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# Interference of porcine circovirus type 2 ORF2 immunogenicity by ORF1 and ORF3 mixed DNA immunizations in mice

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ABSTRACT

Little is known about the influences of other porcine circovirus type 2 (PCV2) proteins on the immunogenicity of Cap protein. Here we constructed plasmids expressing the ORF1 (pORF1) and ORF3 (pORF3) of PCV2, and mixed either of them with the plasmid expressing ORF2 (pORF2) as combined DNA vaccines, to compare their immunogenicity and protective efficacy. Our data revealed that pORF1 reduced the Cap-specific $CD8^+$cell frequency, and both pORF1 and pORF3 attenuated the Cap-specific Th1 and post-challenge-recall VN antibody responses induced by the pORF2 plasmid, despite successful induction of Rep and ORF3 antibodies by pORF1 and pORF3, respectively. Subsequently, protocols with pORF1 or pORF3 showed significantly decreased protective efficacy compared to pORF2 alone. Overall, our data suggested that the ORF1- and ORF3-encoded Rep and ORF3 proteins may interfere with the cellular, humoral and protective immunity of the ORF2-encoded Cap protein *in vivo*.



## Introduction

Porcine circovirus (PCV) is a nonenveloped, single-stranded, circular DNA virus with a diameter of 17 nm (Tischer et al., 1982). PCV was first discovered as a noncytopathic contaminant of the porcine kidney cell culture PK-15 (Tischer et al., 1974). The PK-15-derived PCV, designated PCV1, did not produce clinical disease in experimentally inoculated pigs and was considered to be nonpathogenic (Allan et al., 1995; Tischer et al., 1986). In contrast, PCV2 was identified as the primary etiological agent of an emerging disease in 1991, named postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998; Allan and Ellis, 2000; Clark, 1997). PMWS is considered an important swine disease and has had a serious economic impact on the global swine industry. This disease affects pigs from 5 to 12 weeks of age, with 5–30% morbidity. Clinical signs of the disease include progressive weight loss, difficult breathing, dyspnea, and jaundice (Clark, 1997). In addition to PMWS, PCV2 is also associated with pneumonia, enteritis, reproductive failure, porcine dermatitis and nephropathy syndrome (PDNS) and a variety of other manifestations (Opriessnig et al., 2007).

The complete genomic sequences of PCV1 and PCV2 have been determined (Hamel et al., 1998; Meehan et al., 1997, 1998; Zhou et al., 2006). The overall DNA sequence homology within PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68 to 76%. PCV2 possesses 3 confirmed ORFs: ORF1 located on the viral plus-strand, ORF2 and ORF3 on the counter-clockwise strand, with lengths of 945, 702 and 315 nts, respectively. PCV2 ORF1 encodes a 35.7 kDa replication protein (Rep) involved in virus replication (Mankertz et al., 1998). PCV2 ORF2 encodes a 27.8 kDa capsid protein (Cap) involved in viral immunogenicity (Mahe et al., 2000; Nawagitgul et al., 2000; Truong et al., 2001). PCV2 ORF3 protein is not essential for PCV2 replication, but involved in PCV2-induced apoptosis (Liu et al., 2005).

As the primary immunorelevant protein, PCV2 Cap protein expressed in insect cells (Nawagitgul et al., 2000) or *Escherichia coli* (Zhou et al., 2005a) could be detected by sera of pigs experimentally infected with PCV2. In addition, multiple immunoreactive regions (Mahe et al., 2000) and epitopes (Lekcharoensuk et al., 2004; Shang et al., 2009) have been identified. Monoclonal antibodies against Cap protein show neutralizing activity against PCV2 (McNeilly et al., 2001; Zhou et al., 2005a), suggesting the protein contains at least 1 neutralizing epitope on the virus. In contrast to Cap, limited information is available on the immunogenicity of Rep and ORF3 proteins. Utilizing PEPSCAN analysis, one immunoreactive area (aa 185–211) was identified in PCV2 Rep protein (Mahe et al., 2000). In another study, 2 immunoreactive T lymphocyte epitopes in Rep protein (aa 81–100 and aa 201–220) and 1 in ORF3 protein (aa 31–50) were demonstrated (Stevenson et al., 2007).

PCV2 Cap protein has been studied intensely as vaccine antigen due to its excellent immunogenicity (Blanchard et al., 2003; Fan et al.,

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2008; Fenaux et al., 2003; Kamstrup et al., 2004; Song et al., 2007; Wang et al., 2006). By using ORF2-based DNA and subunit vaccines in mice, we previously demonstrated that Cap-specific $CD8^+$ T cells and virus-neutralizing (VN) antibody correlating mainly with IgG2a play crucial roles in protective immunity against PCV2 (Shen et al., 2008). In contrast, Rep and ORF3 proteins are generally considered weakly immunogenic and seldom used as vaccine antigens. At this time, their immunorelavent characteristics *in vivo* remain unclear. Since Rep or ORF3 antigens alone may be insufficient to provide adequate immunity against PCV2, combining them with Cap would be an alternative approach to examine their effects on viral immunogenicity. Based on this hypothesis, using a mixed DNA vaccine strategy, we investigated the influences of ORF1 and ORF3 plasmids on the immunogenicity of the ORF2 plasmid. Our results suggest that ORF1 or ORF3 DNA vaccines reduce the immunogenic properties and efficacy of ORF2 DNA vaccines. Our data help elucidate the effect PCV2 Rep and ORF3 proteins play in the onset of immunity in the host.

## Results

### *In vitro expression of mammalian expression vector*

Recombinant plasmids expressing PCV2 ORF1 and ORF3 were constructed for use as DNA vaccines. Plasmids were confirmed by PCR, restriction enzyme digestion, and DNA sequencing. *In vitro* expression of protein was analyzed by transient transfection followed by immunoperoxidase monolayer assay (IPMA). When detected with swine PCV2-positive serum, strong signals in pORF1-transfected cells but lacking in pORF3-transfected cells were observed (Fig. 1). However, both of the expressed proteins, localized in nuclei of transfected cells, reacted strongly with the Rep or ORF3 antisera respectively (Fig. 1). As controls, signals were not observed in cells transfected with pCI-neo vector (Fig. 1). The results indicate that the recombinant vectors, pORF1 and pORF3, may express the Rep and ORF3 proteins respectively *in vivo*.

**Table 1**
PCV2 Cap-specific lymphoproliferative response and FCM analysis of mice splenocytes.

| Group | SI | Immunophenotypic cells | |
|---|---|---|---|
| | | $CD4^+$ (%) | $CD8^+$ (%) |
| pORF2 + pCI | $1.54 \pm 0.32^a$ | $15.80 \pm 0.67^a$ | $14.94 \pm 2.56^a$ |
| pORF2 + pORF1 | $1.38 \pm 0.13^a$ | $14.00 \pm 2.87^a$ | $11.58 \pm 2.41^b$ |
| pORF2 + pORF3 | $1.36 \pm 0.20^a$ | $13.07 \pm 1.75^a$ | $12.08 \pm 1.83^{a,b}$ |
| DNA control | $1.00 \pm 0.00^b$ | $9.40 \pm 3.80^b$ | $11.63 \pm 2.17^b$ |

[a, b]Different superscripts within columns represent significant differences between groups ($P<0.05$).

### *Cap-specific cellular immune responses*

The Cap-specific lymphoproliferative responses, $CD4^+$ and $CD8^+$ cell frequencies of vaccinated mice were determined and compared at 8 weeks post the first immunization (p.i.). As shown in Table 1, splenocytes from all the vaccine groups showed proliferative responses when compared to the control mice, with greater statistical difference in pORF2 + pCI group ($P<0.01$) than the pORF2 + pORF1 ($P<0.05$) and pORF2 + pORF3 ($P<0.05$) groups. For flow cytometric analysis, compared with the control group, significantly higher proportions of $CD4^+$ cells were observed in pORF2 + pCI ($P<0.01$), pORF2 + pORF1 ($P<0.01$) and pORF2 + pORF3 ($P<0.05$) groups; however, in the case of $CD8^+$ cells, only the pORF2 + pCI group had a significantly higher frequency than the control ($P<0.05$) (Table 1). Comparing among the vaccine groups, the $CD8^+$ cells in $pORF2^+$ pORF1 group was significantly lower than that in $pORF2^+$ pCI group ($P<0.05$), suggesting a suppressive effect of pORF1 plasmid to the Cap-specific $CD8^+$ cell frequency induced by the pORF2 plasmid.

### *Total IgG antibody response to PCV2 Cap protein*

Total IgG antibody titers against Cap protein were compared among groups. Plasmids pORF2 + pCI elicited the highest antibody response at 4–16 weeks p.i., with the peak IgG titer of $11.2 \pm 1.3$ log2 at 12 weeks (Fig. 2A). As for the co-administration groups, the combined use of pORF1 and pORF3 induced peak titers of $5.6 \pm 3.8$

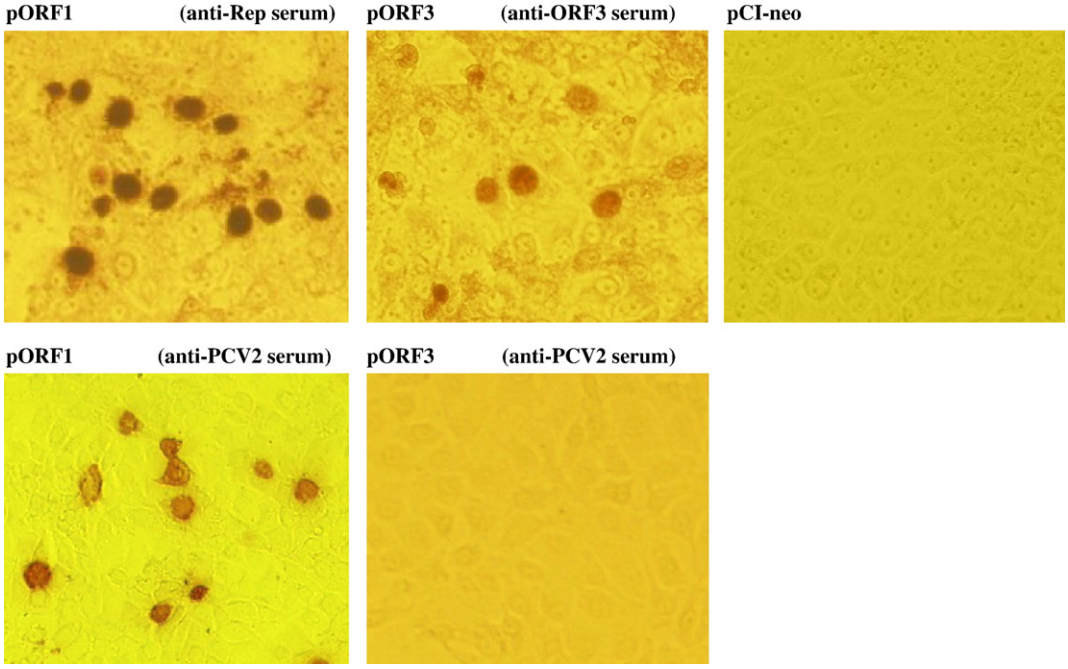


**Fig. 1.** Expression of PCV2 Rep and ORF3 proteins *in vitro*. PCV-free PK-15 cells were transfected with pORF1, pORF3 or pCI-neo, fixed at 48 h post-transfection, and detected by IPMA. The plasmid and antibody (in parentheses) used for each transfection and detection are indicated on top of each panel; cells transfected with pCI-neo were detected with mouse anti-Rep, rabbit anti-ORF3, and swine anti-PCV2 sera separately.

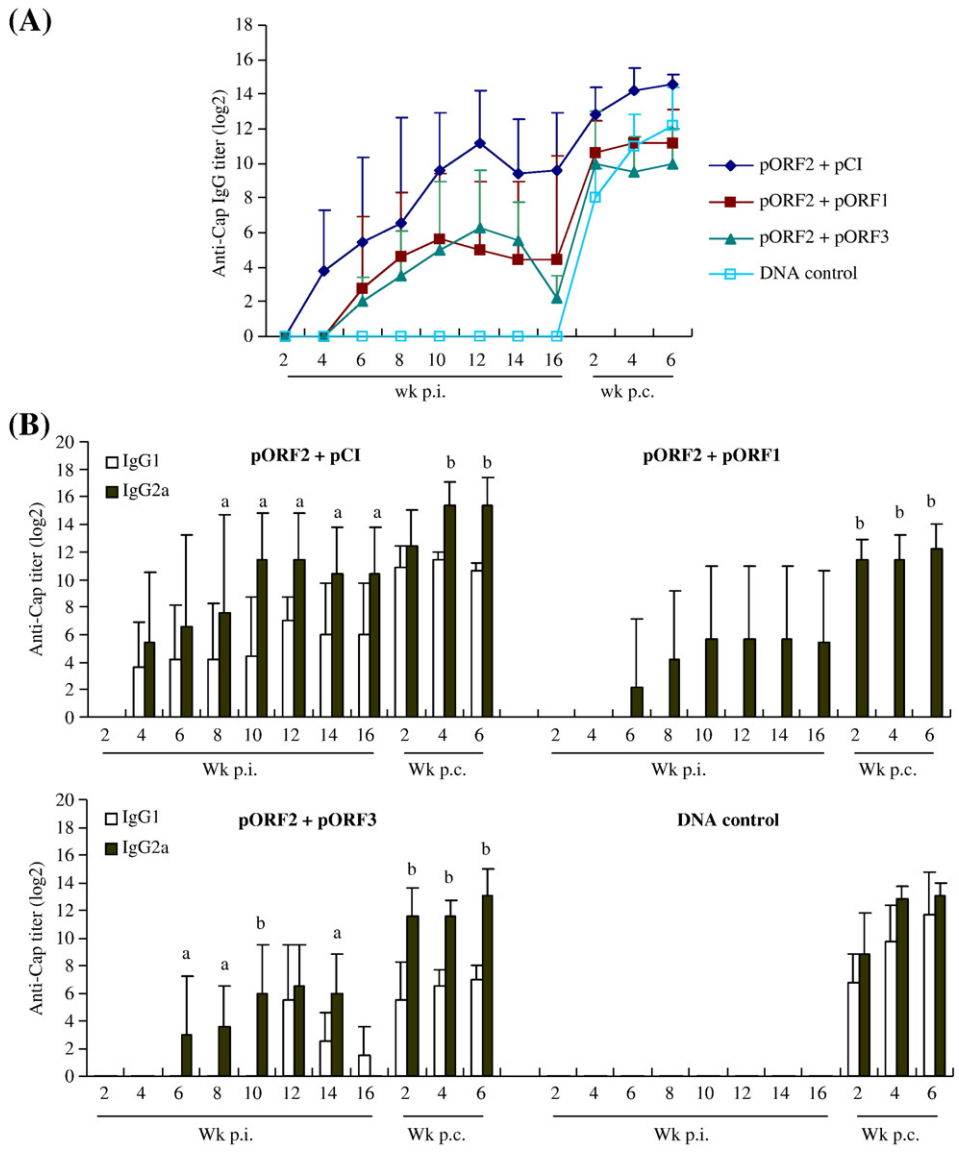


**Fig. 2.** Kinetics of total IgG (A) and IgG isotypes (B) against Cap protein at various times p.i. or p.c. The IgG, IgG1 and IgG2a titers were measured by indirect ELISA. Error bars show the standard deviations ($n=5$). The letters 'a' and 'b' above columns indicate a significantly higher IgG isotype with values of $P<0.05$ and $P<0.01$, respectively, at the same time in a group. Mice were challenged at 16 weeks p.i.

log2 (10 weeks p.i.) and 6.3 ± 3.4 log2 (12 weeks p.i.), respectively (Fig. 2A). Statistically, the Cap-specific IgG in group pORF2 + pCI were significantly higher than that in pORF2 + pORF1 (at 12–16 weeks) and pORF2 + pORF3 (at 10–12 weeks) groups ($P<0.05$). These data suggest that the co-administrations of pORF1 or pORF3 attenuated the Cap-specific antibody responses elicited by the pORF2 plasmid.

*IgG isotype profiles against PCV2 Cap protein*

The different vaccination approaches can affect the antibody isotype and T-helper (Th) cell type of an immune response; IgG2a is produced as a consequence of Th1-cell activation, whereas Th2-cell activation enhances the production of IgG1 and suppresses IgG2a (Mosmann and Coffman, 1989). To better understand the types of immune responses, we examined the IgG isotype profiles against Cap protein. In general, vaccinated mice developed higher IgG2a titers than IgG1, with significant differences in pORF2 + pCI (at 8–16 weeks p.i.) and pORF2 + pORF3 (at 6–10 and 14 weeks p.i.) groups ($P<0.05$) (Fig. 2B), suggesting a Th1 immune response during the corresponding period. Following challenge, titers of IgG1 and IgG2a increased to varying levels in all groups, but the isotype profiles remained similar to that prior to challenge. Interestingly, no anti-Cap IgG1 antibodies were detected in pORF2 + pORF1 vaccinated mice even after the PCV2 challenge; however, the non-significant differences between IgG1 and IgG2a titers revealed a non-biased Th response in this group. Overall, our data suggest that the pORF1 and pORF3 plasmids shorten the duration of Th1 responses induced by pORF2.

*Total IgG and isotype profiles to PCV2 Rep and ORF3 proteins*

The total IgG and isotype profiles against PCV2 Rep or ORF3 proteins were detected in the corresponding groups. As shown in Fig. 3A, low levels of anti-Rep IgG were detected in mice of pORF2 + pORF1 group, with a peak titer of 6.0 ± 3.3 log2 at 16 weeks p.i. Following challenge, control mice also developed Rep-specific IgG, with a peak titer of 6.8 ± 2.7 at 4 weeks post challenge (p.c.). The anti-

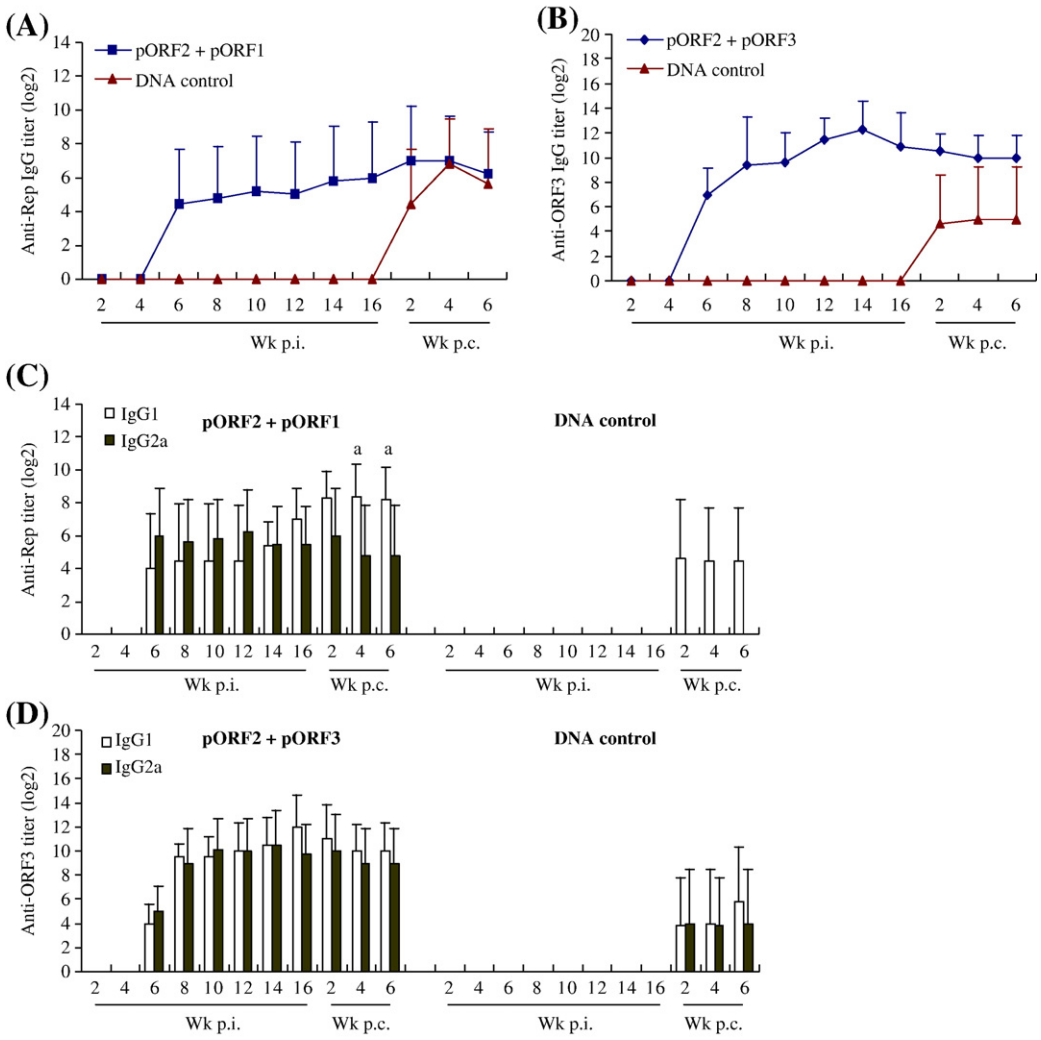


**Fig. 3.** Kinetics of antibody responses to PCV2 Rep and ORF3 proteins induced by different vaccine combinations. The total IgG levels against Rep (A) and ORF3 (B) proteins, and the IgG isotypes specific for Rep (C) and ORF3 (D) proteins are shown for each group. The IgG, IgG1 and IgG2a titers were measured by indirect ELISA. Error bars show the standard deviations ($n=5$). The letter 'a' above column indicates a significantly higher IgG isotype ($P<0.05$) at the same time in a group. Mice were challenged at 16 weeks p.i.

ORF3 IgG levels maintained between 9.4 and 12.3 log2 during 8 to 16 weeks p.i. in pORF2 + pORF3 vaccinated mice, whereas titers as low as 4.6–5.0 log2 were detected in control mice at 2 to 6 weeks p.c. (Fig. 3B). Antibody isotype analysis revealed that, except for the anti-Rep IgG1 in pORF2 + pORF1 group which were significantly higher than IgG2a at 4 to 6 weeks following challenge, no significant differences between IgG1 and IgG2a against Rep or ORF3 proteins were observed, suggesting a non-biased Th response to Rep or ORF3 following immunization in both groups (Figs. 3C–D).

**Table 2**
Neutralizing antibody titers of the mice immunized with different vaccine combinations.

| Group | No. of mice with VN antibody/no. detected (mean VN titer ± SD) at weeks p.c.[a] | | |
|---|---|---|---|
| | 2 | 4 | 6 |
| pORF2 + pCI | 3/5 (320 ± 320)[b] | 5/5 (456 ± 527)[b] | 5/5 (648 ± 630)[b] |
| pORF2 + pORF1 | 0/5 (0)[c] | 3/5 (12 ± 11)[c] | 3/5 (48 ± 44)[c] |
| pORF2 + pORF3 | 0/5 (0)[c] | 0/5 (0)[c] | 0/5 (0)[c] |
| DNA control | 0/5 (0)[c] | 1/5 (4 ± 9)[c] | 2/5 (8 ± 11)[c] |

[a]VN titers are expressed as the maximum serum dilution yielding a 70% reduction in fluorescent focus number. Mice with VN titers ≥20 were considered positive. "0" value is equal to "<20".
[b, c]Different superscripts within columns represent significant differences between groups for each week p.c. ($P<0.05$).

*VN antibody responses to PCV2*

The ability of mouse sera to neutralize PCV2 infection was detected by VNT in parallel with the ELISA test. Prior to virus challenge, there were no detectable neutralizing antibodies in any group (data not shown). After challenge with the PCV2 strain HZ0201 at 16 weeks p.i., strong anamnestic VN antibody responses were observed in pORF2 + pCI group, which increased steadily peaking at 6 weeks p.c. (Table 2). In contrast, mice in pORF2 + pORF1, pORF2 + pORF3 and control groups exhibited weak recall VN antibody responses. Statistically, pORF2 + pCI group showed significantly higher VN titers than pORF2 + pORF1 and pORF2 + pORF3 groups throughout the entire post-challenge period ($P<0.05$) (Table 2). These results suggest that the combined use of pORF1 or pORF3 markedly attenuated the pORF2-primed recall VN antibody responses following PCV2 infection.

*Protection from PCV2 challenge*

No clinical signs or gross lesions were observed in the challenge control group. Microscopic lesions included lymphocyte depletion and infiltration of histiocytes in splenic follicles as described previously (Shen et al., 2008). In the unchallenged control group, no abnormal follicles were found in the spleen at 6 weeks p.c. (Fig. 4).

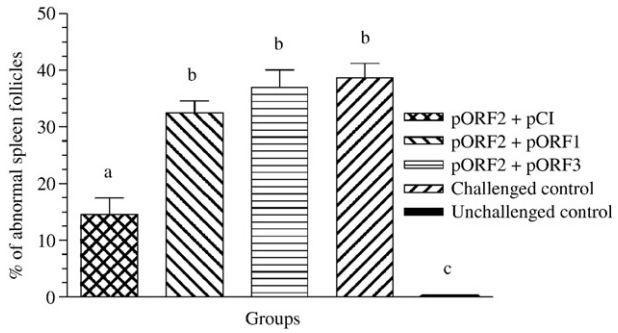


**Fig. 4.** Frequencies of abnormal spleen follicles at 6 weeks p.c. Same letter above columns indicates no significant difference ($P>0.05$); different letters indicate significant differences among the groups ($P<0.05$) ($n=5$).

Among the DNA vaccine groups, mice immunized with pORF2 + pCI had the lowest lesion frequency ($14.6\pm6.4\%$) and showed a significant difference with the challenged control mice ($38.7\pm5.9\%$) ($P<0.01$) (Fig. 4). However, plasmid mixtures of pORF2 + pORF1 and pORF2 + pORF3 demonstrated similar lesion frequencies to that of the challenge control group.

Further, we quantified the PCV2 DNA in serum of challenged mice using real-time PCR. Following PCV2-inoculation, all mice in pORF2 + pORF3 and control groups were positive for PCV2 DNA at 2, 4, and 6 weeks p.c. Fewer mice in the pORF2 + pCI and pORF2 + pORF1 groups were positive for PCV2 DNA throughout the post challenge period except the pORF2 + pORF1 group at 4 weeks p.c. (Table 3). Compared with the challenge control mice, PCV2 DNA copy numbers in pORF2 + pCI group were significantly lower at 2–6 weeks p.c. ($P<0.05$), whereas those in pORF2 + pORF1 and pORF2 + pORF3 groups were lower at 2–4 weeks and 4 weeks p.c. ($P<0.05$), respectively (Table 3).

## Discussion

The ORF1 is the largest ORF of PCV2 and encodes the Rep protein, in which three domains typical for enzymes initiating replication in the rolling circle mode have been identified as well as a P-loop for dNTP-binding (Mankertz et al., 1998). Besides the full-length protein Rep, a spliced isoform of the replication protein called Rep′ has also been identified (Mankertz and Hillenbrand, 2001). Both the Rep and Rep′ proteins are essential for PCV replication; mutations in either Rep or Rep′ proteins resulted in >99% reduction of viral protein synthesis and complete shutdown of viral DNA replication (Cheung, 2003; Mankertz and Hillenbrand, 2001). Small interfering RNAs (siRNAs) targeting PCV ORF1 inhibit viral RNA and DNA replication as well as protein synthesis in PK-15 cells (Feng et al., 2008; Sun et al., 2007), and combined use of siRNAs against both ORF1 and ORF2 showed a synergistic effect (Feng et al., 2008). However, Rep protein is weakly immunogenic compared to the Cap protein, and exhibits a reduced effect on the Cap vaccine efficacy when co-administered to pigs (Blanchard et al., 2003). In recombinant pseudorabies virus systems, Rep-Cap fusion protein failed to (Ju et al., 2005) but Cap alone successfully (Song et al., 2007) induced specific antibody responses, suggesting that the Rep in Rep-Cap fusion antigen has a suppressive effect. In the present study, our data suggest that pORF1 decreased the Cap-specific immune responses induced by pORF2, as evidenced by the reduced Cap-specific $CD8^+$ cell frequency and IgG1 antibody production, and weak protection efficacy in pORF2 + pORF1 group. Similar to ORF1, we found that the co-administration of pORF3 also reduced the Cap-specific antibody response induced by pORF2 plasmid.

Mixed DNA vaccination is an ideal strategy to investigate the immunogenicity of their encoded proteins. Immunization with mixtures of DNA vaccines could induce enhanced or comparable immune responses compared to the single plasmid, which has been demonstrated in the models of malarial (Grifantini et al., 1998), pseudorabies virus (van Rooij et al., 1998), mycobacterium tuberculosis (Morris et al., 2000), and *Plasmodium falciparum* (Jones et al., 2002). On the other hand, in some studies the mixed DNA vaccine led to a decreased response compared to the individual component (Sedegah et al., 2004; Wang et al., 2007). The immune suppression is thought to be caused by antigenic competition that occurs during uptake by the host cells, or competition within cells for translation factors or apparatus, or competition in the antigen processing pathway (Sedegah et al., 2004). However, the attenuation effects of pORF1 and pORF3 plasmids on pORF2 may be closely associated with the pathogenicity roles of their encoded proteins.

It is generally accepted that PCV2 induces immunosuppression in pigs. Flow cytometric analysis in PBMCs revealed decreased $CD4^+$ and $IgM^+$ cells (B lymphocytes) (Segales et al., 2001), or decreased $CD8^+$ and $IgM^+$ cells (Darwich et al., 2003) in naturally PMWS affected pigs. A decrease in $IgM^+$ and $CD8^+$ cells is also found to correlate with the quantity of PCV2 DNA in lymphoid tissues (Darwich et al., 2002). Similar results in PCV2 experimental infections have shown decreased levels of $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ T cells in PCV2-inoculated pigs (Nielsen et al., 2003). Currently little is known regarding the viral proteins by which the PCV2 infection may result in immunosuppression. In the present study, the plasmid-encoded Rep protein decreased the Cap specific $CD8^+$ cells, and both Rep and ORF3 proteins inhibited the Cap specific antibody production, suggesting an immunosuppressive role of these 2 proteins. Since the Rep has also shown suppression of Cap in a mixed subunit vaccine strategy (Blanchard et al., 2003), it is likely that the antigenic competition may occur in the antigen processing or presenting pathways. Also, the pathogenic role of ORF3 protein *in vivo* (Liu et al., 2006) may contribute to its suppression of Cap specific immunity. Since the pORF1 and pORF3 plasmids interfered with the onset of immunity of pORF2 plasmid, the Rep or ORF3 proteins produced during PCV2 infection may also suppress the development of Cap-specific immunity resulting in decreased protection in the host.

A strong Cap specific Th1 immune response is important for protective immunity against PCV2 infection (Shen et al., 2008). IgG isotype profiles were therefore investigated in this study to assess the influences of Rep or ORF3 proteins on the nature of the immune responses against Cap protein. The duration of the vaccine-induced Th1 response against Cap protein was totally abrogated in pORF2 + pORF1 group, and truncated in pORF2 + pORF3 group. In a recent study, ORF1 plasmid immunization elevated IFN-γ and IL-13 on 35 DPI (An et al., 2008). Although IFN-γ is a Th1 cytokine, its activity has been shown to prevent the outgrowth of Th2 cells rather than to promote Thl cells (Seder et al., 1992). IL-13, as a Th2 cytokine, may antagonize the macrophage-activating actions of IFN-γ (London et al., 1998). Therefore, the disturbance of cytokine production may be related to the abrogation of Cap specific Th1 immune response in pORF2 + pORF1 group. In the case of ORF3 plasmid, its inoculation in high dose

**Table 3**
Viremia in mouse serum measured by quantitative PCR.

| Group | No. of mice with viremia / no. detected (mean log PCV2 load ± SD) at weeks p.c. | | |
|---|---|---|---|
| | 2 | 4 | 6 |
| pORF2 + pCI | 2/5 ($5.8\pm5.1$)[a] | 2/5 ($5.6\pm4.9$)[a] | 1/5 ($3.7\pm3.0$)[a] |
| pORF2 + pORF1 | 2/5 ($5.2\pm4.4$)[a] | 5/5 ($7.2\pm6.2$)[a] | 3/5 ($5.5\pm4.5$)[a,b] |
| pORF2 + pORF3 | 5/5 ($6.9\pm5.8$)[b] | 5/5 ($7.0\pm6.0$)[a] | 5/5 ($5.9\pm4.8$)[a,b] |
| DNA control | 5/5 ($6.9\pm5.8$)[b] | 5/5 ($7.7\pm6.6$)[b] | 5/5 ($6.2\pm5.4$)[b] |

[a, b]Different superscripts within columns represent significantly different PCV2 loads (copies/ml) for each week p.c. ($P<0.05$). "0" value is equal to "$<10^3$ copies/ml" (the threshold of sensitivity of the quantitative PCR).

could significantly decrease IL-12 and increase TNF-α level in mice (An et al., 2008). The impaired IL-12 level may affect the development of a Th1 immune response, since IL-12 is thought to directly augment Thl differentiation during Th cell priming (Seder et al., 1993). Further, the elevated pro-inflammatory cytokine TNF-α, clinical signs, and lethal rate (9/15) observed in ORF3 plasmid inoculated mice in the above study (An et al., 2008) demonstrated the pathogenic role of ORF3 protein *in vivo*, which may also account for the weak Cap-specific Thl immunity observed in pORF2 + pORF3 group.

It has been shown that PCV2 vaccines are an effective tool to reduce losses in swine herds caused by PCV2-associated disease. Currently there are several types of antigens available in commercial vaccines, including PCV2 expressed in a killed baculovirus vector, an inactivated PCV2 virus, a killed chimeric PCV1–2 virus, and Cap protein expressed in a baculovirus system (Opriessnig et al., 2007). According to our data, the Rep and ORF3 proteins presented in the first three vaccines may interfere with the Cap-induced protective immunity against PCV2 infection. However, it should be pointed out that Rep and ORF3 proteins may be overexpressed in our study, and the expression abundance of ORF1, 2 and 3 may not reflect the real situation in a virus infection, or a virus antigen stock. Therefore, it needs further study to investigate how great the suppressive effects of Rep and ORF3 proteins may have in a commercial vaccine.

Overall, in the present study, we investigated the influences of the pORF1 and pORF3 on the immunogenicity of the pORF2 by comparing the antigen-specific cellular and humoral immune responses as well as protective efficacy. We demonstrated the suppressive effects of plasmid-encoded Rep and ORF3 proteins on the immunogenicity of Cap in mixed DNA vaccinations. Uncovering such interactions will undoubtedly lead to further understanding of the immunogenicity of PCV2, and ultimately, improved vaccines.

## Materials and methods

### *Cells, virus, proteins, antisera and mice*

A PCV-free PK-15 cell line was maintained in minimal essential medium (MEM, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). The virulent PCV2 isolate HZ0201 was originally isolated from pigs with naturally occurring PMWS (Zhou et al., 2006) and serially passaged in PK-15 cells 15 times. PCV2 Rep and Cap proteins were generated as described previously (Li et al., 2007; Zhou et al., 2005a). PCV2 ORF3 proteins were expressed in *E. coli* BL21 by recombinant pGEX-4T-1 vector (Amersham, Pharmacia Biotech AB, Uppsala, Sweden) inserting PCV2 ORF3 (unpublished data). Swine anti-PCV2 serum was acquired from piglets experimentally infected with PCV2 isolate HZ0201. Mouse anti-Rep and rabbit anti-ORF3 sera were obtained by immunizing animals with the prokaryotic expressed Rep and ORF3 proteins, respectively. Female BALB/c mice were purchased from Shanghai laboratory animal center (Chinese Academy of Sciences, Shanghai, China) and bred in automatic extrusion independent venting isolation cages (Fengshi laboratory animal equipment Co. Ltd., Suzhou, China).

### *Construction and preparation of the DNA vaccines*

PCV2 ORF1 and ORF3 were amplified from the genomic DNA of HZ0201 (GenBank accession no. AY188355) using the specific primers as follows: forward (5′-ATAACGCGTCATGCCCAGCAAGAAG-3′) and reverse primer (5′-GCGGTCGACGACTCAGTAATTTATTTCATATGG-3′) for ORF1, and forward (5′-TAAGTCGACCTTACTGATGGAGTGTGG-3′) and reverse primer (5′-ATAACGCGTATGGTAACCATCCCAC-3′) for ORF3. The PCR products were cloned into a mammalian expression vector pCI-neo (Promega, Madison, WI) to generate the expression vectors pORF1 and pORF3, respectively. To determine the expression of Rep and ORF3 proteins *in vitro*, PCV-free PK-15 cells were transfected with the recombinant plasmids by Lipofectin Reagent (Invitrogen, Carlsbad, CA). At 48 h post-transfection, cells were fixed and detected by an immunoperoxidase monolayer assay (IPMA) (Zhou et al., 2005a) using the swine PCV2-positive serum, or the mouse anti-Rep or rabbit anti-ORF3 sera as the primary antibodies. Recombinant vector expressing PCV2 ORF2 (pORF2) was constructed and confirmed previously (Shen et al., 2008). Finally, the plasmids pORF1, pORF3 and pORF2 were purified by endoFree plasmid Giga kit columns (Qiagen, Valencia, CA) and used as DNA vaccines.

### *Experimental design and samples collection*

All the mice at 8 weeks of age were grouped randomly and injected intramuscularly in the quadriceps with 100 μg of each plasmid in a total of 100 μl PBS as summarized in Table 4. Mice received 3 doses of vaccine at 2-week intervals. Mice given similar injections of 200 μg pCI-neo (pCI) in 100 μl PBS were used as the DNA control. Five mice in each group were euthanized for flow cytometric analysis (FCM) and lymphocyte proliferation assay (LPA) at 8 weeks p.i. At 16 weeks p.i., five mice of each group were challenged intraperitoneally with 0.2 ml of PCV2 inoculum ($10^{4.75}$ $TCID_{50}$/0.1 ml). Clinical observations were recorded daily for 6 weeks and then all mice were euthanized and spleens were collected for pathological analysis. Serum samples were withdrawn from the retro-orbital sinus biweekly for antibody detection and/or viremia evaluation.

### *Lymphocyte proliferation assay (LPA)*

Cap-specific lymphoproliferative response of the splenocytes from the immunized mice was determined by MTT test as described previously (Zhou et al., 2005b). In brief, spleens were aseptically removed from the mice at 8 weeks p.i. to make single-cell suspensions ($4\times10^6$ cells/ml) in RPMI 1640 containing 5% FBS (RPMI1640-FBS), and applied to each well of 96-well culture plates at 100 μl/well. Wells containing no cells were used as blank controls. The PCV2 Cap protein diluted in RPMI1640-FBS was then added at a final concentration of 1 μg/ml (100 μl/well) to stimulate the splenocytes. Cells were cultured at 37 °C in 5% $CO_2$ for 48 h. Twenty μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, St. Louis, MO) (5 mg/ml) was added to each well and incubated for 4 h. The cells were then lysed by adding 100 μl of lysis buffer (10% SDS, 0.01 mol/L HCl) to each well. After 20 h of

**Table 4**
Summary of experimental treatments.

| Groups | Mouse no. | Immunizations (wk 0, 2, 4) | LPA and FCM[a] | Antibody detection[a] | Protection assay[a] |
|---|---|---|---|---|---|
| pORF2 + pCI[b] | 10 | pORF2 and pCI-neo | Mouse splenocytes were prepared at 8 wk p.i. | ELISA and VNT were performed at 2-wk intervals | Mice were challenged at 16 wk p.i., pathology and viremia were evaluated |
| pORF2 + pORF1[b] | 10 | pORF2 and pORF1 | | | |
| pORF2 + pORF3[b] | 10 | pORF2 and pORF3 | | | |
| DNA control[c] | 15 | pCI-neo | | | |

[a] Each treatment includes 5 mice.
[b] Mouse was vaccinated with 100 μg of each plasmid in 100 μl volume.
[c] Mouse was vaccinated with 200 μg of pCI-neo in 100 μl volume.

incubation, optical density (OD) value of each well was measured at 570 nm. The LPA was performed in duplicate and stimulation index (SI) was calculated from the formula: $SI = (OD_{vaccine} - OD_{blank}) / (OD_{DNA\ control} - OD_{blank})$.

*Flow cytometric analysis (FCM)*

The above Cap-stimulated and cultured splenocytes were resuspended in 50 μl PBS ($10^6$ cells) and incubated with 50 μl diluted (1:200) FITC-conjugated anti-mouse CD4 (L3T4) and R-PE-conjugated anti-mouse CD8α (Ly-2) monoclonal antibodies (BD Biosciences, Mountain View, CA) at a concentration of 0.5 μg/million cells on an ice bath. After 20 min incubation, cells were washed and analyzed on a BDLSR cytofluorimeter using the CellQuest software (BD Biosciences).

*ELISA*

Titers of total IgG, IgG1 and IgG2a antibodies against PCV2 Rep, Cap and ORF3 proteins were determined by indirect ELISA. Ninety-six-well plates (Nunc, Roskilde, Denmark) were coated with 100 μl corresponding protein (1 μg/ml for Rep and Cap; 2 μg/ml for ORF3) in 0.05 M Tris–HCl buffer (pH8.5) and left at 4 °C overnight. Next, blocked with PBS containing 5% skimmed dry milk, 100 μl of the serially 2-fold diluted mouse serum samples (lowest dilution, 1:64) were added and incubated at 37 °C for 60 min. The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, or IgG2a antibodies (dilution, 1:6000; Southern Biotechnology Associates). Tetramethylbenzidine (Sigma) was used as a chromogen for color development, and absorbance was measured at 450 nm. Antibody titers were defined as the reciprocal of the highest dilution of sample for which the OD was at least 2 times that of the control serum sample run on the same plate. The data were presented as the log2 of the titer.

*Virus neutralization test*

Virus neutralization tests (VNT) were performed using serum samples that were previously heat inactivated at 56 °C for 30 min. Briefly, an equal volume of $10^{5.0}$ $TCID_{50}$/0.1 ml PCV2 HZ0201 and the serial 2-fold dilutions (1:20 to 1:20,480) of the sera were mixed and incubated at 37 °C for 1 h. The serum–virus mixture was inoculated into 96-well microtitre plates containing semi-confluent monolayers of PCV free PK-15 cells in 10 μl per well at a ratio of 1:10, using 2 wells per serum dilution. The plate was incubated for 48 h at 37 °C. Finally, the 96-well plates were screened by indirect immunofluorescence assay (Zhou et al., 2006). The serum titers were determined as the reciprocal of the highest serum dilution at ≥70% fluorescent focus reduction in the infected cell cultures under a fluorescent microscope.

*Pathology and viremia evaluations*

At 6 weeks p.c., pathological analysis and viremia evaluation were performed as previously described (Shen et al., 2008). Lesion severity was estimated as the frequency of abnormal spleen follicles based on the ratio between the number of the follicles demonstrating lymphoid depletion and histiocytic infiltration by the total follicles counted in each sample of spleen. A quantitative real-time PCR method was used for assessing viremia. PCV2 genomic copies per ml of serum were represented as the mean value of duplicate reactions.

*Statistical analysis*

Data statistical analysis was performed by one-way analysis of variance (ANOVA) using the SPSS software program version 12.0. Results were considered to be statistically significant when the $P$ value was less than 0.05.

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