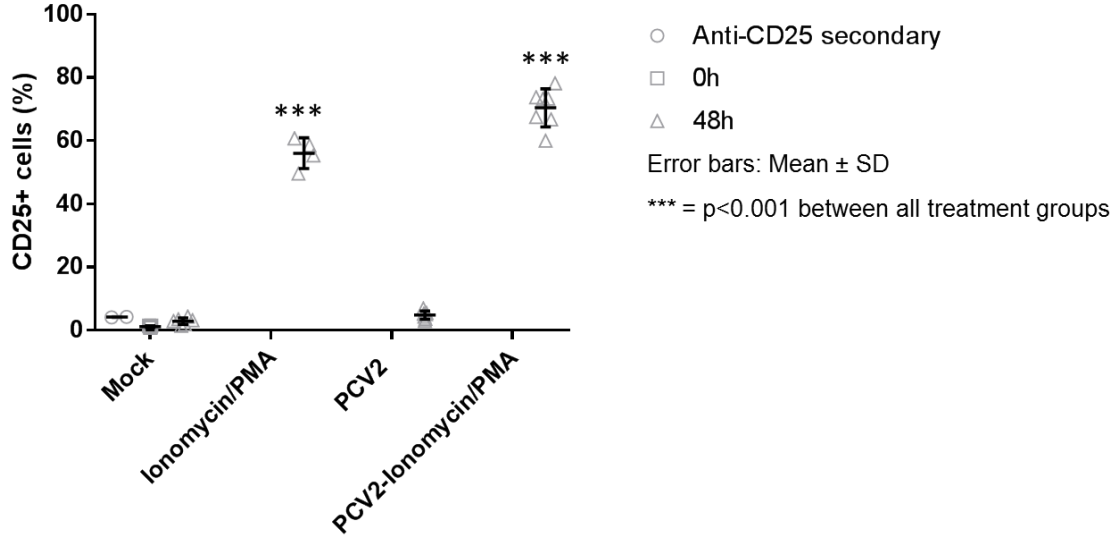


Figure 5-2 Geometric mean fluorescence intensity (MFI) of CD25+ PBMCs after four different treatments. The MFI at baseline (0h) was transformed to 1, and the values normalized for 48h; the graph is constructed based on the results of the LMER model. Error bars equal to mean ± 95% CI, and significance is shown as asterisks between groups (*p<0.05, ***p<0.001).

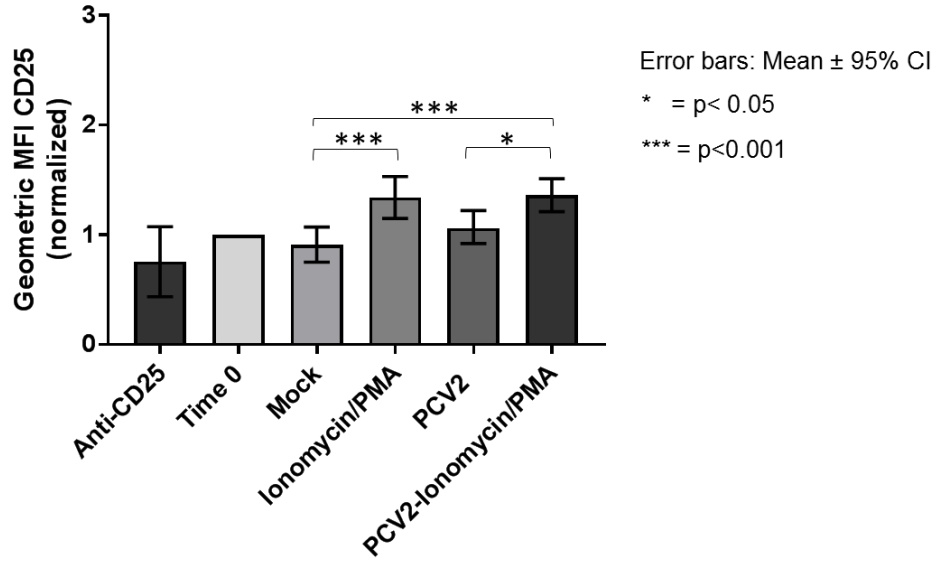


Table 5-1 Percentage and geometric mean fluorescence intensity (MFI) of CD25+ PBMCs after 48h of incubation. Distribution (mean ± SD) of raw data, and parameter estimates of the LMER model (95% CI) are shown; negative values were truncated to 0.

	CD25 + cells (%)		MFI CD25 + cells (norm.)	
Treatment	Mean ± SD	Estimate (95%CI)	Mean ± SD	Estimate (95%CI)
Mock	2.91 ± 1.06	2.94 (0 - 6.27)	1.90 ± 0.63	0.91 (0.75 - 1.07)
Ionomycin/PMA	56.08 ± 4.84	56.16 (52.10 - 60.23)	2.06 ± 0.75	1.34 (1.15 - 1.53)
PCV2	4.85 ± 1.31	4.85 (1.77 - 7.93)	1.25 ± 0.18	1.06 (0.92 - 1.22)
PCV2-Ionomycin/PMA	70.43 ± 6.05	70.43 (67.35 - 73.51)	1.23 ± 0.13	1.36 (1.21 - 1.51)

Figure 5-3 Identification of swine MHC-II receptor in PBMCs after four different treatments. Results are the average of two independent experiments, using seven pigs (pig #: 2, 3, 4, 5, 6, 7, 8). Error bars equal to mean ± SD, and significance is shown as asterisks (***p<0.001, demonstrates significance between indicated treatment groups).

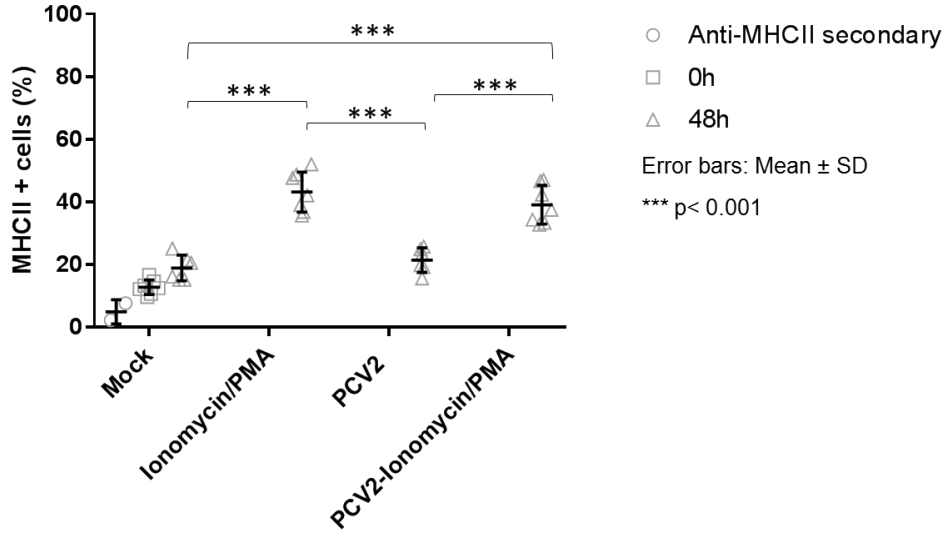


Figure 5-4 Geometric mean fluorescence intensity (MFI) of MHC-II+ PBMCs after four different treatments. The MFI at baseline (0h) was transformed to 1, and the values normalized for 48h; graph is constructed based on the results of the LMER model. Error bars equal to mean ± 95% CI, and significance is shown as asterisks between groups (**p<0.01; ***p<0.001).

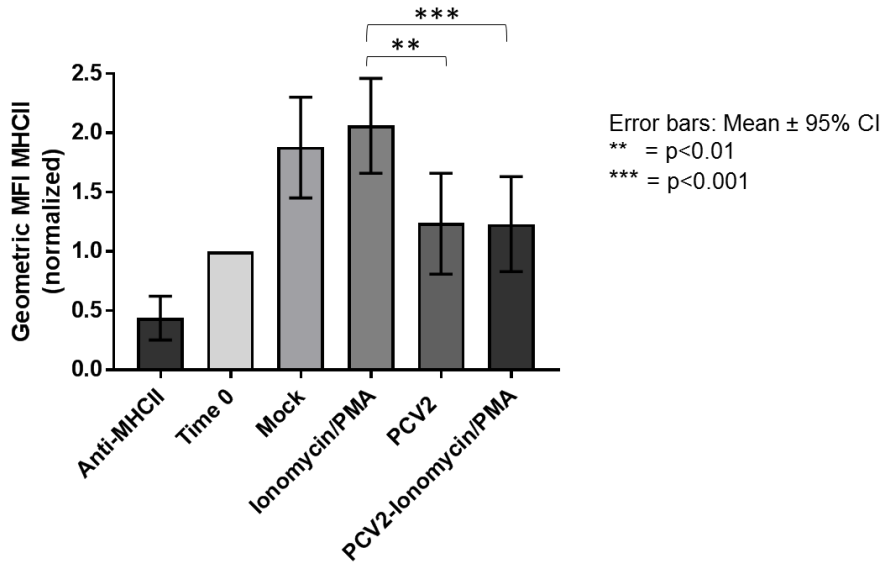


Table 5-2 Percentage and geometric mean fluorescence intensity (MFI) of MHC-II+ cells after 48h of incubation. Distribution (mean ± SD) of raw data, and parameter estimates of the LMER model (95% CI) are shown.

	MHC-II + cells (%)		MFI MHC-II + cells (norm.)	
Treatment	Mean ± SD	Estimate (95%CI)	Mean ± SD	Estimate (95%CI)
Mock	18.97 ± 4.08	18.39 (13.83 - 22.96)	0.91 ± 0.09	1.88 (1.45 - 2.30)
Ionomycin/PMA	43.17 ± 6.38	43.17 (38.81 - 47.53)	1.36 ± 0.39	2.06 (1.66 - 2.46)
PCV2	21.48 ± 3.91	22.00 (17.43 - 26.56)	1.07 ± 0.14	1.24 (0.81 - 1.66)
PCV2-Ionomycin/PMA	39.16 ± 6.18	39.16 (34.79 - 43.52)	1.36 ± 0.14	1.23 (0.83 - 1.63)

Figure 5-5 Percentage of activated cells in PCV2+ or PCV2- populations.

Figure 5-5A Percentage of CD25+ cells in PCV2+ or PCV2- populations. Results are the average of two independent experiments, using seven pigs (pig #: 2, 3, 4, 5, 6, 7, 8). Error bars equal to mean ± SD, and significance is shown as an asterisk between groups (*p<0.05).

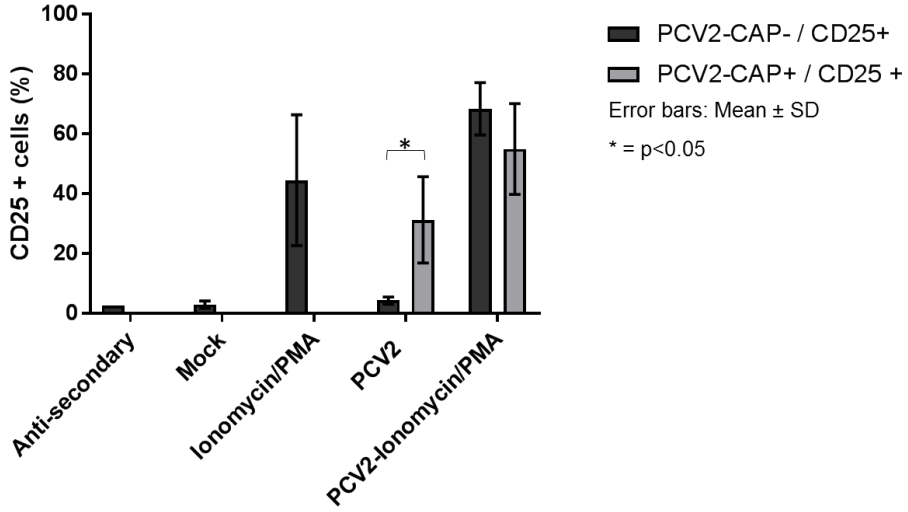


Figure 5-5B Percentage of MHC-II+ cells in PCV2+ or PCV2- populations. Results are the average of two independent experiments, using five pigs (pig #: 2, 3, 4, 5, and 7). Error bars equal to mean ± SD, and significance is shown as an asterisk between groups (*p<0.05).

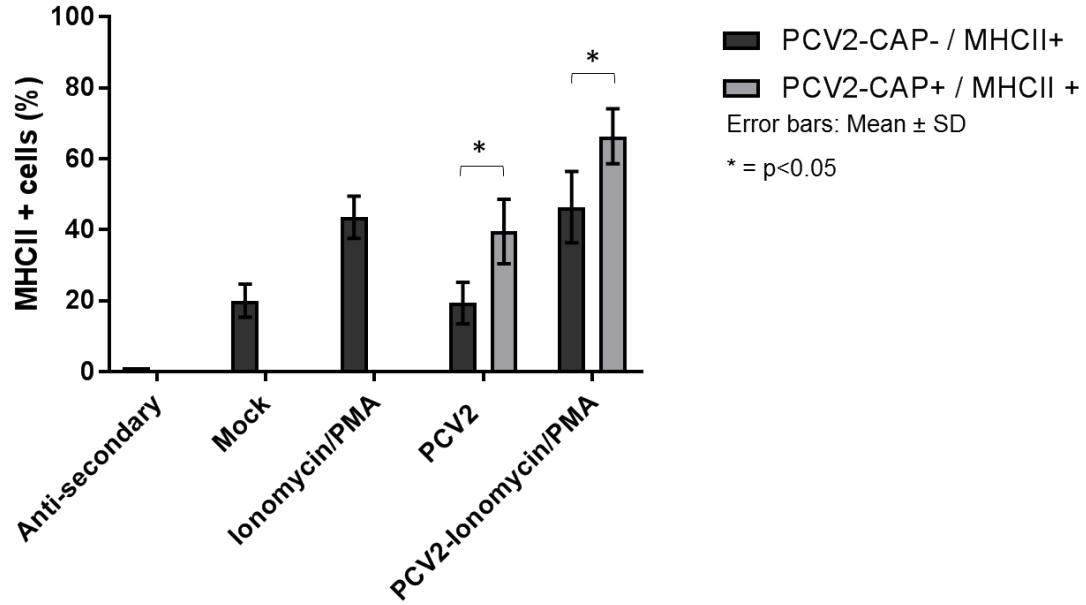


Figure 5-6 Total PBMC count per well of four different treatments after 24h, 48h, 72h, and 96h of incubation. Results are based on the LMER model, with the average of eight 10X images/well of one experiment, using three different pigs (pig #: 5, 6, and 8). Error bars equal to mean ± CI, and significance is shown as an asterisk (*p<0.05).

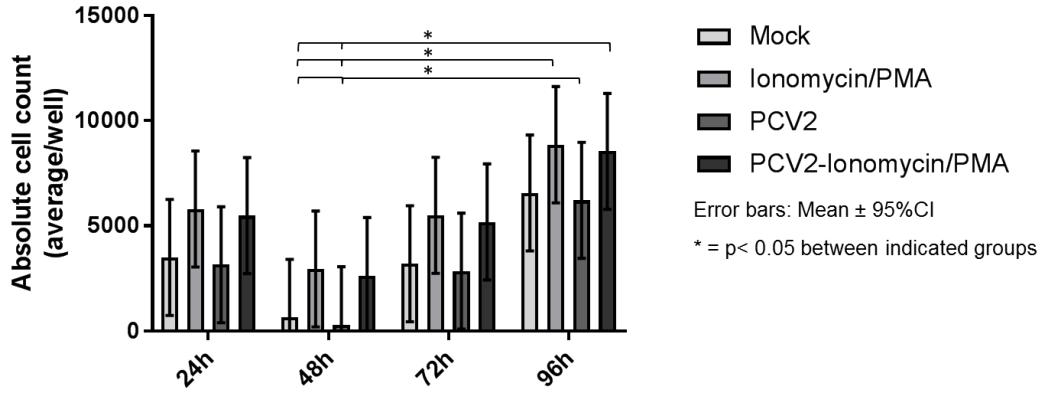


Table 5-3 Absolute cell count (average/well) of three animals using nuclear staining. Results of distribution (mean ± SD) of raw data, and parameter estimates with the 95% CI of the LMER model are shown.

	Absolute cell count (average/well)	
Time / Treatment	Mean ± SD	Estimate (95%CI)
24h Mock	7008.67 ± 3081.26	3501.25 (743.87 - 6258.63)
Ionomycin-PMA	3371.33 ± 583.06	5799.67 (3042.29 - 8557.04)
PCV2	5572.67 ± 2083.02	3152.67 (395.29 - 5910.04)
PCV2-Ionomycin/PMA	1986.33 ± 586.028	5485.417 (2728.04 - 8242.79)
48h Mock	2222.67 ± 725.57	650.25 (0 - 3407.626)
Ionomycin-PMA	1004.33 ± 666.50	2948.67 (191.29 - 5706.043)
PCV2	1641 ± 974.15	301.67 (0 - 3059.04)
PCV2-Ionomycin/PMA	1667 ± 899.98	2634.42 (0 - 5391.79)
72h Mock	3340 ± 1853.33	3200.17 (442.79 - 5957.54)
Ionomycin-PMA	5514 ± 2480.56	5498.58 (2741.21 - 8255.96)
PCV2	3296 ± 1784.39	2851.58 (94.21 - 5608.96)
PCV2-Ionomycin/PMA	4584.67 ± 1983.43	5184.33 (2426.96 - 7941.71)
96h Mock	1338.67 ± 775.78	6558.33 (2426.96 - 7941.71)
Ionomycin-PMA	13214 ± 2594.89	8856.75 (6099.37 - 11614.13)
PCV2	2006 ± 1463.90	6209.75 (3452.37 - 8967.13)
PCV2-Ionomycin/PMA	13608.67 ± 4765.38	8542.5 (5785.12 - 11299.88)

Figure 5-7 Proliferation index in swine PBMCs after mitogen stimulation and PCV2 infection for 3 incubation time points. Results represent the parameter estimates of the LMER model of one (24h, pig #: 6, 7), two (48h, pig #: 5, 6, 7, 8), and three (72h, pig #: 5, 6, 7, 8) independent experiments. Error bars equal to mean ± 95% CI, and significance is shown as asterisks (***p<0.001, *p<0.05).

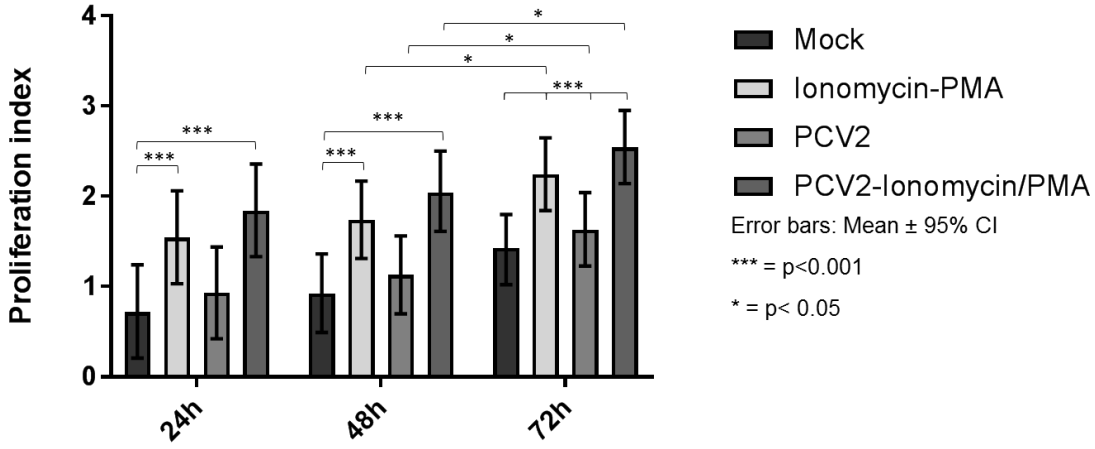


Table 5-4 Proliferation index of PBMCs after four treatments and three incubation time points. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model are shown.

		Proliferation index	
Time/Treatment		Mean ± SD	Estimate (95%CI)
24h	Mock	1.02	0.72 (0.21 - 1.24)
	Ionomycin/PMA	1.03	1.54 (1.03 - 2.06)
	PCV2	1.15 ± 0.01	0.93 (0.42 - 1.44)
	PCV2-Ionomycin/PMA	1.18	1.84 (1.33 - 2.36)
48h	Mock	1.03 ± 0.02	0.92 (0.49 - 1.36)
	Ionomycin/PMA	1.17 ± 0.19	1.74 (1.31 - 2.17)
	PCV2	1.28 ± 0.07	1.13 (0.70 - 1.56)
	PCV2-Ionomycin/PMA	1.52 ± 0.24	2.04 (1.61 - 2.5)
72h	Mock	1.07 ± 0.03	1.43 (1.02 - 1.8)
	Ionomycin/PMA	2.53 ± 0.68	2.24 (1.84 - 2.65)
	PCV2	1.27 ± 0.16	1.63 (1.23 - 2.04)
	PCV2-Ionomycin/PMA	2.83 ± 0.92	2.54 (2.14 - 2.95)

Figure 5-8 Proliferation index of PCV2- and PCV2+ cells, in ionomycin/PMA and PCV2-Ionomycin/PMA treated groups after 48h and 72h. Results represent the average of two independent experiments using four pigs (pig #: 5, 6, 7, and 8). Error bars equal to mean ± SD, and significance is shown as an asterisk (*p<0.05).

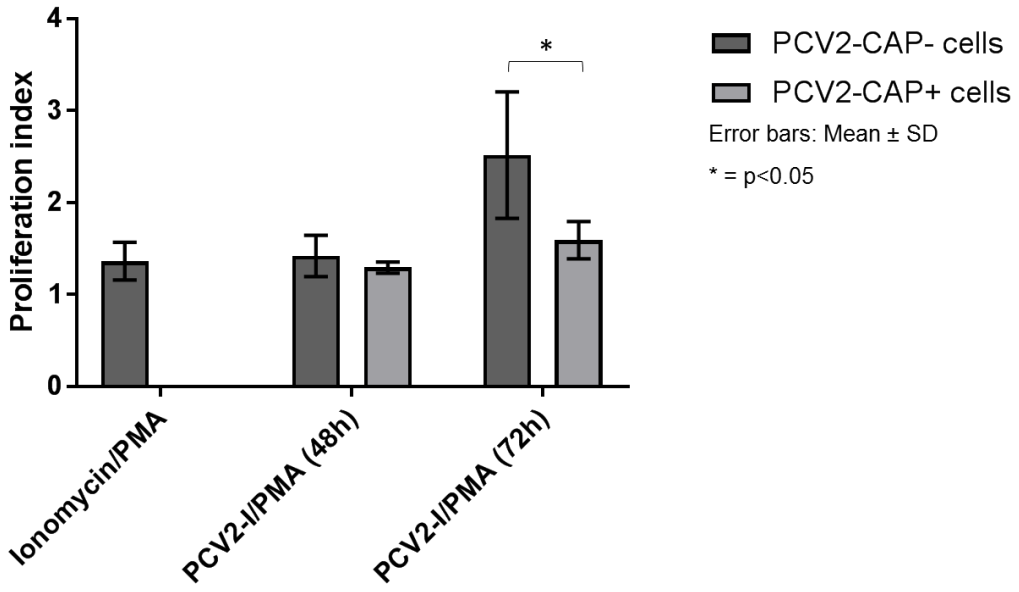


Figure 5-9 Gated population (%) of PBMCs in G0-G1 phase after four different treatments and four incubation time points. Results represent the parameter estimates of the LMER model of two (12h and 24h, pig #: 2, 4, 5, 6), and four (48h and 72h, pig #: 2, 4, 5, 6, 8) independent experiments. Error bars equal to mean ± 95% CI, and significance is shown as an asterisk (*p<0.05).

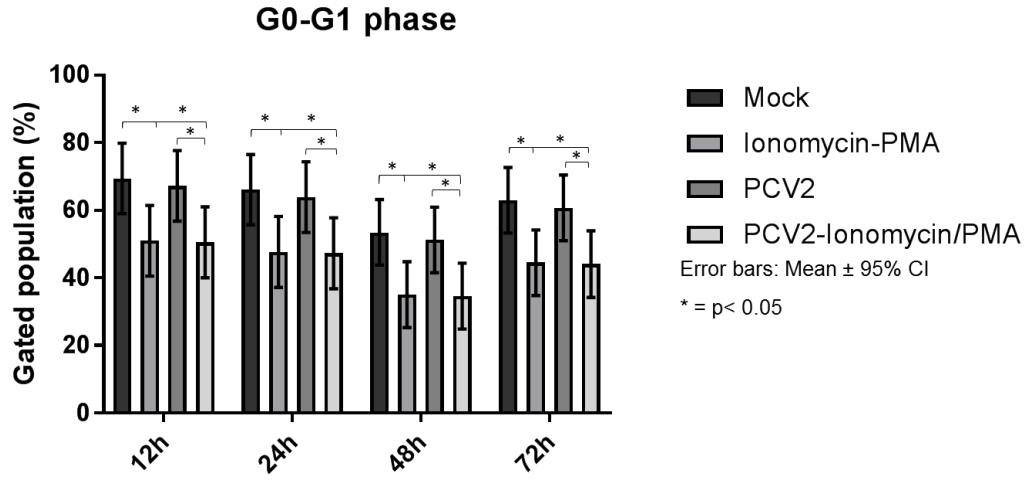


Table 5-5 Percentage of PBMCs in G0-G1 phase after four treatments and four incubation time points. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model are shown.

		G0-G1 phase (%)	
	Treatment	Mean ± SD	Estimate (95%CI)
12h	Mock	56.20 ± 15.69	69.44 (59.03- 79.86)
	Ionomycin/PMA	61.72 ± 11.51	51.01 (40.54 - 61.48)
	PCV2	61.72 ± 15.36	67.22 (56.75 - 77.69)
	PCV2-Ionomycin/PMA	57.59 ± 16.23	50.57 (40.06 - 61.07)
24h	Mock	56.45 ± 25.00	66.13 (55.72 - 76.55)
	Ionomycin/PMA	50.54 ± 25.80	47.70 (37.23 - 58.17)
	PCV2	59.80 ± 14.55	63.91 (53.44 - 74.38)
	PCV2-Ionomycin/PMA	57.19 ± 16.29	47.26 (36.76 - 57.76)
48h	Mock	62.40 ± 8.68	53.48 (43.78 - 63.17)
	Ionomycin/PMA	27.54 ± 14.34	35.04 (25.32 - 44.76)
	PCV2	55.95 ± 11.13	51.25 (41.53 - 60.97)
	PCV2-Ionomycin/PMA	28.79 ± 9.56	34.60 (24.85 - 44.35)
72h	Mock	70.45 ± 17.75	62.96 (53.26 - 72.67)
	Ionomycin/PMA	42.14 ± 17.73	44.53 (34.81 - 54.24)
	PCV2	62.71 ± 8.41	60.74 (51.03 - 70.45)
	PCV2-Ionomycin/PMA	36.49 ± 13.09	44.09 (34.20 - 53.97)

Figure 5-10 Gated population (%) of PBMCs in S phase after four different treatments and four incubation time points. Results represent the parameter estimates of the LMER model of two (12h and 24h, pig #: 2, 4, 5, 6), and four (48h and 72h, pig #: 2, 4, 5, 6, 8) independent experiments. Error bars equal to mean ± 95% CI, and significance is shown as an asterisk.

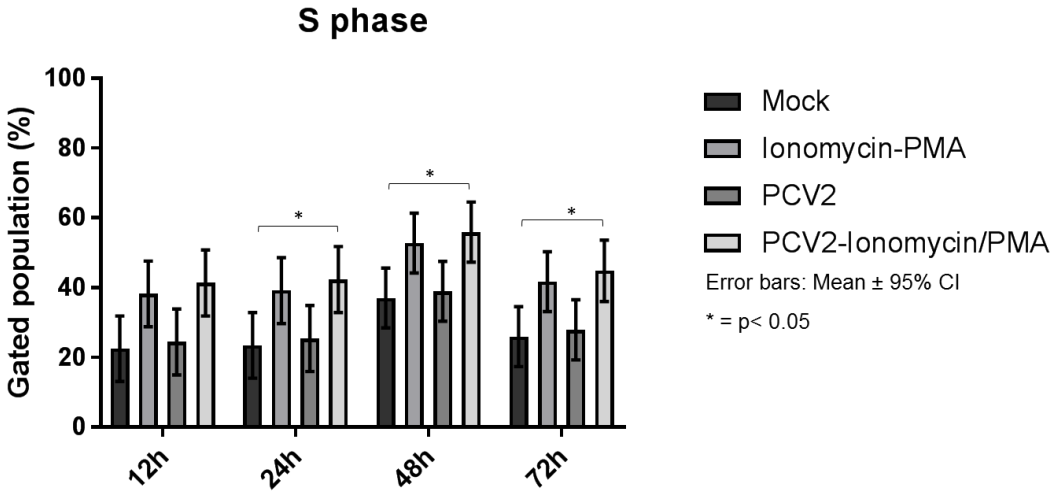


Table 5-6 Percentage of PBMCs in S phase after four treatments and four incubation time points. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model are shown.

		S phase (%)	
	Treatment	Mean ± SD	Estimate (95%CI)
12h	Mock	33.63 ± 9.64	22.50 (13.09 - 31.91)
	Ionomycin/PMA	30.69 ± 12.59	38.20 (28.77 - 47.64)
	PCV2	29.22 ± 17.55	24.42 (14.99 - 33.86)
	PCV2-Ionomycin/PMA	32.62 ± 20.16	41.35 (31.86 - 50.83)
24h	Mock	32.23 ± 20.31	23.48 (14.06 - 32.89)
	Ionomycin/PMA	38.41 ± 20.51	39.18 (29.74 - 48.62)
	PCV2	29.40 ± 13.90	25.40 (15.96 - 34.84)
	PCV2-Ionomycin/PMA	30.03 ± 13.69	42.32 (32.84 - 51.81)
48h	Mock	26.83 ± 13.38	37.06 (28.49 - 45.62)
	Ionomycin/PMA	60.69 ± 17.52	52.76 (44.18 - 61.34)
	PCV2	33.9 ± 14.21	38.98 (30.40 - 47.56)
	PCV2-Ionomycin/PMA	63.18 ± 11.07	55.90 (47.28 - 64.53)
72h	Mock	21.63 ± 12.43	25.99 (17.40 - 34.58)
	Ionomycin/PMA	39.52 ± 22.38	41.69 (33.10 - 50.29)
	PCV2	26.55 ± 8.84	27.91 (19.32 - 36.51)
	PCV2-Ionomycin/PMA	53.53 ± 11.62	44.84 (36.01 - 53.66)

Figure 5-11 Gated population (%) of PBMCs in G2-M phase after four different treatments and four incubation time points. Results represent the parameter estimates of the LMER model of two (12h and 24h, pig #: 2, 4, 5, 6), and four (48h and 72h, pig #: 2, 4, 5, 6, 8) independent experiments. Error bars equal to mean ± 95% CI.

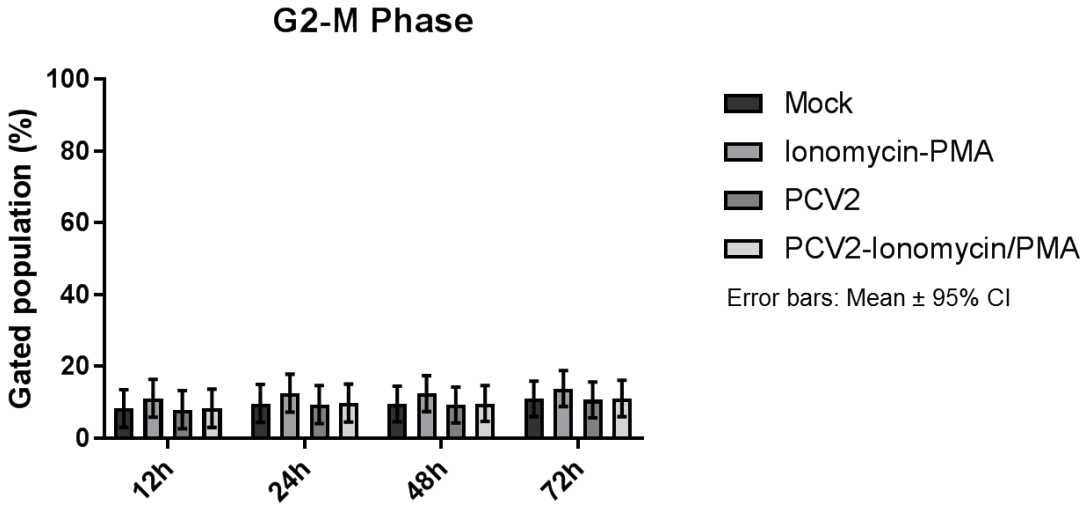


Table 5-7 Percentage of PBMCs in G2/M phase after four treatments and four incubation time points. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the LMER model are shown.

		G2-M phase (%)	
	Treatment	Mean ± SD	Estimate (95%CI)
12h	Mock	10.17 ± 9.30	8.28 (3.01 - 13.55)
	Ionomycin/PMA	7.59 ± 5.74	11.14 (5.85 - 16.44)
	PCV2	9.06 ± 5.89	7.97 (2.68 - 13.26)
	PCV2-Ionomycin/PMA	9.79 ± 5.27	8.39 (3.08 - 13.70)
24h	Mock	11.32 ± 11.52	9.73 (4.46 - 15.0)
	Ionomycin/PMA	11.05 ± 6.76	12.59 (7.30 - 17.89)
	PCV2	7.26 ± 4.87	9.42 (4.13 - 14.71)
	PCV2-Ionomycin/PMA	12.79 ± 8.39	9.84 (4.53 - 15.14)
48h	Mock	10.77 ± 8.09	9.60 (4.63 - 14.56)
	Ionomycin/PMA	11.78 ± 7.73	12.46 (7.48 - 17.44)
	PCV2	10.15 ± 7.33	9.29 (4.31 - 14.27)
	PCV2-Ionomycin/PMA	8.04 ± 4.87	9.71 (4.72 - 14.69)
72h	Mock	7.92 ± 6.31	11.01 (6.04 - 15.99)
	Ionomycin/PMA	18.34 ± 15.24	13.88 (8.90 - 18.85)
	PCV2	10.75 ± 7.10	10.71 (5.73 - 15.68)
	PCV2-Ionomycin/PMA	9.99 ± 4.53	11.12 (6.08 - 16.17)

Figure 5-12 BrdU positive cells (%) after four different treatments and four incubation time points. Results represent the parameter estimates of the LMER model of two (12h and 24h, pig #: 2, 4, 5, 6), and four (48h and 72h, pig #: 2, 4, 5, 6, 8) independent experiments. Error bars equal to mean ± 95% CI and significance is shown as asterisks (***p<0.001).

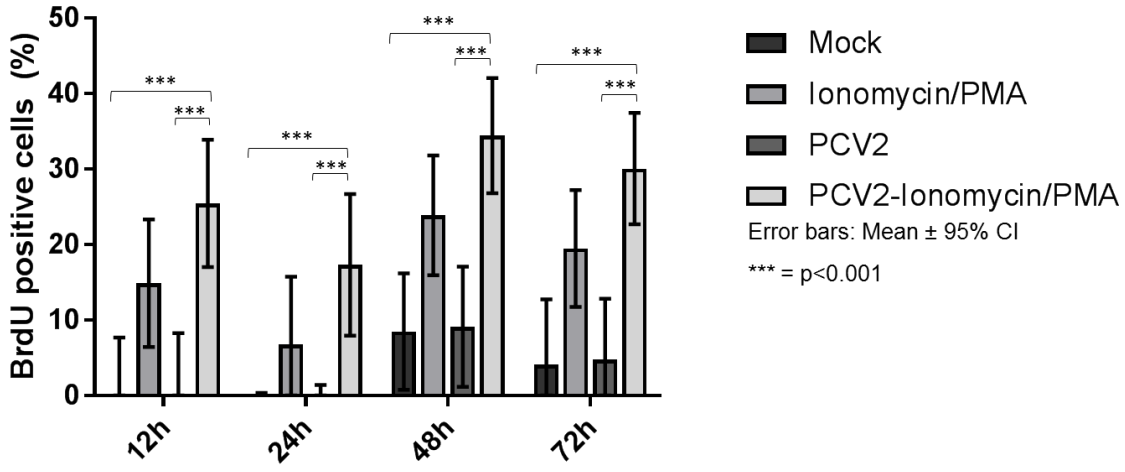
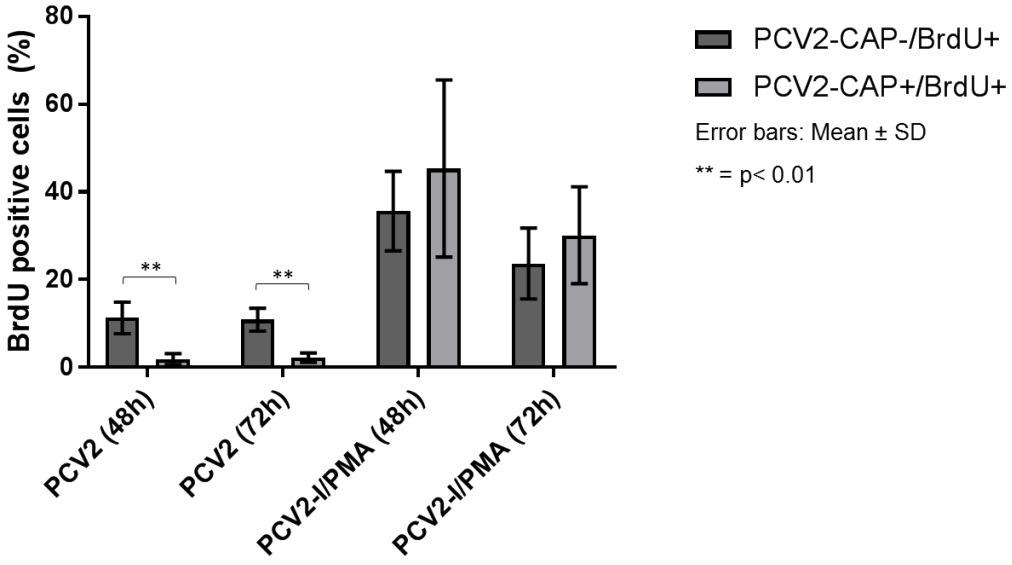


Table 5-8 Percentage of BrdU positive cells in PBMCs. Distribution (mean ± SD) of raw data, and parameter estimates (95% CI) of the model are shown.

		BrdU positive cells (%)	
	Treatment	Mean ± SD	Estimate (95%CI)
12h	Mock	5.65 ± 4.07	0 (0 - 7.69)
	Ionomycin/PMA	10.28 ± 3.65	14.87 (6.43 - 23.31)
	PCV2	5.13 ± 3.66	0.12 (0 - 8.29)
	PCV2-Ionomycin/PMA	15.4 ± 3.25	25.45 (17.01 - 33.89)
24h	Mock	0.21 ± 0.18	0 (0 - 0.36)
	Ionomycin/PMA	1.18 ± 0.79	6.74 (0 - 15.72)
	PCV2	0.40 ± 0.30	0 (0 - 1.38)
	PCV2-Ionomycin/PMA	4.17 ± 1.63	17.32 (7.95 - 26.68)
48h	Mock	0.19 ± 0.10	8.47 (0.75 - 16.19)
	Ionomycin/PMA	27.91 ± 18.76	23.86 (15.92 - 31.80)
	PCV2	1.89 ± 1.27	9.11 (1.16 - 17.07)
	PCV2-Ionomycin/PMA	45.32 ± 20.17	34.44 (26.80 - 42.07)
72h	Mock	0.7 ± 0.01	4.10 (0 - 12.72)
	Ionomycin/PMA	22.17 ± 13.20	19.49 (11.74 - 27.24)
	PCV2	2.25 ± 1.07	4.75 (0 - 12.82)
	PCV2-Ionomycin/PMA	30.12 ± 11.04	30.07 (22.69 - 37.44)

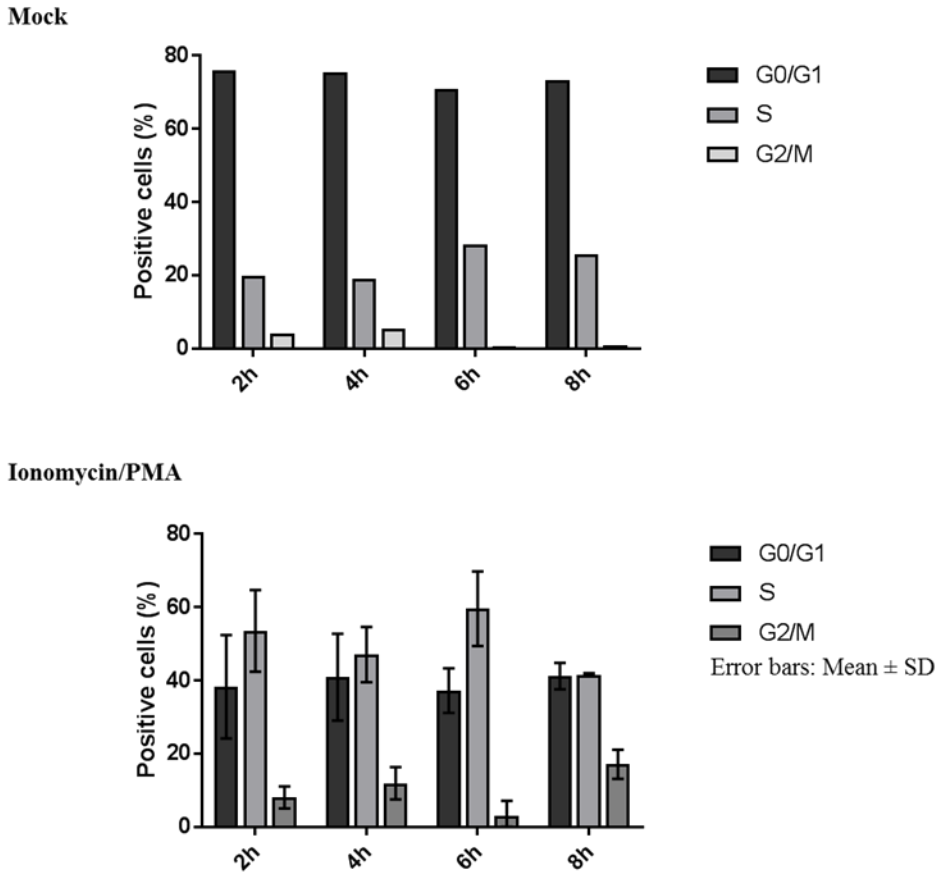
Figure 5-13 BrdU positive cells (%) in PCV2-CAP+ or PCV2-CAP- cells after PCV2 and PCV2-Ionomycin/PMA treatment at 48h and 72h. Results represent the average of five independent experiments using five pigs (pig#: 2, 4, 5, 6, 8). Error bars equal to mean ± SD and significance is shown as asterisks (**p<0.01).



5.5 Appendix

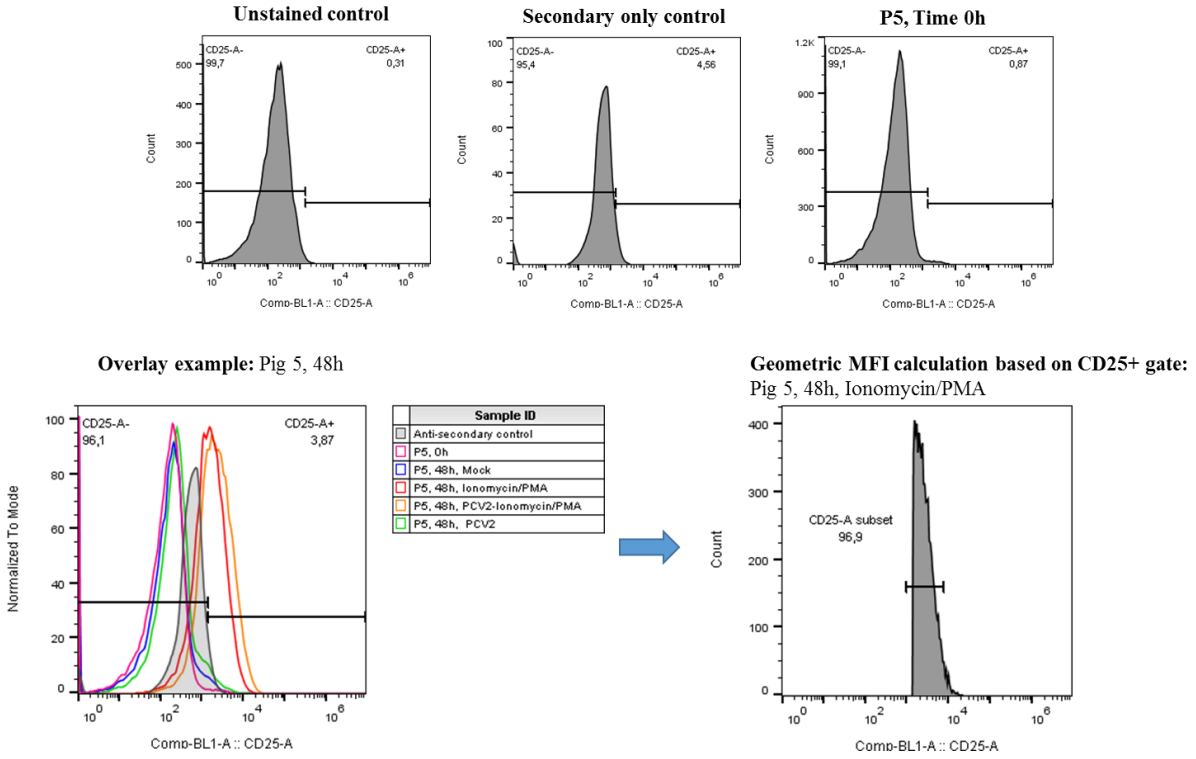
5.5.1 *Optimization of cell cycle analysis*

PBMCs were Mock or Ionomycin/PMA treated (1µg/mL and 1ng/mL, respectively) for half an hour, washed and incubated for 24h. After the first 24h and every 2h afterwards, PBMCs were taken out of the incubator and treated with BrdU (BD Pharmigen, cat #559619) following the protocol described in Section 5.2.5; the cells were analyzed using flow cytometry. The graphs represent the result of one experiment, using PBMCs isolated from three PCV2-free pigs (pig #: 2, 3, 4).



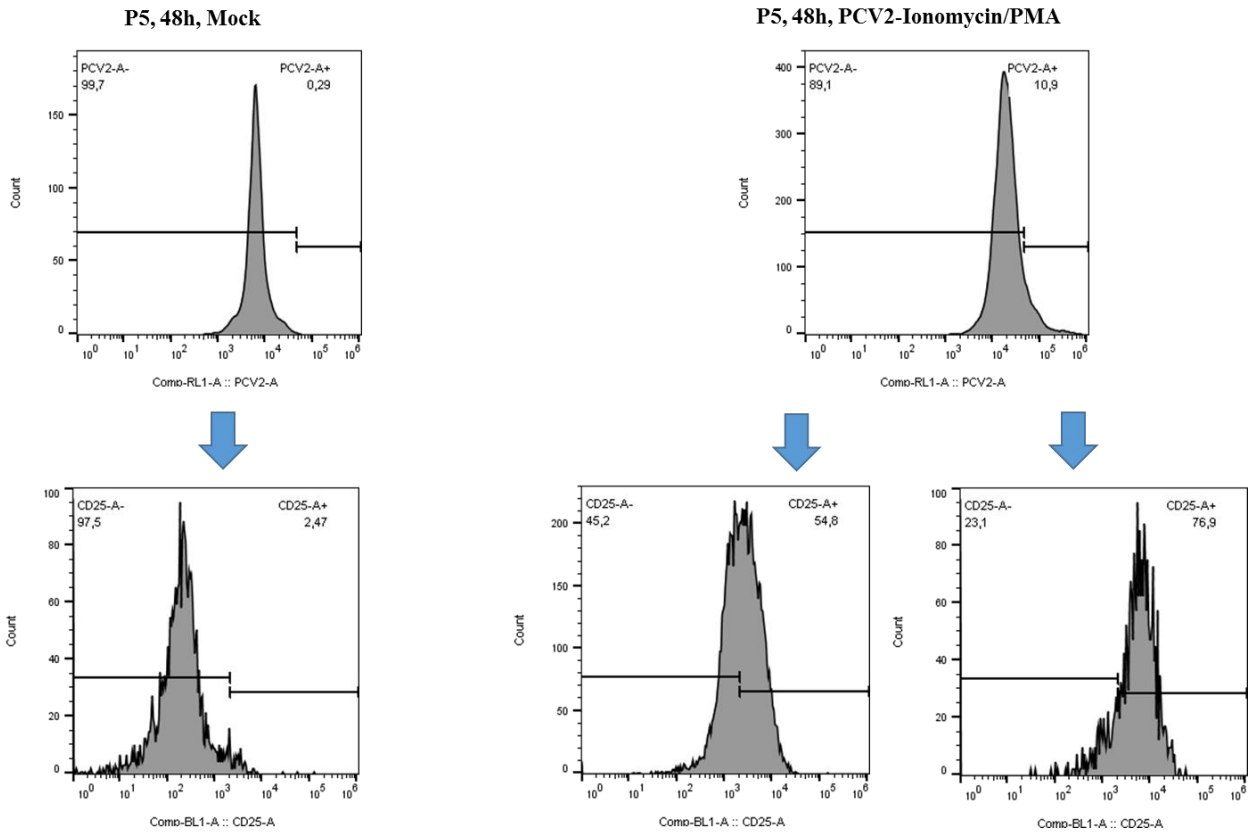
5.5.2 *Gating strategy to determine the CD25+ cells using anti- swine CD25 antibody and the secondary goat anti-mouse IgG-FITC*

Cell debris was gated-out, followed by doublet exclusion in FSC and SSC channels (not shown). Control samples were used to establish a two-parameter histogram and distinguish CD25+ from CD25- cells. The geometric mean fluorescence intensity was determined for the CD25+ population.



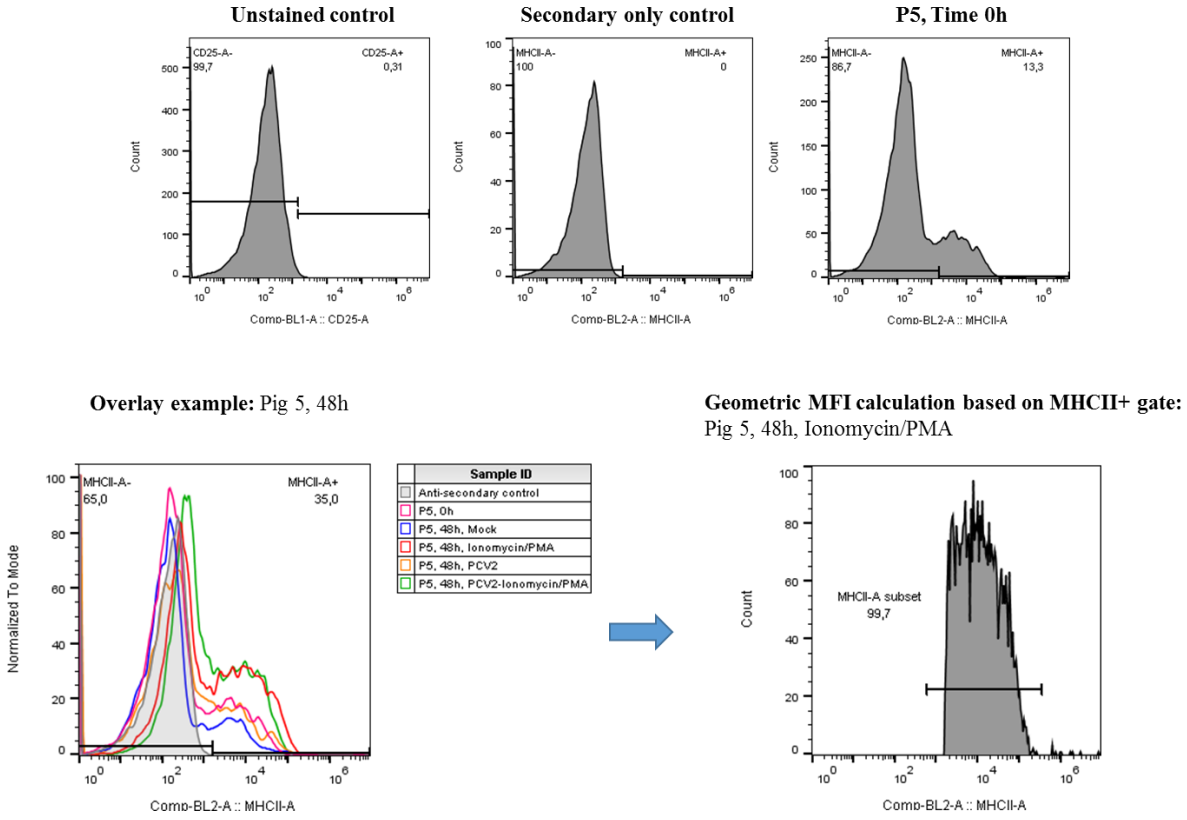
5.5.3 *Gating strategy to determine CD25+/PCV2+ or CD25+/PCV2- cells*

Cell debris was gated-out, followed by doublet exclusion in FSC and SSC channels (not shown). Control samples were used to establish a two-parameter histogram and distinguish PCV2+ from PCV2- cells. The percentage of CD25+ cells was then calculated in a second two-parameter histogram derived form the PCV2+ or PCV2- cells.



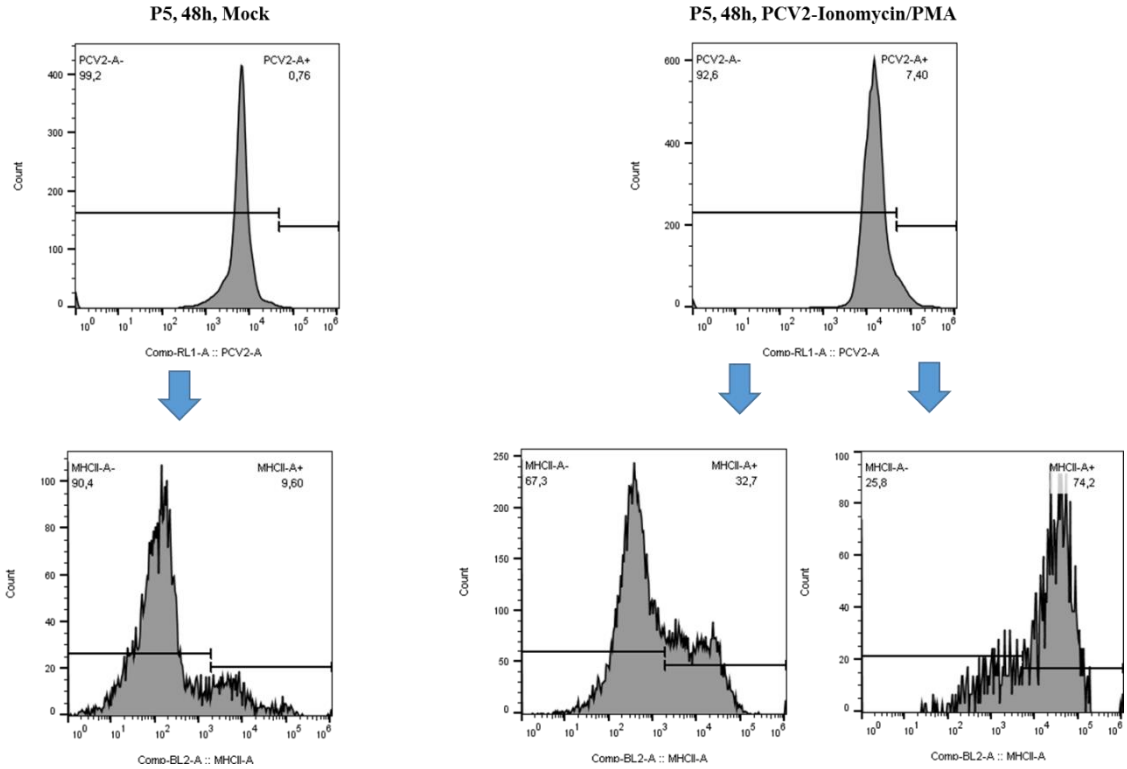
5.5.4 *Gating strategy to determine MHC-II+ cells using a mouse anti-swine MHC-II antibody and the secondary goat anti-mouse IgG2a-PE*

Cell debris was gated-out, followed by doublet exclusion in FSC and SSC channels (not shown). Control samples were used to establish a two-parameter histogram and distinguish MHC-II+ from MHCII- cells. The geometric mean fluorescence intensity was determined for the MHC-II+ population.



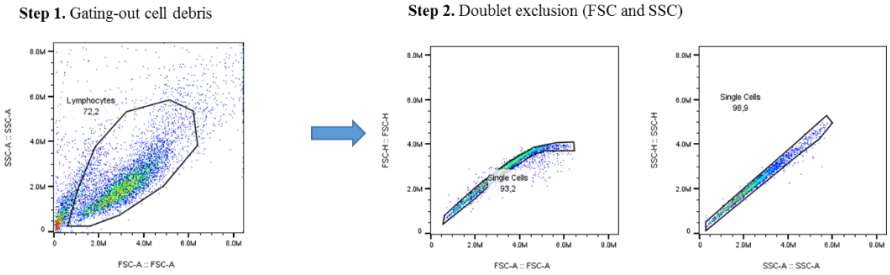
5.5.5 *Gating strategy to determine MHC-II+/PCV2+ or MHC-II+/PCV2- cells*

Cell debris was gated-out, followed by doublet exclusion in FSC and SSC channels (not shown). Control samples were used to establish a two-parameter histogram and distinguish PCV2+ from PCV2- cells. The percentage of MHC-II+ cells was then calculated in a second two-parameter histogram derived form the PCV2+ or PCV2- cells.

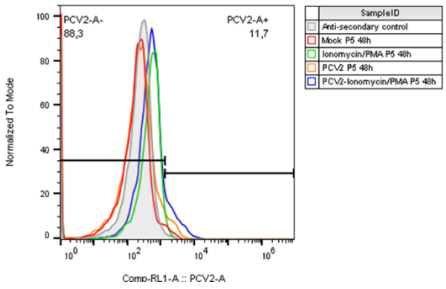


5.5.6 *Gating strategy to determine PCV2+/BrdU+ and PCV2-/BrdU+ cells*

Cell debris was gated-out (step 1), followed by doublet exclusion in FSC and SSC channels (step 2). Control samples were used to establish a two-parameter histogram and distinguish PCV2+ from PCV2- cells (step 3). The percentage of BrdU+ cells was then calculated in a second two-parameter histogram derived form the PCV2+ or PCV2- cells (step 4).

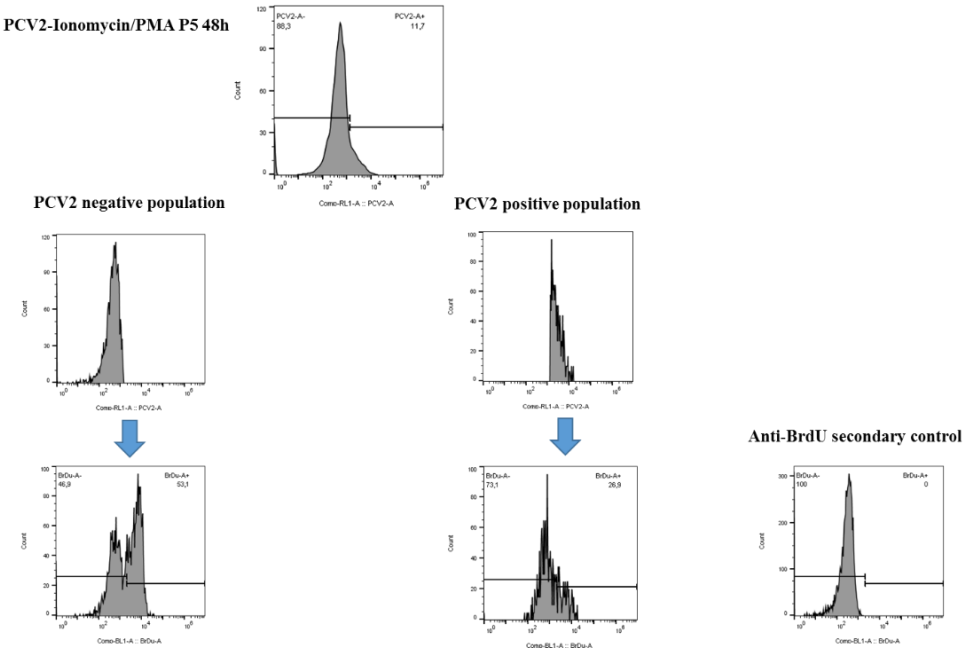


Step 3. Establishment of a two-parameter histogram to distinguish PCV2+ and PCV2- populations (based on control samples).



Overlay example: Pig 5, 48h

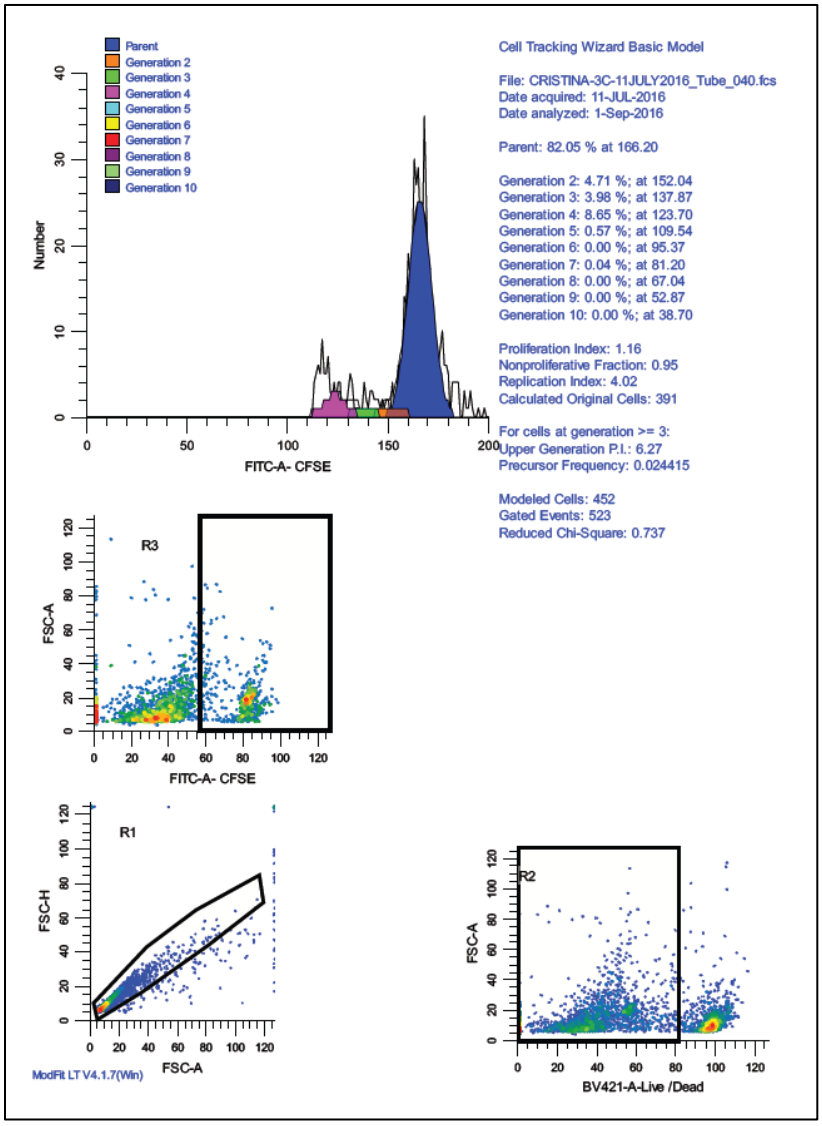
Step 4. Establishment of a two-parameter histogram to distinguish BrdU+ and BrdU- populations (based on control samples).



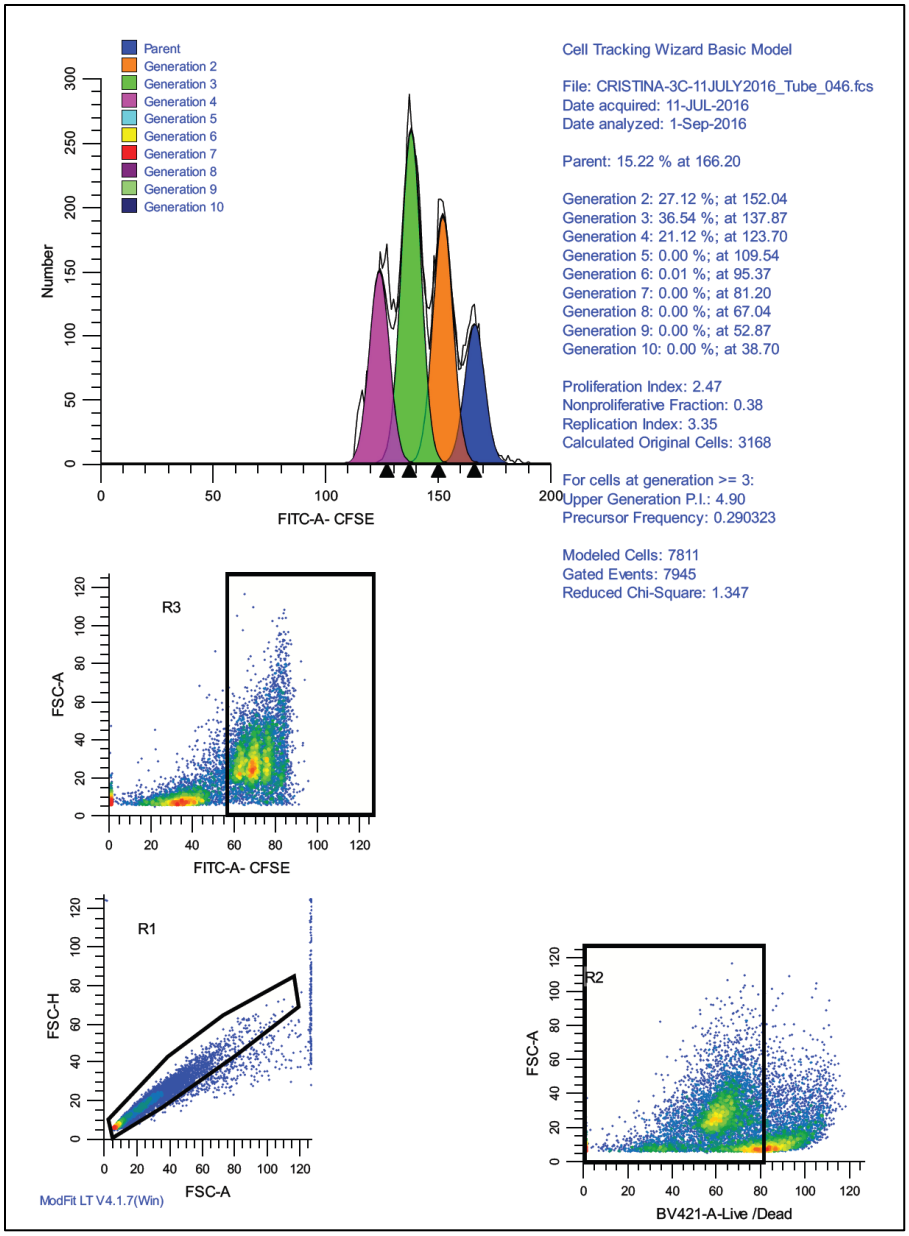
5.5.7 *Analysis report of cell proliferation in CFSE stained cells of Mock (A) and PCV2-Ionomycin-PMA (B) treated samples*

Initial gating was performed to eliminate debris (FSC-A vs FSC-H), followed by gating the live cells (BV-421 Live/Dead vs FSC-A), and a final gate for CFSE positive cells (CFSE-A vs FSC-A). The model was built using the Cell track wizard, with adjustment of the parent population, and determination of the cell generations (ModFit LT, version 4.1).

A.

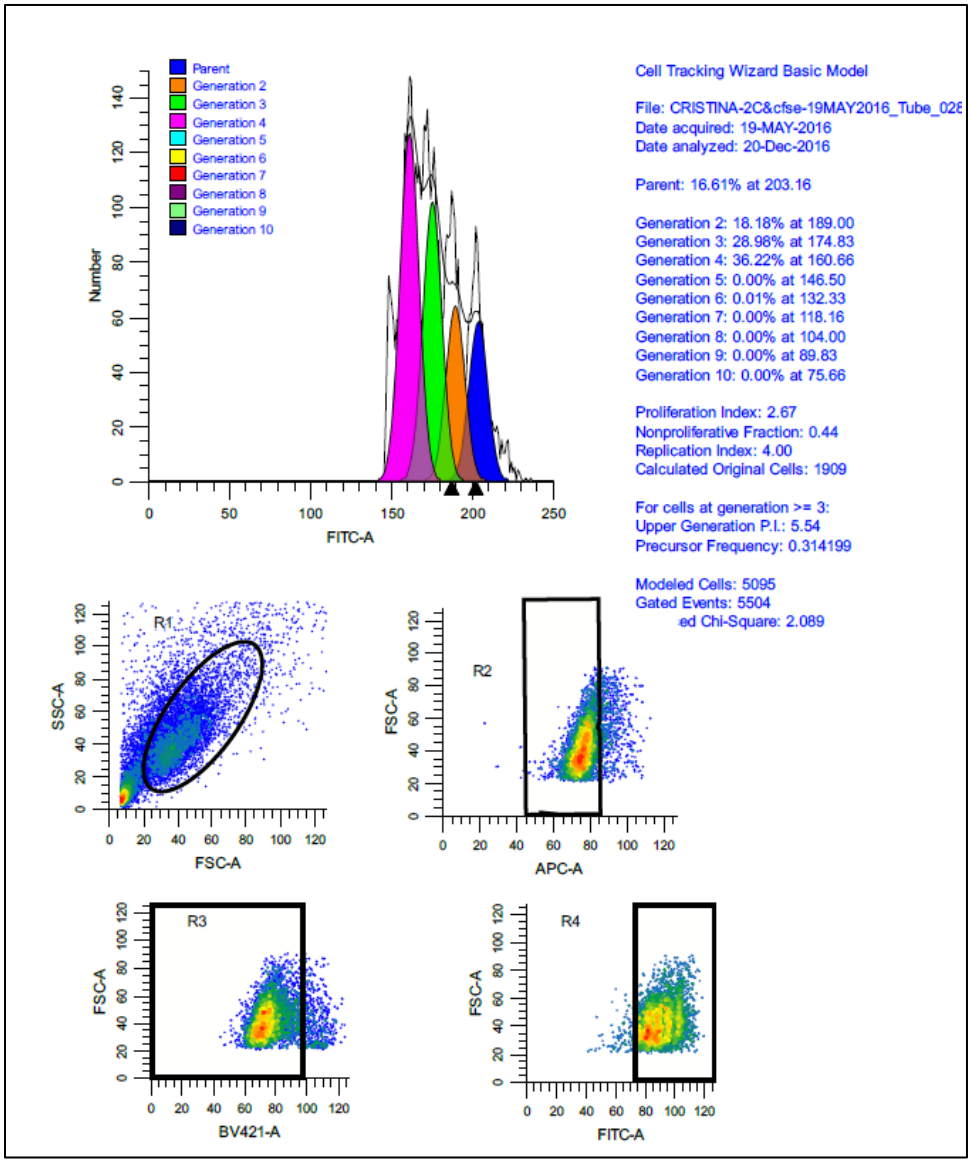


B.


Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10
Number
FITC-A- CFSE
Cell Tracking Wizard Basic Model
File: CRISTINA-3C-11JULY2016_Tube_046.fcs
Date acquired: 11-JUL-2016
Date analyzed: 1-Sep-2016
Parent: 15.22 % at 166.20
Generation 2: 27.12 %; at 152.04
Generation 3: 36.54 %; at 137.87
Generation 4: 21.12 %; at 123.70
Generation 5: 0.00 %; at 109.54
Generation 6: 0.01 %; at 95.37
Generation 7: 0.00 %; at 81.20
Generation 8: 0.00 %; at 67.04
Generation 9: 0.00 %; at 52.87
Generation 10: 0.00 %; at 38.70
Proliferation Index: 2.47
Nonproliferative Fraction: 0.38
Replication Index: 3.35
Calculated Original Cells: 3168
For cells at generation >= 3:
Upper Generation P.I.: 4.90
Precursor Frequency: 0.290323
Modeled Cells: 7811
Gated Events: 7945
Reduced Chi-Square: 1.347
R3
FSC-A
FITC-A- CFSE
R1
FSC-H
FSC-A
ModFit LT V4.1.7(Win)
R2
FSC-A
BV421-A-Live /Dead

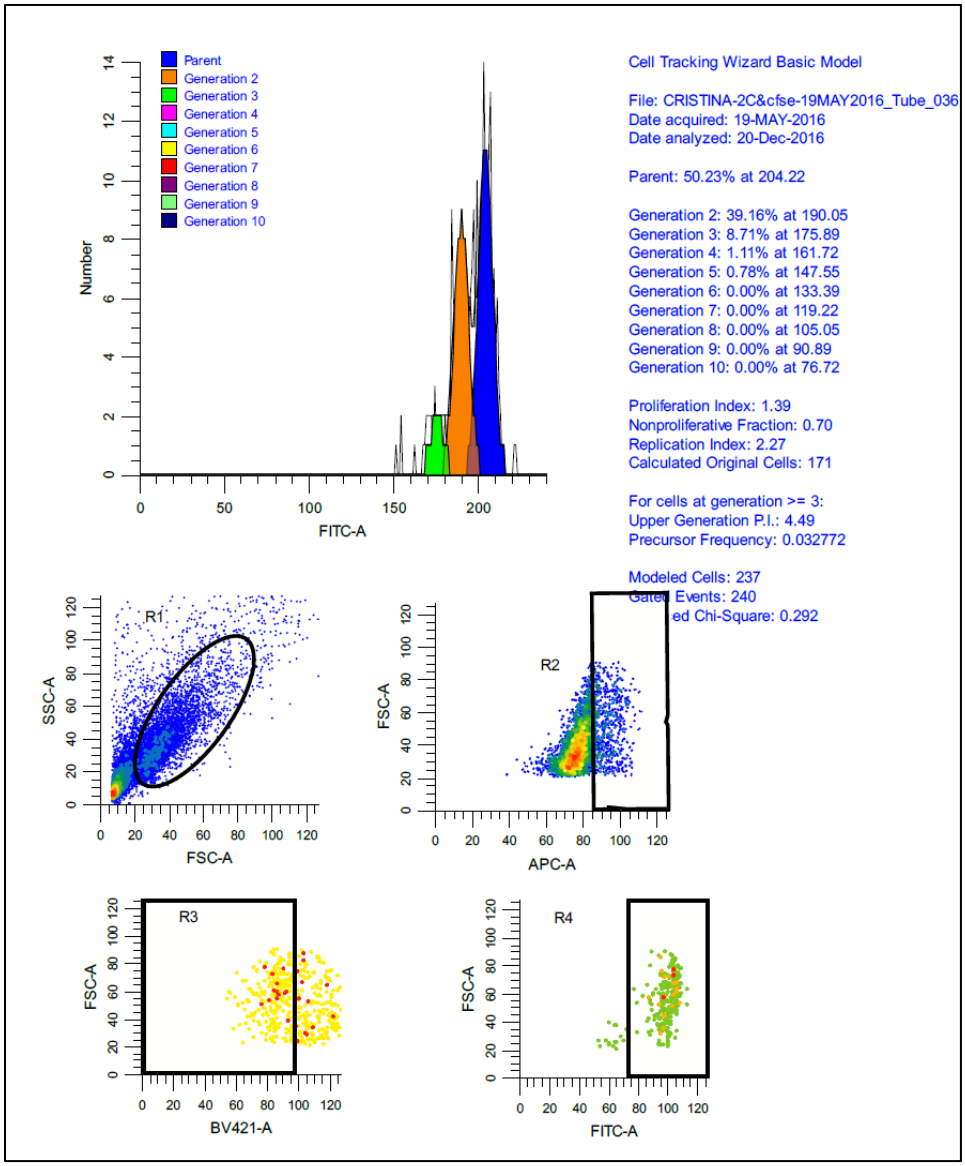
5.5.8 *Analysis report of cell proliferation in CAP- / CFSE+ stained cells of a sample treated with PCV2-Ionomycin-PMA*

Initial gating was performed to eliminate debris (FSC-A vs SSC-A), followed by gating PCV2-CAP- cells (APC-A vs FSC-A), followed by exclusion of dead cells (BV-421 Live/Dead vs FSC-A), and a final gate for CFSE positive cells (CFSE-A vs FSC-A). The model was built using the Cell track wizard, with adjustment of the parent population, and determination of the cell generations by the software (ModFit LT, version 4.1).



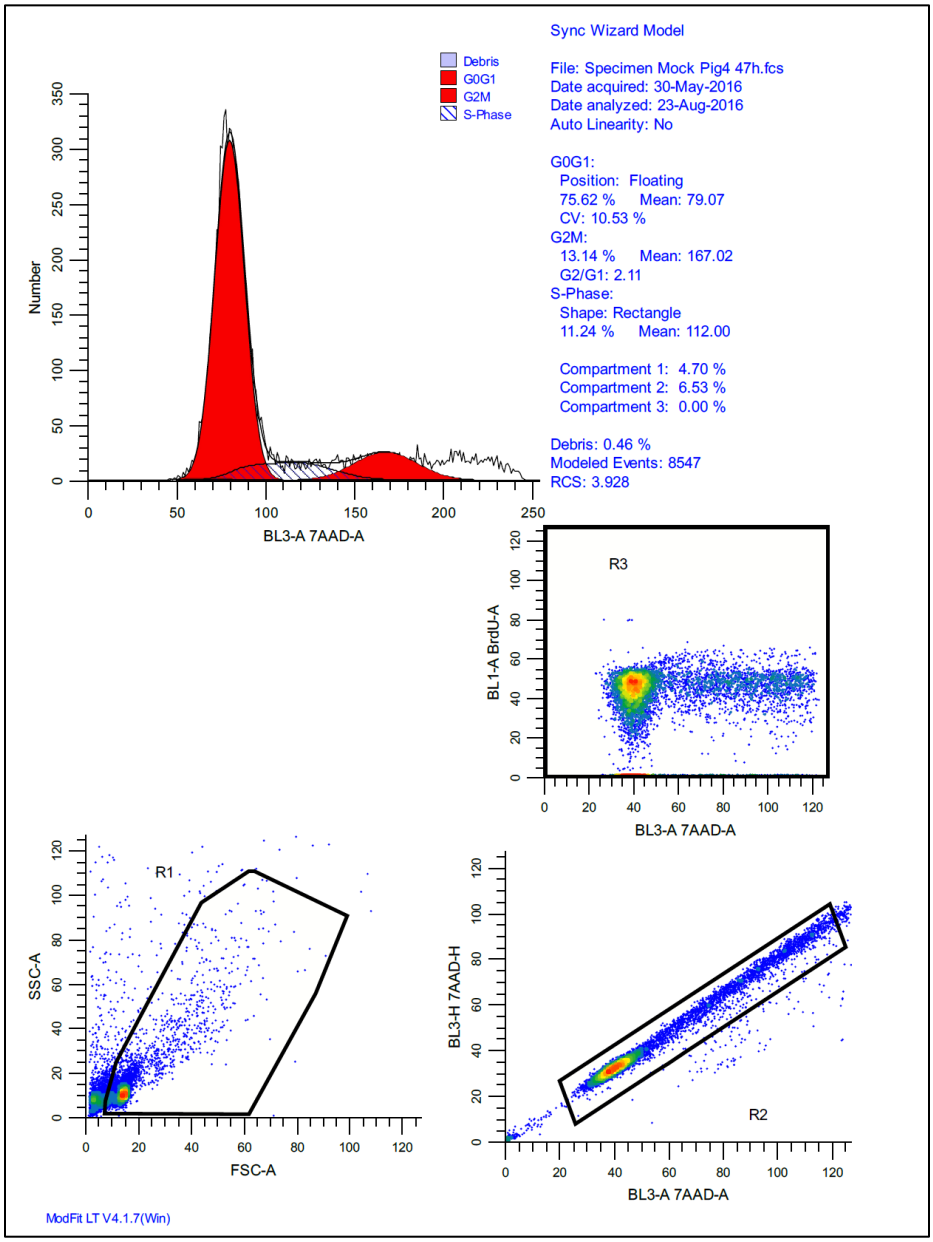
5.5.9 *Analysis report of cell proliferation in CAP+/CFSE+ stained cells of a sample treated with PCV2-Ionomycin/PMA*

Initial gating was performed to eliminate debris (FSC-A vs SSC-A), followed by gating CAP+ cells (APC-A), followed by exclusion of dead cells (BV-421 Live/Dead), and a final gate for CFSE positive cells (CFSE-A vs FSC-A). The model was built using the Cell track wizard, with adjustment of the parent population, and determination of the cell generations (ModFit LT, version 4.1).



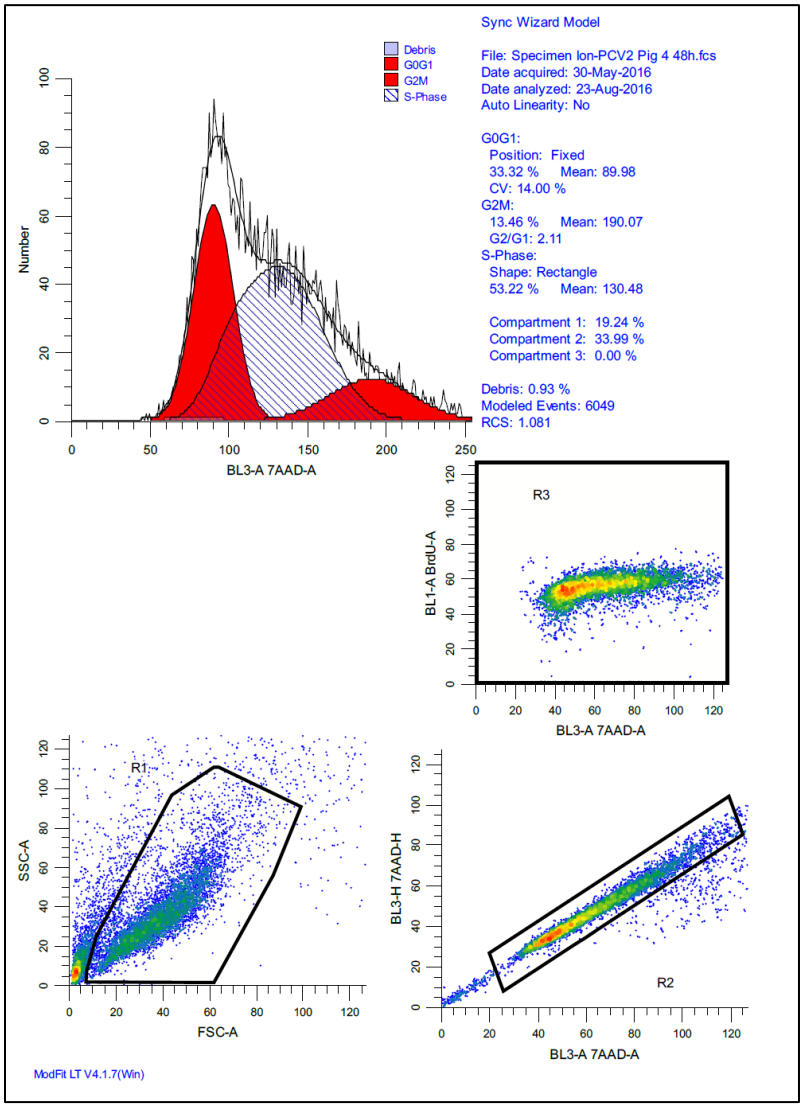
5.5.10 *Cell cycle analysis report of a Mock treated sample*

Initial gating was performed to eliminate debris (FSC-A vs SSC-A), followed by aggregate exclusion (7-AAD-A vs 7-AAD-H), and a final gate to visualize BrdU incorporation (BrdU-A vs 7-AAD-A). The determination of each cell cycle phase was performed using 7-AAD as reference for DNA content (ModFit LT, version 4.1).



5.5.11 *Cell cycle analysis report of a PCV2-Ionomycin/PMA treated sample*

Initial gating was performed to eliminate debris (FSC-A vs SSC-A), followed by aggregate exclusion (7-AAD-A vs 7-AAD-H), and a final gate to visualize BrdU incorporation (BrdU-A vs 7-AAD-A). The determination of each cell cycle phase was performed using 7-AAD as reference for DNA content (ModFit LT, version 4.1).



Chapter Six: Discussion and conclusions

The overall aim of this thesis was to investigate the interaction of PCV2 with the porcine immune system, by studying the PCV2-induced humoral response, and exploring the effect of PCV2 infection on immune cell proliferation and viability. The first part of this thesis compared the PCV2 neutralizing and non-neutralizing antibody levels with regard to the vaccination status, and determined the PCV2 viral load in farmed pigs of different age groups. The second part of this thesis described the SF-pCD methodology used to establish a PCV2-free pig cohort, and the subsequently use of these animals as PCV2-naive whole blood donors to study PCV2 pathogenesis. The third part of this thesis described an *in vitro* cell model of primary PCV2 infection, and brought some insight into the effect of viral infection on cell proliferation and viability (Chapters 4 &5). This last chapter discusses the implications of the findings, the benefits that this study brings to PCV2 knowledge, and consequences for swine industry.

6.1 Evaluating PCV2 vaccine efficacy in farmed pigs

Veterinary vaccines protect animals from a variety of different diseases, and have historically improved the health, welfare, and production capability of farmed animals, while contributing to food safety in humans[185]. Vaccines control the spread of viruses in swine, and they are the main component of eradication campaigns in Europe against viruses as Aujesky's disease virus[186]. In Canada, the licensed antiviral pig vaccines are used to protect against diseases associated with Porcine rotavirus, PRRSV, Swine influenza, Porcine parvovirus, and PCV2[187].

PCV2 vaccination has significantly improved the swine herd health, since it was introduced 10 years ago, by increasing the daily weight gain and reducing PMWS cases[91]. Yet,

the mechanisms of vaccine protection against PMWS and the role of NAbs in vaccinated pigs remain unclear.

In this study, we report no noteworthy difference in the PCV2-NAb titers in the majority of farmed pigs with regard to their vaccination status, however, a significant difference in NAb titer was observed between different groups of age. We also demonstrated the PCV2 genome load in serum in the presence of substantial NAb titers.

The high prevalence of viremic pigs in this study suggests a persistent, subclinical infection in farmed pigs that is only cleared in older animals[188]. PCV2 clearance was only seen in older pigs of the finisher/grower and sow/boar groups, which presented high NAb titers with low or absent PCV2 genome copies in serum. It is important to mention that PCV2 clearance was achieved regardless of vaccine administration, and most likely by specific adaptive immunity due to continuous exposure to PCV2 or PCV2 gene products[189].

PCV2 persistence in serum could be a result of neutralizing antibody-escape, a mechanism observed in infection with highly variable viruses (e.g. PRRSV)[190]. Immune pressure on the neutralizing epitope of the glycoprotein GP4 leads to mutations that are able to escape virus neutralization after PRRSV infection[190]. PCV2 strains are usually very conserved and the presence of immune-escape variants is unlikely[191]. Nonetheless, a change of one alanine to arginine in position 59 of the CAP protein resulted in a PCV2a strain that escapes neutralization, and this could represent a mechanism of viral immune escape *in vivo*[192]. Perhaps another significant reason for PCV2 persistence in serum is that the PCV2 viral load measured in serum corresponds to non-infectious PCV2-DNA genome derived from dying cells.

In this study, the presence of MDA was assessed by measuring the antibodies in piglets of the suckling group. Based on our results (Chapter 2), the presence of MDA does not prevent PCV2 infection perinatally, but they could protect piglets from high viral replication in the first weeks of life. There is a possibility that the presence of maternal antibodies could also interfere with the vaccine efficacy by binding to the antigen and masking the neutralizing epitope from further antigen presentation[193]. Whether the presence of different levels of MDA at the time of PCV2 vaccination could interfere with the induction of high titers of NAbs in farmed pigs still needs to be elucidated[93.]

Experimental[90] and field[84] studies have clearly demonstrated that PCV2 vaccination does not elicit an immune response that protects pigs from the primary PCV2 infection. Other studies, however, claim that the NAb response induced by vaccination lowers or clears PCV2 viral loads in serum[86], and decreases virus shedding[129] in experimentally-challenged pigs. The differences observed between experimental and field PCV2 vaccine efficacy studies could be due to environmental and study design conditions that are very difficult to control in field settings. Some factors that differentiate experimental from field settings are the use of caesarean-derived, colostrum-deprived piglets that have had no previous exposure to PCV2 antigen or antibody, a one-time PCV2 challenge with a unique dose, and the maintenance of pigs in clean, controlled environment[194,195]. In contrast, farmed pigs present different levels of PCV2-MDA that could interfere with NAb induction, they have been challenged with PCV2 since birth, and are subject to handling stress and coinfections, possibly influencing the response of the animals towards vaccination[84,128]. The type of assay used to measure the NAbs could also explain the differences between studies[88]. The use of an automated procedure to analyze the PCV2-VNA, like the one

presented in this thesis[196], could provide greater accuracy to determine the titers of NAbs in future studies.

Although VNAs are useful to the determine the reduction of infectivity by antibodies in the absence of effector cells or complement[197], it is important to consider the limitations of using these tests to determine the titers of NAbs in serum, and the correlation of these titers to *in vivo* protection against infection[198]. VNAs use only one virus strain in a specific concentration to measure the reduction of infectivity *in* vitro; in addition, pre-treatment of the sample inactivates the complement that could interfere with the assay's results. In i*n vivo* situations, antibodies can neutralize virus infectivity by binding to the particle and activating the complement cascade, triggering phagocytosis of the virion[198]. Antibody binding can also activate Fc receptors in NK cells, causing antibody-dependent cellular cytotoxicity (ADCC) of infected cells[197]. These factors should be considered to analyse the role of neutralizing antibodies and the efficacy of vaccination in preventing disease.

Since the NAb induction by PCV2 vaccine is still controversial, a strong T cell immunity should elicit the protection observed after vaccination[129]. In the case of killed or subunit vaccines like PCV2, the induction of CD4+ and CD8+ T cell response after vaccination depends on the local inflammatory reaction elicited by the viral antigen and the adjuvant[73]. Effector CD8+ T cells could control PCV2 replication by recognizing infected cells in the periphery and inducing death by releasing granule contents into cells, or inducing apoptosis via Fas-FasL membrane signaling[75].

The cellular response after PCV2 vaccination has been assessed by studies based on the capacity of IFN-γ secretion by PBMCs after vaccination and challenge[92, 128, 199]. PCV2 vaccination induces IFN-γ-producing cells starting two-weeks after vaccination[128], increasing one-week after experimental challenge and correlating with a decrease in PCV2 viral load in serum[200]. One study reported an increase in CD4+ T cells after PCV2 vaccination, which secreted IFN-γ and TNF-α after *in vitro* re-stimulation with PC2 particles[199]. In addition, the authors reported the induction of CD4+ CD8+ central memory T cells[199], suggesting an important cellular response that could result in protection against disease after vaccination, and should be addressed in future studies.

In summary, this part of the study demonstrated a diversity in the humoral immune response against PCV2 vaccines in farmed pigs, and a high prevalence of viremic pigs, demonstrating the necessity of new vaccine strategies to control PCV2 sub-clinical infection.

6.2 Effect of PCV2 replication on cell proliferation and viability

Viruses are obligate intracellular pathogens that regulate molecular pathways of the host to their advantage[201]. PCV2 is a small virus that is associated with the swine immune system, and depends on the host's machinery for replication and production of virus progeny[201]. To study the interaction between PCV2 and the immune cells, we established an *in vitro* cell model to study primary PCV2 infections in PBMCs, by raising snatched-farrowed, porcine colostrum deprived PCV2-free pigs as blood donors of PCV2-naïve PBMCs. This model helped to elucidate the effect of PCV2 infection in the absence of pre-existing infection, as we and other studies have reported an *in vitro* reactivation of PCV2 persistent infections in PBMCs after mitogen stimulation[72].

Following the clinical observation that immune activation in pigs leads to PMWS[62], previous studies reported that the stimulation of PBMCs *in vitro* led to increased PCV2 infection rates[72,158]. Nonetheless, limitations of these studies included the use of mitogens like Con A and PWM that only stimulate a specific lymphocyte subpopulation[72,158], and low infection rates after stimulation (0-2%)[22].

In the current study, swine PBMCs were stimulated by combining the mitogens ionomycin/PMA, which activate both B and T cells polyclonally (Chapter 4)[159,160]. Ionomycin is an ionophore that directly mobilizes extracellular calcium (Ca^{2+}) through the plasma membrane into the cytosol of cells[202]; ionomycin also facilitates the movement of intracellular stores of Ca^{2+} (e.g. through opening of channels in the endoplasmic reticulum membrane) into the cytosol[202,203]. The increase of Ca^{2+} concentrations initiates intracellular signal pathways, including the activation of calmodurin and calcineurin, resulting in the activation of transcription factors, like nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and NF-κB[204]. PMA additionally activates the protein kinase C pathway[205], which in the presence of Ca^{2+}, also translocates NF-κB into the nucleus, and initiates B and T cell proliferation[206]. In addition, the combined treatment of PBMCs with ionomycin and PMA induces the secretion of IL-2 into cultures, stimulating IL-2 receptor upregulation and T cell proliferation[207, 208]. The activation and subsequent proliferation of swine PBMCs after ionomycin/PMA treatment was confirmed by the upregulation of the activation markers CD25 and MHCII, the increase in the percentage of cells in S phase, proliferation index, and total cell numbers (Chapter 5), demonstrating a suitable *in vitro* model for swine PBMC activation.

Increased PCV2 infection rates were measured after stimulation of PBMCs with ionomycin/PMA *in vitro* (Chapter 4). Although the mechanisms behind this increased infection rates still need to be elucidated, the polyclonal cell activation adopted in our experiments could stimulate cellular pathways that create ideal environments for PCV2 binding, entry, viral protein expression, and virion production.

After binding to GAGs on the cell surface, PCV2 is endocytosed by clathrin-mediated endocytosis (CME) in the monocytic cell line 3D4/31[31]. CME is a mechanism used by eukaryotic cells to internalize material using clathrin-coated pits, which detach from the plasma membrane and fuse into early endosomes[209]. Viruses commonly use CME as an entry pathway to delay immune responses while moving through the cytoplasm, and using the pH acidification environment of the endosome for uncoating[210]. After T cell activation, diverse cellular pathways are initiated, including CME which is used to internalize the IL-2 receptor after cytokine binding[211]. An upregulation of CD25 and MHCII was observed in activated PBMCs (Chapter 5), so future studies should address if PCV2 takes advantage of the increased endocytosis of these or other receptors to enter PBMCs.

Cell activation can also provide an ideal intracellular environment for viral replication, specifically for a virus like PCV2 that depends on the host's DNA polymerase to replicate[212]. In this thesis, a cell cycle dependency for PCV2 replication in PBMCs was suggested by the increased infection rates in mitogen-stimulated cells (Chapter 4), and the decreased proliferation of PCV2-infected cells (Chapter 5). Mitogen stimulation could favor virus replication by initiating cell cycle events that could lead to a specific arrest by viral proteins. For instance, the expression of the oncoproteins E6 and E7 of the *Human papillomavirus* leads to inhibition of

p53 and S phase arrest, resulting in cell proliferation and ultimately contributing to tumor formation[213]. An S phase arrest was observed in PK-15 cells infected PCV2 due to a downregulation of Cyclin A/CDK2[173]. More research is needed to determine if an S phase arrest is needed for PCV2 replication in PBMCs.

After virus replication is completed, new virus particles are assembled and egress the cell[214], although details of this process are still unknown for PCV2[35]. Cell lysis after completion of virion assembly is a common mechanism of egress for non-enveloped viruses, and virus proteins are usually involved in inducing intracellular changes that can lead to cell death[214]. For instance, Parvoviruses are transported out of the nucleus by vesicles towards the endoplasmic reticulum and Golgi apparatus, where cytolysis is induced, leading to virus egress[215]. The high percentage of cell death observed in PCV2-infected cells *in vitro* (Chapter 5) and the generalized depletion of lymphoid cells observed in advanced stages of PMWS affected pigs, suggests a lytic step in the PCV2 replication cycle, and the interaction of viral proteins to induce cell death[98].

PCV2 replication has been associated with the activation of pro-apoptotic cellular pathways that could aid in virion release *in vitro.* PCV2-ORF-3 protein induces the activation of caspases 8 and 3[19], and it's expression is highly cytotoxic *in vitro*[101]. PCV2 replication also activated the nuclear translocation of NF-κB, which resulted in activation of caspase 3 in PK-15[216]. In addition, the expression of the PCV2-CAP induced autophagy[217] and cell death[98] in PK-15 cells.

In lymphoid tissues of PMWS pigs, apoptotic bodies and decreased cell proliferation have been reported, demonstrating that apoptosis is also an important mechanism of PCV2

pathogenesis *in vivo*[164]. Although the mechanisms leading to the generalized lymphoid depletion in PMWS pigs are still unclear, overexpression of IL-10 by invading macrophages[94], decreased expression of activation markers[179], and virus-induced apoptosis could contribute to the cell death. In addition, subcellular localization studies determined that virus factories can be found in mitochondria of lymphoid cells of PMWS pigs, suggesting another pathway of apoptosis induction after PCV2 replication[218].

In this thesis, the apoptotic rate of PBMCs exposed to PCV2 was higher than in the cells exposed to mitogen and PCV2 (Chapter 4), suggesting a direct role of PCV2 on apoptosis induction *in vitro*[66]. In addition, a high percentage of non-infected PBMCs died after exposure to PCV2 (Chapter 4), suggesting a bystander apoptosis induced by viral proteins or cytokines present in the supernatant. Overexpression of the immunosuppressive cytokines IL-10 and TNF-α has been observed in PMWS cases, suggesting a mechanism of PBMC death that should be studied further[95].

In summary, we demonstrated that the effects of a primary PCV2 infection in PBMCs include increased PCV2 infection in activated cells, decreased antigen presentation, bystander cell death in early time points after PCV2 infection, and reduced cell proliferation in infected cells.

6.3 Conclusions and future research

We conclude that farmed pigs are constantly exposed to PCV2 throughout their lives, and that vaccination is not able to protect pigs from PCV2 infection. Thus, new research strategies should be used to develop better PCV2 vaccines that prevent the infection, which will lead to

herd heath improvement. Reverse vaccinology could be used to identify the peptide sequence of the epitope targeted by the neutralizing antibody, and use it to develop an epitope-based vaccine[219]. A vaccine that stimulates the mucosal immunity of the pig could also be tested to induce the production of IgA antibodies to prevent PCV2 oral-nasal infection[220].

The PBMCs isolated from PCV2-naïve pigs helped overcome some difficulties we encountered after *in vitro* stimulation of cells, such as the reactivation of PCV2 infection[72], and the inability to distinguish a primary PCV2 infection *in vitro* from the PCV2 infection already present in the cells. The treatment of these cells with a combination of ionomycin and PMA showed an increase in PCV2 infectivity rate, which makes this mitogen combination an ideal agent to study the effect of the activation on lymphocyte subpopulations in the presence of PCV2.

We also demonstrated that PCV2 infection strongly modulates the function and viability of immune cells, by decreasing antigen presentation and cell proliferation, and inducing cell death rapidly after infection. Future work should be focused on understanding the mechanisms that PCV2 uses to enhance viral replication and induce cell death *in vitro.* It is also necessary to understand which proteins are involved in PCV2-induced cell death, and to identify which cytokines are being secreted into the cell culture, that may explain the bystander cell death.

PCV2 is a small virus with immense consequences on the swine immune system. The implementation of better models to study PCV2 infection and pathogenesis *in vitro* will allow us to understand and prevent the consequences of PCV2 infection in farmed animals.

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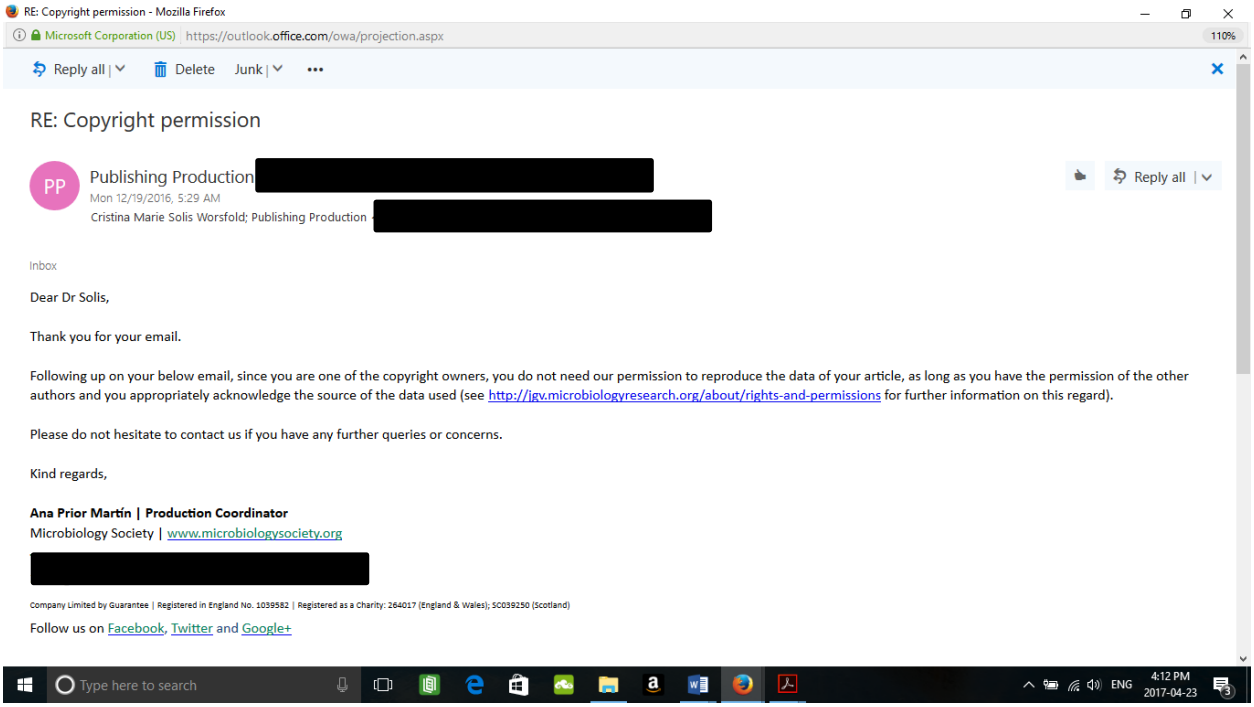

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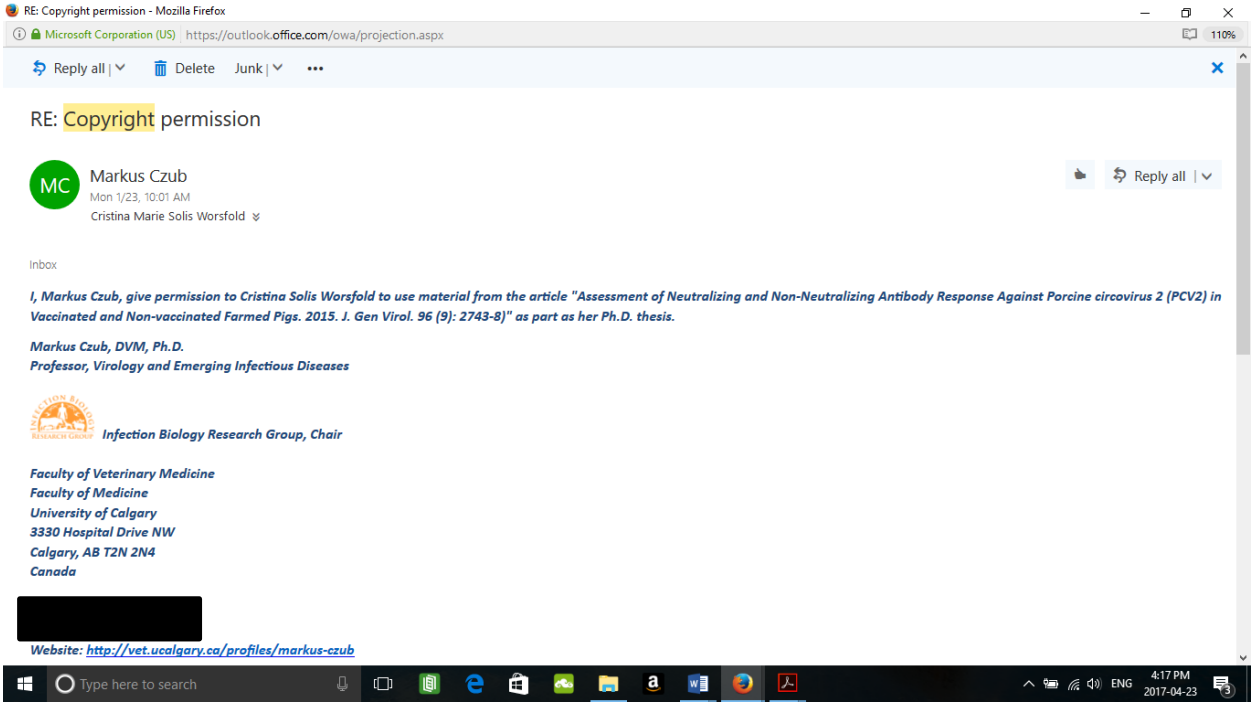
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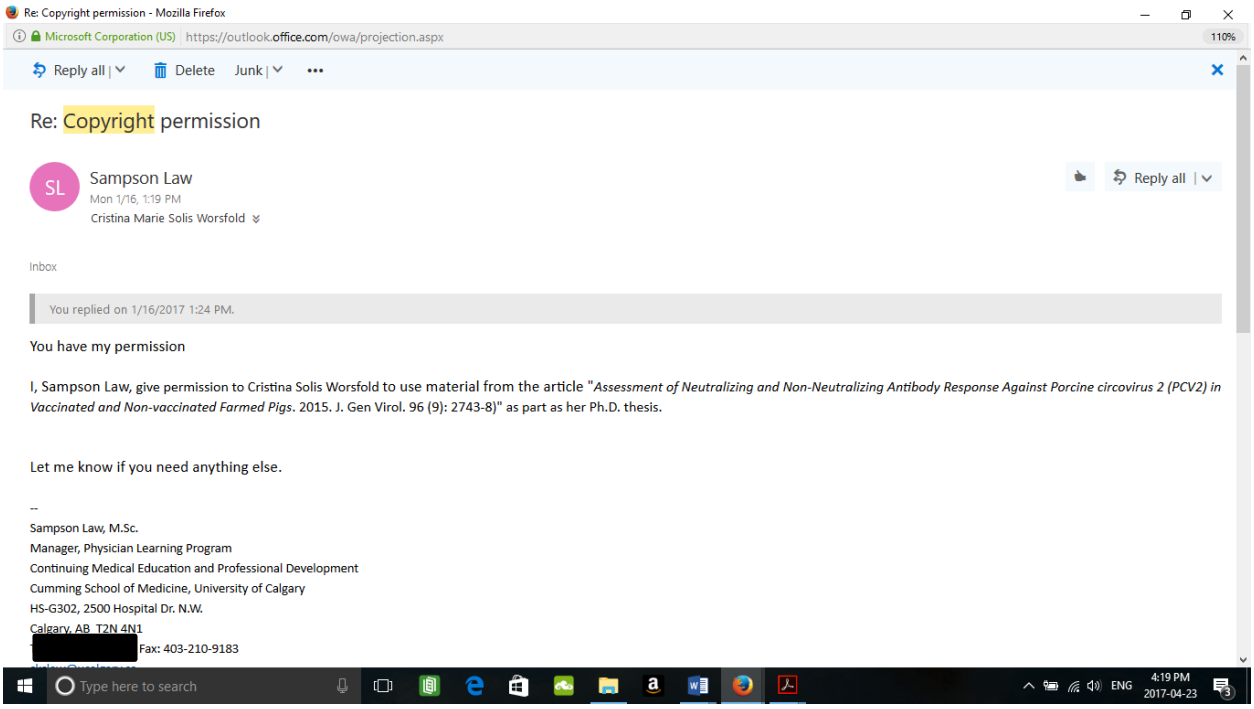
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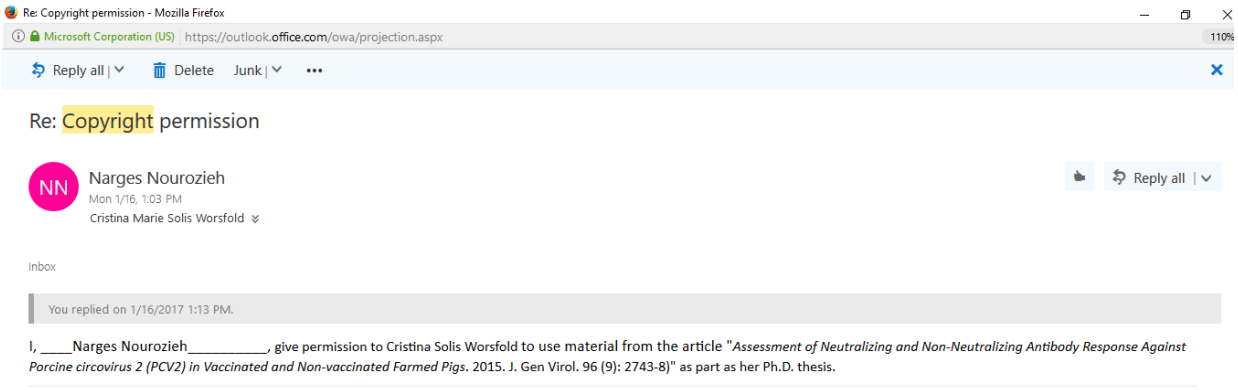
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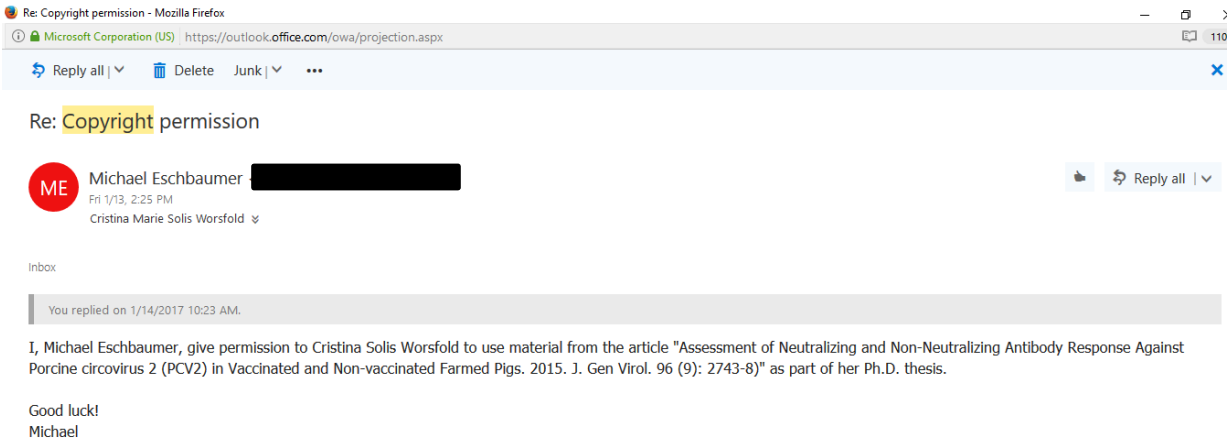
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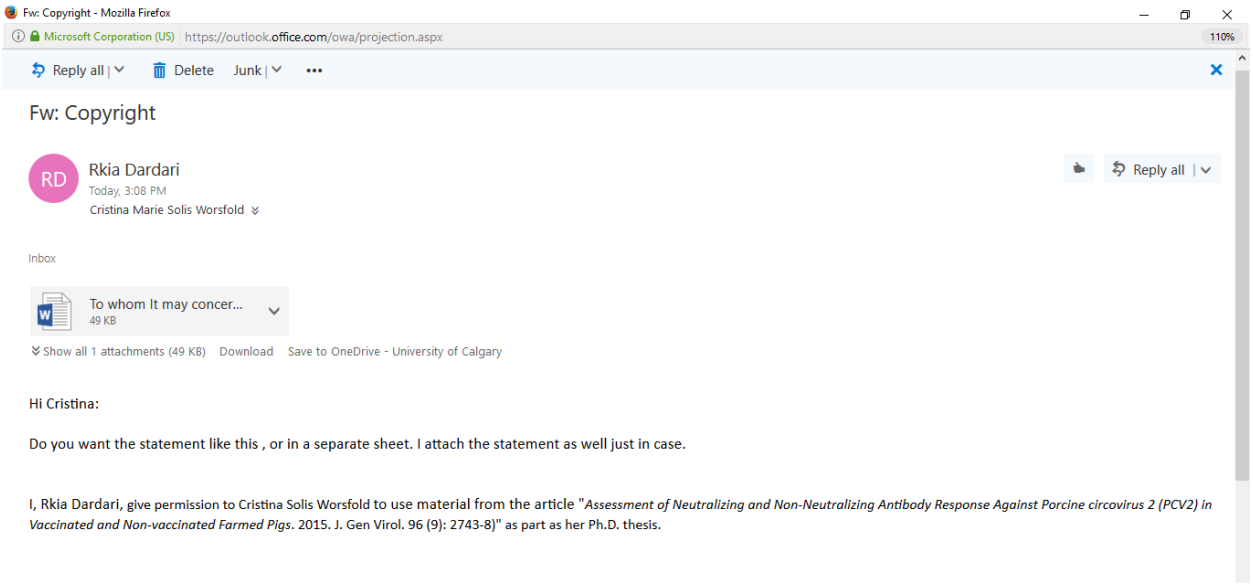
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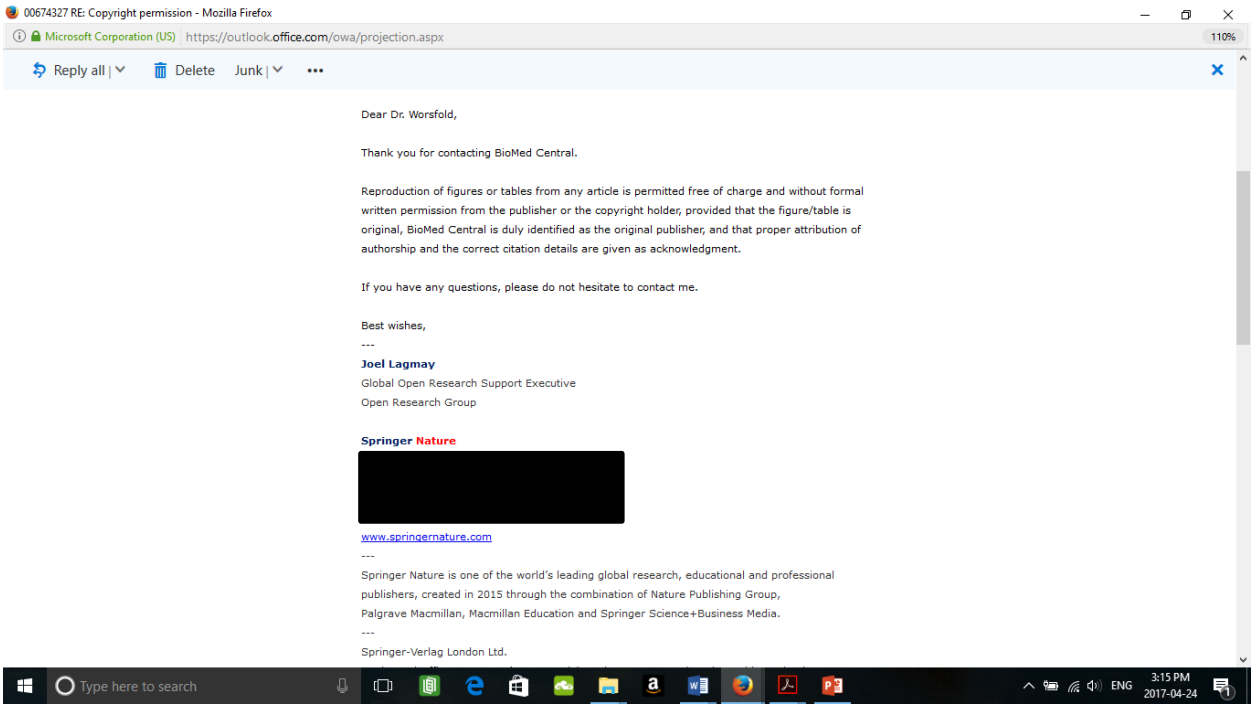
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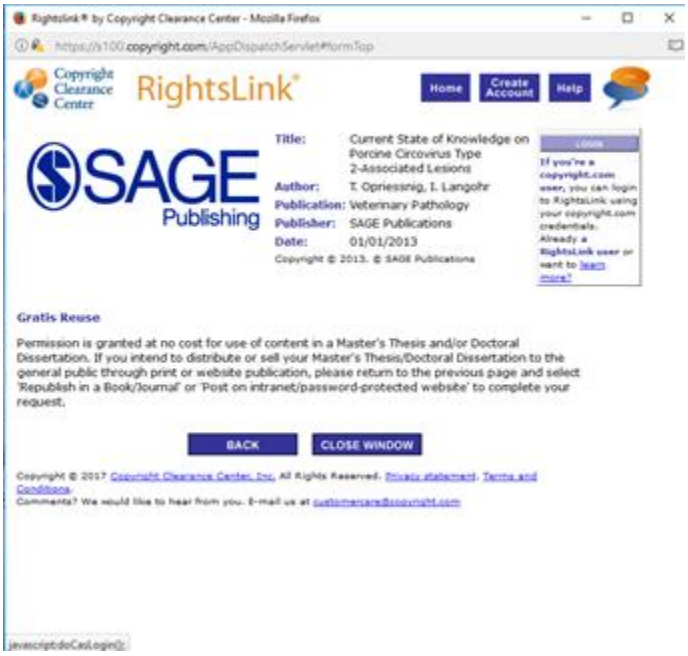
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