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Cytokines and synthetic double-stranded RNA augment the T helper 1 immune response of swine to porcine reproductive and respiratory syndrome virus

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Abstract

Immunization of pigs with a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine initially elicits a weak interferon (IFN)-γ response. To improve the immune response, an adjuvant consisting of plasmid encoding either porcine interleukin (IL)-12 or IFN-α was co-administered during vaccination. In the presence of either adjuvant, at least a three-fold increase in the primary virus-specific IFN-γ response was observed. While this enhancement was only transient (1 week) when the IL-12 expressing plasmid was used, the effect was not only still apparent at 6 weeks after vaccination in the presence of the IFN-α expressing plasmid but even after challenge with a virulent genetically divergent PRRSV. In contrast, no effect of either adjuvant on the production of anti-virus antibodies was noticed throughout the study. Despite the apparent augmentation of a T helper (Th) 1 type response by the inclusion of IFN-α or IL-12 during vaccination, this modulation did not necessarily correlate with a reduction in viremia. Since a similar increase in the degree of the IFN-γ response to the PRRSV vaccine could be achieved by substituting polyinosinic–polycytidylic acid in lieu of either cytokine, exposure to PRRSV in the presence of a variety of Th 1 polarizing molecules can positively influence the development of the cell-mediated immune response of swine to this pathogen. Conceivably, such intervention could be applied to improve the formulation of anti-PRRSV vaccines.
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Keywords: PRRS virus; Interferon-α; Interferon-γ; Interleukin-12; Swine; Cellular immunity

Abbreviations: CMI, cell-mediated immunity; IFN, interferon; SC, secreting cells; IL, interleukin; MLV, modified live virus; pINA, expression plasmid encoding porcine interferon-α; pcPIL12, recombinant plasmid encoding porcine interleukin-12; poly I:C, polyinosinic:-polycytidylic acid; PBMC, peripheral blood mononuclear cells; PRRSV, porcine reproductive and respiratory syndrome virus; Th, T helper; VN, virus neutralizing

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of swine characterized by abortion, stillbirth and weak-born pigs. In its non-reproductive form, this syndrome affects younger pigs more severely than older animals, causing reduced growth and pneumonia that can be made more severe by co-infection with other pathogens (Thacker, 2003). The etiologic agent for this disease is an RNA virus (PRRSV) that belongs to the family *Arteriviridae*, which targets macrophages for infection (Wensvoort et al., 1991; Collins et al., 1992). PRRSV exhibits a significant degree of genetic diversification (Murtaugh et al., 1998; Key et al., 2001; Goldberg et al., 2003) and this characteristic probably contributes to the difficulty in controlling infectious outbreaks caused by it (Meng, 2000). Under experimental conditions, the currently available modified live virus (MLV) vaccine against this pathogen has been shown to provide immunized pigs adequate protection from subsequent challenge with the homologous strain (Lager et al., 1997a, 1997b). However, despite the demonstrated ability of this vaccine to afford pigs partial protection against infection by a genetically divergent PRRSV strain (Osorio et al., 1998; Lager et al., 1999; Mengeling et al., 2003a, 2003b), the overall protective immunity provided to swine in commercial settings is generally inadequate (Halbur, 2003).

Infection of pigs with wild type PRRSV (Nelson et al., 1994; Loemba et al., 1996; Vezina et al., 1996; Yoon et al., 1995; Albina et al., 1998b; Gonin et al., 1999) or their vaccination with a live attenuated form of this virus (Labarque et al., 2000; Ostrowski et al., 2002) elicits an exuberant production of non-neutralizing antibodies. In contrast, a transient T cell mediated PRRSV-specific lymphoproliferative response is detected at 4 weeks post-infection and lasts an additional 9 (Bautista and Molitor, 1997) to 14 weeks (Lopez-Fuertes et al., 1999). Moreover, during this time interval, limited quantities of IFN-γ secreting cells (SC) are also generated (Meier et al., 2003; Xiao et al., 2004). Interestingly, in the absence of additional antigenic stimulation this polarity reverses within the ensuing 5 months, as manifested by a decreasing antibody response and a gradual increase in the intensity of the IFN-γ response (Meier et al., 2003). The initial antibody-dominated immune response is not the result of insufficient antigenic stimulation, since neither the inclusion of a commercial adjuvant during primary vaccination (Meier et al., 2003) nor booster immunizations of previously heavily vaccinated pigs (Bassaganya-Riera et al., 2004) enhances virus-specific cell-mediated immunity (CMI). Thus, PRRSV seems to inherently stimulate an imbalanced immune response characterized by an abundance of humoral immunity and a limited, but potentially protective, Th 1-like IFN-γ response (Murtaugh et al., 2002).

One characteristic of PRRSV infection that probably contributes to the retarded development of a specific cell-mediated immune response is the lack of induced IFN-α production (Albina et al., 1998a; Buddaert et al., 1998; Van Reeth et al., 1999). Usually, virus-infected cells secrete type I IFN and the released cytokine interacts with a subset of naïve T cells to promote their conversion into virus-specific IFN-γ SC (Cella et al., 2000; Cousens et al., 1999; Kadowaki et al., 2000; Biron, 2001; Levy et al., 2003). However, to counteract a virus that is a poor inducer of type I IFN, the development of a Th 1 response can be generated by a pathway that utilizes interleukin (IL)-12 (Cousens et al., 1999; Biron, 2001). Indeed, the injection of recombinant IL-12 (rIL-12) at the time of vaccination and three additional times during the following week enhanced the host cellular immune response to PRRSV (Foss et al., 2002). Based on the success of this intervention and the noted absence of IFN-α generation during PRRSV infection, the present studies were undertaken to determine if the antiviral adaptive immunity induced by vaccination with PRRS MLV could be modified simply by the co-administration of rIL-12 protein directly, or of either porcine IFN-α or IL-12 indirectly via plasmids expressing these cytokines. In addition to these two elements that can deliver Th 1 polarizing signals (Biron, 2001; Kadowaki and Liu, 2002; Kapsenberg, 2003), the effect of stimulation with a known inducer of IFN-α production, namely double-stranded RNA, was examined. Although all three adjuvants positively influenced the intensity and rate of development of the virus-specific IFN-γ response, the results indicate that a stronger immunomodulator will be needed to overcome the tendency of PRRSV not to elicit this type of Th 1 response.

2. Materials and methods

2.1. Viruses and cells

Prior to use for immunization, the Ingelvac PRRS MLV vaccine (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) was reconstituted from lyophilized vials according to manufacturer's instructions. The highly virulent, ‘‘atypical’’ PRRSV strain IA-1-4-2 (97-7895, GenBank accession number AF325691) was originally isolated from a severe case of reproductive failure in an Iowa swine herd in 1996 (Osorio et al., 2002) and was used as the challenge virus in the animal studies. The extent of nucleotide sequence identity in ORF5 between this wild-type strain and the parental strain of the vaccine (VR2332) used in this study is approximately 91%. For ELISPOT assays of PRRSV-specific IFN-γ SC, PRRSV strains VR-2332 (American Type Culture Collection, Manassas, VA) and IA-1-4-2 were used as sources of antigen. When required, PRRSV was propagated and titrated in monolayers of MARC-145 cells as previously described (Meier et al., 2003).

Porcine peripheral blood mononuclear cells (PBMC) were isolated from venous blood that had been anti-coagulated with 5 mM heparin and the cells were subsequently maintained as described by Meier et al. (2003).

2.2. Mammalian expression vectors containing porcine IFN-α or IL-12 cDNA

For generating a plasmid capable of expressing IFN-α in mammalian hosts, an intact cDNA encoding porcine IFN-α was first prepared by RT-PCR using RNA isolated from pig lymphocytes previously infected with pseudorabies virus (to stimulate IFN-α production). Forward (TCTGCAAGGTTCCCAA-TG) and reverse (GTCTGTCACTCCTTCTTCCTG) primers were designed based on the nucleotide sequence of porcine IFN-α cDNA (Lefevre and La Bonnardiere, 1986). Products of the anticipated size (590 bp) were cloned into the pCR®2.1 plasmid (Invitrogen Corp., Carlsbad, CA), and an insert having the predicted restriction enzyme sites was sequenced. A comparison of the coding region within the selected amplicon to the previously reported IFN-α cDNA revealed three nucleotide differences. These variations resulted in two amino acid changes of a tyrosine to a cysteine at position 109 and of an arginine to a leucine at position 141 of the intact protein. The IFN-α cDNA was then excised from the recombinant pCR®2.1 plasmid and placed under the transcriptional regulation of the cytomegalovirus promoter in pcDNA3 (Invitrogen) to generate pINA. To verify that an active cytokine was encoded by the amplified cDNA, Chinese hamster ovary (CHO) cells were transfected with pINA and single cell clones resistant to 1.0 mg geneticin per ml growth medium were prepared. Supernatant medium from the clones were tested for the ability to inhibit the replication of an interferon-inducer negative strain of vesicular stomatitis virus (Sekellick and Marcus, 1979) in Madin Derby bovine kidney (MDBK) cells as previously described (Rubinstein et al., 1981). Clones producing from 0 to greater than 200,000 units (1 unit inhibits 50% of VSV replication in MDBK cell monolayers) of IFN-α were detected.

For generating a plasmid capable of expressing a composite IL-12 moiety consisting of the p35 and p40 subunits joined in tandem by an inert 15 amino acid stretch in mammalian cells, cDNA encoding the 22 amino acid long leader region and 17 amino acids of the mature form of the IL-12 p40 subunit was first derived from plasmid p40–5′ RACE #1 (provided by Dr. M.P. Murtaugh, University of Minnesota) that contains approximately the 5′ half of IL-12 p40 subunit cDNA. This fragment was then inserted into pcDNA3 and ligated to a segment that was excised from the yeast expression plasmid pPic9scIL-12 (Foss et al., 1999) and consisted of the remainder of the IL-12 p40 subunit cDNA linked by a 45 nucleotide stretch to cDNA encoding the mature form of the IL-12 p35 subunit. The resultant plasmid, pcPIL12, utilizes the cytomegalovirus promoter to regulate expression of a complete IL-12 complex consisting of one of each of the two subunits. To verify that a biologically active, secreted cytokine could be produced by pcPIL12, CHO cells were either mock-transfected or transfected with the plasmid and after 48 h the overlaying medium was subjected to a bioassay designed to demonstrate the presence of porcine IL-12 based on its ability to increase the IFN-γ response of memory T cells to recall viral antigen (Meier et al., 2000). By comparison to a yeast-derived porcine rIL-12 standard (provided by Dr. M. Moody, Endogen, Woburn, MA),

>100 ng/ml bioactive cytokine was found in the transfected cell medium whereas no activity was found to be associated with the control supernatant.

2.3. Large scale preparation of pINA and pcPIL12

Escherichia coli strain DH5α (Invitrogen) was transfected with either pINA or pcPIL12 and grown in 1 L of LB medium supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, MO) for 16 h at 37 °C with constant shaking (∼300 rpm). Plasmid purification was conducted using a Qiagen Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

2.4. Preparation of cartridges for intradermal biolistic delivery of cytokine cDNA

Plasmid pINA or pcPIL12 was precipitated onto the surface of gold particles (average diameter of 5 μm; Bio-Rad Laboratories, Inc., Hercules, CA) at a concentration of 1.0 μg DNA/mg gold. Plastic tubing was then coated with 0.5 mg of the DNA-bound gold particles using a Tubing Prep Station (Bio-Rad) following the manufacturer's instructions and cut to yield cartridges containing 0.5 μg DNA.

2.5. Reconstitution of porcine rIL-12

Immediately prior to use as a standard in the IL-12 bioassay or as an adjuvant in the animal studies, lyophilized yeast-derived porcine rIL-12 (Endogen) was reconstituted in low endotoxin-tested PBS (Mediatech, Herndon, VA) to a concentration of 20 μg/ml.

2.6. Stabilization of polyinosinic:polycytidylic acid (poly I:C)

Stabilized polyinosinic:polycytidylic acid (poly I:C) was prepared by the method of Levy et al. (1975) with minor modifications. Briefly, poly I:C (Sigma) at 4 mg/ml in pyrogen-free 0.85% NaCl was denatured at 71 °C for 1 h and allowed to re-anneal while cooling slowly to ambient temperature. The annealed poly I:C solution was then mixed with equal volumes of 6.0 mg poly-L-lysine/ml pyrogen-free 0.85% NaCl and 2% carboxymethylcellulose in pyrogen-free 0.85% NaCl. The final preparation was stored at 4 °C until needed.

2.7. PRRSV vaccination and challenge of pigs

In the first study, 9-week-old Yorkshire x Landrace cross-bred pigs were obtained from a PRRSV-free herd and randomly segregated into five groups ($n = 5$) and a sixth group of only two individuals. The latter group was kept in a PRRSV-free environment and was not vaccinated or challenged. All other animals were immunized in their adductor muscles (inner thigh) with 2.0 ml of Ingelvac PRRS MLV vaccine. At the same time, some of the pigs were inoculated intramuscularly (i.m.) by needle with either 2 ml of saline (group 1), 200 μg pINA (group 2) or pcPIL12 (group 3) per animal or intradermally (i.d.) with 5 μg of pcPIL12/animal (group 4) via biolistic delivery with a gene gun (Bio-Rad) at locations adjacent to the site of vaccination. Twenty micrograms of porcine rIL-12 in a 2 ml volume were co-administered to the members of group 5, which also received a second i.m. injection of the cytokine 24 h later. At 8 weeks post-immunization, all pigs receiving only the vaccine except one (group 1), or the vaccine in conjunction with an i.m. application of either plasmid pINA (group 2) or pcPIL12 (group 3) were transferred to a bio-containment facility together with five additional PRRSV-naïve pigs (group 7) obtained from the same herd mentioned above. At this time all of the transferred animals were challenged with $10^{5.8}$ $TCID_{50}$/2.0 ml (1.0 ml/nostril) of PRRSV strain IA-1-4-2. Fourteen days later all pigs were euthanized.

For the second study, twelve 6-week-old Yorkshire × Landrace cross-bred pigs were obtained from the same PRRSV-free herd described above and were randomly assigned to one of two groups ($n = 6$). While all pigs were immunized i.m. with 2.0 ml of Ingelvac PRRS MLV vaccine, 0.25 mg poly I:C/kg of body weight was co-administered to the animals of one group only. This dose of poly I:C was selected based on its demonstrated ability to induce the maximum presence of IFN-α in the sera of 10-week-old pigs (Loewen and Derbyshire, 1988). Pigs were bled at 6, 12, 24 and 48 h after poly I:C administration and differential white blood cell numbers were determined with a Cell-Dyne 3500 (Abbott Laboratories, Abbott Park, IL) and confirmed by microscopy by an

experienced technician. Eight weeks later, members of both groups were given a secondary "booster" immunization identical in composition to that of the primary vaccination.

2.8. Quantitation of the anti-PRRSV cell-mediated and humoral cellular immune responses of vaccinated pigs

The extent of the host cell-mediated immune response to PRRSV was measured by using a single cell ELISPOT assay (Mateu de Antonio et al., 1998; Meier et al., 2003) to enumerate virus-specific IFN-γ-SC in the PBMC population. The presence of PRRS virus-neutralizing (VN) antibodies in the sera was determined as previously described by Benfield et al. (1992). Briefly, twofold serial dilutions of serum (100 μl) in modified Eagle's minimal essential medium (MEM) were mixed with an equal volume of MEM containing 300 $TCID_{50}$ of the selected PRRSV strain. After incubation at 37 °C for 1 h, the mixture was added to monolayers of MARC-145 cells in 96-well tissue culture plates. The cells were then examined daily during a 7-day interval, and the end point titer was expressed as the reciprocal of the highest serum dilution that neutralized the development of a PRRSV-induced cytopathic effect.

Quantities of non-neutralizing PRRSV-specific antibodies in the sera were measured by using a commercial ELISA assay (Herd-Chek PRRS, IDEXX, Westbrook, ME). Sample to positive (S/P) ratios greater than 0.4 were considered positive and were calculated based on the manufacturer's instructions utilizing the formula: S/P = (test sample A(650) against PRRSV antigen − test sample A(650) against normal host cell antigen)/(positive control against PRRSV antigen − positive control against normal host cell antigen).

2.9. Evaluation of the anti-PRRSV protection afforded to vaccinated pigs

Virus was detected in serum samples collected on days 4, 7 and 11 post-PRRSV challenge and from tonsil biopsies obtained at the termination of the experiment. Prior to use, tonsils (0.2 g) were first homogenized by grinding the tissue with a pestle in a 1.5 ml microfuge tube, adding 500 μl of MEM supplemented with 50 μg of gentamicin per ml, and vigorously shaking the sample with the pestle in the tube. After removal of the pestle, an additional 500 μl of MEM was added, and the homogenate was again mixed vigorously. The suspension was then passed through a 0.20 μm pore-size filter and stored at −20 °C.

To detect infectious PRRSV, quadruplicate sets of MARC-145 cell monolayers in 12- or 24-well plates were incubated with either 300 μl of serial 10-fold dilutions of serum or tonsil homogenate (starting with 1:2 dilution) in MEM supplemented with 50 μg of gentamicin per ml for 1 h at 37 °C in 5% CO_2. Afterwards, the inocula were removed and replaced with 1 ml MEM supplemented with 3% fetal calf serum and 50 μg of gentamicin per ml. The cells were then incubated for 7 days at 37 °C and checked daily for signs of cytopathic effect.

2.10. Statistical analyses

Results are expressed as the mean ± the standard error of the mean (S.E.). Statistical significance was determined by analysis of variance. When significant differences at the 0.05% confidence level were present, individual differences between treatment groups were determined with Fisher's protected least significant difference (PLSD). The comparison between the quantity of virus in sera and tonsils was done by using the Fisher's exact test. The analyses were performed with the Statview program V4.5 (Abacus Concepts, Berkeley, CA).

3. Results

3.1. Anti-PRRSV cell-mediated immune response in pigs vaccinated in the absence/presence of IFN-α or IL-12

Immunization of pigs with only PRRS MLV vaccine induced a relatively mild CMI response during the ensuing 6 weeks as evidenced by the low frequency of PRRSV-specific IFN-γ SC in the vaccinated pigs' PBMC populations (Fig. 1). Although a similar response was evident during this time period for vaccinated pigs also i.m. injected with exogenous IL-12 either directly as protein or

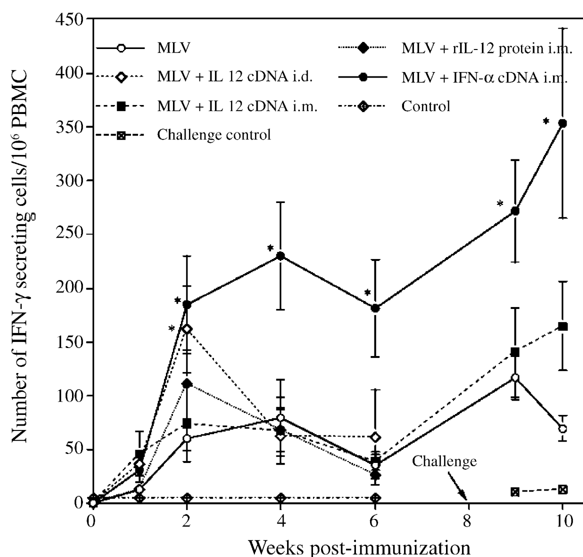

Fig. 1. Comparison of the intensities of the virus-specific IFN-γ SC response of pigs to immunization with a PRRS MLV vaccine in the absence/presence of IL-12 or IFN-α. Groups of pigs were vaccinated with PRRS MLV alone (group 1) or also received i.m. injections of plasmid expressing porcine IFN-α (group 2) or IL-12 (group 3), i.d. injections of plasmid expressing porcine IL-12 (group 4), or 2 i.m. injections of porcine rIL-12 at a daily interval (group 5). An additional group served as unvaccinated controls (group 6). At 8 weeks post-immunization, vaccinated animals in groups 1–3 as well as five, newly acquired PRRSV-naïve pigs (challenge control, group 7) were challenged with PRRSV strain IA-1-4-2. PBMC were isolated from the pigs at the indicated times post-vaccination and the presence of virus-specific IFN-γ SC was determined by using an ELISPOT assay. Asterisks indicate significant differences ($P < 0.05$) between the frequencies of IFN-γ SC in the blood of the animals immunized with the MLV alone as compared to the other immunized groups. Each value represents the mean response of five animals ± S.E.M. except for group 1 that was downsized to four pigs immediately prior to challenge and group 6 that consisted of two animals.

indirectly via IL-12 cDNA, immunized animals that were i.d. inoculated with IL-12 cDNA exhibited an overall 2.7-fold transient increase in the frequency of PRRSV-specific IFN-γ SC at 2 weeks post-vaccination. Likewise, a slightly greater enhancement of three-fold was found in those vaccinated animals that had received IFN-α cDNA. However, in this case, the IFN-γ response remained elevated at about this extent throughout the following 4 weeks.

Since the pigs immunized with PRRSV MLV in the presence of pINA exhibited a significantly enhanced and sustained IFN-γ response as compared to those in the other vaccinated groups, these animals as well as those receiving the vaccine only or the vaccine plus an i.m. injection of pcPIL12 were transferred to a bio-containment facility and challenged with PRRSV strain IA-1-4-2. As controls, an additional group of age matched PRRSV-naïve pigs were included and also infected with the virulent virus. During the 2 weeks after challenge, the animals originally immunized in the presence of IFN-α cDNA continued to display a statistically significant, greater IFN-γ response relative to that measured in the other pigs.

3.2. Anti-PRRSV humoral immune response in pigs vaccinated in the absence/presence of IFN-α or IL-12

All MLV-vaccinated pigs exhibited strongly positive anti-PRRSV antibody titers (S/P ratios ranged from 0.8 to 1.75) at 2 weeks following immunization and their levels of humoral immunity had increased when measured 2 weeks later (S/P ratios ranged from 1.3 to 2.2) (Fig. 2). However, there were no significant differences between the mean S/P ratios of the cohort receiving the vaccine alone and of any of those groups whose vaccination was supplemented with a cytokine adjuvant. In contrast, VN antibodies were not detected until 4 weeks after immunization and then only at low titers (≤1:4; v:v) and in at most one or two pigs per group (Table 1). Differences between the various cohorts in regards to the proportion of pigs that contained VN antibodies were not found to be significant. Immediately prior to challenge with the virulent PRRSV IA-1-4-2 strain at 8 weeks post-vaccination, VN antibodies were not detected in serum collected from any member of the three cohorts being challenged (MLV only, MLV + pcPIL12 i.m., and MLV + pINA i.m.; Fig. 3). Although subsequently by 2 weeks post-challenge the majority of the pigs had developed a detectable VN antibody response, the three groups could still not be statistically differentiated based on their average VN titers. Despite use of the challenge strain in lieu of PRRSV isolate VR-2332 as an antigen source appearing to result in a slightly more sensitive assay for the presence of VN antibody, this visual difference was determined not to be statistically significant. As expected due to the short interval between exposure to virus and performance of the assay, PRRSV-neutralizing antibodies were not

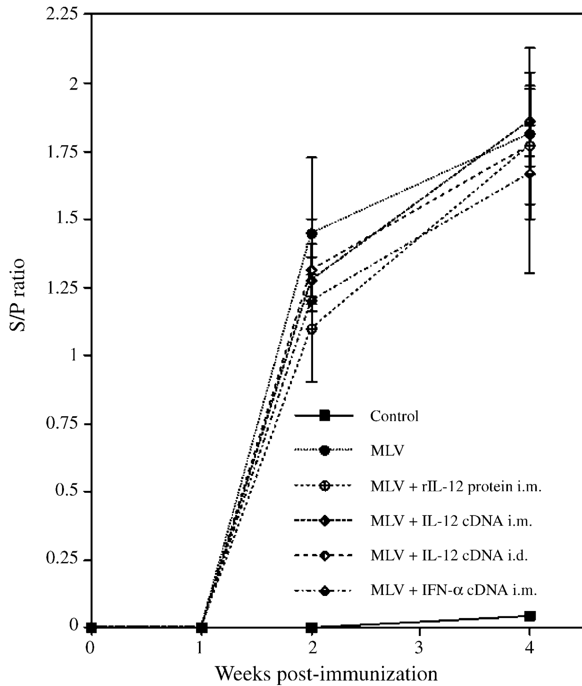

Fig. 2. Comparison of the intensities of the virus-specific humoral immune response of pigs to immunization with a PRRS MLV vaccine in the absence/presence of IL-12 or IFN-α. Serum was drawn from all members of groups 1–6 described in the legend to Fig. 1 and assayed for the presence of anti-PRRSV antibodies. Results are presented as sample to positive (S/P) ratios with a cut-off of 0.4. Each value represents the mean S/P ratio obtained for five animals ± S.E.M. except for the control group consisting of two animals.

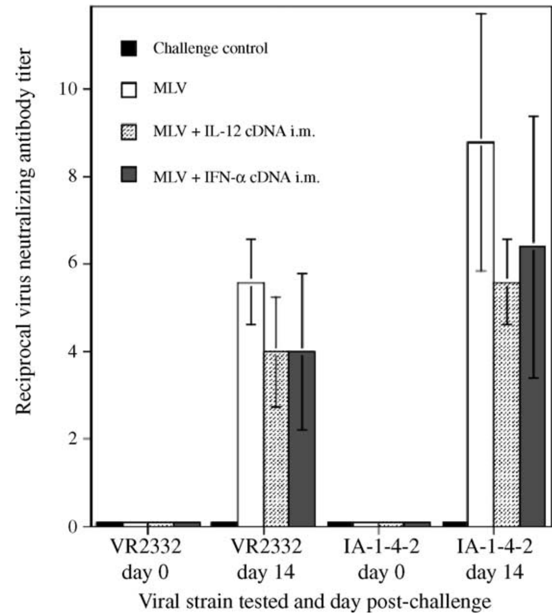

Fig. 3. Comparison of the presence of PRRSV-neutralizing antibodies in the serum of vaccinated pigs after a challenge with wild type PRRSV. Immediately prior to and 2 weeks after challenge with PRRSV strain IA-1-4-2, serum was drawn from four members of group 1 and all pigs in groups 2, 3 and 7 described in the legend to Fig. 1 and assayed for the presence of PRRSV-neutralizing antibodies. Results are presented as the reciprocal of the lowest two-fold dilution of serum that inhibited the replication of either PRRSV strain VR-2332 or IA-1-4-2 in MARC-145 cell monolayers. Each value represents the mean PRRSV neutralizing antibody titer in the blood of five animals ± S.E.M. except for group 1 that was downsized to four pigs immediately prior to challenge.

Table 1

Comparison of the presence of PRRSV-neutralizing antibodies in the serum of pigs after immunization with a PRRSV MLV vaccine in the absence/presence of IL-12 or IFN-α

Group no.[a]	Treatment	Number of VN antibody positive pigs/total pigs[b] (weeks after vaccination)				
		0	1	2	4	8
1	MLV	0/5	0/5	0/5	1/5	0/4
2	MLV + IFN-α cDNA i.m.	0/5	0/5	0/5	0/5	0/5
3	MLV + IL-12 cDNA i.m.	0/5	0/5	0/5	1/5	0/5
4	MLV + IL-12 cDNA i.d.	0/5	0/5	0/5	1/5	ND[c]
5	MLV + rIL-12 protein i.m.	0/5	0/5	0/5	2/5	ND
6	Unvaccinated	0/2	0/2	0/2	0/2	ND

[a] Serum was drawn from all members of groups 1–6 described in the legend for Fig. 1 at the indicated times after vaccination and assayed for the presence of virus neutralizing (VN) antibodies against PRRSV isolates VR2332 and IA-1-4–2.

[b] The numerator values represent the number of VN positive pigs, and the denominator values represent the total number of pigs in each respective group. The VN titer in positive samples was 1:4 against either virus isolate.

[c] Not done.

detected in the sera of the challenged, non-vaccinated pigs.

3.3. PRRS viremia in pigs vaccinated in the absence/presence of IFN-α or IL-12

At 4 days post-PRRSV challenge, one of four pigs immunized with MLV vaccine alone (group 1) and three of five pigs immunized in conjunction with an i.m. injection of pcPIL12 (group 3) had detectable viremia (level of sensitivity $\leq 10^{2.2}$ $TCID_{50}$/ml serum) (Fig. 4). In contrast, at this time all of the unvaccinated pigs (group 7) and all of those that received pINA in combination with the vaccine were viremic (group 2). Three days later, only three of the five animals injected with pINA (group 2) as well as all of the non-immunized pigs (group 7) remained viremic (level of sensitivity $\leq 10^{1.2}$ $TCID_{50}$/ml serum). By 11 days post-challenge, virus could be found in the serum of only one of the unvaccinated pigs and none of the vaccinated pigs (level of sensitivity $\leq 10^{1.2}$ $TCID_{50}$/ml serum). PRRSV was also detected in the tonsils of three of the five non-immunized animals, but not in biopsies removed from any of the vaccinated pigs, when the experiment was terminated at 14 days after challenge (level of sensitivity $\leq 10^{2.2}$ $TCID_{50}$/g tissue). The lower sensitivity of the virus detection assay at 4 days after challenge was due to the toxicity of the serum at a 1:10 dilution. This toxic effect was not present in the serum samples collected 7 or 11 days after challenge.

3.4. Anti-PRRSV cell-mediated and humoral immune responses in pigs vaccinated in the absence/presence of poly I:C

To evaluate the impact of poly I:C on the host response to PRRSV, the immune status of pigs receiving the MLV vaccine alone or in conjunction with this synthetic double-stranded RNA and then revaccinated with the same formulations 8 weeks later was monitored for a total of 11 weeks. A nearly immediate effect of poly I:C on the pigs was observed as evidenced by an approximately 50% reduction in the quantity of white blood cells in their peripheral blood as compared to the other animals at 6 h after the

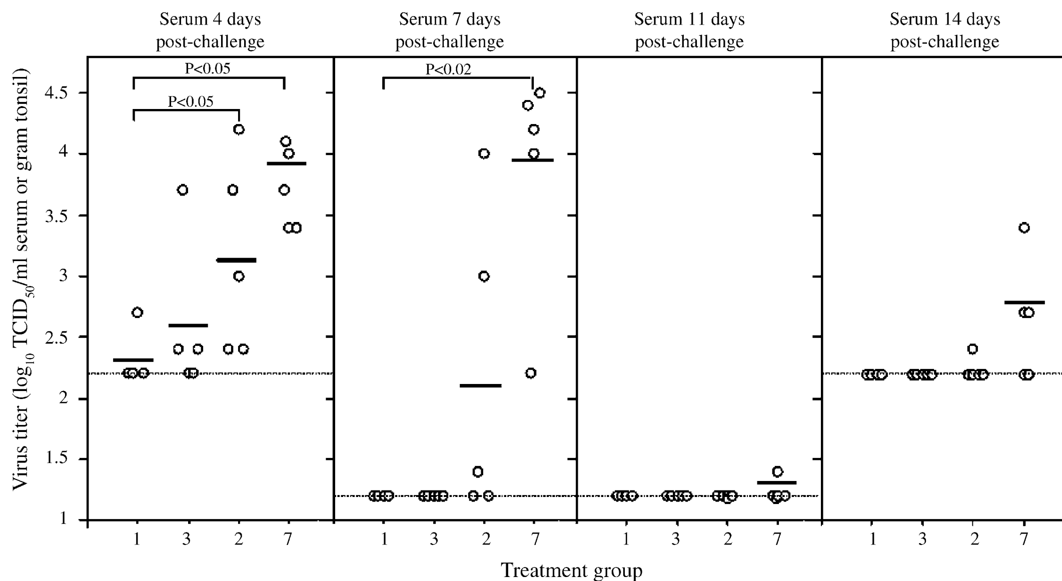

Fig. 4. Comparison of the virus titers in the sera and tonsils of vaccinated pigs after a challenge with wild type PRRSV. Serum was drawn from four members of group 1 and all pigs in groups 2, 3 and 7 described in the legend to Fig. 1 at 4, 7 and 11 days post-challenge with PRRSV strain IA-1-4-2, while tonsil biopsies were obtained at 14 days after challenge for all virus-challenged pigs. Virus titers ($\log_{10}$ $TCID_{50}$ per ml of serum or per gram of tonsil) are presented for each pig (circle). The limit of detection for each assay is indicated by the dotted line and values at this level have been placed on the line. Bars indicate the mean of four or five measurable titers within a group. Statistical significance ($P < 0.02$, $P < 0.05$) is based on differences on the percent of viremic pigs between the indicated treatment groups as determined by Fisher's exact test.

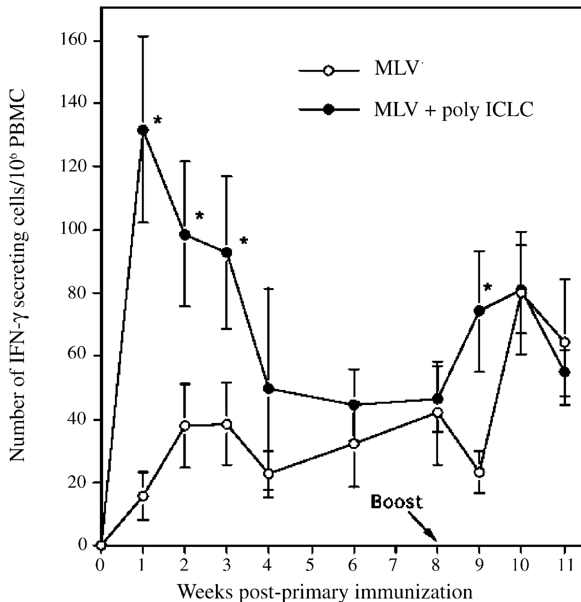

Fig. 5. Comparison of the intensities of the virus-specific IFN-γ SC response of pigs to immunization with a PRRS MLV vaccine in the absence/presence of poly I:C. Groups of pigs were vaccinated with PRRS MLV alone or in conjunction with poly I:C at an 8-week interval. PBMC were isolated from the pigs at the indicated times post-vaccination and the presence of virus-specific IFN-γ SC was determined by using an ELISPOT assay. Significant differences ($P < 0.05$) between the frequencies of IFN-γ SC in the blood of the two groups are indicated by an asterisk. Each value represents the mean response of six animals ± S.E.M.

administration of this compound. This leukopenia was transient as it was no longer apparent 42 h later (data not shown). At 1 week post-immunization, the poly I:C-treated pigs also exhibited a significant 7.3-fold increase in the frequency of their PRRSV-specific IFN-γ SC as compared to the untreated pigs (Fig. 5). This enhancement tapered off but was still >2-fold when measured 1 and 2 weeks later. From then until the time of the second immunization, significant differences between the frequencies of the IFN-γ SC in the two groups were not detected. However, 1 week after the booster immunization, once again the frequency of virus-specific IFN-γ SC in the poly I:C-treated animals increased in comparison to the untreated animals—in this case a statistically significant 2.2-fold. During the ensuing 2-week interval until the termination of the experiment, no differences between the two groups in regards to this parameter were observed. Although PRRSV-specific antibodies were readily detected in all of the pigs' sera when collected at 10 and 11 weeks post-primary immunization, no significant differences were found to exist between the average antibody titers of the poly I:C-treated and untreated groups (data not shown).

4. Discussion

The data presented here demonstrate the adjuvant effects of IFN-α when provided exogenously in the form of an expressible cDNA (pINA) or circuitously via induction of IFN-α synthesis (poly I:C) on the vaccine-induced IFN-γ response to PRRSV. However, despite the positive modulation of Th 1 immunity, no significant alteration in the development of the humoral immune response was observed. Thus, even with such intervention at the initiation of PRRSV immunization, the usual rapid onset of anti-PRRSV antibody production and delayed appearance of VN antibodies (Labarque et al., 2000; Ostrowski et al., 2002; Meier et al., 2003) still occurred. Although in previous studies a similar increased presence of PRRSV-specific IFN-γ SC was afforded to pigs receiving multiple injections of IL-12 at the time of vaccination and during the ensuing week (Foss et al., 2002), the use of a conventional oil-in-water adjuvant was found to be ineffective in this regard (Meier et al., 2003). Moreover, in that study, even though this compound demonstrably enhanced the genesis of VN antibodies recognizing pseudorabies virus in addition to specific IFN-γ SC, the intensity of either type of immune response to PRRSV was not affected. Apparently PRRSV possesses inherent structural elements that prevent the timely development of protective innate or adaptive immunity capable of inhibiting its infectious process. Thus, the ultimate outcome of the interaction between this virus and its host will be determined by the elicited host response which is highly variable, as evidenced by the inconsistency of the clinical outcomes seen upon challenge of naïve or immune pigs with PRRSV (Labarque et al., 2003; Mengeling et al., 2003a, 2003b). This is likely due to the significant variability within the swine population in regards to their innate and adaptive immune responses to PRRSV (Xiao et al., 2004; Royaee et al., 2004).

The ability of PRRSV to not initially elicit protective immunity in an infected host is relatively novel among viruses that even after being inactivated can usually still promote a Th 1-like response (de Wit et al., 2004). In this regard it is notable that the IFN-α response to exposure to PRRSV is nearly non-existent. For example, IFN-α production in the lungs of pigs acutely infected with PRRSV was either almost undetectable, or 159-fold lower than that induced by another pathogen, porcine respiratory coronavirus (PRCV) (Buddaert et al., 1998; Van Reeth et al., 1999). Such lack of efficient stimulation of IFN-α production by a pathogen has a significant impact on the nature of the host's adaptive immune response, since IFN-α up-regulates IFN-γ gene expression, and thus controls the dominant pathway that promotes the development of adaptive immunity, namely, T cell-mediated IFN-γ responses and peak antiviral immune defenses (Cousens et al., 1997; Levy et al., 2003). In this regard, it has become evident that the link between innate and adaptive immunity in viral infections occurs through the interaction of dendritic cells with type I interferon (Montoya et al., 2002; Tough, 2004) and the dendritic-cell controlled polarization of T-cell function (Kapsenberg, 2003). The production of IFN-α by plasmacytoid dendritic cells/natural IFN-α/β-producing cells (NIPC) has an autocrine effect that promotes their functional and phenotypic activation, which is necessary for their optimal expression of co-stimulatory molecules and subsequent ability to cause naïve T cells to differentiate into IFN-γ-SC (Cella et al., 2000; Kadowaki et al., 2000; Fitzgerald-Bocarsly, 2002; Montoya et al., 2002; Honda et al., 2003). Presumably, PRRSV is a poor inducer of IFN-α production by NIPCs since unlike transmissible gastroenteritis virus (Charley and Lavenant, 1990; Nowacki et al., 1993) and type-A CpG oligonucleotides (Guzylack-Piriou et al., 2004) it fails to stimulate the secretion of IFN-α from cultured porcine PBMC (Albina et al., 1998a; Zuckermann et al., unpublished observations). Thus, direct examination of the outcome of the interaction of PRRSV with porcine NIPCs will likely reveal important information on the immunobiology of this virus, especially since this virus is susceptible to the antiviral effects of IFN-α (Albina et al., 1998a). The application of molecular tools such as real-time PCR assays to measure the expression of key immune mediators that regulate the development of Th 1 responses in swine (Dawson et al., 2004) will be particularly useful in this endeavor.

It should be noted that in the absence of IFN-α/β production, the cytokine IL-12 whose synthesis is not induced by most viral infections (Orange and Biron, 1996) can increase IFN-γ production by T cells (Cousens et al., 1999). Thus, two alternative routes (IL-12- or type I IFN-dependent) can lead to an adaptive Th 1 cell-mediated immune response with potent antiviral effects (Biron, 2001). According to a scenario involving the presence of less than a requisite amount of IFN-α, IL-12 could provide the necessary impetus for the development of an anti-viral IFN-γ response. In this regard, IL-12 mRNA has been detected in porcine macrophages infected with PRRSV (Thanawongnuwech et al., 2001), and transiently in the lungs of PRRSV-infected pigs (Chung and Chae, 2003). However, this pathogen is also apparently a poor stimulator of IL-12 production, since a negligible quantity of IL-12 mRNA or protein was produced by porcine PBMC exposed in vitro to PRRSV (Royaee et al., 2004; Zuckermann et al., unpublished observations). The observation that the inclusion of either IL-12 and IFN-α during immunization increased the intensity of the IFN-γ response to PRRSV validates the proposed role of these two innate cytokines in directing the in vivo differentiation of swine Th 1 cells, and helps explain the poor virus-specific IFN-γ response that normally develops as a result of the exposure of pigs to PRRSV (Meier et al., 2003; Xiao et al., 2004).

To compensate for the lack of stimulation of innate cytokine expression by PRRSV, novel adjuvants have been used during immunization. The administration of IL-12 in combination with a live or killed PRRSV vaccine resulted in an increased lymphoproliferative response to this virus (Wee et al., 2001). Inclusion of either a combination of IL-1 and IL-6 or cholera toxin correlated with an enhanced response to PRRSV but only at 6 weeks after the initial exposure (Foss et al., 2002). Moreover, in that study, the frequency of virus-specific IFN-γ SC did transiently increase between 2 and 3 weeks post-vaccination of pigs also injected with porcine rIL-12. Although similar results were obtained in the current study, albeit after biolistic injection of expressible IL-12 cDNA in lieu of protein, the provision of IFN-α cDNA had a more pronounced and sustained effect on the intensity of the cell-

mediated immune response. It was notable that the i.d. administration of IL-12 cDNA with a gene gun was more successful at enhancing the vaccine-induced IFN-γ response than the i.m. injection of the same plasmid. This observation is in agreement with the reported higher efficiency of in vivo DNA transfection by biolistic delivery (Fynan et al., 1993; Colosimo et al., 2000). The greater effectiveness of the plasmid encoding IFN-α rather than IL-12 at enticing an IFN-γ response could be attributed to the relatively low amounts of complete IL-12 receptor on swine lymphocytes and the limited up-regulation of expression of the IL-12 receptor ß2 subunit gene as compared to other species (Solano-Aguilar et al., 2002). In this regard, it should be noted that the injection of bioactive IL-12 into naïve pigs did not stimulate a strong T cell response (Solano-Aguilar et al., 2002). Likewise, the introduction of a known inducer of IFN-α production in pigs, poly I:C (Derbyshire and Lesnick, 1990), during vaccination temporarily amplified the quantities of PRRSV-specific IFN-γ SC, but was not as efficient as the IFN-α encoding plasmid at enhancing the IFN-γ response to the vaccine. This difference could be attributed to the presence of immunostimulatory CpG motifs in the eukaryotic expression vector pcDNA3, which was used in this study to express the cytokine genes, and has been shown to induce IFN-γ expression by porcine leukocytes (Magnusson et al., 2001). It is plausible that the combination of direct stimulation of NIPC by the CpG motifs in the IFN-α-encoding plasmid, presumably through their toll-like receptor 9 (Shimosato et al., 2003), in combination with the plasmid-driven production of IFN-α would provide the necessary stimulatory signals to promote the maturation of dendritic cells and the creation of a microenvironment conducive for a sustained IFN-γ response to PRRSV. Although poly I:C induces IFN-α production, it does not entice porcine NIPC to differentiate (Guzylack-Piriou et al., 2004), and thus might not provide enough impetus to promote a sustained T-cell-mediated IFN-γ response to this virus. This notion is in agreement with the role attributed to CpG-containing oligonucleotides in promoting the maturation of NIPC in humans (Krug et al., 2001) and swine (Guzylack-Piriou et al., 2004).

The inability of IFN-α cDNA to enhance the VN antibody response to PRRSV is in contrast to the positive effect exerted by the co-administration of adenovirus expressing IFN-α in combination with a foot-and-mouth disease virus (FMDV) subunit vaccine into pigs (Chinsangaram et al., 2003; Moraes et al., 2003). The notion of an structural inherent property unique to PRRSV that deters the elicitation of a strong VN antibody response is in accord with the observation that in the case of PRRSV-neutralizing antibodies, the close association of a nearby *N*-glycan with a recognized epitope in the virus's envelope glycoprotein 5 may impede neutralization of this virus (Plagemann et al., 2002). In addition, decoy epitopes that delay the development of VN antibodies also exist (Ostrowski et al., 2002). Remarkably, as we have shown here, the titer of VN antibodies increased equally in all vaccinated groups as a result of the pigs being challenged with a genetically divergent, virulent virus. Similarly, augmented titers of VN antibodies against PRRSV have been observed in pigs previously vaccinated with PRRS MLV after they received a booster immunization with an inactivated genetically divergent virus but not when exposed to the same attenuated virus (Osorio et al., 1998; Bassaganya-Riera et al., 2004).

As compared to the control (PRRSV naïve) pigs, a lower proportion of those vaccinated with PRRS MLV exhibited viremia at 4 and 7 days after challenge. However, no further reduction was observed in animals also receiving either IL-12 or IFN-α. Rather, injection of pINA increased the percentage of viremic pigs, although the mean virus titer in their sera was intermediate between those values determined for the vaccinated and un-vaccinated groups at 4 days after challenge. The significance of this observation with regards to protective immunity is unclear. This is due to the fact that while vaccination can decrease the duration and magnitude of viremia following an experimental challenge (van Woensel et al., 1998; Verheije et al., 2003), the reduction in viremia is not necessarily associated with commensurate amelioration of the severity of other clinical parameters associated with PRRS such as a lessened rate of weight gain, fever, respiratory distress or virus transmission to sentinel pigs (Nodelijk et al., 2001; Labarque et al., 2003; Mengeling et al., 2003a). A similar lack of correlation between viremia and clinical signs was also noted when two different age groups of non-immune pigs were infected with

PRRSV. Whereas a greater frequency of viremia with an accompanying higher virus titer was found in the younger animals (2 months of age), the older pigs (6 months of age) exhibited more severe clinical signs (van der Linden et al., 2003). The difficulty of deciphering PRRSV biology is further revealed by the marked degree of variability and irreproducibility of consecutive trials conducted by the same investigators (Labarque et al., 2003; Mengeling et al., 2003a, 2003b). Moreover, although a positive association between protection from disease and the intensity of the IFN-γ response in swine has been observed in regard to pseudorabies virus (Zuckermann et al., 1998, 1999; Van Rooij et al., 2004), a similar relationship concerning PRRSV was not apparent from the results of this study. Thus, the identification of an immunologic mechanism responsible for mediating protective immunity against PRRSV poses a significant challenge and will perhaps require monitoring the extent of reproductive failure caused by this virus as a measurement of the degree of protection (Lager et al., 1999). Notably, in recent, related field studies we have noticed a positive correlation between the reduction of abortion/still births in sows and the relative frequency of PRRSV-specific IFN-γ SC in their blood (Lowe et al., 2004). In any event, perhaps a more marked enhancement of the vaccine-induced IFN-γ response will be needed in order to improve protective immunity against PRRSV as we found to be the case when IL-12 was used as an adjuvant for an inactivated pseudorabies virus vaccine, which like the PRRS MLV vaccine stimulates a weak IFN-γ response (Zuckermann et al., 1998).

Clearly, further studies will be required to clarify how the outcome of a PRRSV infection is influenced by the intensity of the IFN-γ response by memory T cells. Nevertheless, the potential importance of eliciting a strong IFN-γ response against this pathogen might not be limited to preventing virus replication (Bautista and Molitor, 1999; Rowland et al., 2001). Since IFN-γ can restrain B cell differentiation and even polyclonal B cell activation (Cowdery and Fleming, 1992), it is reasonable to propose that a strong IFN-γ response could mediate protective immunity by thwarting the polyclonal B cell activation that is associated with PRRSV infection and results in immunopathology (Lemke et al., 2004). The uncontrolled B cell activation induced by PRRSV could also contribute to the observed weak cell-mediated immune response, since activated B cells can release IL-10 (Burdin et al., 1997), a cytokine that inhibits both IFN-γ production by porcine T cells (Waters et al., 1999), and IFN-α synthesis by human PBMC in response to viral stimulation (Payvandi et al., 1998). In the accompanying manuscript (Royaee et al., 2004) we demonstrate that PBMC obtained from pigs at 2 weeks after immunization with PRRS MLV spontaneously secrete IL-1, IL-6, and IL-10 and that the production of these cytokines are indeed affected by the administration, at the time of vaccination, of the same IFN-α expressing plasmid utilized in the present study. Such repression may be critical since unabated PRRSV infection will actually stimulate synthesis of IL-6 (Asai et al., 1999), a known inhibitor of Th 1 cell development (Diehl and Rincon, 2002) and promoter of polyclonal immunoglobulin production following B cell activation by the murine arterivirus, lactate dehydrogenase-elevating virus (Markine-Goriaynoff et al., 2001).

Strategies designed to shift the bias of the initial reaction to PRRSV from the strong elicitation of non-neutralizing antibody production towards a greater Th 1-like immune response are worth exploring since they could conceivably lead to the development of an improved vaccine against this pathogen. The need to develop such methodology is made palpable by the unusual kinetics of the immune response to this virus, as evidenced by the lack of a marked CMI response upon vaccination and subsequent challenge with virulent virus. The IFN-γ response to PRRSV therefore appears to be determined at the time of the first exposure to this pathogen and is only minimally affected by re-exposure. Although it is also possible that we did not monitor the immune response long enough post-challenge to detect a significant increase in the number of virus-specific IFN-γ SC, this is unlikely since in the accompanying manuscript a similar minimal enhancement was observed when the immune response was measured for up to 5 weeks after a booster immunization (Royaee et al., 2004). In addition, similar limited changes in the IFN-γ response have been observed upon challenge of vaccinated pigs with wild-type virus (Foss et al., 2002) or booster immunization with MLV vaccine (Meier et al., 2003). Remarkably, the T-cell proliferative response to PRRSV was also not

increased by a booster immunization in pigs that had previously been repeatedly exposed to a MLV vaccine, but rather appeared to be suppressed as compared to that elicited by the same vaccine in naïve pigs (Bassaganya-Riera et al., 2004). The mechanism responsible for this unusual effect is currently unknown, but might be related to the persistence of the virus in lymphoid tissues associated with the site of infection (Xiao et al., 2004). Such sustained presence of virus could adversely affect a subsequent response due to an inherent and yet unknown property of PRRSV.

In summary, it is apparent that PRRSV elicits in swine a polarized immune response characterized by an abundance of non-neutralizing antibodies and a paucity of IFN-γ SC. The molecular pathway responsible for generating this type of immunity is unknown at this time, but based on the results presented it likely involves the limited induction of IFN-α and IL-12 production and/or inherent structural elements of the virus that promote such a response. We propose that the strong humoral immunity bias of the host response to PRRSV is mostly responsible for the difficulties in the development of a vaccine deemed effective in the field. Although our use of porcine cytokines, especially IFN-α, as adjuvants did not radically affect PRRSV's inherent ability to promote this type of polarized immunity, a positive impact on the transition of T lymphocytes to IFN-γ SC was noticed. The limited up-regulation of cellular IFN-γ responses (presented here) or gene expression (described in the accompanying paper, Royaee et al., 2004) indicates that more substantial stimulants of innate immunity are to modify the host response to PRRSV. Perhaps a more potent immunomodulator, such as type A CpG oligodeoxynucleotides (Klinman, 2004), will overcome the tendency of PRRSV to stimulate a strong non-neutralizing antibody response and simultaneously promote a Th 1 (IFN-γ dominated) response.

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References

Albina, E., Carrat, C., Charley, B., 1998a. Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J. Interferon Cytok. Res. 18, 485–490.

Albina, E., Piriou, L., Hutet, E., Cariolet, R., L'Hospitalier, R., 1998b. Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Immunol. Immunopathol. 61, 49–66.

Asai, T., Mori, M., Okada, M., Uruno, K., Yazawa, S., Shibata, I., 1999. Elevated serum haptoglobin in pigs infected with porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 70, 143–148.

Bassaganya-Riera, J., Thacker, B.J., Yu, S., Strait, E., Wannemuehler, M.J., Thacker, E.L., 2004. Impact of immunizations with porcine reproductive and respiratory syndrome virus on lymphoproliferative recall responses of CD8+ T cells. Viral Immunol. 17, 25–37.

Bautista, E.M., Molitor, T.W., 1997. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunol. 10, 83–94.

Bautista, E.M., Molitor, T.W., 1999. IFN gamma inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Arch. Virol. 144, 1191–1200.

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4, 127–133.

Biron, C.A., 2001. Interferons alpha and beta as immune regulators-a new look. Immunity 14, 661–664.

Buddaert, W., Van Reeth, K., Pensaert, M., 1998. In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv. Exp. Med. Biol. 440, 461–467.

Burdin, N., Rousset, F., Banchereau, J., 1997. B-cell-derived IL-10: production and function. Methods 11, 98–111.

Cella, M., Facchetti, F., Lanzavecchia, A., Colonna, M., 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat. Immunol. 1, 305–310.

Charley, B., Lavenant, L., 1990. Characterization of blood mononuclear cells producing IFN alpha following induction by coronavirus-infected cells (porcine transmissible gastroenteritis virus). Res. Immunol. 141, 141–151.

Chinsangaram, J., Moraes, M.P., Koster, M., Grubman, M.J., 2003. Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. J. Virol. 77, 1621–1625.

Chung, H.K., Chae, C., 2003. Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine

reproductive and respiratory syndrome virus (PRRSV). J. Comp. Pathol. 129, 205–212.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., Gorcyca, D., Chladek, D., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4, 117–126.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G., Malone, R.W., Bennett, M.J., Gruenert, D.C., 2000. Transfer and expression of foreign genes in mammalian cells. Biotechniques 29, 314–318.

Cousens, L.P., Orange, J.S., Su, H.C., Biron, C.A., 1997. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. Proc. Natl. Acad. Sci. 94, 634–639.

Cousens, L.P., Peterson, R., Hsu, S., Dorner, A., Altman, J.D., Ahmed, R., Biron, C.A., 1999. Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection. J. Exp. Med. 189, 1315–1328.

Cowdery, J.S., Fleming, A.L., 1992. In vivo depletion of CD4 T cells increases B cell sensitivity to polyclonal activation: the role of interferon-gamma. Clin. Immunol. Immunopathol. 62, 72–77.

Dawson, H.D., Royaee, A.R., Nishi, S., Kuhar, D., Schnitzlein, W.M., Zuckermann, F.A., Urban Jr., J., Lunney, J.K., 2004. Identification of key immune mediators regulating T helper 1 responses in swine. Vet. Immunol. Immunopathol. 100, 105–111.

Derbyshire, J.B., Lesnick, C.E., 1990. The effect of interferon induction in newborn piglets on the humoral immune response to oral vaccination with transmissible gastroenteritis virus. Vet. Immunol. Immunopathol. 24, 227–234.

de Wit, M.C., Horzinek, M.C., Haagmans, B.L., Schijns, V.E., 2004. Host-dependent type 1 cytokine responses driven by inactivated viruses may fail to default in the absence of IL-12 or IFN-alpha/beta. J. Gen. Virol. 85, 795–803.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Mol. Immunol. 39, 531–536.

Fitzgerald-Bocarsly, P., 2002. Natural interferon-α producing cells: the plasmacytoid dendritic cells. Cytokines: receptors and cell signaling (supplement). Biotechniques 16–29.

Foss, D.L., Moody, M.D., Murphy Jr., K.P., Pazmany, C., Zilliox, M.J., Murtaugh, M.P., 1999. In vitro and in vivo bioactivity of single-chain interleukin-12. Scand. J. Immunol. 50.

Foss, D.L., Zilliox, M.J., Meier, W., Zuckermann, F., Murtaugh, M.P., 2002. Adjuvant danger signals increase the immune response to porcine reproductive and respiratory syndrome virus. Viral Immunol. 15, 557–566.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., Robinson, H.L., 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. 90, 11478–11482.

Goldberg, T.L., Lowe, J.F., Milburn, S.M., Firkins, L.D., 2003. Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. Virology 317, 197–207.

Gonin, P., Pirzadeh, B., Gagnon, C.A., Dea, S., 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J. Vet. Diagn. Invest. 11, 20–26.

Guzylack-Piriou, L., Balmelli, C., McCullough, K.C., Summerfield, A., 2004. Type-A CpG oligonucleotides activate exclusively porcine natural interferon-producing cells to secrete interferon-alpha, tumour necrosis factor-alpha and interleukin-12. Immunology 112, 28–37.

Halbur, P., 2003. Factors that influence the severity of clinical disease. In: Zimmerman, J., Yoon, K-J. (Eds.), PRRS Compendium. 2nd ed. National Pork Board, pp. 17–26.

Honda, K., Sakaguchi, S., Nakajima, C., Watanabe, A., Yanai, H., Matsumoto, M., Ohteki, T., Kaisho, T., Takaoka, A., Akira, S., Seya, T., Taniguchi, T., 2003. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. Proc. Natl. Acad. Sci. 100, 10872–10877.

Kadowaki, N., Antonenko, S., Lau, J.Y., Liu, Y.J., 2000. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J. Exp. Med. 192, 219–226.

Kadowaki, N., Liu, Y.J., 2002. Natural type I interferon-producing cells as a link between innate and adaptive immunity. Hum. Immunol. 63, 1126–1132.

Kapsenberg, M.L., 2003. Dendritic-cell control of pathogen-driven T-cell polarization. Nat. Rev. Immunol. 3, 984–993.

Key, K.F., Haqshenas, G., Guenette, D.K., Swenson, S.L., Toth, T.E., Meng, X.J., 2001. Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates. Vet. Microbiol. 83, 249–263.

Klinman, D.M., 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 4, 249–258.

Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A.M., Hartmann, G., 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur. J. Immunol. 31, 3026–3037.

Labarque, G.G., Nauwynck, H.J., Van Reeth, K., Pensaert, M.B., 2000. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J. Gen. Virol. 81, 1327–1334.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2003. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet. Microbiol. 95, 187–197.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1997a. Duration of homologous porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58, 127–133.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1997b. Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58, 113–125.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1999. Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus

(PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. Am. J. Vet. Res. 608, 1022–1027.

Lefevre, F., La Bonnardiere, C., 1986. Molecular cloning and sequencing of a gene encoding biologically active porcine alpha-interferon. J. Interferon Res. 6, 349–360.

Lemke, C.D., Haynes, J.S., Spaete, R., Adolphson, D., Vorwald, A., Lager, K., Butler, J.E., 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by porcine reproductive and respiratory syndrome virus infection in neonatal pigs. J. Immunol. 172, 1916–1925.

Levy, H.B., Baer, G., Baron, C., Buckler, C.E., Gibbs, C.J., Iadarola, M.J., London, W.T., Rice, J., 1975. A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. J. Infect. Dis. 132, 434–439.

Levy, D.E., Marie, I., Prakash, A., 2003. Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. Curr. Opin. Immunol. 15, 52–58.

Loewen, K.G., Derbyshire, J.B., 1988. Interferon induction in piglets with polyinosinic:polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose. Res. Vet. Sci. 44, 132–133.

Loemba, H.D., Mounir, S., Mardassi, H., Archambault, D., Dea, S., 1996. Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch. Virol. 141, 751–761.

Lopez-Fuertes, L., Domenech, N., Alvarez, B., Ezquerra, A., Dominguez, J., Castro, J.M., Alonso, F., 1999. Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res. 64, 33–42.

Lowe, J.F., Husmann, R., Firkins, L.D., Zuckermann, F.A., Goldberg, T.L., 2004. Correlation of cellular immunity to Porcine Reproductive Respiratory Syndrome Virus and clinical disease during outbreaks of PRRS in commercial swine herds. J. Am. Vet. Med. Assoc., in press.

Magnusson, M., Johansson, E., Berg, M., Eloranta, M.L., Fuxler, L., Fossum, C., 2001. The plasmid pcDNA3 differentially induces production of interferon-alpha and interleukin-6 in cultures of porcine leukocytes. Vet. Immunol. Immunopathol. 78, 45–56.

Markine-Goriaynoff, D., Nguyen, T.D., Bigaignon, G., Van Snick, J., Coutelier, J.P., 2001. Distinct requirements for IL-6 in polyclonal and specific Ig production induced by microorganisms. Int. Immunol. 13, 1185–1192.

Mateu de Antonio, E., Husmann, R.J., Hansen, R., Lunney, J.K., Strom, D., Martin, S., Zuckermann, F.A., 1998. Quantitative detection of porcine interferon-gamma in response to mitogen, superantigen and recall viral antigen. Vet. Immunol. Immunopathol. 61, 265–277.

Meier, W., Wheeler, J., Husmann, R.J., Osorio, F.A., Zuckermann, F.A., 2000. Characteristics of the immune response of pigs to PRRS virus. Vet. Res. 31, 41.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18–31.

Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Clouser, D.F., 2003a. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Vet. Microbiol. 93, 25–38.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Koehler, K.J., 2003b. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 93, 13–24.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., Tough, D.F., 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 99, 3263–3271.

Moraes, M.P., Chinsangaram, J., Brum, M.C., Grubman, M.J., 2003. Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine. Vaccine 22, 268–279.

Murtaugh, M.P., Faaberg, K.S., Laber, J., Elam, M., Kapur, V., 1998. Genetic variation in the PRRS virus. Adv. Exp. Med. Biol. 440, 787–794.

Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol. 15, 533–547.

Nelson, E.A., Christopher-Hennings, J., Benfield, D.A., 1994. Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J. Vet. Diagn. Invest. 6, 410–415.

Nodelijk, G., de Jong, M.C., van Leengoed, L.A., Wensvoort, G., Pol, J.M., Steverink, P.J., Verheijden, J.H., 2001. A quantitative assessment of the effectiveness of PRRSV vaccination in pigs under experimental conditions. Vaccine 19, 3636–3644.

Nowacki, W., Cederblad, B., Renard, C., La Bonnardiere, C., Charley, B., 1993. Age-related increase of porcine natural interferon alpha producing cell frequency and of interferon yield per cell. Vet. Immunol. Immunopathol. 37, 113–122.

Orange, J.S., Biron, C.A., 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. J. Immunol. 156, 1138–1142.

Osorio, F.A., Zuckermann, F.A., Wills, R., Christian, S., Galeota, J., Doster, A., 1998. PRRSV: comparison of commercial vaccines in their ability to induce protection against current PRRSV strains of high virulence. Proc. A. D. Leman Conf. 25, 176–182.

Osorio, F.A., Galeota, J.A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F., Laegreid, W.W., 2002. Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology 302, 9–20.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250.

Payvandi, F., Amrute, S., Fitzgerald-Bocarsly, P., 1998. Exogenous and endogenous IL-10 regulate IFN-alpha production by peripheral blood mononuclear cells in response to viral stimulation. J. Immunol. 160, 5861–5868.

Plagemann, P.G., Rowland, R.R., Faaberg, K.S., 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147, 2327–2347.

Rowland, R.R., Robinson, B., Stefanick, J., Kim, T.S., Guanghua, L., Lawson, S.R., Benfield, D.A., 2001. Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma and recovery of virus replication with 2-aminopurine. Arch. Virol. 146, 539–555.

Royaee, A.R., Husmann, R.J., Dawson, H.D., Calzada-Nova, G., Schnitzlein, W.M., Zuckermann, F.A., Lunney, J.K., 2004. Deciphering the involvement of innate immune factors in the development of the host responses to PRRSV vaccination. Vet. Immunol. Immunopathol. 102, 199–217.

Rubinstein, S., Familletti, P.C., Pestka, S., 1981. Convenient assay for interferons. J. Virol. 37, 755–758.

Sekellick, M.J., Marcus, P.I., 1979. Persistent infection. II. Interferon-inducing temperature-sensitive mutants as mediators of cell sparing: possible role in persistent infection by vesicular stomatitis virus. Virology 95, 36–47.

Shimosato, T., Kitazawa, H., Katoh, S., Tomioka, Y., Karima, R., Ueha, S., Kawai, Y., Hishinuma, T., Matsushima, K., Saito, T., 2003. Swine toll-like receptor 9 recognizes CpG motifs of human cell stimulant. Biochim. Biophys. Acta 1627, 56–61.

Solano-Aguilar, G.I., Zarlenga, D., Beshah, E., Vengroski, K., Gasbarre, L., Junker, D., Cochran, M., Weston, C., Valencia, D., Chiang, C., Dawson, H., Urban, J., Lunney, J.K., 2002. Limited effect of recombinant porcine interleukin-12 on porcine lymphocytes due to a low expression of IL-12b2 receptor. Vet. Immunol. Immunopathol. 89, 133–148.

Thacker, B., 2003. Clinical manifestations of PRRS virus. In: Zimmerman, J., Yoon, K-J. (Eds.), PRRS Compendium. 2nd ed. National Pork Board, pp. 7–16.

Thanawongnuwech, R., Young, T.F., Thacker, B.J., Thacker, E.L., 2001. Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. Vet. Immunol. Immunopathol. 79, 115–127.

Tough, D.F., 2004. Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. Leuk. Lymphoma 45, 257–264.

van der Linden, I.F., Voermans, J.J., van der Linde-Bril, E.M., Bianchi, A.T., Steverink, P.J., 2003. Virological kinetics and immunological responses to a porcine reproductive and respiratory syndrome virus infection of pigs at different ages. Vaccine 21, 1952–1957.

Van Reeth, K., Labarque, G., Nauwynck, H., Pensaert, M., 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res. Vet. Sci. 67, 47–52.

Van Rooij, E.M., De Bruin, M.G., De Visser, Y.E., Middel, W.G., Boersma, W.J., Bianchi, A.T., 2004. Vaccine-induced T cell-mediated immunity plays a critical role in early protection against pseudorabies virus (suid herpes virus type 1) infection in pigs. Vet. Immunol. Immunopathol. 99, 113–125.

van Woensel, P.A., Liefkens, K., Demaret, S., 1998. Effect on viraemia of an American and a European serotype PRRSV vaccine after challenge with European wild-type strains of the virus. Vet. Rec. 142, 510–512.

Verheije, M.H., Kroese, M.V., van der Linden, I.F., de Boer-Luijtze, E.A., van Rijn, P.A., Pol, J.M., Meulenberg, J.J., Steverink, P.J., 2003. Safety and protective efficacy of porcine reproductive and respiratory syndrome recombinant virus vaccines in young pigs. Vaccine 21, 2556–2563.

Vezina, S.A., Loemba, H., Fournier, M., Dea, S., Archambault, D., 1996. Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Can. J. Vet. Res. 60, 94–99.

Waters, W.R., Sacco, R.E., Dorn, A.D., Hontecillas, R., Zuckermann, F.A., Wannemuehler, M.J., 1999. Systemic and mucosal immune responses of pigs to parenteral immunization with a pepsin-digested *Serpulina hyodysenteriae* bacterin. Vet. Immunol. Immunopathol. 69, 75–87.

Wee, G.J., Cao, M.J., Liu, W., Kwang, J., 2001. Efficacy of porcine reproductive and respiratory syndrome virus vaccine and porcine interleukin-12. Vet. Therapeutics 2, 112–119.

Wensvoort, G., Terpstra, C., Pol, J.M.A., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Quart. 13, 121–130.

Xiao, Z., Batista, L., Dee, S., Halbur, P., Murtaugh, M.P., 2004. The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J. Virol. 78, 5923–5933.

Yoon, K.J., Zimmerman, J.J., Swenson, S.L., McGinley, M.J., Eernisse, K.A., Brevik, A., Rhinehart, L.L., Frey, M.L., Hill, H.T., Platt, K.B., 1995. Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J. Vet. Diagn. Invest. 7, 305–312.

Zuckermann, F.A., Martin, S., Husmann, R.J., Brandt, J., 1999. Use of interleukin 12 to enhance the cellular immune response of swine to an inactivated herpesvirus vaccine. Adv. Vet. Med. 41, 447–461.

Zuckermann, F.A., Husmann, R.J., Schwartz, R., Brandt, J., Mateu de Antonio, E., Martin, S., 1998. Interleukin-12 enhances the virus-specific interferon gamma response of pigs to an inactivated pseudorabies virus vaccine. Vet. Immunol. Immunopathol. 63, 57–67.