RESEARCH REPORT
SWINE HEALTH

Title: PRRSV protective immunity of broad spectrum: Strategies to induce pan-neutralizing antibodies in a pig - **NPB #12-158**

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Industry Summary:

The betterment of PRRSV vaccines is a national priority for the swine industry and for USDA as well. National Pork Board's Swine Research Program and also USDA-AFRI-NIFA research program assign top priority to the development of cross protective strategies for PRRS immunization, which should result in the enhancement of a vaccine's ability to provide heterologous protection (i.e. protection against PRRSV Strains that are distant or very different from the strain used for vaccination). Such fundamental requirement for improving current vaccines is dictated by the formidable genetic diversity of the multiple strains of PRRSV that simultaneously circulate in the field.

In our laboratories we explore two major alternatives towards the pursuit of broadly protective PRRSV vaccines:

1) The development of consensus-sequence live vaccines that would improve heterologous protection founded on major conserved determinants of protection (epitopes) inducing (T) cell-mediated immunity, and

2) Discovery of key antigenic determinants of protection (conserved B-cell epitopes) that enter in the composition of external glycoproteins of PRRSV that would induce the development of broadly neutralizing antibodies capable of preventing infection by many diverse heterologous PRRSV strains.

This NPB report pertains to experiments on this second category, with results that would directly lead to the development of more effective PRRSV subunit (non-replicating) vaccines for broad protection. The initial results of the NPB project herein reported, have been the basis for our being awarded a substantially higher USDA-NIFA-AFRI award that should direct us, by 2016, to the characterization of the key determinants (B cell epitopes) of protection giving origin to broadly neutralizing antibodies against PRRSV. The existence of such broadly-neutralizing antibodies have been inferred through several different research reports but so far no broadly-neutralizing antibody has been categorically discovered. Such is our proposed target to be achieved in 2016.

Keywords: PRRSV, broad protection, broadly neutralizing antibodies, PRRSV glycoproteins, subunit vaccines, broadly protecting vaccines

Scientific Abstract:

It has been shown by us and others that neutralizing antibodies are important for protection against PRRSV infection in vivo. However, multiple initial trials using GP5 and M as the only PRRSV structural subunits responsible for inducing neutralizing antibodies have shown that these two subunits are of secondary importance at inducing cross protecting antibodies X PRRSV. We postulate that the other "minor" glycoproteins (GP2, GP3, and GP4) of PRRSV are of fundamental importance for induction of protective immunity against PRRSV. Of particular importance to sustain our hypothesis are our findings that demonstrate that the primary viral proteins involved in binding to CD163 (CD163 is the most important cell receptor used by PRRSV to infect the swine macrophage) are at least the minor envelope proteins GP2 and GP4, although the minor GP3 cannot be ruled out at this time either. Since alterations of the GP2 and GP4 contact residues (or contact epitopes) that directly interact with the receptor CD163 would likely impair infectivity and jeopardize PRRSV survival in nature, we hypothesize that such epitopes or residues are likely to be highly conserved and less prone to mutations and that the antibodies targeting these epitopes have higher chance of being broadly neutralizing across the spectrum of PRRSV strains than are antibodies to other viral proteins. In other words, we postulate and plan to demonstrate that these minor glycoproteins of PRRSV are the real structural basis for origination of pan-neutralizing antibodies (a.k.a. broadly neutralizing antibodies) in the swine host.

Initially, the overall goal of this NPB supported research was to discover and characterize high affinity PRRSV neutralizing antibodies derived from B cells of PRRSV-vaccinated/challenged pigs. Central to the success of this project was the contribution of the CellSpot cloning technology patented by our collaborators at Trellis BioScience (San Francisco, CA).

We initiated this project by developing whole baculovirus-expressed PRRSV GPs (GP2, GP3, GP4 and GP5) that could be used for singly immunizing pigs through a prime-boost immunization strategy and"subsequent B cell cloning and identification of B cells secreting high-affinity PRRSV neutralizing antibodies from immunized/challenged pigs by Trellis BioScience". However, we found serious difficulties at producing, out of the baculovirus clones obtained, adequate amounts of purified GPs that would be apt for immunization. We decided to develop an alternative vector expression of the PRRSV GPs by cloning these GPs in human adenovirus. Likewise, initial attempts at B-cell cloning indicated to us that stimulation of B cells and ensuing single cell cloning would be more straightforward (and likely more effective) by directly using the peptides and short polypeptides that contain the epitopes of interest for induction of broadly neutralizing antibodies. To that end we expanded this project to evaluate the residues of GP2 and GP4 that are responsible for the interaction with the cellular receptor CD163, using a mammalian two-hybrid system used to screen site mutated glycoproteins. Therefore, the original NPB proposal with the expanded goal (at no cost to NPB) of identification of contact residues in GP2 and GP4 served for us to be awarded federal competitive funds from the USDA-AFRI-NIFA program (Animal Health, 2013-2016), thus significantly expanding (4X) the original funds awarded by NPB.

Introduction:

The work we present herein centers on one of the 2 major mechanisms to provide protective immunity against PRRSV: PRRSV-neutralizing antibodies. Through our work, it has become clear that neutralizing antibodies are a *bona fide* correlate of protective immunity, although in most cases, the protection conferred by these neutralizing antibodies is predominantly strain-specific, commonly presenting a rather low level of

cross-neutralization towards heterologous PRRSV strains. We have evidence, however, that epitopes inducing antibodies that neutralize across multiple PRRSV strains (a.k.a. “pan-neutralizing” or “broadly-neutralizing” antibodies) indeed exist in the case of PRRSV and that a broader protection is feasible, provided those pan-neutralizing epitopes are appropriately presented to the cells of the pig’s immune system. Our first description of an important neutralizing epitope on the major PRRSV envelope glycoprotein 5 (GP5) contributed to position this protein as one major immunogenic candidate used by the research community to test a great number of vaccine platforms and constructs, in the pursuit of an ideal PRRSV vaccine. It is evident now, however, that GP5, alone or in combination with its other partner, the membrane protein (M), -GP5/M-, is not sufficient to confer effective protective immunity. The formulation of a new generation of PRRSV immunogens should then require the additional contribution of other PRRSV structural proteins. We postulate that the other “minor” glycoproteins (GP2, GP3, and GP4) of PRRSV are of fundamental importance for induction of protective immunity against PRRSV. Of particular significance to this point are our recent findings demonstrating that the primary viral proteins involved in binding to CD163 (CD163 is the most important cell receptor used by PRRSV to infect the swine macrophage) are the minor envelope proteins GP2 and GP4. Research published by the swine virology team at the Shanghai Veterinary Research Laboratory in China, strongly supports our contention about such central role of the minor GPs (2, 3 and 4) in PRRSV infection. These scientists have demonstrated that the minor GPs, but not GP5, are responsible for turning a PRRSV-refractory (that is PRRSV-resistant) cell line into a susceptible one. The data obtained by the Chinese team strongly suggests that those minor GPs are the ones which interact with the main PRRSV receptor in the cells. Since alterations of the GP2 and GP4 contact residues (or contact epitopes) that directly interact with the receptor CD163 would likely impair infectivity and jeopardize PRRSV survival in nature, we hypothesize that such epitopes or residues are likely to be highly conserved and less prone to mutations and that the antibodies targeting these epitopes have higher chance of being broadly neutralizing across the spectrum of PRRSV strains than are antibodies to other viral proteins. In other words, we postulate and plan to demonstrate that these minor glycoproteins of PRRSV are the real structural basis for origination of pan-neutralizing antibodies in the swine host.

Objectives: The original objective of this NPB proposal consisted of **discovering and characterizing high affinity PRRSV neutralizing antibodies derived from B cells of vaccinated/challenged pigs.** We planned to immunize pigs with the selected whole proteins cloned in baculovirus and use the individual proteins as screening antigens to clone single B cells that would produce high affinity PRRSV-neutralizing antibodies derived from B cells of immunized/challenged pigs. As explained below, we have made two major modifications to this protocol, which reflect positive feed-back and suggestions received from the USDA review panel at the time we were awarded , in 2013, our USDA-AFRI-NIFA grant (obtained through our NPB preliminary results, this project):

1) We have switched to adenovirus expression vectors and **2) we plan to map the areas of the GP2 and GP4**

proteins that directly interact with the CD163 receptor, using deletion and amino acid mutagenesis.

Methods and Results:

Cloning and generation of recombinant baculoviruses: PRRSV Open reading frames 2a-5 were PCR amplified and cloned into pENTR TOPO vector. The primers (Table 1, see attached poster by K-Burkgren et al.) were designed to remove signal sequence and transmembrane domains of PRRSV GPs to increase expression and secretion (Fig. 2, attached poster). Correct clones were used to produce recombinant baculoviruses expressing each GPs in collaboration with GlycoBac (U. of Wyoming) using the baculovirus destination vectors under the

promoters p6.9 and polh. Our proteins of interest were successfully cloned and recombinant baculoviruses expressing GP2, GP3, GP4, and GP5 were confirmed by Western blot analysis.

We are using these proteins in animal experiments to confirm the antibody response generated in vivo, and from these animals to start the B cell cloning to identify individual neutralizing antibodies, thus determining the contribution of each PRRSV GP to the induction of a protective immune response in swine.

These preliminary results were presented by our graduate student Kay Kimpston-Burkgren at the 35 th Annual Graduate Student Symposium 2013 of the School of Biological Sciences of the University of Nebraska Lincoln (April12th 2013, Lincoln Nebraska) Poster is attached. The NPB grant support is being acknowledged in such poster.

Cloning of GP3 in Adenovirus for use in prime-boost immunizations:

Due to issues with baculovirus expression and purification of recombinant proteins, we looked for an alternative vector system to express the glycoproteins of PRRSV. Adenovirus vectors have previously been used in pigs and a commercial kit is available to streamline the process. Based on the Gateway cloning technology, the Invitrogen ViraPower Adenoviral Expression System can be used for transient expression of the gene of interest, in our case the glycoproteins of PRRSV. Individual open reading frames 2, 3, and 4 were PCR amplified and then cloned into the pENTR TOPO vector. A LR recombination reaction was used to transfer our gene of interest into the adenoviral vector. The minor glycoprotein GP3 has been successfully cloned into the adenovirus vector and we have confirmed expression of GP3 by Western blot. We are currently working on GP2 and GP4. A companion assay is being developed for the detection of anti-GP3 antibodies in the sera of pigs. Animal studies will begin shortly. Future studies will include expression of the other glycoproteins of PRRSV as well as a study assessing the protection afforded by a prime-boost vaccination schedule using heterologous expression vectors, in this case baculovirus and adenovirus.

Direct identification of CD163 contact residues in GP2 and GP4

In order to evaluate the residues of GP2 and GP4 that are responsible for the interaction with the cellular receptor CD163 a mammalian two-hybrid system is being used to screen site mutated glycoproteins.

GP2 or GP4 and CD163 have been cloned into plasmids containing either a transcriptional activation domain or a DNA-binding domain, thus the glycoprotein-receptor interaction results in the association of both domains and the transcription of the firefly luciferase reporter gene.

293t cells are transfected with the plasmids containing encoding either GP2 or GP4 and CD163 (fused with the above mentioned domains), incubated, harvested, and lysed 24 hours later. Successively firefly luciferase and Renilla luciferase (as transfection control) will be measured.

Subsequently, 10 amino acid-long alanine mutations will be introduced into the GP2 and GP4. The interaction between the mutated glycoproteins and CD163 will be assessed as described before. Once a specific region or regions are identified, smaller mutations will be introduced in order to determine which are the interacting residues. We estimate to construct 15 GP2 (256 amino acids) mutants, and 12 GP4 (178 amino acids) mutants.

Point mutations will be introduced by PCR utilizing primers that include a consecutive alanine mismatches. Posteriorly, the PCR product will be circularized to generate the plasmid for transfection.

The design of these experiments allows for a high-throughput screening of the mutants. Whenever an absence of interaction is detected, SDS-PAGE of the lysates will be run, followed by western blotting. To confirm the presence of the glycoprotein and the receptor, GP2 and GP4 will include a FLAG tag and the proteins will be detected by FLAG antibody, and a monoclonal porcine CD163 antibody will be used to detect CD163. Co-immunoprecipitation followed by SDS-PAGE and western blotting can be used to confirm any of these results.

Discussion:

It is important to state here that we are not ready to announce, yet, that we can offer the swine industry with the "magic bullet" represented by a subunit vaccine that induces cross-neutralizing highly protective antibodies against any strain of PRRSV. However, we believe that we are engaged in the right path to solve the complex issue of heterologous protection with PRRSV immunogens. So far the major outcome that we can cite out of this NPB proposal is having obtained enough credible preliminary results that permitted the award of a significant competitive federal grant to study this issue in a more extensive, detailed and complete manner, throughout the next three years,

By generating the deletion mutants as proposed and examining the interaction of the mutant GP2 and GP4 proteins with CD163, we expect to map the regions of the two viral GPs responsible for interaction with the receptor. Further studies using single amino acid substitution mutants may lead to fine mapping of the critical residues that are important for the interaction.

It is very possible that we may identify several mutants of the proteins that fail to interact with CD163, a result that would be suggestive of the noncontiguous nature of the interaction domain in these proteins. The noncontiguous nature of the contact sites with the cellular receptors has been extensively documented for other viral pathogens such HIV and hepatitis C virus . In this scenario, we will then generate smaller deletion mutants as well as single amino acid substitution mutants to map the minimal amino acid stretches that are required for interaction with the receptor. These noncontiguous stretches of amino acids will be used for synthesis of peptides to generate monoclonal antibodies as described in objective 3. Although we would have preferred to generate mutants with smaller deletions, we have proposed 20-25-residue deletions in the two glycoproteins to maintain a balance between the problems with potential protein folding and handling of large number of deletion mutants. We are optimistic that the extent of deletion proposed here will yield unambiguous results as to the regions of GP2 and GP4 that interact with CD163. Should unexpected problems arise and all the deletion mutants fail to interact with CD163, it would indicate gross misfolding of the protein. In that situation, we may employ smaller deletion of regions of high sequence conservation among PRRSV isolates to examine their interaction with receptor. Another problem that may be encountered is that deletion of sequences from GP2 and GP4 may result in proteins that cannot be detected by the antibodies we currently have. In this case, we can use carboxy-terminal epitope tagged proteins for these studies. We have generated carboxy-terminal FLAG-tagged GP2 and carboxy-terminal HA-tagged GP4 and have shown that each of these tagged proteins interact with CD163. Therefore, use of these constructs and the antibodies to these tags will alleviate the concerns raised above.

Figures/tables:

Next Page: Poster presented by Kay Kimpston-**Burkgren et al** at the **35 th Annual Graduate Student Symposium 2013 of the School of Biological Sciences of the University of Nebraska Lincoln (date: April12th 2013, Lincoln Nebraska)**

Recombinant Baculovirus Production of Porcine Reproductive and Respiratory Syndrome Virus Glycoproteins

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a major problem facing the swine industry. PRRS causes abortions and stillbirths in pregnant sows and respiratory problems in piglets. The most recent study estimated that PRRS costs the United States upwards of 660 million dollars per year. Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent and it is endemic in most swine producing countries. Currently there are no broadly protective vaccines available. An incomplete understanding of the immunogenicity of the individual glycoproteins (GPs) has hindered the development of such a vaccine. Our group is using recombinant baculoviruses to produce our proteins of interest so that we may determine the contribution of glycoproteins 2-5 in the induction of a protective immune response in swine.

Objective

To determine the contribution of each PRRSV glycoprotein to the induction of a protective immune response in swine using the baculovirus expression system

Materials and Methods

Cloning and generation of recombinant baculoviruses

Open reading frames 2a-5 were PCR amplified and cloned into pENTR TOPO vector. The primers (Table 1)were designed to remove signal sequence and transmembrane domains of PRRSV GPs to increase expression and secretion (Murphy *et al.*)(Fig. 2). Correct clones were used to produce recombinant baculoviruses expressing each GPs in collaboration with GlycoBac (U. of Wyoming) using the baculovirus destination vectors under the promoters p6.9 and polh (Fig. 3).

Table1.
Primers used for PCR amplification of PRRSV glycoproteins

Primer name	Sequence (5'→3')	Expected size
GP2ECTOF	CACCTTACCATCGCTGGCTGGC	511 bps
GP2ECTOR	TCATTGCTGAAAATCATGAAGCTTTG	
GP3ECTOF	CACCGATTCCAATACTACGTACTG	691 bps
GP3ECTOR	TTATCGCCGCGCGGCACTG	
GP4ECTOF	CACCTGCAAGCCATGTTTCAGTTC	421 bps
GP4ECTOR	TCACTCAGGTGTCATGAAATGG	
GP5ECTOF	CACCGCGCTCGTCAACGCCAAC	133 bps
GP5delTMR	CTTCGCAAGAGTCTCCACTGCCCAATCAAAT	349 bps
GP5delTMF	CAGTGGAGACTCTTGCGAAGAACTGCATGTC	236 bps
GP5ECTOR	CTAAAGACGACCCCATTGTTC	

Materials and Methods (*Cont'd*)

PCR amplification, cloning of GPs into pENTR TOPO vector and verification of clones by sequencing

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Genes inserted into baculovirus expression vector

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Isolation of recombinant baculoviruses

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Amplification of P1 baculoviruses

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Production and titration of P2 and P3 baculoviruses

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Protein expression and analysis

Figure 1. Outline of approach to produce recombinant baculoviruses expressing PRRSV GPs

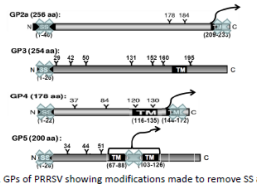

Figure 2. GPs of PRRSV showing modifications made to remove SS and TM domains.

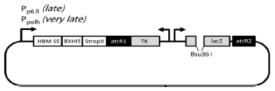

Figure 3. Baculovirus expression vector (Toth *et al.*). HBM SS (honeybee melittin signal sequence), 8XHIS (8X histidine affinity purification tag), StrepII (StrepII affinity purification tag), *attR*1 and *attR*2 (lambda phage *att* sites for recombination).

Protein expression and analysis

Sf9 cells were grown in Sf-900 III serum free media (Gibco) and infected with our recombinant baculoviruses for GPs production. Protein was harvested at 72 hours post infection and analyzed by Western blot.

Results

Baculovirus expressed GP2, GP4, GP5 and GP3

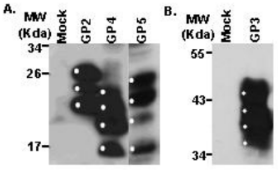

Figure 4. Baculovirus expressed GP2, GP4 and GP5 (A) and GP3 (B) by Western blot analysis

Conclusions

- Our proteins of interest were successfully cloned and recombinant baculoviruses expressing GP2, GP3, GP4, and GP5 were confirmed by Western blot analysis.

Future Directions

- To optimize and scale up production
- To perform animal studies to confirm the antibody response generated *in vivo*
- To determine the contribution of each PRRSV GP to the induction of a protective immune response in swine

References

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Acknowledgement

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