RESEARCH REPORT
SWINE HEALTH

Title: PRRS Immunology Literature Review**, NPB #13-241**

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Industry Summary

PRRSV infection is a chronic infection, persisting in an animal for months. While the early stages of disease are associated with virus in the blood, that virus is eventually cleared; however, virus remains in the lymphoid tissues. This persistence is a problem because the virus can still transmit to naïve animals and cause disease. We've known for some time that a single animal can clear the infection, and the use of load-close-expose (LCE) protocols have been used to return herds to a PRRSV-negative status. Unfortunately, it can take more than 200 days to eliminate PRRSV from the herd. This information is useful because it tells us that the pig's immune system can eventually clear PRRSV from the body. Thus, the immune system "see's" the virus in the context necessary to remove free virus and kill cells infected with the virus; but, we do not completely understand why it takes so long and what immune factors are necessary for the removal of virus.

From the perspective of an immunologist trying to find solutions to combat PRRSV, there are various approaches. First, a primary approach would be the development of a vaccine that induces protective immunity prior to infection. This typically requires knowing which parts of the virus to include in the vaccine so immunity is directed against the portions of the virus that leads to blocking infection, or neutralizing. That said, for many vaccines currently used that work, this hasn't always been determined. But, that's because the vaccine works and therefore, there is little need to know what the immune response targets. But, this is not entirely the case for PRRSV – current vaccines do eventually provide some protection, but it's not ideal. **Thus, to find a solution for the development of an improved vaccine, the portions of the virus that a protective immune response is directed against need to be identified.**

Research has shown that antibody that develops in the late stages of PRRSV infection do prevent PRRSV infection when transferred into naïve pigs that are then challenged. Identifying the regions of the virus that these antibodies bind to neutralize PRRSV infectivity will benefit further development of a vaccine. In addition to identifying regions of the virus that antibodies must target to prevent infection, it will be important to consider the cellular immune component. This arm of the immune system is required to kill cells in the body that harbor PRRSV. If the antibody component of immunity doesn't completely block infection, and some cells are infected, immune cells will kill the infected cells. Immune cells, specifically T cells, use a variety of different mechanisms to kill PRRSV infected cells. The regions of the virus recognized by PRRSV-specific T cells and the mechanism used to kill PRRSV infected cells needs to be identified in order to know that the proper immune response has been initiated bya vaccine. This warrants the development of swine reagents necessary for identifying the T cells, but also development of assays to confirm the PRRSV-specific cells are fully functional. The development of such reagents will benefit PRRSV research, as well as other research focusing on solutions for swine diseases.

Another approach, not exclusive from that described above, is identifying mechanisms that PRRSV uses to dampen the innate immune response and/or interventions that enhance anti-PRRSV immunity. Innate immunity is

responsible for turning on the portion of the immune system that makes antibodies and induces development of PRRSV-specific T cells. This area of research is extremely complex and the area we know little about. That said, it has been shown that administration the immune cytokine IFN-α at the time of infection, can significantly alter disease outcome. The cellular component of anti-PRRSV immunity was enhanced and the pig better controlled the virus. It has been shown also that PRRSV can alter the production of IFN- α, so it's likely these observations are connected. While a significant portion of research has been done on PRRSV pathogenesis, we still know little on the mechanisms PRRSV utilizes to alters the host immune system. **A clearer understanding of the mechanisms used *in vivo*, not just in cell-culture systems, in which PRRSV alters innate immunity and/or adaptive immunity are necessary for moving forward.** This approach could include supplementing with various immune factors to determine if it enhances anti-PRRSV immunity, as well as confirming what happens in cell-culture actually occurs in the pig.

The basic understanding of how PRRSV interferes with the immune system will be beneficial primarily for the development of a vaccine. It's highly likely that a live-attenuated vaccine will be the best approach for PRRSV. Identifying regions of the virus that interfere with PRRSV-specific immunity, and using techniques to remove these portions of the virus, will lead to the development of a rationally attenuated vaccine. In addition, proteins that enhance the protective immune response could be added to the vaccine. Overall, we can use the pig's response to natural infection to teach us which parts of the virus a vaccine should be directed against; however, mechanistic assays will need to be used to confirm the function of isolated immune components. Reserach efforts focused on the mechanism in which PRRSV alters the host immune system (such as T cell development, antigen presentation, and induction of PRRSV-specific lymphocytes) will be necessary to explain the inability of the pig to develop rapid and sterilizing immunity following infection.

Keywords: PRRSV, immunology, antibody, cell-mediated immunity, innate immunity

The long path to PRRSV clearance and protective immunity – how can we get there sooner?

The majority of diseases that have a successful vaccine program are those in which natural immunity, that is immunity that develops after natural exposure, provides significant protection against re-exposure. In such cases we can learn a lot about the immune response to the agent, and identify protective epitopes/immunogens necessary for protection. Then, as opposed to suffering the primary infection for induction of natural immunity, the power of vaccine-induced immunity can be used to provide protection before infection. Diseases in which natural immunity does not provide significant protection, or even clear the initial infection, pose a significant hurdle for clinicians and researchers. PRRSV falls primarily into the latter category, though not entirely.

PRRSV infection is chronic, as numerous research studies have shown that virus can be isolated from lymphoid tissue months after the initial infection. However, the implementation of load-close-expose (LCE) protocols in the field has shown that PRRSV can be eliminated from an individual animal and herd (1). Therefore, the pig immune system is capable of mounting a response that eventually resolves the infection, eliminating the virus from the animal entirely. However, clearance and disease resolution takes a significant amount of time – most LCE protocols indicate more than 200 days. Ultimately, this tells us that the pig eventually "sees" PRRSV in the context necessary to eliminate it from the body. The portions of the virus that the immune system must target are eventually recognized and the immune cells necessary to mediate clearance are induced. Although it takes a long time for clearance, natural infection can be used to correlate immunity (T cell or antibody) to particular PRRSV epitopes. While we can dissect this from natural immunity, the question remains, why does it take so long to get an immune response that can clear PRRSV infection?

The following is a review of the literature related to PRRSV immunity and is structured based on the different arms of the immune system. The review includes work on the adaptive immune response to PRRSV, specifically the

humoral and cell-mediated immune responses, as well as the innate immune response. It deviates from the question above towards understanding what immune cells and factors are necessary for protective immunity and does so because it's difficult to break the concepts apart. The review begins with an examination of the literature on the humoral immune response to PRRSV, as it is well accepted that neutralizing antibody is a key component of sterilizing immunity. For vaccine-based immunity, the rapid induction of neutralizing antibody is the ultimate goal, as it would provide protection against infection. The induction of antibody secretion from B cells requires T cell help, and T cells are required for killing virally-infected cells and work in this area is covered second. This section highlights how little we know about cell-mediated immunity against PRRSV and also, how little we know about T cell phenotype and function for pigs in general. The final section is the innate section, and is presented last because it aims to tie together findings described for adaptive immunity against PRRSV. As mentioned above, we know that pigs do eventually "see" PRRSV and can clear the infection. Thus, identifying the epitopes and immune cells necessary for clearance and pairing those antigens in the context of "proper" innate activation will be necessary to find solutions for enhancing PRRSV immunity.

Pig Humoral Immune Response to PRRSV

PRRSV-specific antibody response during infection

Early after exposure a vigorous anti-PRRSV antibody response can be detected by ELISA starting at 7-9 days post-infection (PI). Such seroconversion can also be detected by indirect fluorescent antibody assay or by immunoperoxidase monolayer assay. However, there is no evidence that this early antibody response plays a role in the protection against PRRSV infection (2, 3). The antibodies that appear during the early PI period do not neutralize PRRSV *in vitro* (3) and when used in passive protection experiments these early PI antibodies (i.e., antibodies collected at 21 days PI) do not mediate passive protection against challenge with virulent antibody-matched PRRSV but rather seemingly enhance the virulence of the infection (4). Antibodies with PRRSV-neutralizing activity appear only at later PI times (specifically at periods equal or higher than 4 weeks PI) (3, 5, 6). Simultaneously with the appearance of an early, non-protective but vigorous homologous antibody response, a polyclonal activation of B cells appears to take place (7, 8) inducing auto-antibodies early after exposure to PRRSV, although it should be noted that this later observation was recorded in a gnotobiotic model, with uncertain relevance for conventionally reared pigs (9).

Kinetics of swine humoral response against PRRSV and its proteins

The kinetics antibody appearance directed to the major structural proteins N, M and GP5 of PRRSV has been studied in infected pigs (10, 11). Specific IgM antibody titers are detected at 7 days PI, with titers peaking between 14 and 21 days PI and decreasing to undetectable levels around 40 days PI. IgG peaks at day 21 to 28 days PI, subsisting then for the entire persistent phase of the infection. As discussed elsewhere in this paper, significant serum neutralizing antibody (SNAb) titers (> 1: 4 or 1:8) are not detected until at least 3 to 4 weeks PI, and not in all animals. The earliest antibodies are directed against the 15 KDa N protein, followed by the 19 KDa M protein then the 26 KDa GP5 envelope glycoprotein. Interestingly, NSP2 contains a cluster of non-neutralizing antibody epitopes suggesting an immunodominant role for this major NS protein (12, 13). The most common PRRSV serologic test (IDEXX Labs, Portland Maine, US) detects antibodies mainly against the N protein. These antibodies appear around the first week PI and persist for several

months, but do not correlate with protection. Although the N protein is a commonly used diagnostic antigen, it has been shown that several of the NS proteins induce antibodies that, based on its kinetics and persistence, can compete with advantage with the N antigen (14, 15)

Antibody enhancement of infectivity: …its importance?

PRRSV is primarily a macrophage-tropic virus, and, as it was previously known for other macrophage-tropic viruses, it was plausible to propose a role for antibody-dependent enhancement (ADE) of infectivity in the case of PRRSV. The occurrence of ADE *in vitro* and *in vivo* has been reported (16, 17). These reports contributed to the establishment of the notion that anti- PRRSV antibodies, particularly the early antibodies that do not neutralize virus and precede the neutralizing response both in time of appearance and robustness, could enhance disease caused by PRRSV and be deleterious for the host. While it appears possible to demonstrate the occurrence of ADE on cultures of swine macrophages infected with PRRSV *in vitro*, the real role of ADE for the host in infection, if any exists, remains unclear and most likely may hold little significance for the overall pathogenesis of PRRS. The actual occurrence of ADE in PRRSV-infected macrophages is, at best, controversial, with different laboratories reporting different success at detecting ADE (16, 18). Such contrast between experimental results of different labs has been ascribed to "virus strain or swine macrophage differences" (18), a contention which per se limits significantly the putative overall pathogenic importance of ADE. Regarding *in vivo* proof for the occurrence of ADE, only two experiments can be cited. In one case it was reported that passive transfer of sub-neutralizing titers of PRRSV followed by PRRSV challenge resulted in enhanced viremia 1-2 logs over the groups receiving only virus, without producing any other clinical exacerbation (16). Another report described that passive transfer of salt-concentrated, non-neutralizing IgGs (IgGs collected at 21 days PI) followed by PRRSv challenge increased rectal temperatures and caused increased interstitial pneumonia. However, the authors could not rule out the possibility that the increased pathology observed was caused by the presence of pro-inflammatory cytokines co-salted out with the IgG fractions collected from the 21 days PI donors sera rather than by the non-neutralizing IgGs. It is known that significant amounts of pro-inflammatory mediators are likely contained in the serum of the PRRSV-infected animals during the first weeks post infection (19, 20). In summary it would appear that the degree of pathogenicity that ADE contributes after PRRSV infection could not be distinguished from the overall pathology caused by the introduction of serum cytokines from PRRSV infected pigs, which would have co-precipitated with the IgG. The contribution of ADE to PRRS pathogenesis has been critically discussed at in a recent review (21).

Where on the PRRSV virion would the neutralizing epitopes be located?

The early observation that virus-specific antibodies in the sera of PRRSV–convalescent pigs displayed the ability to neutralize infectious PRRSV in the absence of complement attracted the interest of many researchers (3, 11). The overall SNAb response against PRRSV has been repeatedly studied and characterized by different groups, although little is still known about the location of the most prominent neutralizing epitopes on the PRRSV virion. The initial investigations directed at locating neutralizing epitopes on the PRRSV virion focused (and logically so) on those viral structures most likely responsible for evoking SNAbs, e.g. the PRRSV envelope glycoproteins, which should interact with host cell proteins and mediate the viral penetration in the cell. Early papers reported that monoclonal antibodies specific for PRRSV GP5

and for PRRSV GP4 (22) possessed PRRSV-neutralizing capacity, which led to focusing initial attention on those two glycoproteins as the main target of SNAbs, mainly GP5 (PRRSV ORF5 gene product). Numerous initial studies proposed that one major neutralizing epitope(s) would be located on GP5 of PRRSV (23-26). Besides the neutralizing epitope on GP5, at least another neutralizing epitope was recognized by a monoclonal antibody on the envelope's GP4 (22, 25) and another on the envelope protein M (25). Initial reverse vaccinology studies and additional reports proposed (27, 28) that the main neutralizing epitope on GP5 targeted by the NAb was located on the GP5 ectodomain. The minimum antigenic area of this epitope comprises amino acids 37 to 44 as the core of the main neutralizing epitope (NE) has been characterized (27). Thus, the notion of the main neutralizing epitope being located on the ecto-domain of PRRSV's GP5 (as it is the case for GL of EAV and VP-3P of LDV) was established, although never further confirmed or thoroughly investigated until recently. While this GP5 neutralizing epitope was assumed to be a linear epitope, early reports (24) as well as more recent publications (29) suggest that the neutralizing epitope in GP5 is conformational rather than lineal. The conformational character of the GP5 neutralizing epitope was first described by Pirzadeh and Dea, (24) who showed that immunizing against PRRSV GP5 by DNA immunization induced the formation of SNAbs, while immunization with recombinant protein produced in bacteria from the same ORF5 gene product failed to induce the production of SNAbs. More recently, two independent reports have concluded that the GP5 ectodomain does not contain any linear neutralizing epitope in either PRRSV type 1 (30) or in PRRSV type 2 (29) strains. In summary, the original observation that DNA immunization with the single GP5 gene alone can evoke antibodies that efficiently neutralize PRRSV infectivity in both macrophages and MARC 145 cells still holds true and has been confirmed by multiple subsequent independent reports that used GP5 as possible subunit candidate to make experimental vaccines (based on GP5 alone or in GP5/M expressed as a dimer) (24, 30-35). In all these cases GP5 alone or GP5/M expressed in multiple different vaccine platforms have induced PRRSV-neutralizing antibodies. While no consistent evidence for sequential epitopes in the ecto-domain of GP5 exists, we should assume that the neutralizing epitope(s) that exist(s) on the PRRSV GP5 is (are) conformational, and that the M protein, when forming the dimer with GP5, contributes to the neutralization capacity of this glycoprotein.

More recently, two important pieces of information have had a significant impact on our view of the role of PRRSV glycoproteins as targets for virus neutralization: 1) One major discovery has been the unequivocal recognition that GP5 *per se* does not determine the host cell tropism of PRRSV thus suggesting that GP5 does not interact with the main cellular receptor for PRRSV. Instead, the cluster of minor PRRSV glycoproteins GP2-GP3-GP4 interacts with the host cell for infection (36). 2) The notion that GP2, GP3 and GP4 interact with each other to form a multi-protein complex that is present in a scarce number on the virus envelope and that at least GP2 and GP4 would be the proteins that interact with the main endosomal and membrane receptor that allows PRRSV penetration (CD163) (37). Such information has stimulated significant interest in these minor glycoproteins and their role as antibody targets for neutralization of PRRSV. Peptide scanning technology led to the identification of peptides in GP2, GP4 and GP3 that define actual linear neutralizing epitopes in these three minor glycoproteins but, interestingly, none in GP5 (30). If the glycoproteins that interact with the main cellular receptor for PRRSV are conserved residues that contact or interact with the viral receptor, these epitopes would be candidates as antigen for induction of broadly neutralizing antibodies, which may be of significance in cross protection. Further work in this area is warranted to determine if this is actually the case.

PRRSV Antibodies and Immune Evasion

As persistence of PRRSV infection in individual animals is one of the biggest obstacles to control the disease in the field, all the possible mechanisms that could explain this persistence have been given great pathogenic significance. Since the initial reports that the N-glycan moieties in GP5 of type-II PRRSV are important for the virus to escape the effect of NAbs (38, 39), "glycan shielding" has been postulated to be a primary mechanism to explain evasion from neutralizing immune response, ensuring *in vivo* persistence of virus, such as had been previously reported for HIV, SIV, and HBV. Furthermore, experiments with GP3 would confirm the previous findings observed in GP5, suggesting that also the N-glycan in GP3 of type-II PRRSV would also be important in protecting the virus from antibody neutralization (40). Recent reports involving deglycosylation experiments of PRRSV type II strains have provided support to the conclusion that glycosylation of GP5 downstream of the putative neutralizing epitope renders PRRSV resistant to neutralization (41, 42)though the same effect on GP3 could not be confirmed Regardless of whether glycosylation of GP3 contributes to the overall glycan shielding of neutralizing epitopes, the overall body of hypo-glycosylation experiments with GP3 by Vu et al firmly confirmed the important role of GP3 in the induction of neutralizing antibodies, a concept that had been already suggested by previous investigators (43-46). More recent work by Vanhee et al.(30) has clearly demonstrated that GP3 contains important linear neutralizing epitopes.

It is also important to mention here that the use of hypo-glycosylated versions of PRRSV obtained by reverse genetics generated with the intent of enhancing of the ability of live vaccines to stimulate neutralizing antibodies (38)is severely limited by the short life span of the hypo-glycoysylated phenotype *in vivo*, as virus replication *in vivo* quickly leads to reversion to wildtype of the glycan shielded version of the GPs (Osorio and Pattnaik unpublished data) . However, the possibility of significant induction of SNabs by immunization with recombinant or inactivated hypo-glycosylated forms of GP5 or GP3 administered as adjuvanted, non-replicating immunogens deserves consideration for additional study, as has been recently published(1)

Role of neutralizing antibodies in protection

At the time various initial systematic studies into PRRSV pathogenesis were being carried out in the early 90's, data showing the simultaneous presence of PRRSV and PRRSV-specific antibodies in serum was interpreted as an indication that the antibodies did not play a role in protection against PRRSV infection (10, 47-51). At the same time, the idea that PRRSV antibodies produced in response to infection were at levels below the endpoint of neutralization (that is, at sub-neutralizing concentrations) could exacerbate PRRSV replication in macrophages *in vitro* and *in vivo* (16), finally led, as explained previously in this article, to the general conclusion that anti-PRRSV antibodies provided a non-protective and deleterious role in the disease (reviewed by Snijder and Meulenberg, 2001). Due to the slow and irregular appearance of PRRSV neutralizing antibodies following PRRSV infection, not much credit was given to the role of neutralizing antibodies in PRRSV protection and/or clearance. Overall, literature related to the role of NAb against PRRSV infection has been, if anything, confusing. For example, reports describing the persistence of infectious PRRSV in lymphoid tissues (e.g., tonsils) for weeks PI in animals with high titers of circulating homologous PRRSV-NAb was interpreted as consistent with the idea that PRRSV NAb are incapable of clearing the virus (52, 53). However, other data indicated that NAb prevented the appearance of viremia (54). Likewise, immunization with PRRSV proteins GP5 and M (23, 24, 55) confer

some degree of protection against infection, and this protection correlates to the appearance of NAb. The ability of the Ab to neutralize in at least one case (24) was evaluated using both MARC 145 cells and PAMs with the same level of efficiency. Collectively, these data suggest that if Nab are present prior to PRRSV infection, either through active or passive immunity, they provide protection against viremia. However, it's unlikely that NAb play a significant role in clearing PRRSV from the tissue and instead, cell-mediated immunity would be necessary for clearance.

In spite of the initial and somewhat continued uncertainties about the actual role of NAb in PRRSV protection, it is known that NAb are important for protection against other arteriviruses. For example, protection against equine arteritis virus (EAV), the prototype for the arterivirus group, is mediated mainly by NAb. The appearance of NAb in serum coincides with the elimination of virus from circulation, and likewise, passive transfer of antibodies to foals lessens or prevents infection with EAV (56)NAb are directed mainly against the glycoprotein GL (the EAV homologue for PRRSV's GP5). The EAV GL glycoprotein has an ectodomain of around 100 amino acids. The main neutralizing epitope (NE) in the GL glycoprotein is located within the second half of the N-terminal hydrophilic ectodomain between amino acids 99-106 (57). Immunization with a recombinant vaccine comprising amino acids 18-122 of EAV-GL expressed in *E. coli* induced NAb in serum. After challenge, a correlation between the level of EAV-specific NAb and the degree of protection was reported (58), confirming the importance of NAb in protection against EAV. Lactate dehydrogenase-elevating virus (LDV) is another well-studied arterivirus and the causative agent of an asymptomatic infection in mice. Transfer of NAb into mice protects them from infection with LDV.

Several unrelated but simultaneous observations pointed to an important role of NAb in protection against PRRSV infection. In initial experiments completed by Osorio et al, PRRSV inactivated autogenous vaccines that failed to induce NAb were not protective, and conversely, attenuated vaccines that induced measurable NAb were protective (59)The onset of NAb after experimental infection has been shown to be associated with clearance of the virus from circulation and from tissues (2)In addition, vaccination of pigs with a DNA vaccine encoding PRRSV GP5 induced moderate levels of Nab and the pigs were partially protected against challenge with the homologous virulent strain. Following challenge, DNA vaccinated pigs presented with a mild fever and virus could be recovered after second passage in MARC-145 cells only from lungs and mediastinal lymph nodes. Two weeks after challenge, the titer of NAb increased to 1:128 in previously vaccinated pigs (24). Thus, the NAb response after vaccination with the DNA vaccine resembles the immune response to an attenuated strain of PRRSV observed in our experiments. After vaccination with a modified live vaccine, there is a low or non-detectable NAb response that rapidly increased in titer upon challenge with heterologous, virulent PRRSV(59). Although both cellular immunity and neutralizing antibodies may be involved in clearance of the virus after infection, there is so far only experimental evidence supporting the role of the latter.

Serum transfer experiments conducted by Osorio et al have provided unequivocal evidence that PRRSV NAb alone can fully prevent transplacental infection by PRRSV and prevent PRRSV infection in the pregnant females (54). Moreover, the transfer of NAb provided sterilizing immunity since no PRRSV could be detected in lymphoid organs by viral isolation, RT-PCR, or swine bio-assay in either the dams or the offspring. Along the same line, we later observed that transfer of NAb to piglets protected against PRRSV infection. The minimal end-point NAb titer in recipient piglets that could protect 100% of the animals against PRRS viremia upon challenge was greater than 1:8 (4). Such passive transfer experiments in piglets

also indicated significant differences with the results obtained in gestating sows. Passive transfer of NAb in piglets under concentrations that would attain an endpoint of around 1:8 did not provide sterilizing immunity to all the piglets. We observed transmission of PRRSV to naïve penmates from piglets that had received NAb and were challenged, although viremia was not detected. Likewise, further experiments indicated that higher concentrations of antibodies transferred (i.e. to attain an end-point of 1:32) were capable of providing sterilizing immunity in piglets, as had been reported with passive transfer of NAb into gestating sows. These paradoxical results likely the consequence of differences in susceptibility and in overall capacity for PRRSV replication in adult sows versus piglets, with piglet target cells (macrophages) being much more permissive to PRRSV replication and consequently the ensuing viral loads in tissues is higher in young pigs than in sows. However, this has not been clearly demonstrated.

A major confounding factor impeding the evaluation of the contribution of NAb in the protection against PRRSV is the fact that many authors attempted interpreting the protective role or value of NAb using the model of kinetics for NAb appearance following infection. When analyzing the NAb response upon natural infection with wild-type PRRSV, it was demonstrated that not all animals induced NAb in a timely manner as to limit the establishment of a chronic infection (11). In addition, the kinetics of appearance of antibodies to the major candidates for NAb (GP5) did not coincide with the clearance of viremia (15), and it was observed that PRRSV persisted in tissues even after the appearance of NAb (60). Collectively, these led to the idea that NAb do not contribute to control of PRRSV. However, the significance of NAb for protection has to be evaluated in it's prophylactic potential, when by different types of intervention, a protective level of NAb that would prevent infection could be established. In that respect, the initial passive protection experiments and additional reports provide compelling evidence that NAb can provide significant protection against PRRSV infection. In that respect an immunization procedure gives rise to protective levels of NAb in blood, subsequently, the serum NAb titer can constitute a true correlate of protective immunity. Recent publications analyzing the response of a large number of animals to different regimens of vaccination against PRRSV (61) indicate that NAb are a possible correlate of vaccine induced protection for PRRSV. However, it should be kept in mind that: 1) NAb protective levels in blood (i.e. 1:8 to 1:32) are hard to obtain with just one application of current vaccines; 2) Most of NAb are specific for the (homologous) originating strain as cross-neutralizing (aka broadly-neutralizing) antibodies are rare; 3) The induction of NAb observed after heterologous challenge in a certain percentage of previously sensitized animals appears to indicate a good prognosis of broad heterologous protection, but the anamnestic NAb response only seems to occur when a certain period of time has elapsed between primary immunization and subsequent secondary challenge. The necessary length of time appears to be 2 to 3 months, if the challenge is one month or less, the anamnestic NAb response is not evident.

How to Broaden Protection through Cross-Neutralizing Ab

Passive transfer experiments demonstrated that NAb are a *bona fide* parameter of PRRSV protective immunity and an important mediator of protection against PRRSV. Many different PRRSV vaccine candidates are now being evaluated by protection against homologous/heterologous challenge plus their ability to induce NAb. Passive protection by NAb has provided unequivocal proof of their value for sterilizing prevention of infection, but this is only consistently true under homologous conditions (i.e. conditions of close identity between the antibody specificity and the challenge strain). As we already discussed, initial studies addressed at identifying the location of neutralizing epitopes in PRRSV positioned GP5 as

an important glycoprotein candidate in such role. From other arterivirus model, as well as from PRRSV studies themselves, GP5 is known to form hetero-dimers with the non-glycosylated M protein that is located on the envelope, which enhances its immunogenicity and capacity to induce NAb. This explains the worldwide use, alone or in combination, of GP5 and M as immunogens for different platforms and constructs tested as subunit vaccine candidates against PRRSV. These attempts have included, amongst others, viral vectors, DNA vaccines as well as genetic adjuvants and other immuno-stimulants. No matter what platform and approach is considered, it has become obvious by now, after these many multiple efforts, that the use of GP5 and M subunits, although immunogenic and somewhat protective, alone are not sufficient to provide complete protection. Those GP5/M subunits have at best reached half of the homologous protection attained by modified live vaccines, and no heterologous protection. Evidence for the need of adding additional PRRSV immunogens to the vaccine formulations is compelling. Particularly important is to find the antigen and/or epitopes that could induce NAb titer against heterologous strains by cross-neutralization.

A significant advancement in the general understanding of the possible role of other PRRSV glycoproteins in protective immunity derives from studies on the interactions amongst the four PRRSV envelope glycoproteins. Das et al (37)looked at either the interaction of PRRSV surface glycoproteins with each other or with the main cellular receptor CD163. They were able to show strong GP4-GP5 interaction and, to a lesser extent, interaction between GP5 with each of the other minor glycoproteins. The GP2 and GP4 proteins, on their part, were found to interact with all the other GPs, resulting in the formation of **a multi-protein complex** (see figure 1). Remarkably, these results showed that the GP2 and GP4 proteins specifically and exclusively interact with the CD163 molecule (37)Recent reports by Wei et al have confirmed the CD163 interaction with GP4 but were unable to confirm GP2 participation in the interaction(42) Overall, Das et al concluded that GP4 protein is critical for mediating inter-glycoprotein interactions and along with GP2, serves as the viral attachment proteins that are responsible for mediating interactions with CD163 for virus entry into susceptible host cells. Thus, it is plausible to conclude that antibodies targeting the receptor binding region of GP2 and GP4 would prevent entry of the virus into the host cell and serve to neutralize infection. Likewise, mutations in the regions of GP2 and/or GP4 that directly interact with the cellular receptor would render the virus incapable of infection and such mutations would not be selected for during an infection. Consistent with this view, the argument could be made that antibodies directed against conserved glycoprotein epitopes (those necessary for viral attachment and entry) would be able to neutralize multiple PRRSV strains, or in other words would be cross neutralizing.

One major piece of evidence supports the occurrence of certain PRRSV epitopes capable of generating pan-neutralizing antibodies in nature: Figure 2 illustrates the recall neutralizing response against a heterologous challenge strain typically displayed by animals that were previously vaccinated with MLV PRRSV vaccines(59)In the fraction of animals that respond to the vaccine, this robust and heterologous strain-specific response correlates closely with protection. We interpret this phenomenon to be clear evidence that **protective cross-neutralization can occur with PRRSV**. These data suggest that the MLV primes the immune system, but the secondary challenge is required for NAb to be detected systemically. The mechanism by which the MLV primes for cross-protection is unclear – it could be memory B cells, memory T cells, or a combination of both. However, the induction of the anamnestic response suggests that the secondary challenge virus replicates for the response, though it may not result in viremia. Further research in this area is warranted to understand what epitopes the NAb are directed against, as well as the immune memory compartment primed by the MLV.

Understanding this mechanism my lead to understanding how to prime against PRRSV.

It should be mentioned that this observation has been reported by others as well, working both with European(62) and US strains as well(63) Recent studies conducted by the Shanghai Veterinary Research Institute, which used chimeric Arteriviruses obtained by reverse genetics, seem to confirm Das at al's original contention suggesting that the PRRSV cluster of GP2-3-4 is responsible for interaction with CD163. In Figure 1, CD163 is shown as a structure with extracellular region having repeating units (9 SRCR domains of CD163) projecting from the plasma membrane of a host cell. Although multi-protein complexes like the one proposed by Das et al can be detected in experiments with transfected cells, the molar ratio of the proteins in such complexes is still unknown. Although GP5 is the major glycoprotein on the envelope and may be distributed uniformly throughout the viral envelope, one can hypothesize that only few of the complexes (possibly two to three per particle) as shown in Fig. 1 may be responsible for binding to CD163. Cryo-electron microscopic studies published by Dr Terry Dokland (U Alabama)(64) suggest that only two to three large protein complexes can be seen on the viral envelope and thus provide additional support for the hypothesis and for the model. This view and the possibility that GP2 and GP4 may indeed be responsible for stimulating antibodies that would block their interaction with the main cell receptor resurfaces interest on these two proteins, which for a while were rather neglected based on the common general interest being centered on GP5, which has been generally believed to be the major PRRSV immunogen for induction of NAb. Although the original finding that GP5 induces neutralizing antibodies still holds, further evidence recently collected at laboratories at the University of Nebraska would indicate that, rather than evoking broadly reactive (pan-neutralizing) antibodies, it would appear that GP5 evokes primarily strain-specific antibodies. Evidence for that comes from our experiments on GP5 de-glycosylation using reverse genetics employing infectious clone PRRSV FL12 strain. In those experiments it was demonstrated the glycan shielding ability of PRRSV and showed that GP5, in its deglycosylated form, remarkably enhances the production of PRRSV-neutralizing antibodies (Ansari et al 2006). As we previously reported, the anti-wildtype (wt) F12 neutralizing titers attained by immunizing pigs with a F12 strain with a deglycosylated GP5 were exponentially enhanced. However, it was reported that the titers in serum from animals with significantly high titers against the wt FL12 strain, was much lower when the same serum is tested against other heterologous wt strain unrelated to FL12 (40).

Collectively, these data suggest that NAb, if present at the time of challenge in a significant enough titer, can provide sterilizing immunity against challenge. Antibody derived through passive or active immunity can provide protection. The specific epitopes that the NAb recognize have not been completely defined, but recent work suggests an important role for antibodies directed against minor glycoproteins and the complexes they generate on the viral surface. What is unclear is why it takes so long for peripheral NAb to appear following challenge and what the role of NAb is in clearance following infection. Shielding of epitopes may be a mechanism by which PRRSV evades the host immune system; however, the pig eventually develops detectable serum NAb or is primed for an anamnestic response that can provide protection against heterologous infection.

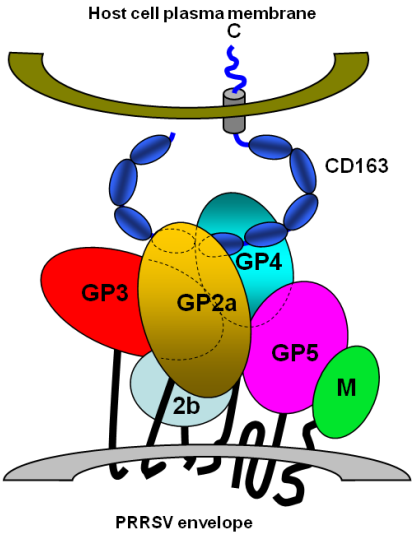
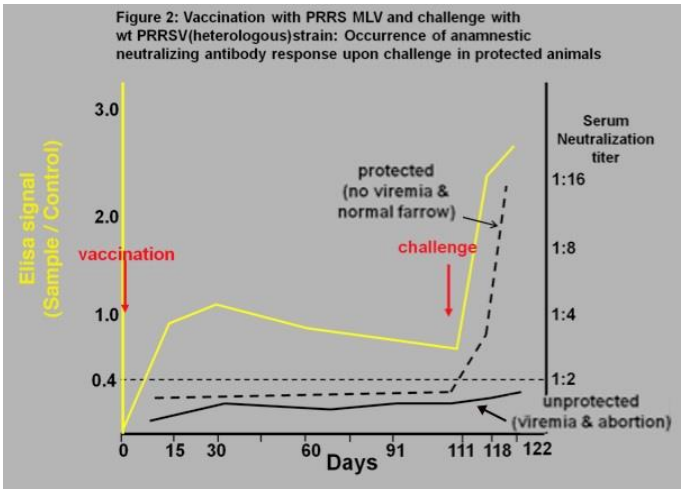


Figure 1: A preliminary model of PRRSV envelope protein complex and its interaction with CD163 on the host cell plasma membrane (37)



From Osorio et al 1998 (59)

Pig Cell-mediated Immune Response to PRRSV

PRRSV T cell Immunity

T cells play a critical role in many aspects of anti-PRRSV immunity due to their central role in development and regulation of antigen-specific immune responses, including education and activation of B cells, determination of the cytokine milieu in the environment of antigen presentation, cytotoxic effector functions to destroy infected cells, and regulation of immune responses to control inflammation and monitor for autoimmune reactions. The expansive toolkit of functional and biochemical assays that are used to characterize T cell responses in mice are largely unavailable in swine due to a paucity of reagents, inability to clone antigen-specific T cells, and inability to expand and immortalize antigen-specific T cells. However, much is known and is reviewed here.

General Features of T cell Response

T cell subpopulations in swine are characterized primarily by cluster of differentiation (CD) marker phenotyping for CD3, CD4, and CD8, and by secretion of interferon-□□□IFN-γ). In addition, swine have a significant population of mature CD4/CD8 double-positive T cells that may play a role in memory responses, but whose functions are relatively

unknown. Working within these limitations, it is known that a substantial, transient decrease in CD4+ T cells occurs in blood at about 3 to 7 days after infection, with a return to normal levels by 7 to 14 days after infection (65, 66). Increased CD8+ T cells were frequently observed, usually after 4-5 weeks of infection (65, 67-69). CD8+ T cells also were increased in infected lungs, whereas CD4+ cells were fewer or rare (70), (71). Increased CD8+ and double-positive T cells also were observed in lymphoid tissues (72). The T cell response to PRRSV also includes γδ T cells, but little is known about their significance (73)

In piglets infected *in utero*, reduced numbers of circulating CD4+ T cells were observed at birth and one week of age, but by 14 days, their numbers had returned to normal and CD8+ T cells were substantially increased (74). *In utero* infection also was associated with mRNA expression of IL-6, IL-10 and IFN-γ (75). Antigen-specific lymphocyte proliferation was first detected at four weeks post-infection (PI), peaked at 7 weeks PI, and declined after 11 weeks PI. The secondary response increased in magnitude. Experiments with blocking antibodies to porcine leukocyte antigens demonstrated that CD4+ T-cells were the major effector cells in the proliferation response. The *in vivo* T cell response to PRRSV was shown by detection of a specific, dose-dependent delayed-type hypersensitivity (DTH) reaction in infected pigs after intradermal administration of UV-inactivated virus (76).

The evolution of PRRSV-specific T cell responses over time following infection has been primarily assessed by the IFN-γ ELISPOT assay which, depending on how the assay is performed, gives a measure of the number of NK cells, helper T cells and/or cytotoxic T cells producing IFN-γ. Peripheral PRRSV-specific T cells were observed at 2 weeks after infection, with extensive variation over time and among animals (77). No significant differences were observed in PRRSV-specific T cells in lymphoid tissues examined during viremic or post-viremic infection, and there was no correlation between PRRSV-specific T cell frequencies and viral loads in lymphoid tissues (77). In nearly all cases, the frequency of specific IFN-γ secreting T cells was low regardless of age (78).

Interferon- secretion also has been used as an indicator of T_{H1} polarization of helper T cell responses in PRRSV infection to identify candidate treatments that potentiate antiviral immunity (79, 80). However, the significance of IFN-γ secretion as an indicator of helper T cell polarization is uncertain in swine since the phenotype of secreting cells usually is not known, multiple lymphocyte populations are capable of IFN-γ secretion, and the basic characteristics of the T_{H1}-T_{H2} paradigm have not been thoroughly established in swine (81).

Cytotoxic T Lymphocyte (CTL) activity

At 35 days after vaccination, a low virus-specific IFN-γ secreting CD8+ T cell response was observed that was consistent with a CTL response (82). Immune activation was influenced by isolate but not route of administration. However, the cytotoxic effector function of CD8-expressing T cells has not been strongly linked to control of primary PRRSV infection. Temporary depletion of CD8+ T cells at the time of infection did not lead to an increase in infection, suggesting that cytotoxic T cells did not have a functional role in control of acute infection (83). Similarly, CTL activity was not detected against PRRSV-infected macrophages until after viremia was cleared (84). Memory CTL proliferation was observed at 14 days after infection but CTL activity was not detected until 49 days after infection (84). While these data suggest that CTL may be involved in the clearance of PRRSV from the tissues (as opposed to acute clearance), the effect of PRRSV infection on CD8+ T cell frequencies in lymphoid tissues has not been established. An extensive survey of cell

frequencies in acute and prolonged infection showed no significant change (77) whereas, in another study, prolonged presence of PRRSV in lymphoid tissues was associated with elevated levels of highly positive CD8 T-cells (85).

Regulatory T cell Activity

PRRSV infection was reported to increase the frequency of putative regulatory T cells (Tregs) that produce TGF-β (86). Type 1 PRRSV strains were observed to induce IL-10 production in infected dendritic cells, but co-culture with lymphocytes did not induce Treg cells or transforming growth factor (TGF)-□ □production (87). By contrast, Type 2 PRRSV infection of dendritic cells can increase Tregs and induce TGF-□ □production□ (88). PRRSV induction of Tregs with suppressive activity was observed both *in vitro* and *in vivo* (89). The induced Tregs suppressed mitogenic proliferation of PBMC. Early induction of Tregs by PRRSV infection of dendritic cells would provide a mechanism facilitating establishment of viral infection. However, it would not account for establishment of type 1 PRRSV infections. The inconsistency in observations might be due to variation in subpopulations of T lymphocytes that express the transcription factor FoxP3, or to immunosuppressive cytokine expression differences (90).

An alternative to IL-10 secretion or Treg induction as an explanation for the difficulty in resolving PRRSV infection is lymphoid apoptosis. Using histology and viral antigen expression, Gomez-Laguna et al showed that mainly lymphocytes in B- and T-cell areas of lymphoid tissues were apoptotic in pigs infected with a type 2 PRRSV (91). This loss of cells may result in the low number of PRRSV-specific T cells detected during infection; however, a direct link has not been clearly established. In addition, the lymphadenopathy associated with PRRSV infection is counterintuitive to this hypothesis, but the finding does warrant additional investigation.

Memory Response

The role of memory T cells in anti-PRRSV immunity has not been studied extensively. It has been reported that a recall response mainly dependent on CD4+ cells and SLA-II was detected from 4 weeks after infection and remained for more than 3 months (92). The majority of studies evaluating T cell responses to PRRSV have investigated the response during the infection as opposed to following clearance. During infection, it's assumed that primarily T effector cells are detected as opposed to memory cells. The distinction between effector versus memory T cells is difficult in swine due to the lack of phenotypic markers and functional characterization of individual T cell populations. Clearly, the evaluation of vaccine immunogenicity and efficacy warrants the measurement of PRRSV-specific memory T cells; however, memory T cells have not been clearly characterized in pigs.

Viral Epitope Targets of T cell Immunity

PRRSV antigens that are targets of cell-mediated immunity have been investigated using various immune assays commonly used to evaluate antigen-specific responses. Incubation of PBMC from PRRSV-infected, but not non-infected pigs, in the presence of envelope glycoproteins (GP) 2 and 5, and matrix (M) protein induced proliferative responses (93). Further analysis of GP5 identified immunodominant epitopes, including in conserved amino acid regions at residues 117-131 and 149-163, using an IFN-γ secreting cell ELISPOT assay (94, 95). Conserved T cell epitopes were identified in N

and GP5 of Type 1 PRRSV, with the GP5 epitope corresponding to epitopes reported in Type 2 PRRSV (96). IFN-γ ELISPOT also was used to identify four conserved T cell peptides in the matrix (membrane) protein of a Chinese highly pathogenic PRRSV strain (97). In the nonstructural protein repertoire, conserved epitopes were identified in nsp2, nsp9 and nsp10 in Type 2 PRRSV that stimulated proliferation and IFN-γ secretion (95, 98). Peptides stimulating IL-10 secretion were also observed, some of which inhibited IFN-γ responses of PBMC stimulated with mitogen. It was noted that low IFN-γ responding pigs tended to be homozygous for SLA haplotypes, but the significance of this observation was not pursued (95).

An interesting model has been developed to evaluate antigen-specific T cell responses to PRRSV proteins in outbred pigs. Swine were immunized with plasmid constructs expressing porcine granulocyte-macrophage colony-stimulating factor (GM-CSF) and PRRSV M or N to generate a source of potentially antigen-specific T cells. Dendritic cells were derived from the same pigs were loaded with the recombinant proteins and used as MHC-matched autologous antigen-presenting cells (APC) to stimulate PBMC from the same pig. T cell proliferation and IFN-γ synthesis were induced in immunized pigs only in response to M and N, with M being more stimulatory. By contrast, they found that serum antibodies were produced only to N (99). This approach, using T cells and MHC-matched autologous APCs, to identify T cell epitopes is likely to be useful in future studies.

Major Gaps in PRRSV T Cell Immunity

The inability of pigs to achieve rapid sterilizing immunity to PRRSV may be due in large part to inadequate T cell responses, since T cells play a central role in direct effector responses to infection, overall immune activation and modulation of appropriate T cell and B cell responses, in regulation of response intensity and duration, and maintenance of immune memory. The essential role of T cells in resistance to viral infection and disease are known primarily from studies of defined cell populations that are specifically expanded and maintained in cell culture, and from genetic deletions of defined cell populations in animals. Examination of cellular and animal responses to infection in these experimental systems has resulted in detailed dissections of cellular properties and molecular mechanisms that explain key features of rapid sterilizing immunity in a variety of viral infections of animals. These features do not operate the same way in pigs infected with PRRSV; however, the ability to perform a detailed investigation to identify the molecular mechanisms gone awry is not possible.

Thus, it is essential to develop a T cell biology toolkit for swine to investigate antigen-specific expansion and growth of normal porcine helper and cytotoxic T cells, as well as immortalization of antigen-specific helper and cytotoxic T cells. At the same time, it is essential to develop MHC class I and class II antigen-presentation culture systems to functionally assess helper and cytotoxic T cell functions *in vitro* under conditions representative of PRRS disease in pregnant sows and growing pigs. Further enlargement of the immune reagents toolkit is essential for phenotypic characterization of T cell subpopulations that is the basis for describing T cell subsets. New and better methods must be found to characterize the responses of activated cells in terms of biologically active secreted molecules, and alterations in expression of surface molecules that can change their ability to respond to the local environment. Genetic tools are needed to introduce and delete targeted gene functions to test potential causal relationships, in place of association studies that are often times difficult to reproduce.

Key investments in basic porcine cellular immunology are essential for unraveling the mechanisms of protection against PRRSV (and likely other swine diseases). The benefit of these investments accrues not only to better control and prevent PRRSV, but also is directly applicable to other viral diseases of swine, which are numerous and ever-expanding.

The innate immune response to PRRSV.

During a viral infection host cell sensor molecules, called pattern recognition receptors (PRRs), located either in the cytosol or endosomes detect the presence of viral nucleic acids. Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (Nod)-like receptors (NLRs) are important pattern recognition receptors that recognize nucleic acids of microbial pathogens (100, 101). Many TLRs detect viral nucleic acids that are found in endosomes following the release of nucleic acids from infected cells. On the other hand RIG-I, MDA5, and LGP2 are important RLRs that detect cytoplasmic viral RNAs produced during the viral replication process. According to the classification of Baltimore, which is based on the mechanism of mRNA production (102), PRRSV, being a positive-sense RNA virus, can be classified as belonging to group IV viruses. Upon infecting a cell, Group IV viruses have the potential to trigger the known endosomal sensor TLR7 and the RLR cytosolic sensors MDA5 and RIG-I (103). Engagement of these sensor molecules initiates a cascade of events that culminate with the secretion pro-inflammatory molecules as well as type I interferon (IFNs), including IFN-α and IFN-β, resulting on the establishment a host antiviral state through the expression of numerous interferon-stimulated genes (104).

The most effective innate anti-viral immune response is the production of type I IFN by virus-infected cells. Although in response to a viral infection most cells types are able to produce type I IFN, this cytokine is most efficiently produced by leukocytes such as macrophages and dendritic cells (DC). A prominent subset of the latter is the plasmacytoid dendritic cell (pDC) producing 3-10 pg/cell of IFN-α, which is 5- to 10-fold higher than the production of IFN-α by macrophages on a per cell basis (105, 106). Notably, pDCs can recognize endocytosed viral molecules without being infected via TLR7, and thus due to their prodigious ability to produce IFN-α, pDCs can generate a systemic wave of type I IFN production (107). Most RNA viruses replicate in the cytoplasm, thus, it is generally accepted that RLRs recognize replicating viral RNA intermediates present in virus-infected cells (108). A report describing the contribution of different types of cells in antiviral immunity demonstrated that alveolar macrophages (AMs) in mice are the primary source of type I IFN in an experimental pulmonary infection with Newcastle disease virus (NDV), while pDCs only produce type I IFN when AM are depleted or after NDV is administered intravenously (109). In addition, since pDC are mainly found in secondary lymphoid tissues and in the blood, it has been proposed that pDCs have a vital role during systemic viral infections where the virus has tropisms for secondary lymphoid organs (110). This notion is supported with the observation that in pigs infected with classical swine fever virus (CSFV), which primarily replicates in secondary lymphoid tissues, pDCs appear to be majorly involved in the massive systemic IFN-α response to the infection with this virus (111). Macrophages, and primarily AMs, are the primary host cell for PRRSV replication. Natural PRRSV infection is typically initiated via the respiratory system and causes a severe interstitial pneumonia. However, within 2 to 4 days of respiratory inoculation with PRRSV, the infection becomes systemic resulting in viremia that persists for 21 days or longer. After this period, PRRSV can be detected in most secondary lymphoid tissues for months. Thus, based on the

distribution of PRRSV in the body and the known triggering signals for a type I IFN response, there are two types of cells that could potentially mediate a type I IFN in response to PRRSV infection - macrophages and pDCs.

The seminal study by Albina et al. (1998) indicated that the IFN-α response of swine to genotype I PRRSV was meager and could even be actively suppressed (67). Since then, a significant amount of research has focused on the possible mechanisms, at the molecular level, by which PRRSV might inhibit the IFN-α response. Since this topic has been recently reviewed extensively by others (112), this review we will mainly focus on what is known about the IFN-α response of swine to PRRSV either *in vivo* or by porcine cells *in vitro*. In this regard, it seems clear that unlike other swine viruses, such as porcine respiratory coronavirus (PRCV) and swine influenza virus (SIV) that are respiratory diseases capable of stimulating significant production IFN-α, PRRSV elicits a moderate to negligible IFN-α response in the respiratory tract. Production of IFN-α in the lungs of pigs acutely infected with PRRSV was either almost undetectable, or >10-fold lower than that induced by PRCV (19, 113, 114).

Evidence indicative of the engagement of virus sensing PRRs in lung cells of PRRSV infected pigs was reported as the increase in the transcription of several viral sensing TLRs (115-117). However, these observations were not confirmed in *ex vivo* experiments using precision cut lung slices exposed to PRRSV (118). While RIG-I and MDA5 transcription in AMs infected with PRRSV was found to be increased, the level of expression was not as strong as that induced by SIV (118). While the transcription of type I IFN genes in AMs and monocyte-derived dendritic cells has been described (119, 120), protein analysis was not always performed. Additional studies using quantitative real-time PCR and ELISA analysis, has shown that even though transcription of type I IFN genes occurs, actual secretion of the corresponding cytokine fails to occur (118, 121, 122). These observations have led to the suggestion that the production of type I IFN by AMs might be regulated at the post-transcriptional level (123). The poor IFN-α response detected in the lungs of PRRSV-infected pigs is consistent with the reported poor IFN-α response of porcine AMs to PRRSV infection *in vitro* (67, 118, 124). The limited type I IFN response could be due to the ability of virus to actively block the response or to the rapid destruction of infected AMs, which is known to occur in the lungs of PRRSV-infected pigs (125). However, the exact mechanism has not been clearly defined.

The IFN-α response of porcine pDC to PRRSV, as compared to other viruses that are able to stimulate a strong response by this cell type, has been characterized from moderate (126) to negligible (127). Although the modest response of porcine pDC to PRRSV reportedly occurs through the TLR7 pathway (126), PRRSV has been shown to have the capacity to strongly inhibit the IFN-α response of pDCs to other porcine viruses, such as transmissible gastroenteritis virus (TGEV), and moderately inhibit the same cytokine response to synthetic immune-stimulatory DNAs (126, 128). In contrast to TGEV, which can stimulate the activation and differentiation of porcine pDC through the up-regulation of IRF-7 expression and acquisition of dendritic cell-type morphology concomitant with an increase in CD80/86 expression, exposure of pDCS to PRRSV did not induce any of these activation pathways (128). The importance of the lack of pDC activation by PRRSV resides in the fact that pDCs appear to play a role in promoting cytotoxic T cell responses that require help or co-stimulation (110). Since pDCs are not permissive to PRRSV and live PRRSV is not required to suppress their function, the inhibitory effect of PRRSV on the pDC's ability to produce IFN-α is unlikely due to the

killing of pDCs by PRRSV. Rather, it appears that PRRSV alters pDC function through a negative signal delivered at the cell surface (128). A negative signal could be mediated via engagement of cell surface receptors on pDCs that are known to negatively regulate type I IFN secretion (110).

Notably, porcine pDCs in the circulation and secondary lymphoid tissues have been found to spontaneously secrete IFN-α (129). Specifically in swine, 8.8 ± 4.3% of tonsil pDC and 15 ± 7.7% of blood pDC can secrete IFN-α at steady state (129). Under experimental conditions the presence of IFN-α at the time of PRRSV infection or vaccination has been found to significantly alter both the innate and the adaptive immune response (130, 131). Given this prominent role for IFN-α in altering PRRSV-specific immune responses it seems likely that, under field conditions, the individually variable presence of spontaneously produced IFN-α in the blood and tissues of pigs may be responsible for the significant level of individual variation that has been observed in the kinetics and intensity of the cell mediated immune response following vaccination with a modified live virus (131). This is supported by the observation of a statistically significant ($p<0.001$) correlation ($R^2 = 0.6$) between the frequency of IFN-α and IFN-γ secreting cells in vaccinated swine (Royaee et al., 2004). Regardless, there is clear data indicating a significant difference in viral clearance and adaptive immune responses when IFN-α is present before or at the time of PRRSV infection (130, 132).

Influence of innate immunity on the adaptive immune response.

The production of IFN-α by pDCs has an autocrine effect that promotes functional and phenotypic activation events necessary for the optimal expression of co-stimulatory molecules and subsequent ability to induce naïve T cell differentiation into IFN-γ SCs (105, 133-136). At this point, the cells express co-stimulatory molecules that promote the differentiation of naïve T cells into IFN-γ secreting cells (SC) (Cella et al., 2000; Kadowaki et al., 2000; Fitzgerald-Bocarsly et al., 2002; Montoya et al., 2002; Honda et al., 2003) and cytotoxic T lymphocytes (110). Thus, it has become increasingly evident that the link between innate and adaptive immunity in viral infections occurs through the interaction of dendritic cells with type I interferon (135, 137) and the dendritic cell controlled polarization of T-cell function (138).

Accordingly, the apparent lack of an adequate IFN□α response of swine upon exposure to PRRSV likely contributes significantly to the inadequate development of a specific cell-mediated immune response (6). Usually, virus-infected cells secrete type I IFN and the released cytokine interacts with a subset of naïve T cells to promote their conversion into virus-specific IFN□γ SC (Cella et al., 2000; Cousens et al. 1999; Kadowaki et al., 2000; Biron, 2001; Levy et al., 2003). In contrast, as we have described above the IFN□α response of swine upon exposure to PRRSV is meager at best. The lack of efficient stimulation of IFN□α□production by host cells after pathogen exposure would be expected to have a significant impact on the nature of the host's adaptive immune response, since IFN□α□up-regulates IFN-γ gene expression, and thus controls the dominant pathway that promotes the development of adaptive immunity, namely, T cell-mediated IFN□γ responses and peak antiviral immune defenses (Cousens et al. 1997; Levy et al., 2003).

Approaches to improve the stimulation of protective immunity to PRRS virus.

To compensate for the apparent inadequate innate cytokine stimulation elicited by the infection of pigs with PRRSV, novel adjuvants have been used during immunization to attempt to overcome the deficit. The administration of IL-12 in combination with live or killed PRRSV vaccine resulted in increased lymphoproliferative responses to the

vaccine virus (139), as well as an enhanced IFN□γ response to a modified live PRRSV vaccine (140). Similarly, an injection of IFN□α□provided exogenously in the form of an expressible cDNA or a replication-defective adenovirus vector was found to exert an enhancing effect on the induction of antigen-specific IFN□γ response to PRRSV (80, 130). Remarkably, no significant alteration in the development of the humoral immune response was observed with either of these treatments. The interventions at the initiation of PRRSV immunization did not alter the usual rapid onset of anti-PRRSV antibody production and delayed appearance of serum virus neutralization antibodies (2, 6, 27, 130). However, a recent study utilizing a strain of PRRSV that induced a strong type I IFN response was able to show an increase in virus neutralizing antibodies both in time to development and levels (141). Thus, the mechanism and/or pathway involved in type I IFN induction may have an effect on subsequent immune responses.

It has been observed that the delivery of IFN□α□cDNA has a pronounced and sustained effect on the intensity of the cell-mediated immune response (80). Likewise, the introduction of the type I interferon agonist poly I:C, a synthetic double-stranded RNA, during vaccination temporarily amplified the number of PRRSV-specific IFN□γ SCs. However, polyI:C was not as efficient as an IFN□α encoding plasmid delivered with vaccine at enhancing the IFN□γ response to PRRSV. The observation that the inclusion of either IL-12 or IFN□α during immunization increased the intensity of the IFN□γ response to PRRSV validates the proposed role of these two innate cytokines in directing the *in vivo* differentiation of swine Th1 cells, and helps explain the poor virus-specific IFN□γ response that develops as a result of the exposure of pigs to PRRSV (6, 77). IFN-γ treatment of MARC cells has been shown to inhibit PRRSV replication; however, the connection between PRRSV-specific IFN-γ secreting cells and antiviral immunity *in vivo* has not been clearly established (142).

Moving Forward

A major control strategy for any infectious disease, including PRRSV, is the development of a vaccine that, upon administration, induces rapid and sterilizing immunity that will prevent infection upon exposure. We all know that hasn't worked so well for PRRSV, or you wouldn't be reading this document. So why not? What is so different about PRRSV that we haven't been able to control it? Obviously there are a lot of opinions, but no obvious answer. From an immunology-centric perspective, this review has focused on key areas related to PRRSV protection and immunity. The path forward may be guided by the primary goal deemed most important, though we will argue that multiple paths pursued simultaneously would likely provide the most benefit.

If the primary goal is to explain the inability of the pig to develop rapid and sterilizing immunity following infection (ie, lack of natural immunity soon after infection) the research approach should be a more basic understanding of PRRSV pathogenesis and identification of PRRSV proteins that alter the induction of the host response. This research area in particular is full of contention, disagreement and conflicting results. Specifically, there are many conflicting publications on the ability of PRRSV infection to alter, or not alter, immune responses to antigens or infectious agents delivered simultaneous to PRRSV infection. Regardless of these research reports, it is well accepted that PRRSV is a predisposing agent for the porcine respiratory disease complex (PRDC), as PRRSV infection leaves pigs extremely susceptible to many different infectious agents. This point, from the field particularly, cannot be denied and highlights the immune-altering capacity of PRRSV. Until more is known about how the virus itself alters basic host immunity, it is

difficult to justify the use of PRRSV itself as a modified-live vaccine and expect anything different than what we already get. The best live-attenuated virus vaccines are those that have been manipulated in a targeted manner to reduce or eliminate a well-characterized immune altering function of the virus. The mechanism of attenuation for currently available commercial modified-live vaccines is not known and induction of immunity following vaccination takes considerable time to develop, similar to natural infection. Thus, research efforts focused on the mechanism in which PRRSV alters the host immune system (such as T cell development, antigen presentation, and induction of PRRSV-specific lymphocytes) will be necessary to explain the inability of the pig to develop rapid and sterilizing immunity following infection.

If the primary goal is the development of a vaccine that provides rapid protection against PRRS, then identification of protective immunogens/epitopes and the mechanism of protection (antibody, T cell) should be the research focus. However, this goal should be approached very carefully. If we rely on natural immunity to identify correlates of protection (epitopes and immune parameters) results will need to be interpreted cautiously because they will be derived in the context of infection. The approach of natural immunity can be informative because PRRSV is eventually cleared from the pig – thus, timing of evaluation will be critical. The use of antigen delivery systems may be of benefit because we know so little mechanistically about how PRRSV collectively alters the host immune response to cause a chronic infection and predispose to secondary infections. Unless the delivery of the protective epitopes/immunogens is outside the context of PRRSV infection, it will be difficult to tease out protection in the cloud of pathogenesis. In other words, the immune-altering effects of the infection itself will remain and make interpretation of results difficult.

To achieve the goal of developing a PRRSV vaccine that induces rapid, sterilizing immunity the two primary goals as outlined above will need to be pursued simultaneously. It's highly likely that the most efficacious vaccine for PRRSV will be a live-attenuated product, though additional platforms should be considered. Thus, supporting applied research to identify protective epitopes to use as vaccine antigen along with supporting basic research to identify immune-altering mechanisms of PRRSV, will likely garner a successful outcome. Regardless of the path forward, PRRSV immunity research **MUST** move beyond observation to mechanism and function. This is highlighted well in the T cell section of the review, but should be reiterated here. The ability to evaluate function of immune cells will require an investment that may not appear to produce applied results immediately. However, the investment will be extremely beneficial for understanding PRRSV immunity, as well as immunity to other swine infectious diseases.

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