Protective immunity against porcine circovirus 2 by vaccination with ORF2-based DNA and subunit vaccines in mice

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The protective immune response against porcine circovirus 2 (PCV2) infection in mice was characterized using flow cytometric analysis (FCM), assays of antibody (of different IgG isotypes) and viraemia, and histopathological examination. An open reading frame 2 plasmid (pORF2) and the capsid protein (Cap) of PCV2 were used as DNA and subunit vaccines, respectively. In FCM analysis, although pORF2 and Cap alone showed comparable efficacy in eliciting lymphoproliferative responses and Cap-specific CD4+ T cells, pORF2 was superior to the Cap protein in triggering CD8+ T cells. A virus neutralization assay showed that pORF2 evoked stronger recall virus-neutralizing (VN) antibody responses than the Cap protein on PCV2 challenge. Correspondingly, VN antibody kinetics coincided with those of Cap-specific IgG2a, but not with the kinetics of IgG and IgG1. Following virus challenge, real-time PCR and histopathological analysis confirmed that only low viral DNA loads and mild microscopic lesions appeared in pORF2-immunized mice. These findings indicate that CD8+ T cells and VN antibody responses correlating mainly with Cap-specific IgG2a play crucial roles in protecting against PCV2 infection, and that the protective immunity induced by the pORF2 plasmid is superior to that induced by the PCV2 Cap protein.

INTRODUCTION

Post-weaning multisystemic wasting syndrome (PMWS), first recorded in pigs in Western Canada in 1991, has been described in pigs from many regions of the world (Allan et al., 1998b; Allan & Ellis, 2000; Clark, 1997; Wen et al., 2005; Zhou et al., 2006). PMWS affects pigs of 5–12 weeks of age, with a morbidity of 5–30%; it is characterized by weight loss, dyspnoea and jaundice. Field and experimental evidence has revealed that PMWS-affected pigs may develop severe immunosuppression (Segales et al., 2004).

Porcine circovirus 2 (PCV2) has been identified as the primary aetiological agent of PMWS (Allan et al., 1998a, b; Allan & Ellis, 2000; Ellis et al., 1998; Fenaux et al., 2002; Meehan et al., 1998). PCV2 is a non-enveloped, single-stranded, circular DNA virus with a diameter of 17 nm (Tischer et al., 1982). The PCV2 genome comprises 1767 or 1768 nt and is assumed to have 11 potential open reading frames (ORF1–11; Hamel et al., 1998; Zhou et al., 2006). PCV2 is a non-enzymatic, single-stranded DNA virus with a diameter of 17 nm (Tischer et al., 1982). The PCV2 genome comprises 1767 or 1768 nt and is assumed to have 11 potential open reading frames (ORF1–11; Hamel et al., 1998; Zhou et al., 2006). Previous studies have demonstrated that ORF1, -2 and -3 encode a 35.7 kDa replication (Rep) protein involved in virus replication (Mankertz et al., 1998), a 27.8 kDa capsid (Cap) protein involved in PCV2 immunogenicity (Mahe et al., 2000; Nawagitgul et al., 2000; Truong et al., 2001; Zhou et al., 2005a) and a protein involved in PCV2-induced apoptosis (Liu et al., 2005), respectively.

Immunization against PCV2 has been studied intensely and found to be the most effective strategy for protecting pigs against PCV2 infection. Based on comparison of the immunogenicity of the PCV2 Cap and Rep proteins, Blanchard et al. (2003) demonstrated that the ORF2-encoded Cap protein was the major immunogen, whilst the ORF1-encoded Rep protein was only weakly immunogenic. Kamstrup et al. (2004) subsequently investigated the potential of a DNA vaccine encoding the PCV2 Cap protein in mice and demonstrated the production of antibody. On the other hand, Fenaux et al. (2003, 2004) focused on a chimeric PCV1–PCV2, which was shown to induce specific antibody responses and protection against PCV2 in pigs. Recent studies on live virus vectors have shown that a recombinant pseudorabies virus (Ju et al., 2005; Song et al., 2007) and adenovirus (Wang et al., 2006, 2007) expressing Cap protein can elicit specific humoral and/or lymphocyte proliferation responses in mice and/or pigs, encouraging further studies of PCV2 vaccines.
However, the mechanisms underlying the immune response and protection against PCV2 have not yet been fully characterized. The purpose of this study was to characterize the protective immune response against PCV2 using a DNA plasmid expressing ORF2 (pORF2) and recombinant Cap protein as PCV2 DNA and subunit vaccines, respectively. Our data suggest that CD8⁺ T cells and recall virus-neutralizing (VN) antibody responses correlating with Cap-specific IgG2a may play important roles in developing protective immunity against PCV2 infection.

**METHODS**

**Cells, virus, protein and mice.** A PCV-free PK-15 cell line was maintained in minimal essential medium (Gibco) 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 15 times in PK-15 cells. The PCV2 Cap protein was generated as a subunit vaccine as described previously (Zhou et al., 2005a). Eight-week-old female BALB/c mice were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China) and raised in automatic extrusion-independent venting isolation cages (Fengshi Laboratory Animal Equipment Co.).

**Construction and preparation of an ORF2-based DNA vaccine.** The entire ORF2 was amplified from the genomic DNA of PCV2 isolate HZ0201 (GenBank accession no. AIY88355) using specific forward (5'-GCGGTGAGCTCAATTAAGGGTATTGGG-3') and reverse (5'-TATACGGGTATATCGTATCCAAGGAG-3') primers, and was subcloned into the mammalian expression vector pcI-neo (Promega) to construct the recombinant expression plasmid pORF2. To determine the expression of Cap protein in eukaryotic cells, PCV2-free PK-15 cells were transfected with pORF2 using Lipofectin reagent (Invitrogen). At 48 h post-transfection, the cells were fixed and examined using an indirect immunofluorescence assay (IFA) with mouse monoclonal antibody (mAB) against Cap protein (Zhou et al., 2005a) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (KPL, Kirkgaard and Perry Laboratories) as the primary and secondary antibodies, respectively. Finally, the pORF2 plasmid was purified using an EndoFree Plasmid Giga kit column (Qiagen) for use as a DNA vaccine.

**Experimental design and sample collection.** Eighty mice were divided randomly into eight groups of ten mice each. The mice in the four vaccine groups were injected intramuscularly in the quadriceps with 100 μg pORF2 plasmid or Cap protein prepared in 100 μl PBS some modifications. In brief, the mice in the pORF2 and Cap groups received vaccination with pORF2 plasmid or Cap protein, respectively, three times every 2 weeks, whilst those in the pORF2/Cap or Cap/pORF2 group were primed with pORF2 plasmid (or Cap protein) and boosted with doses of Cap protein (or pORF2 plasmid) twice every 2 weeks. In the four control groups, the pcI-neo vector and crude lysate of E. coli strain BL21 transformed with pGEX-4T-1 were used as substitutes for pORF2 plasmid and Cap protein, respectively. After the first immunization, mouse serum samples were withdrawn from the retro-oral sinus every 2 weeks until the end of the experiment for antibody detection and viraemia evaluation. At 8 weeks after the first immunization (p.i.), five mice from each group were euthanatized for flow cytometric analysis (FCM) and a lymphocyte proliferation assay. At 16 weeks p.i., the remaining five mice from each group were challenged intraperitoneally with 0.2 ml PCV2 inoculum (10⁵.75 TCID₅₀ ml⁻¹). Clinical signs in the challenged mice were recorded daily for 6 weeks, following which all mice were euthanatized and their spleens collected for pathological analysis.

**Lymphocyte proliferation assay.** Proliferation of splenocytes in the immunized mice was determined using a 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide (MTT) test as described previously (Zhou et al., 2005b). In brief, the spleens were excised aseptically from the mice at 8 weeks p.i. in order to prepare single-cell suspensions (4 x 10⁶ cells ml⁻¹) in RPMI 1640 containing 5% FBS; the suspensions were applied to each well of a 96-well culture plate (100 μl per well). Wells containing no cells were used as blank controls. The PCV2 Cap protein diluted in RPMI 1640 with 5% FBS was then added at a final concentration of 1 μg ml⁻¹ (100 μl per well) to stimulate the splenocytes. After incubation at 37 °C in 5% CO₂ for 48 h, 20 μl MTT (5 mg ml⁻¹; Sigma) was added to each well and the cells were incubated for 4 h. The cells were lysed by adding 100 μl lysis buffer (10% SDS, 0.01 M HCl) to each well. After incubation for 20 h, the absorbance of each well was measured at 570 nm. The stimulation index (SI) was calculated using the following formula: SI=(A_cap−A_blank)−(A_control−A_blank).

**Flow cytometric analysis (FCM).** The cultured and stimulated splenocytes (10⁶ cells) were resuspended in 50 μl PBS and incubated with 50 μl diluted (1:200) FITC-conjugated anti-mouse CD4 (L3T4; BD Biosciences) and R-phycocerythrin-conjugated anti-mouse CD8α (Ly-2; BD Biosciences) mAbs at a concentration of 0.5 μg per 10⁶ cells in an ice bath. After 20 min incubation, the cells were washed and analysed using a BD-LSR cytometer with CellQuest software (BD Biosciences).

**Detection of Cap-specific IgG, IgG1 and IgG2a antibodies.** An indirect ELISA (Shang et al., 2008) was performed to detect the titres of total IgG, IgG1 and IgG2a antibodies against PCV2 Cap protein. In brief, 96-well plates (Nunc) were coated with 100 μl PCV2 Cap protein (1 μg ml⁻¹) in 0.05 M Tris/HCl (pH 8.5) and left at 4 °C overnight. The plates were then blocked with 5% skimmed milk, and 100 μl serially twofold-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-conjugated goat anti-mouse IgG, IgG1 or IgG2a antibody (diluted 1:6000; Southern Biotechnology Associates). Tetrathiazoline (Sigma) was used as a chromogen for colour development and absorbance was measured at 450 nm. Antibody titres were defined as the reciprocal of the highest dilution of sample for which the absorbance was at least twice that of the control serum sample run on the same plate. The data were presented as the log₂ value of the titre.

**Detection of PCV2-neutralizing antibodies.** A virus neutralization test was performed as described previously (Zhou et al., 2005a) with some modifications. In brief, the test sera were inactivated by heating at 56 °C for 30 min. An equal volume of PCV2 HZ0201 (10⁶.₀ TCID₅₀ ml⁻¹) and serial twofold 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 added to 96-well microtitre plates containing semi-confluent monolayers of PCV-free PK-15 cells at 10 μl per well at a ratio of 1:10 using two wells per serum dilution. The plates were subsequently incubated at 37 °C for 48 h and then screened by IFA as described above. The serum titres were determined as the reciprocal of the highest serum dilution resulting in ≥ 70% fluorescent focus reduction in the infected cell cultures viewed under a fluorescent microscope.

**Pathological analysis.** The spleens of mice were collected at 6 weeks post-challenge (p.c.) and processed using conventional histopathological methods. Briefly, the spleens were fixed in 10% neutral-buffered formalin solution, sectioned and stained with haematoxylin and eosin (H&E). Microscopic changes were...
determined by comparing the splenic tissues of the challenged mice with those of the control-group mice. The intensity of lesions was estimated as the frequency of abnormal spleen follicles, which is the ratio between the number of follicles with lymphoid depletion and histiocytic infiltration and the total number of follicles counted in the spleen section of each mouse.

Quantitative real-time PCR for evaluation of viraemia. Viral DNA levels in the mouse serum samples collected after PCV2 challenge were determined using a quantitative real-time PCR. Total DNA was extracted from 10 μl serum by using a UNIQ-10 virus DNA minikit according to the manufacturer’s instructions (Sangon). A forward (5’-TGTAGTATTCAAAAGGCAAGGACG-3’) and a reverse (5’-CGGATATATTCAAGCGAACCAC-3’) primer were used to amplify a 130 bp fragment from the ORF2 of PCV2. The PCR contained a final concentration of 1 × SYBR Premix Ex Taq (TaKaRa), 0.2 μM each primer and DNA equivalent to that of 1 μl serum as template. All reactions were carried out in duplicate on a RealPlex4 (Eppendorf). The program consisted of one cycle at 95 °C for 1 min and 40 cycles at 95 °C for 5 s and 60 °C for 20 s, and was followed by a melting curve analysis to analyse specificity. Serial dilutions of plasmid pORF2 were used to obtain a standard curve. The numbers of virus copies for each sample were presented as the mean value of duplicate reactions. The detection limit of this assay was 10³ copies ml⁻¹.

Statistical analysis. Statistical analysis was performed by one-way analysis of variance using SPSS version 12.0 (SPSS). Results were considered to be statistically significant for P<0.05.

RESULTS

In vitro expression of PCV2 Cap protein in PK-15 cells

The recombinant plasmid pORF2 was identified using PCR and restriction enzyme digestion and was confirmed by DNA sequencing. In vitro expression of Cap protein was analysed by transient transfection followed by an IFA. PK-15 cells transfected with pORF2 were recognized by mAb to Cap at 48 h post-transfection and the expressed Cap protein was found to localize in the nuclei of cells (Fig. 1), as observed in PCV2-infected cells (Allan et al., 1998b), suggesting that the mammalian expression vector pORF2 mimics PCV2 expression of Cap protein in vivo.

Cap-induced cellular immune responses

Mice splenocytes were collected at 8 weeks p.i. and their lymphoproliferative responses to Cap protein were assayed (Table 1). The SI values of all the vaccinated mice were significantly higher than those of the control mice (P<0.01). Although mice in the Cap group exhibited the strongest responses to Cap protein, there was no significant difference among the responses of mice in the various vaccine groups (P>0.05). The proportions of CD4⁺ and CD8⁺ cells in the stimulated splenocytes were determined by FCN. As shown in Table 1, significant upregulation of CD4⁺ cells was elicited in all of the vaccine groups compared with the control group (P<0.01). With regard to the expression of CD8⁺ cells, only the pORF2 (P<0.01) and pORF2/Cap (P<0.05) groups showed significant increases in CD8⁺ cells. These data indicated that the pORF2 plasmid is superior to Cap protein in inducing CD8⁺ cells, whilst both had a comparable capability to trigger CD4⁺ cells.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>SI</th>
<th>CD4⁺ cells (%)</th>
<th>CD8⁺ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00±0.00</td>
<td>9.52±3.68</td>
<td>11.85±1.81</td>
</tr>
<tr>
<td>pORF2</td>
<td>1.54±0.32*</td>
<td>15.36±1.58*</td>
<td>15.01±2.60*</td>
</tr>
<tr>
<td>Cap</td>
<td>1.63±0.14*</td>
<td>15.89±1.65*</td>
<td>12.80±0.99</td>
</tr>
<tr>
<td>Cap/pORF2</td>
<td>1.50±0.11*</td>
<td>14.89±1.59*</td>
<td>13.50±1.32</td>
</tr>
<tr>
<td>pORF2/Cap</td>
<td>1.51±0.20*</td>
<td>16.50±1.67*</td>
<td>14.31±1.51†</td>
</tr>
</tbody>
</table>

Significant differences between the vaccine and control groups are indicated: *, P<0.01; †, P<0.05.
than IgG1 from 10 to 16 weeks p.i. \( (P < 0.05) \), with a peak IgG2a titre of \( 14.0 \pm 1.5 \log_2 \) at 10 weeks (Fig. 3a). In contrast, the Cap protein induced significantly higher IgG1 levels than IgG2a levels from 2 to 16 weeks p.i. \( (P < 0.01) \), with a peak IgG1 titre of \( 17.8 \pm 0.8 \log_2 \) at 6 weeks (Fig. 3b). With regard to heterologous vaccinations, peak IgG1 \( (16.0 \pm 0.8 \log_2) \) and IgG2a \( (14.8 \pm 2.1 \log_2) \) titres appeared at 12 weeks p.i. in the pORF2/Cap group (Fig. 3d), whilst peak IgG1 \( (13.5 \pm 1.7 \log_2) \) and IgG2a \( (14.3 \pm 1.0 \log_2) \) titres appeared at 16 and 14 weeks, respectively, in the Cap/pORF2 group (Fig. 3c). Statistically, the IgG1 titres were significantly higher than those of IgG2a at 2–4 weeks p.i. for Cap/pORF2 and at 4–8 weeks p.i. for pORF2/Cap \( (P < 0.05) \); they subsequently became non-significant (Fig. 3c, d). These results suggested that immunization with the pORF2 plasmid or Cap protein alone induced Th1 or Th2 responses, respectively; based on this, IgG2a was produced as a consequence of Th1 cell activation and IgG1 as a result of Th2 cell activation (Mosmann & Coffman, 1989b).

### VN antibody to PCV2 and its correlation with IgG isotypes

The ability of mice sera to neutralize PCV2 infection was detected using a virus neutralization test together with an ELISA. Prior to virus challenge, no detectable VN antibodies were observed in mice in any group (data not shown). After challenging the mice with PCV2 strain HZ0201 at 16 weeks p.i., strong recall VN antibody responses were observed in the pORF2, Cap/pORF2 and pORF2/Cap groups at 2 weeks p.c., with a seropositivity rate of 100% and mean titres of 640, 805 and 960, respectively (Fig. 4). The VN antibody titres in the pORF2 and pORF2/Cap groups increased steadily and peaked at 6 weeks p.c., whereas those in the Cap/pORF2 group peaked at 2 weeks p.c. and then decreased gradually (Fig. 4). In contrast, mice in the Cap group exhibited...
IgG2a titre of 15 log₂ generated a VN titre of 640, whilst individual mice. As shown in Table 2, mouse no. 12 with a VN antibodies. We then analysed the antibody titres in implicating a probable correlation between the IgG2a and antibodies than the Cap group mice (data not shown), P group mice presented higher VN (P<0.05) and IgG2a antibodies than the pORF2 group mice, whilst the pORF2 group mice exhibited higher IgG and IgG1 (P<0.05) (Fig. 4). These results showed that the protocol using the plasmid was more effective than that with the Cap protein alone in inducing recall PCV2-neutralizing antibody responses in mice.

To investigate the relationship of VN antibodies and IgG isotypes, we compared the different antibody titres following virus challenge. At 2–6 weeks p.c., the Cap group mice exhibited higher IgG and IgG1 (P<0.05) antibodies than the pORF2 group mice, whilst the pORF2 group mice presented higher VN (P<0.05) and IgG2a antibodies than the Cap group mice (data not shown), implicating a probable correlation between the IgG2a and VN antibodies. We then analysed the antibody titres in individual mice. As shown in Table 2, mouse no. 12 with a IgG2a titre of 15 log₂ generated a VN titre of 640, whilst mouse no. 23 with IgG1 titre of 16 log₂ only generated a VN titre of 20. These results suggested that the neutralizing activity of the antibodies was associated with Cap-specific IgG2a rather than with IgG1.

Histopathological examination and evaluation of viraemia

To investigate the protective efficacy of different vaccine protocols, we compared clinical symptoms and pathological changes in the challenged mice. No clinical signs or gross lesions were observed in the mice challenged with virulent PCV2. As shown in Fig. 5(a), lymphocyte depletion, histiocytic infiltration and infrequent apoptotic cells were found in the splenic corpuscles of the PCV2-infected control mice. In contrast, after PCV2 challenge, the extent of the spleen lesions in the immunized mice was obviously smaller, occupying only a small part of the follicles. The frequency of the microscopic lesions (Fig. 5b) in the challenged control mice was 38.6±5.8 %, whereas that of the microscopic lesions in the immunized mice was significantly less compared with the control (P<0.01), with mean values of 10.6±8.5 % for the pORF2 group, 24.7±7.2 % for the Cap group, 13.0±8.4 % for the Cap/pORF2 group and 9.7±7.0 % for the pORF2/Cap group (Fig. 5b). The frequency of lesions was significantly higher for the Cap group compared with the other vaccine groups (P<0.01).

In addition to the histopathological evaluation, we also quantified the serum viral DNA loads of all PCV2-inoculated mice using real-time PCR. The occurrence of viraemia and the mean numbers of PCV2 genomic copies are presented in Table 3. All of the challenged control mice were positive for PCV2 viraemia. Compared with the challenged control mice, those in the vaccine groups exhibited a reduction in the level of viraemia with significantly decreased numbers of PCV genomic copies (P<0.01) at 2–6 weeks p.c. For the different immunization protocols, the Cap group exhibited the highest PCV2 loads throughout the post-challenge period.

Table 2. Isotype analysis and VN antibody titres of individual mice in the pORF2 and Cap groups at 2 weeks p.c.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Mouse no.</th>
<th>IgG1†</th>
<th>IgG2a†</th>
<th>VN titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORF2</td>
<td>11</td>
<td>15</td>
<td>17</td>
<td>1280</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>17</td>
<td>15</td>
<td>640</td>
</tr>
<tr>
<td>13</td>
<td>11</td>
<td>16</td>
<td>0</td>
<td>1280</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cap</td>
<td>21</td>
<td>16</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>15</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>16</td>
<td>13</td>
<td>20</td>
<td>20</td>
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<td>24</td>
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<td>20</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>18</td>
<td>14</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

*Mice were challenged at 16 weeks p.i. †Cap-specific IgG1 and IgG2a titres were detected by ELISA and expressed as log₂ values. ‡VN activity was analysed in a twofold dilution series starting at 1:20.

DISCUSSION

PCV2 is the primary aetiological agent of PMWS. In this study, we characterized the protective immunity of mice against PCV2. Previous studies have reported mice as a model for PCV2 infection (Kiupel et al., 2001; Liu et al., 2006). Although two reports (Quintana et al., 2002; Wang et al., 2006) have described that PCV2 inoculation failed to induce murine lesions, this difference was considered to be related to inoculum dosage and route of administration (Quintana et al., 2002). In the present study, intraperitoneal inoculation of PCV2 successfully produced lesions.
in mice. Furthermore, we chose changes in the spleen as the criteria of pathological analysis, as these are a distinct feature of PCV2 infection. The observed abnormal follicles with lymphocyte depletion and histiocyte infiltration resembled lesions found in PMWS-affected pigs (Chianini et al., 2003; Sarli et al., 2001), as well as those in PCV2-inoculated mice (Kiupel et al., 2001; Liu et al., 2006); therefore, mice were deemed suitable models for this study.

A recent in vivo study reported that B cells may be the primary site of PCV2 replication during early infection (Yu et al., 2007), although in vitro studies have shown that PCV2 can replicate in primary porcine hepatocytes (Hirai et al., 2006) and that PCV2 particles can persist without replication in dendritic cells and porcine alveolar macrophages as well as in monocytes (Gilpin et al., 2003; Vincent et al., 2003). Thus, the target cells for PCV2 replication have not been clearly identified thus far. With regard to PCV2 immunization, no data regarding lymphocyte subsets in cellular immune responses have been recorded recently, although ORF2-based vaccines against PCV2 have been reported previously (Blanchard et al., 2003; Fan et al., 2008; Fenaux et al., 2003; Fig. 5. Microscopic lesions in the spleens of PCV2-challenged mice. (a) Mice were challenged with the virulent PCV2 at 16 weeks p.i. and the presence of splenic lesions was determined by conventional methods of histopathology at 6 weeks p.c. (i) No abnormal follicles were seen in the spleen of unchallenged mice. (ii) Significant depletion of lymphocytes within follicles (arrows) was observed in the spleen of PCV2-inoculated mice. (iii) Infiltration of histiocytes in the splenic follicle of a PCV2-inoculated mouse (arrows). (iv) Apoptotic cells (arrows) in the splenic follicle of a PCV2-inoculated mouse. (v) Mild lymphocyte depletion within a follicle (arrow) in the spleen of a Cap-immunized mouse inoculated with virulent PCV2. Cells were stained with H&E. Bars, 80 μm (i, ii, v); 20 μm (iii, iv).

(b) Frequencies of abnormal spleen follicles at 6 weeks p.c. Shared letters above columns indicate no significant difference (P>0.05), whilst different letters indicate significant differences among the groups (P<0.05) (n=5).

Table 3. Viraemia in mouse serum measured by quantitative PCR

Different superscripts within columns represent significantly different PCV2 loads for each week p.c. (P<0.05). A load value of 0 is equal to <10^3 copies ml^-1 (the threshold of sensitivity of the quantitative PCR).

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice with viraemia/no. tested (mean log PCV2 load ± sd) at the following weeks p.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>pORF2</td>
<td>2/5 (4.8 ± 4.1)*</td>
</tr>
<tr>
<td>Cap</td>
<td>4/5 (5.7 ± 4.5)†</td>
</tr>
<tr>
<td>Cap/pORF2</td>
<td>2/5 (5.2 ± 4.3)*</td>
</tr>
<tr>
<td>pORF2/Cap</td>
<td>1/5 (4.8 ± 4.1)*</td>
</tr>
<tr>
<td>Control</td>
<td>5/5 (7.0 ± 5.9)†</td>
</tr>
</tbody>
</table>
PCV2 Cap protein is related to the induction of PCV2-neutralizing antibody (McNeilly et al., 2001; Zhou et al., 2005a). To date, the protective mechanism of humoral immunity against PCV2 infection has not been fully characterized. It is generally accepted that PCV2-specific antibodies are associated with protection, as field evidence suggests that the decrease in antibodies contributes to the development of PMWS (Allan & Ellis, 2000; Allan et al., 1998b; McIntosh et al., 2006). However, in some cases, the serum antibody level does not appear to influence PMWS occurrence (Carasova et al., 2007; McIntosh et al., 2006). During the PCV2 infection period in this experiment, the IgG2a antibody in the pORF2-immunized mice exhibited higher titres than in the Cap-vaccinated mice (Fig. 3). Correspondingly, we found that the tendency of VN antibody to protect against PCV2 infection coincided with Cap-specific IgG2a but not with total IgG or IgG1 (Figs 3 and 4, Table 2). Therefore, it is reasonable to suggest that VN antibody against PCV2 is composed mainly of IgG2a antibody against Cap protein, and that this Cap-specific neutralizing IgG2a antibody plays an important role in protecting against PCV2 infection. In fact, VN antibodies against dengue virus (Simmons et al., 2001) and herpes simplex virus type 1 (McKendall & Woo, 1988) have also been shown to comprise primarily IgG2a antibody in mice. A recent study reported that recombinant adenovirus expressing the Cap protein generated VN antibody titres ranging from 1:8 to 1:17 in mice (Wang et al., 2006), and all plasmids encoding Cap protein with different subcellular localizations induced VN antibody titres of <1:10 in mice (Fan et al., 2008). Surprisingly, in our experiment, no VN antibody against PCV2 was detected in the immunized mice before virus challenge. This may have been due to the threshold of the virus neutralization test used in our study, which failed to detect VN titres of <1:20. Therefore, a method for improving the induction of VN antibody against PCV2 requires further study.

Th1 cells, which produce interleukin (IL)-2 and gamma interferon (IFN-γ), induce the activation of macrophages, delayed-type hypersensitivity and the production of IgG2a (Abbas et al., 1996; Mosmann & Coffman, 1989a). Unmethylated CpG motifs in the plasmid are one of the pathogen-associated molecular patterns that tend to drive Th1 immune responses via Toll-like receptor 9 (Hemmi et al., 2000). Presently, at least one CpG-containing sequence has been identified as a potent inducer of IFN-α production in PCV2 ORF2 (Hasslunger et al., 2003). In contrast, Th2 cells, which predominantly produce IL-4, IL-5, IL-10 and IL-13, promote the development of eosinophilia as well as the generation of IgG1 and IgE antibody isotypes (Abbas et al., 1996; Mosmann & Coffman, 1989a). Based on the analysis of IgG1 and IgG2a in the immunized mice, our data showed that the pORF2 plasmid and Cap protein induced Th1- and Th2-biased immune responses, respectively, which is similar to reports on other viruses described previously (Ruitenberg et al., 2000; Sin et al., 1999; Tanghe et al., 2001; Zakharchouk et al., 2005). Histopathological examination and real-time PCR results, particularly in the protection assay, also showed that mice immunized using the pORF2, pORF2/Cap and Cap/pORF2 protocols had milder microscopic lesions and lower serum PCV2 loads than the Cap-immunized mice. These results suggest that the Th1-biased immune response and possible cytokines conferred by the pORF2 plasmid benefit PCV2 control. It has been shown that immunity associated with the Th1 response is essential for cytotoxic T lymphocyte production. CD4+ Th1 cells mediate the killing of organisms responsible for a variety of intracellular infections through the production of IFN-γ (Del Prete, 1998; Ogg et al., 1998; Seder & Hill, 2000).

In summary, we have characterized the protective immunity against PCV2 by ORF2-based DNA and subunit vaccines in mice. Our data revealed that Cap-specific CD8+ cells and VN antibody responses, which generally correlated with Cap-specific IgG2a, play crucial roles in protecting against PCV2 infection. To our knowledge, this is the first report of a comparative study involving the cellular and humoral immune responses and protective immunity against PCV2. Our results provide insight for further research on host protective immunity against PCV2 infection.

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