Porcine circovirus type 2: cell tropism and interaction with the immune system

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2 Abbreviations

Ab	antibody
APC	antigen presenting cells
BFDV	beak and feather disease virus
BMDC	bone marrow derived dendritic cells
Cap	Capsid protein
CD	cluster of differentiation
CpG	Cytosine phospho Guanine oligodeoxynucleotide
CTLs	cytotoxic T cells
DC	dendritic cells
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triposphate
ds	double-stranded
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
KLH/ICFA	keyhole limpet haemocyanin in incomplete Freund’s adjuvant
MoDC	monocyte derived dendritic cells
mRNA	messenger ribonucleic acid
NIPC	natural-interferon producing cells
NK cells	natural killer cells
NLS	nuclear localization signals
nt	nucleotides
ODN	oligodeoxynucleotides
ORF	open-reading frame
PBMCs	peripheral blood mononuclear cells
PCV	porcine circovirus
PCV1	PCV type 1
PCV2	PCV type 2
PCVD	PCV diseases
PDNS	porcine dermatitis and nephropathy syndrome

PMWS	porcine multisystemic wasting syndrome
PPV	porcine parvovirus
PRRSV	porcine reproductive and respiratory syndrome virus
RCR	rolling circle replication
Rep	Replicase protein
SPF	specific pathogen free
ss	single-stranded
SWC	swine workshop cluster
Th cells	T helper cells
TLR	toll-like receptor
Tregs	regulatory T cells

3 Abstract

Porcine circoviruses (PCV) are single-stranded circular DNA viruses, classified in the family of *Circoviridae*. PCV are phylogenetically related to plant circoviruses and are currently presumed to have evolved from a recombination event of a plant circovirus and a picorna-like RNA virus. Two types of PCV are known; the non-pathogenic PCV type 1 (PCV1) and PCV type 2 (PCV2), the causative agent of number of diseases in pigs. *In vivo,* PCV2 antigen and DNA have been detected in cells of endothelial, epithelial and monocytic origin in several porcine tissues. Nevertheless, the target cells of PCV2 replication await definition.

Accordingly, the first part of the present thesis work (Dissertation equivalent A) focussed on the identification of PCV2 permissive cells and the characterization of virus replication *in vitro*. Virus binding, entry, protein synthesis, intracellular localisation and progeny production were studied in a number of porcine cells: an endothelial cell line, primary aortic endothelial cells, primary gut epithelial cells, monocyte-derived dendritic cells and fibrocytes.

We could show, that PCV2 was able to bind and enter cells of different origin, relating to published data that PCV2 interacts with glycosaminoglycan structures, which are expressed by many cell types. In all cells, except the monocyte-derived dendritic cells, PCV2 replication was detectable. Moreover, PCV2 replication was clearly dependent on dividing cells. In contrast to other ssDNA viruses, PCV2 did not influence the cellular growth characteristics. Furthermore, we could demonstrate, that the viral proteins co-localised with cell nucleus components during mitosis. This fits to the assumption that the cell nucleus is the replication site of PCV2. In conclusion, the broad cell targeting of PCV2 offers an explanation for its widespread tissue distribution.

PCV2 is the causative agent of a disease in piglets called postweaning multisystemic wasting syndrome, but not all infected animals develop disease – in fact this can often be a minority of the infected animals. However, PCV2-induced diseases increase the pig mortality rate on a farm from 2-3% to 30%, involving higher production costs per animal and the use of antibiotics. Although the specific antibody response has been followed in symptomatic and asymptomatic PCV2 infected piglets, the cellular adaptive immune response against PCV2 is not well characterized. Therefore, the second part of this thesis work (Dissertation equivalent

B) focussed on the characterization of the PCV2-specific cellular adaptive immune response in PCV2 infected SPF piglets. In summary, a PCV2 specific response, represented by an increase in interferon gamma secreting (IFN-γ) cells, was observed from day 7 post-infection. This was mediated by both $CD8^+$ and $CD4^+$ cells. The activation of PCV2 specific T cells was additionally supported by an up-regulation of the IL-2 receptor α chain (CD25) on $CD8^+$ and $CD4^+$ cells. Furthermore, we wanted to address whether differences in the cellular adaptive immune response between PCV2 asymptomatic and PCV2 symptomatic animals explain the clinical outcome of PCV2 infection. As none of the PCV2 infected animals developed clinical symptoms, we could not answer this question.

Considering our observations of an up-regulation of the $CD25^{high}$ cells (containing the porcine Tregs) after PCV2 recall stimulation it would be interesting to analyse the importance of Tregs in the context of PCV2 diseases.

4 Introduction

4.1 Porcine circovirus type 2 (PCV2)

4.1.1 Virus classification

In 1982, Tischer et al. (Tischer et al., 1982) first described a porcine circovirus (PCV) as a contaminant of the porcine kidney cell line PK-15. The virus was named circovirus due to its circular single-stranded (ss) DNA genome; this virus was subsequently termed PCV type 1 (PCV1), to distinguish it from the second porcine circovirus, PCV type 2 (PCV2), identified in 1998 (Meehan et al., 1998). In the sixth report of the International Committee on taxonomy of viruses in 1995, the family of *Circoviridae* was defined. Viruses belonging to this family possess a non-enveloped capsid virion of icosahedral symmetry, with a diameter of 17-25 nanometers (nm) (Figure 1). The virion encapsidates a genome of a monomeric, non-segmented, closed circular, negative sense or ambisense ssDNA molecule, which is 1700-2300 nucleotides (nt) long.

The family of *Circoviridae* is divided in two genera depending on the virion size, genome size and orientation of the DNA. Members of the circovirus genus have 17-22nm virions and an ambisense ssDNA genome of 1700-2000nt. PCV1, PCV2, beak and feather disease virus (BFDV), canary circovirus, goose circovirus, pigeon circovirus, duck circovirus, finch circovirus and gull circovirus belong to this genus (Todd et al., 2005). The second genus – gyrovirus – has to date only one member, chicken anemia virus (CAV). This virus is 25nm in diameter, with a negative-sense DNA molecule of 2300nt (Gelderblom et al., 1989) (Noteborn, 2004) (ICTVdB, the universal virus database, version 4).

PCV1 is currently regarded as a non-pathogenic virus (Krakowka et al., 2000), while PCV2 is the causative agent of postweaning multisystemic wasting syndrome (PMWS) in swine (Allan et al., 1999a ; Ladekjaer-Mikkelsen et al., 2002). The two viruses display a genome homology of less than 80% (Meehan et al., 1998). Their Replicase genes are relatively conserved, but the Capsid gene shows a high sequence variation (Mankertz et al., 2004). No significant nucleic acid or amino acid similarity has been observed between the PCV and other members of the *Circoviridae* family. In contrast, the amino acid sequence of the PCV Replicase protein is related to that of the banana bunchy plant virus (Meehan et al., 1997), a

member of the family *Nanoviridae* (Niagro et al., 1998). Moreover, the non-coding regions of the PCV and BFDV genome display a stem-loop structure similar to a structure found on the geminivirus genome (Niagro et al., 1998). These findings suggest, that PCV and BFDV are evolutionary linked to plant circoviruses of the *Nanoviridae* and *Geminiviridae* families (Niagro et al., 1998).

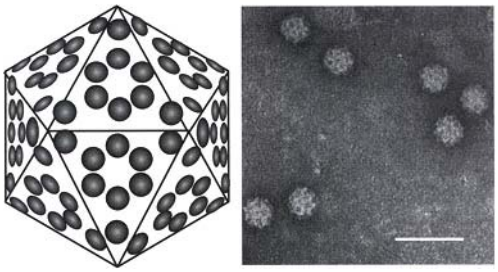

Figure 1: *Circoviridae* family. Schematic figure of the circovirus virion (left) and electron microscopy image (right)
http://www.ncbi.nlm.nih.gov/ICIVdb/ICTVdB/00.016.htm

4.1.2 Genome organization

The 1759nt long PCV genome consists of a circular ssDNA molecule. Porcine circoviruses are among the smallest viruses known – in terms of their coding capacity – replicating autonomously in mammalian cells (Mankertz et al., 1997). Six open reading frames (ORF) on the PCV genome have been identified by computer analysis. So far, the proteins encoded by three of these have been characterized in detail (Liu et al., 2005): (i) ORF1 encodes the Replicase (Rep) proteins (Mankertz et al., 1998), and is located on the genomic DNA strand (Figure 2); (ii) ORF2 encodes the major structural Capsid (Cap) protein (Mankertz et al., 2004), and is located on the complementary DNA strand of the replicative complex (Figure 2) (iii) ORF3 encodes a protein that apparently induces cellular apoptosis *in vitro* by activating the caspase 8 and caspase 3 pathways, and is also located on the complementary DNA strand of the replicative complex (Liu et al., 2005).

The ORF1 transcript encodes the full-length Rep protein (312 amino acids (aa)), with a molecular mass of 35.7 kilo Dalton (kDa), as well as a splice product, the Rep' protein (168aa) (Mankertz et al., 1998). Both proteins bind to the origin of genome replication, are essential for virus replication and propagation, and contain nuclear

localization signals (NLS) (Finsterbusch et al., 2005). Bacterial two hybrid screening and GST-pull-down assays have identified an interaction of Rep with a cellular intermediate filament protein, as well as with the transcriptional factor c-myc and the viral Cap protein (Timmusk et al., 2006). The Rep protein contains three motifs that are found on enzymes involved in initiation of DNA rolling circle replication, together with a P-loop (dNTP binding motif). The Rep protein of PCV shows a high homology with the Rep proteins of plant viruses (nanoviruses and geminiviruses), which has led to the hypothesis that the circovirus Rep evolved phylogenetically from a plant virus (Mankertz and Hillenbrand, 2002).

The Cap protein consists of 234 aa, and has a molecular mass of 27.8kDa (Nawagitgul et al., 2000). During virus replication, the Cap protein is expressed in the cell cytoplasm, nucleus and nucleoli (Finsterbusch et al., 2005). The protein contains NLS at its amino-terminus, and it is known for the Cap protein of BFDV that it displays a DNA-binding domain (Heath et al., 2006; Liu et al., 2001). Bacterial two-hybrid screening has identified an interaction of the Cap protein with the complement factor C1qB (Timmusk et al., 2006).

The ORF3 protein consists of 105aa and has a molecular mass between 6.5kDa and 16kDa (Liu et al., 2005). *In vivo*, the ORF3 protein was shown to be involved in viral pathogenesis when using a BALB/c mouse model of PCV2 infection (Liu et al., 2006). A recent publication showed that the ORF3 protein interacts with a ubiquitin E3 ligase, and may induce cell cycle arrest and cell death in PCV2-infected cell cultures by blocking p53 degradation (Liu et al., 2007).

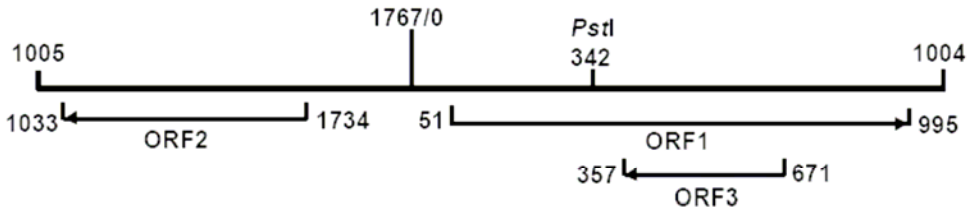

Figure 2: Genetic map of PCV2 strain BJW (Liu et al., 2005).

4.1.3 PCV2 replicative cycle

It is assumed that PCV amplifies its genome via rolling circle replication (RCR), because sequence motifs typical for enzymes that are involved in RCR were found in

the viral Rep protein (Mankertz and Hillenbrand, 2002). The mechanism of RCR is well known and described for other viruses phylogenetically related to PCV (Gutierrez, 1999; Niagro et al., 1998) .

PCV is dependent on the cellular DNA polymerase for replication of its genome. Therefore, viral DNA replication takes place in the host cell nucleus, and is thought to be dependent on mitotically active cells (Tischer et al., 1987). Prior to the replication *per se*, the viral circular ssDNA is converted into a closed-circular double-stranded (ds) form by host enzymes, for which a priming event must occur. In mastreviruses belonging to the *Geminiviridae* family, a small DNA molecule bound to the viral ssDNA serves as a primer for the DNA polymerase. Subsequently, and again prior to the RCR, the circular-closed dsDNA is converted into supercoiled DNA. This happens probably via association of the ds viral DNA with histones of the host cell. From this point on, the viral proteins become involved in the amplification of the viral DNA by RCR. The viral Rep protein binds to a viral DNA stem-loop sequence at the initiation site of replication, and cleaves the DNA via its nicking motif. This provides the cellular DNA polymerase with a 3'-OH end. Elongation and termination of viral DNA replication are not yet fully characterized, but the Rep protein might be involved in the termination of replication. At the end of the RCR, the progeny viral circular sense ss DNA molecules are encapsidated (Gutierrez, 1999).

4.1.4 Target cells of PCV2 infection/replication *in vitro* and *in vivo*

4.1.4.1 In vitro observations

In vitro, PCV2 is reported to infect the porcine PK-15 cell line (Allan et al., 1994; Allan et al., 1999b) as well as a porcine monocytic cell line (Misinzo et al., 2005), foetal porcine cardiomyocytes (Meerts et al., 2005a), porcine alveolar macrophages (Gilpin et al., 2003; Meerts et al., 2005a), porcine monocyte-derived dendritic cells (MoDC) (Vincent et al., 2003), and porcine primary kidney cells and hepatocytes (Hirai et al., 2006). Evidence for PCV2 replication, demonstrated by an increase in infectious viral titres and the *de novo* synthesis of the Cap and Rep proteins, has to date only been demonstrated in the cell lines and in primary hepatocytes (Meerts et al., 2005a; Misinzo et al., 2005). Hence, target cells for PCV2 replication, of relevance *in vivo*, have not been well characterized.

Due to the observation that PMWS diseased animals show decreased B and T lymphocyte levels in the blood (Segales et al., 2004a), PCV2 infection of porcine lymphocytes has been studied. Contradictory reports are published on this topic. Yu et al. (Yu et al., 2007b) detected viral DNA and Cap mRNA in $CD3^+$ sorted lymphocytes, after mitogen-stimulation of PCV2-infected peripheral blood mononuclear cells (PBMCs). These authors did not mention the appearance of viral Cap protein or infectious virus, which would have indicated a productive replicative cycle. In contrast, Gilpin et al. (Gilpin et al., 2003) did not observe any PCV2 protein in $SWC3^-$ sorted lymphocytes by intracellular Cap staining. Such divergent results might be explained if the method for detecting PCV2 DNA or mRNA were more sensitive, although increased sensitivity might also lead to more cross-reactions. An alternative explanation may be that the observations were reflecting the different virus strains used by the two groups for the infection. There may also be a requisite for prior activation of the T lymphocytes. Of course, one has to consider the level of lymphocyte purity in the infected cultures, and the influence of even small amounts of non-lymphoid cells therein. Experiments performed by our own group (unpublished data) have confirmed the results of Gilpin et al. (2003), and also contradict the results from Yu et al (2007b). Concavalin A (ConA) or staphylococcus aureus enterotoxin B (SEB) stimulated PBMCs were infected with PCV2, and the B and T lymphocytes stained for intracellular Cap antigen. The cells clearly identifiable as being infected were the monocytic cells, being positive for the Cap antigen within 24 hours (h). In contrast, PCV2 Cap antigen could never be detected in T or B lymphocytes, even after 72h of infection.

Due to the increasing interest in the field of xenotransplantation, PCV2 infection and replication has also been studied in human cells. Interestingly, human cell lines were infected with PCV, but the infections were non-productive in terms of infectious progeny virus release (Hattermann et al., 2004). Human PBMCs have also been infected, but this required co culture with irradiated PCV2-infected PK-15 cells, being unsuccessful when attempted by direct virus inoculation (Arteaga-Troncoso et al., 2005; Hattermann et al., 2004). The absence of detectable anti-PCV2 Ab in humans provides further evidence that PCV are not commonly infecting humans (Allan et al., 2000). Nevertheless, there are circovirus-related human viruses such as human torque teno virus (TTV) and TTV minivirus (TTVMV), isolated in 1997 and explaining

the detection of anti-PCV1 cross-reactive Ab in humans (Biagini, 2004; Hino, 2002; Tischer et al., 1995).

4.1.4.2 In vivo observations

In vivo, PCV2 antigen and DNA have been detected in pigs by immunohistochemistry and in situ hybridization. Evidence for infection was found in epithelial cells of the lung and kidney, as well as in alveolar macrophages, histiocytes, multinucleated giant cells, follicular dendritic cells and lymphocytes of lymphoid tissue, hepatocytes and Kupffer cells, enterocytes, and endothelial cells of arterioles from several organs(Allan and Ellis, 2000; Darwich et al., 2004; Opriessnig et al., 2006b; Segales et al., 2004b; Shibahara et al., 2000). Although the above-mentioned techniques permitted the detection of $PCV2^+$ cells, it is impossible to draw a conclusion on *in vivo* virus replication; the $PCV2^+$ cells can equally well be identifying an abortive replication, or cells endocytosing material produced at other sites. When Yu et al. (Yu et al., 2007a) analysed the expression of Cap mRNA in tissues and leukocytes of PCV2 infected animals, the mRNA was detected in bronchial and inguinal lymph nodes, as well as in the kidney, liver, lung, spleen, tonsils and PBMCs. With the PBMCs, the Cap mRNA was associated with $CD3^+$ T cells, $CD21^+$ B cells and $SWC3^+$ monocytic cells. Nevertheless, the presence of Cap mRNA can at best only reflect DNA transcription, but provides no information on viral protein synthesis or viral DNA replication. Hence, identification of target cells for PCV2 replication *in vivo* is not straightforward, although staining for the viral Rep protein and the ds replicative form of the viral DNA would be more informative. In this context, a recent publication has described a new method to detect the replicative form of PCV2 in fixed swine tissue (Perez-Martin et al., 2007).

4.2 Pathogenesis and immunology of porcine circovirus diseases

4.2.1 Porcine circovirus diseases (PCVD)

4.2.1.1 Postweaning multisystemic wasting syndrome (PMWS)

In 1997, a wasting syndrome affecting piglets was described in France and Canada (LeCann et al., 1997; Nayar et al., 1997). One year later, a circovirus-like virus was isolated from tissues of diseased piglets in Canada (Ellis et al., 1998), the US and

Europe (Allan et al., 1998). This virus was named PCV type 2, because it possessed less than 80% sequence homology with the formerly described PCV type 1 (Meehan et al., 1998). Although it is now generally accepted that PCV2 is the causative agent of a disease called postweaning multisystemic wasting syndrome (PMWS), full expression of this disease requires additional factors; these include co-infections with agents such as porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV), or immunostimulations such as seen with particular vaccinations (Krakowka et al., 2001; Krakowka et al., 2000; Pogranichniy et al., 2002). Recently, it was speculated that PCV2 strain variation may influence the clinical outcome of PCV2 infection (Lohse et al., 2008). Comparison of strains isolated from cases of PMWS with strains from subclinical PCV2 infections showed a high level of amino acid sequence diversity in the Capsid protein, the major immunogenic protein of PCV2. Additional recent findings are also implying a breed-dependent predisposition of pigs to PCV2-induced diseases (Opriessnig et al., 2006a).

PMWS is now been identified worldwide, as exemplified by the reports from Europe (Allan et al., 1998; (Cadar et al., 2007; Lipej et al., 2005; Vigre et al., 2005; Wallgren et al., 2004) North America (Ellis et al., 1998) and Asia (Choi et al., 2000; Onuki et al., 1999). In Switzerland, the first case of PMWS was detected in 2001 (Borel et al., 2001). Interestingly, retrospective studies showed that PCV2 had existed in Swiss pig herds since 1986 (Staebler et al., 2005). This suggested that the disease had either remained undiagnosed or emerged following changing farming conditions.

PCV2 not only infects domesticated pigs, but also wild boars, which have been found serologically positive for anti-PCV2 antibodies (Vicente et al., 2004). Infected animals can shed viral DNA in bronchial secretions, faeces, urine and semen, observed with both PMWS-symptomatic and healthy PCV2-infected animals (Larochelle et al., 2000; Shibata et al., 2003). Recently, PCV2 DNA has also been detected in the milk of sows (Shibata et al., 2006). Taken together, these findings indicate that pigs can be infected by the oro-nasal route.

On a farm, animals with disease symptoms can be found between the ages of 2 and 4 months, display clinical symptoms of wasting, respiratory distress, diarrhoea, icterus, anaemia and enlarged lymph nodes (Allan and Ellis, 2000; Segales et al., 2005). Considering that such symptoms are not specific to PCV2-induced disease,

other criteria have to be fulfilled for diagnosis of PMWS to be confirmed. Importantly, defined histopathological lesions and the presence of PCV2 antigen must be observed. Lesions are typically described in lymphoid tissues, with a loss of follicular architecture, lymphocyte depletion, and an infiltration with histiocytes and multinucleated giant cells; characteristic basophilic cytoplasmic inclusion bodies are usually seen in histiocytic cells (Rosell et al., 1999). In addition, interstitial pneumonia, mononuclear inflammatory infiltration in the liver, lymphoplasmacytic colitis and peri-endarteritis in several organs are observed (Opriessnig et al., 2006b; Rosell et al., 1999; Segales et al., 2004b).

PCV2-induced disease increases pig mortality on a farm from 2% to between 14% and 30%, thus increasing the production costs per animal and necessitating the use of antibiotics. Accordingly, it is of major economical importance to identify efficient measures for eradicating PCV2-induced disease, for example through vaccination strategies or a change in management and animal husbandry conditions.

4.2.1.2 Porcine dermatitis and nephropathy syndrome (PDNS)

A porcine dermatitis and nephropathy syndrome was first described in the UK in 1993 (Smith et al., 1993). PCV2 was considered as the likely causative agent, because PCV2 DNA was isolated from tissues of PDNS affected animals (Rosell et al., 2000). Nevertheless, confirmatory experimental data reproducing the disease following PCV2 infection is still lacking. Moreover, the situation is further complicated by the observations that viral agents other than PCV2, such as PRRSV, can be found associated with PDNS (Thibault et al., 1998). It is also unknown why PCV2 infection can result in different clinical outcomes, leading in some cases to PDNS, in others to PMWS, or even resulting to subclinical infection. In contrast to the recent work on PMWS, no particular PCV2 strain has been related to PDNS cases (An et al., 2007). Interestingly, PDNS animals show a tendency for higher anti-PCV2 antibody titres when compared with healthy PCV2-infected pen-mates (Wellenberg et al., 2004).

PDNS cases have now been reported worldwide (Chae, 2005). Diseased animals are often 12-14 weeks old, and the mortality rate reaches 20% (Chae, 2005). Symptoms indicative of PDNS are round, red skin lesions on the hindquarters, abdomen and thorax, as well as pneumonia, anorexia, weight loss and swollen lymph nodes (Chae, 2005; Thibault et al., 1998). Typical histopathological findings are vasculitis in the

dermis and the subcutis, and glomerulonephritis (Chae, 2005; Thibault et al., 1998). These histological findings are typical of a type 3 hypersensitivity reaction mediated by immune complex deposition on vascular and glomerular capillary walls. Indeed, an accumulation of immunoglobulins (IgG1+2, IgM) and complement factors (C1q+C3) have been observed in kidneys of PDNS affected pigs (Wellenberg et al., 2004).

4.2.1.3 Experimental models for PMWS

In 1999, an experimental model was established in an attempt to reproduce PMWS. The disease was successfully induced in colostrum-deprived (CD) piglets by co-infection with PCV2 and PPV (Allan et al., 1999a). In parallel, PMWS symptoms were also reproduced in gnotobiotic (germ-free) piglets inoculated intranasally with lymphoid tissue homogenates from PMWS-diseased animals (Ellis et al., 1999). Virus re-isolation and polymerase chain reaction (PCR) analyses on collected tissue material demonstrated the presence of both PCV2 and PPV, indicating that virus co-infections would be important for PMWS induction. It has been speculated that PPV assists PCV2 infection/replication by inducing an immunosuppressive state in the host, or by stimulating cellular DNA synthesis to promote PCV2 replication in its target cells (Krakowka et al., 2001).

Interestingly, several research groups have observed PMWS symptoms in CD or specific-pathogen-free (SPF) piglets infected with PCV2 alone (Ladekjaer-Mikkelsen et al., 2002; Okuda et al., 2003). In contrast, this has never been reported for PCV2 infections in gnotobiotic piglets (Ellis et al., 1999; Krakowka et al., 2000), implying that a second agent or event must have occurred with the PCV2-infected CD or SPF animals for the disease to arise. Certainly, PMWS can be induced in gnotobiotic piglets following infection with PCV2. This requires the additional activation of the immune system with keyhole limpet haemocyanin in incomplete Freund's adjuvant (KLH/ICFA) (Grasland et al., 2005; Krakowka et al., 2001). It has been suggested that the KLH/ICFA induces proliferation of histiocytes and macrophages, leading to enhanced PCV2 replication in these cells (Krakowka et al., 2001). Interestingly, the application of a mineral-oil based adjuvant used with mycoplasma hyopneumoniae bacterin can also result in disease when used with PCV2-infected piglets; this contrasts with non mineral-oil-based bacterin vaccines (Krakowka et al., 2007). An

influence of vaccination or application of immuno-modulatory drugs on PMWS development has also been noted with conventionally reared PCV2-infected pigs (Kyriakis et al., 2002). Such influences may depend on the vaccine and/or adjuvant used, because other groups have not observed a triggering effect of vaccination on PMWS development in conventionally reared piglets. For example, vaccine formulated in a commercial adjuvant containing Montanide 888 and Simusol 5100 did not induce disease in PCV2-infected animals (Opriessnig et al., 2004; Resendes et al., 2004).

An observation which can often be noted is the development of PMWS in PCV2/PPV co-infected gnotobiotic animals (Ellis et al., 1999). In contrast, the number of SPF piglets which will display clinical symptoms following experimental infection is variable, ranging from 5 to 60% depending on the inocula and the experiment (Ladekjaer-Mikkelsen et al., 2002) (Okuda et al., 2003) . It is unknown why only certain of the infected SPF animals develop disease while others remain subclinically infected, but this does reflect the field scenario. It is reported that higher levels of PCV2 DNA, elevated numbers of PCV2 antigen-positive cells, and a delayed antibody response against PCV2 are observed in diseased animals (Ladekjaer-Mikkelsen et al., 2002) (Okuda et al., 2003).

Although the experimental reproduction of PMWS in gnotobiotic piglets by viral co-infection or combined PCV2 infection with immunostimulation is well established, the germ-free animal model does not reflect the field situation. Experiments with SPF or conventionally reared piglets are more relevant models for studying the influence of PCV2 infection *in vivo*. Despite this, the protocols employed for inducing PMWS need to be improved; not all research groups have succeeded in reproducing the disease using similar model systems (for example, Ostanello et al., 2005; Steiner et al., submitted for publication).

4.2.2 Innate immune response

4.2.2.1 In vivo observations

Innate immune responses are the first line of defence against invading pathogens. Neutrophils, monocytes/macrophages, natural killer (NK) cells and DC are important cells mediating innate immunity. A particularly pertinent cell in the context of PCV2

infections is the DC subtype referred to as natural interferon producing cells (NIPC) or plasmacytoid DC (pDC). Studies on innate defences relevant to PCV2 infection are important considering that *in vivo* innate immune responses following PCV2 infection are not well characterized. It has been observed, that PMWS diseased animals displayed higher relative levels of neutrophils and monocytes in the blood compared to asymptomatic PCV2 infected animals (Darwich et al., 2003b ; Sipos et al., 2004). However, these studies monitored the percentage of the cell populations; an increased percentage for one cell population, as seen with the phagocytic cell populations, may have been due to the reduced lymphocyte numbers creating the misconception of an apparent increase in other cell types. Considering the reduced lymphocyte levels in diseased animals, it is to be expected that this will be compensated by a relative percentage increase in other cell populations. In order to ascertain the real existence of an increase or a decrease for a particular cell population, one must monitor actual cell numbers rather than percentages. Nielsen et al. (Nielsen et al., 2003) performed such experiments. They were able to confirm that a T cell and B cell lymphopenia were reality for PMWS animals. These authors also showed that there was in fact no modulation of neutrophil or monocyte numbers in the symptomatic animals. In contrast, there was a reduction in the numbers of γδT cells and NK cells (Nielsen et al., 2003).

Despite these observations, there have been no further studies on the influence of PCV2 infection on γδT cell and NK cell activity. In fact, there have been no analyses on the influence of PCV2 on innate immune activity, such as DC activity *in vivo*. Although monocytic cells may not be depleted during PMWS, there is evidence that their activity may be modulated. During PMWS, an infiltration of histiocytes and multinucleated giant cells into lymph nodes has been observed (Rosell et al., 1999). Additionally, increased mRNA levels of interleukin (IL) 10 were detected in the blood and thymus of diseased animals (Darwich et al., 2003b) (Sipos et al., 2004). It would be interesting to know if this IL-10 is also relevant *in vivo*, and which cells - DC or monocytes/macrophages - are producing the cytokine.

4.2.2.2 In vitro observations

In contrast to the *in vivo* observations, the immunomodulatory activities of PCV2 on innate defences have been more extensively studied *in vitro*. Considering that PCV2 is a monocytotropic virus, the potential immunomodulatory effects of the virus have been studied in monocyte-derived DC (MoDC), bone-marrow derived DC (BMDC) and alveolar macrophages. No modulation of MHC molecules or costimulatory molecules, nor modification of cytokine profiles were observed upon PCV2 infection in these cells (Gilpin et al., 2003; Vincent et al., 2003). Interestingly, PCV2 Cap antigen and infectious virus persisted for the experimental duration of 5 days in absence of apparent virus replication (Vincent et al., 2003). This unresponsiveness of professional APC to intracellular virus might promote anergy with the T lymphocytes (Zheng et al., 2008). Nevertheless, PCV2 infection did not affect the capacity of DC to process and present other antigens such as the foot-and-mouth disease virus antigen or superantigen SEB to T lymphocytes (Vincent et al., 2003). Viral persistence has also been observed in alveolar macrophages, but in this report the macrophage functionality, namely phagocytosis and microbicidal activity, were affected (Chang et al., 2006). Such variations in the outcome of PCV2 infection may relate to a recent report showing differences in infection efficiency of alveolar macrophages (Fernandes et al., 2007). This variation was observed between PCV2 strains, suggesting that strain differences may direct the virus to different target cells, or modulate the cells in a variable manner. Overall, one clear point is that PCV2 can be transported via DC and macrophages from sites of entry to sites of virus replication. If the virus also modulates the activity of the infected cell, this could result in down-regulation of mucosal immune defence mechanisms, increasing the risk of secondary pathogen attack.

With respect to PCV2 impairing innate immune defences, the NIPC or pDC have received particular attention - this cell type is named natural interferon producing cell due to its efficiency at producing high levels of type I interferon (IFN), and is also called plasmacytoid dendritic cell due to its morphology. NIPC have been characterized in humans, mice and pigs. They account for 0.2-0.5% of PBMCs and produce 10-100 times more type I IFN than other cells (Fitzgerald-Bocarsly et al., 2008). NIPC are localised in the blood and secondary lymphoid organs, and migrate to sites of inflammation when activated. C-type lectin receptors, FcγRII and toll-like

receptors (TLR) are expressed on the NIPC surface. Collectively, these provide the cell with the capacity to bind and respond to pathogen-associated molecular patterns (PAMP), as well as interact with and endocytose pathogens. Of course, NIPC are not the only cells capable of producing type I IFN. A property which is particularly found with the NIPC is their ability to sense viral infection in absence of viral replication or viral antigen synthesis (Fitzgerald-Bocarsly et al., 2008). In this context, the NIPC of humans and pigs are the unique cells of innate immune defences capable of responding to unmethylated CpG oligodeoxynucleotides (CpG-ODN). Such DNA motifs of bacteria and viruses interact with TLR9, which is expressed in endosomal structures of NIPC. Activation of TLR9 leads to secretion of type I IFN, mainly of IFN alpha (IFN-α) in the case of NIPC (Perry et al., 2005). This IFN-α is an important mediator in the development of innate and adaptive immune responses. In this respect, important properties of IFN-α are the induction of an antiviral state in cells expressing the type I IFN receptor, enhancement of the maturation of antigen-presenting cells (APC), up regulation of cytokine and chemokine secretion, activation of NK cells and modulation of B and T cell activities (Fitzgerald-Bocarsly et al., 2008; Guzylack-Piriou et al., 2006).

PCV2 was shown to have an inhibitory effect on IFN-α and tumour necrosis factor alpha (TNF-α) secretion by NIPC activated via TLR9 ligand stimulation (Vincent et al., 2005). In addition, when NIPC were together with conventional DC (cDC), the presence of PCV2 promoted an inhibition of the MHC class and CD80/86 costimulatory molecule upregulation on the cDC; this was related to the influence of the virus on the NIPC (Vincent et al., 2005).. These immunomodulatory characteristics were associated with PCV2 infected cell lysates containing both virus and viral DNA. Further studies revealed that it was the PCV2 DNA which was responsible for these activities (Vincent et al., 2007). Moreover, the inhibition of cytokine secretion was not restricted to cells undergoing TLR9 ligand stimulation; stimulation with other TLR ligands as well as viruses could also be impaired by the presence of PCV2 DNA (Vincent et al., 2007). Considering that no colocalisation of the PCV2 DNA and TLR9 ligand in NIPC could be observed, it was speculated that the PCV2 DNA inhibitory signalling was targeting another receptor or element of the signalling cascade (Vincent et al., 2007). One important set of observations in this context comes from the studies on the 65 CpG motifs identified in the PCV2 ssDNA

genome (Hasslung et al., 2003). When CpG-ODNs based on these motifs were synthesised and tested on porcine PBMCs, certain ODNs were found to stimulate the cells, while others inhibited IFN-α secretion (Hasslung et al., 2003). Subsequent analyses reported that the inhibitory effect of these PCV2 ODNs is due to the secondary structure formation rather than the CpG motif *per se* (Wikstrom et al., 2007). Taken together, these data show that the PCV2 DNA is an important immunomodulatory element, capable of inhibiting NIPC function in terms of cytokine secretion, affecting both innate and adaptive immune responses. This would render the host more susceptible to secondary infections, which in turn relates to the known characteristics for inducing PMWS.

An elaboration of PCV2 impairment of innate immune defence processes came from a recent report on the anti-inflammatory cytokine IL-10 (Kekarainen et al., 2008). These authors used PBMCs from animals immune to pseudorabies virus (PRV). When these cells were infected *in vitro* with PCV2 prior to restimulaton with PRV antigen, a down regulation of IFN-γ secretion was observed. This was explained by the presence of the anti-inflammatory cytokine IL-10, which had been induced in the cultures by the PCV2 infection. Considering that macrophages and regulatory T cells produce this cytokine during the control and shutdown of immune responses, the authors proposed that PCV2 infection could render T cells less responsive to activation, leading to the observed decrease in IFN-γ levels.

Although the above work has shown that PCV2 can impair or modulate several innate defence processes, it is clear that the virus does not induce an immunosuppressive state in all infected animals (for example, see (Nielsen et al., 2003)). Therefore, the requirements for PCV2 infection to result in disease are more complex than simply the modulation of innate defences by the virus. It seems probable that a number of interacting events are involved, at the core of which is the immunomodulatory capacity of the virus on innate defence processes.

4.2.3 Humoral adaptive immune response

4.2.3.1 General overview

Appreciation of how a pig can respond immunologically to PCV2 infection requires consideration of how PCV2-specific Ab, the mediators of humoral adaptive immunity,

would arise. Ab are secreted by activated B lymphocytes that recognize specific antigens via immunoglobulin (Ig) receptors, primarily of the IgM and IgD isotypes, expressed on the cell surface (Kim and Reth, 1995). Humoral immune responses are initiated in secondary lymphoid organs. Considering that antibodies are released into the circulation or into the lumen at mucosal sites, important effector functions of the humoral defences occur at these sites.

The initial activation of differentiated B lymphocytes in the follicular zones of secondary lymphoid organs requires that epitopes (antigenic determinant) on the antigen, for which a particular B lymphocyte is specific, bind to and cross-link the antigen-specific Ig receptors on the B cell surface. Via the receptor clustering, two molecules called Igα and IgB that are associated with the membrane IgD and IgM come into close contact (Hsueh and Scheuermann, 2000). Consequently, the tyrosine residues of the immuno-receptor tyrosine based activation motifs (ITAM's) on the cytoplasmic regions of these receptors are phosphorylated, thus serving as docking sites for the adaptor proteins that lead to activation of signalling molecules. Via different signalling cascades transcription factors are activated that regulate B cell gene expression, influencing the proliferation and differentiation of the activated B lymphocyte (DeFranco, 1997). Secondary stimuli are also important for the characteristics of the B lymphocyte response. For example, binding of the complement factor C3d to the receptor CD2 leads to enhanced signalling and an augmented response towards the activating antigen (Toapanta and Ross, 2006). Besides initiation of signalling cascades, antigen binding to the B cell receptors leads to their internalization into endosomal vesicles. By such processes, protein antigens can enter the 'MHC class II processing pathway'. This means that they are degraded into peptide fragments which can bind to MHC class II molecules allowing their presentation on the B cell surface for activation of peptide-specific T helper (Th) lymphocytes (Vascotto et al., 2007).

When most protein antigens activate B lymphocytes, the complete activation towards Ab production (or memory cell production) requires the accessory involvement of peptide-specific Th lymphocytes and the cytokines they produce. In contrast, certain non-protein antigens, such as lipids or polysaccharides, activate B lymphocytes in the absence or Th lymphocyte activity, or with a much reduced dependence on such activities. These are called thymus independent antigens (TI), often characterized by

multiple, identical (“repeating”) epitopes which can cross-link the B cell receptors (Vos et al., 2000). In contrast, the more complex (in terms of their epitope composition) protein antigens are poor B cell activators in the absence of Th lymphocyte accessory activity. This owes much to their more complex construction, particularly the much lower level of identical epitope copies. Therefore, protein antigen activation of B cells towards Ab production requires the involvement of the Th lymphocytes, for which reason they are termed thymus dependent (TD) antigens (McHeyzer-Williams et al., 2003).

When B lymphocytes are activated by TD antigens, the encounter with Th lymphocytes requires that the latter are also activated. This occurs when the Th cells specifically recognize the peptide fragment of the protein antigen – presented in association with MHC Class II on the surface of Antigen Presenting Cells (APC) (see section 4.2.4.1 below) – and interact with costimulatory molecules such as CD80 and CD86 expressed on the B cell surface. Hence, the activated Th cells express CD40 ligand that binds its counterpart CD40 on the B cell surface and release the cytokines that influence the B cell proliferation and differentiation (Gray et al., 1994; Parker, 1993). This T cell help leads to heavy chain isotype switching and affinity maturation of both the B cell receptor and the Ab which the activated cells will ultimately secrete (as plasma cells and plasmacytoblasts). Ultimately, this interaction is also essential for B cell memory, induced when the B lymphocyte interacts with immune complexes of antigen with specific Ab, thus involving both the antigen-specific receptors and the FcR on the B cell surface.

By isotype switching, the secreted antibodies acquire different heavy chains that mediate diverse effector functions. Antigen bound IgM activates the complement system and therefore induces inflammation, mediating the uptake of complement bound antigen by phagocytic cells and lysis of virus-infected cells (by the complement membrane attack complex) that express antigen-antibody complexes on the surface. IgG-antigen complexes also activate the complement system, but in addition the antigen is targeted to Fc-receptor expressing phagocytic cells. These cells bind the Fc portion of the Ab following its interaction with antigen (this reaction has to be of a minimum affinity for ensuring the modification of the Fc structure to promote efficient binding to the cell FcR). In addition to phagocytic cells, NK cells are

also important in the effector Ab response, lysing cells that express IgG-antigen complexes on the surface (Cooper and Nemerow, 1984; Morel et al., 1999).

When IgM, IgA or IgG Ab is of appropriately high affinity, the infectivity of cell-free virus can be neutralised. By such means, the binding of the virus to cell surface receptors and infection of the cells is blocked. Virus neutralisation is measured *in vitro* by a reduction of virus-infected cells. Often, the levels of neutralising Ab positively relate to the protective state from a virus infection *in vivo* (Hangartner et al., 2006). However, the relationship is not absolute (Meyer et al., 2008). Effectively, Ab binding to cell-free virus requires a similar involvement of cellular defences as Ab binding to infected cells. Namely, phagocytic cells are critical for the removal of Ab-virus complexes, a process which can also be enhanced by the additional involvement of the complement cascades (Underhill and Ozinsky, 2002). Of course, for monocytotropic viruses which replicate in such phagocytic cells, the involvement of so-called neutralising Ab can actually enhance the infection. It is known that Ab can mediate the entry of certain viruses into target cells through the involvement of the FcR, leading to enhanced infection/replication. This process is called antibody-dependent enhancement of virus infection, and is usually associated with viruses targeting Fc-receptor expressing cells *in vivo* (Tirado and Yoon, 2003).

4.2.3.2 Humoral adaptive reponse against PCV2

Concerning the immune response following PCV2 infection, a major problem for the animal is the influence of that infection on the immune system. In addition to the lymphopenia referred to in the previous sections (Nielsen et al., 2003; Segales et al., 2004a), histological analyses had shown that lymphocytes were depleted in the lymph nodes and the follicular architecture was disrupted in animals symptomatic for PMWS (Rosell et al., 1999). The observed depletion in lymphocytes was explained by cellular apoptosis, but other mechanisms such as extravasation of cells to sites of inflammation have to be considered (Shibahara et al., 2000). Certainly, the lymphocyte depletion in the lymph nodes and the decrease in circulating B and T lymphocytes in PMWS affected animals are suggestive of an immunocompromised state in the animals (Segales et al., 2004a).

While PCV2 will induce a humoral response, lower anti-PCV2 antibody (Ab) levels and a delayed seroconversion have been observed in PMWS animals compared to

healthy animals (Okuda et al., 2003). On a PMWS affected farm, anti-PCV2 IgM, expected as the first line of B cell-mediated immune defence, were detected at week 8, peaking at week 12, and disappearing by week 16. Anti-PCV2 IgG, which are expected after isotype switching and affinity maturation of the antigen-specific B cells, peaked at week 16. Thereafter, the IgG was found to persist, corresponding to a viral persistence in the serum (Carasova et al., 2007). Hence, at the herd level these authors concluded that the Ab response correlated with a decrease in PCV2 genomic copy numbers in the blood. The authors could not prove this point for highly viremic animals which died before the Ab peaks were expected.

When considering that PCV2 infection does not always induce disease, analyses were performed to focus on neutralising Ab activity in individual PMWS affected animals. In the gnotobiotic experimental model of PMWS, seroconversion against PCV2 was observed at 15 days post infection (p.i.) (Meerts et al., 2005b). With one animal, the increase in anti-PCV2 Ab titre did not correlate with neutralising Ab levels contrasting with the other PCV2 infected animals. This animal also displayed higher levels of infectious PCV2 in the inguinal lymph nodes. The authors therefore concluded that neutralising Ab was important for reduced virus levels *in vivo* (Meerts et al., 2006; Meerts et al., 2005b). Considering that germ-free animals were used for the experimental reproduction of PMWS, together with the observation that only one animal showed a lack of neutralising Ab, the characteristics of neutralising Ab induction were further studied in PCV2 infected SPF pigs and field PMWS cases. In PMWS-diseased SPF animals, PCV2-specific Ab were detected at day 10 p.i., but the rise in neutralising Ab titres did not follow the increase in total anti-PCV2 Ab titres (Meerts et al., 2006). This is in contrast to other studies, where total anti-PCV2 Ab titres were observed to correlate with neutralising Ab titres, even in PMWS animals (Fort et al., 2007). Field studies also showed significantly lower neutralising Ab titres in PMWS animals compared to healthy animals (Meerts et al., 2006). While these authors proposed again that such results showed PCV2 neutralising Ab to be protective *in vivo*, the lower titres may be more a reflection of the PCV2-induced lymphopenia rather than reflecting lack of protection. The methods for measuring neutralising Ab also have to be considered. These may well vary between groups, which could explain some of the differing results. One also needs to consider that “neutralisation” *in vivo* requires involvement of phagocytic cells to remove the

immune complexes, a phenomenon which is not monitored by the *in vitro* neutralisation tests.

Differences were also seen in the IgM, but not IgG levels between PMWS diseased and healthy infected SPF animals (Meerts et al., 2006). Healthy animals displayed higher IgM titres than PMWS diseased animals. The authors suggested that IgM Ab mediate the neutralising effects in the sera. Considering the different roles of IgG and IgM *in vivo*, it is all the more important to determine the mechanisms involved in Ab-mediated immune defence against PCV2. In fact, IgG Ab are important for protection of animals against PCV2 infection. Piglets acquire maternal Ab via the colostrum, which can protect the animals against PCV2-associated disease. During PCV2 infection, a protection from PCV2 viraemia correlated to high levels of PCV2-specific maternal Ab in the piglet blood (McKeown et al., 2005).

In general, the presence of anti-PCV2 Ab, and perhaps high titres of neutralising Ab, may provide some indication of protection from disease *in vivo.* Protection from disease is also clearly dependent on the generation of memory B cells and of long-living plasma cells and therefore persistent secretion of protective antibodies (Dorner and Radbruch, 2007). However, viruses are intracellular pathogens, and if viral antigens are not expressed on the surface of infected cells or found in an extracellular state, antibodies cannot recognize the infection. The generation of both immunological memory and effector immunity against infected cells is dependent on activation of T lymphocytes, including both the Th lymphocytes important for promoting B cell responses and the cytotoxic T cells important in direct attack of virus-infected cells.

4.2.4 Cellular adaptive immune response

4.2.4.1 General overview

The cellular adaptive immunity is mediated by T lymphocytes through their effector functions, either assisting the development of other components of immune defences or directly acting against the pathogen in question. Naïve T helper (Th) cells recognize peptide fragments of antigens that are presented by MHC class II molecules on APC in the lymph nodes (Singh et al., 1997). These peptide fragments are derived either from extracellular protein antigens that entered the MHC class II

pathway following endocytosis, or from intracellular protein antigens that entered autophagosomes (Strawbridge and Blum, 2007). Naïve Th cells specifically bind the peptide-MHC class II complex by the T-cell receptor (TCR) complex and the co-receptor CD4. Again, by expression of costimulatory molecules and cytokine secretion, the APC provide further stimuli to activate the naïve Th cells. Activated Th cells secrete growth factors such as IL-2 and expand, differentiating into effector cell subsets such as Th1 and Th2 cells. Cytokines have a dominant role in the induction of the different Th cell subsets (O'Garra and Arai, 2000). The cytokine IL-12, secreted by activated macrophages and dendritic cells, drives activated Th cells into effector Th1 cells (Del Prete, 1998; Romagnani, 1999). The cytokine IL-4 induces the differentiation into Th2 effector cells (O'Garra and Arai, 2000). Effector Th1 cells can ultimately promote eradication of intracellular pathogens by secretion of the macrophage activating cytokine IFN-γ. Furthermore, effector Th1 cells mediate Ab isotype switching, affinity maturation and B cell memory. In addition, the effector Th1 cells are involved in the activation of naïve cytotoxic T lymphocytes (CTLs) and NK cells. Effector Th2 cells activate mast cells and eosiniphils by secretion of IL-4, IL-5 and IL-13, and are therefore involved in eradication of helminthic and other parasitic infections (O'Garra and Arai, 2000). For both subsets of Th cells, the effector functions promoting innate defence expression occur at sites of inflammation. This relates to the observations that activated Th cells up-regulate the expression of adhesion molecules such as selectin-ligands and integrins that mediate extravasation. Naïve CTLs recognize also peptide fragments of antigens for which they are specific, in association with MHC class I molecules on the surface of APC. Recognition is again by the TCR-complex, but with the CD8 co-receptor (Monaco, 1995). These peptide fragments arise by proteasome degradation of cytosolic protein antigens, including protein antigens that escaped phagosomes. Again, secondary stimuli provided by the APC costimulatory molecules or by cytokines secreted by Th1 cells lead to full activation. Activated CTLs expand and differentiate into effector cells that migrate to sites of inflammation and kill cells that display the peptides for which they are specific in association with MHC class I molecules. The killing is mediated by delivery of cytotoxic granules containing perforins that form channels in the target cell plasma membrane resulting in loss of cytosolic osmolarity and ultimate cell lysis (Berke, 1994).

A T lymphocyte subtype called regulatory T (Treg) cell plays an important role in maintaining homeostasis. Tregs are known to maintain tolerance and to control immune responses. During viral infections, Treg cell activity can result in favouring virus persistence by suppressing virus-specific lymphocyte responses. The precise mechanisms are unclear, but it is thought that direct cell-cell contact and secretion of the cytokine IL-10 play a role in mediating the immunosuppressive activities of Treg cells (Cools et al., 2007).

Naïve porcine Th cells are characterized as $CD4^+CD8^-$ T cells (Saalmuller et al., 2002). When these naïve Th cells are activated to become effector Th cells they also express CD8 (Saalmuller et al., 2002) . Therefore, the phenotype $CD4^+CD8^+$ refers to effector and memory Th cells in the pig (Zuckermann and Husmann, 1996). Porcine CTLs belong to the $CD4^-CD8^+$ T cell population, but express CD8 to a higher level than the effector/memory Th cells. In addition, porcine CTLs express CD8β, whereas both CTLs and effector/memory Th cells express CD8α (Gerner et al., 2008). When both Th cells and CTLs are activated to become effector cells, they up-regulate the receptor binding the growth factor IL-2, called CD25 (Charerntantanakul and Roth, 2006). Recent publications have identified porcine Treg cells within both the $CD4^+CD8^+CD25^+$ and the $CD4+CD8^-CD25^+$ T cell populations. They express Foxp3, an intracellular Treg cell marker, secrete IL-10 upon mitogen stimulation, and suppress the proliferation of activated T cells (Kaser et al., 2008) .

4.2.4.2 Cellular adaptive response against PCV2

In field studies, a decrease in $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ cells was observed in PMWS animals, indicating that certain T cell populations are down regulated during PCV2 infection (Darwich et al., 2002) . During experimental reproduction of PMWS in SPF piglets, PCV2-infected symptomatic animals were distinguished from infected healthy piglets in terms of their leukopenia and lymphopenia from 7d p.i. (Nielsen et al., 2003). By staining for the different T cell subsets, these authors determined that the loss in T lymphocytes involved the naïve $CD4^+CD8^-$ Th cells, the memory/effector $CD4^+CD8+$ Th cells and the $CD4^-CD8^+$ CTLs (Nielsen et al., 2003).

In contrast to the loss of the different T cell subsets in PCV2-infected symptomatic animals, piglets which resisted infection ultimately developed apparently increased

levels of CTLs in the blood (Nielsen et al., 2003). By T lymphocyte epitope mapping, two immunoreactive peptide epitopes have been identified in the ORF1 protein, and one peptide epitope in ORF3 (Stevenson et al., 2007). These were active in terms of inducing proliferation in PBMC cultures from PCV2-immune animals. None of the peptides located in the ORF2 sequence were found to be stimulatory for T cells, suggesting that the anti-PCV2 T lymphocyte responses are mainly oriented against ORF1 and ORF3 peptide fragments (Stevenson et al., 2007).

With respect to T lymphocyte cytokine production, decreased mRNA levels of IL-2 and IFN-γ have been observed in secondary lymphoid organs from PMWS animals (Darwich et al., 2003b). Mitogen-activated cells from PMWS animals released lower amounts of IL-2 than control cells (Darwich et al., 2003a). However, it cannot be ascertained if this were due to the PCV2 infection or other phenomena linked to the development of disease symptoms. If PBMCs from PMWS diseased animals were restimulated with PCV2 *in vitro*, secretion of IFN-γ and IL-10 was observed. From this, it was concluded that the response of PBMCs from PMWS animals was suppressed. However, this cannot be ascertained with confidence when both IFN-γ and IL-10 are induced *in vitro*. Moreover, the lymphopenic state of the animals leading to fewer competent cells being available for the *in vitro* responses would have influenced the results when comparing diseased with healthy animals. Despite this uncertainty over the conclusions from the above work, it is now clear that increased IL-10 levels can be induced in PBMCs and tissues from PMWS animals (Darwich et al., 2003a; Darwich et al., 2003b; Kekarainen et al., 2008; Sipos et al., 2004). The most recent of this work is showing that IL-10 induction is due to the presence of PCV2, but requires a secondary signal such as that from a restimulating antigen when the animals are immune to that antigen (Kekarainen et al., 2008). Such results are now raising the question concerning the involvement of Treg cells in the pathogenesis of PCV2-induced diseases. It has been shown for other viruses, such as hepatitis C virus, that viral persistence is associated with increased levels of Treg cells (Chang, 2007). With the possibility to identify porcine Treg cells by Foxp3 detection, it would be interesting to compare the Treg cell levels in the blood of PMWS-diseased and PCV2-infected healthy animals.

4.2.5 PCV2 vaccination

Vaccines are important for inducing protective immunity in the host against a specific antigen. Under optimal conditions, long-lived humoral immunity and cellular adaptive immunity are induced (Arnon and Ben-Yedidia, 2003). Attenuated live virus vaccines can elicit innate and adaptive immune responses related to that induced by live virus, but without replication and/or disease induction in the host. Virus attenuation is often achieved by growth in cell culture and resultant cellular adaptation of the virus, or by heat-treatment. For PCV2, as for other viruses, the limiting factors for live attenuated vaccine production is the difficulty in preparing sufficient virus material, and the reliability and stability of the attenuation. Therefore, approaches tended to employ inactivated viruses, or to express specific viral proteins or immunogenic peptides by recombinant technology. For eliciting immune responses against these inactivated virus or subunit/peptide vaccines, adjuvants have to be co administered. The role of adjuvants is to activate DC and macrophages at sites of antigen injection, and therefore to provide costimulatory signals to induce efficacious adaptive immunity. Another method is to introduce the protein coding sequence into the genome of a non-pathogenic live virus vector, while more recent approaches have sought to administer plasmid DNA encoding specific antigens. The latter is particularly interesting, because the cells taking up the DNA will transcribe and translate these antigens which can be expressed in the cell cytoplasm in a similar manner as during viral replication Accordingly, association with MHC Class I molecules may become involved, leading to eliciting CTL responses. In order to enhance immunogenicity, genes encoding cytokines such as IL-2, IL-12 or granulocyte-macrophage colony stimulating factor (GM-CSF) have been co-expressed in such plasmid DNA vaccines (Arnon and Ben-Yedidia, 2003).

The first reported PCV2 vaccine candidates were based on protein subunit and DNA vaccines (Blanchard et al., 2003). Immune responses against the viral ORF1 and ORF2 encoded proteins were analysed in piglets vaccinated at 4 and 6 weeks of age with subunit vaccine expressing ORF1 and ORF2 proteins emulsified in the adjuvant Montanide. The experiments also analysed vaccination with plasmid DNA encoding ORF1 and ORF2 proteins in combination with a plasmid encoding GM-CSF. Vaccinated animals were challenged 11 days after the second immunisation. The ORF2 protein was identified as the major immunogenic protein, because ORF2-

specific vaccination protected the animals from clinical disease. Interestingly, all animals vaccinated with the subunit vaccine seroconverted against PCV2 before challenge, in contrast to the DNA-vaccinated animals, and after challenge PCV2 was prevented from entering lymph nodes (Blanchard et al., 2003).

It was demonstrated that the cloned genomic DNA of PCV2 was infectious by injection into liver and lymph nodes of pigs (Fenaux et al., 2002). Therefore, chimeric infectious DNA clones of PCV2 and PCV1 were generated for vaccine approaches (Fenaux et al., 2004). By intramuscular injection of the infectious DNA clone PCV1-2, encoding the PCV2 Cap protein in the PCV1 backbone, no viraemia and a reduced occurrence of histological lesions were observed after challenge infection (Fenaux et al., 2004).

Live virus vector-based vaccines have also been established for PCV2 vaccination. Piglets vaccinated with a recombinant adenovirus expressing PCV2 ORF2 protein could be protected against clinical disease, the animals displaying reduced pathological lesions and levels of viraemia (Wang et al., 2007). Parvovirus-like particles served in another study as antigen carriers of PCV2 ORF2, and induced strong Ab responses in mice (Pan et al., 2008).

Considering that piglets become infected with PCV2 during the first days of life, maternal Ab confer passive immunity. In piglets, maternal Ab are absorbed by uptake of colostrum (first milk), not by transplacental transfer (Pravieux et al., 2007). Therefore, vaccination strategies are not only focusing on the piglets, but also on pregnant sows. The effect of an inactivated oil-adjuvanted PCV2 vaccine for sows was studied on PCV2 infection of newborn piglets. This vaccination clearly reduced the levels of viraemia and improved the piglet health status (Charreyre et al., 2005).

Overall, several PCV2 vaccines are now available that reduce porcine mortality rate on the farm (Horlen et al., 2008). In Europe an inactivated PCV2 vaccine is employed for vaccination of sows, while in the US subunit vaccines expressing viral proteins in the baculovirus system, as well as inactivated PCV1-2 chimera vaccines, are on the market (http://www.vetmed.iastate.edu).

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5 Results

5.1 Dissertation equivalent A

Porcine circovirus type 2 displays pluripotency in cell targeting

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Porcine circovirus type 2 displays pluripotency in cell targeting

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the causative agent of a multifactorial disease associated with immunocompromisation and co-infections. *In vivo*, viral DNA and antigens are found in monocytic, epithelial and endothelial cells. Of these, PCV2 replication has only been studied in monocytic cells, in which little or no replication was identified. Accordingly, PCV2 infection was studied in the endothelial cell line PEDSV.15, aortic endothelial cells, gut epithelial cells, fibrocytes and dendritic cells (DC). In all cells except DC PCV2 replication was detectable, with an increase in the levels of capsid and replicase protein. Variations in endocytic activity, virus binding and uptake did not relate to the replication efficiency in a particular cell. Furthermore, replication did not correlate to cell proliferation, although a close association of viral proteins with chromatin in dividing cells was observed. No alteration in the division rate of PCV2-infected cultures was measurable, relating to replicase expression in only a small minority of the cells. In conclusion, the broad cell targeting of PCV2 offers an explanation for its widespread tissue distribution.

Introduction

Porcine circoviruses (PCV) are small non-enveloped, single-stranded circular DNA viruses (Tischer et al., 1982), classified in the genus *Circovirus*, family *Circoviridae*. According to the eight report on taxonomy of viruses, the genus *Circovirus* consists of PCV and other animal circoviruses such as beak and feather disease virus (BFDV), duck circovirus, canary circovirus, goose circovirus and pigeon circovirus (Todd et al., 2001, 2005). Chicken anaemia virus is the only member of the second genus within the family *Circoviridae—Gyrovirus* (Kato et al., 1995). Two distinct types of PCV have been described: the non-pathogenic PCV type 1 (PCV1), first identified as a contaminant of the porcine kidney cell line PK15 (Tischer et al., 1974); PCV type 2 (PCV2), identified as the causative agent of postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1999). Despite the clear association of PCV2 with diseases such as PMWS, additional factors including co-infections and vaccinations are needed for the disease to be fully expressed (Krakowka et al., 2000, 2001). Primarily 4 to 12-week old piglets are affected, displaying clinical symptoms of wasting, respiratory distress, anaemia, diarrhoea, jaundice and enlarged lymph nodes (Allan and Ellis, 2000). Mortality rates can vary from 1% to 30% in severe cases (Liu et al., 2005). Typical histological findings are lymphocyte depletion and histiocyte infiltration in lymphoid organs (Allan and Ellis, 2000; Segales et al., 2004), as well as lymphohistiocytic and plasmacytic periarteritis and endarteritis in several organs (Opriessnig et al., 2006).

The 1.7 kB PCV2 genome consists of seven open reading frames (ORF) (Meehan et al., 1997). ORF1 encodes the non-structural replicase proteins (Rep and Rep'), which are involved in the replication of the viral genome (Mankertz and Hillenbrand, 2001) while ORF2 encodes the major structural capsid protein (Cap) (Nawagitgul et al., 2000). It was recently shown, that ORF3 encodes a viral protein which can be related to virus-induced cell apoptosis in PK15 cells (Liu et al., 2005). No protein has yet been associated with the other ORFs. Moreover, it is generally assumed that PCV replication depends on host enzymes and entry of the virus into the cell nucleus. Consequently, it has been reasoned that virus replication should only occur in mitotically active cells. Indeed, Tischer et al. (1987) showed that PCV DNA was synthesised during the S-phase of the cell cycle, with viral replication beginning once the cells passed mitosis.

Although the presence of virus antigen has been identified in a number of cell types *in vivo* (Darwich et al., 2004), such observations cannot define the target cell for virus replication. All cell types can endocytose (Willingham and Pastan, 1984), providing the potential to internalise PCV2 antigen and nucleic acids; *in vivo* virus signals have most often been associated with monocytic and endothelial cells (Jensen et al., 2006; Opriessnig et al., 2006; Rosell et al., 1999; Yu et al., 2007). *In vitro* analyses have demonstrated that PCV2 will infect the porcine kidney cell line PK-15 (Meerts et al., 2005a), monocytic cells

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(Gilpin et al., 2003; Vincent et al., 2003), foetal cardiomyocytes (Meerts et al., 2005a), a porcine monocytic cell line (Misinzo et al., 2005) and primary hepatocyte and kidney cell cultures (Hirai et al., 2006). Nevertheless, replication of PCV2 has only been reported in cell lines (Meerts et al., 2005a), and in primary hepatocytes (Hirai et al., 2006). Cell lines do not reflect the characteristics of primary cells; the replication in the hepatocytes raises the question of whether the virus is restricted in its cell targeting. Yet the article of Hirai et al. (2006) on hepatocyte infection does not explain the *in vivo* observations of PCV2 antigen present in monocytic cells, endothelial cells and epithelial cells (Allan and Ellis, 2000; Opriessnig et al., 2006) of several tissues. Accordingly, the present work sought to determine the cell targeting capacity of PCV2, and the level of diversity therein. For this purpose, we analysed and compared PCV2 infection and replication in a porcine endothelial cell line (PEDSV.15), primary porcine aortic endothelial cells (EDC) and gut epithelial cells (GEpC), and two different types of antigen-presenting cells. The latter were monocyte-derived dendritic cells (MoDC) and fibrocytes (Fb); PCV2 is reported to infect but not replicate in DC (Vincent et al., 2003), whereas nothing is known concerning PCV2 infection of Fb, representing cells involved in wound healing processes (Bucala et al., 1994), antigen presentation (Balmelli et al., 2005) and innate immune responses (Balmelli et al., 2007).

Results

Comparative titration of PCV2 on PK-15A and PEDSV.15 cells

The porcine kidney cell line PK-15A – free of PCV1 and PCV2 – is generally used for PCV2 titrations (McNeilly et al., 2001). If PCV2 were to replicate in different porcine cells, the virus may adapt such that the titers obtained on PK-15A cells would not reflect those obtained on the other cells. Hence, cell supernatants (ECV) and the cell lysates (CAV) from PCV2-infected PK-15A and PEDSV.15 cells (MOI of 3 $TCID_{50}$/cell) were titrated on both the homologous and heterologous cell line. Overall, the results showed that the titers for both CAV and ECV arising from PCV2 replication in PK-15A cells or in PEDSV.15 cells were similar when titrated on the homologous or the heterologous cell line (Fig. 1).

Interestingly, the titration of CAV from infected PK-15A cells on PEDSV.15 cells revealed higher CAV titers at 1 and 3 d p.i. (Fig. 1A, dashed line) compared to titration on homologues PK-15A cells (Fig. 1A, solid line), but the difference was only 0.5 to 0.75 $\log_{10}$ $TCID_{50}$/1000 cells. This further demonstrates that the virus does not adapt to the cell type in which it was produced. Therefore all virus titrations in this study were performed on PK-15A cells.

PCV2 replication in the porcine endothelial cell line PEDSV.15

Recent evidence is suggesting that endothelial cells have importance in PCV2 associated disease, following the detection of viral antigen in endothelial cells associated with several organs of infected pigs, and the apparent relationship to arterial wall inflammation (Opriessnig et al., 2006). Therefore, we tested the ability of PCV2 to infect and replicate in the continuous porcine endothelial cell line PEDSV.15. Following infection of the PEDSV.15 cells, 32% of the cells were positive for capsid protein (Cap) at 6 h p.i. — that is at 2 h after the adsorption period and removal of unadsorbed virus (Fig. 2A, left plot; "Cap"). Similar images were obtained with live and UV-inactivated virus, demonstrating that the signal was due to adsorbed and/or endocytosed virion material. By 2 d p.i., 11.5% of the cells remained Cap positive when infected with live virus, whereas the Cap signal had disappeared (<1%) when UV-inactivated virus was employed (Fig. 2A, left plot). From 2 d p.i., the percentage of Cap positive cells began to increase, reaching their maximum of >90% by 7 d p.i. (Fig. 2A, left plot), suggesting that PCV2 replicates in PEDSV.15 cells.

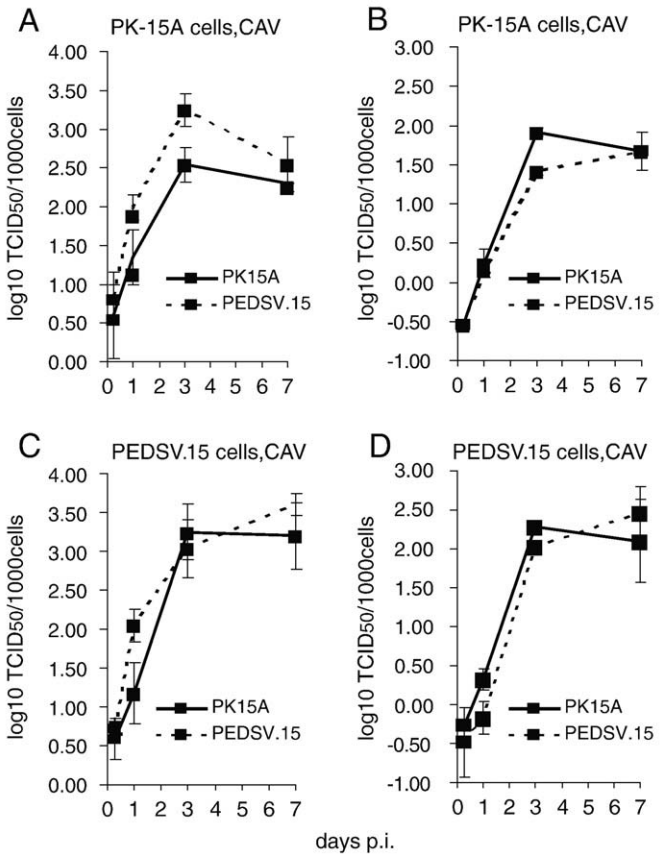

Fig. 1. PCV2 titrations on PK-15A and PEDSV.15 cells. PK-15A (A,B) and PEDSV.15 cells (C,D) were infected at an MOI of 3 $TCID_{50}$/cell with live PCV2 for 4 h at 39 °C, washed, and further incubated at 39 °C for the times shown on the *x*-axis. Cell supernatants (ECV) (B,D) and cell lysates (CAV) (A,C) were collected at the times shown, and titrated on PK-15A (solid lines) or on PEDSV.15 (dashed lines) cells. Means of duplicates +/− SD of one representative experiment are shown.

In order to confirm *de novo* PCV2 replication, the infected cells were stained in parallel for the replicase (Rep) protein, which is essential for the replication of both PCV1 and PCV2 (Mankertz and Hillenbrand, 2001; Sun et al., 2007). The levels of this protein are lower than those for the Cap protein (see Fig. 2). No Rep protein was detected at 6 h p.i., nor did any signal appear during the period of observation when UV-inactivated virus was employed (Fig. 2A, centre plot; "Rep"). In contrast, between 1 and 2% of the cells, on average, were consistently positive for the Rep protein from 2 d after infection with live PCV2 (Fig. 2A, centre plot).

In order to determine if this increased viral protein production translated into virus maturation, cell culture supernatants and cell lysates from the infected PEDSV.15 cells were tested for the presence of infectious virus. The residual infectivity – ECV following the 4 h adsorption period – was less than 10^0 $TCID_{50}$/1000 cells (Fig 2A, right plot – "$TCID_{50}$" – first time point), This was taken to reflect residual inoculum virus and did not change significantly at 1 d p.i. Between 1 d and 2 d p.i., the titers of both ECV and CAV gradually increased to around 10^2 $TCID_{50}$/1000 cells at 7 d p.i., indicating that new viral progeny was produced (Fig. 2A, right plot).

PCV2 replication in EDC, GEpC, MoDC and Fb

PEDSV.15 cells might have lost endothelial cell characteristics, therefore PCV2 infection and replication was studied in primary porcine aortic endothelial cells (EDC). These cells were confirmed to have EDC characteristics in terms of being collagen I negative and VE-cadherin positive (data not shown). After the adsorption period – at

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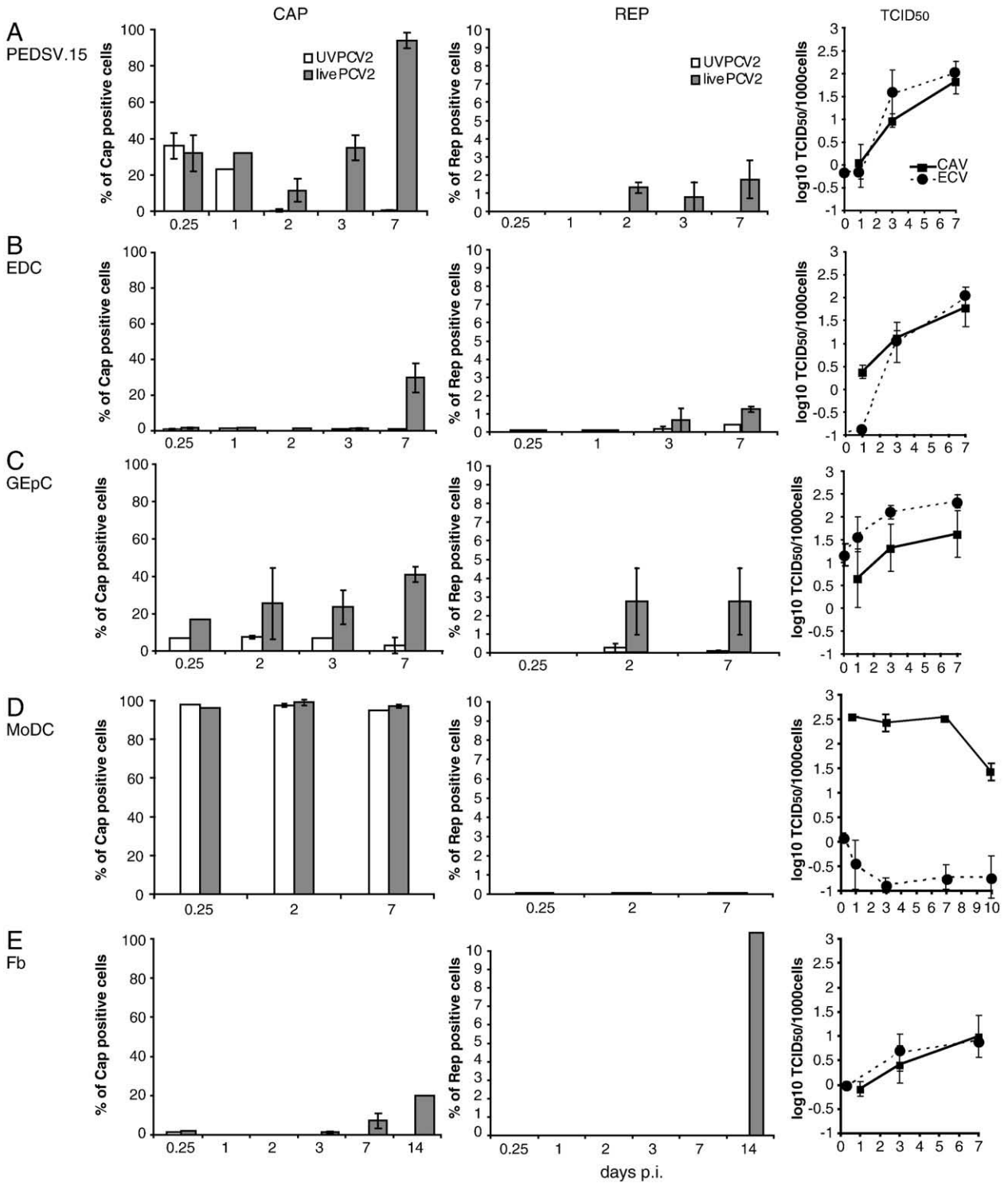

Fig. 2. PCV2 replication kinetics in the PEDSV.15 cell line, EDC, GEpC, MoDC and Fb. PEDSV.15 (A), EDC (B), GEpC (C), MoDC (D) and Fb (E) were infected with UV-inactivated virus (UV PCV2) or live PCV2 for 4 h at 39 °C, washed, and the percentage of Cap (left plots) and Rep (centre plots) positive cells analysed by flow cytometry at the time points indicated (*x*-axis). Mean values+/−SD of two (MoDC, Fb) or three independent experiments are shown. Samples were also collected at the different time points p.i. for ECV and CAV titrations (right plots). The titers are expressed as log 10 $TCID_{50}$/1000 cells (right chart). Mean values of duplicates +/−SD are shown for one representative experiment.

6 h p.i. – very few EDC were positive for the Cap protein, whether infected with live virus or UV-inactivated virus (1.5% and 1.1% respectively; Fig. 2B, left plot). After 3 d, the image had not changed. By 7 d p.i., a significant increase in Cap positive cells was detected in live virus infected cells (29.7% Cap positive cells), while still ≤1% of cells were positive if UV-inactivated virus was employed (Fig. 2B, left plot). Concerning the Rep protein, this was only found in cells infected with live virus (Fig. 2B, centre plot), being first detected at day 3 p.i. (0.65% positive cells) and reaching 1.3% positive cells at 7 d p.i. These results related to the ECV and CAV titers. No ECV titer was detectable after the adsorption period – 6 h p.i – but started to appear at 3 d p.i, rising to $10^{1.98}TCID_{50}$/1000 cells by 7 d p.i. (Fig. 2B, right plot). With the CAV titer, first analysed at 1 d p.i., $10^{0.38}TCID_{50}$/1000 cells were detectable, increasing to $10^{1.8}TCID_{50}$/1000 cells at 7 d p.i. (Fig. 2B, right plot).

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Immunohistological studies have also identified PCV2 antigen in gut epithelial cells and gut-associated lymphoid tissue *in vivo* (Allan et al., 1999). Accordingly, we analysed the characteristics of PCV2 replication in epithelial cells isolated from the small intestine (GEpC) of an SPF pig. The GEpC were controlled to be collagen I negative and E-cadherin positive (data not shown). After the adsorption period, 17% of the cells were positive for the Cap protein when infected with live virus, while 7% were positive following interaction with UV-inactivated virus (Fig. 2C, left plot). No significant increase in Cap positive cells was noted at 2 d p.i., but by 7 d p.i. the number of Cap positive cells had increased to 41% following infection with live virus — again there was no increase when UV-inactivated virus was used (Fig. 2C, left plot). As with the EDC, Rep protein was detectable in the GEpC only when live virus was employed. The Rep was detectable at an earlier time point post-infection with the GEpC – 2 d p.i. – similar to the PEDSV.15 cell line. At this time, 2.9% of the infected GEpC were positive for Rep (Fig. 2C, centre plot). Again the ECV and CAV levels reached their maximum values at 7 d p.i., showing similar titers to those obtained from the PEDSV.15 cell line and EDC.

Next we compared the infection in cells of myeloid origin: MoDC and Fb, which were characterized as described previously (Balmelli et al., 2005; Carrasco et al., 2001; Guzylack-Piriou et al., 2004). We have recently demonstrated that PCV2 efficiently associates with DC and persists in the cells in an infectious form for at least several days, without any evidence of virus replication (Vincent et al., 2003). This relationship of the virus with myeloid cells was elaborated by comparing PCV2 infections of MoDC and fibrocytes (Fb), another cell of myeloid origin. In contrast to the EDC, the uptake of PCV2 antigen by the MoDC was particularly efficient: 96% of the cells were Cap positive after the adsorption period (Fig. 2D). Relating to the previous reports, these high levels of PCV2 antigen persisted in the MoDC for the duration of the experiment — 97.5% of the cells were still Cap protein positive after 7 d (Fig. 2D, left plot). Another major difference between the MoDC and the other cells was that the efficient uptake of antigen and persistence for 7 d was also observed with the UV-inactivated virus (Fig. 2D, left plot). Conversely, there was no detectable expression of the Rep protein in infected MoDC at any time point p.i. (Fig. 2D, centre plot). The ECV and CAV titers related to these observations on the antigen — persisting but not increasing for the duration (10 d) of the experiment (Fig. 2D, right plot).

Although Fb are also of myeloid origin, the characteristics of PCV2 association with these cells was quite dissimilar to that noted with the MoDC. In fact, the Fb related more to the EDC in showing a low number (1.35%) of Cap positive cells at 3 d p.i. (Fig. 2E, left plot), the time when 98% MoDC were Cap positive. Moreover, <1% Fb became Cap positive when UV-inactivated virus was used (Fig. 2E, left plot). On the other hand, the Fb did become Rep positive when infected with live virus, although this took longer than with the EDC and the GEpC — 11% Fb were Rep positive at 14 d p.i. (Fig. 2E, centre plot). There was also a clear increase in ECV and CAV titers with time p.i. (Fig. 2E, right plot), again with a slower kinetic in the Fb compared with EDC and GEpC.

PCV2 binding

The observed variation among the cell types with respect to the number of Cap positive cells at 6 h p.i. raised the question of a variable efficiency in virus binding. Consequently, PCV2 was adsorbed to the different cell types on ice. The CAV titers shown in Fig. 3A reflect the bound infectious virus particles after the 4 h adsorption period on ice. PCV2 bound most efficiently to MoDC and PESDV.15 cells, $10^{1.2}$ and

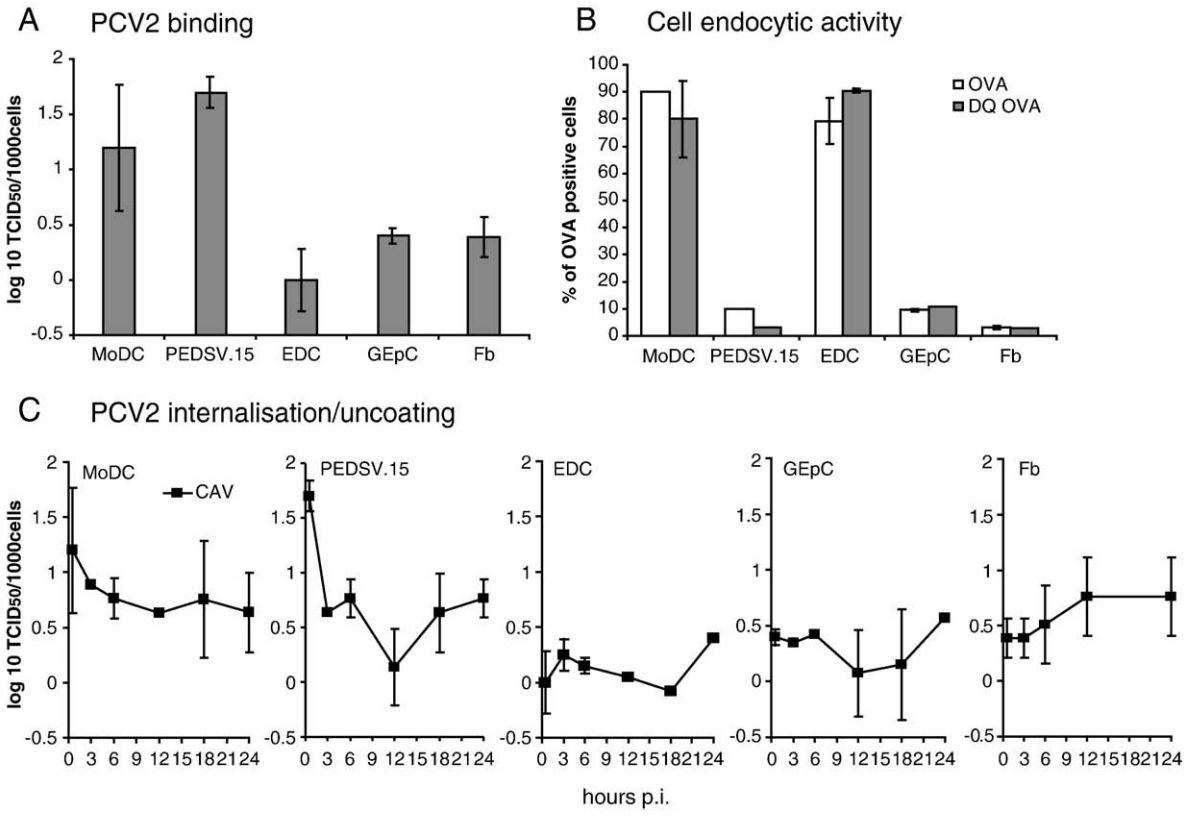

Fig. 3. PCV2 binding (A). MoDC, PEDSV.15 cells, EDC, GEpC and Fb were incubated with live PCV2 for 4 h on ice. The CAV titer at 0 h p.i. represents bound infectious particles after this adsorption period. Mean values of duplicates +/− SD are given for one representative experiment. Endocytic activities of the cells (B). To analyse the endocytic and processing activities of the different cells, the uptake of $Alexa_{488}$-OVA and the dequenching of DQ-OVA were analysed by flow cytometry after 4 h of incubation. Mean values +/− SD of two independent experiments are shown. PCV2 internalisation (C). After the adsorption period (A), the cells were washed and returned to 39 °C to analyse PCV2 internalisation. The 3 h p.i. time point refers to the time at 39 °C, following the 4 h incubation period at 4 °C. Mean values of duplicates +/− SD are given for one representative experiment.

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$10^{1.7}$ $TCID_{50}$/1000 cells respectively (Fig. 3A). The corresponding titers on the EDC, GEpC and Fb ranged from $10^{0.0}$ to $10^{0.4}$ $TCID_{50}$/1000 cells (Fig. 3A).

Relationship of PCV2 uptake to cell endocytic activity

To understand the relationship between endocytic and processing activities of the different cells and PCV2 infection characteristics, the uptake of a fluorescent ovalbumin conjugate (OVA) was used as a measure of endocytosis and DQ-ovalbumin (DQ-OVA) to measure endocytic processing. The DQ-OVA is a reagent heavily labelled with a fluorescent dye, resulting in the total quenching of the fluorescence. Upon uptake and endocytic proteolysis, the protein is cleaved, thus removing the quenching and permitting detection of the fluorescent signal. When OVA uptake was analysed after 4 h incubation, the PEDSV.15 cells and GEpC also gave similar levels — around 10% cells positive (Fig. 3B), relating to the results shown in Fig. 2, where comparable levels of Cap positive cells were found at 6 h p.i.. However, the processing of the OVA – measured with the appearance of the DQ-OVA – appeared to be less efficient in the PEDSV.15 cells (4% PEDSV.15 cells positive compared with 11% of GEpC positive; Fig. 3B). With the Fb, low levels – 3% and 2.5% – of OVA and OVA DQ-positive cells were observed, relating to the low percentage of Cap positive Fb seen at 6 h p.i. (2% — see Fig. 3B). In contrast, the MoDC and the EDC showed high levels of OVA uptake and processing, at this 4 h time point, indicating a more rapid endocytic activity in these cells. However, for the EDC in contrast to the MoDC this high endocytic activity was not manifest with the uptake of PCV2 — only 1.5% of the EDC were Cap positive at 6 h p.i. (see Fig. 2B).

PCV2 internalisation/uncoating

To study the effect of the virus internalisation on the replication efficiency, the cells were incubated with the virus at 4 °C and after this adsorption period washed and shifted to 39 °C. After 3 h, the CAV titers associated with the MoDC and PEDSV.15 cells decreased by 0.3 and 1 logs respectively compared to the 0 h p.i. titers (Fig. 3C). Considering that these titers would have represented both internalised and remaining surface-bound virus, the decrease may have reflected dissociation of the virus from the cell surface. Concerning the other cell types, the titers remained 0.3–0.5 logs lower than the CAV titers associated with MoDC and PEDSV.15 (Fig. 3C).

The CAV titers continued to decrease with the infection of both the MoDC and PEDSV.15 cells until 12 h p.i (Fig. 3C). An apparent titer decline was suggested between 3 h p.i. and 18 h p.i. with the EDC and GEpC (Fig. 3C). An increase in titer following the decline was observed in the PEDSV.15 and EDC cells (Fig. 3C), suggesting that infectious particles were newly produced. Despite the variation shown in Fig. 3C, these results were consistent among the replicate experiments performed.

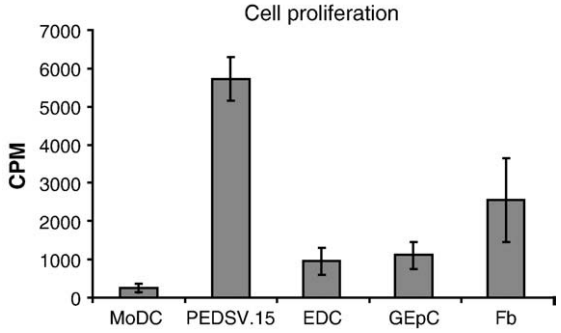

Fig. 4. Mitotic activity of the cells. MoDC, PEDSV.15 cells, EDC, GEpC and Fb were cultured for 24 h at 39 °C and the cell division rates evaluated by ^{3}H-thymidine incorporation assay. Means of triplicates +/−SD are shown.

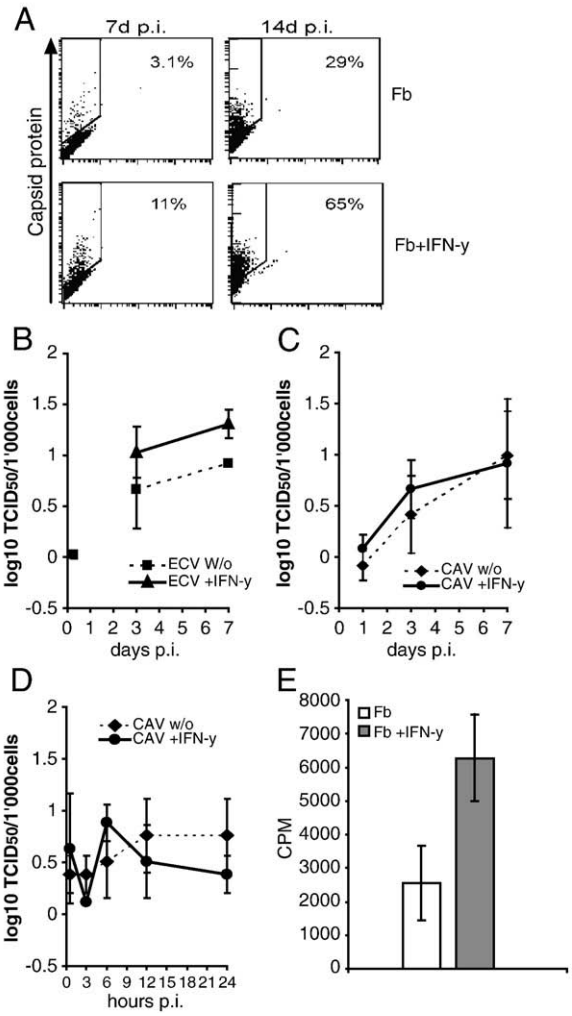

Fig. 5. PCV2 replication in Fb following treatment with IFN-γ Fb were treated or not with 100 ng/ml of recombinant porcine IFN-γ for 24 h at 39 °C, washed and infected for 4 h at 39 °C with live PCV2. The percentage of Cap protein positive cells was analysed by flow cytometry at 7 d and 14 d p.i. (A). The ECV (B) and CAV (C) titers were analysed for IFN-γ treated (+IFN-γ) or untreated cells (w/o). Mean values of duplicates +/−SD of one representative experiment are given. For studying the influence of the cytokine on virus binding and internalisation, the cells were incubated with the virus for 4 h on ice and after washing returned at 39 °C (D). The 0 h p.i. time point refers to the end of the 4 h incubation on ice. Mean values of duplicates +/−SD of one representative experiment are given. In order to analyse the influence of cytokine treatment on the cell proliferation rate, the Fb were treated or not for 24 h with IFN-γ, washed, and for the last 16 h fed with ^{3}H-thymidine. The results are given as counts per minute (CPM); means of triplicates +/−SD are shown.

Efficiency of PCV2 replication does not correlate with the DNA proliferation activity of the cells

Based on the suggestion that PCV2 needs proliferating cells to enter the cell nucleus during mitosis and to advance its replicative cycle in a productive manner (Tischer et al., 1987), the relationship between cell division and virus replication was investigated. The cells with the lowest level of DNA proliferation – MoDC (Fig. 4) – supported little or no virus replication, while the cells with the highest DNA proliferation rate – PEDSV.15 (Fig. 4) – were relatively efficient at producing *de novo* capsid protein (see Fig. 2A). However, a relationship between cell DNA proliferation and virus replication was not observed when the other cell types were brought into the picture. Looking at PCV2 replication kinetics in terms of infectious virus production per 1000 cells demonstrated similar rates in the EDC, GEpC and PEDSV.15 cells were observed (Figs. 2A–C). This contrasted with their DNA proliferation rates — lower for the EDC and the GEpC than for the

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PEDSV.15 cells (Fig. 4). A further discrepancy between the virus replication kinetics and cellular DNA proliferation was also noted with the Fb. These cells displayed the lowest levels of viral replication and slowest production of Cap positive cells. Yet, Fb exhibited a higher DNA proliferation rate compared with EDC and GEpC (Fig. 4).

Considering that IFN-γ is reported to increase Fb proliferation rate (Balmelli et al., 2005) – the cell type in which the virus grew the slowest – we tested the influence of this cytokine on PCV2 replication in Fb. Following stimulation with IFN-γ before infection with live virus, flow cytometric analysis showed that the IFN-γ treatment clearly had a positive influence on the percentage of Cap positive cells after 7 and 14 d p.i. (Fig. 5A).

The IFN-γ dependent increase in Cap positive cells was reflected in higher ECV titers, particularly at 7 d p.i. (Fig. 5B). In contrast, the CAV titers were not significantly increased (Fig. 5C), nor did the IFN-γ enhance virus binding (Fig. 5D, 0 h p.i.). Furthermore, the cytokine did not influence virus internalisation — the CAV titers did not significantly differ from 3 h to 24 h p.i. (Fig. 5D). This was not due to an inability of the IFN-γ to influence cell proliferation — treatment of the Fb showed a clear increase in ^{3}H-thymidine uptake (Fig. 5E).

PCV2 is not influencing cell growth or inducing detectable cell death

Although the cell DNA proliferation rate did not define the characteristics of PCV2 infection or replication, it was possible that the PCV2 infection modified the cell growth characteristics, as observed with other DNA viruses (Schang, 2003; Xie et al., 1995). Accordingly, cell counts following interaction with live virus were compared with cell counts obtained using UV-inactivated virus or mock infection. Figs. 6A–D show that PCV2 infection did not influence the cell numbers in the cultures of any of the cell types, even when the PEDSV-15 cells were passaged (Fig. 6A). Furthermore, PCV2 infection did not influence the cell division or DNA proliferation rates (Fig. 6E).

The above observations tended to conflict with the report of Liu et al. (2005) that the ORF3 protein of PCV2 induces cell death in PK-15 cells during PCV2 infection. Therefore, the percentage of propidium-

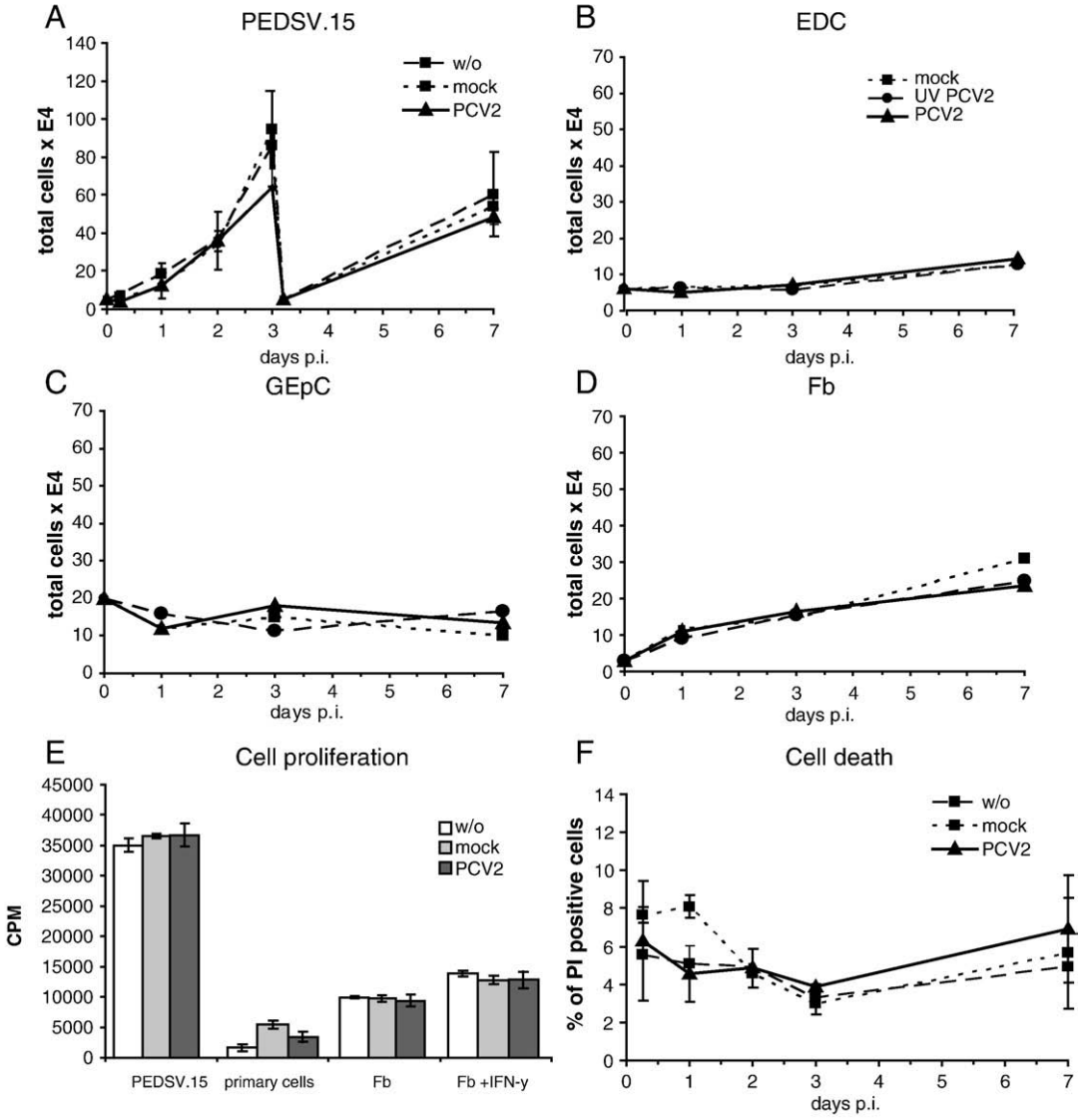

Fig. 6. Growth characteristics of cells following PCV2 infection. PEDSV.15 (A), EDC (B), GEpC (C) and Fb (D) were infected with live virus (live PCV2), UV-inactivated virus (UV PCV2) or mock treated for 4 h at 39 °C. The total cell numbers after harvesting the cells at different time points (*x*-axis) are expressed as $\times 10^4$ cells. Values of one representative experiment are shown. In order to study the influence of virus infection on the cell proliferation rate, the cells were infected with live PCV2 or mock treated for 4 h, washed and cultured in the presence of ^{3}H-thymidine for the last 16 h (E). The values are shown as counts per minute (CPM). Means of triplicates +/− SD are shown. Analysis of the percentage of dead cells in the PEDSV.15 cell cultures – uninfected (w/o), live virus infected (PCV2) and mock treated – were measured by labelling with propidium-iodide at time points p.i. indicated on the x-axis (F). Mean values of duplicates +/− SD of one representative experiment are given.

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iodide (PI) positive cells – reflecting apoptotic and necrotic cells – was analysed by flow cytometry in PCV2-infected PEDSV.15 cells. At 6 h p.i., 5.6% and 7.6% of the cells were PI-positive in the uninfected and mock-treated cultures, respectively (Fig. 6F). With the PCV2 infected cells, 6.3% were PI-positive (Fig. 6F). The percentage of PI-positive cells decreased to 3.8% in the PCV2 infected cells and to 3.0% in the mock-treated cells at 3 d p.i., at which time the uninfected cultures showed 3.3% PI-positive cells (Fig. 6F). The cells were passaged at 3 d p.i. and stained with PI after a further 4 d in culture (7 d p.i.). The PI-positive PCV2-infected cells increased to 6.9%, but were not significantly different from the 5.0% and 5.7% PI-positive cells in uninfected and mock-treated cultures respectively (Fig. 6F).

PCV2 antigen cellular localisation

Confocal microscopy was employed to characterize the cellular localisation of the PCV2 proteins following infection. An adsorption period of 4 h at 39 °C was employed. This was to ensure that the temperature did not influence virus binding to the cells, as it would be the case if the virus were interacting through receptor-independent

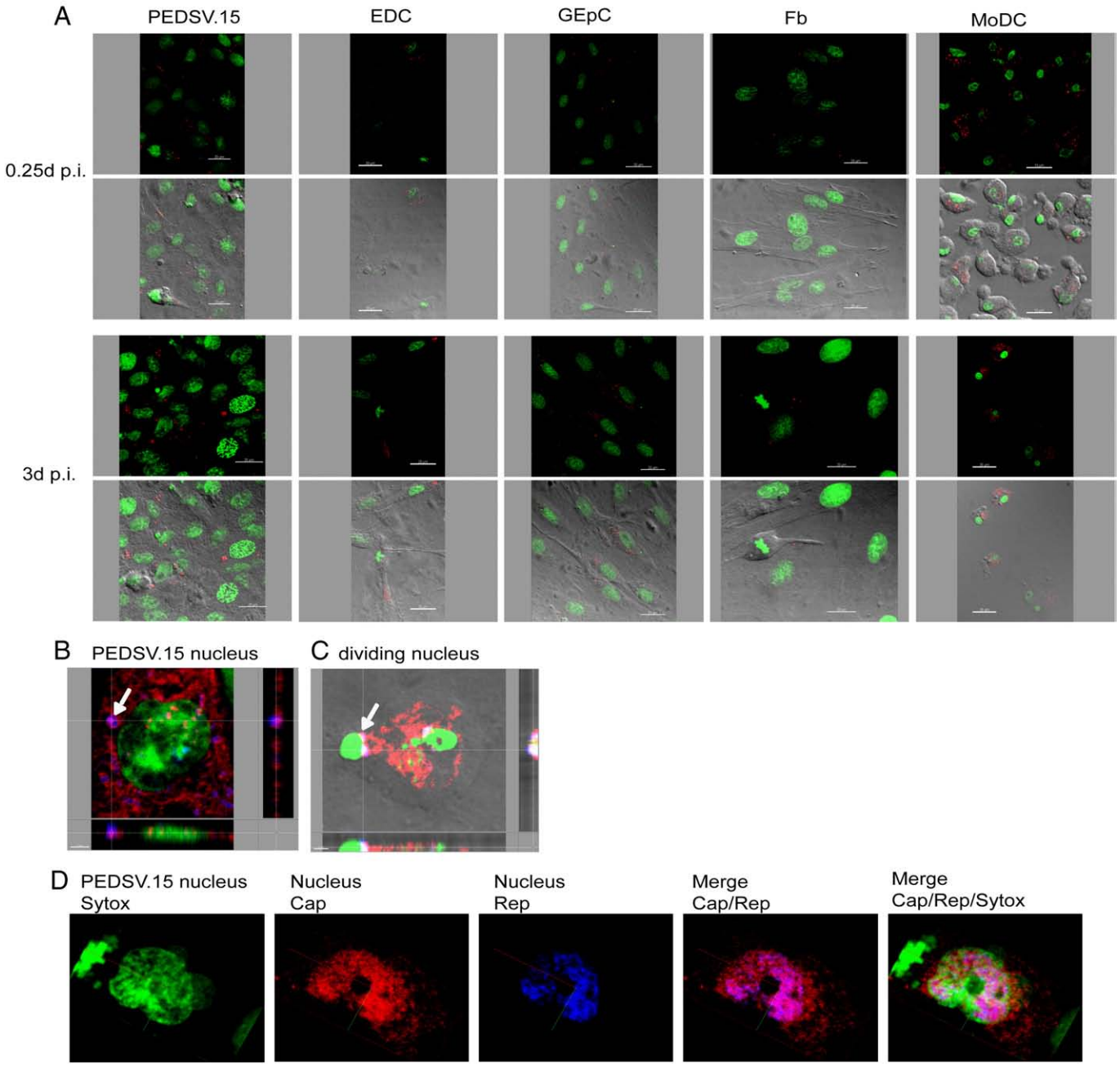

Fig. 7. Detection of Cap and Rep by confocal microscopy (A). For confocal microscopy analysis, live PCV2 virus infected PEDSV.15, EDC GEpC, Fb and MoDC were employed. Following infection for 4 h at 39 °C, the cells were analysed 2 h after washing or after 3-day incubation at 39 °C. At these two time points, the cells were fixed and permeabilised, followed by staining for Cap (red), Rep (blue) and the cell nucleus (green: sytox) at 6 h (0.25 d: lower row: Nomarski interference image) and 3 d p.i. (lower row: Nomarski interference image). A representative experiment is shown. Intracellular localisation of Cap and Rep in PEDSV.15 cells (B–D). Live PCV2 virus infected PEDSV.15 cells were washed after 4 h of infection, incubated for a further 48 h, then fixed and permeabilised, and stained for Cap (red), Rep (blue) and the cell nucleus (green: sytox). Cross-section profiles are shown below and to the right of the main images — by placement of the cross-wire on the cell nucleus as indicated (B,C). Magnification of the cell nucleus shown (B). Dividing cell shown (grey colour = Nomarski interference image) (C). The relative position of Cap and Rep in a cell nucleus is shown (D): cell nucleus labelled with sytox; Cap; Rep; merged image of Cap and Rep; merged image of Cap, Rep and sytox. A representative experiment is shown.

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mechanisms reliant more on membrane fluidity. As expected from the flow cytometry results, the Cap protein associated with most of the PEDSV.15 and MoDC at 6 h p.i. (0.25 d) (Fig. 7A). The amount of antigen per MoDC was often greater than that observed per PEDSV.15 cell, which would explain the lower signal by flow cytometry with PEDSV.15 cells compared with MoDC — 30–40% PEDSV.15 cells positive (see Fig. 2A), >95% MoDC positive (see Fig. 2D) by flow cytometry. With the EDC and GEpC (Fig. 7A), lower levels of antigen were found associated with the cells, although certain EDC could have similar levels to those found with the PEDSV.15 cells. While the image with the EDC would relate to the flow cytometry results (see Fig. 2B), this cannot be said for the images of the GEpC (see Fig. 2C). One explanation might relate to the sizes of the cells in the cultures on the labteks used for microscopy. GEpC are more extensively spread over the slide surface compared with the EDC (Fig. 7A). This would restrict visualisation of positive cells in a single field with the GEpC. Another explanation might relate to a different binding capacity or detection capacity for the anti-Cap antibody when employed with microscopy and flow cytometry. Indeed, the cell-permeabilisation methods do differ for microscopy and flow cytometry. Moreover, the former uses Alexa conjugates for the red channel (due to the laser employed with confocal microscopy), whereas flow cytometry requires phycoerythrin conjugates, which cannot be used with confocal microscopy due to the cross talk between channels. With the Fb the low levels of virus antigen associated with these cells after the 6 h adsorption period relate to the flow cytometry and infectivity results (Fig. 7A).

Interestingly, there was no apparent increase in the Cap antigen signal in the PEDSV.15 cells at 3 d p.i. (Fig. 7A). In contrast, the distribution of the Cap-containing inclusions altered — with more inclusions being seen perinuclear rather than peripheral. Moreover, certain perinuclear inclusions were large, typical of those associated with perinuclear viral antigen factories seen with other DNA viruses. With the EDC and the GEpC, there was a more obvious increase in the Cap protein signal per cell at 3 d p.i. (Fig. 7A). Although this could not be observed with the population analysis through the flow cytometry (see Figs. 2B,C), it did reflect the increase in virus titers obtained from the virus infectivity analyses (see Figs. 2B,C). Moreover, there was again an increase in the number of perinuclear inclusions (Fig. 7A). As for the Fb and MoDC, the amount of Cap antigen in the positive cells had not changed by 3 d p.i. (Fig.7A). This was not surprising, considering that the virus replicated particularly slowly in Fb, while there was no evidence of replication in the MoDC.

The presence of Rep antigen was also noted in the PEDSV.15 cells, EDC and GEpC within 3 d p.i (see Fig. 2). In order to analyse the relative intracellular distribution of the PCV2 antigens, the more rapidly dividing PEDSV.15 cells were used to facilitate antigen localisation in cells with dividing nuclei. At 2 d p.i., both the Cap and Rep proteins were present in the cell nucleus of certain cells (Figs. 7B,C). At this relatively early time point, the bulk of the antigen – both Cap and Rep – showed a cytoplasmic localisation, likely reflecting sites of synthesis. In certain cells, the Rep protein could be found in a perinuclear region, in a close positional relationship to the Cap protein (channel correlation in co-localised volume: Cap/Rep: 0.25)(Fig. 7B, arrow). In cells showing a dividing nucleus, both proteins appeared to migrate with the cell chromatin in the mitotic figures (channel correlation in co-localised volume: Cap/sytox: 0.43; Rep/sytox: 0.72) (Fig. 7C, arrow).

Although the majority of the Cap protein in cells with dividing nuclei (Fig. 7C) was not apparently associated with the chromatids, cells did arise in which the majority of both Cap and Rep proteins were localised in the nucleus (Fig. 7D). Therein, it would appear that the two PCV2 proteins were co-localising in certain areas (purple colour, Fig. 7D). Image analysis confirmed that the Cap and Rep proteins co-localised in the nucleus (channel correlation in co-localised volume: Cap/Rep: 0.61).

Discussion

Although the presence of PCV2 antigen has been described in several types of cells *in vivo* (Allan and Ellis, 2000; Opriessnig et al., 2006), such observations cannot define precisely the cells in which the virus replicates. Accordingly, the present study compared PCV2 infection and replication in cell types of different origin.

Virus attachment and cell endocytic capacity are not the sole limiting factors for PCV2 replication

PCV2 attachment to cells was not restricted to a certain cell type, relating to the reported PCV2 interaction with glycosaminoglycan structures (Misinzo et al., 2006) common to many cell types (Rostand and Esko, 1997). However, PCV2 did not interact equally with all cells. The virus was bound and internalised efficiently by MoDC, but poorly by EDC. In contrast, EDC rather than MoDC supported detectable virus replication. Although this suggests a negative link between endocytic activity and virus replication, the relationship is not so straightforward. Virus binding and internalisation by EDC was also poor compared with PEDSV.15 cells and the GEpC, yet all three were similar for supporting productive virus replication.

While the EDC were poorly endocytic for PCV2, this was not the case for ovalbumin, the endocytosis of which was similar for EDC and MoDC. This discrepancy may reflect inefficient virus/Cap binding by the EDC. Alternatively, EDC may be more efficient at processing the Cap protein, rendering the antigen undetectable. This would relate to the high efficiency of EDC for processing ovalbumin. However, the EDC were similar to the MoDC in this property, suggesting that the EDC handling of ovalbumin and PCV2 cannot be the same as for MoDC, wherein the Cap protein apparently resisted degradation and persisted.

Relationship of de novo Cap synthesis and virus replication

The presence of Cap protein does not equate with *de novo* virus replication. Indeed, the Cap protein signal early after infection reflects more the uptake of the virus particles and Cap protein. Only in cells other than the MoDC – PCV2 infection in MoDC is considered as relatively non-productive (Vincent et al., 2003) – an increase in the percentage of Cap positive cells was observed over time. This increase in the Cap signal did correspond to an elevation in progeny virus titers and therefore productive infection. Nevertheless, an increase in Cap positive cells *per se* did not define the level of PCV2 productivity. PEDSV.15 cells, and to a lesser extent GEpC, were more efficient than the EDC for Cap protein production, but the EDC had a similar kinetics of progeny infectious virus production. This suggests that the EDC were the more efficient at incorporating *de novo* synthesised Cap protein into mature progeny virus.

Relationship of de novo Rep synthesis and replication

Although the Rep protein was only detected at low levels, it was associated with cultures wherein the virus was replicating. Clearly, detection of the Rep protein was more reliable than detection of the Cap protein as an indicator of PCV2 replication. Nevertheless, Rep protein synthesis is only one step in the replicative cycle, although it is essential for assuring initiation of PCV2 genome replication. The involvement of the cellular DNA replication machinery is also indispensable, leading to the formation of the viral replicative form and progeny genomes, upon which the Cap protein condenses to form infectious particles. One can only ascertain complete PCV2 replication by detecting infectious virus progeny. Detection of Rep protein is certainly more indicative of likely virus replication than is detection of Cap protein; detection of replicative forms would be additionally informative.

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The low level detection of Rep protein may relate to how it is involved in the virus replicative cycle. Considering that Rep protein should be found in the cell nucleus during the S-phase of the cell cycle (Mankertz et al., 2004; Tischer et al., 1987), the observed low level of Rep positive cells in the cultures might reflect cells in S-phase at the moment of analysis. If virus replication only occurred in Rep positive cells, the efficiency of virus progeny production is estimated at approximately 5–10 infectious particles per cell with PEDSV.15 cells, EDC and GEpC, which is comparable to other viruses.

Accordingly, the apparently low level of progeny virus production would be reflecting the number of cells in which the Rep protein becomes associated with the cell nucleus, rather than a poor replication *per se*. How the Rep protein becomes associated with the cell nucleus is still a matter of investigation; no intracellular factor to assist PCV2 Rep protein nuclear translocation has yet been identified. Some insight may come from studies on Geminiviruses, with which porcine circoviruses are evolutionarily linked (Niagro et al., 1998). Geminivirus Rep protein interacts with plant and human retinoblastoma protein, which may upregulate S-phase functions required for viral replication (Gutierrez, 1999; Xie at al, 1995). However, the observations on Rep protein association with mitotic figures (see below) may provide an alternative mechanism.

Relationship of mitotic activity to virus replication

PCV2 is reported to require dividing cells for its genome to enter the cell nuclei for replication and transcription (Tischer et al., 1987). Our own observation showed that the cells permissive for productive PCV2 infection were mitotically active, but this is not simply a direct relationship. While the low mitotic activity of MoDC does relate to an apparent absence of virus replication, the level of mitotic activity for the other cells did not reflect the degree of virus replication. Fb were mitotically more active than EDC and GEpC, but showed the slowest virus replicative cycle. PEDSV.15 cells were even more active in terms of cell proliferation, but were similar to the less active EDC for producing infectious progeny virus.

The inflammatory cytokine IFN-γ was reported to enhance Fb proliferation (Balmelli et al., 2005). An increase in the percentage of Cap positive cells was observed with IFN-γ treated Fb, but no change in infectious progeny virus production. This suggested an influence on Fb endocytic activity, relating to the report on enhanced internalisation of PCV2 virus-like particles (VLP's) by an IFN-γ treated monocytic cell line (Meerts et al., 2005b). However, the latter authors maintained the IFN-γ in their cultures, rendering it difficult to define the cellular process being influenced. Our own use of the cytokine only at the initiation of the infection demonstrated that the IFN-γ did not influence PCV2 binding or internalisation by the cells. The influence appeared to be on the production of Cap protein, but not infectious virus.

Virus-induced cell death

PCV2 did not induce detectable cytolysis during the 7-day period of observation, even after passaging the PEDSV.15 cells. This suggests that the extracellular virus was released by exocytosis. Alternatively, if only Rep positive cells released virus by cytolysis, a maximum of 1.5–2% would be lysed at a particular time point. Such low numbers are difficult to discriminate from the "spontaneous" cell death occurring in tissue culture. Further analyses, such as determining the expression of ORF3 – the so-called apoptosis-inducing protein (Liu et al., 2005) – may elaborate on these studies on PCV2 induced cell death.

Subcellular localisation of Cap and Rep proteins

Considering the relationship between PCV2 replication and the detection of Cap and Rep protein, it was of interest to analyse further their compartmentalisation in the cell. Cap and Rep protein synthesis were first detectable in cytoplasmic inclusions, as expected considering the site of protein synthesis. Many of these inclusions – particularly with Rep – were perinuclear, typical of DNA replicating viruses requiring cellular nuclear involvement for virus maturation. The Rep was also seen in close association with the Cap, but the Cap protein dominated the antigen levels in the cell. Considering the replication of beak and feather disease virus (BFDV) (Heath et al., 2006), to which PCV2 is related, the Cap protein may be important for translocating the viral genome and the Rep protein to the cell nucleus. Nuclear association of PCV2 Rep protein is seen together with Cap protein, but porcine circoviruses do differ from BFDV. The PCV1 Rep protein contains its own nuclear localisation signals (NLS) (Finsterbusch et al., 2005). This is important considering our own microscopic observations on PCV2 Rep protein in close proximity to the Cap protein, particularly at cell division. Algorithmic analysis demonstrated that the two proteins were co-localised. Furthermore, both Rep and Cap protein associated with the condensing chromatin in mitotic figures during cell mitosis. This would suggest that Rep and Cap proteins become associated with the nucleus during mitosis, relating to the observations of Tischer et al. (1987).

Conclusions

The present study demonstrates that PCV2 shows a pluripotency for infecting cells of epithelial, endothelial and myeloid origin. This offers an explanation for the widespread tissue distribution of the virus in infected animals (Allan and Ellis, 2000; Opriessnig et al., 2006). Although the virus replication characteristics were variable dependent on the cell type involved, a common feature was an increase in Cap and Rep protein expression correlating with virus replication. Such variation among the different cell types was showing that PCV2 replication is dependent on cellular endocytic processes and mitotic activity. Active endocytic processes are important for the internalisation of the virus and Cap protein, but do not guarantee initiation of the virus replicative cycle. The dominant endocytic processes of MoDC are important for internalisation and accumulation of viral proteins, whereas the processes of EDC and GEpC are more important for virus uncoating and initiation of the virus replicative cycle. Thereafter, successful PCV2 replication appears to be dependent on how the Rep and Cap proteins associate with mitotic figures. A close association with separating sister chromatids suggests that the viral proteins may interact with the microtubules of the mitotic spindle, which in turn could explain how the virus spreads between daughter cells without release from the cell. Further studies on the interaction of PCV2 proteins with such cellular structures, as well as associated proteins, will help clarify how virus infection leads to antigen accumulation or virus replication, and the relevance of the dominant form of endocytosis therein.

Materials and methods

Antibodies

Anti-capsid protein ORF2 mAb 7G5-G4-A1 (IgG2a) and anti-replicase protein ORF1 mAb F210 7G5-C1-G9 (IgG1) were kindly provided by Dr. Allan Gordon QUB, Belfast. Anti-collagen type I mAb (I-8H5) was purchased from Calbiochem/EMD Biosciences, Inc (La Jolla, CA), anti-VE cadherin mAb (F-8; sc9989) from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-E-Cadherin mAb (clone HECD-1) from Zymed laboratories, (Invitrogen, Basel, Switzerland).

PCV2 preparation

A PCV2 isolate from Canada – Stoon 1010 (Ellis et al., 1998) – was used to prepare virus stocks in the PK-15A cell line, as described

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previously (Vincent et al., 2003). PCV2 titers were calculated by titrating the stocks on PK-15A cells, using the Karber formula for calculation, and expressed as 50% tissue culture infectivity doses ($TCID_{50}$/ml) on the basis of the immunofluorescent detection of PCV2 antigen using an antibody directed against the ORF2-encoded capsid protein (7G5-G4-A1) (McNeilly et al., 2001). In some experiments UV-inactivated PCV2 lysate was used for "infection". For this, the infectious lysate from PK-15A cells was inactivated in a UV chamber (Biorad; GS Gene Linker) at 125 mJ for 17 min. The infectivity of the UV-inactivated virus was tested as above by titration on PK-15A cells.

Cells and culture medium

The porcine kidney cell line PK-15A was cultured in minimal essential medium containing Earle's salts (Invitrogen), 10% (v/v) FBS at 37 °C, 6% CO_2.

The porcine aortic endothelial derived cell line (PEDSV.15), kindly provided by Dr. Seebach at the University Hospital Geneva, was cultured in Dulbecco's Modified Eagle's Medium (DMEM), 10% (v/v) FBS, supplemented with Non-Essential Amino Acids Solution (NEAA) and 100 mM sodium pyruvate (all from Invitrogen) at 37 °C, 6% CO_2. The cells were VE-cadherin positive, but negative for PCV1.

Fb were isolated, cultured and phenotypically defined as described previously (Balmelli et al., 2005). In some experiments, cells were activated using 100 ng/ml of porcine IFN-γ (kindly provided by Novartis, Basel, Switzerland).

MoDC were generated and defined as described previously (Carrasco et al., 2001) (Guzylack-Piriou et al., 2004). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of specific pathogen free (SPF) pig blood by density centrifugation over Ficoll-Paque (1.077 g/L) at 1000 ×g for 25 min. CD172a-positive monocytes were selected by magnetic cell sorting using MACS (Miltenyi, Bergisch-Gladbach Germany), and cultured for 5 d in DMEM, 10% (v/v) SPF pig serum, recombinant porcine (rp) GM-CSF (150 ng/ml, kindly obtained from Shigeki Inumaru, Institute of Animal Health, Ibaraki, Japan) and rpIL-4 (100 U/ml; prepared as previously described (Carrasco et al., 2001) (MoDC medium).

EDC were isolated from porcine aorta sections. Surrounding tissue was removed from the aorta, which was kept in cold PBS containing 5× Penicillin/Streptomycin (Invitrogen) and 5× Amphotericin B (Invitrogen) (washing solution). The aorta was rinsed with washing solution and cut in small pieces of approximately 1 cm^2. These were digested at 37 °C for 2 h by constant stirring in PBS containing 5× Penicillin/Streptomycin, 5× Amphotericin B and 1.2 U/ml Dispase II (Roche; nr. 295 825). The resulting suspension was filtered through sterile 4-layer gauze and centrifuged at 800 ×g, 4 °C for 5 min. The cell pellet was washed twice and resuspended in Endothelial-SFM Medium (Invitrogen), supplemented with 7% (v/v) FBS, Glutamax I, 2× Penicillin/Streptomycin, and 2× Amphotericin B (Growth Medium). The endothelial cells were characterized as collagen I negative and VE-cadherin positive. Maintenance medium for the cells was as for Growth Medium, but contained 1× Penicillin/Streptomycin and 1× Amphotericin B. It was changed every 3 d until the cells were ready for use. The cells were employed within 6 passages.

GEpC were isolated from the small intestine (jejunum fragment) of SPF pigs. The tissue sample obtained was rinsed with the same washing solution as for the endothelial cells preparation. It was then cut into small pieces, which were placed in a 75 cm^2 tissue culture flask, and further washed until the supernatant appeared clear. Afterwards, the tissue was digested by shaking at 37 °C for 30 min in DMEM containing 2.5% (v/v) FBS, 1× NEAA, 5× Penicillin/Spreptomycin, 5× Amphotericin B, 1.2 U/ml Dispase II and 500 μg/ml Collagenase D (Roche; nr. 11088858001). The resulting suspension was filtered through sterile 4-layer gauze and centrifuged at 500 ×g, 4 °C for 5 min. The cell pellet was washed twice in DMEM, 2.5% (v/v) FBS, 1× NEAA, 2× Penicillin/Streptomycin, 2× Amphotericin B, 2.5 μg/ml bovine insulin (Sigma Chemicals, Buchs, CH) and 10 ng/ml human epidermal growth factor (PeproTech, Hamburg, Germany) (hEGF) (culture medium). GEpC were characterized as collagen I negative, E-cadherin positive. The cells were used up to passage 7.

Infection of the cells

The different cell types were seeded in 12 well plates in a total volume of 2 ml medium 24 h before infection with live PCV2 or UV-inactivated virus at a MOI of 3 $TCID_{50}$ per cell or mock-treatment. All cultures were maintained at 39 °C, which is the body temperature of the pigs from which the primary cells were isolated. Where indicated in the Results, cells were treated with 100 ng/ml IFN-γ for 24 h before infection. At 4 h post-infection (p.i.), the cells were washed three times with 1 ml of medium/well to remove the majority of unadsorbed virus.

Infection of 5-day old MoDC was performed in either 15 ml tubes or plates. These cells were also infected for 4 h with PCV2 or UV-inactivated virus at MOI of 3 $TCID_{50}$/cell, or for 24 h at a MOI of 1 $TCID_{50}$/cell. After this initial infection period, the cells were washed 3 times with 5 ml of MoDC medium to remove the majority of unadsorbed virus. Cells were further cultured in 12 well plates, and re-fed every 2 to 3 d with fresh MoDC medium. Infection of the different cell types was monitored by flow cytometry at various time points post-infection as indicated in the Results. Mock controls used identical infections to the above, but employing lysates from uninfected PK-15A cells.

Virus binding and internalisation

To study virus binding and entry the cells were kept at 4 °C for the 4 h infection period and washed with cold medium to remove unbound virus. The cells were harvested with cold PBS-EDTA to analyse the virus binding or returned at 39 °C to study virus internalisation. The infectious cell-associated virus (CAV) was measured by virus titration.

Extracellular virus (ECV) and cell-associated virus (CAV) titration

For ECV titration, the cell supernatants were collected at time points p.i. indicated in the Results, and clarified at 500×g, 4 °C for 5 min. The supernatants were stored at −20 °C until titration was performed as described above.

For CAV titration, the cells were harvested at the different time points p.i. indicated in the Results, and counted. At each indicated time point, the same amount of cells was resuspended in 500ul of medium, frozen and thawed 3 times in liquid nitrogen, then centrifugated at 3000×g, 4 °C for 30 min. The supernatants were removed from the pellet and titrated. Virus titers were expressed as $TCID_{50}$/1000cells.

Flow cytometry

Cells were detached by Trypsin-EDTA, then fixed and permeabilised using the cell-permeabilisation kit "Fix and Perm" (ADG An Der Grub, Bio research GMBH, Austria). Viral capsid and replicase proteins were detected using the mAbs against ORF2 and ORF1. The antibodies were detected by fluorescein or phycoerithrin-conjugated goat $F(ab')_2$ anti-mouse isotype-specific immunoglobulins (Southern Biotechnology Associates, Bioconcept, Switzerland).

The percentage of dead cells in culture was assessed by uptake of 100 ng/ml of propidium-iodide (PI; Sigma Chemicals, Buchs, CH).

Confocal microscopy

The adherent cell lines and primary cells were seeded on labtek II (Nunc, Roskilde, Denmark) in 0.5 ml of medium. After 24 h, the cells

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were infected for 4 h with PCV2 or UV-inactivated virus at a MOI 10 and 3 $TCID_{50}$/cell or mock treated in a volume of 200 µl per well. After 4 h of incubation, the cells were washed 3 times with 0.5 ml of medium per well.

MoDC were infected at a MOI of 1 $TCID_{50}$/cell for 24 h, washed three times and the cells plated on collagen-coated labtek II. Cells were washed twice in cold PBS, 1% (w/v) BSA, 0.02% (v/v) NaN_3 (wash medium), fixed in 4% (w/v) paraformaldehyde (EM grade) for 10 min at RT and washed twice in cold wash medium.

Virus antigens were stained with mAb against ORF2 or mAb against ORF1 in 0.3% saponin buffer (PBS, 0.3% w/v Saponin — Sigma) for 30 min on ice. Afterwards, the cells were washed twice in 0.1% saponin buffer (PBS 0.1% w/v Saponin) and incubated with isotype-specific Alexa-conjugated anti-mouse IgG, diluted in 0.3% saponin buffer, for 20 min on ice. The cells were washed again and the cell nuclei were stained with 0.2 µM sytox (Invitrogen), diluted in 0.3% saponin buffer, for 15 min on ice. After washing the cells, they were mounted and analysed with a Leica TCS-SL spectral confocal microscope equipped with Leica LCS software (Leica Microsystems AG, Glattbrugg, Switzerland). The confocal images were further analysed by the Imaris software for co-localisation analysis and the GIMP image analysis program version 2.2.

^{3}H-thymidine incorporation assay

Triplicates of 5,000 cells were seeded in 96 well plates for 24 h with or without 100 ng/ml INF-γ at 39 °C, 6% CO_2, then washed and infected or not for 4 h with live PCV2 or mock treated. After a washing step at 4 h p.i., the proliferative activity of the cells was measured by addition of 1 µCi per well of ^{3}H-thymidine (Hartman Analytica, Zürich, CH) for the last 16 h of culture. Results are shown as counts per minute (CPM).

Endocytotic activity of the cells

The different cell types were seeded in 12 well plates for 24 h at 39 °C. Ovalbumin Alexa Fluor 488 (Invitrogen) or DQ-ovalbumin (Invitrogen) was added at a concentration of 2 µg/1 Mio cell. After 4 h of incubation the cells were washed 3 times with 1 ml of medium, detached with Trypsin-EDTA and after additional washing steps fixed with 1% (w/v) paraformaldehyde. The percentage of OVA or DQ-OVA positive cells was analysed by flow cytometry.

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5.2 Dissertation equivalent B

Cellular adaptive immune response against porcine circovirus type 2 in subclinically infected pigs

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Cellular adaptive immune response against porcine circovirus type 2 in subclinically infected pigs

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ABSTRACT

Porcine circovirus type 2 (PCV2), a non-enveloped circular single-stranded DNA virus, is the causative agent of postweaning multisystemic wasting syndrome (PMWS). For the disease to be fully expressed, additional factors such as co-infections or vaccinations are needed. While a lymphopenia involving B cells and the different T lymphocyte subsets has been noted during PMWS development, nothing is known about the PCV2-specific T cell responses developing in the asymptomatic PCV2-infected pigs. Therefore, the response to PCV2 infection was studied in 3-week old SPF piglets infected with PCV2 (single infection) or PCV2 plus porcine parvovirus (PPV) (dual infection). These animals were asymptomatically infected, confirmed by the absence of clinical disease. At 7 d p.i., B lymphocyte (IgG^+) and T lymphocyte ($CD3^+$) numbers decreased in the dual infected but not the single infected piglets. A specific anti-PCV2 antibody (Ab) response was observed at day 28p.i. and day 24 p.i. in the single (PCV2) and the dual (PCV2/PPV) infected groups respectively. An anti-PPV Ab response was also noted in both the dual infected and PPV alone groups from 10 d p.i., which was not influenced by PCV2 infection. Consistent numbers of PCV2-unspecific IFN-γ secreting cells (SC) were detected by ELISPOT at day 7p.i. in animals infected with PPV alone. Interestingly, this response was absent in the PCV2/PPV dual infected animals. In contrast, PCV2-specific IFN-γ SC were observed in the PCV2/PPV infected group at 7 d p.i.; these were also observed in the PCV2 single infected group, but at 21 d p.i. The nature of the cellular response induced by PCV2 was further analysed by an *in vitro* re-stimulation assay, using α-CD4 and α-CD8 Ab to block specific T cell subtypes. Both α-CD4 and α-CD8 Ab reduced the amount of IFN-γ SC induced by PCV2 re-stimulation, suggesting that cells with functionally active CD4 and CD8 molecules are involved in the response *in vivo* against PCV2 infection. This was supported by an observed increase in the numbers of $CD4^+CD8^-CD25^{high}$ and $CD4^+CD8^+CD25^{high}$ T helper cells, as well as the $CD4^-CD8^+CD25^{high}$ cytotoxic T cells after PCV2 *in vitro* re-stimulation. Overall, these results demonstrate that both B and

T lymphocyte responses develop following PCV2 infection, in which both helper and cytotoxic T cell activities are important components. It is therefore important to consider such immunological characteristics when monitoring PCV2-induced diseases, and also when attempting vaccination against PCV2.

KEYWORDS: PCV2, IFN-γ, CD25, CD4, CD8

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a non-enveloped, circular single stranded DNA virus belonging to the Circoviridiae virus family (Todd et al., 2005). PCV2 is the causative agent of postweaning mutlisystemic wasting syndrome (PMWS) in swine (Allan et al., 1999b). The genome of PCV2 is 1759 nucleotides long, making it one of the smallest viruses replicating autonomously in mammalian cells (Mankertz et al., 1997). It contains three known open-reading frames (ORF), encoding the ORF1 Replicase proteins (Mankertz et al., 1998), the ORF2 Capsid protein (Nawagitgul et al., 2000), and an ORF3 protein shown to activate the caspase 3 and caspase 8 pathways inducing cellular apoptosis *in vitro* (Liu et al., 2005). PCV2 was first isolated from tissues of PMWS diseased pigs in Canada (Ellis et al., 1998), in the US and in Europe (Allan et al., 1998) in 1998. Disease has been reproduced by viral co-infection of colostrum-deprived or gnotobiotic piglets with PCV2 and porcine parvovirus (PPV) (Allan et al., 1999a; Ellis et al., 1999). Particular vaccine adjuvant administration has also been shown to assist development of PMWS disease after experimental PCV2 infection (Grasland et al., 2005; Krakowka et al., 2001). Nevertheless, PCV2 is clearly the causative agent of PMWS (Ladekjaer-Mikkelsen et al., 2002), but the full expression of disease requires additional factors such as co-infections or vaccinations. It is assumed that viral infections or the administration of adjuvant activate PCV2 infected cells to divide (Krakowka et al., 2001) and therefore enable PCV2 to start its own replicative cycle that is dependent on the host DNA polymerase (Tischer et al., 1987) .

On a farm, PCV2-induced diseases are increasing the pig mortality rate from 2-3% to 14-30%. PMWS diseased animals are most often in the age range of 8-12 weeks old. They display clinical symptoms of wasting, diarrhoea, jaundice, respiratory distress and enlarged lymph nodes (Allan and Ellis, 2000). Typical histological findings are also reported in lymph nodes: The follicular architecture is lost, lymphocytes are depleted, histiocytes and

multinucleated giant cells infiltrate the lymph nodes, and basophilic inclusion bodies are detected in the histiocytes (Segales et al., 2004). In addition, interstitial pneumonia, mononuclear inflammatory infiltration in the liver, lymphoplasmacytic colitis and peri-endarteritis are observed (Opriessnig et al., 2006; Rosell et al., 1999).

The induction of anti-PCV2 neutralizing Ab was shown to correlate with protection from disease (Meerts et al., 2006). While important, Ab are effective at targeting extracellular virus and cell surface antigen (Ag) only. PCV2 has been shown to infect epithelial, endothelial and monocytic cells *in vivo* (Jensen et al., 2006; Opriessnig et al., 2006; Rosell et al., 1999; Yu et al., 2007), confirmed by *in vitro* observations on primary cells (Gilpin et al., 2003; Steiner et al., 2008; Vincent et al., 2003). Accordingly, PCV2 infected cells also have to be removed; considering that PCV2 capsid protein will not be expressed at the surface of infected cells for attack by Ab, the infected cells have to be eradicated by other mechanisms. In this context, natural killer cells and the adaptive cellular immune defences are most effective at eradicating virus-infected cells. To date, no studies have analysed the characteristics of PCV2 specific T cell responses. This is particularly important for asymptomatic animals, to increase our understanding of immune defences in animals which resist disease development. Current knowledge comes primarily from the report on the decrease of naïve $CD4^+CD8^-$ T helper (Th) cells, memory/effector $CD4^+CD8^+$ Th cells and $CD4^-CD8^+$ cytotoxic T cells (CTLs) in diseased animals compared to healthy animals (Nielsen et al., 2003). Interleukin 10 (IL-10) and interferon gamma (IFN-γ) has also been noted following *in vitro* PCV2 re-stimulation (Darwich et al., 2003), demonstrating the existence of both regulatory and stimulatory pathways, which is not too surprising, but does not elaborate on the development of T lymphocyte responses in asymptomatic animals. Therefore, the present study was conducted to analyse and characterise the PCV2 specific T cell response developing during experimental PCV2 and PCV2/PPV infection.

MATERIALS AND METHODS

Virus

Porcine circovirus type 2 (PCV2) pool 1452/3 and porcine parvovirus (PPV) pool 1005 were kindly provided by Francis McNeilly, QUB, Belfast. Virus titres were determined on the PCV2/PCV1-negative porcine PK15-A kidney cell line (also kindly provided by Francis McNeilly). Titres of $10^{4.75}$ 50% tissue culture infectivity dose ($TCID_{50}$)/ml and $10^{6.0}$ $TCID_{50}$/0.5ml were determined for the PCV2 and the PPV inocula respectively. In the ELISPOT assay, a lysate from PK15-A cells infected with the PCV2 strain Stoon 1010 (Ellis et al., 1998) (also kindly provided by Francis McNeilly) was used .

Experimental infection and clinical monitoring

Twenty 3-week old SPF piglets were randomly divided into four groups that were kept apart in separate isolated rooms of the institute BSL4 containment facility. The piglets received PCV2 (group 1), PPV (group 2), PCV2 + PPV (group 3), or Dulbecco's phosphate buffered saline (Invitrogen) (PBS) (group 4), administered intranasally on three consecutive days. At day 0, the piglets of the groups 1 and 3 were infected with PCV2 at a titre of $10^{4.65}$ $TCID_{50}$/animal; piglets of groups 2 and 3 were infected with PPV at a titre of $10^{6.2}$ $TCID_{50}$/animal. The piglets of the PBS control group (group 4) received corresponding volumes of PBS. On day 1 and 2 post infection (p.i.), additional dosed of PCV2 and PPV were applied, at titres corresponding to $10^{4.35}$ $TCID_{50}$/animal and $10^{5.9}$ $TCID_{50}$/animal respectively.

Body temperature and clinical symptoms were recorded daily, and the body weight every second to third day.

Blood sampling

Blood was collected at day 0, 7 days (d), 10d, 14d, 17d, 21d, 24d, 28d and 35d p.i., and diluted in Alsever's anticoagulant solution. By staining of whole blood with Tuerk's solution (Dr. Grogg Chemie Ag, Bern, Switzerland), the total leukocyte numbers were calculated. Plasma samples were collected after a centrifugation step at 1,000x g for 25 min and stored at –70°C. Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat fraction of blood by density centrifugation over Ficoll-Paque (1.077 g/liter) as described previously (McCullough et al., 1993). For long-term storage under liquid nitrogen, the PBMC were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 50% (v/v) FBS (all from Invitrogen) and 10% (v/v) DMSO.

Anti-PPV and anti-PCV2 antibody ELISAs

Anti-PPV antibodies (Ab) were detected using a PPV competitive antibody ELISA kit Svanovir (10-7400-02; Svanova Biotech, Uppsala, Sweden). The percent inhibition (PI) values were calculated with the given formula: PI = 100-(MEAN OD samples/MEAN OD negative control), where OD = optical density as 450nm. Positive plasma samples showed a PI value greater than 50 and samples exceeding a PI value of 80 were considered as strongly positive.

Anti-PCV2 specific Ab were detected with an in-house established indirect anti-PCV2 Ab ELISA. Briefly, Nunc MaxiSorb^{TM} plates were coated with 1μg/ml of PCV2 ORF2 virus-like particles (VLPs) (kindly provided by Dr. C. Andreoni, Immunology, Discovery Research, Merial, Lyon, France) in 100 μl PBS overnight (O/N). The plates were blocked with PBS/1% (w/v) dried skimmed milk for 1h at 37°C, and washed once with PBS/0.05%(v/v) Tween-20 (washing buffer). The plasma samples were added in dilutions from 1:50 to 1:800 in PBS/1% (w/v) bovine serum albumin (BSA, bovuminar cohn fraction V, Intergen Company Serologicals) for 1 hour (h) at room temperature (RT). The plates were washed five times and

horseradish peroxidase-conjugated polyclonal anti-swine IgG F(ab')$_2$ fragments (Jackson ImmunoResearch), diluted in PBS, 0.05% (v/v) Tween-20, 1% (w/v) dried skimmed milk, 1% (v/v) rabbit serum (Sigma) was added. After 1h incubation at 37°C and five washing steps, the substrate O-Phenylenediamine (OPD) (Sigma: P-8787) plus 0.0001% (v/v) H_2O_2 was added for 40 minutes, and the absorbance measured at 450nm. Samples were considered as positive if the optical density (OD_{450nm}) values equalled or exceeded 0.1 at dilutions higher than 1:100. Samples from the PBS control animals were always below OD_{450nm} 0.1.

PCV2 TaqMan real time PCR

Viral DNA was extracted from the plasma samples on a TECAN Freedom EVO robot (Tecan, Männedorf, Switzerland) using the NucleoSpin 96 Virus extraction kit (Macherey-Nagel, Oensingen, Switzerland). Samples were tested in triplicates, and PCV2 DNA excised from a plasmid expressing this DNA (kindly provided by Dr. Brian Meehan, QUB, Belfast, UK) used as positive control.

The PCV2 TaqMan real time PCR was designed in-house by Dr. Martin Hoffman (IVI), based on a previously described methodology (Olvera et al., 2004). Briefly, the Cap gene region was used for primer and probe design. For the reaction, 2ul of the collected plasma samples were mixed with 0.5µl of 20µM PCV2 forward and PCV2 reverse primer, 0.5µl of 5µM PCV2 probe (labelled with FAM^{TM} and TAMRA^{TM}), and TaqMan Universal Master Mix (amplitaq gold DNA polymerase, dNTP's, $MgCl_2$, ROX and buffers). Milli-Q water was added to a final volume of 25ul. The amplification reaction was carried out at 95°C for 10min, followed by 50 cycles at 95°C for 15 seconds (s), 60°C for 30s and 72°c for 30s.

Restimulation of PCV2 specific cells

For *ex vivo* IFN-γ ELISPOT assays, freshly isolated PBMC were resuspended in DMEM, 10% (v/v) FBS, 1x Penicillin/Streptomycin, 20µM 2-mercaptoethanol (2-ME) (all from

181 Invitrogen). 200µl of 2.5x10^6 PBMC/ml were seeded in 96-well multiscreen plates, and the
182 cells restimulated with PCV2 virus lysate of the strain Stoon 1010 (Ellis et al., 1998) or with
183 mock lysate, diluted to give an MOI of 1 $TCID_{50}$/cell (or equivalent dilution for the mock) for
184 24h.
185 Thawed PBMCs were also employed for the IFN-γ ELISPOT. These were seeded at a
186 concentration of 3 x 10^6 cells/ml in 24 well plates, and restimulated with PCV2 lysate or
187 mock lysate at an MOI of 0.1 $TCID_{50}$/cell (or equivalent dilution) for 5 days. The cells were
188 harvested, counted, and stained for flow cytometry analysis or re-plated on 96-well
189 mutliscreen plates at a concentration of 1.25 x 10^6cells/ml for IFN-γ ELISPOT analysis.
190 When indicated in the results, 50U/ml of the recombinant porcine cytokine IL-2 (R&D
191 systems) were added to the cultures.
192 In order to block the CD4 or CD8 dependent IFN-γ secretion (by T cells expressing
193 functionally active CD4 or CD8), 50µl of anti-CD4 Ab (74-12-4), anti-CD8 Ab (11/295/33),
194 or control anti-CD1 Ab (76-7-4) were incubated with the cells for 1h at 37°C, prior to PCV2
195 restimulation (Balmelli et al., 2005).
196
197 *IFN-γ ELISPOT assay*
198 The ELISPOT was performed with minor modifications as described previously (Laval et al.,
199 2002). Briefly, 96-well multiscreen plates (Millipore MAIPS4510) were coated with 0.5
200 µg/ml of mouse anti-porcine IFN-γ mAb (P2G10, BD bioscience, Allschwil, Switzerland)
201 diluted in PBS at 4°C O/N. The plates were washed twice with 200µl of cold DMEM, 10%
202 (v/v) FBS and blocked for 2h at 37°C. PBMC were seeded at the above mentioned
203 concentrations into the plates, and incubated at 39°C. Reference positive control cells were
204 activated with 10µg/ml of the mitogen ConA (Amersham Pharma Biotech, Uppsala, Sweden).
205 After 24h, the medium was aspirated and the cells lysed by addition of cold distilled water for

5 minutes. After washing three times with PBS, 0.5% (v/v) Tween-20 (washing buffer), the plates were incubated with 0.17μg/ml biotinylated anti-porcine IFN-γ Ab (P2C11, BD Biosciences, Allschwil, Switzerland) in PBS at 4°C O/N. The plates were washed five times in washing buffer, and Streptavdin HRP (DakoCyomation, Dako AG, Baar, Switzerland) diluted in PBS added for 1h at 37°C. After an additional washing step, the plates were revealed with SIGMAFAST^{TM} 3,3`Diaminobenzidine tablets (Sigma, Buchs, Switzerland) dissolved in distilled water. After 20min incubation at RT in the dark and drying of the plates, the number of spots was determined with a computer-assisted video image analyzer (ELISPOT reader, AID, GmbH, Strassberg, Germany).

Antibodies

For phenotyping of cells, the following Ab were used: fluorescein (FITC) conjugated goat anti swine IgG (H+L)(Jackson ImmunoResearch, Milan Analytica, Magden, Switzerland); mouse anti-swine CD8 beta chain (PG164A, Veterinary Medical Research & Development, Inc., Pullman, WA); mouse anti-swine IFN gamma (IFN-γ) (P2G10 BD biosciences, Allschwil, Switzerland); anti-swine workshop cluster 3a (SWC3a; 74-12-15A); mouse anti-swine CD4 (74-12-4); mouse anti-swine CD8 (11/295/33); mouse anti-swine CD25 (K231.3B2); mouse anti-swine CD3a (PTT3/FyH2). The latter five monoclonal Ab (mAb) were produced from hybridomas kindly provided by Dr. Armin Saalmüller, University of Veterinary Medicine, Vienna, Austria. Anti-Capsid protein ORF2 mAb 7G5-G4-A1 (IgG2a) was kindly provided by Dr. Allan Gordon QUB, Belfast.

Flow cytometry

The PBMC were stained with anti-CD3a and anti-IgG (H+L) Ab to analyse the relative levels of T- and B-lymphocytes respectively. The absolute numbers of lymphocytes was calculated relative to the total leukocyte numbers. The PBMC were stained with anti-SWC3a, anti-CD4

and after a fixation and permeabilisation step (Fix and Perm kit, ADG An Der Grub, Bio research GMBH, Austria) with anti-Capsid Ab to analyse the presence of Capsid antigen in the monocytic cells.

PCV2 restimulated PBMCs were stained with anti-CD8 or anti-CD8β Ab, together with anti-CD4 and anti-CD25 Ab, to analyse the percentage of the different T cell subsets expressing high levels of CD25. For intracellular IFN-γ detection, PBMC were treated with 2μg/ml of Brefeldin A (Sigma, Buchs, Switzerland) 4h prior to staining with anti-SWC3a, anti-CD8 and anti-IFN-γ Ab. The cells were fixed and permeabilised with the Fix and Perm kit (ADG An Der Grub, Bio research GMBH, Austria) before staining for the intracellular cytokine IFN-γ. Antibodies were detected by fluorescein, phycoerythrin or biotin-conjugated goat $F(ab')_2$ anti-mouse isotype specific immunoglobulins (Southern Biotechnology Associates, Bioconcept, Switzerland). Streptavidin RPE-Cy5 (DakoCyomation, Dako AG, Baar, Switzerland) was used to develop the biotinylated conjugates.

Statistical analysis

The statistical significance of the differences between groups was analysed with SigmaStat version 3.0, employing ANOVA on ranks and t-tests.

RESULTS

Clinical monitoring and daily weight gain

The 3-week old SPF piglets were inoculated intranasally with PBS (control group), PCV2, PPV or PCV2/PPV as described in Materials and Methods. Body temperature, body weight and the general condition were monitored from day 0 (before infection) to day 35 pi. The PPV single and PCV2/PPV dual infected animals displayed enlarged inguinal and popliteal lymph nodes from day 10 to day 17 p.i., indicating that the presence of the PPV infection related to these symptoms. None of the animals displayed fever during the 35 days of experiment (Fig. 1A). The rectal temperature in the PCV2 single, PPV single and PCV2/PPV dual infected animals was 0.5-0.7 degrees below the average levels of the PBS group until day 13 p.i. (Fig. 1A). This was considered to be influenced by differences in food management until day 13 p.i., because the PBS control animals had remained in an SPF unit while the infected animals were all in the containment facility. The ad libitum food management can also explain the higher average daily weight gain in the PBS group (Fig. 1B). Overall, the average daily weight gain did not significantly differ among the remaining groups, and reached an average of 0.41, 0.42 and 0.44 kg/day in the PCV2/PPV dual, PCV2 single and PPV single infected animal groups respectively (Fig. 1B).

PCV2/PPV dual infected animals display a transient decrease in leukocyte and lymphocyte counts

The total leukocytes were counted for individual animals in each group, and the group values compared statistically. A significant decrease in the leukocyte levels was detected in the PCV2 single and PCV2/PPV dual infected groups between days 7 and 10 p.i. (42.55 and 46.3 $x10^6$ cells/ml respectively) compared with the PBS controls. On the contrary, the PPV group showed the highest levels of leukocytes at this time (78.95 $x10^6$cell/ml) (data not shown).

In order to analyse the leukocytes in more detail, and to relate to previous publications, the B- and T-lymphocyte numbers were determined by staining for surface IgG and CD3a as described in Materials and Methods. Consistent with the observed decrease in the leukocyte numbers, the B cell counts declined significantly in the PCV2/PPV dual infected group at day 7 p.i. (Fig 2A). However, this was not observed with the PCV2 single infected group. Interestingly, a significant rise in B cell levels was noted subsequent to this point of lower counts, in the PCV2/PPV dual and PPV single infected groups, at day 14 p.i. (Fig. 2A); this related to an overall increase in leukocyte counts for the PPV single infected animals (data not shown). The higher B cell numbers persisted in the PPV infected animals until day 35, whereas the B cell numbers of the animals in the PCV2/PPV dual infected group had significantly decreased (Fig. 2A).

Concerning the T lymphocyte numbers, a significant decrease was also observed at day 7 p.i. in the PCV2/PPV dual infected group, but not in the other groups. While an increase was noted at day 14 p.i., this was statistically significant from the PBS control group only for the PPV single infected group (Fig. 2B). Again similar to the B cell counts, the T cell numbers significantly decreased at day 35 p.i. in the PCV2/PPV dual infected animals (Fig. 2B), but remained at a similar elevated level to the PBS controls for the PCV2 and PPV single infected groups.

Overall, the data shows a decrease in leukocyte and lymphocyte numbers in the PCV2/PPV dual infected animals, even though the animals were clinically asymptomatic. Moreover, although there was a "recovery" of the leukocyte numbers thereafter, this did not continue to increase with the PCV2/PPV dual infected animals in the same fashion as in the PBS control group and other infected groups. This was particularly the case for the T lymphocytes.

Analysis of anti- PCV2 antibodies and PCV2 viraemia in the blood of infected, asymptomatic piglets

When assayed for anti-PCV2 specific IgG, the PBS control group values were always below background level. The plasma samples from the PPV single infected animals also remained below background levels. Seroconversion against PCV2 was clearly observed at day 24 p.i. in the PCV2/PPV dual infected group, and 4 days later in the PCV2 single infected group (Fig.3A). At day 28 p.i., all the PCV2 infected animals had seroconverted against PCV2; the Ab titres increased to comparable levels at day 35 p.i. in both groups (Fig.3A).

The presence of PCV2 DNA in the piglet plasma was analysed by TaqMan real time PCR to seek any evidence of PCV2 viraemia. Samples from the PBS control and the PPV single infected animals all tested negative for the presence of PCV2 DNA (data not shown). In contrast, PCV2 DNA was detected in plasma samples from four of the PCV2 single and five of the PCV2/PPV dual infected animals at day 7 p.i. (data not shown). Interestingly, this apparent viraemia was transient; all other time points tested gave negative results (data not shown). Further analysis of PCV2 viraemia was attempted in terms of the presence of viral antigen in PBMC. Intracellular staining for the viral Capsid (Cap) protein by flow cytometry failed to detect antigen; the Cap protein was never detected in $SWC3^+$ monocytes or $CD4^+$ lymphocytes (data not shown).

PPV specific antibodies are present from day 10 p.i. in the PPV infected animals

The presence of anti-PPV Ab was analysed by a commercial competitive Ab ELISA. At day 10 p.i., all PCV2/PPV dual and PPV single infected animals had seroconverted against PPV (Fig. 3B). The PI values reached 84% and 89% respectively (Fig. 3B), and increased consistently to 94% and 95% at day 21 p.i., remaining high during the remainder of the observation period of 35 days (Fig. 3B). While the animals of the PBS group were always negative for anti-PPV Ab, the animals of the PCV2 single infected group seroconverted at day 28 p.i. against PPV (Fig. 3B). This can be explained by the fact that the PCV2 single infected animals were handled by the same persons in the same building as the PPV infected animals,

336 even though the animals were in separate rooms with separate ventilation; in contrast, the
337 PBS control group was housed and handled separately in an SPF unit. Clearly, this shows the
338 highly contagious nature of PPV.
339
340 *PBMC of PCV2 infected animals respond to PCV2 re-stimulation by IFN-γ secretion*
341 An in-house IFN-γ ELISPOT assay for *ex-vivo* PCV2 re-stimulation of PBMC, described in
342 Materials and Methods, was employed to measure the amount of PCV2 specific IFN-γ SC
343 arising following infection. Firstly, PBMC were freshly isolated from two PCV2 immune
344 adult pigs, to determine the levels and range of IFN-γ SC which could be expected. The cells
345 were re-stimulated with increasing amounts of PCV2 or mock for 24h. A clear PCV2-specific
346 response was observed in terms of the numbers of IFN-γ SC/10^6 cells; this increased in a
347 dose-dependent manner when the re-stimulation employed PCV2 (Fig. 4A).
348 In order to analyse the time-dependent appearance of IFN-γ SC following PCV2 infection, the
349 above assay was employed for the direct *ex vivo* determination of IFN-γ SC using freshly
350 isolated PBMC from the PBS control, PCV2 single, PPV single or PCV2/PPV dual infected
351 piglets. The number of IFN-γ SC/10^6 cells at day 0 remained at background levels for all
352 groups (Fig. 4B). In three of the five PCV2/PPV dual infected animals, PCV2 specific IFN-γ
353 SC were detected at day 7 p.i. (Fig. 4C). This was clearly PCV2 specific, because re-
354 stimulation with mock did not lead to IFN-γ secretion. In contrast, the number of IFN-γ SC in
355 the PCV2 single infected animals was similar to the background levels obtained with a mock
356 antigen stimulation. Related to this, both the PCV2 and mock antigens similarly re-stimulated
357 PBMC from four of the five PPV single infected animals, seen as an increase in IFN-γ SC at
358 day 7 p.i. (Fig. 4C). Although this observed response was the highest for all the groups, it
359 was not PCV2-specific; moreover, the level of activation did not relate to that obtained with
360 cells from the PCV2/PPV dual infected group (Fig. 4C). By day 21 p.i., PBMC from both

PCV2/PPV dual and PCV2 single infected groups gave a PCV2-specific IFN-γ SC response upon *in vitro* re-stimulation (Fig. 4D). In both groups, four out of five animals responded to the virus re-stimulation, giving IFN-γ SC numbers similar to or slightly higher than the numbers obtained for the PCV2/PPV dual infected group PBMC from day 7 p.i. The PBMC from the PPV single infected group again responded to both PCV2 and mock antigens in a similar manner, but now the response was lower than that obtained with the PBMC from the PCV2 infected (single and dual) stimulated with PCV2.

Overall, it can be seen that the PCV2/PPV dual and the PCV2 single infected groups did generate PCV2-specific lymphocytes, in terms of IFN-γ SC. These were clearly identifiable at 7 and 21 days p.i., respectively. There was variation in the number of IFN-γ SC/10^6 cells, both between the groups and between animals, ranging from 4-16 IFN-γ SC/10^6 cells between individual piglets responding in a positive and PCV2-sepcific manner.

Confirmation of the PCV2-specific IFN-γ response using in vitro expanded frozen PBMC

Further analyses on the T cells involved in the IFN-γ response required the use of PBMC that had been stored under liquid nitrogen. Therefore, cells from the PCV2-immune adult animals shown in Fig.4A were again employed, but after storage in liquid nitrogen. It has been published that recall viral antigen-induced proliferation of porcine $CD8^+$ T cells is enhanced by addition of the recombinant human cytokine IL-2 (Zuckermann and Husmann, 1996). Accordingly, 50U/ml of rpoIL-2 was added to cultures using PBMC which had been stored under liquid nitrogen, to enhance the amount of IFN-γ SC (data not shown). Next, the IFN-γ SC levels following virus re-stimulation were determined for freshly isolated compared with thawed cells that had been stored under liquid nitrogen. In the example shown in Fig. 5, when PBMC directly after isolation from a PCV2-immune animal were re-stimulated for 24h with PCV2, an average of 88 IFN-γ SC/10^6 cells were detectable. If the same procedure were

performed with thawed cells that had been stored under liquid nitrogen, only background levels were obtained (Fig. 5). In contrast, when the thawed PBMC were expanded in the presence of the virus plus rpoIL-2, and average of 20 IFN-γ SC/10^6 cells and 40.5 IFN-γ SC/10^6 cells were detectable after 3 days and 5 days respectively (Fig. 5). These results demonstrated that when PBMC had been stored under liquid nitrogen, there was a clear need to expand the cells in the presence of IL-2, for the detection of the PCV2-specific IFN-γ SC response. Accordingly, the subsequent analyses employing thawed PBMC used an expansion period of 5 days in the presence of 50U/ml rpoIL-2.

$CD4^+$ and $CD8^+$ cells are responsible for the IFN-γ secretion upon PCV2 recall stimulation

In order to characterize the nature of the IFN-γ SC detected by the above *ex vivo* analyses, the functionality of the CD4 and CD8 co-receptor on Th and Tc lymphocytes respectively was blocked by the addition of anti-CD4 and anti-CD8 Ab to the cultures; this employed the established method of Balmelli et al. (Balmelli et al., 2005). A control Ab targeting CD1 was also employed; this did not decrease the number of IFN-γ SC induced by the PCV2 stimulation (Fig. 6A). In contrast, both the CD4 and CD8 Ab impaired the development of IFN-γ SC following PCV2 re-stimulation *in vitro* (Fig. 6A). The results were demonstrating that both $CD4^+$ and $CD8^+$ cells are involved during the response against PCV2, at least with respect to the IFN-γ response.

Phenotype of the cells activated by PCV2 re-stimulation

Considering the above results on the inhibitory effect of anti-CD4 and anti-CD8 Ab, the PBMC from the PCV2-immune animals were analysed further with respect to their phenotype. By staining for intracellular IFN-γ after PCV2 re-stimulation, both $CD8^+SWC3^-$ and $CD8^-SWC3^-$ cells were found to be expressing the IFN-γ (Fig. 6B). When considered in

411 terms of the groups response, the differences between PCV2 re-stimulated and mock antigen
412 re-stimulated cells were not statistically significant (Fig. 6B). This was due to certain animals
413 responding specifically to the PCV2 antigen stimulation, while others gave a similar response
414 to that induced by the mock antigen. This is not surprising, because the number of antigen-
415 specific T cells in the circulation of a host varies considerably with time, and can actually
416 become undetectable depending on the time elapsed and the individual involved (Barnard et
417 al., 2005; Blanco et al., 2000; Piersma et al., 2006). Nevertheless, it is clear that when PCV2-
418 specific cells are present in the PBMC, they are of both the $CD8^+$ and $CD8^-$ phenotype,
419 relating to the ability of both anti-CD4 and anti-CD8 Ab impairment of the PCV2-specific
420 IFN-γ response. Interestingly, the animals which gave the highest numbers of $CD8^+$IFN-γ^+
421 cells in their PBMC also showed the highest numbers of $CD8^-$IFN-γ^+ cells (Fig. 6B).
422 In addition to producing IFN-γ, it is reported that activated T lymphocytes up-regulate the IL-
423 2 receptor α chain upon antigen stimulation (Charerntantanakul and Roth, 2006).
424 Accordingly, the T lymphocyte subsets being activated by PCV2 were further analysed by *in*
425 *vitro* re-stimulation of the PBMC followed by simultaneous staining for CD4, CD8 and the
426 IL-2 receptor α chain CD25 expressions. When considered at the group level, the PCV2 re-
427 stimulation induced an upregulation of CD25 on a mean of 1.1% of the $CD4^-CD8^+$ cell
428 population (Fig. 6C, "bar"). In contrast, the $CD4^+$ cells appeared to be more responsive,
429 particularly the $CD4^+CD8^-$ population in which 8.3% of the cells showed upregulated CD25
430 expression; the $CD4^+CD8^+$ cell population showed 0.9% of cells with upregulated CD25
431 expression (Fig. 6C, "bar"). As with the IFN-γ response, when analysed at this group level,
432 the differences between the numbers of PCV2-stimulated cells and mock-stimulated cells
433 were not statistically significant. Again, this was seen to be due to certain individuals not
434 showing a difference between the PCV2 and mock stimulations. Nevertheless, PBMC from
435 particular individual animals were responding specifically to the PCV2 (Fig. 6C, individual

436 symbols). Interestingly, these were the same animals that provided PBMC responding to
437 PCV2-stimulation in terms of IFN-γ production.
438 The results in Fig. 6C are implying that cells responding more specifically to the PCV2 can be
439 found in the $CD4^{-}CD8^{+}$ population, although one cannot rule out an accessory role for the
440 $CD4^{+}$ cells. Accordingly, the PBMC from the different PCV2-infected animals were
441 compared in terms of CD25 expression on $CD8\alpha^{+}$ and $CD8\beta^{+}$ cells, the latter to highlight the
442 responsiveness of the cytotoxic T cells. The analyses focused on PBMC that did not show a
443 clear CD25 upregulation following PCV2 stimulation in Fig. 6C. An example of the
444 comparative CD8α and CD8β labellings is shown in Fig. 6D. Therein, the $CD8\alpha^{+}$ cells
445 responded similarly to PCV2 and mock stimulations with respect to $CD25^{hi}$ expression,
446 whether the cells were $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{+}$. In contrast, $CD8\beta^{+}$ cells (these were only
447 $CD4^{-}CD8\beta^{+}$) showed a clear PCV2-specificity in their CD25 upregulation. Moreover, the
448 CD4+ cells (that is $CD4^{+}CD8\beta^{-}$) also showed a PCV2-specificity in their CD25 upregulation.
449 These results are demonstrating that a significant up-regulation of CD25 expression in
450 response to PCV2-stimulation does occur on $CD8^{+}$ cells, but most particularly the $CD4^{-}$
451 $CD8\beta^{+}$ population, contrasting with the $CD4^{-}CD8\alpha^{+}$ cells (Fig. 6D).
452 Considering these results showing PCV2-stimulation of the $CD8\beta^{+}$ cells, it was of interest to
453 determine if such responsiveness required live PCV2 capable of replication. This has
454 implications for vaccination of animals, and also considers the characteristics of PCV2
455 infection in immune cells (Vincent et al., 2003). Accordingly, PBMC from the PCV2 infected
456 animals were stimulated *in vitro* with baculovirus-derived VLPs of PCV2, in comparison with
457 stimulation by a control calicivirus VLP. The results, looking at the % $CD25^{hi}$ cells in the
458 different T lymphocyte population gates (Fig. 6E), showed that the VLPs would induce CD25
459 upregulation in all the T cell groups: $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$ and $CD4^{+}CD8^{-}$. The most

460 significant increases in the $CD25^{hi}$ cells was noted in the $CD4^{-}CD8^{+}$ cytotoxic and
461 $CD4^{+}CD8^{-}$ helper T cell populations.
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DISCUSSION

Although PCV2 will cause the development of disease such as PMWS, not all PCV2-infected animals develop disease symptoms (Ladekjaer-Mikkelsen et al., 2002; Okuda et al., 2003). Considering the reports showing that PCV2 infection leads to modulation of T lymphocyte activity (Darwich et al., 2003; Kekarainen et al., 2008; Nielsen et al., 2003), the present work sought to analyse how the T lymphocyte response was developing in asymptomatic animals following infection with PCV2. This is particularly pertinent considering the reports on T lymphocytes in sow colostrum (Bandrick et al., 2008; Le Jan, 1994; Tuboly et al., 1988), and the reported transfer of antigen-specific T-cell immunity to piglets, either by transplacental passage or via the colostrum (Bandrick et al., 2008). The study was also designed to relate observations on the T lymphocytes to the development of specific anti-PCV2 humoral immunity, considering the reported relationship between anti-PCV2 neutralising Ab and protection against disease (Meerts et al., 2006).

Accordingly, samples were employed from an experimental infection by PCV2 alone or in combination with PPV, in which none of the virus-inoculated animals developed PMWS. This is not an unknown scenario, because other reports using such a model of PCV2/PPV co-infection in SPF piglets also failed to reproduce disease symptoms (Ostanello et al., 2005). Moreover, our own experimental data is in agreement with these authors in that the development of subclinical PCV2 infection was confirmed by detection of PCV2-specific antibodies and PCV2 DNA in the blood. No differences in the levels of the PCV2 Ab titres were observed in the PCV2/PPV dual infected animals compared to the PCV2 single infected animals. In contrast, there were differences between the groups in terms of the kinetics of the Ab response. Detectable anti-PCV2 Ab were found in the blood from the PCV2/PPV dual infected animals 4 days earlier than with the PCV2 single infected animals. Whether such a short time delay has any significance would require further experimentation. Nevertheless, it

512 is clear that all PCV2-infected animals were able to generate anti-PCV2 Ab within 3 to 4
513 weeks after infection. This may well be an important characteristic; the anti-PCV2 Ab
514 response appears to be delayed, particularly when one compares the kinetics of this response
515 with the more rapid kinetics of the anti-PPV response. If the 4-day delay in the anti-PCV2 Ab
516 response between the PCV2/PPV and the PCV2 alone groups is significant, this may be due
517 to the concomitant PPV infection activating the immune system to respond more rapidly
518 against the PCV2. Alternatively, the presence of the PPV infection may influence the
519 replication characteristics of the PCV2 infection, perhaps by increasing the number of
520 dividing cells required by PCV2 for its replication. This seems to be unlikely considering that
521 the two viruses would have to be in close proximity. Indeed, the PCV2/PPV dual and PCV2
522 single infected animals displayed equal levels of PCV2 DNA in the blood.
523 If the PPV were having an influence, it is more likely to be on the capacity of the immune
524 system to respond in terms of the processing and presentation capacity of PCV2 antigen. On
525 the contrary, the PCV2 infection did not apparently influence the humoral response against
526 PPV: The titres and kinetics of the anti-PPV Ab response did not differ between the PPV
527 single and PCV2/PPV dual infected groups.
528 Further analysis of the immune defence development following PCV2 infection turned to the
529 T lymphocytes, due to the aforementioned characteristic of the influence of PCV2 on T
530 lymphocyte numbers and activities. A decrease in $CD4^+CD8^-$ naïve Th cells, $CD4^+CD8^+$
531 memory/effector Th cells, and $CD4^-CD8^+$ CTLs – as well as the $CD21^+$ B cells – was reported
532 for PCV2 diseased animals compared to healthy animals (Nielsen et al., 2003). In our own
533 experiment, a significant decrease in $CD3^+$ T cells and IgG^+ B cells was observed in the
534 PCV2/PPV dual infected animals. Nevertheless, this was only a transient decrease, followed
535 by a significant rise in the B and T cell numbers compared to the PCV2 single infected group.
536 Moreover, the animals did not develop PMWS.

537 It has been reported that PBMC from PCV2-infected, clinically symptomatic animals display
538 elevated levels of IFN-γ mRNA and secrete IFN-γ upon *in vitro* recall antigen stimulation,
539 compared with cells from infected, healthy animals (Darwich et al., 2003). However, it is
540 difficult to interpret these results, because the capacity of the PBMC to produce IFN-γ
541 secretion was tested only with PCV2 restimulation; no mock antigen was included to
542 ascertain that the IFN-γ induction was PCV2-specific. Our own results have shown that
543 porcine PBMC can show a relatively strong IFN-γ response to mock antigen. Indeed, cells
544 other than antigen-specific T lymphocytes – NK cells, γ/δ T cells, or macrophages – will
545 respond in an antigen non-specific manner to cellular components in the PCV2 preparation,
546 producing IFN-γ (Pintaric et al., 2008). It is essential that PCV2 stimulations be performed in
547 comparison with mock antigen stimulations.
548 When cytokine secretion is only measured by ELISA, as in the report of Darwich et al
549 (Darwich et al., 2003), this can be misleading: It has been shown that the levels of secreted
550 cytokine do not correlate with the amount of IFN-γ SC (Diaz and Mateu, 2005). For this
551 reason, the present work focused on the measurement of IFN-γ SC, with the aim determining
552 the levels of the PCV2-specific IFN-γ secreting cells. The analyses employed both PCV2-
553 stimulation and mock antigen-stimulation, to ascertain if the numbers of detectable cytokine
554 secreting cells were indeed virus-specific. PCV2 specific IFN-γ SC were detected at an earlier
555 time point – 7d p.i. – in the PCV2/PPV dual infected animals than in the PCV2 single
556 infected animals. All PCV2-infected animals did eventually respond to PCV2 recall antigen
557 stimulation with IFN-γ secretion, by 21 days post-infection. Similar kinetics upon
558 experimental viral infection of pigs have been reported, with a peak of IFN-γ SC at day 7
559 followed by relatively stable IFN-γ SC levels (Souza et al., 2007). In comparison with
560 antigen-specific responses against other viruses, such as porcine reproductive and respiratory
561 syndrome virus (49 IFN-γ SC /10^6 cells) (Diaz et al., 2005), the levels of PCV2-specific IFN-γ

562 SC was lower (4-16 IFN-γ SC/10^6 cells). This may be indicating that PCV2 is not a good T
563 cell immunogen. Certainly, PCV2 alone does not activate professional antigen presenting
564 cells or lymphocytes *in vitro* (Vincent et al., 2003) . However, the levels of IFN-γ SC may be
565 reflecting the kinetics of the response against PCV2. The duration of the animal experiment
566 did not exceed 5 weeks, and when PBMC from the other PCV2-immune animals were re-
567 stimulated at 12 weeks after infection, 88 IFN-γ SC/10^6 cells were obtained.
568 Early after infection, an aspecific IFN-γ response was observed in the PPV single infected
569 animals: Similar levels of IFN-γ SC were induced by *in vitro* re-stimulation with the negative
570 mock control antigen and PCV2 antigen. On this basis, it was assumed that the detected
571 response had been induced by the PPV *in vivo*. Interestingly, this response was absent in the
572 PCV2/PPV dual infected animals, indicating that the PCV2 infection did indeed have an
573 immunomodulatory effect, interfering with what appeared to be the PPV-dependent IFN-γ
574 secretion in the dual infected animals. Although the nature of this PPV-dependent response
575 was not characterized further – in terms of being PPV-specific, or due to NK cells, γ/δ T cells
576 or macrophages – it has been reported that PPV infected pigs develop cytotoxic and
577 lymphoproliferative activity detectable by recall antigen stimulation late during infection,
578 around 80-100 days p.i. (Ladekjaer-Mikkelsen and Nielsen, 2002). In our own study, PBMC
579 from the PPV single and PCV2/PPV dual infected animals were re-stimulated with PPV
580 antigen *in vitro*. Only one out of the 10 PPV infected animals provided PBMC capable of
581 generating IFN-γ SC in a PPV-specific manner, from day 21 p.i. (data not shown). It is
582 considered that NK cells or antigen non-specific γ/δ T cells may well be the sources of the
583 IFN-γ detected in PBMC cultures from the PPV infected animals. The observation that this
584 response was only identifiable with cells from the PPV single, and not the PCV2/PPV dual
585 infected animals, does show that the PCV2 infection had an influence. This relates to a recent
586 publication showing that *in vitro* PCV2-infected PBMC displayed an impaired IFN-γ response

upon recall stimulation with pseudorabies virus (Kekarainen et al., 2008). The reported effect was apparently mediated by IL-10, which can influence antigen-specific and antigen non-specific responses, as mediated by NK cells and γ/δ T cells.

Concerning the anti-PCV2 T lymphocyte response, this was found by blocking experiments to be a CD4- and CD8-dependent T cell response. Considering that the blocking antibodies would interfere with functional CD4 and CD8, the results were showing that both helper and cytotoxic T cells were involved. This was supported by experiments wherein an increase in IFN-γ secreting $CD8^+$ cells was observed. Moreover, an up-regulation of the IL-2 receptor α chain (CD25) was detected on $CD4^+CD8^-$ naïve Th lymphocytes, $CD4^+CD8^+$ memory/effector Th lymphocytes, and $CD4^-CD8^+$ CTLs upon recall antigen stimulation. The relatively low percentages of IFN-γ^+ and $CD25^{hi}$ T cells in the cultures is not surprising, considering that similar values have been reported with classical swine fever virus infections (Suradhat et al., 2005). Moreover, one has to consider that PCV2 infection of PBMC can lead to the production of the immunosuppressive cytokine IL-10 (Kekarainen et al., 2008). There is also the possibility that a number of the induced $CD25^{hi}$ cells are porcine regulatory T cells (Treg), which have been identified in the $CD4^+CD8^-CD25^{hi}$ and $CD4^+CD8^+CD25^{hi}$ T cell populations (Kaser et al., 2008). It would be interesting to analyse the levels of Treg in the PBMC of PCV2 immune animals, but our attempts to detect the Treg marker Foxp3 have so far provided poor results (data not shown).

In conclusion, asymptomatic PCV2 infected animals develop antigen-specific T cell responses involving both CD4 and CD8 T lymphocyte populations. These cells were confirmed as being PCV2-specific through comparison with stimulations using mock antigen. The responsive cells are to be found in both the helper and cytotoxic T cell populations, but the kinetics of their responses are dependent on the individual animal and the time after infection at which the cells were tested. Using CD8β as a marker for CTLs increased the clarity of detecting the PCV2-specific T cells, in terms of both $CD4^-CD8\beta^+$ cells and

613 $CD4^+CD8\beta^-$ cells. Interestingly, VLPs based on PCV2 Cap protein were also capable of
614 restimulating $CD4^-CD8^+$ CTLs, as well as $CD4^+CD8^-$ naïve Th cells and $CD4^+CD8^+$
615 memory/effector Th cells. Such results suggest that the PCV2-specific CTLs were capable of
616 recognising antigen processed from VLPs as well as from live virus. This has implications for
617 vaccination of animals, particularly sows, considering the observation of both specific
618 antibodies and T lymphocytes in the colostrum (Bandrick et al., 2008; Le Jan, 1994; Tuboly et
619 al., 1988). If such T cells were to include CTLs, which is possible for an anti-PCV2
620 vaccination based on the ability of the non-viable VLPs to induce CTL reposes *in vitro*, one
621 has the potential for the transfer of both humoral and cytotoxic T cell immunity to piglets. It
622 will be interesting to follow this development of anti-PCV2 cytotoxic immunity, particularly
623 now that the application of CD8β staining, together with $CD25^{hi}$ monitoring and analysis of
624 IFN-γ secreting cells offers the potential for detecting such T cell activity.
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FIGURE LEGENDS

Figure 1: Body temperature and average daily weight gain of animals infected with PCV2 and/or PPV. (A) Body temperature. The rectal body temperature was measured for all animals at daily intervals. Mean values of the groups are shown. (B) Weight gain. The average daily weight gain (kg) is shown for all animals within a group (filled and open symbols); group mean values are represented by the bar symbol.

Figure 2: B- and T-lymphocyte counts. (A) B lymphocyte counts. Isolated PBMC were stained with anti-IgG H+L Ab to analyse the B cell numbers. The absolute B cell counts were calculated and expressed as $x10^3$ cells/ml, for time points indicated on the x-axis. Individual animals are represented by the open and filled symbols, with the mean values for the groups shown by the continuous line. (B) T lymphocyte counts. The PBMC were stained for CD3a expression, to analyse the T lymphocyte counts. These counts are given as $x10^3$ cells/ml for the individual animals (open and filled symbols), with the group mean values shown as the continuous line. *$P<0.05$.

Figure 3: Anti-PCV2 and anti-PPV Ab titres. (A) Anti-PCV2 IgG Ab titres were measured by an indirect ELISA. The $\log_{10}$ of the reciprocal end-point dilution is given for the time points indicated on the x-axis. Mean values for the groups is shown by the histograms, +/- SD. (B) Anti-PPV specific antibodies were measured with a commercial competitive Ab ELISA, at time points indicated on the x-axis. The values are given as percent inhibition (PI), calculated as described in Materials and Methods. Means of the groups, +/- SD, are shown.

Figure 4: IFN-γ Secreting Cells after *ex vivo* PCV2 re-stimulation. (A) PBMC from two adult PCV2-immune animals were used as positive controls for the ability of the test to detect

the IFN-γ SC, and as reference for the samples from the infected piglets. The PBMC were re-stimulated for 24h with PCV2 infected cell lysate, or mock cell lysate as negative control, at increasing MOI based on the titre of the PCV2 (TCID50/cell, x-axis). The IFN-γ SC were measured by ELISPOT assay as described in Materials and Methods, and expressed per 10^6 cells. (B-D) PBMC were isolated from the piglets which had received PBS (control), PCV2 single infection, PCV2/PPV double infection and PPV single infection. The cells were re-stimulated with PCV2 or mock antigen for 24h, and the IFN-γ SC detected by ELISPOT assay. The mean values of the groups, +/-SD, are shown for PBMC isolated before infection (0 days) (B), 7d p.i. (C) and 21d p.i. (D). The IFN-γ SC are calculated per 10^6 cells.

Figure 5: Comparison of the PCV2 re-stimulation profile for freshly isolated compared to frozen and *in vitro* expanded PBMC. PBMC were re-stimulated with PCV2 or mock antigen-treated directly after isolation ("PBMC fresh; 24h") or were expanded from frozen (under liquid nitrogen) cells in the presence of rpoIL-2 and virus for 24h ("PBMC thawed; 24h"), 3 days ("PBMC thawed; 3days") and 5 days ("PBMC thawed; 5days"). Means of triplicates +/- SD of two experiments are shown.

Figure 6: Dependency of the anti-PCV2 response on $CD4^+$ and $CD8^+$ T cell responses. (A) Influence of CD4 and CD8 expression on IFN-γ SC. The CD4 and CD8 T cell receptors on PBMC from PCV2-immune animals were blocked by specific mAb, or treated with control anti-CD1 mAb, for 1h prior to PCV2 or mock antigen re-stimulation for 5 days. The IFN-γ SC were measured by ELSPOT assay, and calculated per 10^6 cells. Mean values of triplicates of one representative experiment +/- SD are shown. (B) IFN-γ synthesis relative to CD8 expression. PBMC from the PCV2-infected piglets (3 months after infection) were re-stimulated with PCV2 or mock antigen for 5 days prior to staining for the presence of

intracellular IFN-γ. The cells were also labelled for the surface markers CD8 and SWC3a. Values represent the percentage of positive cells in the gated lymphocytes, shown on the x-axis, under the line as "= gate". Open symbols represent the individual animals, while the bar shows the mean value for each cell phenotype gate. (C) $CD25^{hi}$ expression relative to CD4 and CD8 expression. T cells from the PBMC of the PCV2 piglets (3 months after infection), activated as under "B", were analysed by staining for the IL-2 receptor α chain CD25 in combination with CD4 and CD8. The percentage of CD25 expressing cells is given for the cells in the lymphocyte gate. Open symbols represent the individual animals, while the bar shows the mean value for each cell phenotype gate. (D) $CD25^{hi}$ expression on $CD8\alpha^{+}$ and $CD8\beta^{+}$ cells following re-stimulations with PCV2 or mock antigen as described under "B". Mean values of triplicates of one representative experiment +/- SD are shown. (E) $CD25^{hi}$ expression relative to CD4 and CD8 expression following re-stimulations with 3μg/ml VLPs of recombinant PCV2 ORF2 antigen compared with a control calicivirus VLP. Otherwise, the stimulations were as described under "B". Mean values of triplicates of one representative experiment +/- SD are shown.

Figure 1

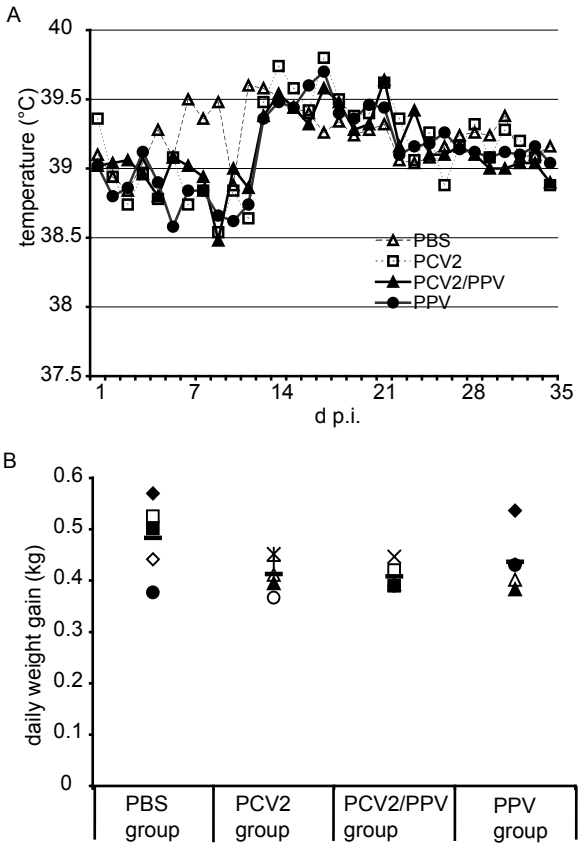

A
40
39.5
39
38.5
38
37.5
temperature (°C)
1
7
14
21
28
35
d p.i.
PBS
PCV2
PCV2/PPV
PPV
B
0.6
0.5
0.4
0.3
0.2
0.1
0
daily weight gain (kg)
PBS group
PCV2 group
PCV2/PPV group
PPV group

Figure 2

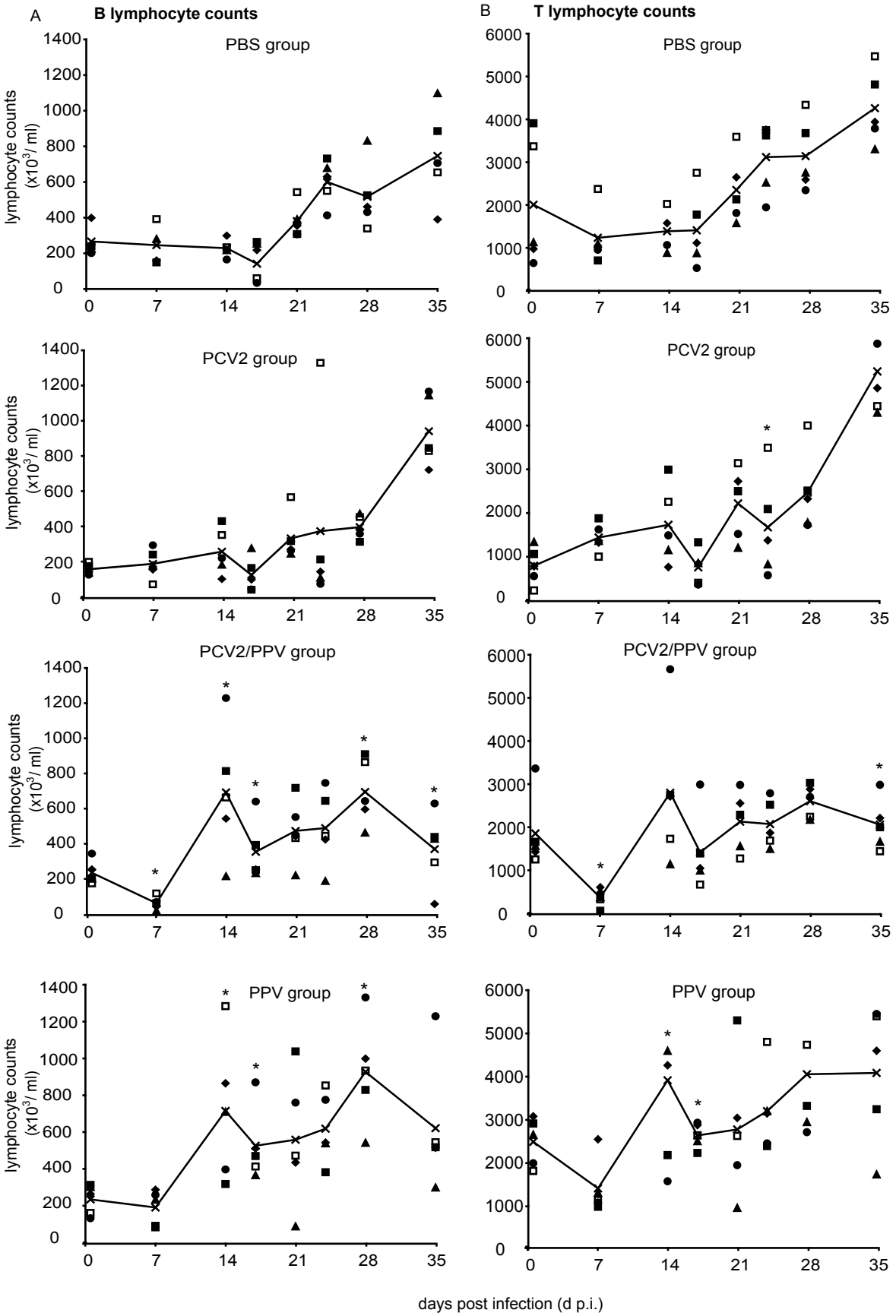

A
B lymphocyte counts
B
T lymphocyte counts
PBS group
PCV2 group
PCV2/PPV group
PPV group
lymphocyte counts (x10³/ ml)
days post infection (d p.i.)

Figure 3

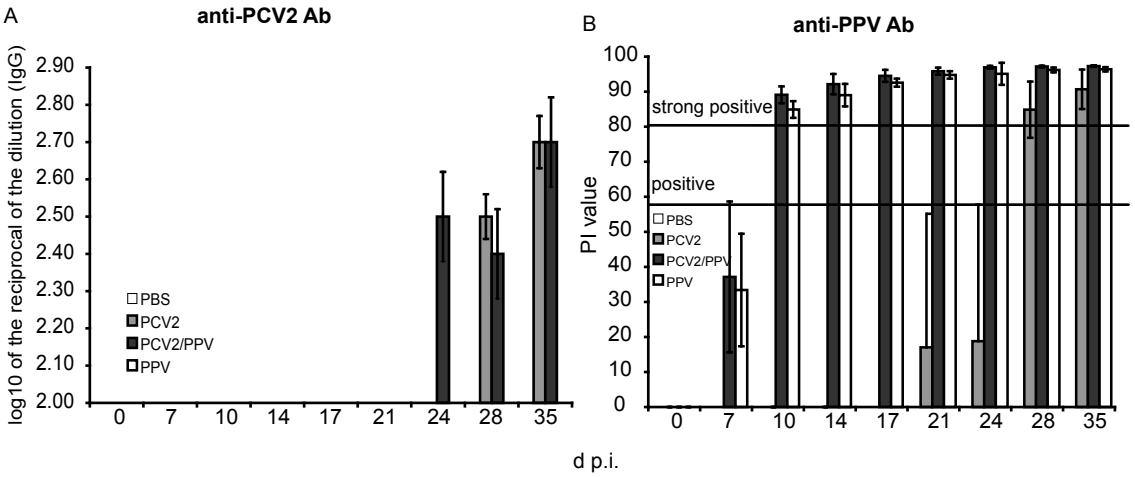

A
anti-PCV2 Ab
log10 of the reciprocal of the dilution (IgG)
2.90
2.80
2.70
2.60
2.50
2.40
2.30
2.20
2.10
2.00
PBS
PCV2
PCV2/PPV
PPV
0
7
10
14
17
21
24
28
35
B
anti-PPV Ab
PI value
100
90
80
70
60
50
40
30
20
10
0
strong positive
positive
PBS
PCV2
PCV2/PPV
PPV
0
7
10
14
17
21
24
28
35
d p.i.

Figure 4

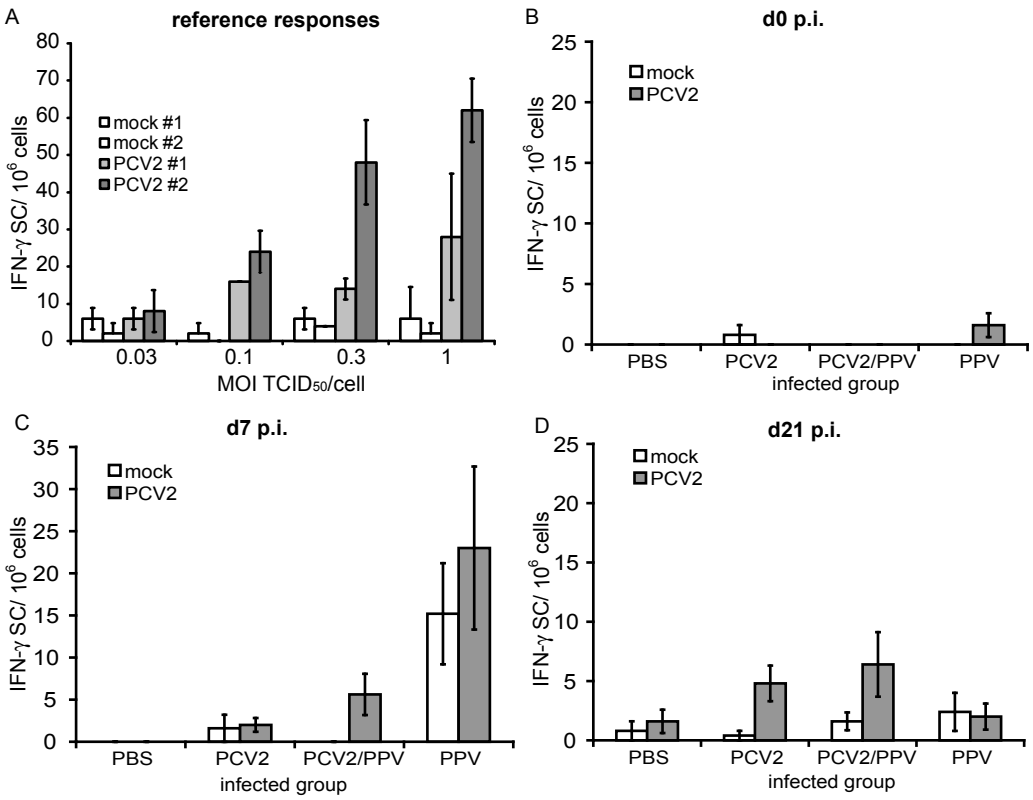

A
reference responses
IFN-γ SC/ 10^6 cells
mock #1
mock #2
PCV2 #1
PCV2 #2
0.03
0.1
0.3
1
MOI TCID50/cell
B
d0 p.i.
mock
PCV2
PBS
PCV2
PCV2/PPV
PPV
infected group
C
d7 p.i.
D
d21 p.i.

Figure 5

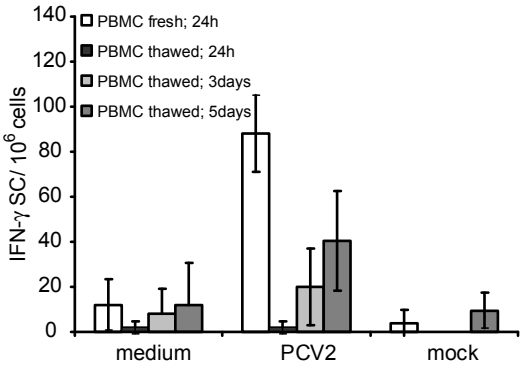
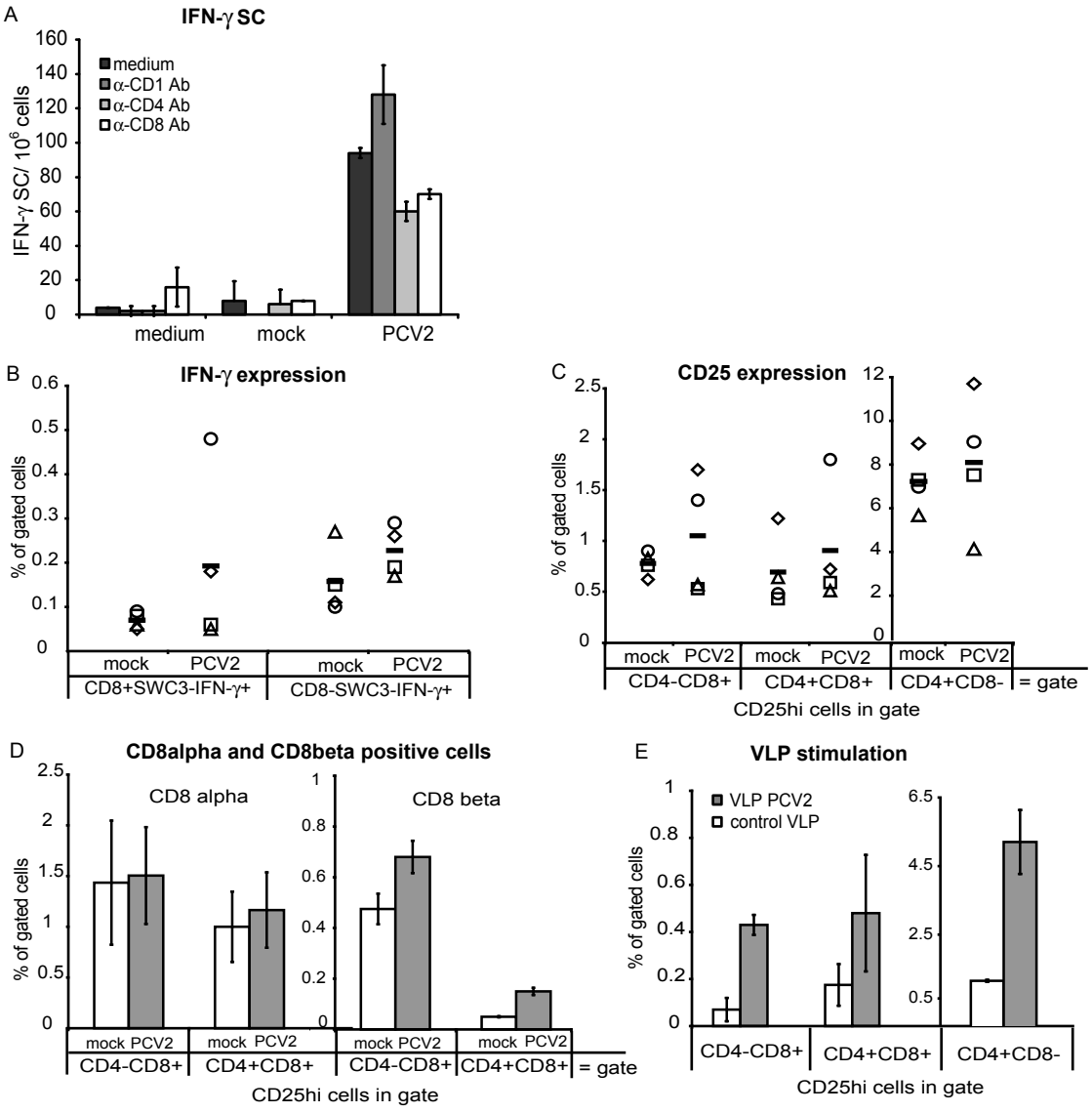

PBMC fresh; 24h
PBMC thawed; 24h
PBMC thawed; 3days
PBMC thawed; 5days
IFN-γ SC/ 10^6 cells
140
120
100
80
60
40
20
0
medium
PCV2
mock

Figure 6


A
IFN-γ SC
IFN-γ SC/ 10^6 cells
medium
α-CD1 Ab
α-CD4 Ab
α-CD8 Ab
medium
mock
PCV2
B
IFN-γ expression
% of gated cells
mock PCV2 mock PCV2
CD8+SWC3-IFN-γ+
CD8-SWC3-IFN-γ+
C
CD25 expression
% of gated cells
mock PCV2 mock PCV2 mock PCV2
CD4-CD8+ CD4+CD8+ CD4+CD8- = gate
CD25hi cells in gate
D
CD8alpha and CD8beta positive cells
CD8 alpha
CD8 beta
% of gated cells
mock PCV2 mock PCV2 mock PCV2 mock PCV2
CD4-CD8+ CD4+CD8+ CD4-CD8+ CD4+CD8+ = gate
CD25hi cells in gate
E
VLP stimulation
VLP PCV2
control VLP
% of gated cells
CD4-CD8+ CD4+CD8+ CD4+CD8-
CD25hi cells in gate

6 Discussion

The first aim of the work was to identify PCV2 permissive cells and characterize virus replication. PCV2 viral antigen and DNA have already been detected in what appear to be endothelial cells, epithelial cells and monocytic cells of several organs *in vivo*. Therefore, a porcine endothelial cell line, primary porcine aortic endothelial cells, primary porcine gut epithelial cells and porcine cells of monocytic origin such as fibrocytes and monocyte derived dendritic cells (MoDC) were included in our studies. We could show that PCV2 was able to bind and enter cells of different origins, relating to published data that PCV2 can interact with glycosaminoglycan structures carrying heparan sulphate, which are expressed by many cell types. In all cells except MoDC, PCV2 replication was detectable. This was characterized in terms of an increase in the levels of viral Capsid and Replicase proteins, together with the production and release of infectious virus progeny. Differences in replication efficiency between the cells could not be explained by differences in virus binding or entry. Moreover, although PCV2 was dependent on dividing cells for replication, the cellular mitotic activity did not correlate directly with the virus replication efficiency. In contrast to other ssDNA viruses, PCV2 did not influence the cellular growth characteristics. Furthermore, we could show viral proteins associating with cell nucleus components during mitosis. This relates to the assumption that the cell nucleus is the replication site of PCV2. Accordingly, the first part of this thesis work can conclude that a broad cell targeting of PCV2 offers an explanation for its widespread tissue distribution *in vivo.*

Secondly, we focussed on the characterization of the cellular adaptive immune response towards PCV2, using subclinically infected piglets, which were shown previously to develop slightly elevated blood T lymphocyte levels. None of the PCV2 infected animals displayed disease symptoms, but PCV2 infection was confirmed by virus specific seroconversion and a transient PCV2 viraemia in the blood. Peripheral blood mononuclear cells (PBMC) of PCV2 infected animals responded to PCV2 re-stimulation by interferon gamma (IFN-γ) secretion from day 7 post-infection. Both $CD4^+$ and $CD8^+$ cells were involved in the production of this cytokine. The cytokine was seen to be associated with $CD8^+$ cells, and restimulation assays identified CD8β cells as being particularly sensitive to the PCV2 antigen in terms of $CD25^{hi}$ responses. Interestingly, virus-like particles were also capable of inducing

$CD8^+CD25^{hi}$ cells during *in vitro* restimulation assay, implying that live virus was not necessary for the lymphocyte activation. Such results raise the question of cross-presentation being involved in induction of CTL immunity against PCV2, at the same time providing evidence that inactivated vaccines may have the potential to induce cytotoxic as well as humoral immunity. Certainly, the work from this second part of the thesis indicates that both CD4 and CD8 T cells are activated during PCV2 infection, and are likely to be involved in mediating immunity against the virus.

7 Outlook/Perspectives

We could show that requirements for PCV2 replication are virus binding, virus entry and cellular mitotic activity. However, these characteristics do not offer an explanation for observed differences in PCV2 replication efficiency between the cells. Therefore, we speculate that other cellular mechanisms or factors are affected during PCV2 infection/replication. With the PCV2-related Geminiviruses (Niagro et al., 1998), it was shown that the viral Replicase protein interacts with plant and human retinoblastoma protein, which may up-regulate S-phase functions required for viral replication (Gutierrez, 1999; Xie et al., 1995). It would be interesting to analyse the interaction of the PCV2 viral proteins with such described cellular factors. Furthermore, we observed a close association of the PCV2 Capsid and Replicase proteins with the chromatin of diving cells. This might explain how PCV2 spreads between daughter cells without cellular release. Therefore, it would be of interest to analyse the interaction of PCV2 proteins with cellular structures such as the microtubules forming the mitotic spindle.

With respect to the immune defences against PCV2, we could demonstrate that following PCV2 infection $CD4^+$ and $CD8^+$ T lymphocytes become activated in terms of IFN-γ secretion. Our characterization of the T cell response with respect to time after infection, and the identification of the T cell subtype mediating the response, was performed with cells from PCV2 infected, but healthy animals. Therefore, it would be interesting to compare the T cell response using the same assays between diseased and asymptomatic PCV2 infected animals. Recently, Tregs have been identified in the porcine $CD4^+CD8^+CD25^+$ and $CD4^+CD8^-CD25^+$ cell populations by detection of the marker Foxp3 (Kaser et al., 2008). Considering our observation of an up-regulation in the $CD25^{hi}$ expressing $CD4^+CD8^-$ and $CD4^+CD8^+$ cells, it would

be interesting to analyse the presence of Tregs in the PBMC of PCV2-infected animals. Our preliminary attempts were unsuccessful, due to difficulties with the Foxp3 reagent. Certainly, increased levels of Tregs could offer an explanation for the immunosuppressive state observed in PCV2 infected animals, and the increased susceptibility to secondary infections.

Besides the presented work in this thesis, I was involved in the other main effort of our group analysing the influence of viral DNA on cellular functions. We could show that the PCV2 DNA inhibited cytokine secretion by activated plasmacytoid dendritic cells, when the cells were stimulated by either TLR-ligands or other viruses. Accordingly, we are currently analysing which form of the viral DNA – replicative or non-replicative form – mediates the observed inhibitory function. Furthermore, studies will focus on the identification of the cellular interaction partner of PCV2 DNA.

8 Curriculum vitae/list of publications

Publications

2008 **Esther Steiner,** Carole Balmelli, Heidi Gerber, Artur Summerfield, Kenneth McCullough:
Cellular adaptive immune response against porcine circovirus type 2 in subclinically infected pigs
submitted

2008 **Esther Steiner**, Carole Balmelli, Brigitte Herrmann, Artur Summerfield, Kenneth McCulllough
Porcine circovirus type 2 displays pluripotency in cell targeting
Virology, accepted

2007 Carole Balmelli, Marco P. Alves, **Esther Steiner,** Daniel Zingg, Nadja Peduto, Nicolas Ruggli, Heidi Gerber, Artur Summerfield, Kenneth McCullough:
Fibrocytes responsiveness to toll like receptor danger signals
Immunobiology; 212 (9-10); 693-9

Research experience

2005-2008 **PhD in immunology at the Institute of Virology and Immunoprophylaxis in Mittelhaeusern, Switzerland**

- project: “interaction of porcine circovirus type 2 with the host immune sytem”

- main topics: identify target cells of virus replication; define cellular adaptive immune response towards PCV2

- methods: isolation of peripheral blood mononuclear cells and magnetic cell sorting; isolation and culture of dendritic cells and NIPC, isolation of primary porcine endothelial cells and gut epithelial cells; virus replication kinetic studies; flow cytometry; confocal microscopy; antibody and cytokine ELISAs; ELISPOT assay; proliferation assay;

Meetings

March 2008 **meeting of the swiss immunology PhD students, Schloss Wolfsberg, Switzerland**
- Abstract title: Interaction of porcine circovirus type 2 with the host adaptive immune system

March 2008 **Gesellschaft für Virologie, annual meeting, Heidelberg, Germany**
- Abstract title: Porcine circovirus type 2 displays pluripotent cell targeting

March 2007 **meeting of the swiss immunology PhD students, Schloss Wolfsberg, Switzerland**
- Abstract title: Porcine circovirus type 2 displays pluripotent cell targeting

September 2006 **9th international conference on dendritic cells, Edinburgh, UK**
- Abstract title: Dendritic cells carry circovirus for prolonged periods leading to dysfunction of danger recognition

March 2006 **EU meeting: control of porcine circovirus diseases, Prague, CZ**
- Work package 4: virus replication and molecular pathogenesis factors

Practical courses Graduate School Bern (PhD Program)

10.-14.07.2006 course in handling laboratory animals, LTK module 1 in Zürich

19.-23.06.2006 vascular cell biology course: “isolation and culture of primary endothelial and epithelial cells, tight junctions versus adherens junctions, analysis of vascular development in transgenic mice”

23.-27.01.2006 course in molecular biological methods: ”Northern blot, RT-PCR, PCR, Southern blot, Western blot, cDNA cloning, sequencing, gene transfer into eukaryotic cells”

21.-25.11.2005 cell migration course: “cell adhesion, transmigration and chemotactic assay, in vitro flow assay, Stamper-Woodruff frozen section-adhesion assay”

10.-14.10.2005 course in biomicroscopy at the ISREC in Lausanne (Switzerland): “laser scanning and spinning disk confocal microscopy, structurated illumination – ApoTome”

Education

2005-2008 Graduate School for Cellular and Biomedical Sciences at the University of Bern (Switzerland): main topics: immunology and cell biology

2004 final examination in veterinary medicine at the University of Bern

1999-2004 University education in veterinary medicine at the University of Bern

Languages

Swiss German	Mother tongue	
German	Fluent	
English	Verbal: very good	Written: very good
French	Verbal: good	Written: good
Italian	Basics	

PC-knowledge

1992-2008

Operating Systems
MS Dos / Windows 3.11, 95, 98, 2000, Me, XP, Vista
Windows NT 3.1, NT 4.0

Programs
WinWord, Excel, Power Point, Access, Cellquest Pro, Flow Jo GIMP, SigmaStat, Reference manager, EndNote; Leica LCS, Adobe Illustrator, Adobe Photoshop;

E-mail Systems
Netscape Mail, Outlook, Outlook Express

Intercultural competences

05.2007-06.2007	working in the immunology department at the Veterinary University Copenhagen, Denmark
10.2004-11.2004	Externship at the SF/SPCA animal hospital in San Francisco, US

Networks

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Member of Swiss Veterinary Society

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Reference persons

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Bern, 26.06.08

9 Declaration of Originality

Last name, first name: **Steiner Esther**

Matriculation number: 99-120-891

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the “Statut der Universität Bern (Universitätsstatut; UniSt)”, Art. 20, of 17 December 1997.

Bern,08.08.2008……