The Veterinary Journal 197 (2013) 438–444

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Comparison of different vaccination schedules for sustaining the immune response against porcine reproductive and respiratory syndrome virus

I. Díaz [a,*,1], M. Gimeno [a,b,1], A. Callén [c], J. Pujols [a,d], S. López [a], C. Charreyre [e], F. Joisel [e], E. Mateu [a,b]

[a] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[c] Merial laboratorios, S.A., Barcelona, Spain
[d] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain
[e] Merial SAS, Lyon, France

ARTICLE INFO

Article history:
Accepted 7 February 2013

Keywords:
Porcine reproductive and respiratory syndrome
Vaccination
Cell-mediated immunity
Neutralizing antibodies

ABSTRACT

In order to better understand how immunization against porcine reproductive and respiratory syndrome virus (PRRSV) can be improved using commercial vaccines, different strategies of immunization were applied in the field using an inactivated vaccine (INV), a modified live vaccine (MLV) or a combination of the two and the responses compared. In experiment 1 (E1), 21 piglets were distributed in three groups. Group A was vaccinated with a commercial INV at 2.5, 3.5 and 6.5 months old; group B pigs received the INV at 1.5, 2.5, 5.5 and 6.5 months old, while pigs in group C were kept as unvaccinated controls. At 7.5 months of age all pigs were challenged with PRRSV and followed for 21 days. In experiment 2 (E2), 32 piglets were distributed evenly in four groups. Groups A, B and C were vaccinated with a commercial MLV at 1.5 months old, while group D pigs were kept as controls. At 4.5 months old, groups A and C received the INV while B received a second MLV, 1 month later group C pigs received a third INV. At 6.5 months old all pigs were challenged as in E1. In both experiments, total antibodies, neutralizing antibodies (NA) and cell-mediated immunity (CMI) were evaluated, and viraemia was determined after challenge.

In E1, immunization with an INV induced high interferon-γ responses after the second and subsequent vaccinations. Development of NA after challenge was faster in INV vaccinated pigs compared to unvaccinated controls. In E2, re-vaccination with INV induced NA responses similar to re-vaccination with MLV; however, a significant increase in NA titres after challenge was only detected in group C pigs. The use of combined protocols (MLV + INV) was superior to the use of MLV alone in inducing cell mediated immunity. In conclusion, the highest immune responses against PRRSV after a single shot were achieved with MLV; after that, INV re-vaccination should be considered as the best strategy to induce significant boosters.

Introduction

More than 20 years after the first emergence in North America of porcine reproductive and respiratory syndrome virus (PRRSV) it is still one of the most important infectious agents in pigs. PRRSV belongs to the genus Arterivirus and currently comprises two genotypes: I (formerly known as European) and II (formerly known as American) (King et al., 2012). When it affects sows, PRRS is characterized by abortions, stillbirths and mummification of fetuses and other reproductive disorders. In weaned and grower pigs, PRRS is a major component of the porcine respiratory disease complex (Chand et al., 2012).

Left to evolve by itself on a farm, PRRS becomes an endemic problem that frequently re-emerges as the proportion of non-immune sows increases or when a completely new and virulent strain is introduced onto the farm. For sows, it is only necessary to maintain a sufficient level of immunity against PRRSV to minimise the consequences of infection. Commercial PRRS vaccines could induce sufficient immunity to minimise the development of reproductive disorders, at least for genotype I strains. However, protection from commercial vaccines is often only partial because of the diversity of PRRSV genotypes and strains within a given genotype (Mengeling et al., 2003; Labarque et al., 2004). New vaccination strategies are needed to achieve an acceptable level of immunity that could partly overcome the genetic diversity (Díaz et al., 2012).

* Corresponding author. Tel.: +34 93581 4565.
E-mail address: ivan.diaz@cresa.uab.es (I. Díaz).
[1] These authors contributed equally to the work.

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http://dx.doi.org/10.1016/j.tvjl.2013.02.008

Modified live (MLV) and inactivated vaccines (INV) are commercially available for PRRSV genotype I. When choosing which vaccine to use in sows, the choice is between the higher safety but lower immunogenicity of INV against the greater immunogenicity (on a dose-by-dose basis) of MLV and the concern of using any viable virus in pregnant sows (Zuckermann et al., 2007). Selection of a vaccination schedule also strongly depends on how the gilts are acclimatized to the farm, particularly the age at which gilts are selected to be replacements and their potential contact with PRRSV before and after entering quarantine/acclimatization facilities.

It is generally accepted that one dose of INV is not sufficient to induce a significant immune response (Zuckermann et al., 2007); however, repeated INV vaccinations can induce a high cell-mediated immunity (CMI) (Piras et al., 2005). The immune responses in INV vaccinated pigs that were previously infected or which had been vaccinated using a MLV are also high (Nilubol et al., 2004, 2007). This has led to vaccination schedules combining MLV and INV being applied in the field against PRRSV, imitating schedules used in other species (Alexander, 2003).

In the present paper, different strategies for immunization of pigs against PRRSV were compared including the use of INV or MLV and their combinations in order to gain understanding on how acclimatization of gilts and revaccination of sows can be done as effectively and safely as possible using commercial vaccines, and also to ascertain the effect of repeated vaccinations on PRRSV-specific immunity.

Material and methods

All experiments were approved by the Commission for Ethics in Animals and Human Experimentation of the Universitat Autònoma de Barcelona (CEEAH) (approval number 665).

Design of experiment 1

Table 1 summarizes the design of experiments 1 (E1) and 2 (E2). In E1, 21 4-week-old piglets (Landrace × Large White) were randomly selected from a high health farm free of PRRSV and of all major pig diseases, including classical and African swine fever and Aujeszky's disease. Animals were moved to an experimental farm, ear-tagged and randomly (random numbers) distributed in three groups ($n = 7$ each). Pigs in group A were vaccinated intramuscularly at 2.5, 3.5 and 6.5 months old with 2 mL of a commercial PRRSV INV (Progressis, Merial: Strain P120, $\geqslant 2.5 \log 10$ antibodies titres by immunofluorescence after two administrations in laboratory conditions) adjuvanted with an oil-in-water adjuvant. Group B animals were vaccinated with the same INV at 1.5, 2.5, 5.5 and 6.5 months old and, group C animals received the adjuvant of the vaccine as a placebo each time that a vaccination was performed in the other groups.

Three weeks after the last vaccination, pigs were moved to the experimental biosafety level 3 facilities at Centre de Recerca en Sanitat Animal (CReSA) and kept for 7 days to allow adaptation to the new conditions. Then, at 7.5 months of age, pigs were intranasally challenged with 2 mL containing 1×10^6 tissue culture infective dose$_{50}$ ($TCID_{50}$) of the PRRSV strain 2749, 99% similar to Lelystad virus based on ORF5 sequences. The pigs were followed for the next 21 days and daily monitored for the development of clinical signs including fever.

Table 1
Design of experiments 1 and 2.

	Months of age							
	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5
Experiment 1 (n = 21)								
A		INV	INV			INV	Ch	End
B	INV	INV			INV	INV	Ch	End
C	Placebo[a]	Placebo	Placebo		Placebo	Placebo	Ch	End
Experiment 2 (n = 32)								
A	MLV			INV		Ch	End	
B	MLV			MLV		Ch	End	
C	MLV			INV	INV	Ch	End	
D	Placebo[b]			Placebo	Placebo	Ch	End	

INV, adjuvanted inactivated PRRSV vaccine (PROGRESSIS, Merial); MLV, adjuvanted modified-live PRRSV vaccine (PORCILIS PRRS, MSD Animal Health); Placebo[a], adjuvant of the INV vaccine; Placebo[b], sterile saline; Ch, intranasally challenged with 1×10^6 $TCID_{50}$ (2 mL) PRRSV strain 2749.

Design of experiment 2

Thirty-two piglets from the same high health farm were randomly selected at weaning and moved to the same experimental farm as in E1 (Table 1). At 6 weeks of age, the piglets were randomly separated into four groups ($n = 8$ each). Pigs in groups A, B and C were vaccinated intramuscularly with a commercial adjuvanted MLV PRRSV vaccine (Porcilis PRRS, MSD AH: Strain DV, $\geqslant 10^4$ $TCID_{50}$ by dose) while pigs in group D received sterile saline as a placebo. At 4.5 months of age, pigs in groups A and C received a dose of the same INV vaccine used in E1 while pigs in group B received a second dose of the MLV vaccine. One month later (5.5 months of age) animals in group C were revaccinated with the INV vaccine while all other pigs received the placebo. The nucleotide sequence of the INV ORF5 gene is more than 95% homologous to the MLV vaccine, whereas the homology for ORF7 is 100%. At 6.5 months of age, all pigs were challenged as in E2 and followed for the next 21 days.

Pigs in both experiments were confirmed to be free of antibodies to PRRSV (HerdCheck 2XR, Idexx Laboratories), *Mycoplasma hyopneumoniae* (Ideia Mycoplasma Hyopneumoniae EIA KIT, Oxoid,) and swine influenza virus (Civtest suis influenza, Hipra Laboratories) before the onset of the experiments. Animals were positive to PVC2 antibodies (Ingezim PCV2, Ingenasa) at the onset of the experiments but later became seronegative indicating the colostral origin of those antibodies.

Sampling

Blood samples were collected in duplicate (heparinised and siliconised blood-collecting tubes) during vaccination periods and then on days +0, +3, +7, +14, and +21 post-inoculation (PI) of challenge virus. Sera were used to determine viraemia, PRRSV-specific antibodies and virus neutralizing antibodies (NA). Heparinized blood samples were used to obtain peripheral blood mononuclear cells (PBMC) to measure IFN-γ by ELISPOT and IL-10 by ELISA.

Humoral response

Development of PRRSV-specific antibodies was measured by ELISA (HerdCheck 2XR, Idexx Laboratories). At the moment of challenge, sera were tested by the viral neutralization test (VNT) (Yoon et al., 1994; Jusa et al., 1996) for the presence of NA against the PRRSV isolate used in the in vivo inoculation (i.e. isolate 2749). Briefly, 50 μL of each whole serum to be tested were diluted serially from 1/2 to 1/128 in cell culture medium. Dilutions were mixed with 50 μL viral suspension containing 200 $TCID_{50}$ of the PRRSV strain 2749. Virus–serum mixtures were incubated for 1 h at 37 °C and then added to MARC-145 cultures in triplicate (96-well plates) and incubated for 3 days at 37 °C in 5% CO_2. Infection of cell cultures was revealed by using IPMA. Additionally, pigs in E1 were tested for NA at days 14 and 21 post-inoculation (PI), and pigs in E2 at 4.5 and 5.5 months old, and at day 14 PI.

Evaluation of the cell-mediated immune response

In both experiments, pigs were monitored once a month during the immunization period and after the challenge (+7, +14 and +21 PI) for the development of the cell-mediated PRRSV-specific immune response by using ELISPOT for the enumeration of interferon (IFN)-γ secreting cells (IFN-γ-SC) using commercial monoclonal Antibodies (mAbs) (Porcine IFN-γ P2G10 and biotin P2C11, BD Biosciences Pharmingen), as reported elsewhere (Zuckermann et al., 1998; Díaz and Mateu, 2005). Briefly, 5×10^5, 2.5×10^5 and 1×10^5 PBMC were stimulated with PRRSV isolate 2749 at a multiplicity of infection (MOI) of 0.01. Unstimulated and PHA-stimulated cells (10 μg/mL) were used as negative and positive controls, respectively. PRRSV-specific frequencies of IFN-γ-SC were calculated by subtracting counts of spots in unstimulated wells from counts in virus-stimulated wells. Frequencies of IFN-γ-SC were expressed as number of responding cells in 10^6 PBMC.

In order to assess if the INV used for immunization was able to prime the interleukin (IL)-10 induction, supernatants from PRRSV-stimulated PBMC were measured by IL-10 ELISA in E1. Briefly, cells were seeded at a density of 5×10^5 and were mock-stimulated or stimulated with live 2749 PRRSV strain at an MOI of 0.01 for 20 h. IL-10 expression in cell culture supernatants were measured as reported previously (Díaz and Mateu, 2005) using commercial pairs of mAbs (IL-10 Swine Antibody Pair, Live Technologies). Cytokine concentrations (pg/mL) were calculated by using the cytokine standards provided by the manufacturer. For a given culture and animal, the virus-specific IL-10 was calculated by subtracting the concentration of IL-10 in the mock-stimulated wells to the values obtained in virus-stimulated cultures.

Virological analysis

In both experiments, blood samples were taken on the day of the challenge and afterwards at days 3, 7, 14 and 21 PI. Blood was allowed to clot and serum was examined by a first round RT-PCR and a subsequent nested RT-PCR (Díaz et al., 2006) to determine the presence of PRRSV. In E1 PRRSV presence was also determined in nasal swabs at 0, 3 and 7 days PI.

Statistical analysis

Statistics were performed using StatsDirect v2.7.7. The Kruskal–Wallis test and the Conover–Inman method for multiple comparisons were used for comparisons of means between groups; the Friedman test was used for comparisons of means inside the same group; and comparison of the proportion of positive pigs was determined by the χ^2 test (Fisher's exact test).

Results

Clinical follow-up and temperatures

In both experiments, respiratory signs were absent or very mild in all pigs, irrespective of treatment group. No pig showed any pyrexia.

Humoral response

Table 2a and b summarizes the humoral response of pigs as measured by ELISA. In E1, where only INV schedules were compared, 9/14 (64%) of the vaccinated pigs seroconverted before the challenge. After the challenge, all pigs in groups A and B seroconverted at the end of the experiment. Based on S/P ratios, no significant increase in antibodies occurred after PRRSV challenge in previously positive pigs. In E2, all vaccinated pigs (groups A, B and C) were positive at 2.5 months old (1 month after the MLV vaccination). S/P ratios were not significantly increased by further vaccination or by challenge. In both experiments, all pigs in the control groups (C in E1 and D in E2) remained negative for both ELISA and VNT during the immunization period.

Table 3a and b summarizes the VNT results. In E1, NA were detected in four vaccinated pigs before the challenge but with low titres (1–3 $\log_2$) of which 1/7 in A (14.3%) and 3/7 in B (42.9%). After the challenge, an anamnestic response occurred in groups A and B. Thus, at day 14 PI, both vaccinated groups showed higher responses than those of controls in terms of the proportion of positive pigs (A = 7/7; B = 7/7 and C = 2/7; $P = 0.02$) as well as in terms of the mean titre at day 21 PI (A = 4.0 ± 0.6; B = 4.6 ± 0.8; and C = 3.0 ± 0.6; A vs. C; $P = 0.07$; and B vs. C $P = 0.003$).

In E2, few animals developed NA after a single dose of the MLV and again with low titres (titre $\log_2 = 1$). Later on, 4/8 (50%) of the MLV re-vaccinated pigs (group B) and 7/16 (43.8%) of the INV re-vaccinated pigs (groups A and C) were positive at 5.5 months of age. At the time of challenge, there were no statistical differences between vaccinated groups in the proportion of NA positive pigs or in the average NA titres in positive animals. At day 14 PI, all pigs that have previously received a combination of MLV and INV had NA (8/8 and 8/8 in groups A and C, respectively) while in the group receiving only MLV or in the unvaccinated-challenged groups some still remained had no detectable NA (proportion of positive pigs: 7/8 in B and 4/8 in D; proportion of positive pigs in A = C > D; $P = 0.03$). Regarding NA titres, the highest mean titre was observed in group C at day 14 PI (C = 3.6 ± 1.2; A = 3.2 ± 0.7; B = 2.1 ± 1.3; and D = 2.2 ± 0.5; C > B and D; $P = 0.02$ and 0.04, respectively). Furthermore, Group C also showed a faster increase in NA titres after the challenge than the other vaccinated groups ($P = 0.04$).

Evolution of the PRRSV-specific IFN-γ-SC

Fig. 1a and b depicts the evolution of the PRRSV-specific IFN-γ-SC in E1 and E2, respectively. In E1, virus-specific IFN-γ responses were established after two INV doses (5.5 months of age; mean ± standard error: A = 33 ± 5; B = 98 ± 5; and C = 2 ± 0 IFN-γ-SC; B > A, $P = 0.005$; B > C, $P < 0.0001$; A > C, $P = 0.001$). Maxima means of IFN-γ-SC before the challenge were reached for both vaccinated groups at 6.5 months old (A = 83 ± 23 and B = 124 ± 34; A = B; A > C, $P = 0.0014$; B > C, $P < 0.0001$). Challenge resulted in an increase of PRRSV-specific IFN-γ-SC frequencies in both vaccinated groups: A, from 49 ± 10 at day 0 post-challenge to 107 ± 19 at 7 days post-challenge ($P = 0.07$); B, from 26 ± 8 to 59 ± 10 ($P = 0.004$) for the same sampling period; afterwards (days 14–21 PI), no differences between groups were detected. In both experiments, a high level of spontaneous production of IFN-γ, which was subtracted from the specific results, was observed in INV vaccinated pigs.

In E2, significant differences between vaccinated groups and the control group were first observed at 5.5 months of age. At that time, pigs receiving MLV as a first immunization and one dose of INV as a revaccination dose reached the highest values (A = 155 ± 16; B = 75 ± 5; C = 139 ± 19; D = 9 ± 2; A = C > B > D; $P < 0.0001$). At day 0 PI, all three vaccinated groups showed higher values than controls ($P < 0.05$) with group C (MLV plus 2 INV doses) showing the highest mean PRRSV-specific IFN-γ-SC (C > D, $P = 0.0002$; B > D, $P = 0.01$ and A > D, $P = 0.04$). Challenge resulted in a significant increase in both INV revaccinated groups: A, from

Table 2
Serological evolution as measured by ELISA.

	Months of age/days post-inoculation (dpi)							
	−7 dpi	7.5 (0 dpi)	7 dpi	14 dpi	21 dpi			
2a: Experiment 1								
A	5/7[a]	5/7[a]	6/7[a]	7/7	7/7			
	0.58 ± 0.25	0.97 ± 0.35	0.79 ± 0.28	0.73 ± 0.19	0.74 ± 0.24			
B	4/7[a]	4/7[a]	4/7[a]	4/7	7/7			
	0.55 ± 0.17	0.7 ± 0.22	0.60 ± 0.22	0.60 ± 0.22	0.51 ± 0.12			
C	0/7[b]	0/7[b]	0/7[b]	3/7	7/7			
	–	–	–	0.87 ± 0.35	0.66 ± 0.30			
	Months of age/days post-inoculation							
	1.5	2.5	4.5	5.5	6.5 (0 dpi)	7 dpi	14 dpi	21 dpi
2b: Experiment 2								
A	0/8	8/8[a]	8/8[a]	8/8[a]	8/8[a]	8/8	8/8	8/8
	–	1.23 ± 0.14	2.80 ± 0.65	2.93 ± 0.60	2.75 ± 0.54	2.72 ± 0.56	2.34 ± 0.71	2.45 ± 0.54
B	0/8	8/8[a]	8/8[a]	8/8[a]	8/8[a]	8/8	8/8	8/8
	–	1.14 ± 0.27	2.00 ± 0.60	2.42 ± 0.85	2.46 ± 0.85	2.17 ± 0.93	2.29 ± 0.45	2.49 ± 0.18
C	0/8	8/8[a]	8/8[a]	8/8[a]	8/8[a]	8/8	8/8	8/8
	–	1.23 ± 0.13	1.92 ± 0.85	2.84 ± 0.14	2.66 ± 0.37	2.41 ± 0.41	2.26 ± 0.23	2.33 ± 0.29
D	0/8	0/8[b]	0/8[b]	0/8[b]	0/8[b]	0/8[b]	8/8	8/8
	–	–	–	–	–	–	1.76 ± 0.49	2.06 ± 0.48

Results are expressed as proportion of positive pigs and mean S/P ratios ± standard deviation for positive pigs. Different superscript letters indicate statistical differences ($P < 0.05$) between groups for proportions of positive pigs at a given time point.

Table 3
Titres of neutralizing antibodies against PRRSV at different times.

	Months of age/days post-inoculation (dpi)			
	7.5 (0 dpi)		14 dpi	21 dpi
3a: Experiment 1				
A				
Positive pigs	1/7		7/7[a]	7/7
Mean titre (log_2) ± SD	1		3.7 ± 0.5	4 ± 0.6[‡]
Range of titres	(1)		(3–4)	(3–5)
B				
Positive pigs	3/7		7/7[a]	7/7
Mean titre (log_2) ± SD	2.3 ± 0.6		3.8 ± 1.5	4.6 ± 0.8[†]
Range of titres	(2–3)		(1–5)	(3–5)
C				
Positive pigs	0/7		2/7[b]	6/7
Mean titre (log_2) ± SD	–		4 ± 0	3 ± 0.9
Range of titres	–		(4)	(2–4)
	Months of age/days post-inoculation (dpi)			
	4.5	5.5	6.5 (0 dpi)	14 dpi
3b: Experiment 2				
A				
Positive pigs	1/8	2/8[ab]	5/8[a]	8/8[a]
Mean titre (log_2) ± SD	1 ± 0	2 ± 1.4	2.8 ± 0.8	3.2 ± 0.7[ab]
Range of titres	(1)	(1–3)	(2–4)	(2–4)
B				
Positive pigs	0/8	4/8[a]	5/8[a]	7/8[ab]
Mean titre (log_2) ± SD	–	1.5 ± 1	1.6 ± 0.9	2.1 ± 1.3[b]
Range of titres	–	(1–3)	(1–3)	(1–4)
C				
Positive pigs	3/8	5/8[a]	6/8[a]	8/8[a]
Mean titre (log_2) ± SD	1 ± 0	1.4 ± 0.5	2.2 ± 1.6	3.6 ± 1.2[a,*]
Range of titres	(1)	(1–2)	(1–5)	(2–6)
D				
Positive pigs	0/8	0/8[b]	0/8[b]	4/8[b]
Mean titre (log_2) ± SD	–	–	–	2.2 ± 0.5[b]
Range of titres	–	–	–	(2–3)

Results are expressed as proportion of positive pigs, mean titre (log_2) ± standard deviation and range of titres (log_2) for positive pigs. Different superscripts in the same column indicate statistical differences ($P < 0.05$) between groups for proportions of positive pigs or for mean titres at a given time point.
[†] Significantly different from mean titre of group C ($P < 0.05$).
[‡] Difference from group C almost significant at 5% level ($P = 0.07$).
[*] Significantly different from the titre at 0 dpi ($P < 0.05$).

50 ± 25 at day 0 PI to 103 ± 18 at 7 days PI ($P = 0.02$); C from 72 ± 16 to 173 ± 15 ($P < 0.05$) for the same sampling periods. Two weeks after challenge, only groups A and C showed higher levels ($P = 0.02$ and $P = 0.03$, respectively) than unvaccinated and infected controls. Later, mean IFN-γ-SC for all groups decreased although group A remained with values significantly higher than the other groups ($P < 0.02$).

When the virus-specific IL-10 concentrations were measured in PBMC from E1, only two pigs from group A were positives at day 0 PI (mean of positive pigs 22.7 ± 9 pg/mL). After the challenge, only one pig in each group was positive at day 7 PI, whereas five animals in group A, six in B and four in C were positive at day 14 PI (mean of positive pigs in A = 74.7 ± 36.3; B = 110.9 ± 52 and C = 138.5 ± 75.4 pg/mL).

Virological analysis

In E1, viraemia could not be detected at 7, 14 or 21 days PI and PRRSV were detected in nasal swabs from just one pig. In E2, the outcome was different. In both first round and nested RT-PCR three pigs in group A (one at day 3 PI and two at day 7) were viraemic; in group B, only one animal was positive at day 7 PI; in group C two were viraemic at day 3 PI. In group D, all pigs became viraemic at least once after the challenge (proportion of positive pigs: D > A = B = C at day 3 PI; $P < 0.02$).

Discussion

One of the key elements for the control of PRRS in a sow herd is the presence of some level of immunity against the virus in the herd. As shown in previous reports, although immunization of gilts and sows does not necessarily provide full protection against the infection it can have beneficial effects in terms of protection against abortion and can reduce the duration of the viraemia or nasal shedding of the virus if the sow becomes infected. Once gilts or sows have been immunized for the first time, the problem is how to assure that immunity is maintained throughout all the productive life of the pig. Thus the development of efficient strategies for sustaining immunity in sows is a very relevant point for PRRS control.

In a previous study (Piras et al., 2005) it was observed that two INV vaccinations 1 month apart were able to induce a high level of CMI (IFN-γ producing cells after re-stimulation). In E1, repeated INV immunizations (three or four doses) were evaluated. A primer immunization with a double injection of an INV was used. Later, simple revaccination was compared with a double revaccination to assess whether the latter improved the immunological response to vaccination schedules based exclusively on INV.

The 3 month interval between the primer immunization and booster vaccinations is commonly used in the field as a way of intensifying vaccination schedules. In the present study, INV was

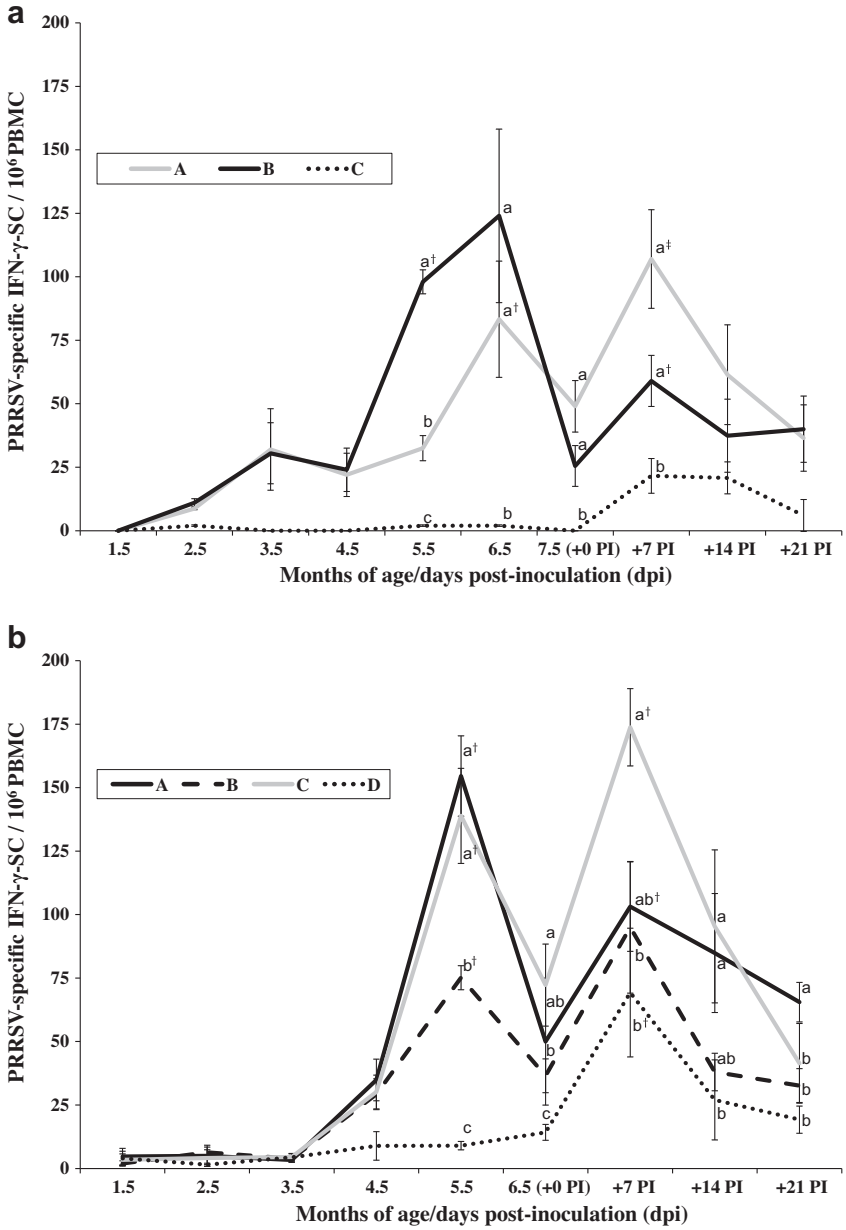

Fig. 1. (a and b) PRRSV-specific cell-mediated immune response as determined by the IFN-γ ELISPOT. Mean frequencies and standard error (SE) of PRRSV-specific IFN-γ-secreting cells. Different superscripts indicate significant differences ($P < 0.05$) between groups at the same time point. †, statistically significant differences between a given sampling and the previous one within a group ($P < 0.05$); ‡, indicates a trend ($P = 0.07$).

not able to induce significant humoral or cell-mediated responses after a single dose, as was shown previously (Meier et al., 2003; Zuckermann et al., 2007). However, after the second immunization, development of a strong CMI occurred.

The dynamics and means of IFN-γ-SC were similar in the different vaccinated groups with the shifts in timing resulting from the different vaccination schedules. This observation is in agreement with that of Piras et al. (2005) who reported that the administration of the same INV used in the present study induced the development of IFN-γ-SC, mainly within the $CD4^{+}CD8\alpha^{int}$ and $CD4^{-}CD8\alpha^{high}$ compartments that are supposed to account for antigen-primed T helper cells and classic cytotoxic T-cells.

It is interesting to note that in the present study, IFN-γ-SC rose rapidly after the second administration of the INV but their numbers decreased after approximately 1 month. Challenge PRRSV induced a significant increase in IFN-γ-SC in a pattern consistent with the development of memory cells. Thus, repeated vaccination with INV was able to produce a sustained basal level of immunity. Moreover, although the INV was scarcely efficient in inducing humoral responses before challenge, after the experimental inoculation INV vaccinated pigs developed NA faster than unvaccinated pigs indicating that priming occurred also for B-cells. This can be interpreted as a faster ability to respond in vaccinated sows which became infected.

One surprising feature observed in cultures of unstimulated PBMC from INV vaccinated pigs was the high level of spontaneous production of IFN-γ. The same phenomenon has been reported previously (Zuckermann et al., 2007). The component of the INV responsible for such spontaneous release of IFN-γ has not been elucidated yet but it was suggested that it could be the vaccine adjuvant (Zuckermann et al., 2007). In the present study the unvaccinated control animals received the adjuvant alone as a placebo, and spontaneous IFN-γ could not be observed in unstimulated PBMC from those animals, suggesting that it was not the

adjuvant per se that was responsible for the phenomenon. Furthermore, it has been previously reported that INV has the ability to elicit spontaneous secretion of IL-10 by PBMC (Zuckermann et al., 2007). In our study, spontaneous production of this interleukin was only observed in those PBMC cultures (data not shown) that were also positive after PRRSV stimulation (4/14 INV vaccinated pigs) at 1 month after the last INV immunization. These results are consistent with Zuckermann et al. (2007) who reported that spontaneous secretion of IL-10 by PBMC declined at 28 days post-vaccination. In our study, IL-10 responses after the challenge were similar, in terms of dynamics and quantity, between unvaccinated pigs (C), and vaccinated pigs (A and B) – a fact that could indicate that IL-10 production might be related mainly to the innate response against the infection (Gimeno et al., 2011; Mateu and Díaz, 2008).

In previous studies, it has been shown that combining a MLV and an INV could be an alternative strategy for improving the immune responses to PRRSV (Nilubol et al., 2004, 2007). In E2 we evaluated a vaccination protocol based on an INV vaccine (simple revaccination or double revaccination) after a MLV primer immunization. Simultaneously, one group of pigs receiving MLV as a primer immunization and as a revaccination was also evaluated in order to compare it with the combined strategy. As expected, all pigs from E2 were positive by ELISA after the primer immunization with MLV and further doses of either vaccine did not substantially increase S/P ratios.

However, for all three vaccinated groups re-vaccination produced an increase in the number of animals with NA, indicating that a booster effect occurred but only against some viral antigens and not specifically against those used for coating of ELISA plates. These observations are in agreement with previous reports (Baker et al., 1999; Joo et al., 1999). In particular, Baker et al. (1999) concluded that enhanced NA responses in sows previously vaccinated with MLV could be only achieved if an INV vaccine is used as a recall stimulus. Similarly, another study evaluating the combination of both types of vaccines (Nilubol et al., 2007) demonstrated that primer inoculation with a MLV followed with a INV recall vaccination was better with regards to NA production than other vaccination protocols examined (INV or MLV alone, or INV as primer inoculation followed with a MLV recall vaccination). Although it is difficult to ascertain, we suggest that in our study the adjuvant of the INV vaccine or the augmented antigenic mass could be the reason behind this phenomenon.

In regard to IFN-γ responses, it is interesting to note that in this second experiment a clear anamnestic response was detected 1 month after revaccination in groups A and C where an INV was administered. Although the boost was also observed for group B (MLV + MLV), the intensity of the response was lower in the latter case. When groups were compared after the revaccination, there were statistically significant differences between INV revaccinated groups and group B. Reasons for such differences among groups are difficult to ascertain. Potential reasons include the different adjuvants used in each vaccine or a possible effect of the live virus in regulating the immune response. However, further investigations are needed to clarify this point.

Conclusions

The present study demonstrated that repeated immunization with INV primed the immune responses in terms of production of NA and CMI as demonstrated by results after challenge. Secondly, a vaccination schedule consisting of the combination of a MLV vaccine that could be used as a primer inoculation for gilts and INV revaccination could reach similar or even superior levels of NA and CMI, and with similar virological protection as repeated vaccination protocols with an attenuated strain. In future experiments the minimum vaccination doses necessary to obtain this level of protection should be ascertained.

Conflict of interest statement

A. Callén, C. Charreyre and F. Joisel are employees of Merial. None of the other authors received any funding or honoraria from Merial nor had any financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. This study was funded by Merial. The study was designed by researchers of CReSA in consultation with Merial. All procedures, data collection, records, manipulation and analysis of samples and data were conducted at CReSA by CReSA researchers.

Acknowledgements

We would like to thank Núria Navarro, Esmeralda Cano, Raquel Maeso and the personnel of BSL-3 facilities of CReSA for assistance. Ivan Díaz has been funded by project PORCIVIR CDS-0007 of program CONSOLIDER 2010 (Spanish Ministry of Education and Science). Mariona Gimeno is funded by the fellowship program of the Universitat Autònoma de Barcelona.

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