Characterization of immune responses to porcine circovirus type 2 (PCV2) infection and vaccination in pigs

Tesi doctoral presentada per Maria Fort de Puig per accedir al grau de Doctor en Veterinària dins del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. Enric Mateu de Antonio i del Dr. Joaquim Segalés i Coma.

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FACULTAT DE VETERINÀRIA DE BARCELONA

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Certifiquen:

Que la memòria titulada, "**Characterization of immune responses to porcine circovirus type 2 (PCV2) infection and vaccination in pigs**" presentada per Maria Fort de Puig per l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la seva direcció a la Universtitat Autònoma de Barcelona.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 15 de juny de 2009.

Dr. Enric Mateu de Antonio Dr. Joaquim Segalés i Coma

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Especialment dedicada als meus pares

"M'ho van explicar i ho vaig oblidar,
m'ho van ensenyar i ho vaig entendre,
ho vaig fer i ho vaig aprendre"
Confuci, 551-479 a.C.

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SUMMARY / RESUM

SUMMARY

Porcine circovirus type 2 (PCV2) is the causative agent of Postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease of nursery and fattening pigs that causes considerable economic losses to the swine industry worldwide. The histopathological features of PMWS and the fact that secondary and opportunistic infections are common in PMWS-affected pigs indicate that this disease involves a deep alteration of the immune system. For years, control of PMWS was limited mainly to the improvement of management strategies and to the control of risk factors thought to influence the infection outcome. At present, four vaccines against PCV2 are being sold on the international market and their application in the field drastically reduced the incidence of PMWS. This Thesis aimed to characterize the immune responses developed by pigs upon PCV2 infection and vaccination. In the first part (Studies I and II), the immune features of PCV2 experimental sub-clinical infections were characterized, with particular emphasis on cell-mediated responses, and on the role of PCV2 capsid (Cap) and replicase (Rep) proteins on it. Results from these studies showed that whole PCV2, but not Cap or Rep, induces the release of interleukin (IL)-10 in peripheral blood mononuclear cells (PBMC), even in those cultures obtained from PCV2-*naïve* pigs, a fact that indicates the innate origin of this response. In addition, it was found that interferon (IFN)-α can be detected in serum at early stages of the sub-clinical infection (5 days post inoculation (PI)). In contrast, IL-10 in serum could only be sporadically detected, suggesting that increased levels of this cytokine are not characteristic of PCV2 sub-clinically infected pigs. With regards to the acquired immunity to PCV2, humoral responses were developed mostly between the second and third week PI, characterized by the production of PCV2 total and neutralizing antibodies (NA), appearing NA later than total antibodies. Cell-mediated immunity developed within the first two weeks PI and it was demonstrated that both Cap and Rep proteins of PCV2 are involved in its development. In the second part of this thesis (Studies III and IV), the immunogenicity and efficacy of a commercial PCV2a-based sub-unit vaccine

used in one- and two-dose schedules was evaluated in conventional piglets. Results from these studies demonstrated that vaccination induced the development of humoral and cell-mediated responses and significantly reduced viremia, shedding, and viral load in tissues upon challenge with either PCV2a or PCV2b isolates. It was also found that maternally derived PCV2 antibodies (MDA) protect against PCV2 infection and influence the humoral response developed after vaccination. Results from study IV suggest that pigs with IPMA titres below 5 $\log_2$ are potentially more susceptible to PCV2 infection. Besides, IPMA titres beyond 10 $\log_2$ were seen to interfere with the development of antibodies following PCV2 vaccination. Based on these observations a "vaccination window" was proposed, defined as the range of antibody titres at which piglets should be vaccinated to minimize interference with MDA and, at the same time, ensure the development of protective immunity before PCV2 exposure.

RESUM

El *Circovirus porcí tipus 2* (PCV2) és l'agent causal de la circovirosi porcina (CP), una malaltia multifactorial que afecta porcs en fases de transició i engreix i que causa importants pèrdues econòmiques a la indústria porcina d'arreu del món. Les característiques histopatològiques de la CP i el fet que els animals malalts pateixin sovint infeccions secundàries i oportunístiques indica que aquesta malaltia implica una greu alteració del sistema immunitari del porc. Durant molts anys, el control de la CP es centrava principalment en la millora d'estratègies de maneig i en el control dels factors de risc que influeixen la presentació clínica de la malaltia. A dia d'avui, s'han introduït al mercat internacional quatre vacunes enfront PCV2 i la seva implementació al camp ha disminuït dràsticament la incidència de la CP. La present Tesi tenia com objectiu la caracterització de les respostes immunitàries desenvolupades pel porc en el curs de la infecció i vacunació per PCV2. En la primera part (Estudis I i II), es va caracteritzar el perfil immunològic de porcs sub-clínicament infectats amb PCV2, amb especial èmfasi en les respostes mediades per cèl·lules i el paper de les proteïnes capside (Cap) i replicasa (Rep) de PCV2 en el seu desenvolupament. Es va demostrar que el virus sencer, però no Cap i Rep indueix l'alliberació d'interleuquina (IL)-10 en cèl·lules mononuclears de sang perifèrica (CMSP), fins i tot en els cultius procedents d'animals verges, indicant l'origen innat d'aquesta resposta. Addicionalment, es va observar que, en fases inicials de la infecció per PCV2, es pot detectar interferó (IFN)-α en sèrum (dia 5 post-inoculació (PI)). Per altra banda, nivells detectables d'IL-10 només s'observaren de forma esporàdica, suggerint que els animals infectats sub-clínicament per PCV2 no es caracteritzen per tenir nivells elevats d'aquesta citoquina en sèrum. En relació a la immunitat adaptativa enfront a PCV2, es va veure que la resposta humoral es desenvolupa entra la segona i tercera setmana PI i que es caracteritza per la producció d'anticossos totals i neutralitzants, essent els neutralitzants d'aparició més tardana. La immunitat mediada per cèl·lules apareix entre la primera i segona setmana PI, i tant Cap com Rep estan implicades en el seu

desenvolupament. A la segona part d'aquesta Tesi (Estudis III i IV), es va avaluar la immunogenicitat i eficàcia d'una vacuna comercial (una i dues dosis) basada en la Cap d'una soca de genotipus PCV2a en porcs convencionals. Els resultats d'aquests estudis van demostrar que la vacunació indueix el desenvolupament d'immunitat humoral i cel·lular i redueix la viremia, l'excreció i la càrrega vírica en teixits després de la infecció amb PCV2, tant amb soques de genotipus PCV2a com PCV2b. També es va observar que els anticossos maternals (AM) protegeixen enfront la infecció per PCV2 i influeixen en el desenvolupament de la resposta humoral després de la vacunació. Els resultats de l'estudi IV suggereixen que porcs amb títols d'IPMA per sota 5 $\log_2$ són potencialment més susceptibles a infectar-se amb PCV2. D'altra banda, es va veure que títols d'IPMA per sobre 10 $\log_2$ poden interferir amb el desenvolupament d'anticossos en resposta a la vacunació. En base a aquestes observacions, es proposa una "finestra de vacunació", definida com el rang de títols d'anticossos en el que els porcs s'haurien de vacunar per minimitzar la interferència amb els AM i, al mateix temps, assegurar el desenvolupament d'immunitat protectiva abans que els animals s'exposin a PCV2.

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Fort, M., Fernandes, L. T., Nofrarías, M., Díaz, I., Sibila, M., Pujols, J., Mateu, E. & Segalés, J. (2009). Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. *Vet Immunol Immunopathol* 129, 101-7.

Fort, M., Sibila, M., Pérez-Martín, E., Nofrarías, M., Mateu, E. & Segalés, J. (2009). One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model. *Vaccine* 27, 4031-7.

Fort, M., Sibila, M., Nofrarías, M., Pérez-Martín, E., Olvera, A., Mateu, E. & Segalés, J. Porcine circovirus type 2 (PCV2) Cap and Rep proteins are involved in the development of cell-mediated immunity upon PCV2 infection. Submitted for publication.

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LIST OF ABBREVIATIONS

Aa	amino acid
AM	alveolar machrophages
BSA	bovine serum albumin
Cap	capsid protein
CD	cluster of differentiation
CDCD	colostrum-deprived, caesarean derived
ConA	concanavalin A
CpG	cytosine-phosphate-guanine
CyA	cyclosporine A
DC	dendritic cells
ds	double stranded
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GEP	Good Experimental Practices
GLM	general linear model
ICTV	International Committee for the Taxonomy of Viruses
IFN	interferon
SC	secreting cells
Ig	immunoglobulin
IL	interleukin
IPMA	immunoperoxidase monolayer assay
ISH	*in situ* hibridization
LPS	lypopolysaccharide
m.o.i	multiplicity of infection
mAb	monoclonal antibody
MDA	maternally derived antibodies
mDC	myeloid dendritic cells
MEM	minimum essential medium
NA	neutralizing antibodies
NIPC	natural interferon producing cells
OD	optical density
ODN	oligodeoxynucleotides
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCV	porcine circovirus
PCVD	porcine circovirus diseases
pDC	plamocitoid dendritic cells
PE	phycoerythrin
PHA	phytohaemagglutinin
PI	post-inoculation
PK	porcine kidney
PMWS	postweaning multisystemic wasting syndrome
Post-C	post-challenge

Pre-C	pre-challenge
PRR	pattern recognition receptor
PRRSv	porcine reproductive and respiratory syndrome virus
Q-PCR	quantative PCR
Rep	replicase protein
RF	replicative form
SPF	specific pathogen free
ss	single stranded
TA	total antibodies
$TCID_{50}$	50% tissue culture infectious dose
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	tumor necrosis factor
VLP	virus-like particles
VNT	virus neutralization test

CHAPTER 1

INTRODUCTION

1.1. Porcine circovirus type 2 (PCV2)

1.1.1. History of PCV2 and porcine circovirus diseases (PCVD)

In 1974, Tischer and co-workers discovered a virus contaminating the porcine kidney cell line PK-15 (ATTC-CCL33) that was morphologically similar to a pircornavirus (Tischer *et al.*, 1974). Later on, it was shown that the contaminant agent was a circular single-stranded DNA (ssDNA) virus that was accordingly named porcine circovirus (PCV) (Tischer *et al.*, 1982). Although serological surveys revealed the presence of PCV antibodies in the pig population, no disease could be attributed to the infection with that virus (Tischer *et al.*, 1986; Dulac & Afshar, 1989; Allan *et al.*, 1994), and it was consequently considered non-pathogenic.

In 1991, a new emerging disease designated as postweaning multisystemic wasting syndrome (PMWS) was reported in Western Canada (Clark, 1997; Harding, 1997). The new syndrome was clinically characterized by progressive loss of weight, respiratory distress and skin pallor. Pathologically, diseased pigs revealed a multiorganic affectation including generalized lymphadenopathy, interstitial pneumonia, hepatitis, splenomegaly and gastric ulcers (Clark, 1997; Harding, 1997). One year later, a PCV-like agent was isolated from tissues of PMWS-affected pigs in North America and Europe (Allan *et al.*, 1998a; Ellis *et al.*, 1998). Comparison between the original non-pathogenic PCV and the newly discovered circovirus associated with PMWS outbreaks revealed that these viral agents were genetically and antigenically distinct (Allan *et al.*, 1998b; Meehan *et al.*, 1998). Therefore, PCV was subsequently divided into two types; the non-pathogenic PK-15 cell culture-derived virus was designated as PCV type 1 (PCV1) and the circovirus isolated from wasted pigs as PCV type 2 (PCV2) (Hamel *et al.*, 1998; Meehan *et al.*, 1998)

Besides PMWS, PCV2 has been also associated with other disease conditions including porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure and porcine respiratory disease complex (PRDC). In 2002, Allan *et al.* (2002) proposed the term *porcine circovirus diseases* (PCVD) to group all the diseases and syndromes associated with PCV2. Similarly, in March 2006, the American Association of Swine Veterinarians (AASV) proposed a similar nomenclature to collectively name the same conditions: *porcine circovirus associated disease*s (PCVAD). At present, there is still no consensus with regard the disease nomenclature and both terms, PCVD and PCVAD, are used in Europe and North America, respectively.

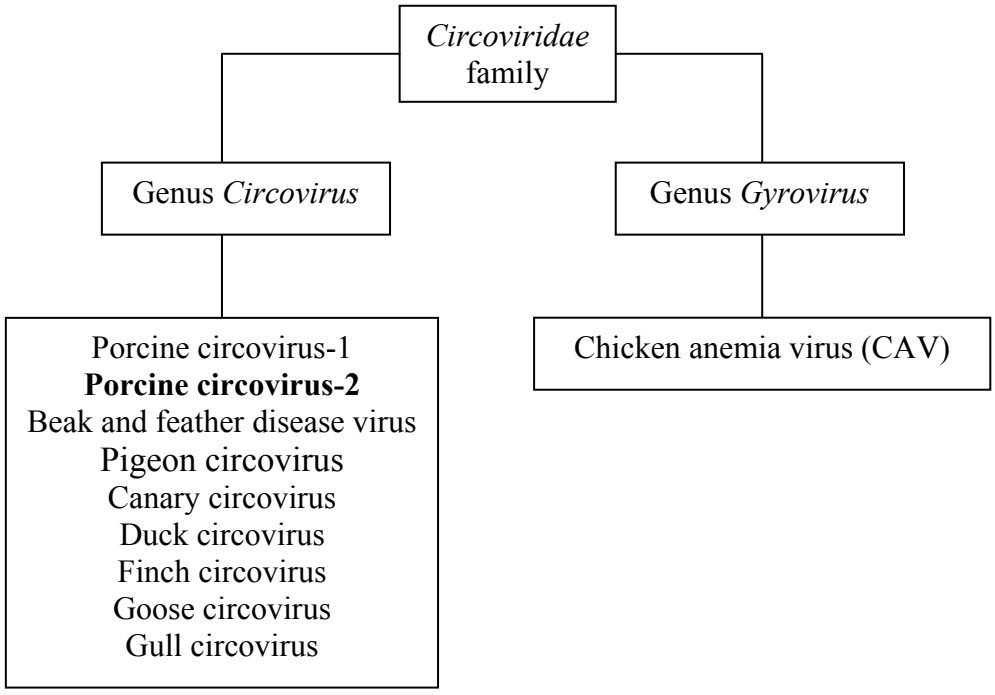
To date, experimental studies demonstrating the link between PCV2 and the development of disease have been successful only for PMWS (Allan *et al.*, 1999a; Bolin *et al.*, 2001) and reproductive failure (Sanchez *et al.*, 2001b; Mateusen *et al.*, 2007). The association between PCV2 and all the other conditions is based on retrospective studies and/or clinical cases, but experimental evidences of such connection have not been obtained so far (Segalés *et al.*, 2005a).

1.1.2. Taxonomy

PCV2 is a member of the *Circoviridae*, a family of icosahedrical, small, non-enveloped ssDNA viruses that infect vertebrates (Lukert *et al.*, 1995). Due to continuous descriptions of new cirvovirus-like agents during the last years, the classification of the *Circoviridae* family has been, and might be, subjected to new reorganizations (Davidson & Silva, 2008). At present, according to the International Committee for the Taxonomy of Viruses (ICTV) (www.ictvonline.org), this family is divided, based on their virion size and genomic organization, into the genera *Circovirus* and *Gyrovirus* (Figure 1.1). Besides PCV, seven avian viruses have been described within the genus C*ircovirus*: beak and feather disease virus (Ritchie *et al.*, 1989), pigeon

circovirus (Woods *et al.*, 1993), canary circovirus (Phenix *et al.*, 2001), goose circovirus (Todd *et al.*, 2001), duck circovirus (Hattermann *et al.*, 2003), finch circovirus (Shivaprasad *et al.*, 2004) and gull circovirus (Smyth *et al.*, 2006). Although not included in the ICTV taxonomy list yet, novel circoviruses infecting ravens (Stewart *et al.*, 2006), starlings (Johne *et al.*, 2006) and swans (Halami *et al.*, 2008) have been reported. Chicken anemia virus is the only species included in the genus *Gyrovirus.* (Todd, 2004).

Figure 1.1: Taxonomical classification of PCV2



1.1.3. Morphology and molecular organization

PCV2 is one of the smallest viruses known, with an icosahedric, non-enveloped virion particle of 17±1.3 nm of diameter (Tischer *et al.*, 1982). The genome molecule is a covalently closed circular ssDNA of approximately 1.76-1.77 kilobases (Meehan *et al.*, 1998).

After PCV2 infects the cell, the ssDNA is converted to a double stranded DNA (dsDNA) intermediate, also known as the replicative form (RF). The RF is ambisense, with genes encoded by both the viral (positive) and the complementary (negative) strand. PCV2 genes are arranged in 11 putative open reading frames (ORFs) (Hamel *et al.*, 1998); however, protein expression has been described for only three of them. The ORF1, also called *Rep* gene, is located on the positive strand and clockwise oriented; it codes for the non-structural replicase proteins Rep and Rep', of 314 and 178 amino acids (aa) in length, respectively (Mankertz *et al.*, 1998; Cheung, 2003). ORF2 or *Cap* gene, located on the complementary strand and counterclockwise oriented encodes for the only structural protein (233-234 aa), the capsid (Cap) (Mankertz *et al.*, 2000; Nawagitgul *et al.*, 2000). ORF3 is completely overlapped with ORF1 gene, located in the complementary strand and counterclockwise oriented. It codes for a non-structural protein of 105 aa length. *In vitro*, the ORF3 protein was found to be involved in virus-induced apoptosis of PK-15 cells (Liu *et al.*, 2005). Very recently, it was also reported that piglets inoculated with an ORF3-defficient PCV2 mutant showed a decreased viremia and less PCV2-associated pathological lesions in comparison to pigs inoculated with the wild type PCV2. It was suggested, therefore, that the ORF3 protein might play a role in viral replication and pathogenicity (Karuppannan *et al.*, 2009).

1.1.4. Biological and physico-chemical properties

PCV2 is very stable at environmental conditions; however, infectivity can be reduced by exposing the virus to high temperatures. Thus, after pasteurization, namely 60ºC, 10', PCV2 infectivity decreased 1.6 log, 0.75 log after dry heat treatment (80 ºC, 72h) and 1.25 log after extreme dry heat treatment (30', 120ºC) (Welch *et al.*, 2006). Virus titres can be reduced also by commercial disinfectants based on alkali (i.e. sodium hydroxide), oxidazing agents (i.e. sodium hypochlorite) or quaternary ammonium (Royer *et al.*, 2001; Martin *et al.*, 2008).

1.1.5. Genetic and antigenic variation

Genetic homology among PCV2 isolates is relatively high (Mankertz *et al.*, 2000; Meehan *et al.*, 2001; Larochelle *et al.*, 2002) although diversity within the PCV2 population exists. The earliest phylogenetic analyses showed that PCV2 isolates from different geographic origins varied in their genomic sequence (Hamel *et al.*, 1998; Fenaux *et al.*, 2000; Mankertz *et al.*, 2000; Meehan *et al.*, 2001). Later on, based on PCV2 aa/genome sequences or restriction fragment length polymorphisms, two differentiated genogroups of PCV2 were identified (de Boisseson *et al.*, 2004; Timmusk *et al.*, 2005; Carman *et al.*, 2006; Gagnon *et al.*, 2007; Olvera *et al.*, 2007; Grau-Roma *et al.*, 2008). Depending on the author and the laboratorial technique implemented, different nomenclatures were initially used to refer to PCV2 genogroups (Table 1.1).

Table 1.1: Published nomenclatures referring to the two main PCV2 genogroups

Author	De Boissesson et al., 2004	Timmusk et al., 2005	Carman et al., 2006	Olvera et al., 2007	Grau-Roma et al., 2008	Gagnon et al., 2007
Grouping	I	SG3	Pattern 321-like	Clade or group 1	Genotype 1	PCV2b
	II	SG1/SG2	Pattern 422-like	Clade or group 2	Genotype 2	PCV2a

The two phylogenetic groups were commonly referred as genotypes 1 and 2 in Europe and PCV2a and PCV2b in North America. At present, the North American nomenclature has been adopted, therefore the European genotypes 1 and 2 have finally been designated as PCV2b and PCV2a, respectively (Segalés *et al.*, 2008). Recently, a new genotype has been identified in archived Danish samples (Dupont *et al.*, 2008) and designated, accordingly, as PCV2c (Segalés *et al.*, 2008). In Denmark, it was found that PCV2c was circulating predominantly during the 80s, PCV2a during the 90s, and PCV2b from 2001-02 onwards, suggesting a potential genotype switch in the predominance of PCV2 isolates over time (Dupont *et al.*, 2008). In the same study, the analysis of PCV2 sequences available at the National Center for Biotechnology Information

(NCBI) database in February 2007 evidenced that PCV2b became predominant over time in several countries, pointing out to a global genotype switch from PCV2a to PCV2b. Such temporal distribution of PCV2 genotypes and the current predominance of PCV2b in the field is supported by several epidemiological studies worldwide (Allan *et al.*, 2007; Cheung *et al.*, 2007b; Gagnon *et al.*, 2007; Takahagi *et al.*, 2008; Timmusk *et al.*, 2008; Chiarelli-Neto *et al.*, 2009).

The detection of different PCV2 strains in the same pig, either belonging to the same or different genotype, has been also reported (de Boisseson *et al.*, 2004; Cheung *et al.*, 2007b; Gagnon *et al.*, 2007; Grau-Roma *et al.*, 2008; Hesse *et al.*, 2008). Recent studies provided evidence of potential homologous recombination between PCV2 strains that are co-infecting the same animal (Hesse *et al.*, 2008; Cheung, 2009) or concurrently replicating in cell culture (Lefebvre *et al.*, 2009). The authors of these studies suggested that the emergence of new isolates and the potential shift from PCV2a to PCV2b genotype could have been the result of recombination between strains coexisting in the same animal.

Based on the similar reactivity of monoclonal and polyclonal antibodies to different PCV2 strains (Allan *et al.*, 1999b; McNeilly *et al.*, 2001), it was initially thought that no antigenic differences did exist among PCV2 strains. However, recent studies have concluded the opposite. Lefebvre *et al.* (2008) found that 4 out of 16 monoclonal antibodies (mAbs) produced against the Cap protein of a PCV2a strain did not react with PCV2b strains, or showed a reduced affinity compared to PCV2a strains, when tested with an immunoperoxidase monolayer (IPMA) and viral neutralization assays. In addition, none of the tested mAbs was able to neutralize all the seven strains included in that study. Further characterization of the antigenic diversity of PCV2 isolates was provided by Shang *et al.* (2009). In that study, mAbs specific to the Cap protein of PCV1 and PCV2 were used to map type-specific and common epitopes between PCV1 and PCV2, and to identify antigenic differences among PCV2 strains of distinct genotype. Two linear B-cell epitopes specific for PCV2 Cap protein, and two

epitopes shared by PCV1 and PCV2 Cap proteins were identified. The analysis on antigenic diversity in those epitopes revealed three antigenic phenotypes of PCV2 with different genome length, named $PCV2^{1766}$, $PCV2^{1767}$ and $PCV2^{1768}$.

Despite the existence of antigenic differences, the immunity induced after infecting pigs with one genotype conferred protection against subsequent challenge with the other genotype (Opriessnig *et al.*, 2008b).

1.2. PCV2 infection and postweaning multisystemic wasting syndrome (PMWS)

PCV2 infection does not equal to PMWS. In the field, the vast majority of PCV2 infections are sub-clinical, and only a small proportion of PCV2-infected pigs develops the clinical form of disease. At present, the full expression of PMWS is thought to require from other "not yet clearly identified" co-factor(s) that will trigger the progression of PCV2 infection towards disease (Segalés *et al.*, 2005a).

1.2.1. Epidemiological and clinical features

Although PMWS was not reported until 1991 (Clark, 1996), retrospective studies performed on archived samples showed evidence of PCV2 infection from 1962 in Germany (Jacobsen *et al.*, 2009), 1969 in Belgium (Sanchez *et al.*, 2001a), 1970 in the United Kingdom (Grierson *et al.*, 2004), 1973 in Ireland (Walker *et al.*, 2000) and 1985 in Canada and Spain (Magar *et al.*, 2000b; Rodríguez-Arrioja *et al.*, 2003). Nowadays, PCV2 infection is so widespread in the domestic pig population that almost no seronegative farms can be found in epidemiological studies (Larochelle *et al.*, 2003; López-Soria *et al.*, 2005; Grau-Roma *et al.*, 2009). In contrast, PMWS prevalence is generally low, ranging from 4 to 30%, although up to 60% morbidity has been reported in some farms (Segalés & Domingo, 2002). From its first description in Canada to date, PMWS has been

diagnosed in countries from all five continents, and is nowadays considered an endemic disease with a major economic impact in most pig-producing countries.

PCV2 infection can occur during the whole pig productive life, but PMWS usually affects animals from 8 to 16 weeks of age (Sibila *et al.*, 2004; Grau-Roma *et al.*, 2009). The syndrome is clinically characterized by growth retardation, loss of weight and death. Other clinical signs such as respiratory distress, diarrhoea or pallor of the skin are also frequently described in affected pigs. Between 70%-80% of diseased animals die (Segalés & Domingo, 2002). Figure 1.2 shows the wasting condition of a PMWS-affected pig compared to a clinically healthy pen mate.

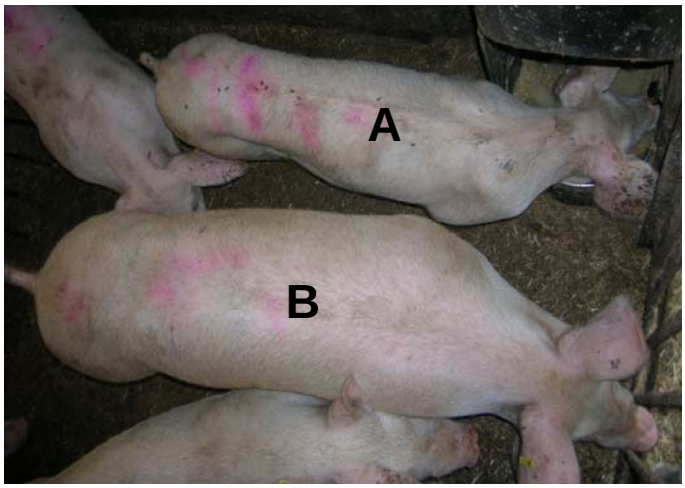


Figure 1.2: PMWS-affected pig (A) and an age-matched clinically healthy animal (B). Note the growth retardation and the marked spinal cord in the affected pig.

1.2.2. PCV2-associated pathology

The most evident pathological findings associated with PMWS, although not always present, are enlarged lymph nodes and non-collapsed lungs. To a lesser extent, atrophic thymus, white-spotted kidneys and ulceration of *pars* oesophagea of the stomach can also be reported (Rosell *et al.*, 1999).

Unlike gross findings, which are unspecific and variable, microscopic lesions associated with PCV2 are unique. Typically, lesions are found in lymphoid

tissues and consist of lymphocyte depletion combined with histiocytic and/or multinucleated giant cell infiltration. Basophilic intracytoplasmatic inclusion bodies can be found in monocytic lineage cells (Rosell *et al.*, 1999). The severity of these lesions is directly related to the status of disease (Segalés *et al.*, 2004b; Opriessnig *et al.*, 2007). Thus, PMWS-affected pigs can be differentiated from sub-clinically affected ones by the intensity of PCV2-associated lesions.

1.2.3. Diagnosis

The complexity of PMWS diagnosis relies on the fact that the sole detection of PCV2 in serum or tissues of a pig, even in the presence of clinical signs compatible with PMWS, is not conclusive to establish a diagnosis. Thus, sub-clinical infections are common, and the clinical signs and gross findings of the disease are non-specific and shared with several other infectious and non-infectious pig diseases.

At present, three diagnostic criteria must be fulfilled to establish an individual diagnosis of PMWS (Segalés *et al.*, 2005a):

A. Clinical signs compatible with PMWS (growth retardation and wasting).
B. Moderate to severe histopathological lesions characterized by lymphocyte depletion together with granulomatous inflammation (Figure 1.3A).
C. Moderate to high amount of PCV2 genome/antigen within lesions (Figure 1.3B).

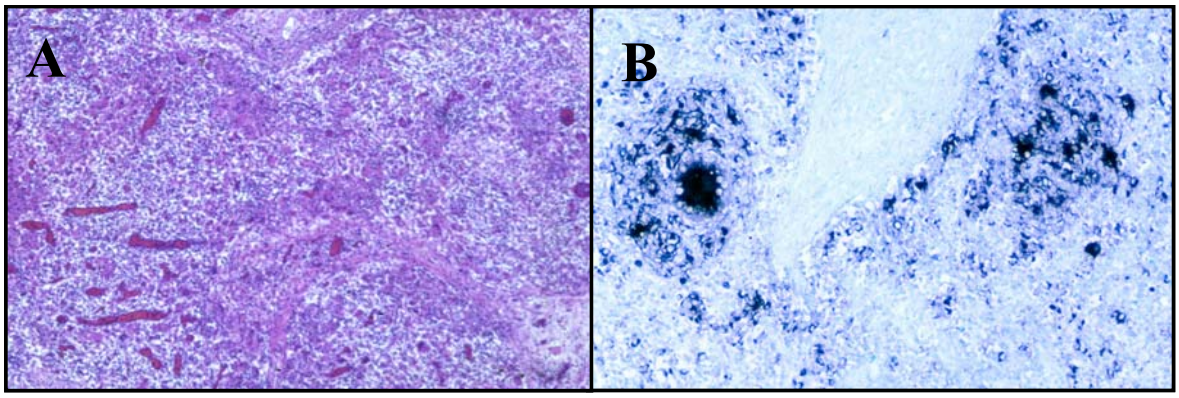


Figure 1.3: (A) Moderate to severe lymphocyte depletion and granulomatous infiltration in lymphoid tissues. Hematoxylin and eosin stain. (B) PCV2 DNA (moderate to high amount) detected in lymphoid tissues by *in situ* hybridization (ISH). Fast green counterstain.

A diagnosis of sub-clinical PCV2 infection is established when, although PCV2 is detected in blood and/or tissues, the amount of viral load is low and associated with no or minimal lesions (Opriessnig *et al.*, 2007).

Diagnostic performance of serological and virological techniques aimed to differentiate between sub-clinical infections and PMWS cases on living pigs has been assessed. By means of PCV2 load quantification in sera, Olvera *et al.* (2004) suggested 10^7 DNA copies/ml as potential threshold to distinguish between PMWS and sub-clinically affected pigs. Using the same technique in combination with serological tests (IPMA or seroneutralization) allowed the confirmation or exclusion of PMWS diagnosis (Fort *et al.*, 2007; Grau-Roma *et al.*, 2009). Nevertheless, those studies concluded that such techniques might be useful to refine PMWS diagnosis, but cannot be used alternatively to histopathology and detection of PCV2 in tissues.

Since individual PMWS cases can be diagnosed in herds with no increased mortality rates and no economic losses associated with PMWS (Nielsen *et al.*, 2008), the need to formally establish a herd case definition arised. Therefore, the European Union (EU) Consortium on PCVD (www.pcvd.org, 2005) wrote a proposal in which two criteria to diagnose PMWS at the level of a farm were defined:

1. Significant increased postweaning mortality. This situation is considered when current mortality (over a period of 1-2 months) is equal or higher than the mean historical mortality (over a period of at least 3 months) plus 1.66 times the standard deviation. Alternatively, the increase might be also determined statistically by means of the chi-square test. If historical data is not available, increased mortality is determined when the herd postweaning mortality exceeds the national or regional level by 50% or more.
2. Individual pigs fulfilling the individual PMWS case definition (at least one out of five necropsied pigs has to be reported).

1.2.4. Pathogenesis

Figure 1.4 shows an overview of the events taking place in the course of PCV2 infection and the reported differences between pigs that develop PMWS and those that remain sub-clinically infected. Little is known about the events taking place at very early stages of infection and target cells for PCV2 early replication have not been identified yet. In PCV2-infected pigs, the highest amount of PCV2 is found in the cytoplasm of monocyte/machrophage lineage cells (Rosell *et al.*, 1999; Sanchez *et al.*, 2004) and *in vitro* studies showed that PCV2 is able to infect these cells, remaining persistently for extended periods of time with no apparent active replication (Gilpin *et al.*, 2003; Vincent *et al.*, 2003). It was therefore suggested that, although monocytic cells may not be the primary target for PCV2 replication, they may represent a dissemination mechanism for PCV2 throughout the host (Vincent *et al.*, 2003). In addition, PCV2 is able to induce functional impairment of *in vitro* cultured dendritic cells (DC) (Vincent *et al.*, 2005). This later fact might be critical due to the central role of these cells in mediating innate and virus-specific immune responses. However, innate immunity mounted by pigs following PCV2 infection has not been studied so far, and thus, whether the PCV2-induced impairment of DC functionality observed *in vitro* does also occur *in vivo* remains unknown. In this respect, this Thesis will

provide information to clarify the immune events taking place at early stage of PCV2 infection.

PCV2 viremia is first detectable around 7 days post inoculation (PI), and viral titres increase and reach a peak between days 14 and 21 PI (Rovira *et al.*, 2002; Resendes *et al.*, 2004a; Opriessnig *et al.*, 2008b). By that time, PCV2 can be present in several organs, although the highest viral loads are typically detected in lymphoid tissues. Besides monocytic lineage cells, PCV2 has been detected in epithelial cells from kidney and the respiratory tract, endothelial cells, lymphocytes, enterocytes, hepatocytes, smooth muscle cells, pancreatic acinar and ductular cells (McNeilly *et al.*, 1999; Rosell *et al.*, 1999; Shibahara *et al.*, 2000; Sanchez *et al.*, 2004). Since PCV2 does not code for its own DNA polymerases and cells in the S-phase are necessary for the virus to complete its infectious cycle (Tischer *et al.*, 1987), it is assumed that the cells with higher mitotic rates are the most efficient supporting replication of PCV2. Although earlier studies suggested that lymphocytes are not targets for PCV2 replication (Gilpin *et al.*, 2003; Vincent *et al.*, 2003), recent studies suggest the opposite. Thus, by measuring Cap mRNA, PCV2 replication was revealed in lymphocyte populations from PBMC and bronchial lymph nodes from experimentally PCV2-inoculted pigs (Yu *et al.*, 2007a). In addition, *in vitro* studies showed that Concanavalin A (ConA)-stimulated PBMC are susceptible to PCV2 replication (Yu *et al.*, 2007b; Lefebvre *et al.*, 2008b; Lin *et al.*, 2008). Further characterization of the PCV2-infected leukocyte subpopulations indicated that mainly circulating T ($CD4^+$ and $CD8^+$) and, to a lesser extent, B lymphocytes (IgM^+) might support PCV2 replication, whilst PBMC-derived monocytes apparently do not (Yu *et al.*, 2007b; Lefebvre *et al.*, 2008b; Lin *et al.*, 2008).

Between the second and third week PI, specific immune responses to PCV2 develop (Pogranichnyy *et al.*, 2000; Resendes *et al.*, 2004a; Meerts *et al.*, 2005). The ability of a pig to mount an adequate adaptive immune response has been suggested as the determinant to avoid progression of PCV2 infection towards

PMWS. Thus, the onset of PCV2 antibody development is typically followed by a decrease of viral titres that usually ends up with the resolution of viremia (Resendes *et al.*, 2004a; Meerts *et al.*, 2005; Fort *et al.*, 2007; Opriessnig *et al.*, 2008b). In those sub-clinically affected pigs, the amount of PCV2 detected in tissues is low, resulting in no or minor alterations of the immune system, from which they recover. It is important to note, however, that long-lasting PCV2 viremia and/or detection of PCV2 in tissues have been reported in experimentally sub-clinically infected pigs despite the presence of high PCV2 antibody titres (Allan *et al.*, 1999a; Pogranichnyy *et al.*, 2000; Resendes *et al.*, 2004a). Conversely, poor humoral responses (Bolin *et al.*, 2001), and, in particular, the absence of PCV2-specific neutralizing antibodies (NA) (Meerts *et al.*, 2006), are related to an increased viral replication, resulting in severe lymphoid lesions and significant changes of the immune system. Such events lead pigs to the immunosuppressive status characteristic of PMWS.

As seen under experimental conditions, field studies performed on PMWS and non-PMWS affected farms also reveal important differences between the events taking place during clinical and sub-clinical PCV2 infections. From lactation to the growing-finishing period, the proportion of PCV2-positive pigs and viral loads increase gradually and coincidentally with the waning of maternally derived PCV2 antibodies (MDA). The highest infectious pressure is found at the time the disease appears (Calsamiglia et al., 2002; Grau-Roma et al., 2009). Diseased pigs display higher viral loads in sera and in sites of potential excression, together with a weaker antibody response compared to sub-clinically infected ones (Fort et al., 2007; Grau-Roma et al., 2009; Olvera et al., 2004). In those sub-clinically infected pigs, the duration of viremia is variable, ranging from 5 to 21 weeks, and a high proportion of pigs might be viremic and, at the same time, have high levels of PCV2 antibodies (Sibila *et al.*, 2004).

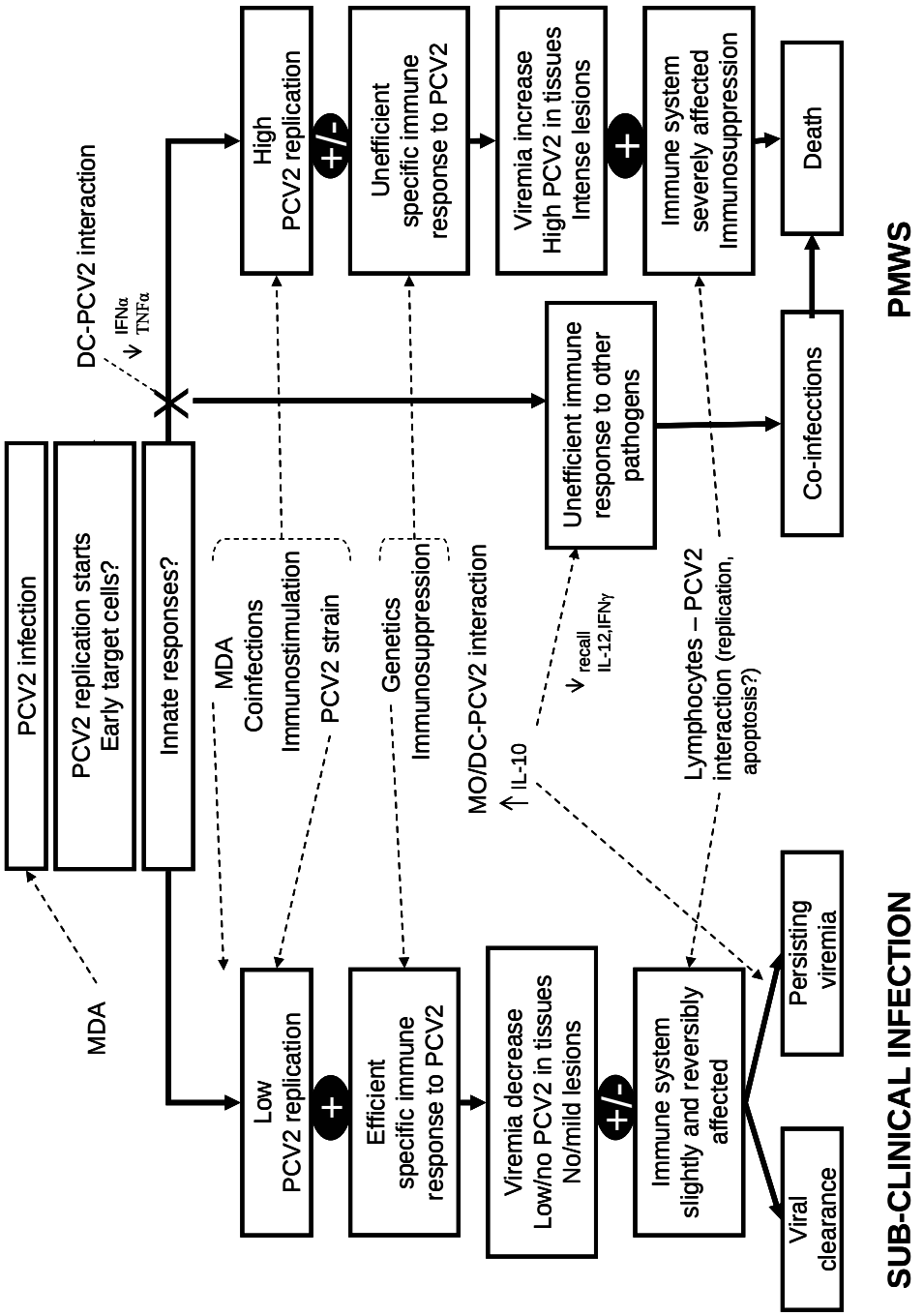


Figure 1.4. Overview of the events taking place in the course of PCV2 infection in an experimental model. Potential factors involved in its progression into either sub-clinical infection or PMWS are indicated in dashed lines. MDA: maternal derived antibodies; DC: dendritic cells; MO: macrophages

1.2.5. Factors influencing the progression of PCV2 infection into PMWS

To date, the mechanisms by which pigs develop PMWS or remain PCV2 sub-clinically infected remain unclear. Several factors have been identified to influence the progression of PCV2 infection towards PMWS (Figure 1.4) and, consequently, the pathogenesis of the disease is not yet fully understood. Its complexity is further evidenced by the lack of a universal reproducible experimental model of disease. Attempts to reproduce PMWS with PCV2 alone yielded successful results in a limited number of studies (Allan *et al.*, 1999a; Kennedy *et al.*, 2000; Bolin *et al.*, 2001; Harms *et al.*, 2001). In most occasions, the sole inoculation with PCV2 results in sub-clinical infections (Balasch *et al.*, 1999; Ellis *et al.*, 1999; Magar *et al.*, 2000a; Pogranichnyy *et al.*, 2000; Albina *et al.*, 2001). Chances to achieve clinical disease increase when PCV2 is inoculated in combination with another swine pathogen, such as porcine parvovirus (PPV) (Allan *et al.*, 1999a; Ellis *et al.*, 2000; Hasslung *et al.*, 2005), porcine reproductive and respiratory syndrome virus (PRRSv) (Allan *et al.*, 2000; Rovira *et al.*, 2002) or *Mycoplasma hyopneumoniae* (Opriessnig *et al.*, 2004). Enhancement of PCV2 replication and development of PMWS has been induced as well after the stimulation of the immune system, either by the injection of immunostimulating products (Krakowka *et al.*, 2001; Kyriakis *et al.*, 2002; Grasland *et al.*, 2005; Wang *et al.*, 2007) or vaccination (Opriessnig *et al.*, 2003). Based on all these data it has been postulated that stimulated lymphoid tissues – either by concomitant infections or any other immunostimulating factor – are the optimum environment for PCV2 replication. Interestingly, the induction of an immunosuppressive status by injecting pigs with cyclosporine or dexamamethasone also resulted in up-regulation of PCV2 replication in tissues of infected pigs (Krakowka *et al.*, 2002; Kawashima *et al.*, 2003; Meerts *et al.*, 2005). In this context, increased viral loads were attributed to the artificially induced impairment of immune responses of treated pigs.

To gain insight into the pathogenesis of the disease, several experimental and field studies aimed to identify factors influencing the PMWS outcome. Thus, factors inherent to the individual, to the virus and factors influencing the host-virus interaction have been reported.

With regards to host-dependent factors, it has been postulated that susceptibility to PMWS might be genetically determined since some field and experimental data point towards certain breeds or genetic lines for being more frequently affected (López-Soria *et al.*, 2004; Opriessnig *et al.*, 2006a; Opriessnig *et al.*, 2009b). In addition, the fact that pigs with identical genetic background might display different clinical outcomes following PCV2 experimental inoculation was suggestive of the existence of interindividual genetic variability as well. Very recently, it has been reported that porcine chromosome 13 might contain genes conferring susceptibility to PMWS and one potential gene candidate has been identified (Karlskov-Mortensen *et al.*, 2008). This gene encodes for *MyRIP,* a protein involved in vesicle trafficking and exocytosis. The authors speculated that persistence of PCV2 in cellular vesicles of the innate immune system cells could be related to the lack of cytosolic trafficking mediated through *MyRIP*. However, PMWS susceptibility could not be related to any polymorphism in the coding part of *MyRIP*, neither with a differential expression of this gene between healthy and disease pigs.

Regarding virus-dependent factors, it was demonstrated that pigs experimentally inoculated with a PCV2 subjected to a high number of passages in cell-culture had reduced viremia and PCV2-associated lesions compared to pigs inoculated with the wild-type PCV2 (Fenaux *et al.*, 2004b). Those differences were attributed to two aa mutations in the Cap protein, suggesting that minimal changes in the genome of PCV2 might account for differences in virulence and PCV2-associated pathology. It has been also postulated that the genotype of PCV2 might influence the progression of PCV2 infection. Several epidemiological studies found that PCV2b isolates were associated with PMWS

occurrence to a higher frequency compared to isolates belonging to the PCV2a genotype, suggesting the existence of differences in virulence among PCV2 genotypes (Carman *et al.*, 2006; Cheung *et al.*, 2007b; Gagnon *et al.*, 2007; Grau-Roma *et al.*, 2008; Wiederkehr *et al.*, 2009). Such association, however, could not be reproduced in a controlled study designed for this purpose using two PCV2 isolates from each genotype (Opriessnig *et al.*, 2008b).

The presence of passively acquired PCV2 antibodies at the time of PCV2 exposure is also an important factor to consider. Several studies performed on naturally and experimentally PCV2 infected pigs revealed that viral replication and the expression of PMWS is highly influenced by the presence of PCV2 antibodies. Under field conditions, it has been shown that pigs do not usually develop PMWS before four weeks of age (Rodríguez-Arrioja *et al.*, 2002; Grau-Roma *et al.*, 2009), neither do they get infected while MDA are present (Grau-Roma *et al.*, 2009). The authors of these studies suggested that maternally derived immunity might protect against PCV2 infection and disease. Under experimental conditions, the protective effect of MDA has been proved and demonstrated to be dependent on the antibody titre attained through the colostrum intake (McKeown *et al.*, 2005; Ostanello *et al.*, 2005).

Besides virus- and host-dependent factors, other factors, mainly related to the management routines implemented in the herd, have been reported to increase the risk of suffering PMWS (Rose *et al.*, 2003; López-Soria *et al.*, 2005). No scientific data are available in regards to how management measures might influence the appearance of disease. However, it is known that certain routines such as mixing pigs from different ages or large pens may increase the stress level of the pig. Stress-related down-regulation of the immune system might explain the higher occurrence of PMWS following certain management measures.

Despite all the research done, the fine mechanism(s) by which the abovementioned factors trigger the development of PMWS is not known. However, when all the accumulated data are considered, it becomes evident that PMWS-affected pigs mainly differ from sub-clinically affected ones in the PCV2 load and in the severity of the damage of the immune system (Figure 1.4). Both facts point towards a balance between the pig capability to mount an efficient immune response to PCV2 and the ability of the virus to evade/dampen the immune control as the key events in the pathogenesis of PMWS.

1.2.6. Control measures

Prior to the availability of PCV2 vaccines, control measures of PMWS were focused on the control of risk factors involved in the progression of the disease. A 20-point plan of recommendations to help producers identify and adjust management practices that favour disease appearance was proposed (Madec *et al.*, 2001). Such recommendations were mainly focused to reduce the infection pressure of PCV2 and other infectious agents and to minimize stress of pigs. Main points of Madec's plan include 1) limiting pig-to-pig contact between different litters, 2) reduction of stress, 3) optimize hygienic conditions by improved disinfection and cleaning procedures, and 4) good nutrition. The implementation of Madec's plan significantly reduced PMWS-associated losses, and is nowadays still considered a guideline for intervention strategies to control PMWS.

At present, besides controlling the triggering factors of PMWS, prevention of disease outbreaks can be effectively achieved by controlling PCV2 infection through vaccination. The available information on the commercial PCV2 vaccines is reviewed in section 3.2.2.1.

1.3. PCV2 and the immune system

1.3.1. Immunopathogenesis of PCV2 infections

1.3.1.1. Immunomodulatory activity of PCV2

The notion that PMWS-affected pigs suffer from an acquired immunodeficiency (Darwich *et al.*, 2004) led to the speculation that PCV2 infection might modulate the host's immune defences. Several *in vitro* studies have recently supported this hypothesis and clarified some aspects of the complex interaction between PCV2 and the cells of the immune system.

In DC, PCV2 infection induces functional impairment of their activity depending on the subpopulation (Vincent *et al.*, 2005). In myeloid DC (mDC), infection with PCV2 did not alter their ability to process and present antigen to T lymphocytes, nor did it interfere with DC maturation (Vincent *et al.*, 2003; Vincent *et al.*, 2005). Conversely, the interaction of PCV2 with plasmocitoid DC (pDC), also known as natural interferon producing cells (NIPC), induced impaired responsiveness to danger signals. Thus, PCV2 induced inhibition of interferon (IFN)-α and tumor necrosis factor (TNF)-α normally produced by NIPC upon interaction with oligodeoxynucleotides (ODNs) with central CpG motifs (CpG-ODN), thereby interfering with NIPC maturation as well as the paracrine maturation of mDC (Vincent *et al.*, 2005). Due to the importance of DC cells in mediating innate defences, the ability of PCV2 to interfere with their functionality might represent a major barrier to the development of an adequate immune response, either against PCV2 itself or against any other pathogen (Vincent *et al.*, 2007).

When PCV2 is added to *in vitro* cultured alveolar machrophages (AM), an altered production of certain cytokines and/or chemokines is observed. Thus, PCV2 infection decreased production of O_2 free radicals and H_2O_2, and increased

TNF-α, interleukin (IL)-8, alveolar macrophage-derived neutrophil chemotactic factors-II (AMCF-II), granulocyte colony-stimulating factor (G-CSF), and monocyte chemotactic protein-1 (MCP-1) (Chang *et al.*, 2006a). It was speculated that this alteration of the functionality of PCV2-infected AM may favour spread of PCV2, as well as render pigs more susceptible to opportunistic and secondary pulmonary infections.

Addition of PCV2 to PBMC obtained from both healthy and diseased pigs suppressed IL-4 and IL-2 responses to phytohaemagglutinin (PHA) and promoted secretion of IL-10, IL-1β and IL-8 (Darwich *et al.*, 2003a). Interestingly, contrary to what was observed for pDC, PCV2 induced the production of IFN-α in PBMC (Wikstrom *et al.*, 2007). In addition, PCV2 seems to modulate the specific-immune responses developed by pigs to other pathogens (Kekarainen *et al.*, 2008b). Thus, IL-12, IFN-α, IFN-γ and IL-2 recall responses of PBMC after pseudorabies virus stimulation were down-regulated by PCV2. The inhibitory effect on IL-12, IFN-α and IFN-γ responses was mediated by the release of PCV2-induced IL-10. *Ex vivo*, increased levels of this cytokine in serum of PCV2-infected pigs was associated with development of PMWS (Stevenson *et al.*, 2006).

The implication of different components of PCV2 in the modulation of the immune response has been also investigated. Vincent *et al*. (2007) found that PCV2-induced impairment of DC function did not require viral replication and was mediated by the viral DNA. The same authors demonstrated that a minimum concentration of dsDNA (replicative form) was necessary to mediate such inhibition. In PBMC, the PCV2-induced suppression of IL-2 and IFN-γ released upon recall antigen was associated to the whole virus and certain CpG-ODNs derived from its genome. In contrast, PCV2 virus like particles (VLP) did not show any suppressive effect, neither did they modulate IFN-α responses (Kekarainen *et al.*, 2008a). In fact, the modulation of IFN-α production by PBMC could be attributed to the presence of CpG-ODN in the PCV2 genome.

Sequences with both IFN-α-inducing and -inhibitory activities were detected, but IFN-α inducers were predominant (Wikstrom *et al.*, 2007; Kekarainen *et al.*, 2008a). The majority of inhibitory CpG-ODNs were found to be within the *Rep* gene (Kekarainen *et al.*, 2008a). Regarding the IL-10 inducing ability of PCV2, it was only maintained as far as the whole virus was used for stimulating the cells. Neither VLPs nor any of the studied CpG-ODNs were found to be IL-10 inducers (Kekarainen *et al.*, 2008a).

Altogether, these data suggest that PCV2 has the potential to evade the immune control and mediate immunosuppression by impairing the host's immune mechanisms. At present, it is not known why only a small proportion of PCV2-infected pigs become immunocompromised and unable to counteract the immunomodulatroy effect of PCV2.

1.3.1.2. Immunosuppression in PMWS-affected pigs

The most striking evidence of immunosuppression is given by the extensive lesions observed in lymphoid tissues of PMWS affected pigs. These include depletion of B and T lymphocytes combined with an increase in the number of macrophages and loss or redistribution of interfollicular dendritic cells (Chianini *et al.*, 2003). In lymphoid tissues, depletion of T lymphocytes was found to involve mainly $CD4^+$ cells and, to a lesser extent, $CD8^+$ cells (Sarli *et al.*, 2001).

Another feature of immunosuppression in PMWS affected pigs is the alteration of PBMC subsets. In a cross-sectional study in which natural cases of PMWS were compared with healthy pigs, a decrease on IgM^+, $CD8^+$ and $CD4^+/CD8^+$ subpopulations was related to disease (Darwich *et al.*, 2002). The kinetics of such lymphopenia as well as the phenotype of the cells involved was further characterized by Nielsen et al. (2003) under experimental conditions using specific pathogen free (SPF) pigs. Thus, depletion of B ($CD21^+$) and T ($CD3^+$) lymphocytes was observed only in PCV2-inoculated pigs that later developed

PMWS, starting from day 7 PI, and becoming severe at the time of clinical signs occurrence. Changes in T cell subsets involved mainly $CD3^+CD4^+CD8^+$ memory T cells. Conversely, in those PCV2-inoculated pigs that did not show clinical signs, the number of cytotoxic ($CD3^+CD4^-CD8^+$) and γδ ($CD3^+CD4^-CD8^-$) T lymphocytes was increased in comparison to that of control pigs, thereby suggesting an active response to PCV2 infection.

Lymphoid depletion and lymphopenia are consistent features of PMWS affected pigs. However, it is still unknown whether the loss of lymphocytes is a direct effect of PCV2 infection or an indirect consequence of responses to PCV2 infection. Some authors postulated that lymphoid depletion was a result of virus-induced apoptosis (Shibahara *et al.*, 2000; Kiupel *et al.*, 2005). However, contradictory results were found by others (Mandrioli *et al.*, 2004; Resendes *et al.*, 2004b). In a recent study it was observed that PCV2-infected PBMC presented morphological changes typical for cellular degeneration. Those changes were correlated with an increase of viral titres, suggesting that PCV2 infection of PBMC may lead to cell death (Lefebvre *et al.*, 2008b). It has been shown *in vivo* that B and T lymphocytes support PCV2 replication (Pérez-Martín *et al.*, 2007; Yu *et al.*, 2007a). Whether this latter fact underlies the severe lymphoid depletion observed in PMWS-affected pigs remains to be elucidated.

1.3.2. Protective immunity to PCV2

1.3.2.1. Protective immunity developed upon PCV2 infection

1.3.2.1.1. Humoral responses

Most of the published serological surveys for PCV2 are based on the detection of total anti-PCV2 antibodies (TA), without determining their neutralizing activity. In the field, seroconversion for TA occurs in both sub-clinically- and PMWS-affected pigs (Rodríguez-Arrioja *et al.*, 2002; Larochelle *et al.*, 2003; Sibila *et*

al., 2004; Grau-Roma *et al.*, 2009). Whereas some studies found no differences between non-PMWS- and PMWS-affected pigs regarding titres of TA (Larochelle *et al.*, 2003), other works reported weaker responses in diseased pigs (Meerts *et al.*, 2006; Grau-Roma *et al.*, 2009). Under experimental conditions, delayed responses or low titres of TA have been related to the expression of PMWS (Bolin *et al.*, 2001; Ladekjaer-Mikkelsen *et al.*, 2002; Rovira *et al.*, 2002; Okuda *et al.*, 2003; Meerts *et al.*, 2006). Several field and experimental studies have shown that PCV2 might persist in tissues and blood in the presence of high titres of TA (Rodríguez-Arrioja *et al.*, 2002; Larochelle *et al.*, 2003; Sibila *et al.*, 2004; McIntosh *et al.*, 2006) (Krakowka *et al.*, 2000) (Magar *et al.*, 2000a); however, those studies did not discriminate between neutralizing and non-neutralizing antibodies.

It has been demonstrated that PCV2-infected pigs develop PCV2-specific NA (Pogranichnyy *et al.*, 2000; Meerts *et al.*, 2005; Meerts *et al.*, 2006; Fort *et al.*, 2007). Under experimental conditions, NA develop between days 10 to 28 PI (Pogranichnyy *et al.*, 2000; Meerts *et al.*, 2006; Fort *et al.*, 2007) and low titres have been related to an increased PCV2 replication and development of PMWS (Meerts *et al.*, 2006). So far, only one work has investigated the dynamics of NA in naturally infected pigs (Meerts *et al.*, 2006). Longitudinal serum samples from two different field studies, one conducted in Belgium and the other in Denmark, were analysed for the presence of NA. It was shown that maternally derived NA were passively transferred to all piglets. Maternal-derived NA titres faded out around 10 weeks of age in Belgian pigs and at three weeks post-mingling in Danish pigs. In both cases, none of the pigs that developed PMWS seroconverted for NA. In addition, another study showed that the levels of NA were correlated with the clinico-pathological status of naturally infected pigs (Fort *et al.*, 2007). Thus, PCV2- positive pigs with NA titres equal or beyond 1:512 were found to be more likely sub-clinically infected and those with titres ≤ 1:16 had a higher likelihood to suffer from PMWS. It is important to note that not all pigs with low NA titres had low levels of TA (Meerts *et al.*, 2006; Fort *et al.*, 2007). This latter

fact suggests that either some animals develop a humoral response lacking NA or that NA develop later than non-NA. A delay on NA responses has been reported in PCV2 sub-clinically infected pigs (Pogranichnyy *et al.*, 2000; Fort *et al.*, 2007). In addition, there is one study reporting the coexistence of high NA titre and a high viral load in serum and tissues (Fort *et al.*, 2007). Taking together, these data suggest that the sole presence of PCV2 antibodies does not fully guarantee viral clearance when infection has taken place and points out to the role of other immune mechanisms different than humoral responses.

1.3.2.1.2. Cell-mediated responses

The role of adaptive cell-mediated responses on controlling PCV2 infection and disease has not been studied in depth. However, the fact that PMWS-affected pigs have impaired T cell responses (Darwich *et al.*, 2002; Nielsen *et al.*, 2003) is suggestive of their contribution to the protective immunity against PCV2 infection. In addition, it has been shown that artificially induced immunosuppression may potentiate viral replication (Krakowka *et al.*, 2002; Kawashima *et al.*, 2003; Meerts *et al.*, 2005). In gnotobiotic pigs, treatment with cyclosporine A (CyA) before PCV2 inoculation resulted in an increased viral replication. Such effect was suggested to be partly mediated by a CyA-induced impairment of cellular responses (Krakowka *et al.*, 2002; Meerts *et al.*, 2005). Furthermore, IFN-γ mRNA expression levels in PBMC from experimentally PCV2-inoculated pigs were found to correlate with viral replication and CyA immunosuppressed status. Thus, a higher expression of IFN-γ mRNA apparently made pigs less susceptible to PCV2 replication (Meerts *et al.*, 2005). Another study, in which pigs were immunosuppressed with dexametasone, provided similar results. Thus, PCV2 inoculation of dexametasone-treated pigs induced viral replication and PCV2-associated lesions, whereas neither PCV2 genome nor PCV2-associated lesions were detected in pigs inoculated with PCV2 alone (Kawashima *et al.*, 2003).

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1.3.2.2. Protective immunity conferred by PCV2 vaccination

1.3.2.2.1. Commercially available vaccines

At least four commercial vaccines for use in growing pigs and breeding-aged animals are currently available in all major swine producing regions worldwide (Table 1.2).The first vaccine on the market was CIRCOVAC® (Merial), an inactivated PCV2, oil-adjuvanted vaccine for use in sows and gilts 2-4 weeks prior to farrowing. CIRCOVAC® was first used in Europe under temporary license in 2004 and by 2006-07 was already available in most European countries and Canada. The other three commercial products are recombinant vaccines designed for use in growing pigs, around 3-4 weeks of age. Suvaxyn PCV2 One Dose® (Fort Dodge) is based on a chimeric infectious virus containing the immunogenic ORF2 Cap gene of PCV2 into the non-pathogenic genomic backbone of PCV1 (Fenaux *et al.*, 2003). Ingelvac CircoFLEX® (Boehringer Ingelheim) and the vaccines from Intervet-Schering Plough (Porcilis PCV® and Circumvent®) are sub-unit vaccines based on the product of the ORF2 expressed on baculovirus. All four vaccines are based on PCV2a strains.

Data from field studies have demonstrated that all vaccine products show remarkable efficacy. A drastic reduction on PCV2-associated productive losses has been observed in growing pigs, either vaccinated or originated from vaccinated breeding herds. Thus, improved average daily gain and feed conversion, decreased mortality rates and reduced medication cost are some of the benefits observed in vaccinated herds (Fachinger *et al.*, 2008; Kixmoller *et al.*, 2008). Vaccination of sows has been demonstrated to have a beneficial effect on the reproductive performance as evidenced by the improvement of reproductive parameters of vaccinated breeding stocks. Thus, sow and gilt vaccination has been reported to increase the number of live born pigs and the number of pigs per sow per year, and to reduce the number of mummies per sow (Thacker *et al.*, 2008; Villa, 2008). Recently, the potential protective effect of

dam vaccination on preventing PCV2 foetal infection and reproductive failure was investigated under experimental conditions (Madson *et al.*, 2009). PCV2-*naïve* pregnant sows were either vaccinated or given placebo on day 28 of gestation, and by day 56 were inoculated with a PCV2b isolate. Reproductive failure could not be reproduced, but PCV2 infection of *naïve* pregnant sows resulted in foetal infection, from which sow vaccination did not protect. Vaccination of the dam did not prevent colostral shedding of PCV2, suggesting that sow vaccination might not prevent vertical transmission.

Table 1.2: Commercial PCV2 vaccines.

PCV2 Vaccine	**Ingelvac CIRCOFLEX®**	**Suvaxyn® PCV2 One Dose**	**Porcilis PCV® Circumvent®**	**CIRCOVAC®**
Company	Boehringer Ingelheim	FORT DODGE®	Intervet Schering-Plough Animal Health	MERIAL
Antigen	PCV2 Cap protein	Inactivated PCV1-2 chimera	PCV2 Cap protein	Inactivated PCV2
Licensed for	Piglets (2 weeks and older)	Piglets (4 weeks and older)	Piglets **Porcilis PCV®:** 3 days and older **Circumvent®:** 3 weeks and older)	Female breeding-age pigs
Dose and Posology	1 ml IM* Single dose	2 ml IM Single dose	2 ml IM **Porcilis PCV®:** Two doses (3 days and 3 weeks of age) /Single dose (3 weeks of age) **Circumvent®:** Two doses (3 and 6 weeks of age)	2 ml IM **Primary vaccination:** Two doses (3-4 weeks apart), at least 2 weeks before mating (gilts) or farrowing (sows) **Revaccination:** One dose at each gestation, at least 2-4 weeks before farrowing

*IM = intramuscularly

To date, few studies have dealt with the mechanism(s) underlying vaccine-induced protection. It is generally assumed that the main basis of vaccine

efficacy relies on the protective effect of PCV2 antibodies, either passively acquired (sow vaccination) or actively induced (piglet vaccination). However, low antibody responses or lacking antibody development after vaccination apparently does not always rule out protection. Fenaux *et al.* (2004a) found that after the immunization with a chimeric PCV1-2 virus, although not all pigs seroconverted to PCV2, they were still protected from developing PCV2-viremia and clinical signs after challenge with PCV2. The authors of that study suggested a potential role of cell-mediated immunity in vaccine-induced protection. Recently, it was reported that colostrum of vaccinated SPF sows contained PCV2-specific interferon-gamma secreting cells (IFN-γ-SC) (Goubier *et al.*, 2008). The transfer of those cells to their offspring was proved, but their protective effect could not be elucidated since IFN-γ-SC were only detected in newborn piglets within a very short period of time.

1.3.2.2.2. Experimental vaccine prototypes

Besides commercial vaccines, other vaccine prototypes have been designed and tested in several *in vivo* models. Those include inactivated vaccines (Pogranichniy *et al.*, 2004), DNA vaccines (Blanchard *et al.*, 2003a; Kamstrup *et al.*, 2004; An *et al.*, 2008; Fan *et al.*, 2008b; Shen *et al.*, 2008) and recombinant sub-unit vaccines expressing PCV2 viral proteins (Liu *et al.*, 2001; Blanchard *et al.*, 2003a; Ju *et al.*, 2005; Wang *et al.*, 2007; Fan *et al.*, 2008a; Wang *et al.*, 2008; Aravindaram *et al.*, 2009). Data from the abovementioned vaccination studies provide insight into the immune responses generated by the different PCV2 ORFs and their corresponding encoded proteins. Blanchard *et al*. (2003) found that the ORF2-encoded Cap protein was a major immunogen and induced protection against subsequent challenge with PCV2, whilst the product of the ORF1, the Rep protein, was weakly immunogenic. Protective immunity was attributed to the early strong antibody response induced by the Cap but not by the Rep. In the same study, the protection induced by DNA and sub-unit vaccines was compared as well. Interestingly, whereas the immune response elicited by a

sub-unit vaccine neutralized PCV2 infection, DNA vaccination appeared to promote PCV2 replication in pigs (Blanchard *et al.*, 2003b). In contrast, using a murine model, Shen *et al.* (2008) found that vaccination with an ORF2 plasmid (ORF2p) vaccine resulted in a higher efficacy upon challenge with PCV2 compared to the Cap protein. In addition, although both systems had comparable efficacy in eliciting lymphoproliferative responses and Cap-specific $CD4^+$ T cells, ORF2p was superior to the Cap protein in triggering $CD8^+$ T cells and recall virus-neutralizing antibody responses. It was suggested, therefore, that $CD8^+$ T cells and NA responses played crucial roles in the vaccine-induced protection against PCV2. Other studies on live virus vectors reported that recombinant viruses expressing the Cap can elicit specific humoral and/or lymphocyte proliferation responses to PCV2 (Ju *et al.*, 2005; Song *et al.*, 2007; Wang *et al.*, 2007; Fan *et al.*, 2008a; Pan *et al.*, 2008; Wang *et al.*, 2008). However, the immunogenicity of those products was again mostly tested in mice, and the efficacy on pigs was only demonstrated in one of them (Wang *et al.*, 2007).

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Development of PMWS seems to be related with a massive PCV2 replication, while sub-clinical infection – the most common outcome in PCV2 infected pigs – apparently implies the control of viral loads. Weak or delayed antibody responses to PCV2 and, in particular, the lack of NA, have been related to high PCV2 loads and development of PMWS. In contrast, very little is known on the role of cell-mediated immunity. However, certain features of the PCV2 infection, such as the co-existence of persistent viremia and high levels of PCV2 antibodies or the fact that PCV2 is a strongly cell-associated virus, suggest that humoral responses might not be enough to deal with PCV2 infection. Therefore, it was hypothesized that the immune control of PCV2 replication implies the involvement of cell-mediated immunity.

At present, PCV2 vaccines are available, or will become available soon in all countries with a relevant swine industry. Although their efficacy has been largely tested (and proved) in field trials, very few data are available on the immununological mechanisms underlying vaccine-induced protection. On the basis that PCV2-specific NA are protective against PCV2 infection and development of PMWS, it was hypothesized that the primary mechanism behind the protection conferred by vaccination was the induction of NA.

Recent epidemiological studies suggest a shift in predominating PCV2 genotypes over time and PCV2b strains have been postulated to be more virulent than those belonging to PCV2a genotype. All PCV2 commercial vaccines are based on PCV2a strains but their implementation in the field drastically reduces PMWS incidence in farms where PCV2b is predominant. Therefore, PCV2a-based vaccines seem to equally protect against infection with either PCV2a or PCV2b strains.

This Thesis aimed to characterize the immunological responses developed in the course of PCV2 infection and following PCV2 vaccination. The specific objectives were as follows:

- To characterize the immunological profile developed by pigs upon PCV2 infection and to investigate the role of different viral components on the induction of innate and PCV2-specific adaptive responses.
- To characterize the immunological responses developed by conventional pigs after vaccination with one and two doses of a PCV2a Cap based sub-unit vaccine (Porcilis PCV®) and to assess its efficacy upon challenge with PCV2a and PCV2b isolates.
- To investigate the effect of maternally derived antibodies on PCV2 piglet vaccination, and thereby provide information to be used as a guideline for the proper implementation of vaccination programs in the field.

CHAPTER 3

STUDY I:

Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets

3.1. Introduction

Several studies have suggested the interaction between PCV2 and the immune system as a key event in the pathogenesis of PMWS. PCV2 infects monocytic lineage cells (Rosell *et al.*, 1999; Gilpin *et al.*, 2003; Vincent *et al.*, 2003), and its long-lasting persistence within macrophages and DC has been suggested as a potential mechanism of dissemination for PCV2 throughout the body (Vincent *et al.*, 2003). In addition, PCV2 impairs the ability of PBMC to respond to mitogens (Darwich *et al.*, 2003a) and viral DNA has been shown to block the activity of NIPC to produce IFN-α, which in turns affects the ability of these cells to mediate antiviral responses upon the infection (Vincent *et al.*, 2007). Besides, PCV2 has been demonstrated to induce IL-10 secretion in *in vitro* cultured PBMC (Darwich *et al.*, 2003a; Kekarainen *et al.*, 2008b), leading to down-regulation of other cytokines produced during recall antigen responses (Kekarainen *et al.*, 2008b). The authors of these latter studies suggested the involvement of IL-10 in the suppressed Th_1 responses observed during the course of PMWS. This hypothesis is also supported by other works performed with PCV2-experimentally inoculated pigs, in which the elevation of IL-10 in serum was correlated with either development of PMWS (Stevenson *et al.*, 2006) or increased PCV2 load in serum (Darwich *et al.*, 2008). Altogether, these data suggest that the mechanisms used by PCV2 to counteract the host immune defences are likely to lie under the immunomodulatory activity of its viral components.

Most PCV2 infections are sub-clinical and different factors have been postulated as potential triggers for the development of the disease. Experimentally, the most successful models used to reproduce PMWS include co-inoculation with PPV (Allan *et al.*, 1999a) or the use of substances modulating the immune system such as keyhole limpet haemocyanin in incomplete Freund's adjuvant (Krakowka *et al.*, 2001). Occasionally, PMWS has been also reproduced using PCV2 alone (Allan *et al.*, 1999a; Kennedy *et al.*, 2000). Although none of these models has

been consistently and repeatedly demonstrated in conventional pigs, evidences point towards an alteration of the immune response - induced by PCV2 itself or by other yet unknown factors - as the triggering factor for PMWS development.

Lipopolysaccharide (LPS) has been used in previous works aimed to reproduce viral-induced multifactorial respiratory disease, by being inoculated in pigs infected with either PRRSV or porcine respiratory coronavirus (PRCV) (van Gucht *et al.*, 2003; Van Gucht *et al.*, 2006). Results from those studies demonstrated that the inmunomodulatory effect of LPS, seen as an over-expression of certain pro-inflammatory cytokies, was found to correlate with clinical disease. In addition, another study performed by Chang *et al.* (2006) demonstrated that LPS was able to *in vitro* induce up-regulation of PCV2 replication in swine pulmonary macrophages.

The aim of the present work was to gain insight into the immune response generated upon PCV2 infection, by studying the innate and adaptive immune responses in experimentally inoculated pigs, either using PCV2 alone or in combination with LPS as a potential immunostimulant. The parameters evaluated were used to set a reference pattern to be later compared with the events taking place in the course of PMWS.

3.2. Materials and methods

3.2.1. Experimental design

All details on source and housing of animals as well as the experimental conditions have been previously described (Fernandes *et al.*, 2007). Briefly, fifty-four one-week-old colostrum-deprived, caesarean-derived (CDCD) piglets were included in this study. Pigs were randomly distributed into four groups, namely A (n=10), B (n=8), C (n=18) and D (n=18). Group A pigs were kept as uninoculated controls; group B animals were intraperitoneally inoculated with 50

µg/kg of LPS from *Salmonella typhimurium* (Sigma-Aldrich, L7261); group C pigs were inoculated with $10^{5.2}$ 50% tissue culture infectious dose ($TCID_{50}$) of the Burgos strain of PCV2 (1 ml orally and 1 ml nasally) produced in PK-15 cells, and group D pigs received simultaneously PCV2 and LPS at the doses stated above. In order to study the events taking place at the early stages of infection, thirty two piglets were sequentially necropsied within the first 8 days of the experiment for the collection of tissues, serum and PBMC. The remaining pigs were followed up throughout the experimental period, being bled on days 0, 7, 14, 21 and 29 PI for serum (all pigs) and PBMC collection [2 from each control group (A and B) and 5 from each PCV2-inoculated group (C and D)]. During the experimental period, clinical signs were monitored daily and pigs were weighted three times a week until day 29 PI (euthanasia). Table 2.1 summarizes the experimental design.

Animal care activities and study procedures were conducted in accordance with the guidelines of the Good Experimental Practices (GEP), under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona*

Table 2.1: Summary of the experimental design.

Group	Inoculum	Dose	Days post infection								
			Num. piglets sampled per day								
			0	**1**	**2**	**5**	**7**	**8**	**14**	**21**	**29**
A	**MEM**	**2 mL**	6	1	1	1	4	1	4	4	4
B	**LPS**	**50 µg/kg**	4	1	1	1	4	1	4	4	4
C	**PCV2**	**$10^{5.5}TCID_{50}$**	6	3	3	3	6	3	6	6	6
D	**PCV2+LPS**	**B+C**	6	3	3	3	6	3	6	6	6
Total number of piglets			22	8	8	8	20	8	20	20	20

* Minimum Essential Medium

3.2.2. Virological and serological studies

Viral load was determined in serum by means of a quantitative PCR (Q-PCR) (Olvera *et al.*, 2004) and in tissues using an ISH procedure (Rosell *et al.*, 1999).

Humoral responses to PCV2 were assessed by an IPMA and a viral neutralization test (VNT) as described elsewhere (Fort *et al.*, 2007).

3.2.3. Ex vivo cytokine profile determination

Levels of IFN-α, IL-10, IL-1β, IL-8 and TNF-α in plasma samples were examined by means of capture ELISAs developed using commercially available antibodies (anti-IFN-α antibodies from PBL Biomedical Laboratories, Piscataway, NJ, USA; anti-IL-1β, anti-IL-8 and anti-TNF-α from R&D Systems, Spain; and IL-10 from Biosource, Spain). For each ELISA, the cut-off value was calculated as the average of the optical density of negative controls plus three standard deviations. Cytokine concentrations were determined using a regression line built up with the optical densities of the cytokine standards used in each test.

3.2.4. In vitro cytokine profile determination

PBMC were separated from whole blood by gradient density centrifugation using Histopaque 1.077 (Sigma-Aldrich). Cells were cultured for 20 hours at 37ºC in 5% CO_2 ($5x10^5$ cells/well) in presence of either PCV2 (multiplicity of infection (m.o.i) of 0.01 $TCID_{50}$/cell), PHA (10 μg/ml), or were mock-stimulated with cell culture supernatants of uninfected PK-15 cells. Cultures were done at least in triplicate and supernatants corresponding to the same animal and stimulus were mixed for the analysis. Capture ELISAs for IFN-γ (BD, Madrid, Spain), IL-2, IL-4 and IL-10 (Biosource, Spain) were performed and cytokine concentrations were calculated as explained above.

Frequencies of PCV2-IFN-γ-SC in PBMC were determined by ELISPOT at 7, 14, 21 and 29 days PI, by using commercial mAbs (Swine IFN-γ Cytosets kits, Biosource, Spain) following a previously described protocol (Díaz & Mateu, 2005). Briefly, 96-well flat bottom plates (Costar 3590, Corning USA) were coated overnight with anti-IFN-γ antibody at 8.3 µg/ml in carbonate-bicarbonate buffer (pH 9.6). Plates were washed three times with phosphate-buffered saline (PBS) and blocked with PBS containing 10% of foetal bovine serum (FBS) for 1 hour at 37ºC. After removing the blocking solution, 100 µl containing 5 x 10^5 PBMC were dispensed per well and stimulated with either PCV2 (m.o.i=0.01), PHA (10 µg/ml) or mock-stimulated for 20 hours at 37ºC in presence of 5% CO_2. Cells were removed and wells were then incubated 1 hour at 37ºC with 50 µl of the biotinylated antibody at 2.5 µg/ml in PBS containing 0.05% of Tween 20 and 0.5% of bovine serum albumin (PBS-T-BSA). After three washings with PBS-T, plates were incubated 1 hour with Streptavidin-horseradish peroxidase (HRP) (Biosource, Spain) at 0.5 µg/ml in PBS-T-BSA, and finally, insoluble 3,3′,5,5′-tetramethylbenzidine (TMB) blue (Calbiochem, Spain) was used to reveal the reaction. PCV2-IFN-γ-SC were calculated by subtracting the number of spots counted in mock-stimulated wells from PCV2-stimulated ones. Results were expressed as numbers of IFN-γ-SC per million PBMC.

3.2.5. Leukocyte subsets determination

PBMC subsets were phenotypically characterized by flow-cytometry for $CD3^+$ (BD #559582), $CD4^+$ (BD #12516), $CD8^+$ (Southern Biotech #4520-09), $CD21^+$ (Southern Biotech #4530-02) and SWC3/CD172a (Southern Biotech #4525-09) cells. Fifty µl of a cell suspension adjusted at 4x10^6 cells per ml in flow cytometry medium (FCM; PBS containing 0.1% BSA) were placed into a 96 well-plate and incubated for 30 minutes with 50 µl of each mAb, followed by two washes with FCM. Except for $CD21^+$, which was already conjugated with fluorescein isothiocyanate (FITC), and $CD8^+$and $SWC3^+$ with phycoerythrin (PE), a rabbit F(ab´)2 anti-mouse Ig-FITC (Dako, Denmark) was used as

secondary antibody. Finally, PMBC were washed twice and fixed in FMC containing 0.3% paraformaldehyde. The analysis was performed using an EPICS_XL MCL flow cytometer (Beckman-Coulter, USA). Irrelevant isotype-matched antibodies were used as background controls.

3.2.6. Statistical analysis

Statistical analyses to compare means of the different parameters among groups were performed using the general linear model (GLM) procedure (SAS 9.1 software, SAS Institute Inc., Cary, North Carolina, USA). When no differences attributed to the effect of LPS were detected, data were analysed considering PCV2 inoculation as the only classificatory variable, being groups A+B considered as control group and groups C+D as PCV2-inoculated one. Significance level (α) was set at 0.05

3.3. Results

3.3.1. Clinical and pathological outcomes

Details on clinical and pathological data are found in Fernandes *et al.* (2007). At the end of the study (day 29 PI), PCV2-inoculated groups showed lower mean body weight (6.6±2.3 kg in group C and 6.6±2.7 kg in D) compared to control ones (8.0±1.72 kg in group A and 7.5±1.6 kg in B) ($P<0.05$) However, none of the pigs developed clinical signs compatible with PMWS throughout the whole study. One day after inoculation, all pigs receiving LPS (groups B and D) had significantly higher rectal body temperatures compared to groups A and C (39.2±0.9 vs. 38.4±0.4; $P<0.01$); from then onwards, no differences in rectal temperatures were observed among groups. Regarding pathological studies, animals euthanized within the first 8 days PI had no lesions and no detectable virus in tissues by ISH. In contrast, from the 20 piglets necropsied at the end of the study (day 29 PI), 9/12 PCV2-inoculated animals showed presence of mild

PCV2-like lesions in lymphoid tissues together with low to moderate amounts of PCV2 DNA in those lesions as determined by ISH (5/6 in group C and 4/6 in group D).

3.3.2. Viremia and humoral response to PCV2

Results of the Q-PCR showed that PCV2 DNA firstly appeared in sera of PCV2-inoculated pigs by day 7 PI, with increasing titres until day 14 PI in group C ($6.4 \times 10^5 \pm 1.3 \times 10^5$ viral copies/ml) and day 21 PI in group D ($1.3 \times 10^6 \pm 2.8 \times 10^5$ viral copies/ml) ($P>0.05$). PCV2-uninoculated pigs remained free of virus all along the study.

Development of humoral response started in PCV2-inoculated pigs (groups C and D) with the appearance of IPMA antibodies mostly between 7 and 14 days PI, with increasing titres until day 29 PI (mean IPMA titre: 10.3 ± 1.2 $\log_2$). Seroconversion for NA occurred between 21 and 29 days PI, with a mean titre of 5.0 ± 1.1 $\log_2$. No differences between groups C and D were noticed regarding IPMA or NA titres.

3.3.3. Ex vivo cytokine responses

On day 1 PI, all PCV2-inoculated piglets had a transient increase of IL-8 in serum (3/3 in group C: 170±38 pg/ml and 3/3 in group D, 200±23 pg/ml), whereas uninoculated controls remained negative. At the time IL-8 faded out, levels of IFN-α started to be detectable in serum of PCV2-inoculated pigs. Thus, on day 2 PI, one animal in group C and one in D were positive for this cytokine, and, on day 5 PI, sera from all PCV2-inoculated pigs had detectable IFN-α (3/3 in group C, 151±12.7 pg/ml and 3/3 in group D, 149.7±39.1 pg/ml). Later on, positive results were only sporadically detected (Figure 2.1).

Regarding IL-10 detection in serum, only one animal (No 51) from group C was positive (day 7 PI; 154 pg/ml). For IL-1β and TNF-α, most piglets were negative and these cytokines were only detected sporadically regardless of the PCV2 inoculation status (data not shown).

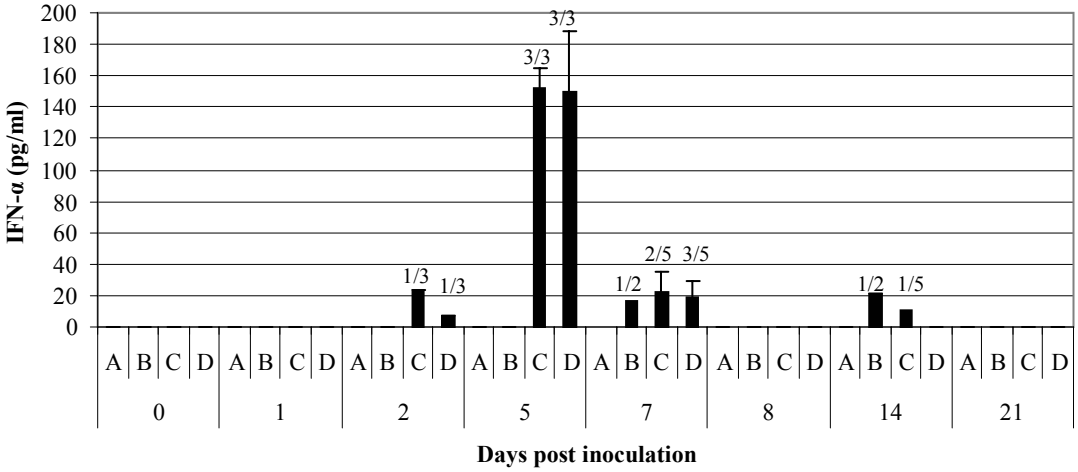


Figure 2.1: Levels of IFN-α (pg/ml) detected in plasma, expressed as mean ± S.D. of positive pigs in each experimental group (A to D), from 0 to 29 days PI. The number of positive pigs at each time point is indicated.

3.3.4. In vitro cytokine responses

After *in vitro* treatment of PBMC with PCV2, no induction on IL-2, IL-4 or IFN-γ was observed. On the other hand, the levels of IL-10 detected in supernatants of PBMC stimulated with PCV2 were significantly higher than those detected in mock-stimulated cultures ($P<0.01$). Indeed, the IL-10 release induced by the virus was observed at all sampling days, with no differences among treatments, although individual results showed certain variation within the same group along the study. Thus, in group A, 9/10 pigs were detected positive (range: 17.2-273.9 pg/ml), 8/8 in group B (range: 19.3-194.4 pg/ml), 16/18 in group C (range: 13.1-298.3 pg/ml), and 15/18 in group D (range: 36.3-282.4 pg/ml).

Development of PCV2-specific IFN-γ-SC was only observed in PCV2-inoculated groups (C and D) and started mostly between 14 and 21 days PI.

Thus, on day 21 PI, 4/5 pigs in group C and 3/4 in group D were positive with mean frequencies of 77±30 and 84±49 PCV2-IFN-γ-SC per million of PBMC (P>0.05), respectively. On day 29 PI, all pigs were positive (mean PCV2-IFN-γ-SC of 73±30 and 59±49 in groups C and D, respectively; P>0.05). Figure 2.2 shows mean values of IFN-γ-SC in PCV2-inoculated pigs (groups C and D), together with mean PCV2 antibody titres and viremia. Individual data are shown in annex of study I.

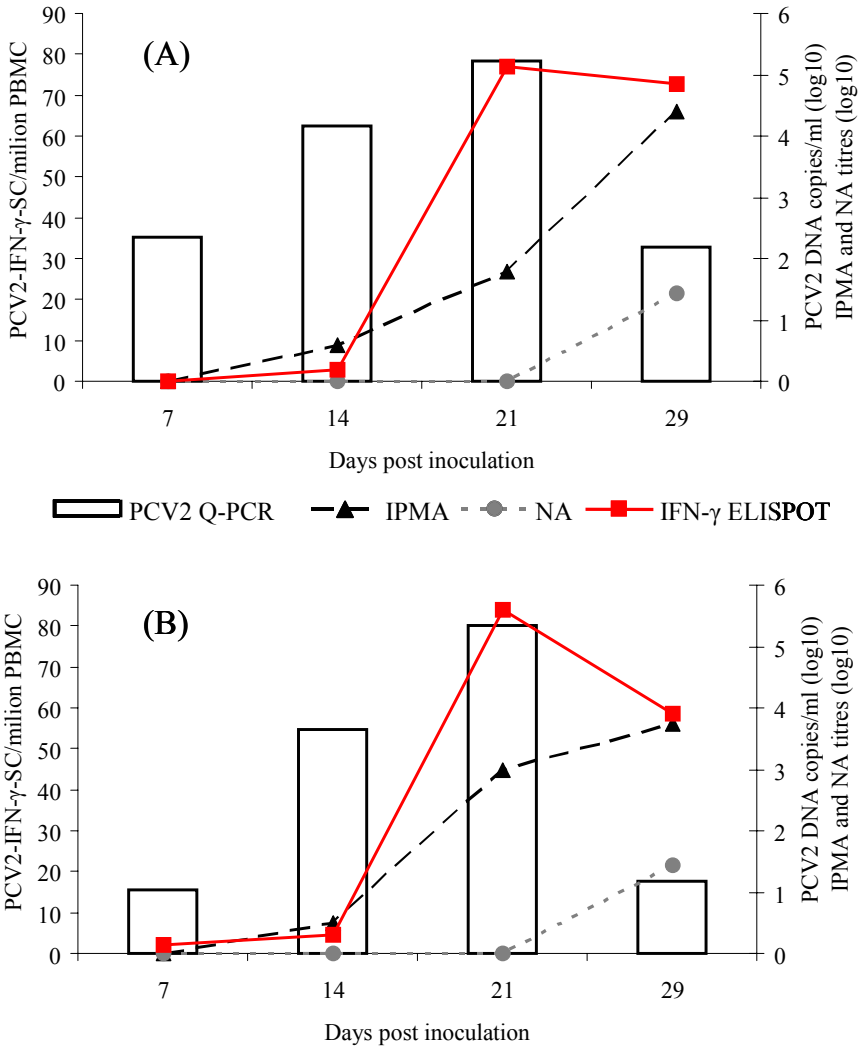


Figure 2.2. Mean values of PCV2-IFN-γ-secreting cells (PCV2-IFN-γ-SC), IPMA antibodies (IPMA), neutralizing antibodies (NA) and viremia (Q-PCR) of PCV2-inoculated groups C (A) and D (B), from 7 to 29 days PI (Only pigs from which PBMC were collected are included).

3.3.5. Leukocyte subsets determination

In the flow cytometry analyses, most changes in the relative proportions of cell subsets were sporadic and transient and could not be attributed to treatments received. However, PCV2-infected pigs showed a decrease in the relative proportions of $CD4^+SwC3^+$ cells (0.3±0.2 vs. 1.1±0.4; $P<0.01$) as well as $CD4^+CD8^+$ lymphocytes (3.5±0.8 vs. 5.9±1.9; $P<0.01$) on days 14 and 21 PI, respectively compared to PCV2-free pigs. In addition, a downshift in $CD21^+$ cells was observed in PCV2-inoculated groups compared to controls at same days (1.5±0.7 vs. 3.3±1.0; $P<0.05$ and 2.1±0.7 vs. 3.0±0.5; $P=0.056$; respectively). By day 29 PI those differences disappeared.

3.4. Discussion

Pathogenesis of PMWS is not fully understood yet and there is not a single parameter or group of parameters to be used as reliable predictors for the development of the disease. Previous reports suggested that the complex host-virus interaction and the subsequent immune response generated might be critical determinants for the understanding of the disease (Meerts *et al.*, 2006; Stevenson *et al.*, 2006; Vincent *et al.*, 2007). In the present study we tried to characterize some of the parameters of the innate and adaptive immune responses against PCV2 in animals that withstand the infection without developing clinical disease.

To date, although reproduction of PMWS has been achieved using several experimental models (Tomás *et al.*, 2008), none of them has been demonstrated to be consistently repeatable in conventional pigs. Among them, the use of immunostimulants and co-infections following PCV2 infection is apparently the most successful strategy to experimentally reproduce clinical disease, suggesting the activation of the immune system as a potential triggering factor of PMWS (Tomás *et al.*, 2008). In the present study, the effect of immunostimulation on the course of PCV2 infection was evaluated in one-week old CDCD piglets by

injecting them with LPS, simultaneously to PCV2-oronasally-inoculation. Although LPS was claimed to have a positive effect on PCV2 replication in pulmonary macrophages (Chang *et al.*, 2006b), our data showed no significant effect of LPS on the evolution of PCV2 infection and the immunological parameters evaluated throughout the experiment. These results suggest that the LPS-induced viral replication reported *in vitro* does not apparently occur *in vivo*, or not extensively enough to trigger PMWS under the present experimental conditions. Nevertheless, further studies such as the local effect of LPS on PCV2 target tissues should be performed to conclude that, since no significant induction of pro-inflammatory cytokines attributed to LPS administration was detect.

Regarding the innate immune responses against PCV2, the earliest event detected following PCV2 infection was an increase in IL-8 in serum (day 1 PI). The ability of PCV2 to induce IL-8 production in porcine alveolar macrophages or PBMC has been previously reported (Darwich *et al.*, 2003a; Chang *et al.*, 2006a) and agrees with the inflammatory nature of the lesions usually seen in PCV2-infected animals. In the present study, other pro-inflammatory cytokines such as IL-1β or TNF-α were not detected in serum with a consistent pattern. However, the lack of detection of these cytokines in plasma does not exclude them from playing a role in early phases of the infection, since they could have been acting locally at the site of viral replication without reaching levels high enough to be detected in serum. In contrast, an IFN-α response was observed in PCV2 inoculated pigs on day 5 PI. IFN-α is considered a crucial cytokine for the host antiviral defences, being involved not only in innate responses but also in regulating the adaptive immunity generated upon viral infections. Indeed, the ability to counteract IFN-α-mediated responses through different pathways, and thereby interfere with the immune mechanisms of the host, has been reported for several viruses (García-Sastre & Biron, 2006). In the case of PCV2, recent studies showed that PCV2 or PCV2-CpG motifs may inhibit or induce IFN-α responses (Vincent *et al.*, 2005; Wikstrom *et al.*, 2007) depending on the cell subset studied and on the structure of CpGs. Vincent et al. (2007) reported that

PCV2 was able to block IFN-α induction in NIPC, suggesting the inmunomodulatory activity of PCV2 as a key event in the pathogenesis of PMWS. In this respect, those animals withstanding the infection without developing clinical disease should be able to counteract the inhibitory activity of PCV2, probably by means of strong innate responses. In the present study, the detection of IFN-α in serum of all PCV2-inoculated pigs at early stage of infection indicated that development of an innate response against the virus was generated. The fact that no PCV2 genome neither PCV2-associated lesions were found in tissues of the early necropsied pigs might be attributed to the ability of PCV2 to persist in monocytic-lineage cells without active replication (Vincent et al., 2003), and therefore being undetectable by a standard HIS technique. These results, together with the fact that PCV2 is known to mediate inhibition of NIPC responsiveness, suggest that the balance between the host ability to mount a proper innate antiviral response and the virus ability to dampen it might be determinant for the infection evolution and triggering of the disease.

Elevated levels of IL-10 in serum were detected only in one PCV2-inoculated pig, on day 7 PI. In contrast, *in vitro* results showed an induction of IL-10 release in response to PCV2 stimulation of PBMC. This occurred regardless of the treatment administrated to the pigs. The involvement of IL-10 in the pathogenesis of PMWS has been suggested in several studies. Darwich *et al.* (2003b) found an overexpression of IL-10 mRNA in thymus of PMWS-affected pigs in correlation with lymphoid lesions. Also, cytokine profile evaluation of blood samples from PCV2-experimentally inoculated pigs showed an association between elevated plasma levels of IL-10 and development of PMWS (Stevenson *et al.*, 2006). *In vitro*, PCV2 has been demonstrated to induce IL-10 secretion in PBMC cultures, which, in turns, lead to repression of other cytokines (Kekarainen *et al.*, 2008b). Altogether, these data suggest that the ability of PCV2 to induce IL-10 might contribute to the immunosuppressive status observed in the course of PMWS. In our study, none of the pigs developed clinical disease and viremia decreased by day 29 in all pigs but one (No. 51).

Interestingly, this pig was the only one with detectable IL-10 in serum. These results suggest that some pigs - those controlling the progression of the infection – might counteract the ability of PCV2 to induce an IL-10 release.

Regarding adaptive responses, IPMA antibodies to PCV2 appeared around 14 days PI, whereas NA appeared 7-14 days later. Inefficient or delayed development of antibody-mediated immunity to PCV2 has been previously correlated with high level of PCV2 replication and the outcome of clinical disease (Okuda *et al.*, 2003; Meerts *et al.*, 2006; Fort *et al.*, 2007) However, certain delay on the neutralizing response has been also observed in PCV2 sub-clinically infected pigs in which PMWS was not developed (Fort *et al.*, 2007).

The cytometric analysis indicated that infected pigs suffered a downshift in $CD4^+SwC3^+$, $CD4^+CD8^+$ and $CD21^+$ cells at 14 and 21 days PI, and these values returned to normality by the end of the study. Changes in PBMC subsets in PCV2 infected pigs that do not developed the disease have been previously reported (Darwich et al., 2004; Nielsen et al., 2003) and differ from those ones observed in PMWS-affected ones (Nielsen et al., 2003); whereas leukopenia observed in pigs infected sub-clinically seems to be transient, leukopenia in PMWS-affected ones appears earlier and is stronger, being correlated with the appearance of clinical disease.

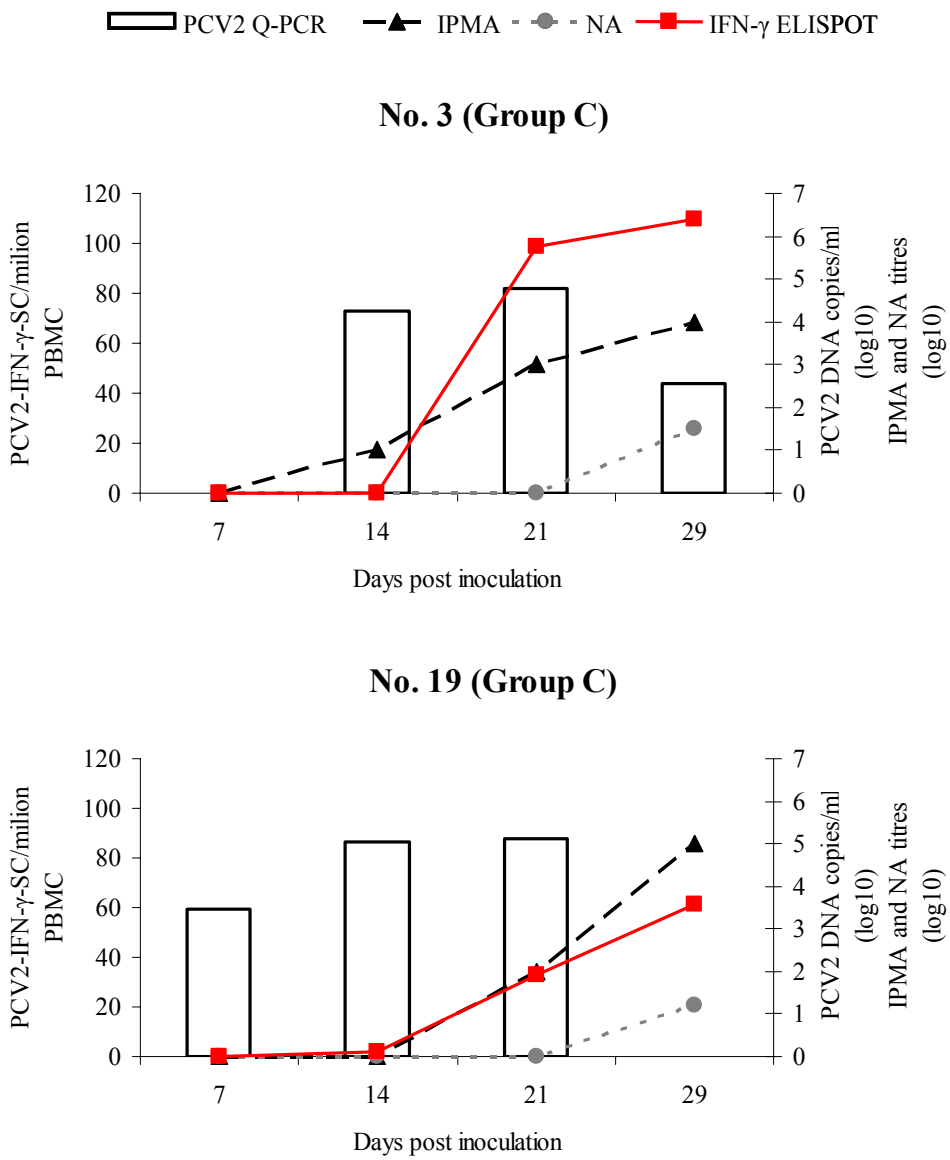
The role of specific cellular defense mechanisms in providing protection against PMWS has not been elucidated yet. In the present study, we describe for the first time the development of IFN-γ-SC in response to PCV2 infection. IFN-γ is considered to be a key cytokine for Th_1 polarisation; by controlling the differentiation of naïve CD4 T cells into Th_1 effectors, this cytokine mediates cellular immunity against viral infections. In the present study, development of IFN-γ-SC in PCV2 sub-clinically infected animals started mostly between days 14-21 PI and was coincidental with the decrease of viral load in blood in all pigs but one. Our results suggest that cell-mediated responses, measurable as IFN-γ-

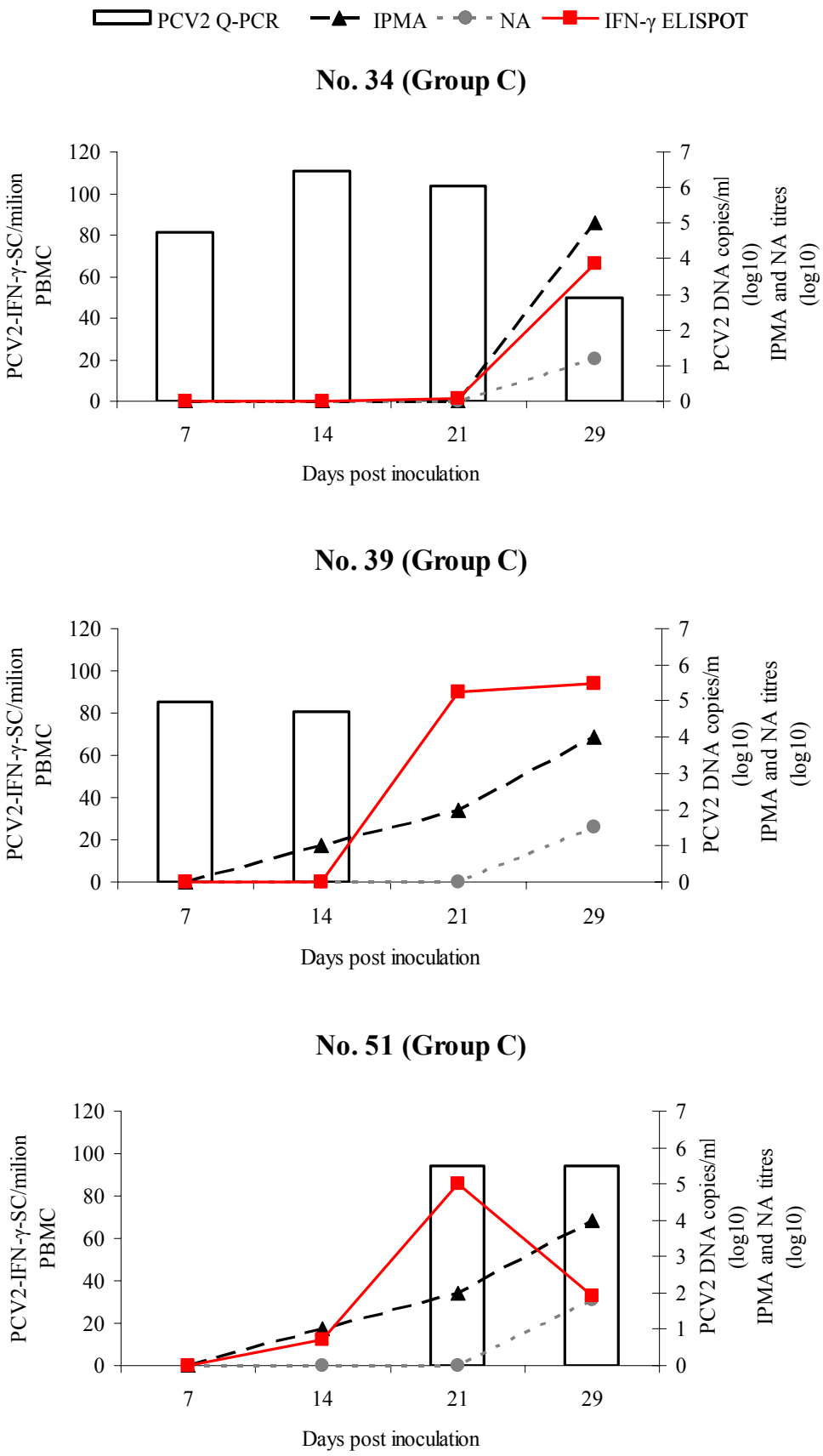
SC, might also contribute, together with development of PCV2 NA, to the clearance of PCV2.

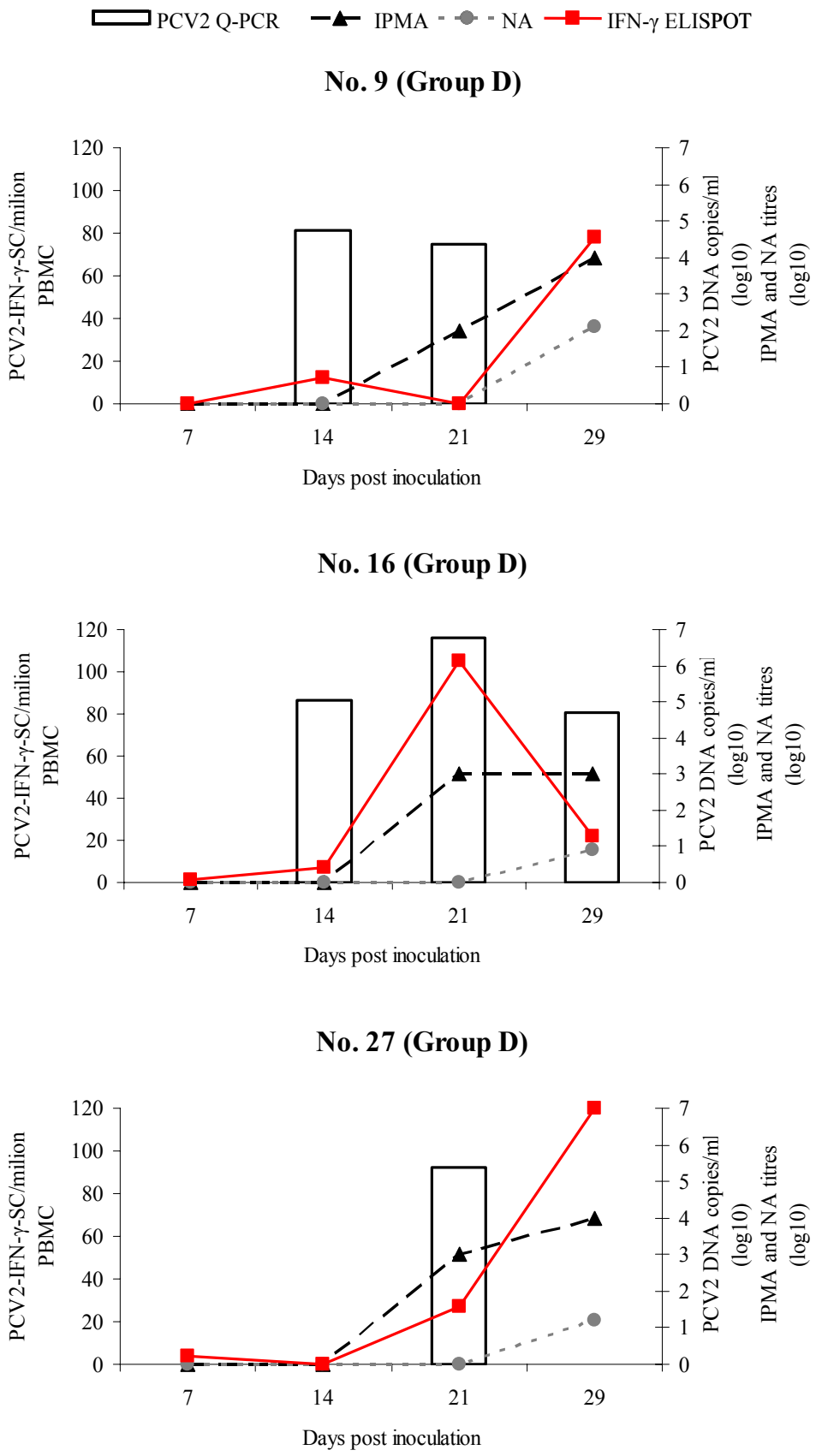
In conclusion, the results of the present study suggest that in PCV2-infected pigs, an early IFN-α response, together with the development of PCV2-IFN-γ-SC and NA may be adequate predictors of the evolution of PCV2 infection and should be explored in experiments aimed to elucidate the mechanisms lying behind the different clinical outcomes of PCV2 infections in pigs.

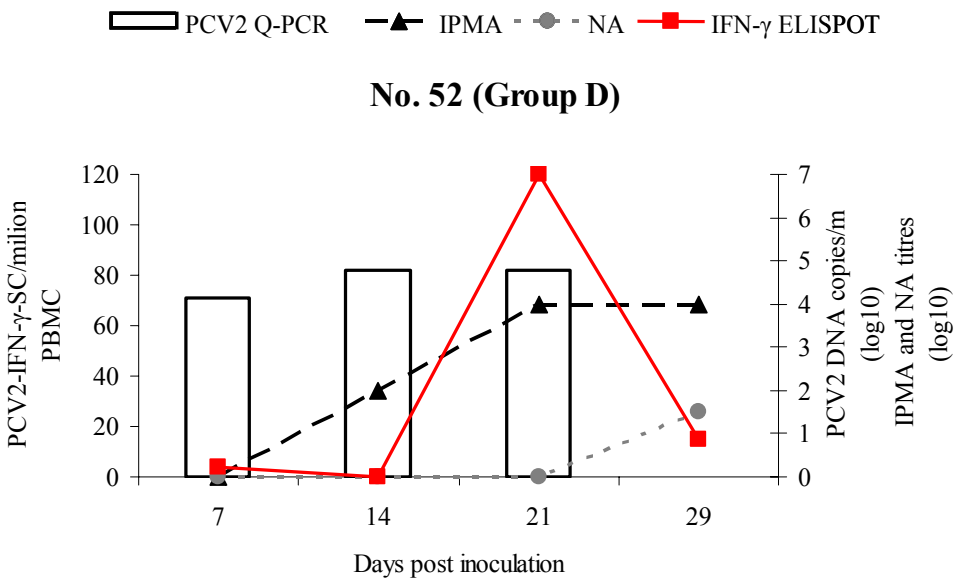
Annex of study I

Individual data on the evolution of PCV2-IFN-γ-secreting cells (PCV2-IFN-γ-SC), IPMA antibodies (IPMA), neutralizing antibodies (NA) and viremia (Q-PCR) of PCV2-inoculated groups C (A) and D (B), from 7 to 29 days PI.




PCV2 Q-PCR
IPMA
NA
IFN-γ ELISPOT
No. 34 (Group C)
PCV2-IFN-γ-SC/milion PBMC
PCV2 DNA copies/ml (log10)
IPMA and NA titres (log10)
Days post inoculation
No. 39 (Group C)
PCV2-IFN-γ-SC/milion PBMC
PCV2 DNA copies/m (log10)
IPMA and NA titres (log10)
Days post inoculation
No. 51 (Group C)
PCV2-IFN-γ-SC/milion PBMC
PCV2 DNA copies/ml (log10)
IPMA and NA titres (log10)
Days post inoculation


PCV2 Q-PCR
IPMA
NA
IFN-γ ELISPOT
No. 9 (Group D)
No. 16 (Group D)
No. 27 (Group D)
PCV2-IFN-γ-SC/milion PBMC
PCV2 DNA copies/ml (log10)
IPMA and NA titres (log10)
Days post inoculation
7
14
21
29
0
20
40
60
80
100
120
1
2
3
4
5
6


PCV2 Q-PCR
IPMA
NA
IFN-γ ELISPOT
No. 52 (Group D)
PCV2-IFN-γ-SC/milion PBMC
PCV2 DNA copies/m (log10)
IPMA and NA titres (log10)
120
100
80
60
40
20
0
7
6
5
4
3
2
1
0
7
14
21
29
Days post inoculation

CHAPTER 4

STUDY II:

Porcine circovirus type 2 (PCV2) Cap and Rep proteins are involved in the development of cell-mediated immunity upon PCV2 infection

4.1. Introduction

PCV2 genome is composed of 1,767-1,768 nucleotides codifying for 11 putative ORF (Hamel *et al.*, 1998). So far, protein expression has been only described for three of those ORFs. ORF1 encodes for the Rep protein and its splicing variant Rep', both considered essential proteins for viral replication (Cheung, 2003). ORF2 encodes for the Cap protein, the only structural protein of PCV2 (Nawagitgul *et al.*, 2000). The product encoded by ORF3 is a non-structural protein that has been recently associated with viral replication and pathogenesis *in vivo* (Liu *et al.*, 2006; Karuppannan *et al.*, 2009). At present, Cap is considered to be the most immunogenic protein of PCV2 (Pogranichnyy *et al.*, 2000; Blanchard *et al.*, 2003a) and at least three conformational neutralizing epitopes within this protein have been identified so far (Lekcharoensuk *et al.*, 2004; Shang *et al.*, 2009). The role of Rep protein in the immune responses against PCV2 has not been extensively studied yet. Under experimental conditions it was demonstrated that CDCD piglets inoculated with PCV2 developed antibodies against three different viral proteins with molecular masses of 28 (Cap), 28.5 (Rep') and 35 (Rep) Kd (Pogranichnyy *et al.*, 2000). In addition, a serological survey performed on PMWS-affected farms revealed the presence of Cap and Rep antibodies in both PCV2 sub-clinically infected and PMWS-affected pigs (Pérez-Martín *et al.*, 2008). In both studies, Cap-specific antibodies appeared earlier and reached higher titres than anti-Rep antibodies. The fact that all commercial PCV2 vaccines are based on either inactivated virus or Cap protein (Opriessnig *et al.*, 2007) suggests that the protective immunity against PCV2 involves the development of Cap antibodies.

Up to now, most studies on the immune responses taking place during PCV2 infection or after vaccination have focused mainly on the development of antibodies (Pogranichnyy *et al.*, 2000; Bolin *et al.*, 2001; Ladekjaer-Mikkelsen *et al.*, 2002; Rovira *et al.*, 2002; Blanchard *et al.*, 2003a; Fenaux *et al.*, 2004a; Meerts *et al.*, 2006; Opriessnig *et al.*, 2008a). However, both humoral and

cellular compartments of the immunity are apparently involved in the immune responses developed upon PCV2 infection (Meerts *et al.* 2005, Study I), and high levels of viral replication leading to PMWS have been associated with impaired neutralizing and T-cell immune responses (Krakowka *et al.*, 2002; Nielsen *et al.*, 2003; Meerts *et al.*, 2006).

The aim of the present study was to gain insight into the immunological responses developed by pigs infected with a PCV2a or a PCV2b isolate, with emphasis on cell-mediated immunity and the role of Cap and Rep proteins in its development.

4.2. Materials and methods

4.2.1. Viruses and viral proteins

Stoon-1010 (PCV2a genotype) and Sp-10-7-54-13 (PCV2b genotype) isolates were used as challenge viruses. For the inoculation of pigs, the isolates were propagated in PCV-free PK-15 cells and harvested after lysis and centrifugation of cell cultures. For *in vitro* studies, only cell culture supernatants were collected. Uninfected PK-15 supernatants were used as mock-stimulus. To evaluate whether the passage number at which the strains were used (passage 30^{th} and 14^{th} for Stoon-1010 and Sp-10-7-54-13, respectively) could account for changes in the aa composition, a comparative analysis of the ORF2 sequences among the challenge viruses and their parental viral seeds was performed. Since a very early passage of Stoon-1010 was not available, the first sequence of this strain submitted to the GenBank database (accession number AF055392) was considered as its parental sequence. For the Sp-10-7-54-13 isolate, the ORF2 was sequenced from the PCR product obtained from the pig serum (Fort *et al.*, 2007).

Baculovirus PCV2 VLPs (Cap) and baculovirus preparations without VLPs (Bac) were kindly provided by Boehringer Ingelheim Vetmedica Inc (St. Louis, MO,

USA). The Rep protein and the mock baculovirus extract (Ni) were expressed in baculovirus and produced in *Trichoplusia ni* larvae (Pérez-Martín *et al.*, 2008).

4.2.2. Experimental design and sampling

Seventeen conventional piglets obtained from a PCV2 seropositive farm were included in the experiment. Animals were checked for the presence of PCV2 antibodies by an IPMA (Fort *et al.*, 2007), and distributed into three groups balanced according to weight and antibody titres. At 6 weeks of age, pigs were intranasally inoculated with a total of $5x10^{6.5}$ $TCID_{50}$/pig of either Stoon-1010 (Stoon group; n=6) or Sp-10-7-54-13 (Sp group, n=7) isolates. Four pigs were kept as controls and were given an equal volume of PBS (PBS group) by the same route of administration.

Blood samples were taken in duplicate (siliconized and heparinized tubes) at challenge, 7, 14 and 21 (necropsy) days PI. Serum was stored at -80ºC until further use. PBMC were immediately isolated from heparinised blood by density gradient centrifugation using Histopaque 1.077 (Sigma-Aldrich).

The animal care activities and the study procedures performed in the present work were authorised and by the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona*.

4.2.3. Clinical and pathological studies

Pigs were monitored daily for clinical signs from day 0 to 21 PI. Rectal temperatures were measured every two days and body weight weekly. The experiment was terminated on day 21 PI when pigs were euthanized by an overdose of sodium pentobarbital. Animals were necropsied and samples of mesenteric, inguinal superficial and mediastinal lymph nodes, tonsil and lungs were collected and fixed by immersion in 10% buffered formalin. Tissues were

subsequently embedded in paraffin, and two consecutive sections cut at 4 μm thick. One slide was stained with haematoxylin-eosin and the second one subjected to an ISH technique to detect PCV2 nucleic acid (Rosell *et al.*, 1999). Three parameters were evaluated per lymphoid tissue: lymphocyte depletion, granulomatous inflammation and presence of PCV2 genome by ISH. Lung tissue was evaluated for presence of inflammation and PCV2 nucleic acid as well. All parameters were scored from 0 (no lesion/negative to ISH) to 3 (severe lesion/high amount of PCV2 genome) (Rosell *et al.*, 1999).

4.2.4. PCV2 viremia

PCV2 DNA quantification was performed on serum samples as previously described (Olvera *et al.*, 2004). Results were expressed as $\log_{10}$ PCV2 genome copies / ml of serum.

4.2.5. PCV2-specific IPMA antibodies

Levels of PCV2-specific antibodies were measured in serum collected at all sampling days by means of an IPMA (Fort *et al.*, 2007).

4.2.6. PCV2 Cap-specific lymphoproliferation

For all experiments, PBMC were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS), 1% glutamine, 1% non-essential aa, 1% sodium pyruvate, 1% peniciline-streptamicin, 0.5% gentamicin and $5x10^{-5}$M 2-mercaptoethanol. Viability of PBMC was assessed with Trypan blue staining.

Proliferative responses of PBMC induced by PCV2 Cap protein were measured on days 0, 7 and 21 PI using a commercial cell proliferation kit (Cell proliferation Biotrack ELISA System, Amersham Biosciences). Briefly, PBMC were cultured at 37ºC and 5% CO_2 ($1x10^5$ cells/well) in the presence of Cap

protein (0.6 µg/ml). PHA (10 µg/ml) and Bac were used as positive and negative controls, respectively. After 48 h of incubation, 5-bromo-2'-deoxyuridine (BrdU) was added to each well (final concentration 10 µM) and plates were incubated in the dark for 16 additional hours at 37 ºC in 5% CO_2. Plates were then centrifuged, dried and fixed using absolute ethanol. Finally, the BrdU incorporated during the DNA synthesis of the proliferating cells was quantified by an ELISA performed following the manufacturer recommendations. Lymphoproliferative responses specific of Cap were calculated as the optical density (OD) obtained using Cap as stimulus minus the OD obtained using Bac. For each animal and day, the result was given as the average of two replicates.

4.2.7. IFN-γ ELISPOT

Frequencies of IFN-γ-SC in PBMC were detected on days 0, 7, 14 and 21 PI by an ELISPOT performed as described in study I. Anti-IFN-γ (clon P2G10) at 5µg/ml and biotinylated anti-IFN-γ (clon P2C11) at 0.5 µg/ml, from BD Pharmingen, were used as capture and detection antibodies, respectively. Whole PCV2 (PCV2-Stoon and PCV2-Sp) (m.o.i of 0.05) and Cap and Rep (0.6 µg/ml) were used as stimuli. As negative controls, supernatants from mock-infected cell cultures, Bac and Ni were used. For each individual and stimulus, the ELISPOT count was reported as the average number of spots of replicates minus the average number of spots in the corresponding negative control wells (namely, mock-infected supernatants for PCV2, Bac for Cap protein and Ni for Rep protein). For each stimulus, a cut-off value was calculated as the average of the ELISPOT counts of PBS-inoculated pigs plus three standard deviations. Results of responding pigs were expressed as the number of IFN-γ-SC cells per 10^5 PBMC.

4.2.8. IL-10 ELISPOT

IL-10 secreting cells (IL-10-SC) were measured by ELISPOT at the same times and using the same stimuli mentioned above for the IFN-γ ELISPOT. Anti-IL-10 (clone 945A 4C4 37B1) and biotinylated anti-IL-10 (clone 945A1A926C2) antibodies from Biosource (Spain) were used as capture (10 μg/ml) and detection (2 μg/ml) antibodies, respectively. Since PCV2 induces IL-10 in *in vitro* cultured PBMC (Study I, Kekarainen *et al.* 2008b), a preliminary assay was performed to set up the optimal cell concentration that allowed a proper reading of the test (data not shown); this value was established at $2.5x10^4$ PBMC/well. Apart from that, the technique was performed following the same protocol mentioned in study I for the IFN-γ ELISPOT. The ELISPOT count for each stimulus was reported as the average number of spots of replicates minus the average number in the corresponding negative control wells. Results were expressed as the number of responding cells per $5x10^5$ PBMC.

4.2.9. Statistics

Statistical analyses were done using SPSS v.15. Shapiro-Wilk test was used to evaluate the normality of the distribution of the examined variables. ANOVA and the Tukey's follow-up test were used for comparison of means of rectal temperature, weight gain, viremia, Cap-limphoproliferation and IFN-γ and IL-10 ELISPOTs. Data on serology (IPMA) was analysed by the Kruskall-Wallis non-parametric test. The Chi-square test was applied to compare the proportion of positive and negative results in viremia, lesions and ISH, and IFN-γ ELISPOT. A linear regression model was built to correlate IFN-γ- and IL-10-SC detected between PCV2-Sp- and PCV2-Stoon-stimulated PBMC. The significance level (α) for all analyses was set at 0.05.

4.3. Results

4.3.1. Sequencing of ORF2

At the aa level, the ORF2 sequences from the Sp-10-7-54-13 used as inoculum and its parental strain obtained from the pig were identical. Stoon-1010 differed from the parental strain in one aa change (I200A).

4.3.2. Clinical and pathological results

None of the pigs showed wasting or other clinical signs compatible with PMWS during the whole study period. The Stoon group had higher rectal temperature at 4, 7, 9 and 11 days PI compared to control group ($P<0.05$). Mean rectal temperature of the Sp group was higher than control group at 9, 11 and 14 days PI ($P<0.05$) (data not shown). No differences in rectal temperature were observed between the Stoon and the Sp group. In the whole experimental period, average daily weight gain was higher in Stoon-inoculated compared to Sp-inoculated pigs (0.68±0.05 vs. 0.55±0.07 kg/day; $P<0.05$). At the end of the study, mean body weight of the Stoon group (28.1±2.6 kg) tended to be higher ($P=0.096$) than mean body weight of the Sp group (23.7±3.1 kg). No differences between challenged and control pigs (25.9±3.8 kg) were observed ($P>0.05$).

One pig from group Stoon developed colibacillar diarrhoea within the first week PI and was euthanized on day 10 PI. Histopathological studies revealed no evidences of PCV2-associated lesions or PCV2 genome in tissues of this pig. Ten out of the twelve PCV2-inoculated pigs that finished the study had PMWS-like lesions and/or presence of PCV2 DNA in the studied tissues. PCV2-associated microscopic lesions, characterized by low to moderate depletion of follicles, granulomatous inflammation in lymphoid tissues and lung inflammation (interstitial pneumonia), were detected in 2/5 of the Stoon-inoculated and in 7/7

of the Sp-inoculated pigs (P<0.05). PCV2 DNA was revealed in tissues of 1/5 and 6/7 pigs in Stoon and Sp groups, respectively (P=0.07).

4.3.3. PCV2 viremia

All PCV2-inoculated pigs but one from the Stoon group became viremic during the study. However, the duration of viremia depended on the virus received at challenge (Figure 4.1). Thus, whereas 4/7 pigs in the Sp group were viremic from day 7 PI until the end of the study, only 1/5 Stoon-inoculated pigs was positive at all three consecutive sampling days (7, 14 and 21 PI). No significant differences were detected in the mean viral load of positive pigs between Stoon and Sp groups. The proportion of PCV2 positive pigs and mean viral loads of positive pigs from day 7 to 21 PI are shown in table 4.1.

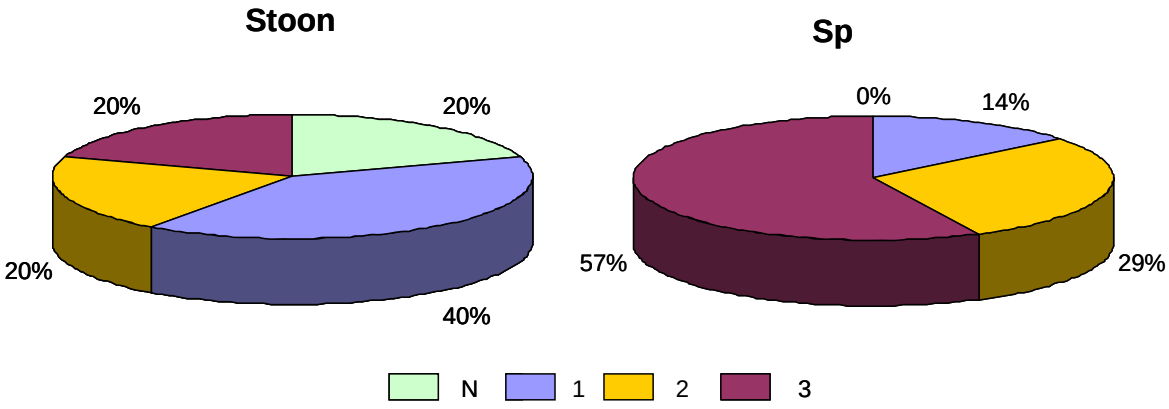


Figure 4.1: Percentage of pigs detected negative (N) at all three sampling points, or one (1), two (2) or three (3) sampling days as PCV2-positive by Q-PCR in Stoon and Sp groups.

Table 4.1: Percentage of PCV2 Q-PCR positive pigs in serum and mean viral load (log_{10} PCV2 DNA copies/ml ± SD) of positive pigs from day 7 to 21 days PI.

Group		Days post inoculation		
		7	14	21
PBS	**% of positive pigs**	0 (0/4)	0 (0/4)	0 (0/4)
Stoon	**% of positive pigs**	33.3 (2/6)	60 (3/5)	60 (3/5)
	Viral load	4.8± 0.1	4.4± 0.5	5.4± 0.7
Sp	**% of positive pigs**	71.4 (5/7)	100 (7/7)	71.4 (5/7)
	Viral load	5.0 ± 0.6	5.0± 0.6	4.7±0.7

4.3.4. PCV2 antibodies

At the beginning of the study, piglets had MDA titres ranging from 4.3 to 6.3 log_2 (mean titre of 5.6 ±1.0 log_2) and no significant differences among groups. Seroconversion against PCV2 occurred in all PCV2-inoculated pigs between days 7 and 14 PI. By the end of the study, the levels of PCV2 antibodies in Sp-inoculated pigs were higher than those in Stoon-inoculated animals (11.3 log_2 vs. 8.8 log_2, $P<0.05$). The evolution of PCV2 IPMA titres is shown in Figure 4.2.

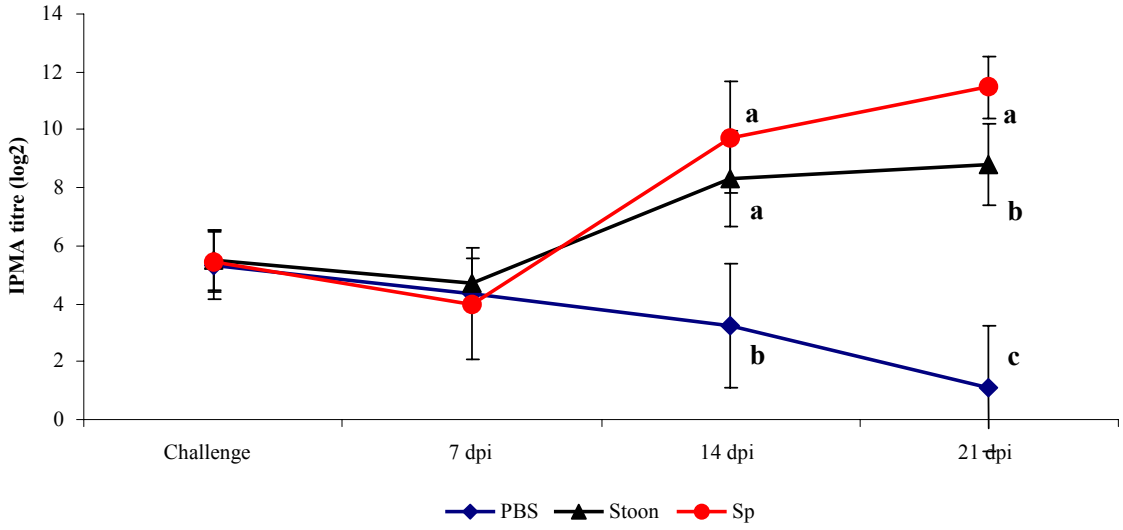


Figure 4.2. Serological profile obtained by IPMA in each of the experimental groups from day of challenge to the end of the study. Different letters (a-c) indicate significant differences among groups ($P<0.05$).

4.3.5. Cap-specific limphoproliferation

When PBMC were stimulated with PHA, no differences in the proliferative responses were detected among groups, neither among sampling days (mean OD: 2.3±1.0). In contrast, using the Cap protein, ODs obtained from PBMC of Sp-inoculated pigs were significantly higher than those from controls from day 7 PI onwards ($P<0.05$). For the Stoon-inoculated animals, although the ODs observed in response to Cap stimulation increased with time (0.4±0.16 at 21 PI versus 0.06±0.06 at challenge, $P<0.05$), values did not differ significantly from those of control pigs. Proliferative responses of Sp-inoculated pigs were higher than those of Stoon-inoculated ones both on day 14 PI (0.72±0.47 vs. 0.22±0.27, $P=0.06$) and 21 PI (0.73±0.24 vs. 0.41±0.16, $P=0.05$). Lymphoproliferation in response to the PCV2 Cap protein is summarized in Figure 4.3.

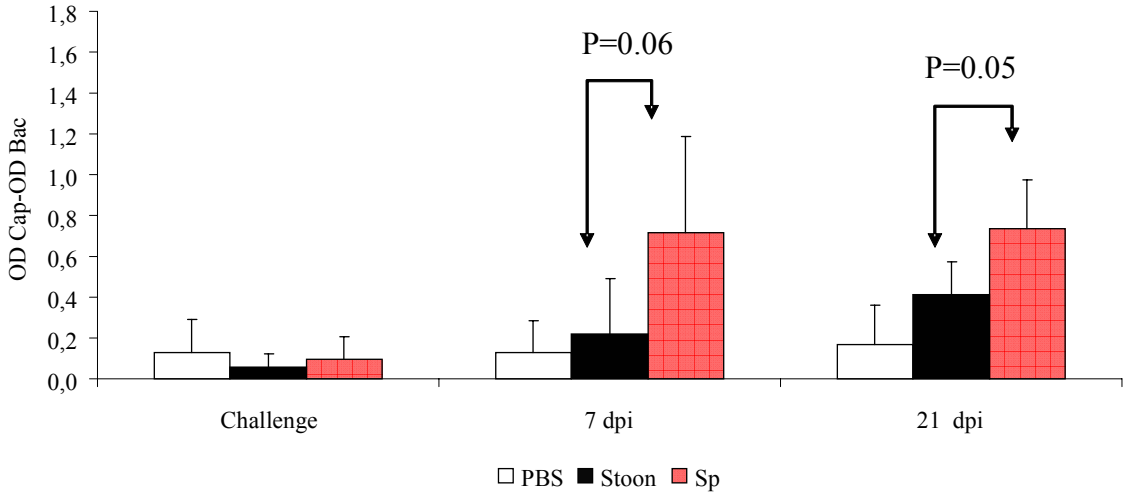


Figure 4.3. Lymphoproliferative responses to the Cap protein of PCV2 detected on day of challenge, 7 and 21 days PI in each experimental group.

4.3.6. IFN-γ ELISPOT

Development of IFN-γ-SC in response to the different stimuli (PCV2-Stoon, PCV2-Sp, and Cap and Rep proteins) for each experimental group is displayed in figure 4.4. The cut off value for each stimulus (mean of control group ± 3 x

standard deviation) was 9, 6 and 2 for PCV2 (both isolates), Cap and Rep proteins, respectively.

In response to the whole virus, no significant differences were detected when either PCV2-stoon or PCV2-Sp were used as recall antigen, and frequencies induced by both stimulus were correlated ($r=0.94$, $P<0.01$). Thus, when PBMC were stimulated with the PCV2-Stoon (PCV2a), IFN-γ-SC were firstly detected on day 7 PI in 2/6 Stoon-inoculated pigs, and in 7/7 Sp-inoculated ones ($P<0.05$). Similarly, using the PCV2-Sp (PCV2b) as stimulus, 1/6 Stoon and 6/7 Sp pigs responded ($P<0.05$). On days 14 and 21 PI, 4/5 Stoon pigs responded to the PCV2-Stoon and 3/5 to the PCV2-Sp, with low IFN-γ-SC frequencies and no significant differences between both stimuli. In the Sp-inoculated group, all pigs (6/6) responded with high frequencies to both PCV2-Stoon and PCV2-Sp stimuli.

When Cap protein was used as stimulus both the number of responding pigs as well as the IFN-γ-SC frequencies of positive pigs were higher compared to the results obtained with the whole virus stimulation. Thus, on day 7 PI, 7/7 Sp-inoculated pigs responded and displayed significantly higher frequencies than the 4/6 responding Stoon-inoculated pigs (98 ± 70 vs. 15 ± 4, $P<0.05$). From then onwards, all PCV2-inoculated pigs responded to the Cap protein. Pigs inoculated with the Sp-10-7-54-13 isolate showed significantly higher counts of IFN-γ-SC than those that received Stoon 1010, both on day 14 PI (187 ± 39 vs. 59 ± 40; $P<0.05$) and 21 PI (203 ± 90 vs. 41 ± 31, $P<0.05$).

In the Stoon group, Rep-IFN-γ-SC could be detected only in one pig and at very low frequencies (3 and 5 IFN-γ-SC on days 7 and 14 PI, respectively). In contrast, all pigs of the Sp group but one responded to the Rep protein. In this latter group, the highest response was detected on day 14 PI, when 6/7 pigs were positive with frequencies ranging from 25 to 194 Rep-specific IFN-γ-SC. By the end of the study, Rep-IFN-γ-SC frequencies slightly decreased in all Sp-inoculated pigs but one (mean Rep- IFN-γ-SC: 78 ± 65).

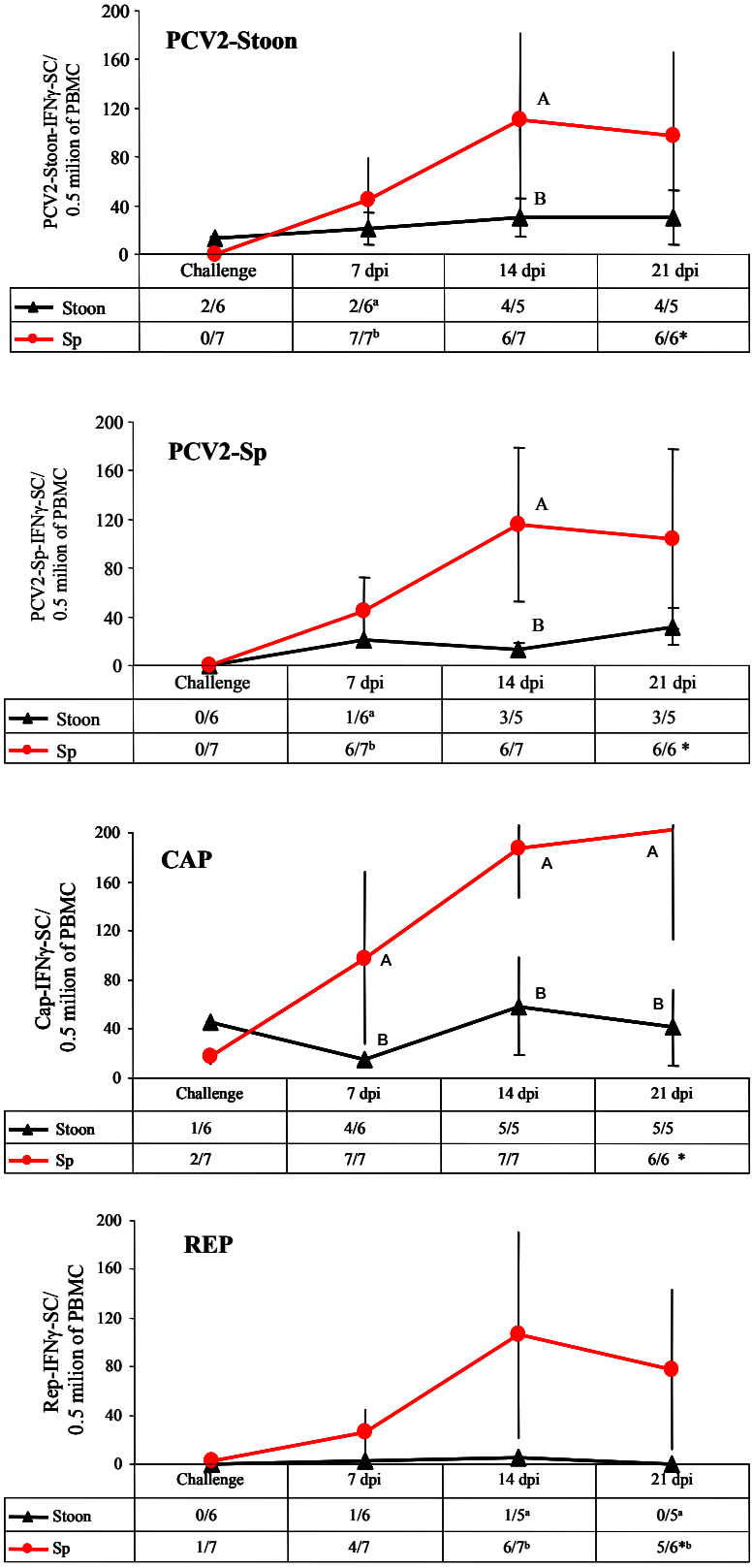


Figure 4.4 Proportion of pigs with positive results at the IFN-γ ELISPOT and mean frequencies of IFN-γ-SC of positive animals in Stoon and Sp groups at challenge, 7, 14 an 21 days PI in response to PCV2 isolates (PCV2-Stoon and PCV2-Sp), and Cap and Rep proteins. Different letters indicate significant differences ($P<0.05$) among groups in

the proportion of positive pigs (A,B) and the mean frequencies of IFN-γ-SC of positives (a,b). * PBMC from 1/7 Sp-inoculated pigs were not available on day 21

4.3.7. IL-10 ELISPOT

Stimulation of PBMC with the whole virus (either PCV2-Stoon or PCV2-Sp) induced IL10 responses in both inoculated and non-inoculated pigs. These responses were detected at all days, with no differences between groups at any time (Figure 4.5). IL-10-SC induced by both isolates were correlated (r=0.55, r=0.83, r=0.73 and r=0.89 on days 0, 7, 14 and 21 PI; P<0.01). Although frequencies induced by PCV2-Stoon were slightly higher, they did not differ statistically from those induced by PCV2-Sp. Contrarily to the results obtained in the ELISPOT for the detection of IFN-γ-SC, stimulation with either Cap or Rep protein did not result in any significant induction of IL-10-SC throughout the whole experiment.

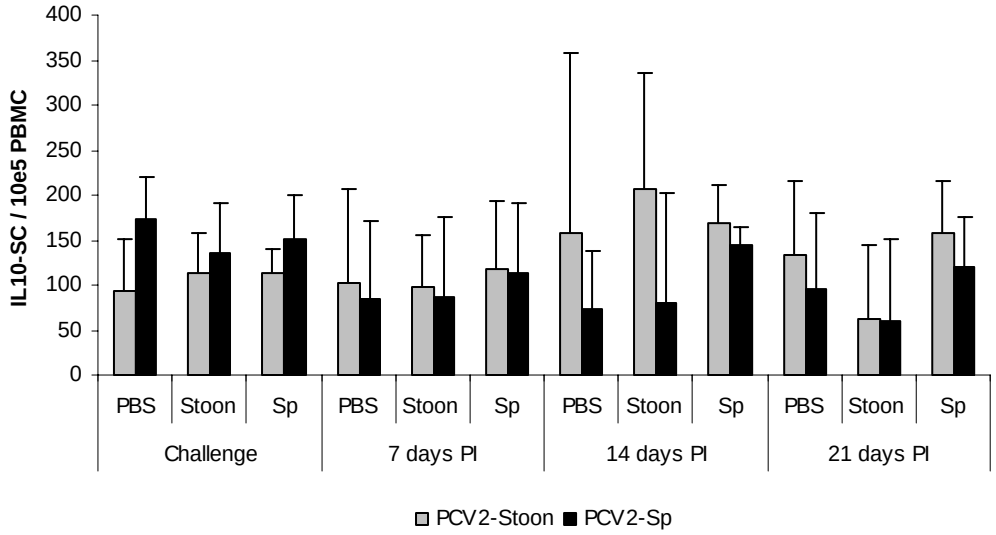


Figure 4.5: Frequencies of IL-10-SC detected in PBS, Stoon and Sp groups at challenge, 7, 14 and 21 days PI in response to PCV2 isolates Stoon-1010 (PCV2-Stoon) and Sp-10-7-54-13 (PCV2-Sp).

4.4. Discussion

The present study aimed to gain insight into the cell-mediated immunity developed upon PCV2 infection and the involvement of the different viral

proteins in its development. To represent genetic variability within PCV2, one PCV2a (Stoon-1010) and one PCV2b (Sp-10-7-54-13) isolates were used for challenge as well as for *in vitro* assays. Since viral stocks were produced at relatively high passages (30^{th} and 14^{th} for Stoon-1010 and Sp-10-7-54-13 isolates, respectively), ORF2 sequencing was performed to elucidate potential differences in the aa composition between the viruses used as inocula and their corresponding parental seeds. Thus, for Sp-10-7-54-13 isolate, no change was detected after 14 passages in cell culture. Conversely, the ORF2 of Stoon-1010 used as inoculum and the first sequence of this strain submitted to GenBank differed in one aa (I200A). Whether this mutation might account for differences in antigenicity and/or replication cannot be rule out, since it has been demonstrated that minimal changes (P110A and R191S) in the Cap of a PCV2 isolate after 120 passages in cell culture enhanced PCV2 replication *in vitro* and attenuated the virus *in vivo* (Fenaux *et al.*, 2004b). Recently, Opriessnig *et. al* (2006b) observed differences in virulence between two isolates with 100% identical Cap proteins but a single aa change at position 41 of the Rep protein. In the present study, only ORF2 was sequenced and, therefore, potential differences in the aa composition of non-structural proteins between the inocula and their corresponding parental sequences could not be elucidated.

Although PMWS was not reproduced, challenge with both Stoon-1010 and Sp-10-7-54-13 isolates resulted in the development of a sub-clinical PCV2 infection. However, comparison among challenge groups revealed substantial differences in the progression of PCV2 infection. Thus, the Sp group had higher proportion of individuals with PCV2-associated lesions and presence of PCV2 DNA in tissues compared to the Stoon group. In addition, although all PCV2-inoculated pigs but one from Stoon group had PCV2 in serum, most Stoon-inoculated pigs developed a short viremic phase (figure 4.1). Previous studies found that reduced viremia and PCV2-associated lesions of inoculated pigs were related to the presence of high levels of PCV2 MDA at challenge (McKeown *et al.*, 2005; Ostanello *et al.*, 2005). In the present work, the levels of MDA are not apparently

at the base of the above-mentioned differences between Stoon- and Sp-inoculated pigs, since all of them had IPMA titres below 6.3 $\log_2$ at the start of the experiment and no differences in antibody levels were detected among groups. On the other hand, a potential explanation might be the existence of differences in virulence among both isolates, which would be coherent with the postulated higher pathogenicity of PCV2b strains (Gagnon *et al.*, 2007; Grau-Roma *et al.*, 2008). However, since only one isolate from each genotype were compared, whether the observed differences are related to genotype or to the particular strains used in the present study cannot be determined.

The adaptive immunity developed by pigs to PCV2 infection depended on the isolate used for challenge. Although the onset of humoral response occurred in both groups between 7 and 14 days PI, Sp-inoculated pigs showed significantly higher antibody titres compared to Stoon-inoculated ones at the end of the study. In addition, remarkable differences were observed in regards to cell-mediated immunity. Thus, in the Sp group, lymphoproliferative responses to the Cap were detected as early as day 7 PI, with increasing levels until the end of the study. In contrast, values for Stoon-inoculated pigs were much lower and similar to those of control pigs. In the IFN-γ ELISPOT, the Sp group had significantly higher proportion of responding pigs as well as frequencies of IFN-γ-SC in responders compared to the Stoon group. The distinct immunological profiles observed between Sp- and Stoon-inoculated pigs could be attributed to the observed differences in replication between both isolates but also to differences in antigenicity. However, the fact that a similar profile of IFN-γ-SC was developed when either one or the other PCV2 isolate was used as recall antigen is suggestive of the existence of conserved T-cell immunodominant epitopes between both strains. This hypothesis would be supported by the highest responses in pigs inoculated with Sp-10-7-54-13 (PCV2b genotype), taking into account that the stimuli used in the lymphoproliferative (Cap) and ELISPOT (Cap and Rep) assays were based on PCV2a strains. The same applies for the serological results, since IPMA was performed on PCV2a-infected PK-15 cells.

It can be speculated, therefore, that the higher level of replication exhibit *in viv*o by Sp-10-7-54-13 is at the base of the stronger immunological response developed by Sp-inoculated pigs. Interestingly, differences in the appearance and intensity of cell-mediated responses to Cap and Rep proteins were observed. Thus, all PCV2-inoculated pigs, even those showing low virus replication, developed IFN-γ-SC in response to the Cap, indicating that this protein is a good T-cell immunogen. Taking into account that PCV2 is able to infected and persist in monocyte-machrophage lineage cells for long periods of time with no apparent active replication (Gilpin *et al.*, 2003; Vincent *et al.*, 2003), the induction of cell-mediated immunity against Cap might be of crucial importance to avoid viral persistence in those cells. Contrary to the results obtained with Cap, only pigs with detectable viral load and presence of PCV2-associated lesions in tissues developed significant IFN-γ responses when Rep was used as recall antigen. This finding suggests that certain level of virus replication is required to trigger detectable responses to this protein. Since the Rep is highly expressed in cells supporting PCV2 replication, cellular responses to this protein might be important to constrain PCV2 replication and prevent the progression of PCV2 infection towards PMWS. In a recent field study, it was shown that PMWS-affected pigs could be differentiated from healthy and wasted non-PMWS affected animals by having impaired anti-Cap and anti-Rep antibody responses, and in the case of anti-Rep antibodies, lower titres were already detected prior to the appearance of disease (Pérez-Martín *et al.*, 2008).

Stimulation of PBMC with either the whole PCV2 or the Cap protein yielded different results at the IFN-γ ELISPOT. These differences might be the result of the distinct immunomodulatory activity of PCV2 viral components (Kekarainen *et al.*, 2008a). It is possible that IFN-γ responses developed upon PCV2 infection might have been partly inhibited when PCV2 was used as stimulus, since the whole virus and its DNA repress the production of IFN-γ in PMBCs upon recall antigen (Kekarainen *et al.*, 2008a). Conversely, the lack of such repressive effect by the Cap might explain the high frequencies observed when this protein was

used as recall antigen. These observations should be considered and used advantageously for refining this and other assays aimed to measure the immune responses to PCV2.

In contrast to the results obtained in IFN-γ ELISPOT, frequencies of IL-10-SC were not associated with the challenge group, neither were they related to the sampling day, further indicating that IL-10 was released mainly as a result of a natural immune response to PCV2. In addition, induction of IL-10-SC depended on whether the whole PCV2 or the viral proteins were used as stimuli. Neither Cap nor Rep protein induced IL-10-SC when added to PBMC cultures. In contrast, very high frequencies were observed when the whole virus was used. These results are in agreement with a previous study in which PCV2 but not PCV2-derived VLPs induced IL-10 in PBMC (Kekarainen *et al.*, 2008a). Present data indicates that, as reported for the Cap, the Rep is not directly involved in the PCV2-induced up-regulation of IL-10. Comparison among stimuli revealed no significant differences on the frequencies of IL-10-SC induced by either the Stoon-1010 or Sp-10-7-54-13 isolates. Since those two strains belong to genotypes PCV2a and PCV2b, respectively, this finding suggests that the potentially higher virulence of PCV2b isolates is not apparently related to a major IL-10 inducing capability. In a previous work, however, differences on the levels of IL-10 released after stimulating PBMC with two PCV2a strains were described (Fort *et al.*, 2008). Further studies including a larger set of PCV2a and PCV2b isolates would clarify whether differential interactions with the immune system do exist among PCV2 strains.

In summary, the present study indicates that cell-mediated immunity developed upon PCV2 infection involves both Cap and Rep proteins. Further studies using a model of clinical disease are required to elucidate the relevance of Cap and Rep T cell responses in the protective immunity against PMWS.

CHAPTER 5

STUDY III

Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins

5.1. Introduction

Recently, different PCV2 vaccines have been shown to protect against the development of PMWS and their use resulted in a significant improvement of nursery and fattening mortality rates of vaccinated farms (Allan & Mc Neilly, 2006). Commercially available vaccines against PCV2 can be used in piglets or in sows. Under field conditions, piglet vaccination has been demonstrated to effectively reduce mortality rates and to improve pig performance when administered at 3-4 weeks of age (de Grau *et al.*, 2007; Desrosiers *et al.*, 2007; Ritzmann & Kixmoeller, 2007). Vaccination of sows with the aim of inducing colostral transfer of immunity to piglets is also effective (Joisel *et al.*, 2007). This latter fact reinforces the notion that maternal immunity is protective (McKeown *et al.*, 2005; Ostanello *et al.*, 2005; Calsamiglia *et al.*, 2007). Vaccine prototypes such as those based on inactivated virus (Pogranichniy *et al.*, 2004) or recombinant products expressing the Cap protein (Blanchard *et al.*, 2003a; Wang *et al.*, 2007) produce clinical protection after a PCV2 challenge, reduce viremia and induce a strong antibody response. Up to now, the main basis for the efficacy of PCV2 vaccines has been thought to lie in the development of antibodies although the development of NA in vaccinated pigs has been scarcely studied (Pogranichniy *et al.*, 2004; Wang *et al.*, 2007).

Several studies reported that two distinct phylogenetic groups of PCV2 viruses may be distinguished (Carman *et al.*, 2006; Olvera *et al.*, 2007) and, although not fully demonstrated, those genotypes might vary in pathogenicity (Cheung *et al.*, 2007a; Grau-Roma *et al.*, 2008). A recent proposal to standardise the nomenclature defined three PCV2 genotypes; namely PCV2a, PCV2b and PCV2c (Segalés *et al.*, 2008). Genotype PCV2b has been suggested to potentially group more virulent isolates than genotype PCV2a (Grau-Roma *et al.*, 2008). Also, genetic diversity of PCV2 may affect the Cap protein where the main neutralizing epitope is located, as demonstrated by the different reactivity of mAbs to the Cap protein when confronted to different PCV2 strains (Lefebvre *et*

al., 2007). Thus, those mAbs were able to discriminate geographically (Europe versus North-America) PCV2 isolates, as well as isolates from different clinical presentations of the disease. These facts open the question on the efficacy of PCV2 vaccines against viral strains belonging to different genotypes or geographic origins.

The aim of this study was to test the development of immunity after vaccination with a PCV2 sub-unit vaccine containing the Cap protein expressed in a baculovirus system and to ascertain the efficacy of the vaccine in a challenge model using 4 different PCV2 isolates of different genotype and different geographical origin.

5.2. Materials and methods

5.2.1. Animals

Seventy-two 2-weeks-old piglets were obtained from a commercial 2000-sow farm free from Aujeszky's disease virus and *Brachyspira hyodysenteriae* and seropositive but stable at PRRSv. The absence of PCV2 in the selected animals was confirmed by means of a PCR (Quintana *et al.*, 2002) on serum, nasal and rectal swabs. Piglets were transported to the biosafety level 3 animal facilities of the Centre de Recerca en Sanitat Animal (CReSA) and were allowed to acclimatize for 10 days before the first immunization. During that period, piglets where bled from the vena cava to determine their PCV2 serological status by means of an IPMA technique as previously described (Fort *et al.*, 2007).

5.2.2. Viruses and vaccine

Four different PCV2 isolates obtained from lymphoid tissues of PMWS-affected pigs were used as challenge viruses. The *cap* gene (ORF2) region of each strain was sequenced and classified according to Grau-Roma *et al*. (2008) into

genotypes PCV2a or PCV2b (Figure 5.1); virus A (strain I-12/11) and D (strain MO/S-06), isolated in The Netherlands and United States, in 2003 and 2006 respectively, were classified into genotype PCV2b; viruses B (strain Burgos) and C (strain Stoon-1010), isolated from Spain and Canada, respectively, belonged to genotype PCV2a. Inocula were checked to be free from viral, bacterial and fungal contaminants prior to be used.

A PCV2 sub-unit vaccine containing the Cap protein of a PCV2a strain in an oil-in-water emulsion was used (Porcilis® PCV, manufactured by Intervet International, The Netherlands). This Cap protein is expressed in a baculovirus expression system and after inactivation and purification forms VLP morphologically indistinguishable from PCV2 particles (data not shown).

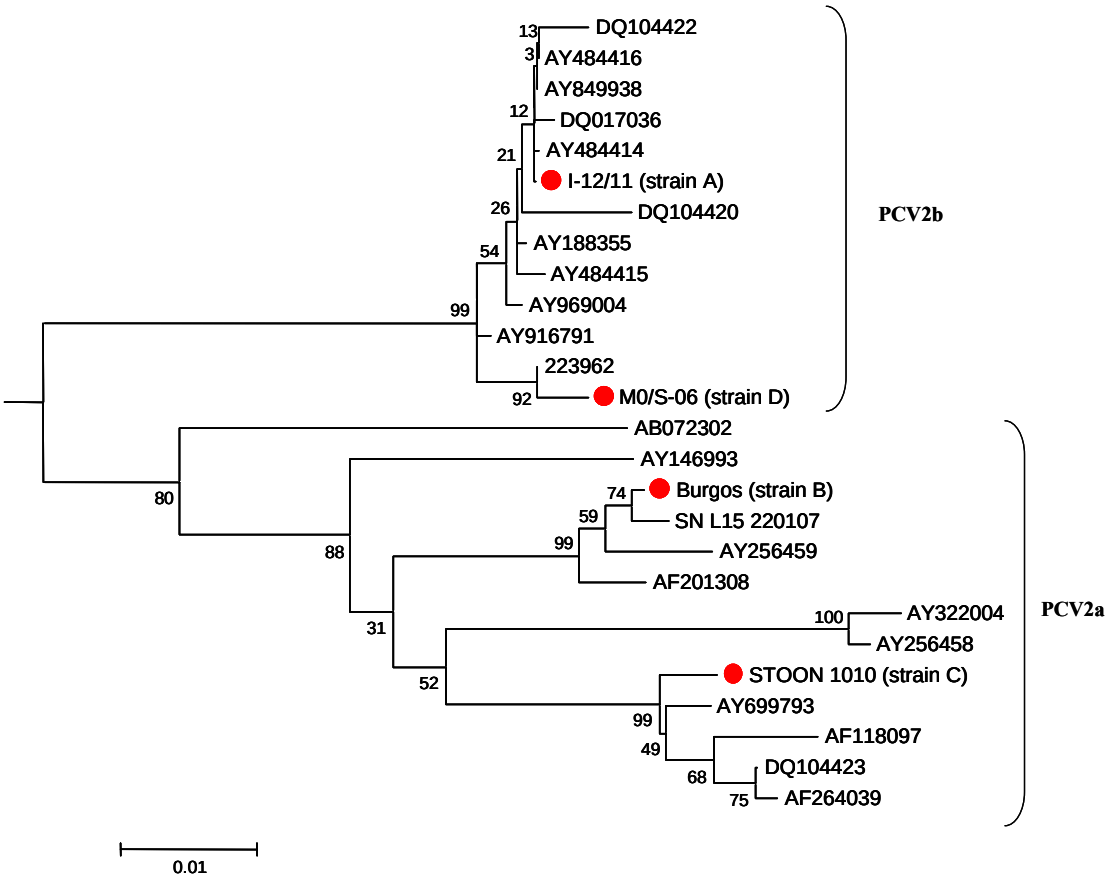


Figure 5.1: Phylogenetic tree including the four PCV2 isolates used in this study together with representative PCV2 ORF2 sequences available in the GenBank database (Neighbour-Joining Tree, 10000 bootstraps). Accession numbers and classification are also reported.

5.2.3. Experimental design

A summary of the experimental design is shown in table 5.1. After arriving to the animal facilities, pigs were tagged and distributed into five groups, namely A, B, C, D and E, and balanced according to weight and serological status of PCV2. Groups were housed in different isolation boxes and contained 14 (groups A to D) or 16 (group E) piglets allocated into two different pens. The pens allowed nose-to-nose contact between animals within the same box. At four weeks of age (day 29 pre-challenge, Pre-C), seven piglets of each group were intramuscularly vaccinated on the right side of the neck. The remaining seven (or nine in group E) animals in each group received 2.0 ml of sterile PBS as a placebo. Two weeks later (day 15 Pre-C) piglets were either revaccinated or received a second dose of sterile PBS on the left side of the neck according to their assigned group. Fifteen days later (day of challenge, day 0), animals were intranasally inoculated with 2.0 ml of MEM containing $2x10^{4.2}$ $TCID_{50}$ (1 ml/nostril) of either PCV2 strains A, B, C or D (each group received the homonymous PCV2 strain). Pigs from group E (control group) were not challenged and received 2 ml of PBS by the same inoculation route.

Body weight was recorded from all piglets at the time of first and second vaccinations, and at weekly intervals from the day of challenge onwards. Rectal temperatures were recorded three times per week, on days 0, 2, 5, 7, 9, 12, 14, 16 and 19 post-challenge (Post-C). Blood samples were collected from vena cava at the start of the acclimatisation period, at the time of first and second vaccinations, at the time of challenge and also on a weekly basis thereafter. Sera were obtained and stored at -80ºC until serological and virological determinations. Nasal and faecal swabbing was performed concurrently with blood sampling. Nasal swabs were taken from both nostrils by swabbing the nasal mucosa deeply into turbinates. Faecal swabs were obtained by swabbing the surface of the colo-rectal region. Swabs were suspended in 1 ml of PBS, vortexed and stored at -80ºC until further use. On day 20 Post-C, animals were

euthanized and a complete necropsy was done. Samples from inguinal and mesenteric lymph nodes, tonsil and spleen were collected and fixed by immersion in 10% buffered formalin for further pathological studies.

Animal care and procedures used in this study agreed to the guidelines of the GEP, under the supervision of the Ethical and Animal Welfare Committee of the Universitat Autònoma of Barcelona.

Table 5.1: Summary of the experimental design

Group	Treatment		Num of pigs	Inoculum	Dose
A	Vaccinated	A V	7	Virus A (I-12/11)	
	Non-vacc.	A NV	7		
B	Vaccinated	B V	7	Virus B (Burgos)	$2x10^{4.2}$ $TCID_{50}$
	Non-vacc.	B NV	7		
C	Vaccinated	C V	7	Virus C (Stoon 1010)	
	Non-vacc.	C VN	7		
D	Vaccinated	D V	7	Virus D (MO/S-06)	
	Non-vacc.	D NV	7		
E	Vaccinated	E V	7	PBS	2 ml
	Non-vacc.	E VN	9		

5.2.4. Pathological studies

Fixed tissue samples were dehydrated through graded alcohols, embedded in paraffin and stained with hematoxylin-eosin stain. Each tissue was assessed for the presence of PMWS-like lesions (lymphocyte depletion together with granulomatous inflammation) (Segalés *et al.*, 2004a) and scored from 0 (no lesions) to 3 (severe lesions) (Rosell *et al.*, 1999). Besides, an ISH was also performed on same tissues. Presence of PCV2 genome within lesions was semi-quantified using a visual score from 0 (absence of PCV2 genome) to 3 (high amount of PCV2 genome) (Rosell *et al.*, 1999). The final histopathological and ISH scores per pig resulted from the average scores obtained in the 4 studied tissues (inguinal and mesenteric lymph nodes, tonsil and spleen).

5.2.5. Virological studies

Q-PCR to detect PCV2 genome was performed on sera, nasal and faecal swabs according to a previously described protocol (Segalés *et al.*, 2005b). Viral concentrations were expressed as the mean viral DNA copy numbers per ng of total DNA (nasal and faecal swabs) or ml (serum).

5.2.6. Serological studies

PCV2 IPMA antibodies were measured on days 29 and 15 Pre-C, day of challenge and days 6, 13 and 20 Post-C. PCV2-specific NA were measured on days 29 Pre-C, at challenge and 20 days Post-C (Fort *et al.*, 2007). Briefly, 200 $TCID_{50}$ of PCV2 were mixed with serial dilutions of each serum (1:2 to 1:4096) in a 96 wells-plate. After 1 h of incubation at 37ºC, $2x10^4$ swine kidney (SK) cells were added to each well and incubated for 72 h at 37ºC in 5%CO_2. Plates were fixed and the positive cells were detected by an IPMA, using a porcine hyperimmune serum against PCV2. Neutralization titres were calculated as the highest dilution of each serum giving a reduction on 50% of the infected cells when compared to control wells.

5.2.7. Statistical analyses

Serological titres (IPMA and NA) as well as viral loads were transformed to log_2 and log_{10} respectively prior to the analyses. Shapiro-Wilk test was used to evaluate the normality of the distribution of the examined variables. ANOVA according to the general linear model procedure was used to compare means of different parameters and the Bonferroni adjustment procedure was used for multiple comparisons. The Fisher exact test was applied to compare the proportions of positive and negative Q-PCR results between vaccinated and non-vaccinated groups. A linear regression model was built to correlate level of antibodies on day 29 Pre-C to antibody titres on day 0. PMWS-like lesions and

PCV2 ISH were evaluated as the total score (sum of scores of the lymphoid tissues) and statistically analysed by means of the Friedman Rank Sum test, controlling for the four challenge isolates. Also, virological data were categorized as a binary response i.e. positive or negative and data over time were analysed by means of a Generalized Estimating Equations (GEE), including the type of isolate and treatment as factor, and accounting for the correlation structure of the data from the repeated measurements on an animal. In this analysis the odds ratio (OR) was estimated as a relative measure of the reduction in the risk of being viremic or a shedder after vaccination. Statistical analyses were performed using SAS 9.1 software (SAS institute Inc., Cary, North Carolina, USA). The significance level (α) was set at 0.05.

5.3. Results

5.3.1. Clinical and pathological outcomes

None of the animals developed clinical signs compatible with PMWS during the whole experiment. No differences in body weight or in rectal temperatures were observed among treatments and among groups at any time of the experiment (data not shown). From the total of 56 PCV2-inoculated pigs, only 7 non-vaccinated animals had PMWS-compatible lesions in lymphoid tissues (2 pigs from group A, 4 from group B and 1 from group D). All these 7 pigs but one from group B were also positive by ISH with low to moderate amounts of PCV2 genome within the specific lesions. No significant differences in PMWS lesions were detected when vaccinated and non-vaccinated piglets were compared within groups. However, when data from all piglets (independently of the group) were used, vaccinated pigs had significantly less PMWS-like lesions in lymphoid tissues compared to non-vaccinated ones (Friedman Rank Sum test $P<0.01$).

5.3.2. Viremia and shedding

None of the vaccinated pigs developed viremia throughout the whole study, nor did any of the non-vaccinated pigs before challenge. After inoculation, slight differences were noticed among challenge groups regarding the starting of viremia in non-vaccinated pigs. Thus, PCV2 DNA was firstly detected by day 6 Post-C in 1/7 pigs in group A (viral load of 4.0 $\log_{10}$) while in groups B and D viremia was firstly detected by day 13 Post-C in 5/7 and 6/7 pigs, respectively (mean viral loads 5.3±1.9 $\log_{10}$ and 5.4±1.1 $\log_{10}$); at the same day, 5/7 pigs from group A were also positive (mean viral load 6.4±1.7 $\log_{10}$). In group C, only two pigs developed viremia by day 20 Post-C, with a mean viral load of 4.5±1.3 $\log_{10}$. At that time, the quantity PCV2 genome detected in the rest of the groups was slightly decreased (5.3±13 $\log_{10}$ in group A, 5.0±0.9 $\log_{10}$ in B and 4.0±0.7 $\log_{10}$ in D) in comparison to results from day 13 Post-C. Control pigs (Group E) remained negative until the end of the experiment. Percentage of viremic pigs along the experiment is shown in Figure 5.2A. The evolution of mean PCV2 viral load in serum of vaccinated and non-vaccinated pigs for each group, measured by Q-PCR, is shown in Figure 5.2B

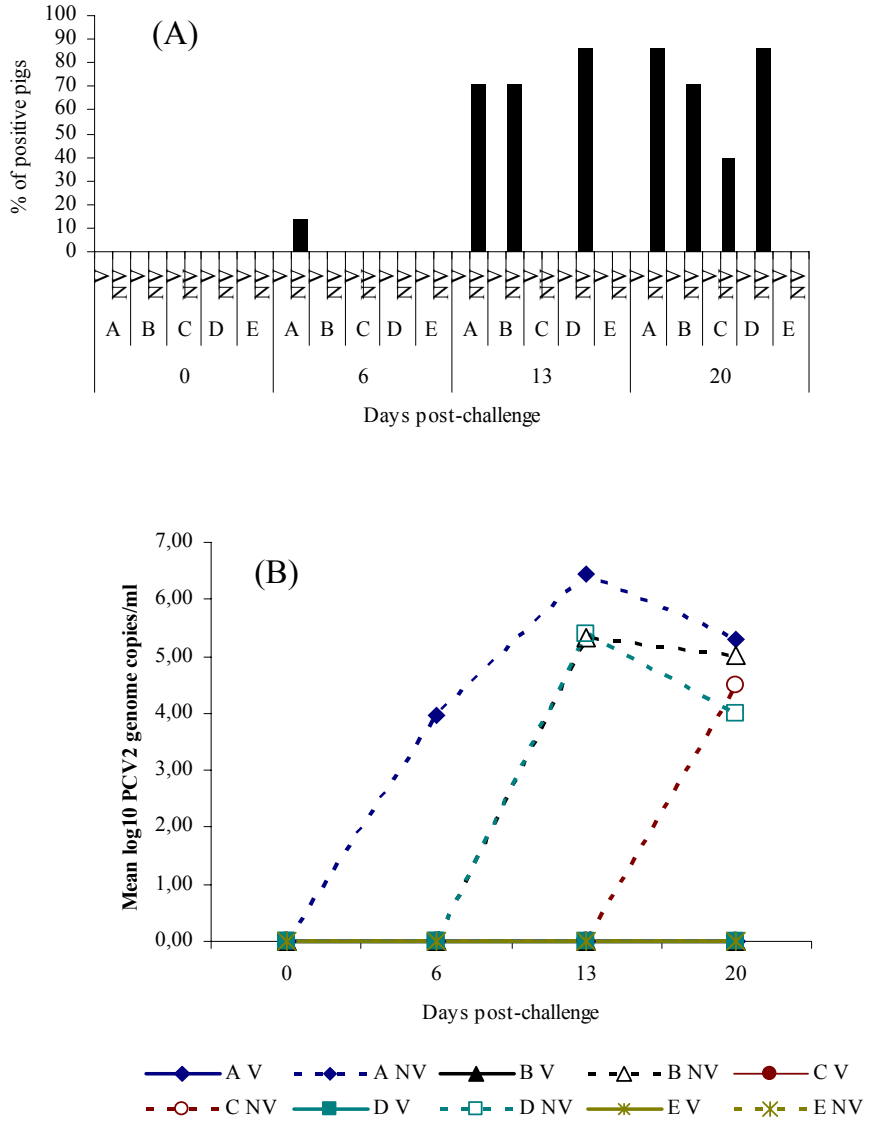


Figure 5.2: Percentage of serum PCV2 Q-PCR positive pigs (A) and mean PCV2 viral load of positive animals (B) in vaccinated and non-vaccinated groups (A-E), from days 0 to 20 post-challenge.

None of the pigs had PCV2 in nasal swabs, from day 29 Pre-C to day of challenge. After inoculation, pigs were firstly detected as positive by day 6 Post-C; on day 13 Post-C, all but one pig had detectable PCV2 genome in the nasal cavity. However, by day 20 Post-C, the proportion of shedders was significantly lower (P=0.01) in vaccinated animals compared to non-vaccinated ones. Moreover, viral quantity in nasal swabs was consistently higher in non-vaccinated pigs from day 13 Post-C onwards, and by day 20 Post-C all vaccinated groups had significantly lower ($P<0.05$) amounts of PCV2 in nasal

cavities than the non-vaccinated counterparts. Figures 5.3A and 5.3B summarize these results.

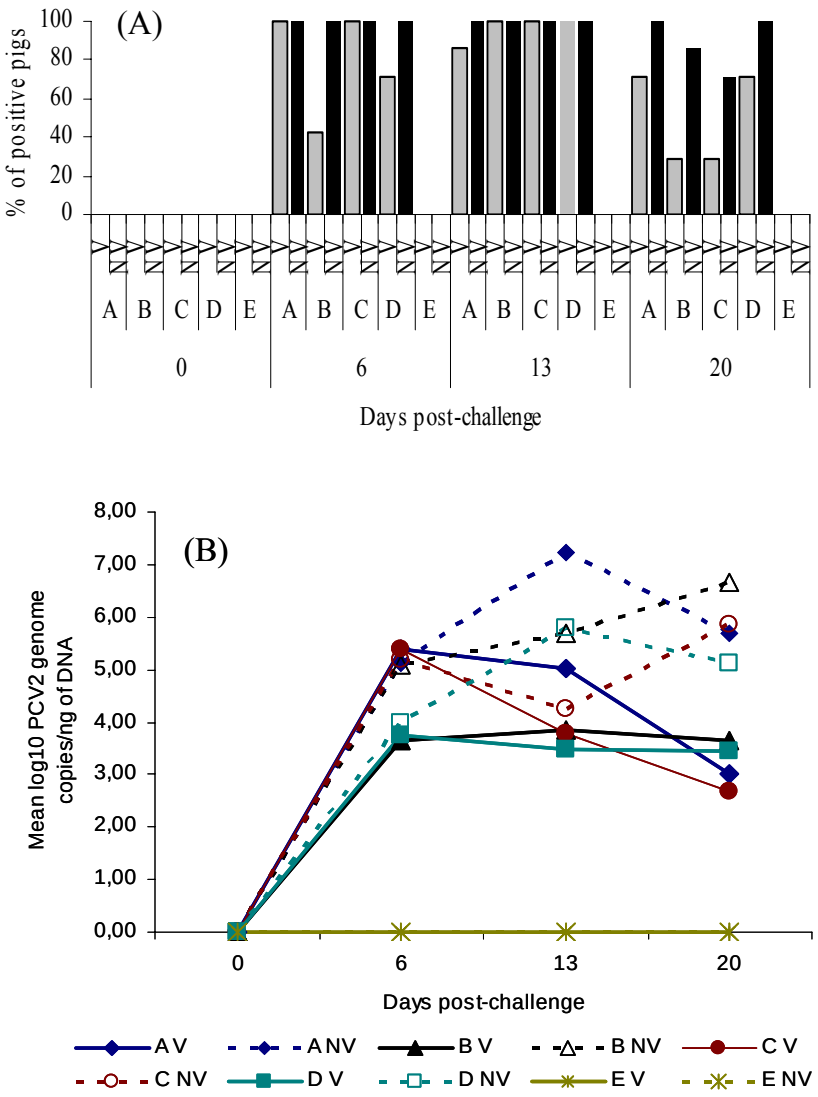


Figure 5.3: Percentage of PCV2 Q-PCR positive pigs in nasal swabs (A) and mean PCV2 viral load of positive animals (B) in vaccinated and non-vaccinated groups (A-E), from days 0 to 20 post-challenge.

None of the pigs had PCV2 in faeces from day 29 Pre-C to day of challenge. On day 6 Post-C only 4/56 of the inoculated pigs had detectable PCV2 in faeces, corresponding to one non-vaccinated pig from group A and three vaccinated pigs, two from group A and one from B. By day 13, percentage of positives within non-vaccinated pigs compared to vaccinated ones was significantly higher ($P<0.05$) in groups A, B and D. At that time, all non-vaccinated subgroups had positive pigs; however, while in group A all but one non-vaccinated pigs were

PCV2 faecal shedders, in group C only one was positive. On day 20 Post-C all non-vaccinated groups still had positive pigs, while only one vaccinated animal was positive in groups A and D. Comparison among groups detected significantly higher ($P<0.05$) proportion of positive pigs in non-vaccinated subgroups compared to vaccinated ones in groups A, B and C. No significant differences could be detected on viral load when positive pigs were compared among treatments in any of the groups. Evolution on PCV2 detection in faeces is shown in figures 5.4A (percentage of Q-PCR positive pigs) and 5.4B (mean viral load of positive pigs).

Finally, the odds ratio of detecting PCV2 by PCR in serum, nasal cavity and faeces in vaccinated pigs in relation to that one in non-vaccinated pigs was 0.11 ($CI_{95\%}$ = 0.00–0.27, $P<0.01$), 0.066 ($CI_{95\%}$ = 0.018–0.24, $p<0.01$) and 0.069 ($CI_{95\%}$ = 0.020–0.24, $p<0.01$) respectively.

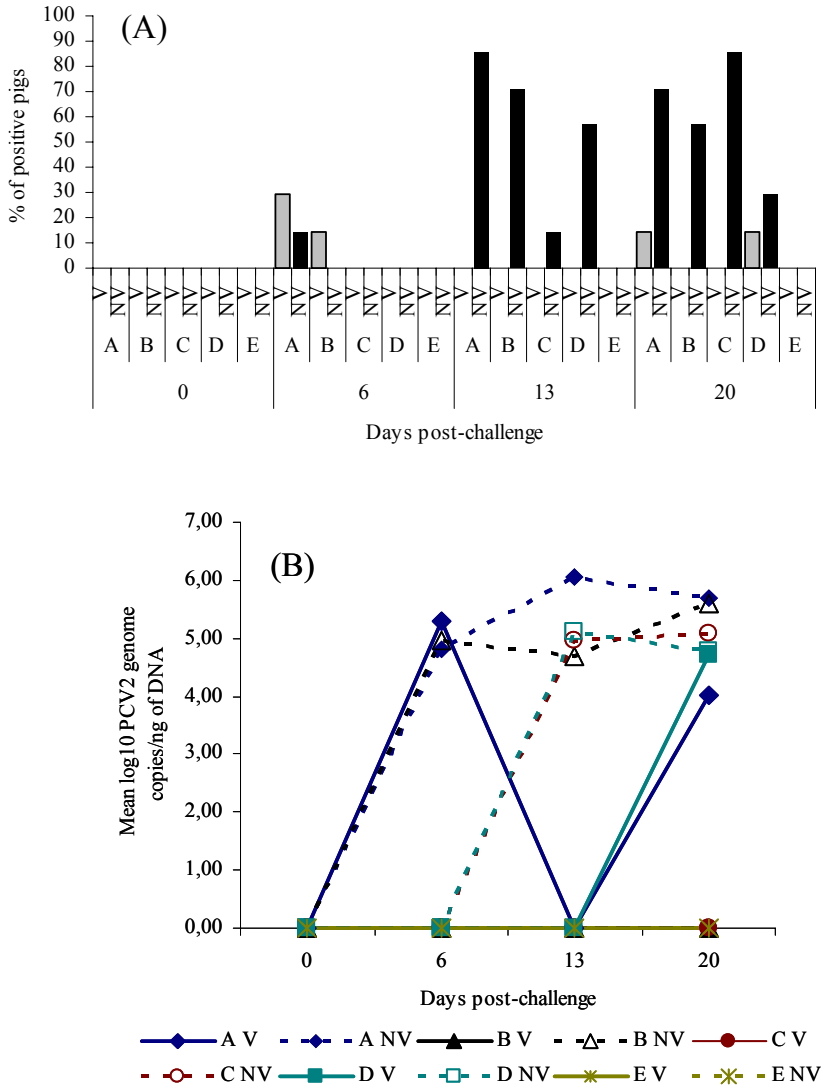


Figure 5.4. Percentage of PCV2 Q-PCR positive pigs in faecal swabs (A) and mean PCV2 faecal viral load of positive animals (B), in vaccinated and non-vaccinated groups (A-E), from days 0 to 20 post-challenge.

5.3.3. *Humoral response*

5.3.3.1. PCV2 IPMA antibodies

Evolution of IPMA titres to PCV2 of vaccinated and non-vaccinated pigs for each group is displayed in figure 5.5A. On day 29 Pre-C (first vaccination), all pigs were seropositive with mild to medium IPMA titres (6.8±1.9 $\log_2$). No significant differences between groups and between vaccinated and non-vaccinated pigs within groups were observed at that time point. When pigs

received the second vaccine dose (day 15 Pre-C), significantly higher titres were detected in vaccinated pigs compared to non-vaccinated ones (6.8±1.6 log_2 vs. 4.9±2.1 log_2; P<0.01). By day 0 (PCV2 challenge), seroconversion had occurred in 33/35 vaccinated pigs (mean titre: 11.8±1.9 log_2). For vaccinated animals, titres did not change significantly after challenge. In contrast, on day 0, non-vaccinated pigs showed a decrease of their titres (4.4±1.9 log_2) compared to day 29 Pre-C and titres did not increase clearly until day 20 Post-C. In group C, only 2/7 non vaccinated pigs had seroconverted by that time. IPMA titres on day 20 Post-C were significantly higher in vaccinated pigs compared to their non-vaccinated counterparts (12.4±1.5 log_2 vs. 6.3±3.5 log_2; P<0.01)

In order to ascertain whether or not maternal immunity might have interfered in the development of the humoral response after vaccination, a linear regression was performed to evaluate the correlation between IPMA results at the day of the first immunization and the IPMA titres at the day of challenge. Results showed a slight interference of maternal antibodies (r=-0.53; P<0.01).

5.3.3.2. Neutralizing antibodies to PCV2

Evolution of NA titres to PCV2 of vaccinated and non-vaccinated pigs for each group throughout the experiment is displayed in figure 5.5B. At the time of first vaccination, no differences in NA titres were detected neither among groups nor treatments. On day 0, 26/35 vaccinated pigs had increased their PCV2-specific NA titres (average: 7.5±1.7 log_2) while for non-vaccinated NA titres had declined (average: 3.9±1.9 log_2) (P<0.01). On day 20 Post-C, NA titres of vaccinated pigs were still raising while for non-vaccinated pigs only 9/37 pigs had detectable NA at that time (average titres: 8.9±1.4 log_2 vs. 3.9±1.9 log_2; P<0.01). A positive correlation was observed between IPMA and NA titres at all sampling days (P<0.05, r ranging from 0.54 to 0.82) in both vaccinated and non-vaccinated pigs. Similar to the results observed for IPMA titres, regression analysis showed a

slight interference of maternal antibodies in the development of NA after vaccination (r=-0.45; P<0.01).

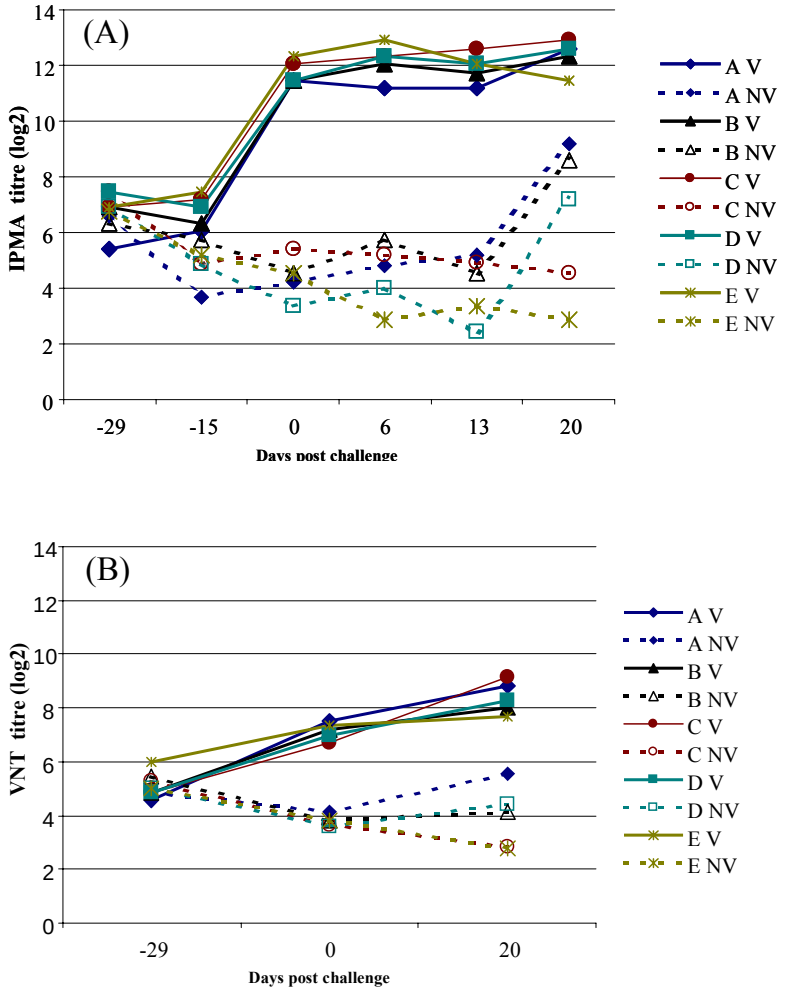


Figure 5.5: Serological profile of IPMA antibodies (A) and neutralizing antibodies (B) to PCV2, in vaccinated and non-vaccinated groups (A-E), from day 29 pre-challenge to 20 post-challenge.

5.4. Discussion

PMWS has an important economic impact on swine industry worldwide (Segalés *et al.*, 2005a). Although several co-factors or triggers are thought to be necessary for the development of the disease, PCV2 is considered the essential etiological agent (Ellis *et al.*, 1998) and therefore, effective vaccines seem the most straightforward method for controlling PMWS. Up to date, several vaccine

prototypes such as DNA vaccination (Blanchard *et al.*, 2003a; Kamstrup *et al.*, 2004), the use of recombinant subunit vaccines expressing viral capsids or non-structural (replicase) PCV2 proteins (Blanchard *et al.*, 2003a; Fenaux *et al.*, 2004a; Ju *et al.*, 2005; Song *et al.*, 2007) or inactivated vaccines (Pogranichniy *et al.*, 2004) have been demonstrated to be immunogenic when injected in mice or pigs. However, vaccine efficacy has been only assessed in some studies (Blanchard *et al.*, 2003a; Fenaux *et al.*, 2004a; Pogranichniy *et al.*, 2004; Wang *et al.*, 2007), and none of them evaluated the efficacy of the vaccine against a set of different PCV2 strains. Therefore, this is the first study that tests the efficacy of PCV2 vaccination upon challenge with different PCV2 isolates.

The lack of a consistent, precise and reproducible model of PMWS (Segalés *et al.*, 2005a) is one of the main drawbacks for the experimental evaluation of PCV2 vaccines. Although the prevention of the disease is the ultimate parameter to be evaluated, vaccine efficacy may be validated beyond that by evaluating prevention of the infection, reduction in lesion severity or by determining the ability of the vaccine to induce an immune response that significantly reduces viremia and viral shedding. A vaccine able to prevent infection and shedding of the virus should prevent the disease as well as reduce PCV2 transmission.

In the present study, the experimental model using PCV2 alone as inoculum did not succeed in causing PMWS; however, the results clearly showed that the vaccine was able to prevent viremia in all vaccinated animals regardless of the virus used for the challenge. In contrast, nasal and faecal shedding was not fully prevented, although in both cases the percentage of positive pigs and the viral load in nasal cavities and faecal swabs were significantly reduced in vaccinated pigs compared to their non-vaccinated counterparts. As shown by odds ratios obtained for a positive nasal or faecal PCV2 excretion, vaccination reduced the chance on shedding via these routes on average 14 times. In fact, vaccination prevented faecal shedding in all pigs inoculated with virus C (strain Stoon-1010) and in the rest of the groups faecal shedding was only sporadically observed in

one vaccinated pig from groups B (strain Burgos) and D (strain MO/S-06), and two from group A (strain I-12/11), respectively. This is the first report indicating that vaccination may also reduce PCV2 nasal and faecal shedding. This fact suggests that vaccination may be beneficial not only in terms of reducing viral load in excreta and, potentially, disease on an individual basis, but also on an epidemiological scale. These results open the question of whether vaccination might be devised as an eradication tool in the future.

The examined PCV2 vaccine induced a strong humoral response with induction of IPMA and NA between first vaccination and challenge. Since maternally derived immunity has been demonstrated protective against PMWS (Ostanello *et al.*, 2005; Calsamiglia *et al.*, 2007), any PCV2 vaccination strategy based on piglet vaccination should induce early seroconversion and minimum interference with maternal antibodies to protect the piglet all throughout its productive life. In the present study, the use of conventional, colostrum-fed piglets allowed the possibility to make a proper approach to field conditions since piglets had remaining maternal immunity at the beginning of the study. Although a slight interference was observed, the vaccine was able to effectively overcome maternal immunity and, as early as 13 days after the first vaccination, this effect was already noticeable on IPMA titres of vaccinated pigs. Similar results were obtained with other recombinant vaccine prototypes based on the PCV2 ORF2 protein (Blanchard *et al.*, 2003a; Wang *et al.*, 2007); however, the potential interference of maternal antibodies with the vaccine was not elucidated in those studies in which all piglets were seronegative at the time of the first vaccination. It is worth to comment here that the vaccine induced high titres of NA. Recent studies have associated the lack of PCV2 NA, or an impaired production, with the development of PMWS (Meerts *et al.*, 2006; Fort *et al.*, 2007) and thus, NA are thought to be one of the key elements in protection against PMWS. Wang *et al*. (2007) described PCV2 NA starting by 27 days after priming with an adenovirus expressing the ORF2. In that study, some delay was observed in the development of NA compared to IPMA antibodies; the latter ones appeared

shortly after vaccination. In the present work, viral neutralization test was only performed on day 29 Pre-C, day of challenge and 20 days Post-C, and therefore no data is available on NA titres at the day of booster vaccination. However, certain delay on neutralizing response was observed at least in 9 out of 35 pigs, which had already seroconverted for total antibodies by day 0 Post-C, but not for NA. Those 9 pigs yielded positive results on the NA test by day 20, when all vaccinated pigs showed moderate to high NA titres.

In this vaccine experimental trial, the effect of vaccination upon challenge with different PCV2 isolates was evaluated. The challenge strains included in the study came from different geographic origin; viruses C and D were isolated in North America and viruses A and B in Europe. Viruses A and D corresponded to PCV2b genotype and B and C to PCV2a genotype, which are thought to differ in virulence (Cheung *et al.*, 2007a; Grau-Roma *et al.*, 2008) and which may also have antigenic differences (Lefebvre *et al.*, 2007). Our results indicate that the vaccine had a similar effect in all cases indicating that, most probably, critical epitopes are not affected by the genetic differences displayed by the isolates used. Interestingly, although this vaccine contained the Cap protein from a PCV2a strain, it was found to be equally effective against both challenge strains from genotype PCV2a and genotype PCV2b.

Comparison among non-vaccinated challenged groups revealed differences in the studied parameters, further supporting distinct biological behaviour between PCV2 isolates potentially related to virulence and pathogenicity. Virus A (PCV2b) was considered the potentially most virulent one since it caused more severe PMWS-like lesions and also the longest period of viremia with the highest viral loads. On the other hand, the group of pigs inoculated with virus C (PCV2a) had no pigs with PMWS-like lesions in lymphoid tissues and developed very low levels of viremia, with only few pigs that had seroconverted by day 20 Post-C; these results may suggest that virus C could be less virulent or have slower growth pattern compared to the other isolates. Unfortunately, the failure to

reproduce clinical PMWS did not allow elucidating the relation between genetic variation among challenge viruses and severity of disease, as observed by Cheng et al. (2007a), who reported differences in virus pathogenicity after PMWS reproduction with two isolates from different genotype.

In conclusion, the results demonstrated that vaccination with a PCV2a based vaccine was able to prevent PCV2 viremia in all four different challenge situations representing both PCV2a and PCV2b strains, resulting in a decrease of nasal and faecal PCV2 shedding and, although PMWS was not reproduced, significantly reducing histopathological lymphoid lesions and PCV2 load within lesions. In addition, vaccination induced a strong humoral response with development of NA even in the presence of maternal immunity. Taken together these results suggest that the vaccine may be useful to control PCV2 infections both at individual and population basis, even in the presence of maternal derived immunity.

CHAPTER 6

STUDY IV

One dose of a porcine circovirus 2 (PCV2) sub-unit vaccine administered to 3-week-old conventional piglets elicits cell-mediated immunity and significantly reduces PCV2 viremia in an experimental model

6.1. Introduction

The protective immunity induced by commercial PCV2 vaccines is thought to rely on the development of a strong humoral response, whereas little information is available on the role of cell-mediated immunity. Recently, Goubier *et al.* (2008) reported the presence of PCV2-specific IFN-γ-SC in colostrum of PCV2 naturally infected and vaccinated sows, being their numbers significantly higher in the latter ones and proven to be passively transferred to their offspring. However, in that study, no conclusion could be established on their protective effect against PCV2 since virus-specific IFN-γ-SC could be only detected in piglets within the first five days post farrowing. Studies I and II demonstrated that in the course of PCV2 infection, pigs develop cell-mediated immunity specific to the virus. It was also suggest that the development of PCV2-specific IFN-γ-SC might mediate viral clearance in contribution with NA.

Protection provided by colostral antibodies against PCV2 infection or development of PMWS is documented in the literature (McKeown *et al.*, 2005; Ostanello *et al.*, 2005; Calsamiglia *et al.*, 2007), and seems to be associated with their neutralizing activity (Meerts *et al.*, 2006; Fort *et al.*, 2007). Conversely, the potential interference of MDA with vaccination is still unclear. Under experimental conditions, although high levels of maternal antibodies partially interfered with the development of a humoral response after vaccination, such interference was not enough to significantly hamper vaccine efficacy (Opriessnig *et al.*, 2008a). On the other hand, under field conditions, the lack of vaccine interference with maternal-derived immunity has been described (Kixmoller *et al.*, 2008), but there is at least one study reporting decreased vaccine efficacy to be dependent on the antibody levels at the time of vaccination (Henry *et al.*, 2008).

In study III, the humoral response and the efficacy of a PCV2 sub-unit vaccine using a two-dose schedule in commercial pigs was evaluated. The results of that

study showed that double vaccination was able to prevent viremia. In the present study we investigated whether the degree of immunity induced after one-dose schedule of the same product was efficient enough in protecting 3-week old piglets against PCV2 infection. For that, vaccine immunogenicity was assessed in terms of development of humoral and cell-mediated immune responses, and PCV2 infection by measuring PCV2 viremia, shedding and viral load in tissues. Besides, the potential effect of MDA on vaccine efficacy was also investigated.

6.2. Material and methods

6.2.1. Animals and housing

One hundred and six 2-week-old male piglets were randomly selected from a PCV2-seropositive, conventional 2,000-sow farm located in the North-eastern Spain. Piglets were selected from a total of 35 sows (1-6 piglets/sow). The herd was free from Aujeszky's disease virus, *Brachyspira hyodysenteriae*, *Mycoplasma hyopneumoniae*, and seropositive but stable to PRRSv. PCR (Quintana *et al.*, 2002) was performed on blood, nasal and faecal swabs to confirm that piglets were not infected with PCV2. Selected animals were transported to the experimental facilities of CReSA and let to acclimatize for 7 days before PCV2 vaccination. During that period, animals were weighted and bled to assess their serological status to PCV2 using a previously described IPMA technique (Fort *et al.*, 2007). Piglets were then distributed into four groups balanced according to weight and titres of PCV2 IPMA antibodies.

6.2.2. Vaccine and PCV2 inoculum

The vaccine product was a commercial PCV2a (Segalés *et al.*, 2008) based sub-unit vaccine (Porcilis PCV®, Intervet International, The Netherlands). In the present experiment the vaccine was administered as a single dose at 3 weeks of age.

PCV2 strain I-12/11 (PCV2b genotype) was used as inoculum. This strain was isolated from lymphoid tissues of a field case of PMWS in year 2003 in The Netherlands. The virus was propagated in PCV-free PK15 cells to a titre of 10^5 $TCID_{50}$/ml.

6.2.3. Experimental design and sampling

At 21 days of age, 51 piglets were intramuscularly vaccinated (vaccinated, V), whereas the remaining animals (n=55) received PBS (non-vaccinated, NV). Three weeks after immunization (42 days of age), piglets were challenged intranasally with either 2 ml of a suspension containing 10^5 $TCID_{50}$ of PCV2/ml (challenged, C; n [V-C] =26, n [NV-C] =18) or 2 ml of sterile PBS (non-challenged, NC; n [V-NC] =25, n [NV-NC] =37). After inoculation, pigs were monitored for 21 days. During that period pigs were clinically examined on a daily basis and rectal temperatures were recorded three times per week. Body weight was measured before the immunization, at challenge and at weekly intervals thereafter.

Blood samples were taken at the time of vaccination, at challenge and on days 7, 14 and 21 PI. Sera were stored at -80ºC until used. For the evaluation of cell-mediated immunity, blood samples with lithium heparin were taken from 24 pigs (six from each of the four experimental groups) and isolation of PBMC was performed by density gradient centrifugation using Histopaque 1.077 (Sigma-Aldrich). To assess PCV2 shedding throughout the experiment, nasal and faecal swabs were also taken at all sampling days, re-suspended in 1 ml of PBS and stored at -80ºC until further analysis. On day 21 PI, all pigs were euthanized with an intravenous overdose of sodium pentobarbital and subjected to necropsy. For histopathological studies, samples of tonsil and mediastinal, superficial inguinal and mesenteric lymph nodes were collected and fixed in 10% buffered formalin

Animal care activities and study procedures were conducted in accordance with the guidelines of the GEP, under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma of Barcelona*.

6.2.4. Pathological studies

Formalin-fixed, paraffin-embedded tissue samples were cut at 4 μm thick, stained with hematoxylin-eosin stain and examined for lesions compatible with PMWS. For each individual and tissue, lesions were scored from 0 (no lesions) to 3 (severe lesions) (Rosell *et al.*, 1999). The final histopathological score was the average of the four studied tissues (tonsil, superficial inguinal, mesenteric and mediastinal lymph nodes). ISH was also performed on the same tissues for the detection of PCV2 genome (Rosell *et al.*, 1999). The amount of PCV2 DNA was semi-quantified using a score from 0 (lack of PCV2 genome) to 3 (high amount of PCV2 genome) in each individual examined tissue. The final ISH score was the average of the four studied tissues.

6.2.5. Detection of PCV2 DNA

Serum samples, nasal and faecal swabs were analysed for the presence of PCV2 genome by means of a real-time quantitative PCR (Q-PCR) using the method reported by Olvera et al. (Olvera *et al.*, 2004). Viral concentrations were expressed as PCV2 DNA copy numbers per ml of serum or ml of PBS (nasal and faecal swabs) (Hjulsager *et al.*, 2008)

6.2.6. Serology

Sera were examined for PCV2-antibodies by means of an IPMA technique at all sampling days. PCV2 NA were determined on day of vaccination, at challenge and at the end of the study using the VNT described by *Fort et al.* (2007). With the aim to estimate the decay of passively acquired antibodies to PCV2, an

additional analysis by an indirect enzyme-linked immunosorbent assay (ELISA) for PCV2 antibodies (Ingenasa-PCV, Ingenasa) was performed using serum samples of NV-NC pigs.

6.2.7. Rate of decay of passively acquired PCV2 antibodies

Half-life of MDA was defined as the time needed for a decline of the levels of MDA resulting in a 50% decrease in ELISA optical density. Thus, for each individual and pair of consecutive time-points, decline rate was calculated according to the following formula: $T_{1/2}= (t_1-t_2)* [ln_2/ln (OD_{t=1}-OD_{t=2})]$, where t_1 and t_2 represent consecutive sampling times (in days) and $OD_{t=1}$ and $OD_{t=2}$ are the optical densities obtained in the ELISA. Assuming a constant rate of decay, this rate can be calculated from the following equation: $0.5 = (1-RD)^{T}_{1/2}$ where *RD* is the rate of decay and $T_{1/2}$ is the average half-life of MDA.

6.2.8. Determination of IFN-γ-SC specific to PCV2

Frequencies of IFN-γ-SC in isolated PBMC were detected on day of vaccination, day of challenge and on days 7, 14 and 21 PI by an ELISPOT performed as reported in study I. Anti-IFN-γ (clon P2G10) at 5µg/ml and biotinylated anti-IFN-γ (clon P2C11) at 0.5 µg/ml, from BD Pharmingen, were used as capture and detection antibodies, respectively. The PCV2 strain used for challenge and an extract of recombinant Cap protein expressed in baculovirus and produced in *Trichoplusia ni* insect larva (Pérez-Martín *et al.*, 2008) were used as stimuli. Supernatant of non-PCV2 infected PK-15 cells (mock-stimulus) and wild type baculovirus extract (Ni) were used as their corresponding negative controls. For each individual and stimuli, the ELISPOT count was reported as the average number of spots of replicates minus the average number in the corresponding negative control wells (mock-stimulated cells or Ni-stimulated cells). Results were expressed as the number of responding cells per million of PBMC.

6.2.9. Statistical analyses

Prior to the analyses, values of the number of copies of viral DNA/ml and serological titres (IPMA and VNT) were transformed to $\log_{10}$ and $\log_2$ values, respectively. Normality of the distribution of the examined variables was evaluated by the Shapiro-Wilk test. One-way ANOVA and the Tukey's test were used for mean comparison of the normally distributed variables between groups. Non-normally distributed variables were analysed using the non-parametric Kruskal-Wallis and Mann-Whitney tests. The Chi-square test was applied to evaluate the proportion of positive and negative results in serology, ELISPOT, viremia, lesions and ISH. A linear regression model was built to correlate IPMA to NA or to ELISA results. Statistical analyses were performed using SPSS v.15. The significance level (α) for all analyses was set at 0.05.

6.3. Results

6.3.1. *Clinical presentation and histopathologial evaluation*

None of the pigs developed clinical disease or fever during the whole study. No statistically significant differences in body weight were observed among groups at any of the sampling times.

In the histopathological examinations, mild PMWS-like lesions (lymphocyte depletion and granulomatous inflammation) were observed in 3/18 (16.7%; $CI_{95\%}$: 4.4%-42.7%) NV-C pigs and in 1/26 (3.9%; $CI_{95\%}$: 0.2%-21.6%) V-C pigs, with no significant differences among groups. Also, PCV2 was detected in lymphoid tissues by ISH in 7/18 (38.9%; $CI_{95\%}$: 18.3%-63.9%) NV-C pigs, whereas no positive tissues were observed in pigs from V-C group (0/26) ($P<0.05$).

6.3.2. *Detection of PCV2 DNA in sera, nasal and faecal swabs*

The proportion of PCV2 Q-PCR positive pigs in sera, nasal and faecal swabs for each of the challenged groups during the study is shown in table 6.1.

In the NV-C group, viremia was firstly detected on day 7 PI in 2/18 (11.1%; $CI_{95\%}$: 1.9%-36.1%) pigs (viral load of 6.0 and 6.6. log_{10}), being the proportion of viremic pigs increased up to 7/18 (38.9%; $CI_{95\%}$: 18.3%-63.9%) pigs on day 14 PI and 10/18 (55.6%; $CI_{95\%}$: 31.3%-77.6%) by the end of the study. Mean viral loads were 5.3±1.0 log_{10} and 4.8±0.6 log_{10} PCV2 copies/ml, respectively. In contrast, of the 26 V-C pigs, only two developed viremia throughout the study and in both cases PCV2 DNA was only detected on day 7 PI (viral load of 4.3 and 3. 9 log_{10}); from then onwards, all pigs from V-C group remained negative.

One week after challenge, all pigs from the NV-C group (18/18), and 21/26 (80.8%; $CI_{95\%}$: 60.0%-92.7%) in the V-C one were positive by Q-PCR in nasal swabs (mean viral loads 5.3±1.4 log_{10} and 4.8±0.6 log_{10}) and, on day 14 PI, all pigs from both groups were nasal shedders. However, by the end of the study the proportion of positive pigs was reduced to 17/26 (65.4%; $CI_{95\%}$: 44.4%-82.1%) in the V-C group, whereas 15/18 (83.3%; $CI_{95\%}$: 57.7%-95.6%) NV-C pigs were still positive (non-significant). At that time, viral quantity detected in nasal cavity of V-C pigs was significantly lower compared to NV-C counterparts (4.5±1.0 log_{10} vs. 5.3±1.2 $log_{10;}$ $P<0.01$).

PCV2 load in faeces was lower than in nasal swabs. In the NV-C group, both the percentage of faecal shedders and viral load detected in positive pigs increased from 2/18 (11.1%; $CI_{95\%}$: 1.9%-36.1%) on day 7 PI (mean viral load: 4.6±0.2 log_{10}) to 9/18 (50.0%; $CI_{95\%}$: 26.8%-73.3%) on day 21 PI (mean viral load: 5.0±0.7 log_{10}). Out of 26 V-C pigs only one (3.9%; $CI_{95\%}$: 0.2%-21.6%) had detectable PCV2 DNA in faeces (21 days PI, viral load: 4.5 log_{10}). These differences between NV-C and V-C groups were significant ($P<0.01$).

Serum samples, nasal and faecal swabs from V-NC and NV-NC pigs were all PCV2 negative throughout the study.

Table 6.1: Percentage of PCV2 Q-PCR positive pigs in serum, nasal and faecal swabs from day of PCV2 vaccination to 21 days PI, in non-vaccinated and vaccinated challenged groups.

		Percentage of PCV2 Q-PCR positive pigs				
	Group	**Vac**	**Ch**	**7 d PI**	**14 d PI**	**21 d PI**
Serum	**NV-C**	0 (0/18)	0 (0/18)	11.1 (2/18)	38.9 (7/18)	55.6 (10/18)
	V-C	0 (0/26)	0 (0/26)	7.7 (2/26)	0 (0/26)	0 (0/26)
Nasal swabs	**NV-C**	0 (0/18)	0 (0/18)	100 (18/18)	100 (18/18)	83 (15/18)
	V-C	0 (0/26)	0 (0/26)	80.8 (21/26)	100 (26/26)	65.4 (17/26)
Faecal swabs	**NV-C**	0 (0/18)	0 (0/18)	11.1 (2/18)	16.7 (3/18)	38.9 (9/18)
	V-C	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	3.9 (1/26)

6.3.3. *Humoral immune response to PCV2*

The evolution of serological titres to PCV2 measured by IPMA and VNT tests of the different animal groups is displayed in Figure 6.1.

At the time of PCV2 vaccination, no significant differences in IPMA and NA titres were detected among groups. At challenge (21 days after vaccination), IPMA titres in vaccinated pigs (mean titres: 9.6±1.7 $\log_2$ and 9.5±1.3 $\log_2$, respectively, for V-NC and V-C groups) were higher ($P<0.05$) than those of their non-vaccinated counterparts (mean titres: 7.4±2.6 $\log_2$ and 7.8±3.5 $\log_2$, respectively, for NV-NC and NV-C groups). These differences were evident until the end of the study. NV-C pigs seroconverted between days 14 and 21 PI, reaching an average IPMA titre of 8.3±3.0 $\log_2$ at that latter time. In contrast, by day 21 PI, IPMA titres of NV-NC pigs had decreased to 3.8±3.2 $\log_2$ ($P<0.05$).

PCV2 NA titres in vaccinated pigs (V-NC and V-C groups) at challenge were 6.0±1.9 log_2 and 6.3±1.5 log_2, respectively, while in non-vaccinated pigs (NV-NC and NV-C groups) NA titres were 5.1±1.8 log_2 and 5.7±2.7 log_2, respectively. Although those differences were not statistically significant, a trend was observed (P=0.09). After challenge, seroconversion for NA was observed in both vaccinated groups (average titres: 7.0±1.3 log_2 and 6.7±1.7 log_2, for V-C and V-NC groups respectively), but no significant differences were detected when vaccinated groups were compared with NV-C group (average titre: 5.4±2.8 log_2). In NV-NC group, titres still decreased by that time, reaching significantly lower titres (3.1±1.7 log_2) compared with all the other groups ($p<0.05$). A positive correlation was observed between IPMA and NA titres at all sampling days (day of PCV2 vaccination: $r^2=0.63$, $P<0.01$; day of challenge: $r^2=0.52$, $P<0.01$; day 21 PI: $r^2=0.68$, $P<0.01$).

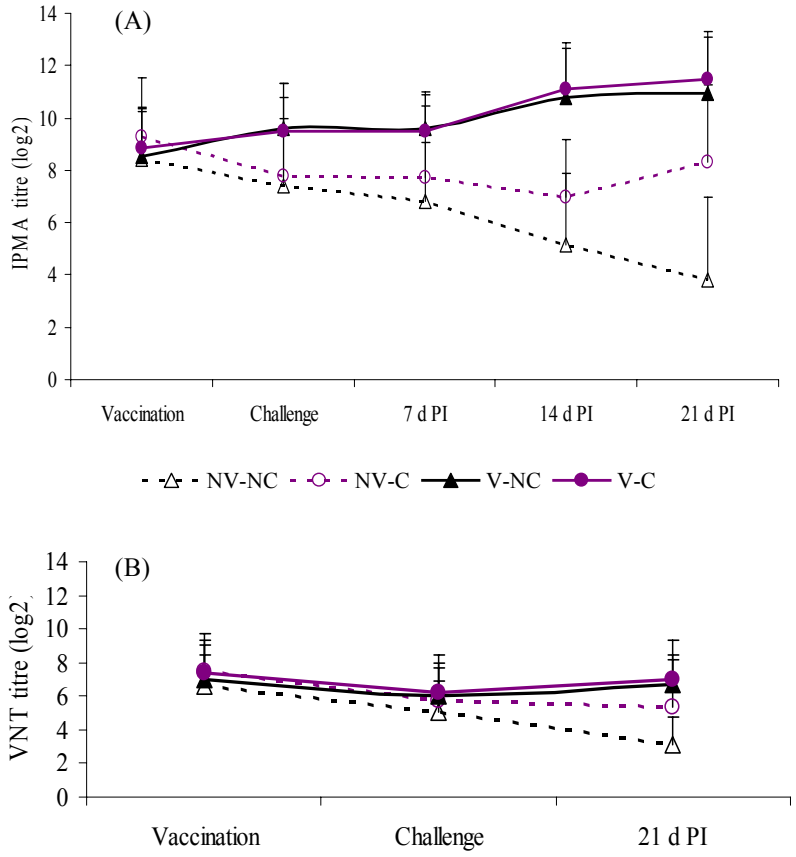


Figure 6.1. Serological profile to PCV2 measured by IPMA (A) and VNT (B) for each experimental group, from day of PCV2 vaccination to day 21 PI.

6.3.4. *Rate of decay of passively acquired PCV2 antibodies*

NV-NC pigs allowed the calculation of the decay rate of MDA. In order to improve the calculation accuracy, serum samples from NV-NC pigs were analysed using an indirect ELISA (Ingenasa-PCV, Ingenasa). Correlation between IPMA and ELISA results was 0.722 ($P<0.01$). Thus, for NV-NC group, the average half-life was calculated to be 15.9 days and the antibody decay ratio was 0.044. If a linear correlation is assumed to exist between optical densities and antibody titres, the decay in the maternal antibody titres would be approximately 4.4% per day.

6.3.5. Effect of maternally derived antibodies on PCV2 infection

Since not all PCV2 inoculated pigs became viremic, the serological profiles of pigs that became viremic and those that did not were compared. For that, NV-C pigs were classified into two categories (viremic and non-viremic) depending on whether or not they had become viremic at any time during the experiment. Then, antibody titres (IPMA and NA) at challenge were compared. Non-viremic pigs had significantly higher IPMA titres than viremic animals (10.6 ± 1.3 $\log_2$ versus 5.5 ± 3.3 $\log_2$, $P<0.01$). For NA, the picture was similar (8.0 ± 2.1 $\log_2$ in non-viremic versus 3.9 ± 1.7 $\log_2$ in viremic animals, $P<0.01$).

6.3.6. Effect of maternally derived antibodies on PCV2 vaccination

Since a significant correlation was determined between antibody titres (both IPMA and NA titres) at challenge and prevention of viremia, the possible interference of maternal antibodies with the development of an active humoral immune response after vaccination was examined. Animals from the V-NC group were categorized according to their serological profiles obtained using

both IPMA and VNT in response to vaccination (seroconversion or lack of seroconversion). Antibody titres at the time of vaccination were compared between responding and non-responding pigs. Results indicated that pigs that did not seroconvert against PCV2 had significantly higher IPMA titres ($P<0.01$) at the time of vaccination (10.0±1.3 log_2) compared to those animals that seroconverted (8.0±1.6 log_2). For NA, differences were also significant (8.8±1.8 log_2 versus 5.5±1.6 log_2, respectively; $P<0.01$). Figure 6.2 shows the antibody-profile of PCV2 IPMA (2A) and NA (2B) titres of V-NC pigs according to their humoral immune response after vaccination.

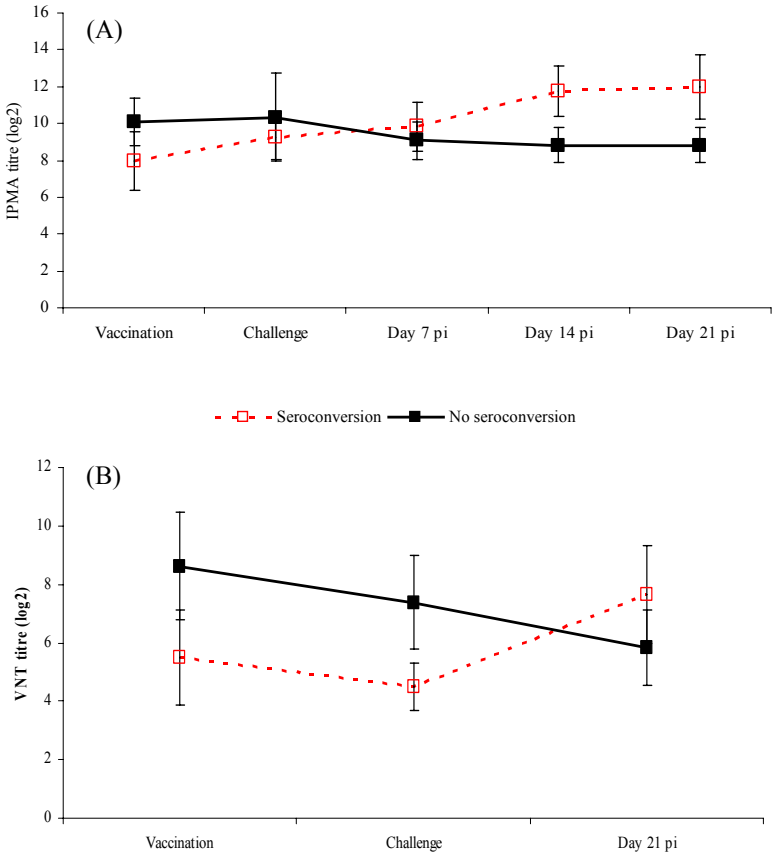


Figure 6.2: Antibody-profile of PCV2 IPMA (A) and NA (B) titres of V-NC pigs according to their humoral immune response after vaccination.

6.3.7. Cell-mediated immune response to PCV2 using whole virus or Cap protein

Development of PCV2- and Cap-specific IFN-γ-SC for each experimental group is shown in Figure 6.3. Before vaccination, frequencies of IFN-γ-SC for PCV2 or Cap were negligible (0-6/10^6 PBMC). At challenge, 4/6 pigs in V-C group and 5/6 in V-NC responded to the whole virus, with average frequencies of PCV2-specific IFN-γ-SC of 20±12/10^6 PBMC and 18±10/10^6 PBMC, respectively. Two weeks after challenge, no response was observed in V-NC animals (0-2 PCV2-IFN-γ-SC), while 6/6 pigs responded in the V-C group (average frequency of PCV2-specific IFN-γ-SC 35±22/10^6 PBMC), being this difference significant ($P<0.05$). In the V-C group, IFN-γ responses remained constant on day 21. For NV-C pigs, development of PCV2-specific IFN-γ-SC started on day 14 PI (3/6 pigs with an average frequency of 72±66 PCV2-IFN-γ-SC) and increased by day 21 PI (4/6 pigs with an average frequency of 148±199 PCV2-IFN-γ-SC).

When using the Cap-protein as recall antigen, results of the IFN-γ ELISPOT were slightly different than using whole virus. Thus, in V-C and V-NC groups at challenge, 5/6 and 6/6 animals responded to Cap protein with average frequencies of 22±7/10^6 PBMC and 38±20/10^6 PBMC IFN-γ-SC, respectively. Challenge of vaccinated pigs (V-C group) produced a booster in the frequencies of Cap-specific IFN-γ-SC. Thus, two weeks after inoculation with the wild virus, frequencies of Cap-specific IFN-γ-SC/10^6 PBMC increased up to 53±27 in V-C pigs while decreased to 11±6 in V-NC animals ($P<0.05$). In the NV-C group, only two pigs responded, with average Cap-specific frequencies of 29±15/10^6 PBMC on day 14 PI and 11±8/10^6 PBMC on day 21 PI; contrary to the results observed in vaccinated groups, in NV-C group frequencies developed in response to the Cap protein were lower in comparison to those obtained using the whole virus.

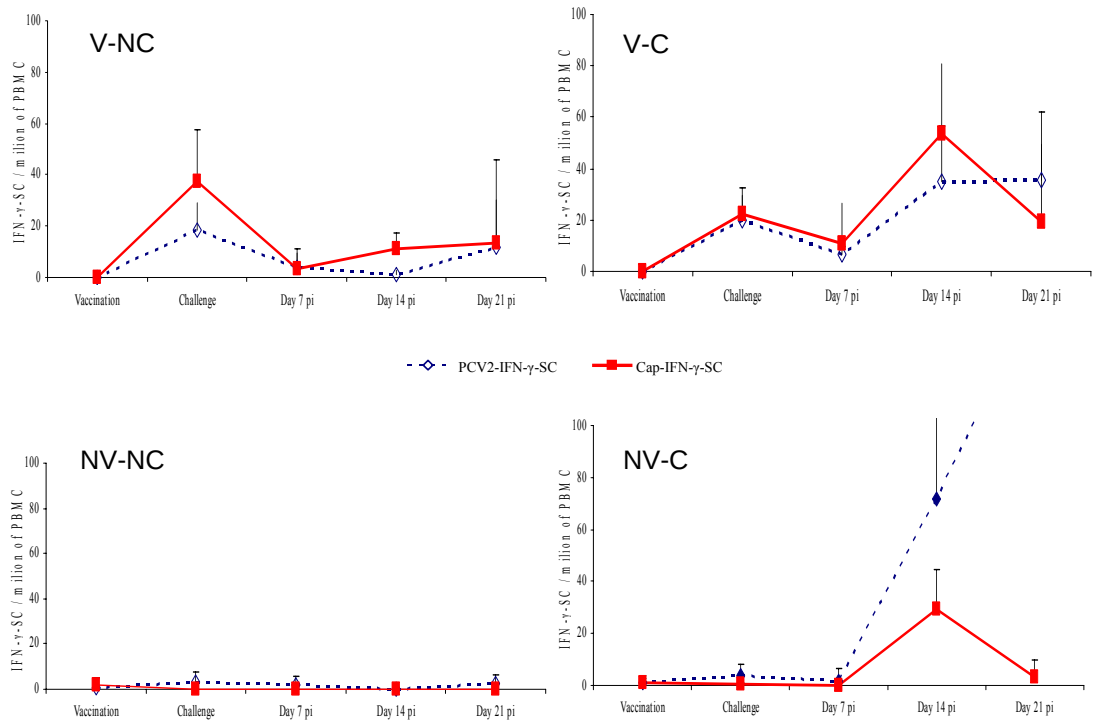


Figure 6.3: Development of PCV2- and Cap-specific IFN-γ-SC for each experimental group, from day of PCV2 vaccination to day 21 PI. V=vaccinated, NV=non-vaccinated, C=challenged, NC=non-challenged.

6.4. Discussion

PCV2 vaccines seem to be very efficient based on the results from experimental infections and field reports (Kixmoller *et al.*, 2008; Opriessnig *et al.*, 2008a). However, several aspects related to the practical use of PCV2 vaccination for piglets such as the interference of MDA, the need for a second dose and the precise mechanisms involved in post-vaccination immunity are still poorly known. In the present study, with the aim to investigate whether the administration of one dose of a PCV2 sub-unit vaccine (Porcilis PCV®) might be efficient enough to control PCV2 infection, conventional piglets were vaccinated at 21 days of age and challenged three weeks later with a genotype PCV2b isolate. Vaccine efficacy was assessed in terms of reducing PCV2-associated lesions and viral load in serum, nasal and faecal swabs. To evaluate vaccine

immunogenicity, both humoral and cell-mediated responses were measured. Besides, the potential interference effect that maternal antibodies might have on the immunity induced by one dose schedule vaccination, and therefore on its efficacy on controlling PCV2 infection, was also investigated.

PMWS is considered a multifactorial disease in which the participation of PCV2 is the essential element (Segalés *et al.*, 2005a). However, the disease is hardly reproducible under experimental conditions. A meta-analysis performed by Tomás et al. (2008) indicated that the highest likelihood to experimentally reproduce PMWS includes the use of PCV2-seronegative piglets younger than 3-weeks of age. However, such models do not fit with the natural conditions in which pigs develop PMWS in the nurseries or fattening units. Therefore, conventional colostrum-fed piglets were used in the present study to test the vaccine product mimicking field conditions. It was assumed that chances to reproduce PMWS by using such type of animals and PCV2 alone as inoculum were low and, therefore, the evaluation of vaccine efficacy in terms of protection against clinical disease was not feasible. This situation applies to this study, but also to earlier ones in which only sub-clinical infections were developed (Fenaux *et al.*, 2004a; Opriessnig *et al.*, 2008a, Study III). As performed in those studies, parameters related to PCV2 infection such as viremia, shedding, presence of microscopic PMWS-like lesions in lymphoid tissues and viral load within lesions were considered for the assessment of vaccine efficacy in the present work.

In study III, vaccination of conventional pigs with two doses of Porcilis PCV®, administrated at 4 and 6 weeks of age, resulted in prevention of viremia. In the present study, in which a single dose of the same vaccine was evaluated, 24 out of 26 V-C animals did not develop viremia either. In addition, the two pigs that were found PCV2 PCR positive in serum on day 7 PI were negative on day 14 PI, and remained so until the end of the study. In contrast, in the NV-C group, 10 out of 18 pigs were PCV2 positive in sera throughout the study. Comparison of the Q-PCR results in sera between those two groups indicated that a single dose

vaccination was able to reduce the proportion of viremic pigs, and, when not fully prevented, viremia was shortened. In terms of vaccine efficacy, this represents 86.1% PCV2 viremia reduction. Additionally, a positive effect of vaccination on PCV2 shedding was also observed, significantly reducing the proportion of PCV2 faecal shedders and viral load in nasal cavity on day 21 PI, in comparison to the NV-C group. Herein, it was shown that one dose was effective in reducing the effects of sub-clinical PCV2 infection, limiting virus replication and thereby decreasing the amount of virus released. Nevertheless, further studies are needed to perform side by side comparison between one and two dose regimens as well as to assess one dose vaccination efficacy on clinical PCVD control.

At challenge, development of a humoral immune response to the vaccine was observed since significantly higher IPMA titres in vaccinated pigs compared to controls were observed. In contrast, although NA titres were also higher in vaccinated groups at the same time, no clear seroconversion for the latter ones was observed until day 21 PI. Also, in the V-C group, a boost on PCV2 antibodies was detected firstly on day 14 PI using an IPMA technique, whereas neutralizing response was mostly low and even undetectable in some pigs by the end of the study. These results support earlier data reporting low and delayed production of NA to occur sporadically in some pigs in response to both, PCV2 infection (Fort *et al.*, 2007) and PCV2 vaccination (Wang *et al*., 2007, Study III). In a recent study by Opriessnig *et al.* (2008a), no clear seroconversion for NA after a single PCV2 vaccination was observed in presence of high level of antibodies at the time of immunization. The present data shows that, although one dose was able to induce a neutralizing immune response in most of the pigs, mean NA titres developed (6.1±1.7 $\log_2$) were apparently lower in comparison to the levels detected using the same technique in study III, in which piglets were given the same product twice (7.5±1.2 $\log_2$). These results suggest that two doses might be necessary to induce a strong neutralizing response in pigs that have high antibody titres at vaccination. Further analysis of the serological data showed

interference of passively acquired antibodies with PCV2 infection and active seroconversion to vaccination. According to our results, animals with IPMA titres beyond 10.6 log_2 (or NA > 8 log_2) are likely to be protected against PCV2 viremia when PCV2 challenged oro-nasally with 10^5 $TCID_{50}$, whereas those having titres below 5.5 log_2 at IPMA or 3.9 log_2 at NA are potentially more susceptible. Regarding the effect of MDA on vaccination, the performed analysis suggests that IPMA titres equal or beyond 10 log_2 (or NA> 8.8 log_2) seem to interfere with the development of the humoral immune response after vaccination, while levels below 8 or 5.5 log_2 at IPMA and VNT techniques respectively do not.

Ideally, PCV2 vaccination should be administrated when residual maternal antibodies are minimal and before pigs become naturally infected. Since PCV2 infection and seroconversion dynamics greatly vary among farms (Grau-Roma *et al.*, 2009), antibody-profiling of a representative number of piglets prior to vaccine implementation seems therefore a good tool to estimate proper vaccination timing. Based on our results, to ensure a humoral immune response development after vaccination, PCV2 IPMA titres at the time of vaccine administration should stay below 10 log_2.. Considering the fact that pigs with IPMA titres of 5.5 log_2 or lower are potentially susceptible to PCV2 infection and that the half-life of passively acquired antibodies is around 15 days, an estimation of the pig age at vaccination can be established, and vaccination scheduled accordingly.

In the present study, we first describe the development of cell-mediated immunity in response to PCV2 piglet vaccination. Thus, at challenge (3 weeks after immunization), only vaccinated pigs showed induction of IFN-γ-SC specific to PCV2 and the Cap protein. This response was increased in the vaccinated pigs which were later challenged, highly suggesting the presence of effector memory T cells in immunized animals. These results are in accordance with those obtained by Shen *et al.* (2008) using a murine model, in which it was

demonstrated that protective immunity against PCV2 in mouse was mediated by Cap-specific $CD8^+$ cells and seroneutralization responses. In pigs, the development of virus-specific IFN-γ-SC in response to PCV2 infection has been demonstrated (Studies I and II). Interestingly, in the present study, the intensity of responses generated using either the Cap protein or the whole PCV2 as stimulus differed among groups. Thus, whereas in vaccinated groups (V-NC and V-C) responses to the Cap protein were similar or slightly higher than to the whole PCV2, in NV-C pigs, IFN-γ responses obtained by stimulating PBMC with the whole virus were higher than with the Cap protein. These findings suggest that infected animals in which PCV2 is replicating might respond strongly to other viral components different from the Cap protein. Kekarainen *et al.* (2008a) demonstrated the ability of PCV2 viral components to differently modulate cytokine responses *in vitro*. Thus, stimulation of PBMC with the whole PCV2 resulted in IL-10 induction, while no production was observed using PCV2 virus-like particles (VLPs) or oligodeoxyribonucleotides containing CpG motifs (CpG-ODNs) based on the PCV2 genome. Also, PCV2 and some CpG-ODNs but not VLPs were found to inhibit IFN-γ and IL-2 produced *in vitro* during recall antigen responses. These results, together with our findings and the fact that the Cap protein has been demonstrated to be a good immunogen (Blanchard et al. 2003a, Study II), suggest that this protein is a suitable stimulus for *in vitro* assays aimed to assess cell-mediated immune responses to PCV2 vaccination.

In summary, the results of the present study demonstrate that one dose administration of Porcilis PCV® significantly reduced PCV2 viremia, shedding, as well as viral genome loads within PMWS-like lymphoid lesions. Also, high levels of MDA were seen to interfere with seroconversion to vaccination, while apparently no effect was observed on vaccine efficacy. The data presented here provide new information regarding the mechanism by which PCV2 vaccines may confer protective immunity against PCV2, describing for the first time the development of cell-mediated immunity in response to PCV2 piglet vaccination.

CHPATER 7

GENERAL DISCUSSION

Since PCV2 was first linked to PMWS in 1997, several experimental models including infectious or non-infectious co-factors have been shown to trigger the progression of PCV2 infection into the clinical form of disease (Segalés *et al.*, 2005a). However, the mechanisms by which those co-factors influence the development of PMWS are partly unexplained, and consequently, a universal reproducible experimental model of the disease is still lacking today. As a result, most of the data obtained in experimental trials are based on a limited number of PMWS-affected pigs or even only on PCV2 sub-clinical infected animals. The abovementioned considerations also apply to the results discussed here since PMWS could not be reproduced in any of the four experimental inoculations performed in this Thesis, thus supporting the fact that reproduction of PMWS by using PCV2 alone is difficult.

Although PMWS was not reproduced, substantial differences were observed when virological and pathological outcomes were compared among studies (Table 7.1). The most remarkable difference among studies was the serological status of the piglets against PCV2. Piglets from study I were caesarean-derived colostrum-deprived and, therefore, all negative to PCV2 antibodies. In contrast, the use of conventional pigs from a PCV2-seropositive herd in studies II-IV implied the presence of remaining passively acquired PCV2 antibodies at challenge. In study I, all pigs became infected and all but two had PCV2-associated lesions and virus in tissues. Conversely, in studies II-IV, infection of all inoculated pigs was never achieved and the proportion of animals with lesions consistent with a PCV2 infection was always lower compared to study I. In addition, comparison among studies II, III and IV revealed that pigs from study IV had the highest levels of MDA and the lowest proportion of viremic animals. It has been demonstrated in this Thesis (study IV) as well as by other authors (McKeown *et al.*, 2005; Ostanello *et al.*, 2005) that MDA protect against PCV2 infection on a titre-dependent manner. It can be concluded, therefore, that the observed differences among studies might be partly explained by the distinct PCV2 serological status of pigs at challenge.

Besides the levels of PCV2 antibodies, the dose of PCV2 in the inoculum might have had also an impact on the final outcome of PCV2 infection. Pigs from studies I, III and IV were inoculated with PCV2 at a titre around 10^5 $TCID_{50}$, whereas 10^7 was used in study II. Since pigs from studies II and III had similar backgrounds and levels of maternal antibodies, the different infectious dose seems a likely explanation for the substantial differences exhibited between both studies on the evolution of PCV2 infection. In addition, the high dose of the inoculum administered in study II might also explain why, despite having different serological status, pigs from studies I and II displayed comparable results at viremia and histopathology.

Table 7.1. Summary of the experimental set-up and virological and pathological results for each of the four PCV2 experimental inoculations performed in this Thesis. Only non-vaccinated pigs were considered in studies II and III.

		Study I	**Study II**	**Study III**	**Study IV**
Experimental set-up	**Age at challenge (weeks)**	1	6	8	6
	IPMA titre at challenge	negative	5.6±1.0	4.4±2.6	7.8±3.5
	PCV2 genotype	a	a or b	a or b	b
	$TCID_{50}$/pig	$10^{5.5}$	$10^{7.2}$	$10^{4.5}$	$10^{5.3}$
Virological and pathological outcome	**% viremic pigs**	100 (12/12)	92 (11/12)	83 (20/24)	56 (10/18)
	% of pigs with PCV2 lesions	75 (9/12)	75 (9/12)	29 (7/24)	17 (3/18)
	% of pigs with positive ISH	75 (9/12)	58 (7/12)	25 (6/24)	39 (7/18)

Altogether, these data suggest that the occurrence and progression of PCV2 infection is directly influenced by the balance between the infectious pressure at which piglets are subjected and the presence of protective levels of PCV2 antibodies. The observations made in this Thesis are in accordance with the results obtained from a meta-analysis performed on published data from PCV2 experimental infections, in which the main common characteristics shared by

successful experiments (i.e., experiments that reproduce PMWS clinical signs in at least one PCV2-inoculated animal) were the inoculation of colostrum-deprived pigs during the first week of age with high doses of PCV2 inoculum (>10^5 $TCID_{50}$) (Tomás *et al.*, 2008).

When exposing a pig to PCV2, three different situations might follow: 1) PCV2 infection does not occur, 2) PCV2 infection occurs but low amount of virus is detected and no clinical disease develops, or 3) PCV2 infection occurs, followed by the accumulation of high viral loads and development of PMWS. The ability of a pig to mount an efficient immune response to PCV2 infection seems critical to keep it as sub-clinical (situation number 2), whereas impaired immune responses presumably lead pigs to PMWS. Some of the immunological mechanisms behind the ability of a pig to control PCV2 replication were investigated in the present Thesis.

In the first part of this Thesis (Studies I and II), innate and adaptive immune responses of pigs upon PCV2 experimental inoculation were investigated.

Innate immunity constitutes the first line of host's defences against pathogens. It involves cells possessing pattern recognition receptors (PRR), which detect molecules present in the pathogen but not in the host. Through pathogen-PRR interaction, the expression of cytokines is induced resulting in the activation of an immediate immflamatory process and the initiation of adaptive immunity responses against the pathogen. In study I, an early IFN-α response was detected in PCV2-inoculated pigs, as seen by the transient detection of this cytokine in serum on day 5 PI. IFN-α underlies the main antiviral defences initiated following activation of the innate immunity. Not only does it mediate direct inhibition of virus replication in infected cells, but it also stimulates the initiation of adaptive responses through the activation of T helper and T citotoxic cells (García-Sastre & Biron, 2006). The fact that pigs from study I had the ability to

mount an efficient specific response to PCV2 is also suggestive of the existence of effective innate responses.

Recent *in vitro* studies indicated that PCV2 inhibits the function of pDC to produce IFN-α and other cytokines with important antiviral effects released during innate responses (Vincent *et al.*, 2005). Although such inhibition apparently occurs regardless of the infection status of the pig, only a small proportion of PCV2 infected animals develops PMWS, thus suggesting that this mechanism does not seem to be acting as the sole trigger of the disease. Results of study I suggest that some pigs might be able to counteract PCV2-induced suppression of innate immunity through the induction of early IFN-α-mediated antiviral responses. If this is at the base of the susceptibility of individuals to PMWS, it is possible that genetic variants within the pig population might trigger innate responses more or less effectively. This has already been described for the progression of some human viral diseases, such as hepatitis B virus (HBV) and human immunodeficiency virus 1 (HIV-1) infections, in which clinical disease has been associated with certain genetic variants of the IFN-α receptor-I (Diop *et al.*, 2006; Song le *et al.*, 2008).

Besides its ability to inhibit the function of pDC to produce IFN-α, PCV2 can also modulate the secretion of other cytokines, such as IL-10 (Kekarainen *et al.*, 2008b). In studies I and II, it was demonstrated that PCV2 promotes the expression of IL-10 regardless of the immunological and infection status of the pig, indicating the innate nature of this response. The fact that PCV2 Cap or Rep proteins alone do not promote IL-10 induction (Study II) suggests that IL-10 release is mediated by either the PCV2 DNA and/or other viral elements. Over-expression of IL-10 has been detected in serum and tissues of PMWS-affected pigs (Darwich *et al.*, 2003b; Stevenson *et al.*, 2006). In study I, measurements of this cytokine in serum revealed detectable levels only in one pig, suggesting that sub-clinically affected pigs do not have a significant increase on IL-10 expression. This would be consistent with the lower amounts of PCV2 typically

detected in pigs coursing a sub-clinical infection compared to PMWS-affected ones (Olvera *et al.*, 2004; Fort *et al.*, 2007; Grau-Roma *et al.*, 2009).

To clarify the role of innate responses in the pathogenesis of PMWS, the observations made in this Thesis need corroboration in a model of clinical disease, by comparing the immunological profiles between sub-clinically and PMWS affected pigs.

When innate responses are not sufficient to control a pathogen, acquired immunity develops. Adaptive immunity has the ability to recognize the pathogen and to mount a specific attack against it, by means of humoral and cell-mediated responses. The adaptive immunity developed upon PCV2 infection was investigated in this Thesis. In all four studies, seroconversion for total PCV2 antibodies measured by IPMA occurred in most piglets between the second and third week PI, in agreement with the observations made by other researchers (Pogranichnyy *et al.*, 2000; Bolin *et al.*, 2001; Resendes *et al.*, 2004a). In contrast, remarkable differences on the NA kinetics were detected among studies and individuals. In study I, NA were not detectable until the fourth week PI, two-three weeks after the onset of total antibodies. In studies III and IV, by the time pigs were euthanized (20 days PI), clear seroconversion for NA was only detected in 9/20 and 3/10 infected pigs, respectively. It can be concluded, therefore, that NA to PCV2 might develop slowly, at least during primary infections. This phenomenon might explain the prolonged viremia detected in a high proportion of pigs naturally or experimentally infected with PCV2 (Bolin *et al.*, 2001; Larochelle *et al.*, 2003; Resendes *et al.*, 2004a; Sibila *et al.*, 2004). Whether delayed neutralizing responses are caused by PCV2-induced immunosuppression, poorly immunogenic neutralizing epitopes or even both mechanisms, remains to be elucidated. One possible explanation would be that non-neutralizing epitopes display immunogenic dominance over neutralizing epitopes. This would explain the positive results detected at IPMA in absence of NA when the immune system first encounters PCV2 in a primary infection

(Study I) or vaccination (Study IV). As seen in study III, strong NA responses are developed after repeated immunizations.

The involvement of NA in protection against PMWS has been demonstrated (Meerts *et al.*, 2006). However, in the present Thesis, it was shown that viremia decreased in some pigs prior to the appearance of NA, thus suggesting the role of additional immune mechanisms on controlling PCV2 replication.

NA mediate the clearance of cell-free viruses. However, target and elimination of virus-infected cells requires activation of cell-mediated responses. Such responses may even become crucial for the complete clearance of non-cytopathic viruses (Recher *et al.*, 2004). The present Thesis demonstrates that adaptive immunity developed over the course of a sub-clinically PCV2 infection also involves cell-mediated responses, as seen by the development of PCV2-specific IFN-γ-SC. In addition, such responses were apparently related to the viral replication. Thus, in pigs in which PCV2 viral load was low, IFN-γ-SC were rather low or even not detected (Study II). In contrast, pigs exhibiting high levels of viremia and lesions consistent with PCV2 replication developed higher frequencies of IFN-γ-SC that appeared earlier than PCV2 antibodies (Study I and II). The appearance of IFN-γ-SC was mostly found coincidentally with the decrease on viral titres and, in some cases, this occurred before the appearance of detectable NA (Study I). Based on these observations, it seems that development of PCV2-specific cell-mediated responses might help to avoid progression of PCV2 infection. Further studies focused on acquired immunity of PCV2-infected pigs that develop clinical disease are required to elucidate whether impairment of cell-mediated responses might be also a recurring phenomenon in diseased animals, as it has been already demonstrated for impaired humoral responses (Meerts *et al.*, 2006). Such situation would be expectable in a strong DC responsiveness scenario, such as the one induced by PCV2 (Vincent *et al.*, 2005; Vincent *et al.*, 2007), since immature DC are unable to stimulate effector and memory T-cell responses.

The role of the distinct PCV2 viral proteins in the development of adaptive immunity to PCV2 has only been assessed for humoral responses (Mahe *et al.*, 2000; Pogranichnyy *et al.*, 2000; Cheung & Bolin, 2002; Meerts *et al.*, 2005; Pérez-Martín *et al.*, 2008). All authors of these studies came to the conclusion that PCV2 antibodies are mainly directed to Cap, whereas anti-Rep antibodies are not always detectable and, when produced, appeared later and with lower titres than anti-Cap antibodies. In this Thesis, the role of Cap and Rep proteins in cell-mediated responses to PCV2 was investigated. It was demonstrated that PCV2 sub-clinically infected pigs develop IFN-γ-SC in response to both proteins (Study II). Low viral load triggered the development of IFN-γ-SC against the Cap but not against the Rep, thus suggesting differences in immunogenicity between the two proteins. In contrast, high frequencies of Rep-IFN-γ-SC were associated with high viral load, most probably reflecting the increased levels of this protein during the replication cycle of the virus (Mankertz *et al.*, 2004). Since the replicase is indispensable for viral replication, targeting this protein seems also an effective strategy to limit progression of PCV2 infection.

The second part of this Thesis focused on the immunological mechanisms underlying vaccine-induced protection (Studies II and III).

The protective effect of PCV2 antibodies, either passively acquired or induced by vaccination, was evidenced in this Thesis. Study IV demonstrated that the presence of PCV2 antibodies in colostrum protects against challenge with PCV2 on a titre dependent manner. Such protective effect was associated with their neutralizing activity. Based on the results of study IV, a titre of 5.5 $\log_2$ at IPMA and 4 $\log_2$ at the neutralizing assay, could be proposed as potential thresholds for susceptibility to PCV2 infection. In study III, piglet vaccination with a PCV2 vaccine containing the Cap (PCV2 Cap vaccine) completely prevented viremia after PCV2 challenge, presumably as a result of the induction of high levels of PCV2 antibodies (11.8±1.9 $\log_2$), which were far beyond the protective titre

established in study IV. Therefore, present results demonstrate that antibodies are of major importance on protection against PCV2 and point to the induction of sterilizing immunity as the primary goal to be achieved for PCV2 vaccines.

Study IV described for the first time the development of cell-mediated immunity after vaccination with a PCV2 Cap vaccine. The relevance of this finding requires further research, but the role of cell-mediated responses during the course of PCV2 infection is suggestive of their contribution also on vaccine-induced protection. Since the protective effect of PCV2 antibodies is titre-dependent, the sole induction of a humoral response might not guarantee full protection against PCV2 infection (Blanchard *et al.*, 2003a; Opriessnig *et al.*, 2009a). In this context, cellular responses might become important to block PCV2 replication and avoid progression of PCV2 infection towards PMWS. Upon PCV2 infection, Cap- and Rep-IFN-γ-SC are developed. However, following vaccination with the commercial available PCV2 vaccines, only responses to the structural protein of PCV2 should be expected, since all of them are based on Cap proteins or inactivated viruses. It would be of interest, therefore, to investigate whether potential attenuated PCV2 vaccine prototypes induce Rep-specific T cell responses.

Up to now, vaccination has been proven highly efficacious in the field (Fachinger *et al.*, 2008; Kixmoller *et al.*, 2008). However, the fact that PCV2 genome is evolving over time (Olvera *et al.*, 2007) arises concerns about their efficacy on future new variants of PCV2. In Canada, during years 2004 to 2006, farms known to be serologically positive to PCV2a experienced severe outbreaks of PMWS that were linked to the emergence of PCV2b isolates (Gagnon *et al.*, 2007). Nonetheless, although genetic and antigenic diversity among PCV2 strains has been demonstrated (Olvera *et al.*, 2007; Lefebvre *et al.*, 2008a; Shang *et al.*, 2009), the existence of potential distinct PCV2 serotypes has not been reported so far. Study III demonstrated that vaccination with a PCV2a Cap vaccine equally protects against challenge with PCV2 belonging to the

homologous or the heterologous genotype. This agrees with field observations, since all commercial vaccines are based on PCV2a strains but are apparently able to protect against PMWS in farms in which PCV2b is predominant. On the basis of these results, it can be speculated that there is at least one immunodominant neutralizing epitope conserved among genotypes to which Cap vaccine-induced antibodies are presumably directed.

On a practical level, one of the main problems for vaccinating piglets is the interference with MDA. In the present Thesis, the effect of MDA on PCV2 vaccination was assessed, thereby providing a practical approach to help establish effective vaccination programs under field conditions.

The effect of MDA on vaccine-induced immunity was first evaluated in study III. It was found that maternal immunity slightly interfered with the development of PCV2 antibodies following double vaccination with a Cap vaccine. However, since vaccinated pigs elicited significantly higher humoral responses compared to controls, such interference was not studied in depth. The effect of MDA was assessed in more detail in study IV, in which pigs were vaccinated once with the same product. It was shown that, in the face of high levels of MDA (≥ 10 $\log_2$), piglets did not seroconvert to vaccination. Given the results of that study, a deeper analysis of the data from study III was performed. Figure 7.1 shows the serological profile developed by vaccinated, non-challenged pigs from study III, grouped according to the titres the animals had at the time they were given the first dose of vaccine (> 8 or ≤ 8 $\log_2$). As it was observed in study IV, seroconversion after the administration of one dose depended on the MDA titres at vaccination. However, after revaccination, all pigs seroconverted for IPMA and NA, indicating that double vaccination overcame the interference with MDA. Interestingly, figure 7.1, as well as figure 5.2 from study I, clearly shows that regardless of the seroconversion status, the antibody titres at the end of the study differed according to the initial MDA levels. Thus, the higher the MDA titres at vaccination, the lower the levels of vaccine-induced antibodies. These

observations indicate that high levels of MDA might influence the duration of the immunity induced by vaccination. Whether this might eventually result in reduced vaccine efficacy for late infections deserves further study.

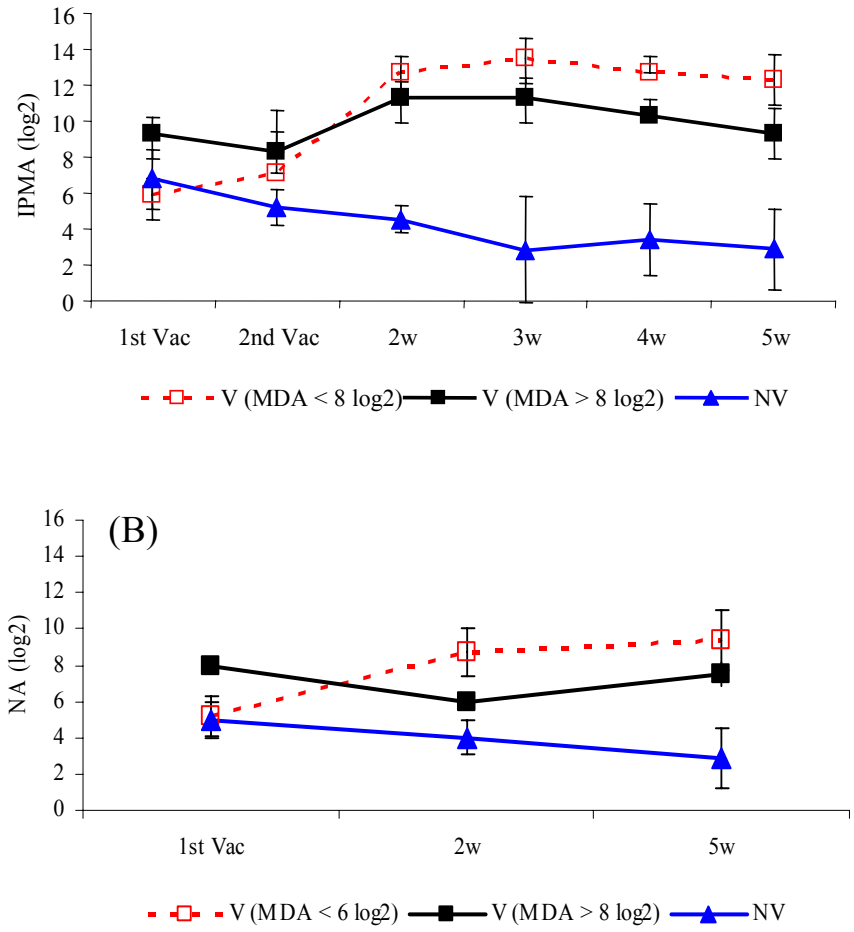


Figure 7.1: Serological profile of vaccinated, non-challenged pigs from study III grouped according to IPMA (A) and NA (B) titres they had at the time they were given the first dose of Cap vaccine. NV= non-vaccinated pigs

In studies III and IV, the efficacy of a Cap vaccine was evaluated at two and one doses respectively. In both trials, the immunization induced after vaccination significantly reduced the virus load in blood and lymphoid tissues, indicating that both one- and two- dose approaches are efficient in controlling PCV2 infection, even in the presence of MDA. Concomitantly to the development of this Thesis, another research group performed two experimental studies in which some of the parameters evaluated in studies III and IV were also investigated. In the first one, the effect of MDA on the efficacy of another one dose commercial PCV2 Cap

vaccine was evaluated upon challenge with PCV2 using pigs with different levels of passively acquired antibodies (Opriessnig *et al.*, 2008a). It was found that, although pigs with high titres of MDA differed from the seronegative ones in the humoral response developed after vaccination, the vaccine was equally effective in reducing PCV2-associated lesions and viremia following PCV2 challenge. Nevertheless, it is important to note that in that study, as well as in studies III and IV of the present Thesis, PCV2 challenge was performed a few weeks post vaccination (4, 2 and 3 weeks post-vaccination, respectively) and, thus, it is not representative of all potential field scenarios. Piglet vaccination is usually administered in the field at around three weeks of age, but exposure to PCV2 might occur during the whole pig productive life. In the second study by Opriessnig and co-workers (Opriessnig *et al.*, 2009a), the duration of immunity and the efficacy of one- and two-dose PCV2 commercial vaccines was evaluated upon challenge 2 or 3 months post-vaccination, respectively. As expected, pigs vaccinated with two doses had higher PCV2 antibody levels one week after revaccination but, besides that, no other difference in the humoral response developed to vaccination was observed. In addition, both, one- and two-dose approaches significantly reduced PCV2 viremia and overall microscopic lymphoid lesions following challenge at 2 and 3 months post-vaccination, respectively. This fact indicates that the immunity induced after the administration of either one or two doses of Cap vaccine is effective in protecting from late infections. However, all pigs included in that study were PCV2 seronegative at vaccination and therefore, whether the presence of maternal antibodies might have an effect on the long term efficacy could not be elucidated. Taking into account that the antibody profile developed following the administration of PCV2 Cap vaccine, either at one or two doses, depends on the MDA titres at vaccination (study II, III, Opriessnig et al 2008c), the potential interference of high levels of passively acquired PCV2 antibodies with vaccine efficacy at late challenge warrants further study.

Since the levels of MDA and PCV2 infection dynamics vary among farms (Grau-Roma *et al.*, 2009), the optimum vaccination timing might also differ from farm to farm. Therefore, the best vaccination strategy would be the one adapted to the epidemiological situation of each particular herd. An intention of the present Thesis was to provide information to help practitioners identify the proper PCV2 vaccination timing. This would be of particular interest when dealing with potential situations of vaccine failure due to inappropriate vaccination programs. Apparently, vaccinating too early is not necessary, since maternal-derived immunity is protective, and might be even counterproductive due to potential interference of MDA with the vaccine. On the other hand, a too late vaccination might constitute a risk of vaccine failure, since, as soon as MDA wane, pigs might become infected with PCV2. Therefore, based on the results of this Thesis, a "vaccination window" is proposed, defined as the range of antibody titres at which piglets should be vaccinated to minimize interference with MDA and, at the same time, avoid a gap of immunity (Figure 7.2). By means of serological and virological herd profiling, the age at which piglets fall into that "vaccination window" can be estimated and, therefore, vaccination implemented accordingly.

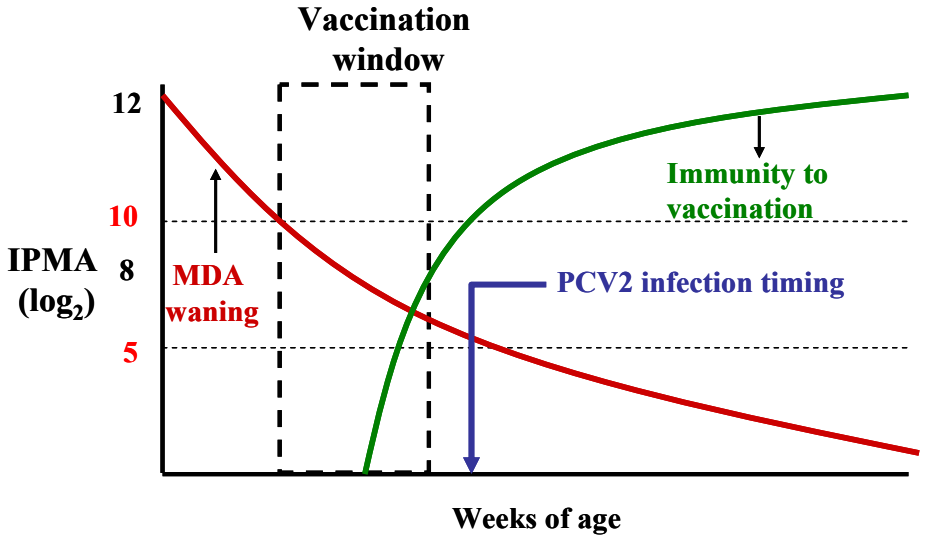


Figure 7.2. Maternally derived and vaccine-induced antibody profiles and PCV2 vaccination window

CHAPTER 8

CONCLUSIONS

1. *In vitro* stimulation of PBMC with the whole PCV2 but not with Cap or Rep proteins induced the release of IL-10. This occurred regardless of whether or not pigs have been primed for PCV2, a fact that suggests the innate origin of this response.

2. In the course of a sub-clinical PCV2 infection, IFN-α can be detected in serum on day 5 post-inoculation (PI). In contrast, detectable IL-10 in serum can be detected only sporadically, suggesting that increased levels of this cytokine in serum are not characteristic of PCV2 sub-clinically infected pigs.

3. In the course of a sub-clinical PCV2 infection, PCV2-specific cell-mediated immunity develops mostly within the first two weeks PI and both Cap and Rep proteins of PCV2 are involved in its development.

4. Sub-clinical PCV2 infected pigs develop humoral responses mostly between the second and third week PI, which are characterized by the production of PCV2-specific IPMA and neutralizing antibodies (NA); however, NA appear later than non-neutralizing antibodies.

5. PCV2a and PCV2b genotypes induce similar frequencies of PCV2-specific IFN-γ secreting cells when used as recall antigens in PCV2 infected pigs, indicating that both genotypes share immunodominant T-epitopes.

6. Vaccination of conventional pigs with a PCV2a-based sub-unit vaccine induces the development of cell-mediated and humoral responses and significantly reduces viremia, shedding, and viral load in tissues upon challenge with either PCV2a or PCV2b isolates. These observations indicate that the immunity induced by PCV2a-based vaccines protects against strains belonging to both genotypes.

7. Maternally derived antibodies (MDA) protect against PCV2 infection and influence the development of humoral responses to vaccination on a titre-dependent manner. High levels of MDA interfere with the seroconversion for PCV2 IPMA and NA developed following one-dose vaccination. Such interference is overcome by the administration of a second dose of the tested vaccine.

CHAPTER 9

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