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The protective immune response against Pseudorabies virus induced by DNA vaccination is impaired if the plasmid harbors a functional *Porcine circovirus* type 2 *rep* and origin of replication

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ABSTRACT

A plasmid rendered replicative in mammalian cells by inserting the *Porcine circovirus* 2 (PCV2) origin of replication and replicase gene (Ori-*rep*) has been previously constructed. The aim of the present study was to evaluate if the replication capacity of this plasmid could be advantageously used to improve the protective immunity induced by DNA vaccination. In this case we used the porcine Pseudorabies virus (PrV) DNA vaccination model. The replicative capacity of the DNA vaccine did not improve the protective immunity against PrV in pigs, but on the contrary the presence of the PCV2 Ori-*rep* sequence was harmful in the induction of this immunity compared to an equivalent but non-replicative DNA vaccine. In addition, the distribution and the persistence of the replicative and non-replicative plasmids inside the body were the same. This is the first study showing an *in vivo* deleterious effect of the replicative active PCV2 Ori-*rep* on the natural and specific protection against PrV infection.

1. Introduction

DNA vaccination, a potential alternative to conventional vaccination, consists of the injection of circular plasmids that encode the vaccinating antigen, this antigen being synthesized within the injected body. These plasmids do not replicate in mammalian cells. For example, we and others have developed a model of DNA vaccination of pigs against swine alpha herpesvirus Pseudorabies virus (PrV) infections (Dory et al., 2005b; Dufour et al., 2000; Gerdts et al., 1999; van Rooij et al., 2000). PrV causes Aujeszky's disease, a serious illness characterised by nervous disorders, respiratory distress, severe weight loss, high morbidity and mortality leading to severe economic losses in the pig industry (Mettenleiter, 2000). Among the strategies tested to improve the effectiveness of DNA vaccines, efforts have been made to increase the quantity of messenger RNAs encoding the vaccinating antigen (Dubensky et al., 1996). With this strategy for example, improvements of anti-herpes simplex virus (Hariharan et al., 1998), anti-*Mycobacterium tuberculosis* (Kirman et al., 2003) and in our hands anti-PrV (Dory et al., 2005b) vaccinations were obtained. These results emphasize the value of multiplying the vaccinating molecule inside the cells. Recently, we rendered a plasmid replicative in porcine cells *in vitro* by inserting the *Porcine* circovirus type 2 (PCV2) origin of replication and replicase gene (Ori-*rep*) (Faurez et al., 2010). Indeed, the PCV2 genome, which is a circular, single-stranded, positive-sense DNA, replicates by a rolling circle mechanism (for review see (Faurez et al., 2009)). PCV2, a member of the genus *Circovirus*, family *Circoviridae*, affects swine and wild boars and is the causal agent of post-weaning multisystemic wasting syndrome (PMWS), associated with several diseases designated as *P. circovirus*-associated diseases (PCVD or PCVAD) (Gillespie et al., 2009; Madec et al., 2008).

The aim of the present study was to assess whether this replicative plasmid could be advantageously used in a DNA vaccine formulation against PrV infection in pigs. For this purpose, the PrV-gC gene was used as the vaccinating antigen. PrV-gC, a 479 amino-acid protein, is involved in the binding of the virus to heparan sulfate proteoglycans located on the surface of target cells. Contrary to PrV-gB and PrV-gD, PrV-gC is not involved in immune evasion (Favoreel et al., 1999; Van de Walle et al., 2003). The resulting plasmid was called pOri-*rep*-gC. The protective immunity induced by this vaccine was compared to the one induced by the same plasmid rendered non-replicative (=pOri-*rep*^{KO}-gC). Furthermore, the body

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distributions of pOri-*rep*-gC and pOri-*rep*^{KO}-gC were compared over time.

2. Materials and methods

2.1. Constructs and plasmids

A pcDNA3-based replicative plasmid harboring the PCV2 origin of replication and the *rep* gene (Fig. 1A) sequence has been previously constructed by us (Faurez et al., 2010). The PrV NiA3 strain gC sequence (Dory et al., 2005a; Gravier et al., 2007b) was introduced in the multicloning site to generate the pOri-*rep*-gC plasmid (Fig. 1B). Two mutations in motif III of the *rep* (Y96 → F96 and S98 → A98) (Fig. 1C) were introduced using the QuickChange XLsite mutagenesis kit (Stratagene-Agilent Technologies, Waldbronn, Germany) and the following primers: 5′-ACTCCATCAGTA AGTTGCCTTCTTTA**GC**GCA**AA**ATTCTTTATTCTGCTGATC- TGTTCC-3′ and 5′-GGAACAGATCAGCAGAATAAAGAAT**TT**TGC**GC-** TAAAGAA GGCAACTTACTGATGGAGT-3′. The exchanged nucleic acids are underlined. The resulting plasmid was named pOri-*rep*^{KO}-gC (Fig. 1B). The expression of PrV-gC was verified by immunoperoxidase monolayer assay (IPMA) staining of transfected PK15 cells using a serum of a PrV-infected pig as described (Gravier et al., 2007b). The replication rates of the different constructs were evaluated as described (Faurez et al., 2010). The quantitative real-time PCR-based replication assay was based on the methylation status of plasmids transfected into eukaryotic cells. Methylation status depends on whether or not replication has occurred within eukaryotic cells. Dam-methylation of the GATC site occurs in input plasmids (i.e. generated in prokaryotic cells), whereas the GATC site is not methylated in plasmids that replicate in eukaryotic cells. Therefore, to differentiate replicated from non-replicated plasmids, low molecular-weight DNA extracted from PK15 cells was incubated either with DpnI that cuts the dam-methylated GATC site or with MboI that, on the contrary, cuts the non-methylated GATC site. To reduce the background level that is observed in quantitative real time PCR due to incompletely digested DNA, Exonuclease III (ExoIII) was added to digest any incompletely cut DNA. This quantitative PCR-based method is based on the discriminative quantitation of *Mbo*I-resistant, non-replicated input plasmids and *Dpn*I-resistant, replicated plasmids. Briefly, 4.5×10^5 of PK15 cells were plated onto 6-well tissue culture plates. Twenty-four hours later, PK15 cells were co-transfected with 10 ng of plasmids of interest and with 1 μg of pcDNA3-Rep used as a replication booster. Twenty-four hours later, plasmids were extracted using the QIAamp MinElute Virus Spin Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and incubated either with *Dpn*I + ExoIII or with *Mbo*I + ExoIII. Thereafter, quantitative PCR was performed on both digested products in an ABI Prism 7000 SDS (Applied Biosystems, Foster City, USA) using the SYBR Green Master Mix (Applied Biosystems, Foster City, USA) and 300 nM of the following primers to amplify a fragment of the neomycin resistant gene: 5′GCTCCTGCCGAGAAAGTATCC3′ and 5′TTCGCTTGGTGGTCGAATG3′. qDE represents the quantity of plasmids after *Dpn*I + ExoIII digestion thus the replicated plasmids. qME corresponds to the quantity of plasmids after *Mbo*I + ExoIII digestion corresponding to input plasmids. The replication rate expressed as a percentage was calculated by using the following formula: qDE/(qME + qDE). The assay was performed 3 times. Rep and Rep' mRNA productions were assessed by RT-PCR 24 h after the transfection of PK15 cells. The primers and RT-PCR conditions were those previously described (Faurez et al., 2010). For the animal experiments, each plasmid was amplified into an *Escherichia coli* DH5α strain and purified using the NucleoBond® Xtra Maxi Endotoxin Free kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. Potential endotoxin contaminations were researched using the E-TOXATE™ (*Limulus* Amebocyte Lysate) test kit according to the manufacturer's instructions (Sigma–Aldrich, Saint-Louis, MO).

2.2. Animal experiments

Specific pathogen-free (SPF) pigs were housed and treated in accordance with local veterinary office regulations ("*Direction Départementale de la Protection des Populations des Côtes d'Armor*", France).

2.2.1. Experiment #1: protection against PrV infection

Four groups of eight, 8-week-old pigs weighing 25.0 + 2.6 kg were i.m. (right neck) injected twice with 200 μg of pOri-*rep*-gC, pOri-*rep*^{KO}-gC, pOri-*rep* or empty pcDNA3, respectively, using 0.8 × 40 mm needles. A 3-week interval between injections was applied. Three weeks later, pigs were intranasally infected with 3.16×10^4 $TCID_{50}$ of the NIA3 PrV strain per nostril (Gravier et al., 2007b). A fifth group of non-injected and non-challenged pigs was used as a control group. Mortality rate and body temperature were assessed daily. Body temperature was also measured 4 h after each injection of plasmids. Behavioral symptoms and respiratory distress were scored daily 0, 1, 2 or 3 for no, mild, intermediate or severe symptoms, respectively. Mean relative daily weight gain during the 7-day post-challenge period was determined (Stellmann et al., 1989). The injected muscles and other organs were examined in pigs that died from Aujeszky's disease or were euthanized at the end of the assay.

2.2.2. Experiment #2: biodistribution of plasmids

Two groups of 18, 7–11 weeks-old pigs were i.m. injected with 200 μg of pOri-*rep*-gC or pOri-*rep*^{KO}-gC, respectively. A third group of 6 pigs received PBS. Blood, liver, spleen, kidney, ovaries (when available), lung, left pre-scapular lymph node, left non-injected muscle, right pre-scapular lymph node draining the injection site, injected muscle and brain samples were collected from 3 pigs of each plasmid group and 1 pig of the PBS group 10 min, 1 h, 24 h, 1 week, 3 weeks and 6 weeks after injection. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until quantification of plasmids.

2.3. Virus excretion

Nasal fluid samples were harvested by swabbing and added to 2 mL of Eagle's Minimum Essential Medium. Serial 10-fold dilutions were incubated with PK15 cells, and viral titers were determined 5 days later according to Kärber's calculation (Kärber, 1931).

2.4. Determination of PrV-specific IgG serum antibodies

Anti-PrV IgG serum antibody titers were determined by an indirect ELISA as described (Gravier et al., 2007b). Briefly, Maxisorb 96-well plates (Nunc, Naperville, IL) were coated with PrV glycoproteins and successively incubated with serial threefold dilutions of serum, with mouse anti-porcine IgG (Serotec Ltd, Oxford, UK), with peroxidase-labeled rabbit anti-mouse IgG (Jackson Laboratories, West Grove, Pennsylvania, USA) and finally with TMB (Pierce, Rockford, IL, USA). IgG titers ($\log_{10}$) were expressed as the highest dilution giving an OD value higher than the threefold OD of control sera from naïve pigs.

2.5. PrV neutralizing antibodies

Serum-neutralizing antibody (NAb) titers were determined as described (Gravier et al., 2007b). Briefly, 50 μL of twofold dilutions

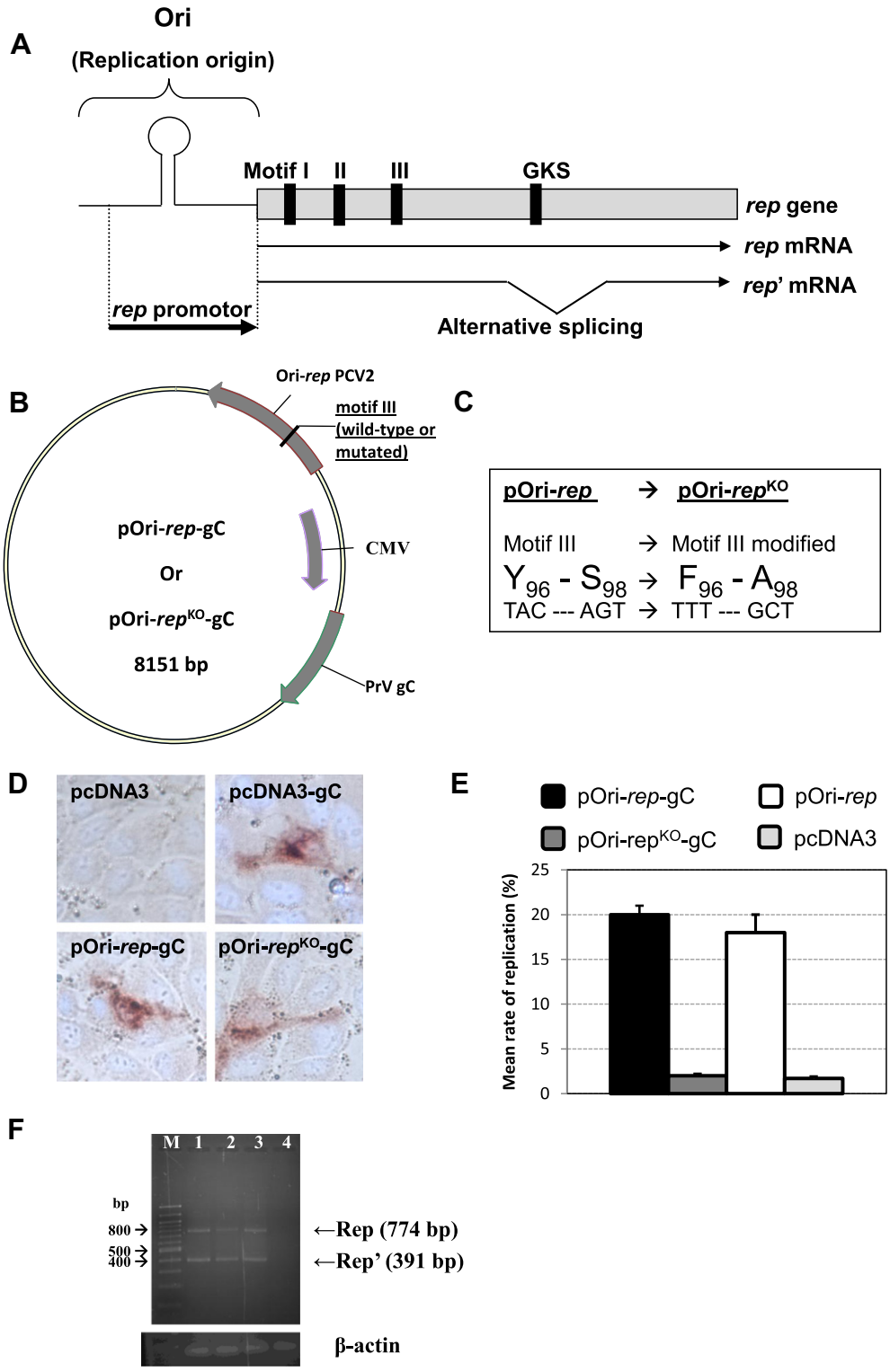

Fig. 1. Constructs (A) PCV2 Ori-*rep* DNA sequence with the origin of replication, the *rep* promotor and the *rep* gene. GKS is a glycine-rich sequence ended with glycine-lysine-serine involved in the formation of a P-loop nucleoside triphosphate-binding domain. (B) pOri-*rep*-gC and pOri-*rep*^{KO}-gC plasmids. (C) Mutations rendering the PCV2 Ori-*rep* DNA sequence unable to replicate (Ori-*rep*^{KO}). (D) IPMA on PK15 cells transfected with pcDNA3, pcDNA3-gC, pOri-*rep*-gC and pOri-*rep*^{KO}-gC and stained with a serum from a PrV-infected pig (magnification: ×40). Nucleus were counterstained with Hoescht solution. (E) Measurements of the *in vitro* replication of pOri-*rep*-gC, pOri-*rep*^{KO}-gC, pOri-*rep* and pcDNA3 in the presence of the replication booster pcDNA3-Rep. Error bars correspond to standard deviations. (F) Rep and Rep' mRNA expressions detected by RT-PCR in PK15 cells transfected with pOri-rep-gC (line 1), pOri-repKO-gC (line 2), pOri-rep (line 3) and pcDNA3 (line 4). B-actin mRNA was measured as an internal control. *M* is the 100 bp marker size.

of decomplemented serum were incubated with 50 μL of 100 $TCID_{50}$ of NiA3 PrV strain in 5% CO_2 in 96-well plates for 1 h at 37 °C. Then, 2.25×10^4 PK15 cells were added. Five days later, NAb titers (log_{10}) were expressed as the highest serum dilution inhibiting the cytopathogenic effect in 50% of the wells.

2.6. Expressions of porcine IL-4 and IFN-γ mRNA

Peripheral blood mononuclear cells (PBMC) were isolated from blood, re-stimulated or not for 16 h with the PrV strain NIA3 (multiplicity of infection 10), and total RNA was isolated as described using the 96 RNeasy kit (Qiagen, Hilden, Germany) (Dory et al., 2005a). Expression of porcine IFN-γ mRNA was determined by quantitative real-time polymerase chain reaction (RT-qPCR) using primers, probes and PCR conditions, as previously described (Dory et al., 2005a). For each sample, cytokine mRNA and β-actin mRNA threshold cycles (Ct) were determined simultaneously and relative quantities were determined according to the User Bulletin number 2 of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The amount of cytokine mRNA was normalized to the internal β-actin mRNA reference (ΔCt = cytokine Ct – β-actin Ct) and quantified with respect to the non-stimulated sample ΔΔCt = ΔCt of the stimulated sample – ΔCt of the non-stimulated sample) according to the equation $2^{-\Delta\Delta Ct}$.

2.7. Quantification of plasmids

DNA extractions were performed on 25 mg of tissue sample using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) after overnight proteinase K digestion or on 200 μL of serum using the QIAamp DNA MiniElute Virus Spin Kit (Qiagen) according to the manufacturer's instructions. qPCR was performed as described (Gravier et al., 2007a). Due to different DNA extraction efficiencies, qPCR was performed with 50 to 70 ng of DNA.

2.8. Statistical analysis

Clinical, immunological and biodistribution data were analyzed using the non-parametric Mann–Whitney test (Mann and Whitney, 1947) included in Systat 9 software (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

3.1. In vitro characterizations of the plasmids

PCV2 genome replicates using a rolling circle replication (RCR) strategy. The *rep* gene encodes the Rep and Rep' proteins. These two proteins form the RCR initiator protein complex that binds to the PCV2 stem loop structure, destabilizes the replication origin and initiates the RCR phenomenon (for review see (Faurez et al., 2009)). pOri-*rep*-gC is a pcDNA3-based plasmid carrying the PCV2 origin of replication (comprising the *rep* promotor) and *rep* gene (Fig. 1A) as well as the PrV-gC (Fig. 1B). pOri-rep^{KO}-gC corresponds to the plasmid pOri-*rep*-gC with two mutations in motif III of the *rep* gene (Fig. 1B and C), rendering this PCV2-based DNA molecule non-replicative (Faurez et al., 2010; Mankertz and Hillenbrand, 2002; Steinfeldt et al., 2007). Constructs were validated by sequencing. Moreover, porcine PK15 cells transfected with pOri-*rep*-gC, pOri-rep^{KO}-gC or pcDNA3-gC, but not with pcDNA3, stained equivalently with serum from a PrV-infected pig (Fig. 1D), indicating that PrV gC is expressed. The pOri-*rep*-gC and pOri-*rep* plasmids showed levels of replication of around 20% in PK15 cells when a replication booster corresponding to a plasmid encoding the PCV2 Rep protein was co-transfected (Fig. 1E). The pOri-rep^{KO}-gC and pcDNA3 plasmids were not able to replicate. Rep and Rep' mRNA were detected in similar levels in PK15 cells transfected with pOri-*rep*, pOri-*rep*-gC and pOri-rep^{KO}-gC plasmid (Fig. 1F). Endotoxin levels were below 0.1 EU/μg of DNA for each plasmid production.

3.2. PCV2 Ori-rep sequence impairs the clinical protection against PrV

One pig in the pOri-*rep* group had to be euthanized before the PrV challenge due to rectal prolapse. No body temperature was observed neither 4 h, nor 24 h after each injection of the plasmids.

No signs of Aujeszky's disease were observed in the non-injected and non-challenged group: no behavioral symptoms and respiratory distress, no mortality, no nasal excretion of PrV and the pigs gained 1.39 ± 0.23 kg/day/100 kg (Figs. 2 and 3 and Table 1). Starting day 7 after infection, pigs in the pOri-*rep* group presented severe behavioral symptoms and respiratory distress (Fig. 2A). From day 8 to day 10, the two remaining pigs presented clinical scores of 3, i.e. the highest score possible. During the 1st week after PrV-infection, the pigs lost 1.92 ± 0.40 kg/day/100 kg (Table 1). All the pigs presented a body temperature superior to 40 °C and 4 out of 7 presented a body temperature superior to 41 °C (Table 1). Nasal excretion of PrV was detected from day 4 to day 11 post-infection (Fig. 3). All the pigs in this group died between day 6 and 13 post-infection (Fig. 2B and Table 1). Pigs injected with pcDNA3 were less affected by the PrV-infection. The pigs presented less severe behavioral symptoms and respiratory distress, and this is particularly true at day 7 post-infection ($p < 0.05$) (Fig. 2A). Thereafter, the symptoms are still lower, but due to the small number of pigs at this time point, the difference between these 2 groups was not anymore significant. Only 5 out of 8, i.e. 62.5% died (Fig. 2B and Table 1). There were no differences in terms of body weight loss and body temperature between the pOri-*rep* and pcDNA3 groups. Nasal excretion of PrV was detected in the pcDNA3 group from day 4 to day 8 post-infection, but not at day 11 (Fig. 3).

Pigs in the pOri-*rep*-gC group presented a significant peak of mild to intermediate clinical signs at day 6 post-infection (when compared to pOri-rep^{KO}-gC, pcDNA3 and no injection and no challenge, but not to pOri-*rep*, Fig. 2A), then these signs became blurred. They lost 1.30 ± 0.56 kg/day/100 kg during the first week of infection, but this was not significantly less than for the pOri-*rep* and pcDNA3 groups. Notably, 2 pigs presented a body temperature superior to 42 °C. Nasal excretion of PrV was detected from day 4 to day 11 post-infection (Fig. 3). Four out of 8 pigs, i.e. 50%, died after the PrV-infection (Fig. 2B and Table 1). In the pOri-rep^{KO}-gC group, pigs presented mild behavioral symptoms and respiratory distress significantly lower than the ones observed in the pOri-*rep*-gC group at day 6 post-infection ($p < 0.05$). There were no significant differences in terms of body weight loss and temperature between pOri-*rep*-gC and pOri-rep^{KO}-gC groups. As for the pcDNA3 group, nasal excretion of PrV was detected in the pOri-rep^{KO}-gC group from day 4 to day 8 post-infection, but not at day 11 (Fig. 3). In terms of mortality, the pOri-rep^{KO}-gC group presented the lowest mortality rate of 25% since only 2 out of 8 pigs died (Fig. 2B and Table 1). At day 13 post-infection, the difference of survivors between the pOri-*rep* group and the two groups encoding PrV-gC (pOri-*rep*-gC and pOri-rep^{KO}-gC) were significant ($p < 0.05$) (Fig. 2B).

3.3. PCV2 Ori-rep sequences modify the PrV-specific production of IFN-γ mRNA, but not the PrV-specific humoral immune response

Specific IgG-type and neutralizing antibodies against PrV were not detected in the 4 injected groups until day 4 after PrV-challenge (Fig. 4A and B). Roughly similar levels of these antibodies were detected on day 11 after the PrV challenge in these four groups. In addition, only PBMCs from the four groups isolated 11 days after PrV infection and stimulated *in vitro* with PrV produced IL-4 mRNA (data not shown), which plays a key role in TH2 responses (Finkelman et al., 1988; Wood and Seow, 1996). IFN-γ mRNA, which has several immunoregulatory roles and

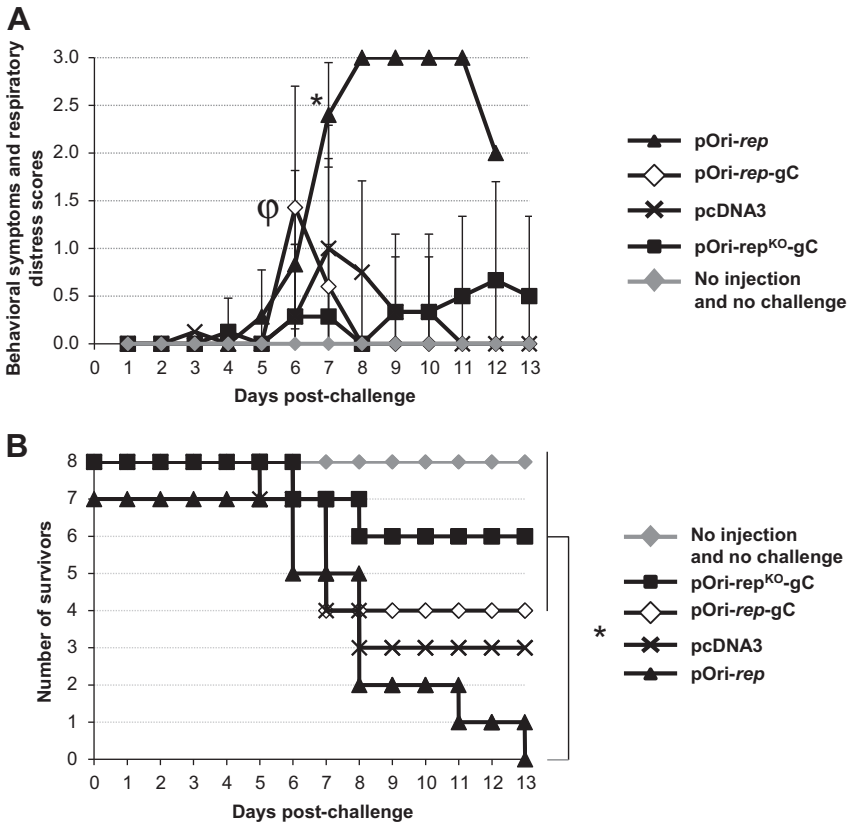

Fig. 2. Clinical protection of pigs against PrV-infection. Pigs were injected twice with the plasmids pOri-*rep*-gC, pOri-*rep*^{KO}-gC, pOri-*rep* or pcDNA3. They were then subjected to a virulent PrV-challenge and clinical parameters were observed daily. A fifth group of non-injected and non-challenged pigs was observed in parallel. Behavioral symptoms and respiratory distress (A) and mortality (B) were observed at different times after PrV-challenge. Behavioral symptoms and respiratory distress were scored as indicated in the materials and methods section. Error bars correspond to standard deviations. $^{*}p < 0.05$ compared to all the other groups. $^{\varphi}p < 0.05$ compared to pOri-rep^{KO}-gC, pcDNA3 and no injection and no challenge, but not to pOri-*rep*.

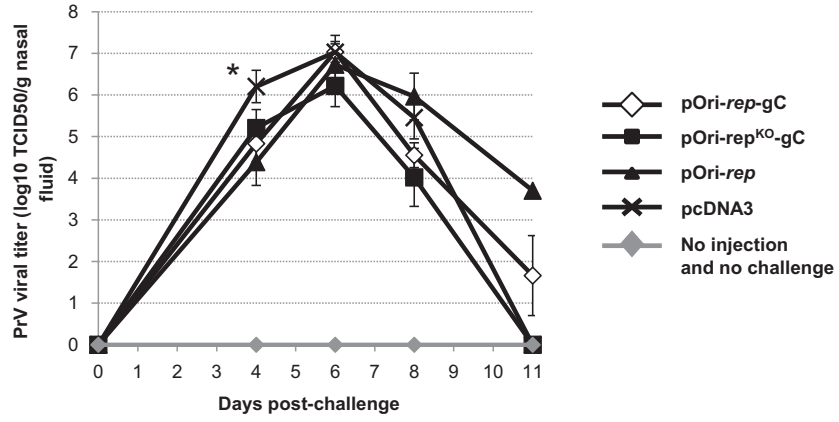

Fig. 3. Nasal excretion of PrV after PrV infection. Pigs were injected twice with the plasmids pOri-*rep*-gC, pOri-*rep*^{KO}-gC, pOri-*rep* or pcDNA3. They were then subjected to a virulent PrV-challenge. A fifth group of non-injected and non-challenged pigs was observed in parallel. Nasal excretions of PrV were measured at different times after PrV-challenge in nasal fluids and were expressed as $\log_{10}$ TCID50/g of nasal fluid. Error bars correspond to standard deviations. $^{*}p < 0.05$.

effector functions involved in TH1 responses (Wood and Seow, 1996), started to be detected 4 days after PrV infection only in the group injected with pOri-*rep*^{KO}-gC ($p < 0.05$) (Fig. 4C). At day 11 post-PrV, the four challenged groups produced IFN-γ mRNA, with the pOri-*rep*^{KO}-gC group seeming to produce more (but not significantly) of this cytokine mRNA (Fig. 4C). IFN-γ and IL-4 proteins were detected in supernatants of PBMCs isolated from the four challenged groups only at day 11 after PrV-challenge (data not shown). In summary, the cellular immune response against PrV, and not the humoral one, is delayed in the presence of the PCV2 Ori-*rep* sequence.

3.4. Similar body distribution and persistence of pOri-rep-gC and pOri-rep^{KO}-gC

The pOri-*rep*-gC and pOri-*rep*^{KO}-gC plasmids were i.m. injected once in pigs and were measured in blood and different organs at several times after injection (from 10 min to 6 weeks). No visible

Table 1
Mean relative daily weight gain during the 7 day post-challenge period (MRDG7) and post-PrV challenge body temperatures and mortality.

Group	MRDG7 ± SD (kg/100 kg/day)	Body temperature[a]			Mortality rate (%)
		>40 °C	>41 °C	>42 °C	
pOri-*rep*	−1.92 ± 0.40	7/7	4/7	0/7	100
pcDNA3	−1.71 ± 0.40	8/8	6/8	0/8	62.5
pOri-*rep*-gC	−1.30 ± 0.56	8/8	6/8	2/8	50
pOri-*rep*^{KO}-gC	−1.28 ± 0.40	8/8	6/8	0/8	25
No injection, no challenge	+1.39 ± 0.23	0/8	0/8	0/8	0

[a] Number of pigs at the given body temperature/number of pigs in the group.

signs of local adverse reactions were observed in any of the injected muscles and at any time-point. The 2 plasmids were found at similar levels in the injected muscle (Fig. 5). The highest concentrations (around 10^9 copies/μg DNA) were found at 10 and 60 min after injection. Thereafter, concentrations decreased gradually and similarly, reaching a very low level at day 21 and an undetectable level at day 42.

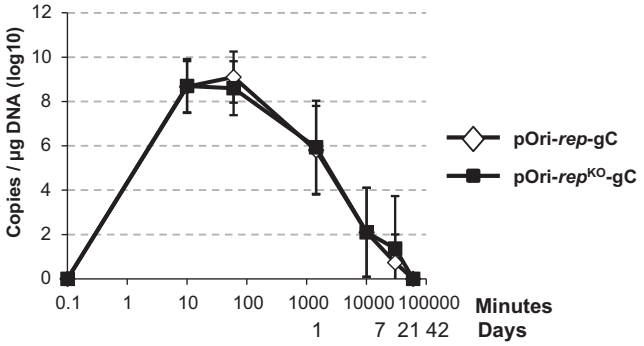

Fig. 5. Detection of pOri-*rep*-gC and pOri-*rep*^{KO}-gC in injected muscles from 10 min to 42 days after a single intramuscular injection. Pigs were injected once with pOri-*rep*-gC or pOri-*rep*^{KO}-gC. At different time points after injection, injected muscle samples were obtained from groups of 3 pigs. DNA was extracted from these samples and subjected to quantitative PCR specific of the injected plasmids. Results are presented as number of copies of plasmid/μg of total DNA ± standard deviations over the time.

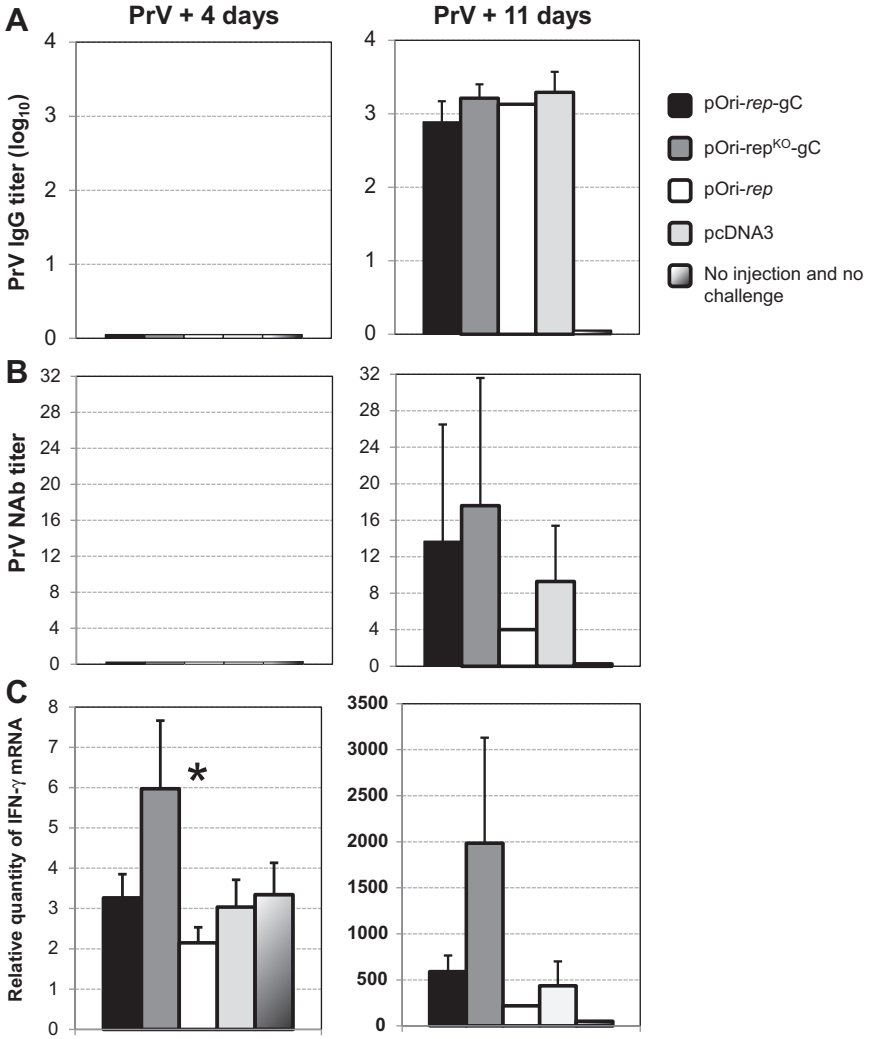

Fig. 4. Immune responses of pigs after injection of plasmids and PrV-challenge Pigs were injected twice with the plasmids pOri-*rep*-gC, pOri-*rep*^{KO}-gC, pOri-*rep* or pcDNA3. They were then subjected to a virulent PrV-challenge. A fifth group of non-injected and non-challenged pigs was observed in parallel. Here are represented blood anti-PrV IgG1 type antibodies (A) and neutralizing antibodies (B) at days 4 and 11 after PrV-challenge. PBMCs were also isolated at 4 and 11 days after PrV-challenge and incubated or not with live PrV for 16 h. Quantity of IFN-γ mRNA in response to PrV-stimulation was normalized to the internal β-actin mRNA reference and quantified with respect to the non-stimulated cells at days 4 and 11 after PrV-challenge (=relative quantity of IFN-γ mRNA) (C). Standard deviations are presented in each graph. $^*p < 0.05$.

Both plasmids were transiently detected at similarly low levels (maximum: 10^4 copies/μg DNA) up to 60 min in the blood, the kidney, the pre-scapular lymph node draining the non-injected muscle and the lung, and until day 1 in the pre-scapular lymph node draining the injected muscle, the liver, the spleen, the brain and the opposite muscle (data not shown). Plasmids were not detected in the ovaries (only 1 female per condition). No plasmids were detected in any of the organs of the pigs of the PBS group (data not shown).

The quantities of plasmids extracted from the injected muscles 24 h after injection were too low (10^3 copies) to determine whether replication occurred or not *in vivo*. To do so, at least 10^5 - copies of plasmids are needed (Faurez et al., 2010).

4. Discussion

It was previously shown that the use of a plasmid that enhances the quantity of mRNAs encoding the vaccinating antigen was beneficial for several DNA vaccination models (Dubensky et al., 1996; Hariharan et al., 1998), including the one against PrV-infection in pigs (Dory et al., 2005b). The idea here was to take advantage of a replicative plasmid in mammalian cells we have developed (Faurez et al., 2010) to improve DNA vaccination efficacy. In fact, with this strategy, the whole plasmid replicates, multiplying therefore the number of gene copies encoding the vaccinating antigen. The plasmid was rendered replicative by harboring the PCV2 origin of replication (containing the replicase promotor) and replicase gene (Ori-*rep*). For this purpose, we choose to use the porcine DNA vaccine model against PrV-infection currently developed in our laboratory (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007b).

In the present study, we choose to use PrV-gC as vaccinating antigen, since it confers only partial protection when it is carried by conventional plasmids (Gerdts et al., 1997), and a moderate PrV-challenge (confirmed by the fact that 3 pigs out 8 survived in the group injected with empty pcDNA3, the negative control group). Under these conditions, there is room to observe potential beneficial effects of the novel DNA vaccination strategy tested. Six out of 8 pigs survived PrV-infection when they received the non-replicative DNA vaccine encoding PrV-gC (i.e. pOri-*rep*^{KO}-gC), which is therefore the protection of reference within this study. It is nevertheless note wise that behavioral symptoms and respiratory distress are not statistically different between the pOri-*rep*^{KO}-gC group and the pcDNA3 group. These clinical parameters do not take into account the mortality of the pigs. If mortality at day 8 is being taken into account and a score of 4 is attributed to the dead pigs, pigs injected with pOri-*rep*^{KO}-gC are more protected against PrV-infection than pigs of the pcDNA3 group ($p = 0.009$). Furthermore, at the end of the assay, 6 pigs out of 8 were still alive in the pOri-*rep*^{KO}-gC group, and only 3 out of 8 in the pcDNA3 one. Taking all these parameters together, there is a beneficial effect of PrV-gC on the protective immunity against PrV. For the pigs vaccinated with pOri-*rep*-gC, Aujeszky's disease was more severe and closer to one observed for the pcDNA3 group: increased mortality and behavioral symptoms and respiratory distress and some pigs presented a fever superior to 42 °C. Similarly, infection of pigs with PCV2 virions also moderately influences the induction of protective immunity against classical swine fever virus (CSFV) induced by vaccination (Huang et al., 2011). Furthermore, in our study, the period of the viral excretion tended to be prolonged in the presence of the active PCV2 Ori-*rep* sequence (pOri-*rep* and pOri-*rep*-gC groups) (superior to 11 days) compared to the pcDNA3 and pOri-*rep*^{KO}-gC groups (inferior to 11 days), although this effect does not have statistical support. Comparing the (pOri-*rep*-gC + pOri-*rep*) groups to the (pOri-*rep*^{KO}-gC + pcDNA3) groups, pigs receiving the PCV2 Ori-*rep* sequence excreted PrV for a significant longer period after the PrV challenge ($p < 0.05$). In previous studies with the same PrV-strain, the duration of viral excretion was lower than 10 days (Dory et al., 2005a,b), and therefore was more similar to what was observed in the pcDNA3 and pOri-*rep*^{KO}-gC groups. Therefore, pigs receiving the pOri-*rep* cannot effectively control the proliferation and the spread of PrV. The extension of the viral excretion period is not always correlated with the decreased survival. In fact, the single pig still alive at day 11 in the pOri-rep group still excreted viruses. He died two days later. On the other hand, pigs of the pOri-rep-gC showed also an extension of the viral excretion, but they survived.

From an immunological point of view, there were no differences in the production of neutralizing and specific antibodies against PrV; these antibodies were detected only at day 11 after the challenge. Furthermore, after *in vitro* incubation of PBMCs with live PrV, no differences were observed in the production of IL-4 mRNAs or proteins, which play a key role in TH2 responses (Finkelman et al., 1988; Wood and Seow, 1996). In the CSFV model, PCV2 infection induced a transient delay in the establishment of the humoral immune response, not an attenuation of it (Huang et al., 2011). Therefore, PCV2 has no or few impact on the development of the humoral immune response against PrV or CSFV, respectively.

On the other hand, the production of IFN-γ mRNA in PBMCs incubated *in vitro* with live PrV was observed only in the pOri-*rep*^{KO}-gC group, not the pOri-*rep*-gC one, when these cells were isolated 4 days after PrV-challenge. This cytokine has several immunoregulatory roles and effector functions involved in TH1 responses (Wood and Seow, 1996). This response is observed early in the PrV-immunization process (Dory et al., 2007), and is necessary, although not sufficient, to participate in the effective clinical protection of pigs (Dory et al., 2006). Here, the absence of IFN-γ production coincides with the worsening of symptoms and higher pig mortality in the pOri-*rep*-gC group. By day 11 post-challenge, all the PBMCs isolated from the 4 PrV-challenged groups produced IFN-γ response to PrV stimulation. PCV2 inhibited partially the cytotoxic immune response against CFSV (Huang et al., 2011).

In addition, not only the memory response, but also the innate response to PrV infection is affected by the presence of the replicative-active PCV2 Ori-*rep* sequence. All the pigs died in the pOri-*rep* group at the end of the assay. This is correlated with a dramatic increase in behavioral symptoms and respiratory distress starting day 6 post-infection. Furthermore, as in the pOri-*rep*-gC group, the PrV excretion seemed to last longer compared to the pcDNA3 group.pOri-*rep*-gC or pOri-*rep*^{KO}-gC were still detected in the injected muscles 3 weeks after their injections, i.e. at the time of PrV challenge. PCV2 has been shown to be detrimental to the *in vitro* innate response to PrV (Hasslung et al., 2003) and to *in vitro* PrV recall antigen immune responses (Kekarainen et al., 2008a,b). More importantly, the PCV2 DNA molecules (Vincent et al., 2007), and more specifically CpG DNA sequences (Hasslung et al., 2003; Kekarainen et al., 2008a), are essential players in the detrimental role of PCV2. Virus-like particles corresponding to the structural PCV2 capsid protein has no effect on the *in vitro* PrV-induced response (Kekarainen et al., 2008a), showing that really the DNA of PCV2 is essential in this detrimental role.

The pOri-*rep* and pOri-*rep*-gC plasmids, i.e. with the Ori-rep sequence, showed levels of replication of around 20% in PK15 cells when a replication booster corresponding to a plasmid encoding the PCV2 Rep protein was co-transfected. For the *in vivo* assays, we did not co-inject the replication booster, in order to study only the effect of the replicative DNA molecule on the induction of the protective immunity against PrV. In the absence of the replication booster, no replication could be detected in PK15 cells (Faurez et

al., 2010). Therefore, the replication rate *in vivo*, if replication occurs, may be very low, and this may explain why we could not either detect replicated plasmids, nor show that the quantity of plasmid increases in the body with the time. Nevertheless, some elements would indirectly support the occurrence of *in vivo* plasmid replication. The PCV2 *rep* gene and its origin of replication contain CpG sequences that have deleterious effects on the production of IFN-γ by PMBCs stimulated with PrV (Kekarainen et al., 2008a). To knock-out the replicative capacity of the PCV2 Ori-*rep* sequence, four nucleotides were substituted in the motif III sequence, resulting in the introduction of one additional and detrimental CpG (5′-TGCGCT-3′) (Kekarainen et al., 2008a). Therefore, if the injected plasmid is directly responsible for the attenuation of the protective response against PrV infection, the protection induced by pOri-*rep*^{KO}-gC would be worse than the one induced by the pOri-*rep*-gC plasmid (since the same quantity of plasmids in different organs was detected throughout the assay). However, the opposite result was observed. We can speculate that the replicated DNA may be the intermediary that causes the negative effect in PrV immunization. In fact, during the replication of the PCV2 genome, two forms of DNA molecules are produced. The replicated PCV2 DNA form is a double-stranded DNA molecule that has been described to modulate the production of IFN-α (Vincent et al., 2007). The infectious form of PCV2 is a single-stranded DNA molecule. Therefore, it is possible that the replication of the plasmid induces a modulation of the production of cytokines, and in particular IFN-α, this modulation being potentially involved in the modification of the induction of the protective immune response against PrV. Moreover, we cannot exclude the possibility that the PCV2 wt Rep protein acts as a deleterious protein for the induction of immune responses against PrV. From a gene expression point of view, the Rep and Rep' mRNA were expressed in similar manner if the *rep* gene was mutated or not. Co-expression of *rep* gene and gC seems not to be involved in the overall differences in ability of DNA vaccine to elicit protection against PrV since the presence of the Ori-rep sequence did also modified the natural protective immune response against PrV. On the other hand, the mutation in motif III of the Rep proteins (YCSK → FCAK) could be involved in the modification of an interaction between the PCV2 Rep or Rep' with components of the immune system. However, there are no data available in the literature to support this suggestion. Therefore, the co-expression of gC and Rep genes seems not to contribute to the overall differences in ability of the DNA vaccines to elicit protection against PrV. Finally, ORF3, that encodes a protein involved in apoptosis (Liu et al., 2007) and production of TNF-α (An et al., 2008), is not modified by the knock-out of the *rep* function. It can be possible that, in the case of the replicative plasmid, more proteins encoded by ORF3 are produced, which then may be deleterious for the induction of protection against PrV.

In conclusion, negative effects of the presence of a PCV2 Ori-*rep* sequence in a DNA vaccine against PrV on the specific and innate protective responses against PrV infection were observed in pigs. To our knowledge, this is the first study showing such a deleterious effect *in vivo*. Additional studies are now needed to explain why this phenomenon was observed. In fact, this can be due either to the presence of replicated plasmid DNA, to the presence of the Rep and Rep' proteins or to the expression of the PCV2 ORF3. The induction of protection against PrV first involves the induction of the cellular immune response, followed by the humoral immune response (Dory et al., 2007). In the presence of PCV2 Ori-*rep* DNA, the cellular immune response, but not the humoral response, induced by vaccination or by the natural innate immune response can be negatively modulated. This can be deleterious if the cellular response is essential to fight the pathogen (such as PrV), but will have no or few consequences if the humoral immune response is essential.

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