RESEARCH REPORT
SWINE HEALTH

Title: Identifying PRRSV structural components that activate regulatory T cells and diminish protective immunity - **NPB #08-193**

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Industry Summary

This study was designed to test the **hypothesis** that **certain structural components of PRRSV drive the activation of regulatory T cells (Tregs).** Activating these T cells would thereby diminish the protective immune response. The long term goal is to design vaccines containing the necessary components for producing protective immunity rather than immune suppression, and heterologous protection. This hypothesis was proposed to be tested by two objectives, only one of which was funded by this grant (the second objective will be submitted for subsequent funding). To determine which structural proteins activated Tregs, we expressed the individual structural protein open reading frames (ORF 2-7, corresponding to GP2-5, M, and N) in bacteria. We used these proteins in a Treg-activation assay in vitro to determine which structural proteins stimulate Tregs. Our results show that both GP4 and GP5 are capable of stimulating Tregs. Synthetic peptides will then be used to fine map the Treg-activation epitopes in GP4 and GP5. Activation of Tregs not only dampens the immune response to the virus and secondary infections, but may also play a role in preventing heterologous protection by vaccines. Therefore, knowing which structural proteins activate Tregs and knowing where the Treg-activation epitopes are will allow us to design vaccines that avoid a Treg-response. These vaccines will not only provide protective immunity against a homologous strain, but will confer protection against heterologous strains as well. For more information, contact Tanya LeRoith (tleroith@vt.edu).

Keywords: PRRSV, regulatory T cell, structural proteins, protective immunity, vaccine

Scientific Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) accounts US swine industry losses of up to $600 million each year. Protective immunity is delayed and weak because of virus-mediated immune-modulation, leading to virus persistence and severe secondary respiratory infections. Infection and vaccination with PRRSV induces a rapid, non-neutralizing antibody response, and an early, weak non-specific gamma interferon (IFN-γ) response. A PRRSV-specific T cell IFN-γ response does not appear until at least 2 weeks after infection, gradually increases and then plateaus at 6 months postinfection, and is associated with a slow increase in neutralizing antibody. Protective immunity requires both an IFN-γ and neutralizing antibody response; however, peak viremia and shedding occur before development of neutralizing antibody and IFN-γ. Current

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commercial vaccines provide good homologous protection; however, heterologous protection is often incomplete. The virus activates regulatory T cells (T_{regs}) and delays IFN-γ production leading to immune suppression. Vaccines that induce IFN-γ rather than IL-10 confer better heterologous protection. The objective of this study was to test the **hypothesis** that **certain structural components of PRRSV drive the activation of regulatory T cells.** Stimulating these T cells would thereby diminish the protective immune response. Our long term goal is to design improved vaccines containing the necessary components for producing protective immunity rather than immune suppression. Since some investigators have shown that cross protection depends more on the ability of a vaccine to induce IFN-γ than on virus homology, these vaccines should provide cross protection as well. To test this hypothesis, we expressed structural proteins GP2-5, M, and N in *E. coli* and used them in an *in vitro* T_{reg}-activation assay. Our results show that both GP4 and GP5 are capable of activating Tregs. We are currently using synthetic peptides to fine-map the T_{reg}-epitopes to determine which epitopes should be mutated for development of a more efficacious vaccine that does not activate T_{regs} and provides heterologous protection.

Introduction

The major obstacles in stimulating protective immunity against PRRSV are 1) the ability of the virus to modulate and dampen the immune response, leading to virus persistence, and 2) the lack of heterologous protection by currently available vaccines. Immune modulation not only allows PRRSV to replicate and cause disease, but it also leads to severe secondary respiratory infections as well. One way the virus is able to do modulate the immune system is by activating regulatory T cells. Regulatory T cells (T_{regs}) not only dampen the immune response to the antigen that activated them, but they also non-specifically dampen the immune response to other antigens as well. T_{reg} induction is associated with inhibition or downregulation of IFN-γ production, as is seen in acutely infected pigs. Importantly, vaccines that induce a strong IFN-γ response provide better protection against pathogenic strains regardless of homology. Current vaccines provide good protection against homologous strains but fail to protect against other strains, even strains in the same genotype, which may be due to their ability to activate T_{regs}. Therefore, determining which viral components are necessary for regulatory T cell activation will be vital in designing future effective vaccines that avoid this arm of the immune response. If we can eliminate T_{reg} activating epitopes from modified live vaccines, the vaccines will provide protective immunity, protect against persistent and secondary infection, and may provide better heterologous protection than vaccines currently in use. Therefore, our **hypothesis** was that **certain structural components of PRRSV drive the activation of regulatory T cells.** Stimulating these T cells would thereby diminish the protective immune response. Our long term goal is to design improved vaccines containing the necessary components for producing protective immunity rather than immune suppression.

Objectives

Objective 1: Determine which structural components activate regulatory T cells *in vitro*

a. Determine which structural protein(s) activate T_{regs}
b. Map T_{reg} activation epitopes in the proteins determined in Objective 1a

Only objective 1 was included in this proposal. We will submit an additional proposal for funding objective 2.

Objective 2: Determine if eliminating T_{reg} epitopes from structural components in subunit vaccines produces a protective immune response.

a. Construct subunit vaccines with mutations in T_{reg} epitopes of structural components
b. Immunize pigs with the subunit vaccine and evaluate T_{reg} numbers, IFN-γ or IL-10 production
c. Challenge immunized pigs with wild-type PRRSV to determine if the immune response is protective

Materials and Methods

Construction of recombinant protein

RNA was extracted from PRRSV strain 2385 and reverse transcribed with gene-specific reverse primers corresponding to open reading frames 2-7 (Table 1). PRRSV structural protein genes were amplified with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) using primers with 14 or 15 nucleotide 5' extensions complementary to the plasmid later used for cloning. Genes were amplified on a Biometra thermocycler with a 9min initial denaturation at 95ºC followed by 34 cycles of 94ºC for 1min, 51ºC for 1min, and 72ºC for 2min 30sec, with a 7min final extension at 72ºC. PCR products were electrophoresed on a 1% TBE-agarose gel to confirm product size and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA). Purified product was treated with T4 DNA polymerase in the presence of dATP and cloned into a pET-30 Ek-LIC vector (Novagen) which contained a kanamycin resistance gene and a His-tag marker. Cloned plasmids were transformed into NovaBlue GigaSingles Competent Cells (Novagen), plated on LB media containing 30ug/ml kanamycin and incubated overnight at 37ºC. Transformed colonies were PCR screened with T7 promoter and T7 terminator primers and the products electrophoresed on a 1% TBE-agarose gel to confirm uptake of the gene of interest. Colonies were PCR screened with a 5min denaturation at 99ºC followed by 34 cycles of 95ºC for 30sec, 50ºC for 30sec, and 72ºC for 1min, followed by a 5min final extension at 72ºC. Screened plasmids were grown overnight at 37ºC in 100ml LB containing 30ug/ml kanamycin with shaking at 250rpm. Plasmids were purified from overnight cultures with the PureYield Plasmid MidiPrep System (Promega) and sequenced at the Virginia Bioinformatics Institute at Virginia Tech. Plasmids containing inserts with the correct sequence were transformed into BL21(DE3) cells for expression. Single colonies of transformed BL21(DE3) were inoculated into 5ml LB starter cultures containing 30ug/ml kanamycin and incubated overnight at 37ºC with shaking at 250rpm. 100ml LB cultures containing 30ug/ml kanamycin were inoculated with the starter cultures and incubated as previously until the OD600 reached 0.5-1.0. IPTG was added at 100mM and the incubation continued for three additional

Primer Name	Orientation	Sequence	Expected Product Size (bp)
PRRSV-orf2LIC-F	forward	5' GAC GAC GAC AAG ATG AAA TGG GGT CTA TGC AAA 3'	797
PRRSV-orf2LIC-R	reverse	5' GAG GAG AAG CCC GGT TAC CGT GAG TTC GAA AGA AA 3'	
PRRSV-orf3LIC-F	forward	5' GAC GAC GAC AAG ATG GCT AAT AGC TGT ACA TTC 3'	791
PRRSV-orf3LIC-R	reverse	5' GAG GAG AAG CCC GGT TAT CGC CGT GCG GCA CTG AG 3'	
PRRSV-orf4LIC-F	forward	5' GAC GAC GAC AAG ATG GCT GCG TCC CTT CTT TTC 3'	563
PRRSV-orf4LIC-R	reverse	5' GAG GAG AAG CCC GGT TAA ATT GCC AAC AGA ATG GT 3'	
PRRSV-orf5LIC-F	forward	5' GAC GAC GAC AAG ATG TTG GGG AAA TGC TTG ACC 3'	629
PRRSV-orf5LIC-R	reverse	5' GAG GAG AAG CCC GGT TAA GGA CGA CTC CAT TGT TC 3'	
PRRSV-orf6LIC-F	forward	5' GAC GAC GAC AAG ATG GAG TCG TCC TTA GAT GAC 3'	551
PRRSV-orf6LIC-R	reverse	5' GAG GAG AAG CCC GGT TAT TTG GCA TAT TTA ACA AG 3'	
PRRSV-orf7LIC-F	forward	5' GAC GAC GAC AAG AGT CCA AAT AAC ACC GGC AAG 3'	398
PRRSV-orf7LIC-R	reverse	5' GAG GAG AAG CCC GGT TAT GCT GAG GGT GAT GCT GT 3'	

Table 1: Primers designed for amplification of PRRSV open reading frames prior to vector ligation. Underlined regions are complementary to the ligation vector. Expected product sizes are shown in base pairs (bp).

hours, as determined for GP4 (ORF4 product) and GP5 (ORF5 product) in an earlier pilot study (results not shown). Cultures were centrifuged 10min at 10,000g, supernatants decanted, and cell pellets resuspended in 5ml BugBuster Master Mix (Novagen) per gram of pellet wet weight. Soluble and inclusion body proteins were

extracted with BugBuster Master Mix according to the manufacturer's recommendations. His-tagged recombinant proteins were purified from the soluble fraction using nickel charged His-bind resin (Novagen) according to the manufacturer's instructions. Inclusion body proteins and purified soluble proteins were electrophoresed 45min at 200 volts on 12% XT Bis-Tris SDS-PAGE gels in a Criterion electrophoresis cell using XT MES Running Buffer (Bio-Rad). Proteins were transferred via Western blotting to a PVDF membrane using Tris/Glycine transfer buffer. Transfer proceeded 30min at 100 volts. After transfer, the gel was back-stained with Bio-Safe Coomassie Stain (Bio-Rad), transferred total protein was detected with Colloidal Gold protein stain (Bio-Rad), and transferred recombinant proteins were detected with the Protein Detector Western Blot Kit TMB System (KPL) using a His-tag monoclonal antibody (EMD Chemicals).

Peptides

Synthetic 20-mer peptides that overlap by 2 amino acid residues were constructed. The peptides were purchased commercially and dissolved in 10% (vol/vol) acetic acid-20% (vol/vol) acetonitrile in water or 15% (vol/vol) *N*,*N*-dimer (Table 2).

Infection of monocyte-derived dendritic cells and induction of regulatory T cells

Two hundred mililiters of peripheral blood was collected from pigs that were PCR and sero-negative for PCV2, PRRSV, and swine influenza. The blood was collected into heparinized syringes, diluted 1:2 with sterile PBS, overlaid on Ficoll-Hypaque and centrifuged at 400xg and 18° C for 35 minutes. PBMCs was collected from the buffy coat into ice-cold complete RPMI medium (10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 55uM B mercapto-ethanol), washed two additional times and resuspended in complete RPMI 1640 medium. Freshly isolated PBMCs were seeded in T75 tissue culture flasks and incubated overnight in complete RPMI 1640 medium at 37° C, 5% CO_2 to allow monocytes to adhere. Non-adherent cells were removed and cryopreserved. Adherent cells were cultured at 37° C, 5% CO_2 in complete RPMI medium supplemented with 20ng/mL recombinant porcine-interleukin 4 and 20ng/mL recombinant porcine GM-CSF for for 5 days and then harvested. After 5 days, DC differentiation was confirmed by typical veiled morphology and phenotyping with monoclonal antibodies specific for MHC I, MHC II, CD 172 (SWC3), CD 14 and CD 1. Monocyte-derived DCs were infected with PRRSV at a multiplicity of infection (m.o.i.) or approximately 2.5ug/ml of UV-sterilized or 0.2um-filtered PRRSV recombinant structural protein or peptide will be added. MoDC were cultured in the presence of recombinant protein for 24 hours. Autologous cells from the non-adherent fraction obtained during monocyte isolation were added at $1.25x10^5$ cells/ml to UV-sterilized protein samples in 6-well plates or at $2x10^6$ cells/ml to filtered protein samples in 96-well plates. After an additional 48 hours in culture cells were centrifuged 10min and stained for flow cytometry using Staining Buffer (BD Pharmingen).

Flow cytometric and cytokine analysis

After 3 days of co-culture with MoDCs, lymphocytes were evaluated for expression of CD4, CD25 and FoxP3 using flow cytometry. Cells were sequentially stained with CD4/IgG_{2b} (VMRD), Alexa-fluor647 goat anti-mouse IgG2b (Invitrogen), mouse anti-pig CD25 (Serotec), and FITC-labeled goat anti-mouse IgG (Serotec). For intracellular staining, cells were permeabilized with permeabilization/ fixation buffer (eBioscience) at 4° C for 12-18 hours followed by staining with PE anti-mouse FoxP3 (eBiosciences clone FJK-16s) for 30 minutes at 4° C. Flow cytometric analysis was conducted using FACSCalibur cytometer (BD) and analyzed using FloJo software. Levels of IFN-γ, IL-10, and TGF-β were analyzed by cytokine ELISAs specific for porcine cytokines, as well as by real-time RT-PCR. The cytokine ELISAs were done on the culture supernatants.

Results

Derivation of monocyte-derived dendritic cells

Dendritic cells were derived from the monocyte fraction by incubating purified monocytes for 7 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, and 10 ng/ml recombinant porcine GM-CSF (R&D systems). The culture medium was replenished every 3 days. Cells were characterized by

veiled morphology and by immunophenotyping with mAb specific for CD14, MHC I, MHC II, CD1, SWC3, CD11b/c and CD 80/86. The dendritic cells were then infected with PRRSV and again immunophenotyped. We saw a downregulation of MHC II, CD 1, CD 11b, and CD 172 after infection with PRRSV (Fig 1).

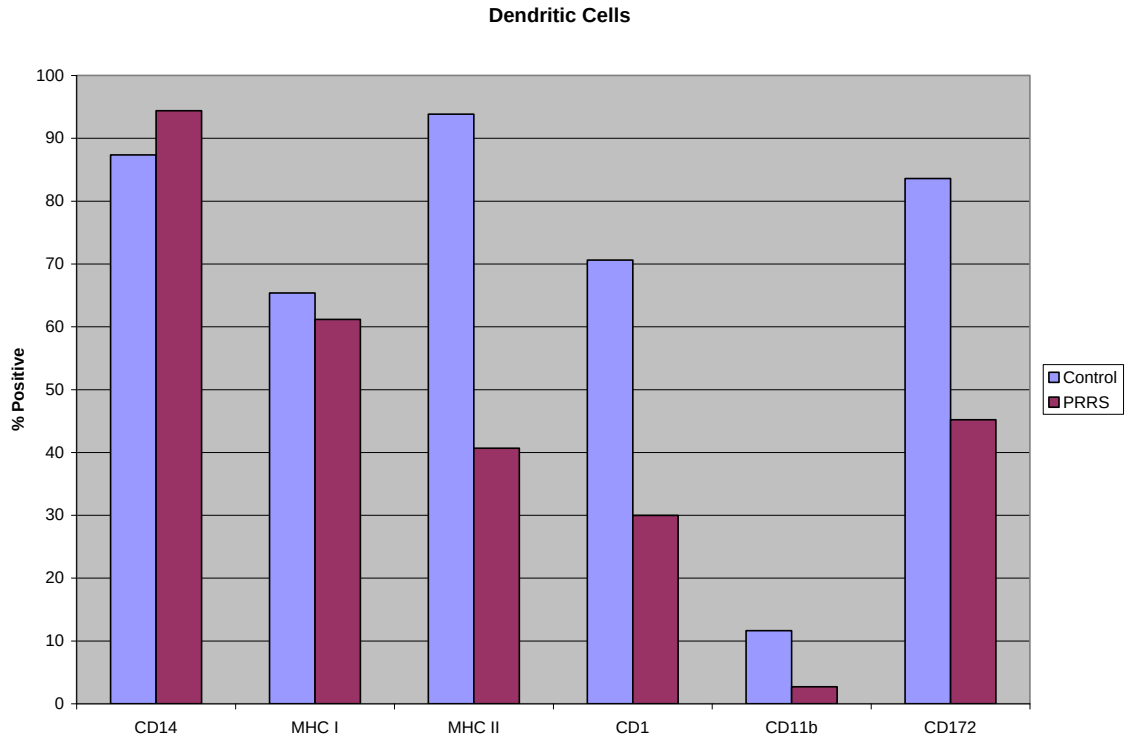

Fig 1. PRRSV-infected dendritic cells. Values in blue show the expression of surface molecules on sham infected dendritic cells, while values in maroon show the expression of surface molecules on PRRSV-infected dendritic cells.

Sequencing data (ORF expression)

Serial passage of the virus *in vitro* has resulted in some sequence mutations that are different from the published strain; however, our *in vivo* experiments show that the virus retains pathogenicity. To determine if these base changes reflect actual mutations rather than sequencing errors, we are re-cloned the ORF's to ensure the correct sequence. The sequences were confirmed and shown to be the correct sequence rather than sequencing errors.

ORF2

primers designed from NCBI accession PRU20788 (VR2385)

090213 sequence differences (PRU20788→090213):

bp289:	A→G	met→val	medium non-polar→small non-polar
bp292:	C→T	leu→phe	medium non-polar→large non-polar
bp294:	T→A	leu→leu	no change
bp511:	C→A	his→asn	large basic→medium polar

ORF3

primers designed from NCBI accession PRU20788 (VR2385)

090213 sequence differences (PRU20788→090213):

bp150:	T→C	asn→asn	no change
bp210:	C→T	gly→gly	no change
bp320:	C→T	ala→val	small non-polar→small non-polar
bp525:	G→C	gly→gly	no change

ORF4 *ready for expression*
primers designed from NCBI accession PRU20788 (VR2385)
the last two bases of the reverse primer (GGT) differ from PRU03040 (GCC)
090107 sequence differences (PRU20788→090107):
bp191: A→G
090205 sequence shares **100% identity** with PRU20788
090326 freezer stock sequence matches 090205 sequence

ORF5
primers designed from NCBI accession PRU03040 (VR2385)
090107 sequence differences (PRU03040→090107):
bp585: A→__ poor chromatogram; instrument called bases incorrectly; other seqs correct
090205 and 090213 sequences share 100% identity to one another
090205 and 090213 sequence differences (PRU03040→090205/090213 consensus):
bp106: G→A gly→ser small polar→small polar
bp333: G→C met→ile medium non-polar→medium non-polar

ORF6
primers designed from NCBI accession PRU03040 (VR2385)
090129 sequence differences (PRU03040→090129):
bp145: G→A val→ile small nonpolar→medium non-polar
090326 freezer stock matches 090129 sequence

ORF7
primers designed from NCBI accession PRU03040 (VR2385)
090107 sequence differences (PRU03040→090701):
bp96: C→G his→tyr large basic→large polar
bp216: T→G arg→arg no change
bp269: G→A cys→tyr small polar→large polar

Regulatory T cell activation

Using an in vitro T_{reg} activation assay, we tested the ability of the structural proteins to individually activate T_{regs}. Consistent with data that showed that GP5 induces a delayed and weak B cell response in pigs, we found that GP4 and GP5 consistently produced the highest numbers of CD4+CD25+ FoxP3+ lymphocytes (Fig 2 and 3).

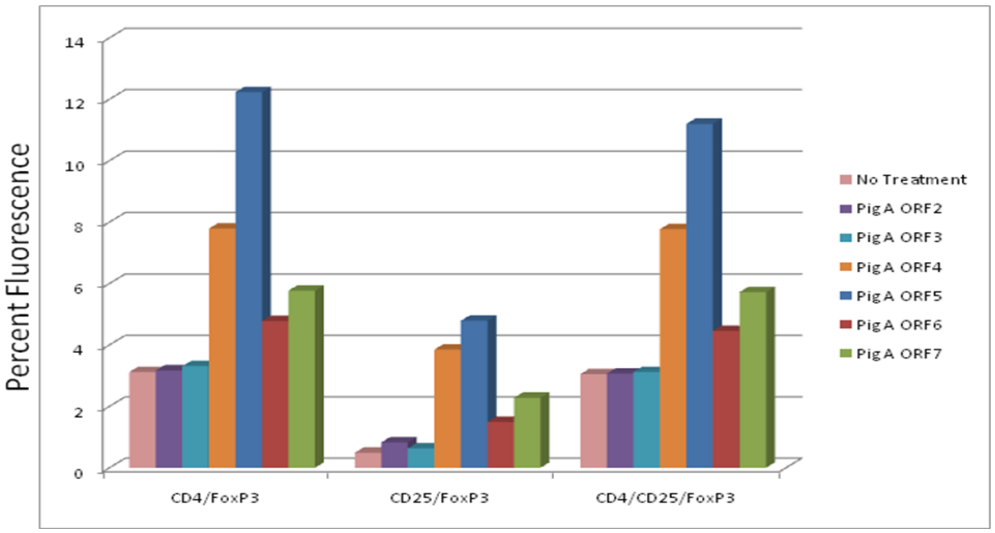

Figure 2: Flow Cytometry CD4+CD25+FoxP3+ Expression, Pig A. Percent CD4+FoxP3+, CD25+FoxP3+, and CD4+CD25+FoxP3+ fluorescence after in vitro culture with porcine MoDC and UV-sterilized PRRSV protein is shown. PRRSV protein.

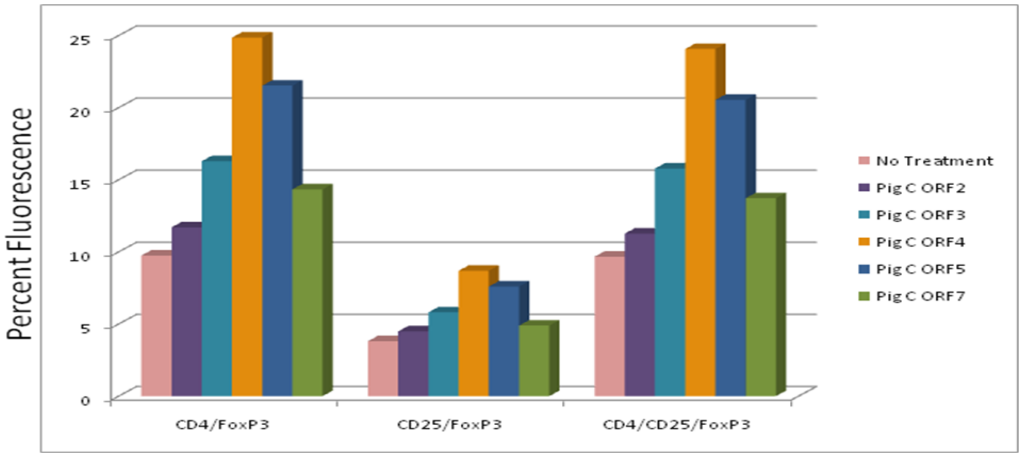

Figure 3: Flow Cytometry CD4+CD25+FoxP3+ Expression, Pig C. Percent CD4+FoxP3+, CD25+FoxP3+, and CD4+CD25+FoxP3+ after in vitro culture with porcine MoDC and UV-sterilized PRRSV protein.

Synthetic peptides

Based on the data that GP4 and GP5 were most effective at activating T_{regs}, 21 synthetic peptides were designed based on the sequence of our virus (Table 2). The synthetic peptides overlap by 2 amino acids and contain putative T cell epitopes.

Table 2: Synthetic Peptides: (plus sign indicates partial HLA-A2 site)

orf4 A: MAASLLFLLVGFKCLLVSQA (contains 2 putative HLA-A2 site)
orf4 B: QAFACKPCFSSSLSDIKTNT (contains 0 putative HLA-A2 site)
orf4 C: NTTAAAGFAVLQDISCLRHR (contains 0 putative HLA-A2 site)
orf4 D: HRNSASEAIRKVPQCRTAIG (contains 0 putative HLA-A2 site)
orf4 E: IGTPVYITVTANVTDENYLH (contains 0 putative HLA-A2 site)

orf4 F: LHSSDLLMLSSCLFYASEMS	(contains 1+ putative HLA-A2 site)
orf4 G: EMSEKGFKVVFGNVSGIVAV	(contains 1 putative HLA-A2 site)
orf4 H: VCVNFTSYVQHVKEFTQRSL	(contains 0+ putative HLA-A2 site)
orf4 I: SLVVDHVRLLHFMTPETMRW	(contains 1 putative HLA-A2 site)
orf4 J: PETMRWATVLACLFTILLAI	(contains 1 putative HLA-A2 site)
orf5 A: MLGKCLTAGCCSQLLFLWCI	(contains 0+ putative HLA-A2 site)
orf5 B: CIVPSCFVALVSANGNSSSN	(contains 0 putative HLA-A2 site)
orf5 C: SNLQLIYNLTLCELNGTDWL	(contains 0 putative HLA-A2 site)
orf5 D: WLANKFDWAVECFVIFPVLT	(contains 0 putative HLA-A2 site)
orf5 E: LTHIVSYGALTTSHFLDTVG	(contains 0 putative HLA-A2 site)
orf5 F: VGLVTVSTAGFVHGRYVLSS	(contains 0 putative HLA-A2 site)
orf5 G: SSIYAVCALAALICFVIRLA	(contains 2 putative HLA-A2 site)
orf5 H: LAKNCMSWRYSCTRYTNFLL	(contains 0+ putative HLA-A2 site)
orf5 I: LLDTKGRLYRWRSPVIIEKR	(contains 1+ putative HLA-A2 site)
orf5 J: KRGKVEVEGHLIDLKRVVLD	(contains 1 putative HLA-A2 site)
orf5 K: LDGSAATPVTRVSAEQWSRP	(contains 0 putative HLA-A2 site)

These peptides are currently being tested in the in vitro T_{reg}-activation assay fine-map T_{reg}-activation epitopes.

Discussion

PRRSV infection and persistence currently are major problems in the field. However, the mechanism by which the virus evades the immune system leading to immune suppression and persistent infection is not currently known. This immune modulation not only allows PRRSV to replicate and cause disease, but leads to severe secondary infections as well. One way the virus is able to do this is by activating regulatory T cells. Regulatory T cells not only dampen the immune response to the antigen that activated them, but also non-specifically dampen the immune response to other antigens as well. This research addressed two important questions in PRRSV immunology – why is the protective immune response to the virus weak, and why is it delayed? This was done by determining which components of the virus stimulate regulatory T cells, resulting in diminished and delayed protective immunity. Our data indicates that the structural proteins GP4 and GP5 are critical to T_{reg} activation. We are currently fine-mapping the T_{reg} -activation epitopes so that we can mutate these epitopes to design vaccine strains that prevent T_{reg} activation. Determining which viral components are necessary for regulatory T cell activation will be vital in designing vaccines that avoid this arm of the immune response, thereby providing protective immunity while also avoiding both persistent and secondary infection. Additionally, because heterologous protection relies on a robust IFN-γ response, vaccines that do not activate T_{regs} may also provide protection against heterologous challenge.