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Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination

Atabak R. Royaee[a,1,2], Robert J. Husmann[b,2], Harry D. Dawson[c], Gabriela Calzada-Nova[b], William M. Schnitzlein[b], Federico A. Zuckermann[b,*], Joan K. Lunney[a,d,*]

[a]*Animal Parasitic Disease Laboratory, Animal and Natural Resources Institute, Beltsville Human Nutrition Research Center, ARS, BARC, USDA, Beltsville, MD 20705, USA*
[b]*Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802, USA*
[c]*Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, ARS, BARC, USDA, Beltsville, MD 20705, USA*
[d]*APDL, ANRI, ARS, USDA, Building 1040, Room 103, BARC-East, Beltsville, MD 20705, USA*

Abstract

The natural response of pigs to porcine reproductive and respiratory syndrome virus (PRRSV) infections and vaccinations needs to be altered so that better protection is afforded against both homologous and heterologous challenges by this pathogen. To address this problem, real-time gene expression assays were coupled with cytokine Elispot and protein analyses to assess the nature of the anti-PRRSV response of pigs immunized with modified live virus (MLV) vaccine. Although T helper 1 (Th1) immunity was elicited in all vaccinated animals, as evidenced by the genesis of PRRSV-specific interferon-gamma secreting cells (IFNG SC), the overall extent of the memory response was variable and generally weak. Peripheral blood mononuclear cells (PBMC) isolated from these pigs responded to PRRSV exposure with a limited increase in their expression of the Th1 immune markers, IFNG, tumor necrosis factor-alpha and interleukin-15 (IL15), and a reduction in the quantity of mRNAs encoding the innate and inflammatory proteins, IL1B, IL8 and IFNA. Efforts to enhance Th1 immunity, by utilizing an expression plasmid encoding porcine IFNA (pINA) as an adjuvant, resulted in a temporary increase in the frequency of PRRSV-specific IFNG SC but only minor changes overall in the expression of Th1 associated cytokine or innate immune marker mRNA by virus-stimulated PBMC. Administration of pINA, however, did correlate with decreased IL1B secretion by cultured, unstimulated PBMC but had no effect on their ability to release IFNG. Thus, while exogenous addition of IFNA during PRRSV

Abbreviations: IFN SC, interferon-gamma secreting cells; C_t values, cycle threshold C_t values; DDAB, dimethyldioctadecylammonium bromide; MLV, modified live virus; pINA, expression plasmid encoding porcine IFNA; pANI, recombinant plasmid having the IFNA insert in the opposite orientation relative to that in pINA; LSD, least significant difference; PRRSV, porcine reproductive and respiratory syndrome virus; PRCV, porcine respiratory coronavirus; PRV, pseudorabies virus; SIV, swine influenza virus

* Corresponding authors. Tel.: +1 301 504 9368 (J.K. Lunney)/+1 217 333 7767 (F.A. Zuckermann); fax: +1 301 504 5306 (J.K. Lunney)/+1 217 244 7421 (F.A. Zuckermann).
E-mail address: fazaaa@uiuc.edu (F.A. Zuckermann), jlunney@anri.barc.usda.gov (J.K. Lunney).
[1] Present address: Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA.
[2] Atabak R. Royaee and Robert J. Husmann contributed equally to this work.

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vaccination has an impact on the development of a Th1 immune response, other alterations will be required for substantial boosting of virus-specific protection.
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Keywords: Swine; Cytokine gene regulation; T helper 1 immunity; anti-PRRSV immunity; IFNA adjuvant; Inflammatory cytokines

1. Introduction

Current vaccines are only partially effective in providing pigs protection against infection by porcine reproductive and respiratory syndrome virus (PRRSV). Since attenuated, modified live virus (MLV) is clearly superior to inactivated PRRSV in regards to eliciting an immune response, the former is preferentially used for immunizations of swine. In an attempt to increase the pigs' resiliency against both homologous and heterologous PRRSV challenge, research worldwide has been directed towards improving vaccine formulations. For instance, in the last decade numerous groups have evaluated the use of cytokines and co-stimulatory molecules as adjuvants during immunization of swine (Pasquini et al., 1997; Zuckermann et al., 1998, 1999; Somasundaram et al., 1999; Murtaugh et al., 2002; Foss et al., 2002; Boyaka et al., 2003; Wu et al., 2004). Initially, exogenous recombinant interleukin-12 (rIL12) was found to enhance the cellular immune response to PRRSV, both in vitro and in vivo (Foss et al., 2002; Meier et al., 2003). However, our recent data has shown that better stimulation of anti-PRRSV immunity was provided by exposure to a different cytokine, interferon-alpha (IFNA) (Meier et al., 2004).

Like other pathogens, infection by PRRSV clearly stimulates cytokine production. The T helper 1 (Th1) cytokine, IFNG, as well as innate immune markers, the inflammatory interleukins, IL1B, IL6 and IL8, have been detected in virus-infected pigs (Aasted et al., 2002; Bassaganya-Riera et al., 2004; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003; Thanawongnuwech et al., 2001, 2003; Thanawongnuwech and Thacker, 2003). However, when the immune responses in pigs' lungs to different swine viruses were compared, the quantities of numerous secreted cytokines appeared to be much lower in the presence of PRRSV than when either swine influenza virus (SIV) or porcine respiratory coronavirus (PRCV) was present in this organ (Van Reeth et al., 1999, 2002; Van Reeth and Nauwynck, 2000). Similarly, PRRSV has been found to elicit a substantially weaker, peripheral blood IFNG response than pseudorabies virus (PRV) (Meier et al., 2003). Likewise, PRRSV has been shown to be a poor stimulator of innate cytokine production (Albina et al., 1998a,b; Buddaert et al., 1998; Van Reeth et al., 1999; Chung et al., 2004) in contrast to most viruses that elicit copious amounts of IFNA and IFNB. Since these two cytokines are involved in the generation of antiviral IFNG secreting cells (SC) (Levy et al., 2003a,b), a deficit in innate cytokine production following PRRSV infection could be a cause of the weak adaptive IFNG response.

To determine the influence of PRRSV on the production of various regulatory factors governing antiviral immunity, we initially focused on assaying the impact of vaccination on the anti-viral memory responses of swine PBMC by monitoring their expression of a panel of immune genes. Later, we extended this procedure to determine how the administration of an IFNA expression plasmid at the time of immunization altered cellular responses, swine immune gene expression and spontaneous secretion of cytokines. By doing so, we intended to identify target genes whose up-regulated expression would correlate with a more effective PRRSV vaccination.

2. Materials and methods

2.1. Mammalian expression vector containing porcine IFNA cDNA

cDNA encoding porcine IFNA was prepared by RT-PCR and placed under the transcriptional regulation of the cytomegalovirus promoter in pcDNA3 (Invitrogen, Carlsbad, CA) to generate a plasmid expressing the insert (pINA) (Meier et al., 2004). As a control plasmid, the IFNA cDNA was excised from pINA by EcoRI digestion and religated with the dephosphory-

lated plasmid backbone to generate a recombinant having the insert in the opposite orientation relative to that in pINA (pANI). Plasmid structure was confirmed by restriction enzyme analysis. Unlike pINA, Chinese hamster ovary cells transfected with pANI failed to secrete detectable amounts of biologically active IFNA. Large quantities of both plasmids were prepared as previously described (Meier et al., 2004).

2.2. PRRSV vaccination of pigs

For the first study, 6-week-old Yorkshire × Landrace cross-bred pigs ($n = 19$) were obtained from an unvaccinated, specific-pathogen-free (SPF) facility. Each animal was immunized i.m. into the adductor muscle (inner thigh) with 2.0 ml of Ingelvac PRRS MLV vaccine (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). All pigs were given a secondary, booster immunization 8 weeks later and kept for an additional 5 weeks. Pigs in this study were not challenged with virulent virus.

In the second study, groups of 5 PRRSV-naïve pigs were immunized i.m. with 2.0 ml of PRRS MLV vaccine alone, or in combination with 200 μg pINA or pANI. To improve the efficiency of transfection, DNA/lipid vesicle complexes were prepared by mixing the plasmid with the cationic lipid dimethyldioctadecylammonium bromide (DDAB) as previously described (Van Rooij et al., 2002). Animals receiving the vaccine only were also injected with a DDAB solution lacking plasmid. The immune responses of all pigs were monitored for 12 weeks thereafter. No MLV boost or PRRS challenge infection was tested for this experiment.

2.3. Cytokine ELISPOT and ELISA assays

Isolation of PBMC from venous blood and their subsequent culturing (10^7 cells per well of a 24-unit plate) was performed as previously described (Meier et al., 2003). The host cell-mediated immune response to PRRSV was measured by using a single cell ELISPOT assay to enumerate the frequencies of virus specific IFNG (Meier et al., 2003) and IFNA (Splichal et al., 1997) SC. For these assays, the parental virus of the MLV vaccine, PRRSV strain VR-2332 (American Type Culture Collection, Manassas, VA), was used as the recall antigen and results were compared to those obtained using unstimulated (medium only) or mitogen-treated cells.

For cytokine mRNA and protein analyses, PBMC from each animal were cultured (10^7 cells per well of a 24-unit plate) in the presence/absence of PRRSV strain VR-2332. After 24–48 h of exposure to the virus, the cells and culture supernatants were collected and aliquoted. Cell culture supernatants were tested for the levels of T-cell derived cytokine proteins. IFNG protein was measured using the IFNG ELISA (Mateu de Antonio et al., 1998), IL1B and TNFA using swine cytokine ELISAs from R&D Systems (Minneapolis, MN), and IL6, IL8, and IL10 using swine cytokine ELISA assays from Biosource International (Camarillo, CA).

2.4. Real-time PCR for immune and inflammatory genes

Real-time PCR technology (Dawson et al., 2004, and manuscript submitted) was used to measure immune marker gene expression. RNA was extracted from cultured PBMC with Trizol® (Invitrogen, Carlsbad, CA) and its integrity and quantity was assessed using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies, Palo Alto, CA). All RNAs were DNAse-treated prior to cDNA synthesis using Superscript reverse transcriptase (Invitrogen) and oligo-dT as previously described (Dawson et al., 2004). Real-time PCR was performed on 100 ng cDNA (RNA equivalent)/25 μl reaction/well using the Stratagene Brilliant kit (La Jolla, CA) and an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Amplification conditions were: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; then 4 °C. All probes and primers for real time PCR were designed using the Primer Express (Applied Biosystems, Foster City, CA) software package and nucleotide sequences obtained from GenBank or the TIGR porcine EST database to generate amplicons spanning adjacent exons when possible (Table 1). For most of the genes assayed, the intron–exon structures have not been described and, based on human sequence information, approximately 50% of the assays described here are predicted to amplify genomic DNA. Gene names and abbreviations are based on the International Society for Animal

Table 1
Sequence information for swine immune genes

Gene[a]	Sequence F primer	F primer (nM)	Sequence R primer (5′–3′)	R primer (nM)	PCR (bp)	Sequence probe (5′–3′)	Probe (nM)	Accession no.[b]
C19ORF10 (IL25)	CTTGGACAGCTCAGCCTTGAA	900	CCTGAAAAGTGAGGAATTTGAAGTG	900	79	TET-AAAACAGCAGTGTCCCACAGGCCTG-BHQ1	150	TC129279
CD80	ATGGAGAAGAATTAAATGCT-ACCAACAC	900	GCCTGTCACATTGAAATCCAGTT	300	98	TET-TCCCAAGATCCTGAAACTGAGCTCTACATGA-BHQ1	200	AB049760
CD86	TGGGTCGCACAAGCTTTGA	900	TGAGCCCTTGTCCTTGATTTG	50	74	TET-CCACCTGGACCCTGAGACTCCACAAC-BHQ1	200	NM_214222
CSF2 (GMCSF)	AGCGGCTGTGATGAATGAAAC	300	CGCAGGCCCTGCTTGTAC	300	105	TET-TGACCCCCAGGAGCCGACATG-BHQ1	150	NM_214118
IFNA	TCAGCTGCAATGCCATCTG	300	AGGGAGAGATTCTCCTCATTTGTG	300	108	6FAM-TGACCTGCCTCAGACCCACAGCC-TAMRA	200	NM_214393
IFNG[c]	TGGTAGCTCTGGGAAACTGAATG	300	GGCTTTGCGCTGGATCTG	300	79	TET-CTTCGAAAAGCTGATTAAAATTCCGGTAGATAATCTGC-BHQ1	200	NM_213948
IL10	TGAGAACAGCTGCATCCACTTC	300	TCTGGTCCTTCGTTTGAAAGAAA	300	104	6FAM-CAACCAGCCTGCCCCACATGC-TAMRA	150	NM_214041
IL12A	GCCCAGGAATGTTCAAATGC	300	GGGTTTGTTTGGCCTTCTGA	300	84	TET-CAACCACTCCCAAAATCTGCTGAAGGC-BHQ1	200	NM_213993
IL12B	AAGCTGTTCACAAGCTC-AAGTATGA	300	TCTTGGGAGGGTCTGGTTTG	300	81	6FAM-ACCAGCAGCTTCTTCATCAGGGACATCA-TAMRA	200	U08317
IL12RB2	GGCCAGGAAAGGGACAAAG	300	CCCCAGCACCTTGTACAGATC	300	137	6FAM-AAGTCCACCACCTCCAAGGGCTCTCAC-TAMRA	100	NM_214097
IL13	CTGACCACCAGCATGCAGTACT	50	GCTGCAGTCGGAGATGTTGA	900	63	6FAM-TGCCGCCCTGGAATCCCTCA-TAMRA	150	NM_213803
IL15	GATGCCACATTGTATACTGAAA-GTGA	300	GCGTAACTCCAGGAGAAAGCA	300	81	6FAM-CATCCCAATTGCAAAGTAACAGCGATGA-TAMRA	150	NM_214390
IL18	CGTGTTTGAGGATATGCCTGATT	300	TGGTTACTGCCAGACCTCTAGTGA	300	107	6FAM-TGACTGTTCAGATAATGCACCTCAGACCGT-TAMRA	100	NM_213997
IL1B	TTGAATTCGAGTCTGCCCTGT	900	CCCAGGAAGACGGGCTTT	900	76	TET-CCCAGGAAGACGGGCTTT-BHQ1	150	NM_214055
IL2	ACAGTTGCTTTTGAAGGAAGTT-AAGAA	300	CCTGCTTGGGCATGTAAAATTT	300	86	6FAM-CGAGAATGCTGATCTCTCCAGGATGCTC-TAMRA	150	NM_213861
IL23	GCCCTGCTTGGGCTCAA	300	GTAGATCCACATGTCCCATTGGT	300	90	TET-CACGCTGGCCTGGACTGCACATC-BHQ	200	AB030002
IL4	GCCGGGCCTCGACTGT	300	TCCGCTCAGGAGGCTCTTC	300	67	6FAM-CTTCGGCACATCTACAGACACCACACG-TAMRA	200	X68330
IL5	GACTGGTGGCAGAGACCTTGAC	300	CTTCAATGCATAGTTGGTGATTTGT	300	114	6FAM-CTGCTCTCCATTCATCGAACTCTGCTGAT-TAMRA	200	NM_214205
IL6	AATGTCGAGGCTGTGCAGATT	300	TGGTGGCTTTGTCTGGATTCT	900	82	TET-AGCACTGATCCAGACCCTGAGGCAAA-BHQ1	200	NM_214399
IL8	CCGTGTCAACATGACTTCCAA	300	GCCTCACAGAGAGCTGCAGAA	900	74	TET-CTGTTGCCTTCTTGGCAGTTTTCCTGC-BHQ1	200	NM_213867
RPL32[c]	TGGAAGAGACGTTGTGAGCAA	300	CGGAAGTTTCTGGTACACAATGTAA	300	94	TET-ATTTGTTGCACATTAGCAGCACTTCAAGCTC-BHQ1	200	NM_001001636
SLC11A1 (NRAMP1)	GGCCGTGGGCATCGTT	900	GTACATGTTGGCTTCTCGGATGT	900	121	TET-CGCCATCATCATGCCCCACAAC-BHQ1	250	NM_213821
SOCS1	TTCTTCGCCCTCAGTGTGAA	900	GGCCTGGAAGTGCACGC	900	63	TET-TTCGGGCCCCACAAGCATCC-BHQ1	150	TC157850
STAT1[c]	CACAGAAATCAATTCAGTCTT-GATGTATC	300	GAAAGTACTACTCCAGGCCAAAGGA	300	95	TET-CCTTTAGGGCCGTCAAGTTCCATAGGTTC-BHQ1	100	NM_213769
STAT4	GAAAACCCTCTGAAGTACCT-CTATCCT	300	TCACATGGCTGGGAGCTGTA	300	80	TET-TGCTGCCTCCCACTGAACAGGACCT-BHQ	200	AB020984
STAT6	CCTGGGTTGGTGAAGACATGT	300	GCCCCTCCAAGAGAAGCTTAG	300	78	TET-TGCTGCCTCCCACTGAACAGGACCT-BHQ	200	TC139231
TGFB1	AGGGCTACCATGCCAATTTCT	300	CCGGGTTGTGCTGGTTGT	300	101	6FAM-CACTCAGTACAGCAAGGTCCTGGCTCTGTA-TAMRA	200	M23703
TLR2	TATCCAGCACGAGAATACA-CAGTTTAA	300	CGAGTTGAGATTGTTATTGCTAAT-ATCTAAAA	900	86	TET-CATTGGCTTCCCCAGACCCTGGA-BHQ1	200	NM_213761
TLR4	TGGCAGTTTCTGAGGAGTCATG	900	CCGCAGCAGGGACTTCTC	900	72	TET-CGGCATCATCTTCATCGTCCTGCAG-BHQ1	150	AY289532
TNF (TNFA)	TGGCCCCTTGAGCATCA	300	CGGGCTTATCTGAGGTTTGAGA	300	67	6FAM-CCCTCTGGCCCAAGGACTCAGATCA-TAMRA	150	NM_214022

[a] Official HGNC gene names and symbols [www.ncbi.nlm.nih.gov/LocusLink].
[b] GenBank or TIGR Accession Numbers.
[c] Dawson et al. (2004).

Genetics (ISAG) guidelines, i.e., using the human Official Gene Symbol (HGNC) as found at the Locus Link/Gene website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene. Relative quantification of target gene expression was evaluated using cycle threshold (C_t) values. Gene expression data were normalized to the amount of RNA/cDNA amplified (Bustin, 2002).

2.5. Statistical analysis

Elispot results are expressed as the mean + 1 S.E. Statistical significance was determined by analysis of variance. Individual differences between treatment groups were determined with Fisher's protected least significant difference (LSD). This test evaluates pairwise comparison with a multiple *t*-statistic. Correlation analyses were done by Spearman rank correlation (Spearman's ρ). The analyses were performed with the Statview program V4.5 (Abacus Concepts, Berkeley, CA) or PRISM 4 software (Graph Pad, San Diego, CA). Cytokine protein levels were analyzed by repeated measures ANOVA and compared using Tukey's multiple comparison test with PRISM 4 software.

All statistical analyses of immune marker gene expression data were performed using JMP Software (Cary, NC). The effect of PRRSV infection on PBMC mRNA expression (C_t values) was evaluated by one-way ANOVA that compared C_t values obtained for the respective pig samples pre- and post-MLV vaccination. Fisher's LSD post-hoc test was applied to assess differences between groups of pigs at different weeks after vaccination. $P \leq 0.05$ was considered statistically significant for all analyses.

3. Results

3.1. Cell-mediated immune response of pigs immunized with PRRS MLV vaccine

Examination of the temporal development of a memory response to PRRSV in pigs immunized and boosted with a MLV vaccine revealed a wide variation in the frequencies of virus-specific IFNG SC in their PBMC populations, even among pigs from the same litter (Fig. 1). While some pigs exhibited a strong IFNG response, this type of immunity was barely elicited in others. Overall, more pigs in litter A (8/10) than in litter B (4/9) reached an arbitrary threshold of 50 IFNG SC/10^6 PBMC within 8 weeks after primary vaccination. This difference with respect to birth origin became even more apparent after the booster immunization, when the frequency of IFNG SC exceeded 80/10^6 PBMC for 7 of 10 pigs in litter A (nos. 26–31,34), but for only 3 of 9 pigs in litter B (nos. 64,68,69). Remarkably, with two exceptions (nos. 26,30) those pigs that developed the greatest IFNG response to primary vaccination (nos. 29,31,34,68,69) were also the strongest responders to the booster immunization. However, with a few exceptions (nos. 26,28,34) the increase in the IFNG response to the booster immunization was less than 2-fold of the highest response attained by any one pig at some point before the boost.

Since the innate immunity cytokine IFNA promotes the development of an anti-viral Th1 response, the frequencies of IFNA SC and PRRSV-specific IFNG SC were compared on an individual animal basis to establish whether there was an association between these two phenotypes. Measurements of virus activated IFNG SC were performed at the peak of the primary response to PRRS MLV (weeks 4 and 5 post-vaccination), while the occurrences of innate IFNA SC were determined later (week 13 post-vaccination). As seen in Fig. 2, there was a positive correlation ($R = 0.06$, $P = 0.001$) between these innate and adaptive immune parameters on a pig by pig basis, despite the average IFNA response to stimulation with PRRSV in animals from litter A (36.7 ± 8.4 IFNA SC/10^6 PBMC) being significantly higher ($P = 0.05$) than that of the B littermates (17 ± 2.7 IFNA SC/10^6 PBMC). The positive correlation between these two parameters was also present when the IFNG response observed at 10 and 13 weeks after immunization was used to measure this association.

3.2. Immune gene expression by PBMC from PRRS MLV vaccinated pigs

In view of the observed, disparate intensities of the IFNG responses of the pigs to PRRS vaccination, gene expression profiling of selected animals' PBMC recall response to PRRSV was conducted to determine whether differences in the resultant patterns could be

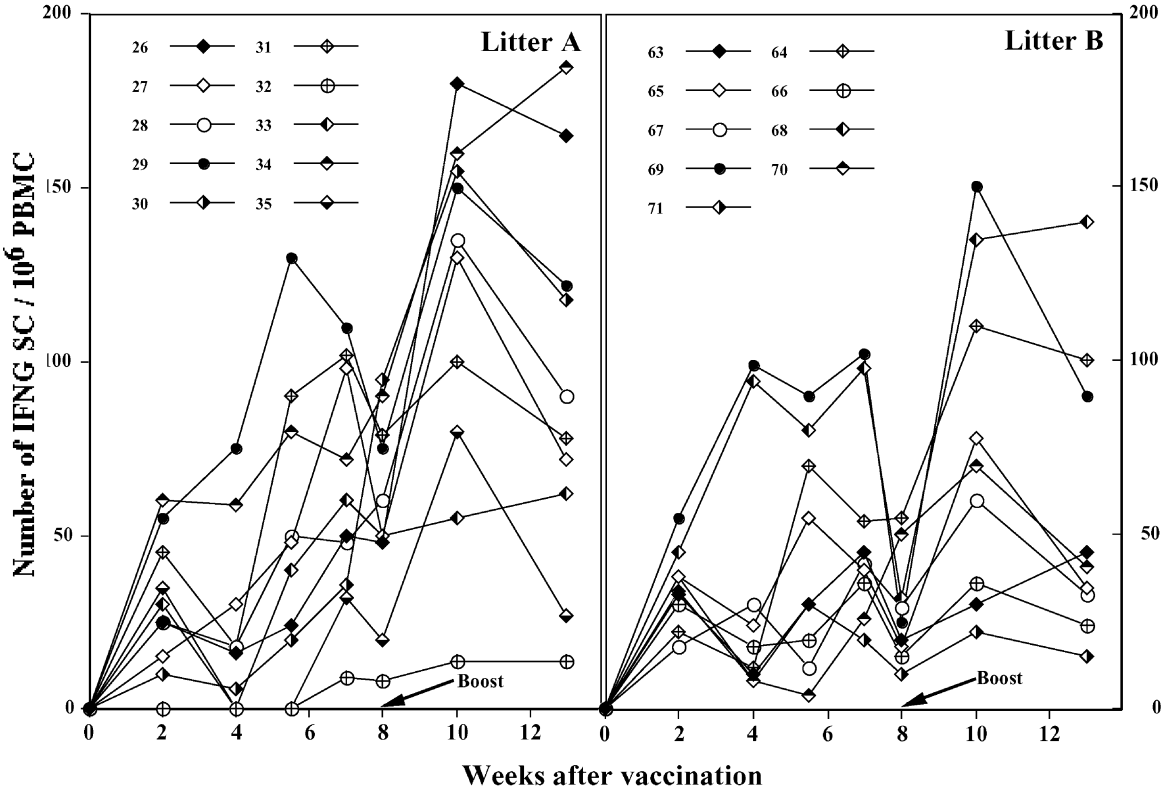

Fig. 1. Kinetics of the development of cell-mediated immune response of individual pigs to PRRSV MLV vaccination. Two litters of 6-week-old pigs ($n = 19$) were immunized with PRRS MLV vaccine and boosted 8 weeks later. At the indicated times post-primary immunization, the frequency of virus-specific IFNG SC in their PBMC was determined using an ELISPOT assay as described in Section 2.

detected. To cover the range of elicited cell-mediated immunity, nine representatives exhibiting either a relatively high (nos. 29,68 and 69) or low (nos. 26,28,32,33,66,67) IFNG response to the PRRSV vaccine at 5 weeks after primary immunization were picked from the two litters (Fig. 3A). For profiling, relative changes in the expression of a panel of 24 immune markers including Th1-associated, Th2-asssociated, innate immune, and regulatory proteins were assessed by using real time PCR and RNA templates derived from PBMC cultured in parallel to those used for the quantitation of PRRSV-specific IFNG SC as described in Fig. 2.

As expected, IFNG mRNA levels increased (2 to 5-fold) in the in vitro restimulated PBMC isolated from most pigs at week 5 after vaccination as compared to those in the cells obtained from the respective, pre-vaccinated animals (Fig. 3B). However, no change was detected for the one pig (no. 32) that had the lowest frequency of IFNG SC. Expression of certain Th1 genes, IL12A (IL-12p35), IL12B (IL-12p40), and IL12RB2, were not consistently up-regulated as had been found previously in swine (Solano-Aguilar et al., 2002). Limited increases in the quantities of transcripts for the other Th1-associated genes, IL15, IL18, and TNFA, were also detected. Expression of genes encoding the Th2 markers was generally down-regulated as were those for the innate immunity markers, especially IL1B and IL6. Regulatory immune gene expression with the exception of the weakly down-regulated IL10 gene was variable at week 5 post-vaccination.

To understand the developing anti-PRRSV immune response more thoroughly, extended temporal comparisons of the gene expression profiles of PBMC isolated from the 9 selected pigs and subsequently

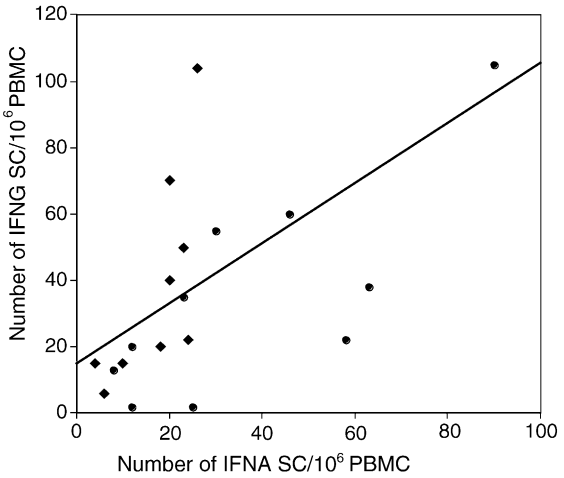

Fig. 2. Comparison of the IFNG SC and IFNA SC responses of individual pigs to vaccination with PRRSV. PBMC were isolated from the pigs described in the legend to Fig. 1 during their peak IFNG response to PRRSV (weeks 4 and 5 post-primary vaccination) and the frequencies of virus-specific IFNG SC in these populations were determined via an ELISPOT assay. The frequency of innate IFNA SC in the pigs' PBMC populations was measured at 5 weeks after the booster immunization (week 13 post-primary vaccination) by using an ELISPOT assay. The average IFNG response was compared to the IFNA response on an individual basis for members of litter A (circles) and B (diamonds). Regression analyses confirmed that these two types of immune functions were correlated on a pig by pig basis with $R = 0.6$ at a $P \leq 0.001$ level.

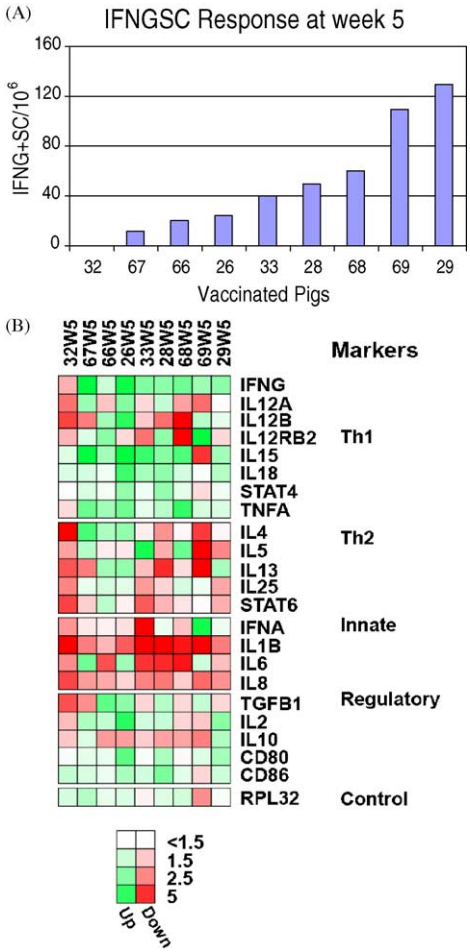

Fig. 3. Comparison of individual pig IFNG SC and immune marker gene expression at 5 weeks post-MLV vaccination. (A) The frequencies of virus-specific IFNG SC in pig PBMC populations, cultured for 48 h in vitro with PRRS viral strain VR-2332, at 5.5 weeks post-PRRSV MLV immunization were determined using an ELISPOT assay. (B) Immune gene expression profiles using real time PCR of cDNA prepared from bulk PBMC cultures, stimulated in vitro with PRRSV for 48 h, and measured as described in Section 2. Results represent the relative ratio of C_t values of the indicated genes at 5.5 weeks to 0 weeks post-PRRSV MLV immunization. Squares are color-coded to denote either up- or down-regulation of gene expression.

restimulated with MLV were performed. In addition to the above mentioned 5 weeks post-primary vaccination, samples were obtained 3 weeks earlier and 2, 5 and 8 weeks later to represent an interval beginning 2 weeks after the initial immunization and terminating 5 weeks after the booster immunization. For each time point and gene the results from the nine animals were averaged (Fig. 4). [For details of average C_t values and statistics refer to Table 2]. Overall, IFNG mRNA levels were up-regulated by 1.8 to 2.9-fold (significantly at 2.5-fold starting at week 4 post-MLV), relative to pre-vaccination levels and, surprisingly, were not substantially increased after the MLV boost at week 8. Significant sustained increases were also noted in the quantities of transcripts encoding TNFA (1.9 to 2.6-fold) and IL15 (2.8 to 4.2-fold). Likewise, there were some increases in IL18 gene expression, in contrast to a weak down-regulation of IL12A, IL12B, and IL12RB2 gene activity (Fig. 4). No substantial changes in the amount of RNAs encoding the Th2 cytokines, IL4 and IL5, were detected throughout the study, whereas IL25 and STAT6 gene expression was significantly down-regulated after the booster vaccination. Although IL13 and IL23 mRNA levels were also analyzed, the obtained values were too low

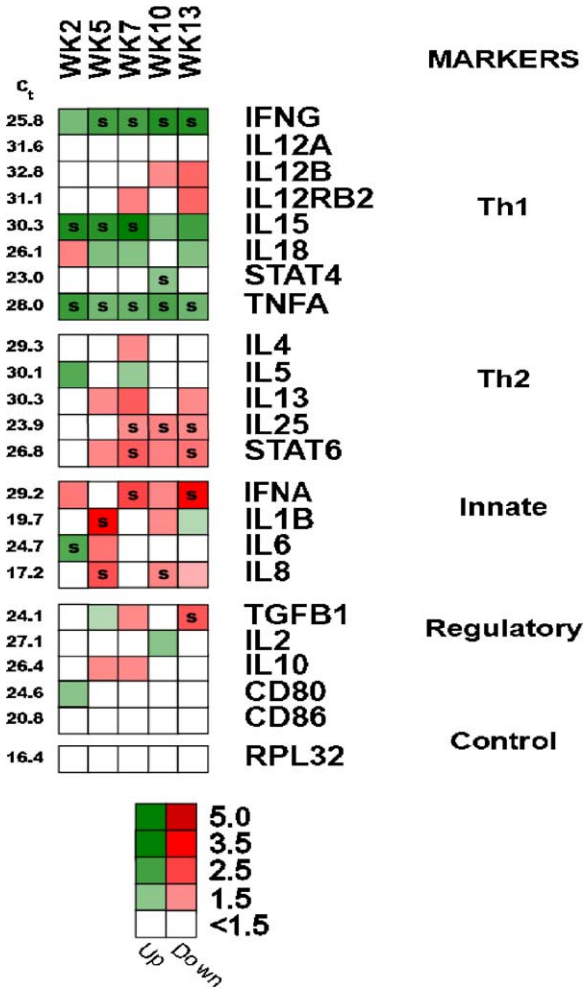

Fig. 4. Temporal comparison of immune marker gene expression in activated PBMC from pigs vaccinated with PRRS MLV. Immune marker gene expression was measured by real time PCR for cDNA prepared from PBMCs stimulated in vitro with PRRSV for 48 h, as described in Section 2. Results represent the relative ratio of averaged C_t values obtained for each marker, with cDNA prepared at the indicated time points post-PRRSV MLV immunization of the nine pigs described in Fig. 3, to the pre-vaccination value. Squares are color-coded to denote either up- or down-regulation, with statistical differences ($P \leq 0.05$) between the values derived at a given time and at 0 weeks post-vaccination indicated by the letter "s".

($C_t > 35$) to be included in statistical comparisons (Table 2).

The previous reports of lack of post-vaccination up-regulation of innate markers were reinforced when the pooled mRNA gene expression was analyzed. Remarkably, IL6 mRNA was the only innate cytokine gene that, although transiently, was up-regulated at 2 weeks after primary vaccination (Fig. 4). No enhancement of this activity was detected after the booster immunization. As seen in Fig. 4, IL1B and IL8 were significantly down-regulated at week 5 with IL8 again down at week 10. Over the full course of vaccination IFNA mRNA levels were down-regulated relative to pre-MLV levels, significantly at weeks 7 and 13 (2.5 to 3.4-fold decrease). Expression of the TGFB1 gene was also down regulated at 13 weeks post-primary immunization. In contrast, no major changes in IL10, CD80, CD86, the T cell growth factor IL2 or the control housekeeping gene, RPL32, were found.

3.3. Adjuvant effect of porcine IFNA on the cell-mediated immune response of pigs immunized with PRRS MLV vaccine

In an accompanying manuscript (Meier et al., 2004), administration of the IFNA-encoding plasmid pINA at the time of immunization with PRRS MLV was shown to enhance the IFNG response. Since IFNA production is presumably reduced after PRRSV vaccination (Fig. 4), the exogenous addition of this cytokine might compensate for this deficiency and thus stimulate cell-mediated immunity. The pINA plasmid was derived from the pcDNA vector that alone can apparently stimulate IFNA gene expression in pigs (Johansson et al., 2002). Therefore, it is not clear what impact is provided by the plasmid DNA itself as opposed to the encoded protein. Accordingly, we wanted to explore in more detail the effects of using the pINA biologic in conjunction with the MLV vaccine. To compensate for a nucleic acid effect on elicited immunity, in addition to the mock-treated and pINA-injected vaccinated groups, a third set of MLV-immunized pigs received a control plasmid, pANI, in which the IFNA cDNA had been inverted to prevent IFNA expression yet maintain the integrity of the DNA.

Although enhancement of the vaccine-induced IFNG response by pINA was apparent as early as 2 weeks after MLV immunization (Fig. 5), this difference was only statistically significant ($P < 0.05$) during the peak increases manifested 2 and 4 weeks later. For instance, at 4 weeks after the primary immunization, the pINA-treated pigs exhibited 3.9

Table 2
Swine immune marker expression (C_t values) of PBMC sample cDNAs following PRRSV vaccination

Gene	Gene	Week 0			Week 2			Week 5			Week 7			Week 10			Week 13		
Group		Mean	S.D.	Stat*	Mean	S.D.	Stat*	Mean	S.D.	Stat*	Mean	S.D.	Stat*	Mean	S.D.	Stat*	Mean	S.D.	Stat*
Th1	IFNG	25.8	0.9		25.0	1.6		24.5	0.9	*	24.5	0.9	*	24.3	0.9	*	24.3	0.9	*
	IL12A	31.6	1.1		31.8	1.0		31.9	1.1		31.2	0.9		31.7	0.7		31.3	0.9	
	IL12B	32.8	1.7		33.7	1.3		33.2	1.1		33.1	1.3		33.4	2.1		33.8	0.9	
	IL12RB2	31.1	2.2		30.8	1.0		31.1	1.5		31.8	1.1		31.1	1.9		32.1	0.7	
	IL15	30.4	2.1		28.8	2.0	*	28.9	1.9	*	28.3	1.1	*	29.6	1.4		29.0	1.7	
	IL18	26.1	0.5		26.8	0.8		25.3	0.7		25.4	1.1		26.4	1.3		25.4	1.2	
	IL23[a]	35.4	1.7		35.7	0.9		35.7	1.6		36.2	1.1		35.9	0.9		37.4	1.5	*
	STAT4	23.0	0.4		23.0	0.7		22.6	0.5		22.9	0.5		22.4	0.8	*	22.8	0.7	
	TNFA	28.0	0.8		26.6	0.9		27.1	0.8		27.0	0.9		26.7	1.3		27.1	0.4	
Th2	IL4	29.2	1.6		29.5	1.7		29.6	0.8		29.8	0.9		28.9	1.4		29.5	1.2	
	IL5	30.1	0.8		28.9	2.2		30.3	1.3		29.6	1.3		30.0	0.8		30.4	1.3	
	IL13	30.3	1.1		30.2	1.0		31.0	1.8		31.4	1.7		30.4	2.2		30.9	1.5	
	IL25	23.9	0.9		24.2	0.6		24.2	0.6		24.5	0.5	*	24.6	0.8	*	24.5	0.3	*
	STAT6	26.8	1.1		27.3	1.0		27.4	0.8		27.9	0.6	*	27.5	1.2		27.7	0.4	*
Innate	IFNA	29.2	1.8		30.1	1.2		29.5	1.6		30.5	1.1	*	29.9	0.8		31.0	1.3	*
	IL1B	19.7	0.7		19.3	1.0		22.1	1.3	*	19.7	1.3		20.3	1.0		19.7	0.6	
	IL6	24.7	1.4		23.5	1.2	*	25.6	0.9		24.3	1.3		24.7	0.6		24.4	1.0	
	IL8	17.2	0.7		16.7	0.9		18.4	0.7	*	16.7	0.9		17.9	0.8	*	17.3	0.4	
Regulatory	TGFB1	24.1	1.3		24.2	1.2		24.1	1.1		24.7	1.1		24.7	1.5		25.3	1.0	*
	IL2	27.1	0.8		26.9	1.0		26.6	0.8		27.0	0.9		26.5	0.7		26.8	1.0	
	IL10	26.4	0.4		26.4	0.8		27.0	0.9		27.0	0.9		26.6	1.0		26.6	0.7	
	CD80	24.6	0.4		24.0	0.9		24.1	0.6		24.2	0.9		24.7	1.1		24.6	0.6	
	CD86	20.8	0.2		20.6	0.7		20.3	0.6	*	20.9	0.4		21.1	0.7		20.8	0.5	
Control	RPL32	16.3	0.3		16.1	0.4		16.2	0.5		16.3	0.2		16.3	0.3		16.6	0.3	

Swine immune marker expression (C_t values) for each gene and cDNA sample is noted. Data were averaged (mean ± S.D.) for each week after vaccination.

[a] Gene expression (C_t values) > 35 are not included in the final analyses because of poor assay sensitivity.

* Indicates results are significantly different at $P \leq 0.05$ from prevaccination (week 0) data.

and 2.6 times the frequency of virus-specific IFNG-SC in their peripheral blood as compared to the vaccinated only or pANI-injected groups. By 3 weeks later, this cell-mediated immune response had subsided in all three groups; no significant differences between groups were observed. Thus, the artificial augmentation of IFNA production during the initial exposure of the pig to PRRSV MLV appeared to be essential for a superior cell-mediated immune response to PRRSV vaccination.

3.4. Effect of IFNA adjuvant on immune marker gene expression by PBMC from pigs immunized with PRRS MLV

To evaluate the effect of using IFNA to adjuvant the immune response to PRRSV, gene expression analyses similar to those described above were conducted on PBMC isolated from each member of the three MLV-vaccinated groups. In this study, cells were procured prior to the pigs being exposed to PRRSV and then at 2 and 4 weeks post-immunization, times when the most significant changes in immune marker expression were expected. Because of the minor changes in gene expression detected when RNA was collected from PBMC after a 48 h exposure to PRRSV (Figs. 3B, 4), the PBMC culturing time was reduced to 24 h. Substantially greater changes in immune gene expression levels were observed (Fig. 6 and Table 3).

Consistent with the observed generation of virus-specific IFNG SC (Fig. 5), a significant increase in the presence of IFNG mRNA was found in PBMC isolated from every group at week 2 (>12-fold) or week 4 (>5-fold) post-vaccination and subsequently exposed

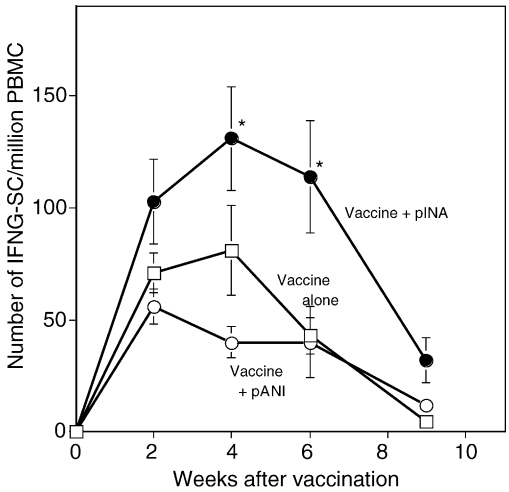

Fig. 5. Effect of co-administration of IFNA plasmids on kinetics of the development of the cell-mediated immune response of pigs to PRRS MLV vaccine. Groups of pigs were vaccinated with PRRS MLV vaccine in the presence of DDAB alone, or co-administered with IFNA expression plasmid, pINA, or with control pANI plasmid. Following vaccination blood PBMC were harvested, restimulated 24 h with PRRSV in vitro and IFNG SC responses quantitated using an ELISPOT assay. Each value represents the mean response of 5 animals ± standard error of the mean (SEM). Significant differences ($P < 0.05$) between the frequencies of virus-specific IFNG SC in the peripheral blood of animals receiving the pINA plasmid vs. the other two groups are represented by an asterisk.

to PRRSV. Likewise, the quantities of transcripts encoding the Th1 markers, TNFA (>9-fold) and STAT1 (>2.9-fold), were greater at week 2 after immunization, although they had lessened by week 4. In contrast to our earlier results (Fig. 4), a significant up-regulation of IL12B, and IL12RB2 gene expression (5 to 13-fold) was found at week 2 after vaccination for all 3 groups but had dissipated 2 weeks later. However it should be noted (Table 3) that these genes were expressed at low levels at week 0 (C_t values = 34 and 31, respectively). Although activity of the Th1 marker IL15 gene had increased in all groups at week 2 post-vaccination, this change was only significant (>30-fold) for the MLV-immunized only group. Two weeks later, a significant increase was noted for the animals injected with pINA. Alterations in the expression of the Th2 cytokine IL4 and the Th1 suppressor SOCS1 exhibited substantial (>20-fold) and significant changes at week 2 after immunization for MLV and pINA groups. In this experiment, the quantity of IL25 mRNA had statistically increased >1.8-fold at week 2 post-vaccination. However, once again the amount of IL13 mRNA was too low for use in statistical analyses.

The expression of genes encoding several innate immune markers, IFNA, IL1B, IL6, IL8, and CSF2, and two regulatory proteins, IL10 and TGFB, also significantly increased at week 2 post-vaccination and then waned 2 weeks later. These increases at 2 weeks post-vaccination were substantial (>25-fold) for IL6 mRNA in all groups, as seen previously (Fig. 3). Up-regulation of IL1B was >5.4-fold at week 2 and IL8 at >3.7-fold; IFNA mRNA increased >30-fold in the MLV and MLV plus pINA groups at this time point (Fig. 6). Similarly, regulatory markers, IL10, and TGFB were both significantly increased. The level of mRNAs encoding the TLR markers and the control housekeeping gene RPL32 showed limited changes.

PBMC mRNA isolated from pigs in this vaccination experiment exhibited greater up-regulation of expression for numerous genes as compared to samples obtained during the first study (Fig. 6 compared to Figs. 3B, 4). Presumably, this phenomenon was due to the mRNA being extracted at 24 rather than 48 h, after in vitro PRRSV restimulation. In this regard, a recent temporal evaluation of mRNA quantities in PBMC cultured in vitro in the presence of PRRSV indicated that the expression of most genes has already increased by 24 h and only a few genes exhibited greater activity after 48 h (data not shown). Further it should be noted that mRNA was not evaluated on cells cultured with no PRRSV.

Based on the IFNG Elispot data (Fig. 5) we expected that there would be substantial differences in Th1 marker gene expression by virus-stimulated PBMC derived from pINA-treated pigs at weeks 2 and 4 post-vaccination as compared to the other animals. Overall only minor variations were detected between the 3 vaccination groups; all had similar increases in IFNG and TNFA mRNA levels. The enhancement of IFNG, IL15 and IL18 gene expression was greater in PBMC from the MLV-immunized only group than from pigs receiving the pINA adjuvant. Surprisingly, the extent of these differences was almost equivalent to those associated with cells from the pANI adjuvanted group, despite the significantly lower IFNG SC responses of these animals to vaccination (Fig. 6). For all pigs in this experiment

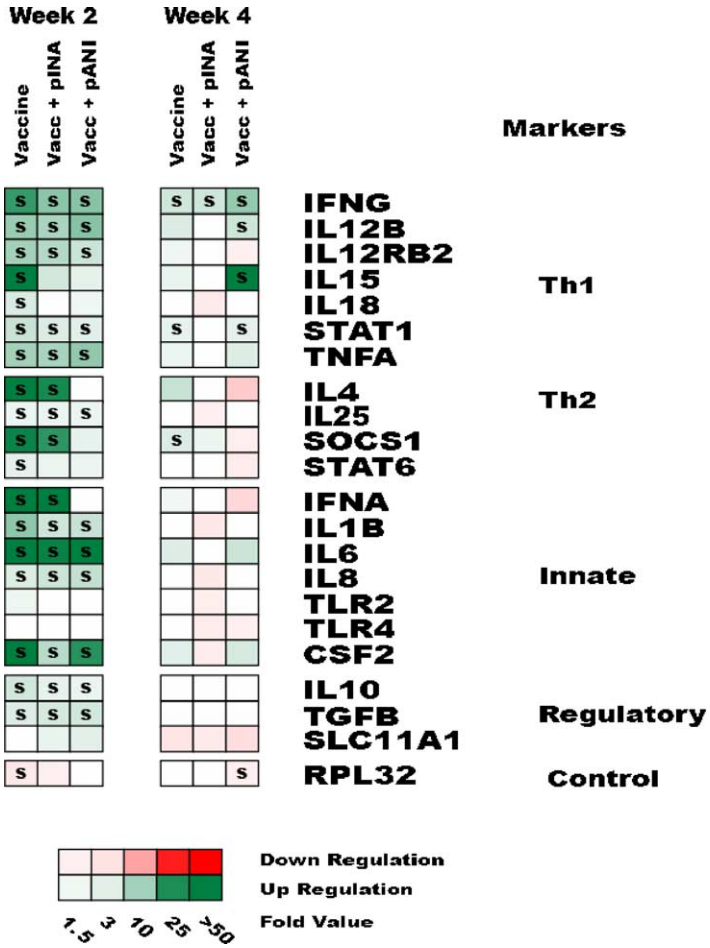

Fig. 6. Temporal comparison of immune marker gene expression in activated PBMC from pigs vaccinated with PRRSV alone or in the presence of adjuvant pINA or pANI. PBMC were prepared from swine vaccinated with PRRS MLV vaccine only, or MLV co-administered with IFNA expression plasmid, pINA, or with control pANI plasmid. PBMC were restimulated 24 h with PRRSV, pelleted and stored at −70 °C until RNA and cDNA were prepared. Immune marker gene expression was analyzed as described in Section 2. Results represent the relative ratio of C_t values obtained for PBMC isolated at 2 or 4 weeks post-PRRSV MLV immunization for 5 pigs/group compared to data acquired prior to vaccination. Squares are color-coded to denote either up- or down-regulation of the indicated genes. Significant differences ($P < 0.05$) between the values derived at either 2 or 4 weeks post-vaccination were calculated relative to week 0 data and noted by the letter "s".

there was a rapid return to homeostatic levels of mRNA expression at 4 weeks after vaccination; the pINA-injected group may have reduced expression the most by returning the closest to week 0 levels at week 4. Separate statistical comparisons of all groups, for week 2 gene expression data only, indicated that there were few genes for which major differences in the extent of expression could be found (data not shown). A greater impact appeared to be imparted by pINA adjuvant, as compared to the MLV only control and pANI adjuvanted groups in that there was an apparent lack of up-regulation of IL4, IFNA and SOCS1 gene expression at 2 weeks post-vaccination for the pANI group (Fig. 6). However, review of the data indicated that the PBMC derived from these pigs, for unknown reasons, had higher levels of the respective transcripts at week 0 (lower C_t values) and thus showed no up-regulation of gene expression (Table 3). To further explore possible differences in innate cytokine gene expression between the groups, and since mRNA levels do not necessarily correlate with protein production, we investigated whether unstimulated

Table 3
Swine immune marker expression (C_t values) of PBMC sample cDNAs following PRRSV vaccination with MLV alone or in combination with pINA or pANI plasmids

Treatment	Vaccine									Vaccine + pINA									Vaccine + pANI									All vaccinated pigs		
	Week 0			Week 2			Week 4			Week 0			Week 2			Week 4			Week 0			Week 2			Week 4			Week 0	Week 2	Week 4
Gene	Mean	S.D.	Stat[a]	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	S.D.	Stat	Mean	Mean	Mean
IFNG	28.6	0.6	A	24.1	1.3	D	26.2	1.2	B	27.9	0.5	A	24.2	0.6	CD	25.4	1.1	BC	28.5	0.5	A	24.8	1.1	CD	25.0	1.5	BCD	28.3	24.4	25.6
IL12B	33.9	1.1	AB	30.5	2.9	D	32.0	2.2	BCD	33.2	0.8	AB	30.0	0.9	D	32.6	0.9	ABC	34.5	1.0	A	30.8	1.2	CD	32.1	1.2	BCD	33.9	30.4	32.3
IL12RB2	30.8	0.5	A	27.5	1.2	C	30.1	1.4	AB	31.7	1.6	A	28.7	1.9	BC	31.6	1.6	A	30.5	1.0	A	28.0	0.9	C	31.1	1.3	A	31.0	28.0	30.9
IL15	33.9	1.3	A	28.9	1.3	DE	32.7	2.0	AB	31.4	0.8	BC	29.1	0.9	CD	31.1	0.9	BCD	32.3	1.2	AB	30.8	2.4	BCD	26.5	3.6	E	32.5	29.6	30.4
IL18	25.1	0.8	AB	23.0	1.2	C	25.4	1.2	AB	24.6	0.9	AB	24.4	1.1	B	25.7	0.3	A	24.9	0.9	AB	24.2	0.8	BC	24.7	0.4	AB	24.9	23.9	25.3
IL23[b]	36.8	2.1	A	29.5	1.1	F	35.1	1.7	ABC	36.1	2.0	AB	30.3	0.7	EF	37.0	1.3	A	32.9	1.8	CD	32.3	1.3	DE	34.1	2.8	BCD	35.3	30.7	35.5
STAT1	20.5	0.8	A	17.9	0.9	DE	19.2	1.5	C	19.4	0.5	BC	17.6	0.6	E	19.0	0.4	CD	20.3	0.6	AB	18.7	0.5	CD	18.8	0.5	CD	20.1	18.1	19.0
TNFA	27.5	1.1	A	24.2	2.1	C	26.6	1.5	A	27.2	1.2	A	24.0	0.8	C	26.7	0.8	A	28.1	0.9	A	24.6	1.2	BC	26.3	1.7	AB	27.6	24.3	26.5
IL4	31.5	1.4	A	25.6	0.8	E	28.9	0.9	BC	30.9	1.0	A	26.3	1.3	DE	30.7	0.5	A	27.8	1.5	CD	27.6	1.4	CD	30.3	2.3	AB	30.1	26.5	29.9
IL13[b]	36.7	2.0	AB	29.2	3.3	D	34.5	2.0	BC	34.4	1.1	BC	29.9	1.7	D	34.0	1.6	C	37.8	2.2	A	30.1	2.3	D	35.1	0.6	ABC	36.3	29.7	34.5
IL25	25.6	0.7	AB	24.6	1.1	CD	25.4	0.9	ABC	24.8	0.3	BCD	23.9	0.8	D	25.5	0.4	ABC	25.6	0.5	AB	24.7	0.5	BCD	25.8	0.3	A	25.3	24.4	25.5
SOCS1	28.3	1.2	A	23.5	1.1	E	26.4	0.6	BC	28.5	1.3	A	24.0	0.8	DE	27.7	0.9	AB	26.6	1.2	BC	25.2	0.8	CD	27.4	1.8	AB	27.8	24.2	27.1
STAT6	26.7	0.6	ABC	25.3	1.3	D	27.3	1.2	AB	26.8	0.9	ABC	25.7	1.1	CD	27.4	1.4	AB	27.1	0.5	AB	26.2	0.5	BCD	28.1	0.3	A	26.9	25.7	27.5
IFNA	30.7	0.8	A	24.2	1.0	E	30.1	1.4	AB	31.0	1.6	A	25.7	1.9	DE	31.1	1.4	A	27.1	1.8	CD	27.6	2.2	BCD	29.1	3.9	ABC	29.6	25.8	30.2
IL1B	21.9	0.9	A	18.2	2.6	B	21.4	2.4	A	21.2	0.5	A	18.7	1.3	B	22.4	1.0	A	21.3	0.3	A	18.7	2.0	B	21.0	1.4	A	21.4	18.6	21.7
IL6	29.3	1.2	A	23.4	3.6	B	27.5	2.1	A	28.1	0.6	A	23.3	1.6	B	28.2	0.6	A	29.8	0.8	A	23.5	2.6	B	27.3	1.6	A	29.1	23.4	27.7
IL8	19.8	1.0	A	17.9	2.0	BC	19.8	2.0	AB	19.2	0.6	AB	16.7	0.9	C	20.4	0.7	A	19.6	0.5	AB	16.9	1.9	C	19.4	1.6	AB	19.5	17.2	19.9
TLR2	21.8	0.7	A	21.1	0.8	A	21.8	1.5	A	21.0	0.6	A	20.7	0.6	A	21.7	0.5	A	21.3	0.6	A	21.2	0.8	A	21.6	0.5	A	21.4	21.0	21.7
TLR4	21.9	0.6	A	21.6	0.7	A	22.3	1.2	A	21.1	0.5	A	21.1	0.4	A	22.1	0.5	A	21.6	0.4	A	22.0	0.6	A	22.2	0.4	A	21.5	21.6	22.2
CSF2	29.3	1.7	A	24.1	3.5	D	27.7	2.8	ABC	27.7	1.0	AB	24.8	1.4	CD	28.8	0.9	A	29.5	1.3	A	24.9	2.7	BCD	27.4	2.3	ABC	28.8	24.6	28.0
IL10	27.7	0.6	A	25.4	1.1	C	27.4	1.5	A	27.0	0.7	A	25.6	0.6	BC	27.0	0.5	A	26.8	0.5	A	25.7	1.0	BC	26.6	0.5	AB	27.2	25.6	27.0
TGFB1	25.3	0.6	AB	23.5	1.7	C	25.4	1.7	AB	25.9	1.1	A	23.8	1.1	BC	25.4	1.4	AB	26.2	0.7	A	23.9	1.1	BC	26.3	0.2	A	25.8	23.7	25.7
SLC11A1	24.0	1.5	BCD	23.7	1.5	BCD	25.6	1.6	AB	24.3	1.5	BCD	23.2	2.1	D	25.5	1.6	AB	25.1	0.5	ABC	23.4	0.7	CD	26.9	0.6	A	24.5	23.4	25.9
RPL32	17.1	0.5	A	15.9	0.6	D	16.8	0.5	AB	16.6	0.2	ABCD	16.0	0.3	D	16.9	0.3	A	16.9	0.3	A	16.6	0.6	ABC	16.2	0.6	BCD	16.9	16.2	16.7

Swine immune marker expression (C_t values) for each gene and cDNA sample is noted. Data were averaged (mean ± S.D.) for each week after vaccination.

[a] C_t values for the same gene noted by different letters are significantly different at $P < 0.05$ from each other.

[b] Gene expression (C_t values) > 35 are not included in the final analyses because of poor assay sensitivity.

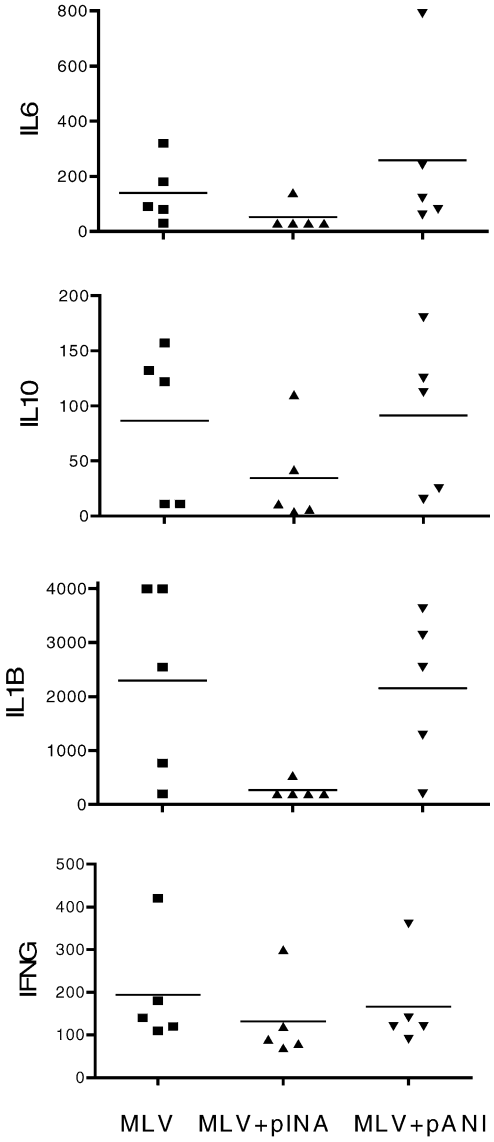

Fig. 7. Cytokine secretion by PBMC from pigs vaccinated with PRRSV alone or in the presence of adjuvant pINA or pANI. Groups of 5 pigs were immunized with PRRS MLV vaccine in the presence of DDAB alone or containing plasmid pINA or pANI, and PBMC isolated at 2 weeks after immunization. After a 48 h culture without virus stimulation, cell-free supernatants were harvested and the amount (pg/ml) of IFNG, IL1B, IL6 and IL10 determined using a specific ELISA as noted in Section 2. Results for each pig are shown separately and the average value for each group designated by a horizontal line.

cultures of PBMC isolated from the various pigs secreted different amounts of cytokine proteins.

3.5. Adjuvant effect of IFNA on cytokine protein expression by PBMC from pigs immunized with PRRS MLV

To compare secretion of immune cytokine proteins, IL6, IL1B, IL10, and IFNG, from pigs at 2 weeks post-vaccination, cell-free supernatants were harvested after 48 hrs from PBMC cultured in the absence of PRRSV. Whereas comparable quantities of IFNG were released by the cells regardless of their source, reduced amounts of IL6, IL1B, and IL10 were present in the medium overlaying PBMC from the pINA-treated pigs as compared to the other two groups (Fig. 7). Due to the small numbers of pigs in each group only the differences in the concentrations of IL1B were found to be statistically significantly lower for cells from the pINA-treated pigs. All cell culture supernatants contained high levels of IL8 but lacked detectable quantities of TNFA regardless of the immunization protocol (data not shown). Overall, it was reassuring that changes in levels of mRNA encoding IFNG, IL10, IL1B and IL6 were mimicked by alterations in the extent of cytokine protein secretion and corroborates the use of gene expression analyses as a fast screening method to identify molecular pathways involved in regulating the immune response.

4. Discussion

These viral immune response studies revealed a significant degree of variability in the intensity of the IFNG response of swine to vaccination with PRRS MLV, as is the case when pigs are deliberately infected with a virulent strain (Xiao et al., 2004). Moreover, the initial cell-mediated immune response to exposure to this virus, regardless of its degree of virulence, is relatively weak when compared to that elicited by immunization against other swine viruses (Van Reeth et al., 1999, 2002; Meier et al., 2003). These are remarkable observations in light of the fact that the intensity of the humoral immune response to PRRSV is fairly predictable, and can be characterized as consistently strong and uniformly present in all pigs

that are either immunized with a MLV vaccine or infected with wild-type virus (Yoon et al., 1995; Mengeling et al., 2003a,b). Our studies showed marked differences in the intensity of the IFNG response even between pigs in litters raised in the same environment, indicating that genetic factors are likely responsible for some of these differences.

Previously, IFNG had been detected in the lungs of PRRSV-infected pigs and postulated to be a necessary component for host control of this pathogen (Bassaganya-Riera et al., 2004; Thanawongnuwech et al., 2003; Thanawongnuwech and Thacker, 2003). This notion is supported by the fact that, like other viruses, PRRSV is susceptible to the inhibitory effect of IFNG (Bautista and Molitor, 1999; Rowland et al., 2001). It is also notable that although PRRSV is also vulnerable to the antiviral effects of IFNA, it differs from most viruses in its inability to stimulate the production of this cytokine in vivo (Albina et al., 1998a). For instance, during an acute PRRSV infection the presence of IFNA in the lung of pigs was either undetectable (Albina et al., 1998b), minimal (Van Reeth et al., 1999), or substantially lower than that induced by PRCV (Buddaert et al., 1998). Indeed, even when alveolar macrophages infected with PRRSV were superinfected with swine transmissible gastroenteritis virus (TGEV), an otherwise strong inducer of IFNA production, secretion of this cytokine was not detected (Albina et al., 1998a). Although, the mechanism responsible for the lack of IFNA response is currently unknown, the data from Albina et al. (1998), suggested the existence of an active suppression of the IFNA response. This important issue remains to be determined.

In our studies, a positive correlation between the number of virus-specific IFNG SC present in the peripheral blood of vaccinated pigs and the number of cells capable of releasing IFNA in response to stimulation with PRRSV was observed. This observation is similar to that reported between the ex vivo production of IFNA and IFNG in HIV-1 infected individuals (Hober et al., 1998). This relationship suggests that despite the poor IFNA induction in response to PRRSV, those animals with the highest capacity to produce IFNA when exposed to this virus are also more likely to develop a relatively stronger IFNG response. In support of this concept is the demonstration that as a group, the pigs in litter A exhibited both higher IFNA and IFNG responses to PRRSV as compared to those in litter B. This connection could be attributed to the known positive effect of IFNA in promoting the differentiation of virus-specific T cells into effector cells capable of secreting IFNG (Biron et al., 2002; Nguyen et al., 2002). Thus, the use of exogenous IFNA as an adjuvant, which in our studies clearly enhanced the development of T cell-mediated immunity, requires further scrutiny. Additionally, investigations concerning the genetic basis of susceptibility of pigs to PRRSV, especially in regards to a greater innate ability to produce IFNA in response to infection with PRRSV or other viruses, should be continued.

Although the importance of the IFNG response in the clearance of PRRSV during a primary infection has recently been questioned, based on the lack of a positive correlation between the frequency of virus-specific IFNG SC and amount of virus found in selected tissues (Xiao et al., 2004), the possibility of a local inhibition of T cell effector function (i.e., IFNG secretion) in the lung or other infected tissue by cytokines such as IL10 was not considered. This cytokine is present in the lungs (Johnsen et al., 2002; Chung and Chae, 2003; Labarque et al., 2003) and bronchoalveolar lavage fluids (Thanawongnuwech and Thacker, 2003) of PRRSV-infected pigs and can decrease anti-viral associated IFNG production (Liu et al., 2003; Marshall et al., 2003). That IL10, whose synthesis is induced by PRRSV, may have a potential role in hampering an adequate Th1 response was made evident by the observation that PBMC isolated from classical swine fever virus (CSFV)-immunized pigs when subsequently incubated with PRRSV had increased amounts of IL10 mRNA, concomitant with decreased IFNG gene expression after stimulation with CSFV recall viral antigen (Suradhat et al., 2003). Thus, the presence of IL10 could adversely affect the potentially protective anti-viral immune response. In this regard, elicitation of IL10 production by macrophages is a common mechanism used by viruses to suppress or delay the host immune response and facilitate the infection process (Redpath et al., 2001). As we have shown here, IL10 is spontaneously secreted by PBMC from pigs exposed to an attenuated PRRSV vaccine 2 weeks earlier. Surprisingly, this process can be partially reversed by the administration of a porcine IFNA-

expressing plasmid at the time of vaccination with PRRS MLV; PBMC from such treated animals secreted relatively less IL10 than cells from MLV only immunized pigs.

Vaccination with PRRS MLV, with or without exogenous IFNA adjuvant, resulted in the expected increases in the Th1 dominant cytokine, IFNG, and associated increases in Th1 related mRNAs, TNFA and IL15. Additionally IFNG protein was produced but TNFA protein was not detected. It is unclear what causes this lack of detectable TNFA protein when gene expression is clearly up-regulated, indicating the potential for reservoirs of this protein following PRRSV vaccination. Sensitivity of protein assays may be a factor. Yet, in agreement with our data, TNFA is either not detectable at all in BAL fluids (Van Reeth et al., 1999) or in very low levels at 14 days post-infection (Labarque et al., 2003).

A remarkable characteristic of the host reaction to PRRSV is the development of a substantial humoral immune response which is associated with a polyclonal B cell activation that results in increased amounts of total immunoglobulins (Lemke et al., 2004). It is interesting to note that since the inflammatory cytokine IL6 promotes the terminal differentiation of activated B cells into antibody-secreting cells (Diehl and Rincon, 2002), it might also be involved in the B cell polyclonal activation that is observed as a result of PRRSV infection. In this regard, elevated IL6 levels in the sera of pigs infected with PRRSV has been previously reported (Asai et al., 1999; Feng et al., 2003) and found to be associated with heightened quantities of serum haptoglobin, but not alpha-1 acid glycoprotein (Asai et al., 1999). Lactate dehydrogenase elevating virus (LDV) is also known to stimulate the production of IL6 by macrophages, in this case in mice, raising the suggestion that this cytokine might be partly responsible for the polyclonal B cell activation seen during infection by this murine virus (Markine-Goriaynoff et al., 2001). In both of our experiments IL6 mRNA from PRRSV activated PBMC was significantly and transiently up-regulated at week 2 after immunization, and was correlated with expression of IL6 protein by PBMC cultured without virus. Notably, IL6 is known to inhibit Th1 differentiation by enhancing suppressor of cytokine signaling (SOCS)-1 expression, which in turn interferes with IFNG signaling and the development of Th1 cells (Diehl et al., 2000; Diehl and Rincon, 2002). In our experiments an increase in SOCS1 gene expression was observed in PBMC at 2 weeks after MLV vaccination. Thus, IL6 production during the development of the adaptive immune response to PRRSV could be contributing to the low frequencies of IFNG SC created in response to vaccination against this virus. It is notable that, although not statistically significant, the amounts of IL6 mRNA present in virus-stimulated PBMC obtained at 2 weeks after vaccination from immunized pigs also receiving the pINA plasmid tended to be lower as compared to when the cells originated from members of the other two vaccinated groups. This agrees with recent work from Larsen and Olsen (2002) who found that the administration of an IL6 expressing plasmid during vaccination for swine influenza did not enhance vaccine efficacy. Based on these results and a higher frequency of PRRSV-specific IFNG SC associated with the group immunized with the pINA-adjuvanted MLV vaccine, it can be speculated that the presence of IFNA at the time of vaccination might have other benefits besides increasing the intensity of the IFNG response. For instance, by diminishing the production of IL6, it is conceivable that the ability of PRRSV to elicit polyclonal B cell activation and a strong but ineffective non-neutralizing antibody response might be moderated. Such a modulation could help maintain the health of the animal by reducing the lymphadenopathy that is associated with PRRSV infection and due primarily to lymphoid follicle hyperplasia (Lemke et al., 2004). Moreover, a lower concentration of IL6 might provide an environment more conducive to the development of a strong IFNG response, which in turn might provide a better degree of protective immunity.

The results of our previous study (Meier et al., 2003) combined with those by Lemke et al. (2004) suggest that the reaction of swine to PRRSV infection is characterized by a strong humoral immune response and a weak to negligible IFNG response. Based on the results presented here, as well as those of others discussed above, we propose that the nature of the initial innate immune response to the virus is likely responsible for this polarized immunity. Specifically, we propose that the combination of a limited IFNA response, and a sustained production of IL1, IL6 and possibly IL10 during the first two weeks after

exposure to the PRRS virus, play critical roles in facilitating the development of a weak Th1 immune response. The observation that the provision of an exogenous source of IFNA at the time of vaccination resulted in an increased presence of IFNG SC and a reduction in the amounts of IL1, IL6 and IL10 readily secreted by PBMC, is consistent with this notion. Together these results suggest that the polarized immune response to PRRS virus is likely regulated in a fashion akin to what has been described in the Th1/Th2 paradigm in humans and rodents, and that this response may be altered by exogenous cytokine administration.

Our results demonstrate that exogenous IFNA introduced in the form of expressible cDNA enhanced the development of vaccine-induced PRRSV-specific cellular immunity but had a limited impact on immune gene expression by PBMC encountering recall antigen. Thus co-administration of cytokine-encoding plasmid and PRRS MLV has the potential to enhance vaccine induced immune responses considered to be protective in nature. Vaccine studies in Sweden indicated that the pcDNA vector alone could help stimulate IFNA responses (Magnusson et al., 2001; Johansson et al., 2002). Adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit provided immediate protection from foot-and-mouth disease (Chinsangaram et al., 2003; Moraes et al., 2003). Further exploration of IFNA adjuvants will likely contribute to deciphering the immunobiology of PRRSV, and indicate the feasibility and complexity of using gene therapy technology to increase the immunogenicity of conventional vaccines. Since it is clear that PRRSV has the intrinsic capacity to polarize the immune response of the pig into one primarily of a non-protective humoral nature, future experiments should include the use of even more aggressive immune-modulating strategies to obtain the timely development of a strong virus-inhibitory immune response that can provide adequate levels of protection to most if not all individuals in a population.

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