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Development of a bivalent PRRSv/PCV2 vaccine capable of inducing broader cross protection against PRRSv- Phase I

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Statement of the problem:

PRRSV strains circulating in the field are constantly evolving and have significant antigenic diversity. Many strains of the virus possess the ability to interfere with the innate immune response, ultimately resulting in a delayed and ineffective adaptive immune response. These two factors complicate the development of effective vaccines that could be used to combat the virus. In a previous study we identified two vaccination strategies that appear to result in higher neutralizing antibody titers in weaned pigs. However, little is known about how these vaccination strategies were able to achieve this result. In phase one of our study our goal was to identify the best sample and time point to use in evaluating how each strategy impacts the innate immune response to vaccination. We will use this information to evaluate the relationship between the innate immune response and the neutralizing antibody response in an *in vivo* study that will comprise phase II of our investigation.

Objectives

1) Identify the best sample to use for evaluating the innate immune response against our vaccines
2) Identify the best time point to take samples following vaccination
3) Identify cytokines or chemokines that are typical of the innate response obtained with each vaccination strategy

Materials and Methods

Experimental Design

Twenty-seven four week old pigs were obtained for use in this study shortly after weaning. The animals were confirmed to be sero-negative for antibody against PRRSV using an ELISA (IDEXX) before they were included in the trial. On day 0 of the study the animals were split into three treatment groups (Table 1). The MLV PRRSV vaccine used in treatments one and two was Ingelvac MLV® (Boehringer-Ingelheim) and the PCV2 vaccine used in treatment 1 was Circovac® (Merial). Immunizations were delivered using a 5/8 inch needle and a 2mL dose of vaccine at each injection site. Vaccination sites were marked with a lab marker and were monitored daily to make sure all marks remained visible until the last day of the study. Sera were drawn from each animal at days 1, 3, and 7 for testing in IFN-α and IFN-γ ELISAs. On days 1, 3,

and 7 three pigs were selected from each group and biopsies were taken from the injection sites using a 4mm biopsy punch. Separate pigs were sampled at each time point and samples were tested using Real Time quantitative Reverse Transcription PCR (qRT-PCR).

Table 1- Treatment groups included in study

Treatment Group	Treatment	Number of animals
1	Inactivated PCV2/MLV PRRS (Injection site 1), inactivated PRRS in Adjuvant A delivered at a separate injection site (Injection site 2)	9
2	Inactivated PRRS in Adjuvant B with MLV	9
3	Mock-vaccination, Phosphate Buffered Saline	9

IFN in serum

Serum samples were evaluated using an ELISA for porcine IFN-α similar to one described previously (Diaz de Arce et al., 1992). Sera were also tested for IFN-γ using a commercially available ELISA (MabTech). All samples were tested in duplicate and the mean of the absorbance from both wells was used to determine the concentration of IFN-α present in the serum by comparison to a standard curve. None of the animals were positive for IFN-γ in serum at any of the time points sampled.

qRT-PCR array testing of injection site biopsies

Biopsy samples were immersed in an RNA preservation solution (Thermo Fisher) immediately after they were taken and placed on ice. Samples were returned to the laboratory and were frozen at -80˚C until they could be processed. Prior to processing, samples were flash frozen in liquid nitrogen and pulverized in lysis buffer. Homogenized samples were filtered through a QiaShredder® column (Qiagen) to remove large debris and RNA was extracted using an RNEasy kit (Qiagen) following the manufacturer's instructions for incorporating a DNA digestion step on the filter membrane. 100ng of RNA from each sample was used for first strand synthesis using an RT^2 First Strand kit (Qiagen). qRT-PCR was carried out using RT^2 SYBR© green master mix and a Porcine Cytokine and Chemokine Array kit (Qiagen-PASS-150Z) designed for 96 well plates according to the manufacturer's instructions. Samples were tested for transcription of 85 immunologically relevant cytokines included in the standard format of the kit along with controls. Data were analyzed using Gene Globe® software (Qiagen). Ct values obtained from all samples were normalized against those obtained against porcine ribosomal protein 13a (RPL13a) at each time point and fold change was determined using the $2^{-\Delta\Delta Ct}$ method. Results were considered significant if the fold change observed was regulated greater than 2-fold and the result of a Student's t-test comparing $2^{-\Delta\Delta Ct}$ values between the control and treatment groups indicated a p-value of 0.05 or less ($\alpha=0.05$).

Results and Discussion

Results from phase I of our study indicated that testing of sera for IFN-α and IFN-γ was uninformative. None of the groups differed significantly in the quantity of these proteins expressed systemically at any time point during the study. However, there was one animal from treatment group 2 that had consistently elevated serum IFN-α levels and this animal exhibited a peak in expression at day 3. IFN-γ was not detected in serum at any time point during the study. Biopsy sampling, when combined with qRT-PCR testing, was very informative. The most significant changes in transcription occurred at three days post-vaccination at all of the injection sites sampled. Samples from animals that were assigned to both treatment groups showed a marked up-regulation of genes associated with chemotaxis and macrophage differentiation compared to the mock-vaccinated animals (Table 2).

Table 2- Genes exhibiting significant changes in transcription in comparison to mock-vaccinated controls

Group	Injection Site	Day	Gene	Fold-Change	P-value
1	PCV2/MLV	1	-	-	-
1	Inactivated PRRSV, Adjuvant A	1	**CCL2**	**8.73**	**0.025515**
			CCL3L1	**4.95**	**0.007913**
			CCL4	**3.62**	**0.045535**
			IL10	**2.53**	**0.033464**
			AMCF-II	**2.42**	**0.030503**
2	MLV with inactivated PRRSV, Adjuvant B	1	**CNTF**	**-2.36**	**0.021166**
1	PCV2/MLV	3	**CXCL8**	**25.34**	**0.021946**
			AMCF-II	**8.46**	**0.000886**
			IL1B1	**6.65**	**0.010437**
			TNFSF13B	**4.17**	**0.041744**
			INHBA	**2.50**	**0.046242**
1	Inactivated PRRSV, Adjuvant A	3	**IL1B1**	**10.58**	**0.024183**
			CCL3L1	**8.71**	**0.049073**
			AMCF-II	**4.91**	**0.029430**
			THPO	**2.42**	**0.022794**
2	MLV with inactivated PRRSV, Adjuvant B	3	**CXCL8**	**14.93**	**0.000034**
			IL1B1	**12.5**	**0.031474**
			CCL3L1	**10.13**	**0.016888**
			SPP1	**9.25**	**0.001551**
			AMCF-II	**9.21**	**0.01813**
			CCL2	**8.53**	**0.000014**
			M-CSF	**5.99**	**0.001895**
			TNFSF9	**5.25**	**0.014392**
			CCL19	**5**	**0.040412**
			IL27	**4.94**	**0.02867**
			TNFSF13B	**4.41**	**0.008423**
			CCL8	**3.38**	**0.036202**
			CCL21	**3.36**	**0.010095**
			IL7	**2.68**	**0.012395**
1	PCV2/MLV	7	**CCL28**	**-4.94**	**0.014051**
1	Inactivated PRRSV, Adjuvant A	7	**CCL28**	**-4.90**	**0.017356**
			CXCL9	**8.69**	**0.012604**
			SPP1	**59.71**	**0.048161**
2	MLV with inactivated PRRSV,	7	**FASLG**	**4.22**	**0.033860**

	Adjuvant B				

Data from all injection sites indicated that transcription of genes encoding chemotactic proteins (CXCL8, AMCF-II, CCL3L1) were significantly up-regulated. This was expected and may serve as a principle mode of action for both adjuvants by attracting antigen presenting cells (APCs) to the injection site. These results can be applied by veterinary practitioners and researchers during the design of studies meant to determine the role that adjuvants play in innate immunity following vaccination.

Literature Cited

Diaz de Arce, H, Artursson K, L'Haridon R, Perers A, La Bonnardiere C, Alm GV. 1992. **A sensitive immunoassay for porcine interferon-alpha**. *Vet. Immunol. Immunopathol.* 30:319-327.