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Porcine reproductive and respiratory syndrome (PRRS): an immune dysregulatory pandemic

J. E. Butler · K. M. Lager · William Golde · Kay S. Faaberg · Marek Sinkora · Crystal Loving · Y. I. Zhang

J. E. Butler

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Abstract Porcine reproductive and respiratory disease syndrome (PRRS) is a viral pandemic that especially affects neonates within the "critical window" of immunological development. PRRS was recognized in 1987 and within a few years became pandemic causing an estimated yearly $600,000 economic loss in the USA with comparative losses in most other countries. The causative agent is a single-stranded, positive-sense enveloped arterivirus (PRRSV) that infects macrophages and plasmacytoid dendritic cells. Despite the discovery of PRRSV in 1991 and the publication of >2,000 articles, the control of PRRS is problematic. Despite the large volume of literature on this disease, the cellular and molecular mechanisms describing how PRRSV dysregulates the host immune system are poorly understood. We know that PRRSV suppresses innate immunity and causes abnormal B cell proliferation and repertoire development, often lymphopenia and thymic atrophy. The PRRSV genome is highly diverse, rapidly evolving but amenable to the generation of many mutants and chimeric viruses for experimental studies. PRRSV only replicates in swine which adds to the experimental difficulty since no inbred well-defined animal models are available. In this article, we summarize current knowledge and apply it toward developing a series of provocative and testable hypotheses to explain how PRRSV immunomodulates the porcine immune system with the goal of adding new perspectives on this disease.

Keywords Immune dysregulation · Pandemic · Economic loss · Arterivirus · Hypothesis

Introduction: what is PRRS?

History and discovery of the causative virus

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in the USA in 1987 as sporadic epidemics of abortions in sows and respiratory disease in pigs. The disease spread rapidly becoming a pandemic within a few years [1, 2]. The causative virus, PRRS virus (PRRSV), was independently discovered in Europe and the USA in 1991 [3, 4]. There are two recognized genotypes: type 1 or European-like (prototype Lelystad) and type 2 or

J. E. Butler (✉)
Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA
e-mail: john-butler@uiowa.edu

K. M. Lager · K. S. Faaberg · C. Loving
Virus and Prion Research Unit, National Animal Disease Center, Ames, IA, USA
e-mail: Kelly.lager@ars.usda.gov

K. S. Faaberg
e-mail: Kay.faaberg@ars.usda.gov

C. Loving
e-mail: Crystal.loving@ars.usda.gov

W. Golde
Foreign Animal Disease Unit, Plum Island Animal Disease Center, Greensport, NY 11944, USA
e-mail: William.golde@ars-usda.gov

M. Sinkora
Institute of Gnotobiology, Czech Academy of Sciences, Novy Hradek, Czech Republic
e-mail: Marek.sinkora@tiscali.cz

Y. I. Zhang
Department of Veterinary Medicine, University of Maryland, 8075 Greenmead Dr. College Park, Baltimore, MD 20742, USA
e-mail: zhangyi@umd.edu

North American-like (prototype VR-2332). The virus is a member of the family *Arteriviridae* in the order *Nidovirales*, which includes lactate dehydrogenase-elevating virus of mice (LDV), simian hemorrhagic fever virus (SHFV), equine arterivirus (EAV) and the recently described wobbly possum disease virus (WPDV) [5, 6]. As the name implies, except for WPDV that appears to be only neurologic, they are associated with some form of vasculitis. The virus can be transmitted across the placenta to infect the fetus [7, 8] despite the fact that the porcine placenta is impermeable to maternal antibodies [9]. PRRS is the number one disease problem in major swine producing areas around the world. It is estimated to cost the industry 660 million dollars a year just in the USA with proportional losses recognized in other countries. This is attributed to the remarkable ability of PRRSV to: (1) infect swine at all stages of production, (2) be shed in the semen of boars for extended periods of time, (3) be easily transmitted between farms, (4) tolerate a high mutation rate, and (5) negatively modulate the host's immune response.

PRRS has been a troubling disease because of its persistence and because >20 years of research has failed to produce an efficacious vaccine. This has been somewhat surprising since EAV infections are resolved in 7–14 days and a number of efficacious vaccines are available [10]. The rapid resolution of EAV is reminiscent of the pattern of sterilizing immunity seen with porcine influenza even in germfree (GF) piglets, so it is not simply a case of neonatal incompetence. Rather, PRRSV is more similar to LDV in which both the virus and the antibody response persist in mice [11]. As implied by its name, PRRS causes two separate pathologies: fetal abortion and respiratory disease in young and older pigs. There is some evidence that PRRSV replicates predominately in the thymus, which results in thymic atrophy [8, 12, 13]. This feature separates PRRSV from both EAV and LDV. While this is especially pronounced with highly pathogenic strains (HP-PRRSV) [14, 15], it is not necessarily the case for all isolates.

More than 2,000 papers have been published on PRRS, nearly all of which describe studies using conventional animals [1, 2, 16–18]. Most initial studies focused on adaptive immunity, although it is well recognized that viral infection also affects the innate immune system [19]. Few studies have focused on immune dysregulation by PRRSV, but recent work describes how PRRSV can suppress innate immunity ("The innate immune response to PRRSV" section). Murtaugh and Genzow propose that "Identification of the viral structures that elicit the protective immunity in pigs and factors that modulate the efficacy of protection in vivo is essential to rational development of immunological tools to prevent and control PRRS." This focus is very important but as General Guderian advised Hitler in 1942 "If what you are doing is not working, try something different" [20]. What is lacking in PRRSV research is a greater effort to determine the mechanisms, whereby the virus modulates the porcine immune response. In this review, we describe testable hypotheses to explain how this virus modulates the host immune system.

Both PRRSV and LDV are immune modulatory and although not retroviruses, may have more in common with HIV than EAV. LDV elevates IgG levels in mice with little production of virus-specific antibodies [11, 21], which is almost identical to what is seen in *isolator piglets* infected with PRRSV [22] ("The effect of age, rearing, complement and the role of mucosal immunity" section). Polyclonal B cell activation is often associated with autoimmunity and is common to a number of viral infections that are genetically unrelated to the arteriviridae [23]. Many viral infections such as bovine viral diarrhea virus [24] interfere with "normal" immune processes, which prolong the replication window for the viruses and thus increase the opportunity for contagious spread. Thus, virus classification may be a poor predictor of the effect of a virus on the immune system.

With rare exception, interference with the immune response is not the cause of death; good parasites rarely kill their host. Rather, secondary bacterial infections are more likely to cause death in PRRSV-infected conventional animals [8, 16, 25, 26]. Renukaradhydad et al. [27] showed that coinfection with PRCV (porcine respiratory coronavirus) reduced NK cell function more than PRRSV alone and dual infection caused more pathology [28]. Likewise, PRRS decreased the efficacy of SIV vaccination and increased clinical disease [29], and *Mycoplasma hyopneumoniae* infection significantly prolonged and increased the severity of PRRS [30].

Pathology

As implied in the name of the disease, the clinical manifestations of PRRS involve reproductive failure in sows and respiratory disease in young and growing pigs. Historically, field reports described "uncomplicated" PRRSV infections in young pigs as a mild-to-moderate pneumonia recognized clinically as an increased respiration rate at rest that would become labored with exertion. These observations were readily demonstrated experimentally. Reproductive failure, which became the hallmark sign of PRRS, included abortion "storms" and a sudden increase in dead fetuses and weak-born pigs that would affect most of the sows in the herd. In experimental sow infections during late gestation, fetal death and weak-born pigs are a predictable outcome, but PRRSV-induced abortions are uncommon.

The course of clinical disease following PRRSV infection has been well chronicled. In the hundreds of animal experiments that have been reported since 1991, it has become clear that there is considerable variation in clinical responses. Most of this is attributed to the use of different

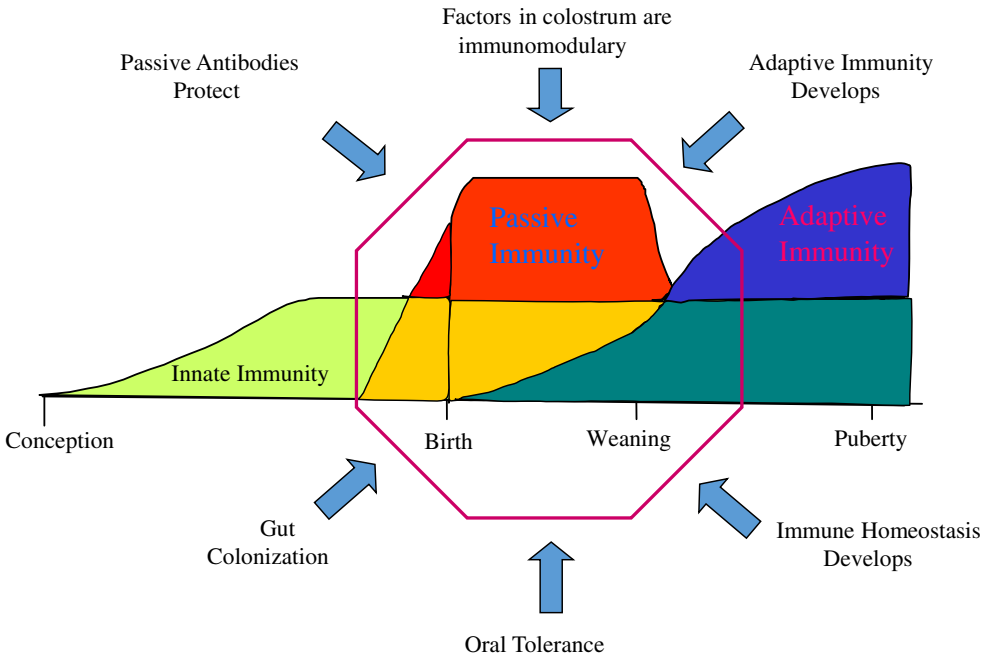

Fig. 1 The critical window of immunological development. Neonates are vulnerable during this period since their adaptive immune system is undeveloped, and they depend on innate and passive immunity. Within this period, healthy gut colonization takes place which drives the development of adaptive immunity and both oral tolerance and immune homeostasis develop. In some mammals, passive maternal antibodies are provided in utero as well as post-natally through suckling. The *colors* are a result of blending overlapping events. Modified from Butler and Sinkora [237]

PRRSV isolates, and collectively, it appears that the isolates from the early 1990s are less pathogenic than isolates from the late 1990s and certainly much less pathogenic when compared to Asian HP-PRRSV. Although differences in viruses may be a major factor in clinical variability, differences do occur when using the same virus under similar conditions suggesting that the host is also an important variable. Fortunately, there is considerable knowledge and expertise in PRRSV genetics to allow this to be further tested ("PRRS the virus" section). At this time, variation in clinical response is attributed to genetics, age, and coinfections [31].

Based on early field reports and experimental data, swine become more resistant to clinical disease with age, and boars and sows exhibit fewer clinical signs. This is not completely accurate since there is growing evidence that as PRRSV mutates overtime, it may gain in virulence. Why adults are more resistant to clinical disease and more likely to resolve the disease with VN antibodies [32] is unclear, but it may reflect the less well-developed immune system of neonates (Fig. 1). Likewise, how the virus develops a chronic infection in the boar and is shed in the semen for extended periods of time is not known. Current swine husbandry practices are almost completely dependent on the use of artificial insemination resulting in a population of boar studs that may supply semen to tens of thousands of sows. This practice dramatically magnifies the danger of using PRRSV-contaminated semen. Similarly, the concentration of sows in large buildings certainly contributes to possible horizontal transmission of virus and subsequent clinical and economic affects.

At a cellular level, PRRSV antigens and nucleic acids have been demonstrated in cells of the monocyte and dendritic cell lineage in a variety of organs. PRRSV in the lung is often associated with lesions; however, the presence of virus and lesions is less frequent in other organs. The observations support a tropism of the virus for the lung, which could lead to pneumonia. However, when compared to other swine pathogens, the presence of PRRSV in the lung and other organs seems minimal in relationship to clinical disease. One explanation for this may be that the pathogenic mechanism(s) of PRRSV is(are) not necessarily a simple cytolytic effect on a tissue with influenza A that infects airway epithelia. Instead, PRRSV may just affect a smaller group of cells that have important regulatory controls, which could lead to a variety of diseases most likely those of hematopoietic/lymphoid tissues.

Immune dysregulation is a common tactic for many viruses

The behavior of good parasites like viruses is to cause a delay in their eviction to allow for reproduction and transfer of their offspring to another host. Others may revert to a low virulence state and continue to survive in the host. Viruses such as those in the herpes family that are persistent for life have all evolved mechanisms that dysregulate the immune system. Few investigative groups have seriously focused on immune dysregulation during PRRSV infections.

A great many viruses foil antigen presentation by interfering with MHC expression. Rapid reduction of MHC

class I surface expression is a common feature of viral infections and is seen with foot-and-mouth disease virus [33]. In Epstein Barr virus (EBV) infection, degraded peptides from the EBNA-1 nuclear antigen are not degraded, and so, these peptides are not presented [34]. Something similar happens with presentation of peptides derived from a 72-kDa transcription factor in human cytomegalo virus (HCMV) [35]. While the complex mechanism in these two examples is incompletely understood, there is better data for several other herpes viruses that inhibit the TAP complex. TAP is required for the transport of cytosolic peptides (including those derived from a virus) across the ER. This step is required in their eventual presentation to CD8 T cells. TAP inhibition is found in herpes infection of swine, dogs, and cattle but not in rodents or lagomorphs [36]. An adenovirus protein (E19) retains degraded peptides in the ER and thus also prevents their presentation to T cells [37]. In HCMV, several gene products target MHC I for proteasome degradation [38]. In HIV, the *Nef* and *Vpu* proteins downregulate expression of surface MHC I [39]. In both human and bovine papilloma viruses, the gene product E6 is believed to interfere with the processing of cellular proteins and could thus affect presentation of peptides [40]. Viruses may also interfere with MHC II expression that is induced by IFN [41]. Viral infection also disrupts cell cycling and interferes with cytokine and chemokine production and also cytokine action. The list of examples is long but in general, IL-1, IL-12, both type I and II interferons are affected. As reviewed above, interference with innate cytokine synthesis may be especially important. These effects have been reported for a wide variety of viruses including pox viruses, herpes viruses, adenoviruses, and others. This further indicates that immune dysregulation is widespread among viral infection and that many families are involved indicating that it is a feature of the type of particular pathogens and but not their place in phylogeny.

Viral gene products also interfere with effector functions of the immune system. For example, they can interfere with apoptosis, and in swine, FMDV has been shown to inhibit the natural killer (NK) cell response to infection [42]. It is known that adenoviruses can cause lysosomal degradation of FAS that is part of the complex used by cytotoxic T cells and NK cells to induce apoptosis of virus-infected cells [43, 44]. More than 30 viral genes affect this part of the anti-viral defense [45].

Infecting viruses may also interfere with virus neutralization. The mechanism of viral neutralization has been a matter of conjecture for >40 years. Do neutralizing antibodies bind those viral epitopes that prevent their recognition by the receptors on potentially permissive cells or do they inhibit the fusion of the viral membrane with the endocytic membrane? If it is simple blocking, multiple antibodies appear to be needed since as many as 25 % of such viral epitopes must be antibody bound to prevent infection [45, 47]. Is simple blocking by antibodies enough or is help needed from an immune complex? In the case of EAV, adding fresh serum as a source of complement, greatly increased the effectiveness of VN. Covalent binding of C3 and C4 can facilitate clearance by cells that express complement receptors. In addition to merely facilitating clearance, complement-containing immune complexes can augment B cell activation [46], whereas IgG complexes without complement can downregulate B cell responses through crosslinking to FcγRIIβ [47]. Non-neutralizing antibodies may also act as a Trojan horse in facilitating virus uptake through FcγRs, a process dubbed as antibody-dependent enhancement that can increase infectivity 10–100 fold [48].

Recently, attention is being given to another immunosuppressive player in cancer and persistent viral infection. Myeloid-derived suppressor cells (MDSC) were first described from a mouse model of lung cancer in which these cells inhibited T cell proliferation [49]. These cells function through reactive oxygen species (ROS), iNOS and arginase-1 [50]. Acting through ROS, TCR can become nitrated preventing peptide binding [51]. ROS-dependent suppression of $CD4^+$ and $CD8^+$ T cells by MDSC in HCV infections [52]. Current understanding suggests that MDSC also inhibit NK cell function. MDSC suppression is also known for HIV, VSV, and vaccinia [50]. Since PRRSV can be persistent, a role for MDSC should not be ignored.

If viral neutralization is complement dependent, viruses that interfere with this mechanism can prolong their replication time in the host. There is evidence that vaccinia, cowpox, and variola secrete proteins that block C3 convertase action [53, 54]. While the mechanism involved is unclear, herpes viruses can also inhibit complement activation [55, 56].

It has been known for some time that many viruses that cause persistent infection including LDV and PRRSV are strong polyclonal B cell activators and often lead to the appearance of autoantibodies, a symptom that the pre-immune repertoire has been expanded [21–23, 57–62]. Tumorigenic viruses like EBV that target B cells give rise to elevated levels of monoclonal antibodies not directed to EBV [63]. In these cases, immunoglobulin (IgG) levels are a poor indicator of the anti-viral response.

The host immune response

The innate immune response to PRRSV

PRRSV interferes with interferon induction in vivo and in vitro

Host innate immune responses play a key role against early viral infection. Host pattern recognition receptors for RNA

viruses include RIG (retinoic-acid-inducible gene)-I-like receptors (RLRs) and Toll-like receptors (TLRs) [64, 65]. Activation of RLR and TLR signaling pathways leads to activation of interferon regulatory factor 3 (IRF-3), IRF7, and NF-κB, followed by induction of type I IFNs (i.e., IFN-α and β) and expression of inflammatory cytokines. Type I IFNs are critical to innate immunity against viral infections and play an important role in the stimulation of adaptive immune response [66, 67].

PRRSV is sensitive to type I IFNs, and the sensitivity is confirmed in vivo. Pigs that were inoculated with recombinant adenovirus for IFN-α expression and challenged with PRRSV 1 day later had reduced lung lesion and delayed viremia and antibody response [68]. The presence of IFN-α at the time of infection alters innate and adaptive immune responses to PRRSV [69]. PRRSV appears to inhibit synthesis of type I IFNs in pigs, while swine transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) induced high level of IFN-α [70, 71]. IFN-α could not be detected in the lungs of pigs in which PRRSV actively replicated. It was estimated that the IFN-inducing capacity of PRRSV is at least 159-fold lower than that of PRCV [71]. PRRSV infection of pulmonary alveolar macrophages (PAMs) does not lead to IFN-α production [70].

Plasmacytoid dendritic cells (pDCs) are thought to be the major source of IFN-α in vivo. PRRSV also fails to induce porcine pDCs to produce IFN-α, while pseudorabies virus (PrV), swine influenza virus (SIV), and TGEV stimulated the pDCs to synthesize IFN-α [72, 73]. However, NF-κB activation occurred in the presence of PRRSV. Loving et al. [74] showed that PRRSV replicated in monocyte-derived DCs but not lung DCs and that DC response to PRRSV was merely limited to IFN-β transcription but no IFN-alpha transcription. PRRSV replication in MARC-145 cells significantly inhibits the double-stranded RNA-induced type I IFN transcription [75].

PRRSV proteins inhibit IFN induction and IFN-activated signaling

The PRRSV proteins that are found to be antagonists of IFN induction include nsp1, nsp2, nsp11, and N (see review [76]). Nsp1 has been studied in more detail than the others. Nsp1 is self-cleaved into nsp1α and nsp1β subunits, both of which mainly localize in the cell nucleus and dramatically inhibit IFN-β expression [77]. Beura et al. [78] showed that nsp1β inhibited double-stranded RNA (dsRNA)-induced IRF3 phosphorylation and nuclear translocation. However, Kim et al. [79] showed that nsp1 inhibited IRF3 association with CREB-binding protein (CBP) in the nucleus but had no effect on IRF3 phosphorylation and nuclear translocation. The discrepancy is possibly because an nsp1β that is 14-residue longer than its authentic form was used in the Beura's study. Another possible reason is that different PRRSV strains were used.

Nsp2 inhibits IFN induction by blocking IRF3 activation, and the ovarian tumor (OTU) protease domain interferes with the NF-κB signaling [80]. Nsp2 also inhibits the antiviral function of ISG15 by the deubiquitinase activity of the OUT domain [81]. Nsp11, an endonuclease, is also an IFN antagonist [78]. The IFN antagonizing activity is not restricted to nonstructural proteins. Nucleocapsid (N) protein inhibits IFN-β induction by interfering with dsRNA-induced IRF3 activation [82]. The multiple components of nsps interfere with IFN induction. The nsps are early proteins, and N is a late one, which may play roles at different stages of viral replication.

PRRSV interferes not only with IFN induction, but also with IFN-activated signaling. IFNs bind to their receptors on cell surface and activate JAK/STAT signaling, resulting in the expression of IFN-stimulated genes (ISGs) [83]. PRRSV inhibits the IFN-activated JAK/STAT signal transduction and ISG expression in both MARC-145 and PAM cells [84–86]. PRRSV replication in MARC-145 cells suppresses JAK/STAT signaling stimulated by addition of IFN-α [84]. PRRSV infection of PAM cells also blocks JAK/STAT signaling, while a vaccine strain Ingelvac PRRS MLV has little effect, possibly due to its less efficient replication in the primary cells [84]. Nsp1β inhibits the JAK/STAT signaling via inducing the degradation of karyopherin-alpha1 (KPNA1, also called importin-alpha5), which is known to mediate the nuclear import of STAT1 [81]. PRRSV infection of MARC-145 cells also reduces KPNA1 expression. Besides nsp1β, other PRRSV proteins including nsp7, nsp12, GP3, and N were also found to be able to inhibit IFN signaling [85].

Strain and cell variability in IFN induction

PRRSV field isolates have variable suppressive effect on IFN-α induction in PAM cultures, and the suppression was found at post-transcriptional stage [87]. This is not unexpected as PRRSV strains are divergent in genomic sequences ("PRRS the virus" section). PRRSV infection of monocyte-derived dendritic cells (Mo-DC) induces the transcription of IFN-α/β but no detectable IFN-α in culture supernatant, suggesting a blockage at post-transcriptional stage [88]. PRRSV infection of MARC-145 cells inhibits IFN expression by interfering with the RLR signaling pathway [89]. A variety of type 1 and 2 PRRSV were found to stimulate IFN-α secretion by pDC via TLR-7 pathway, and the effect did not require live virus [90]. The suppressive effect on pDC was thought to be strain dependent. A novel isolate, A2MC2, induced IFNs in both MARC-145 and PAM cells, and virus replication was needed for IFN

induction [91]. Type 1 IFNs and ISGs were detected in A2MC2-infected cells. A2MC2 infection of pigs resulted in higher level neutralizing antibody than a MLV vaccine strain that is highly homologous in sequence [92].

Variable effect on IFN signaling among PRRSV strains was also found [85]. Among six PRRSV strains (VR-2385, Ingelvac PRRS MLV, VR-2332, NVSL97-7895, MN184, and Lelystad) tested, all but MN184 inhibited IFN signaling in MARC-145 cells, and all but MLV and NVSL blocked the IFN activation in PAMs. Nsp1β from the six strains were cloned, and all but MLV nsp1β inhibited IFN signaling when overexpressed [92].

Humoral responses of conventional animals

There is good agreement that PRRSV infections are not resolved rapidly in piglets, e.g., not in 7–14 days, in contrast to infections with swine influenza, FMDV, or EAV in horses [10, 93, 94]. Further, the carrier state may exist for up to 150 days [95], and viral RNA can be detected out to 251 dpi [95, 96]. Antibodies to PRRSV can be detected as early as 1 week after infection [97] (Fig. 2), yet viral neutralizing (VN) antibodies are not usually detected prior to 4 weeks [98, 99] (Fig. 3). Maximum titers may not be reached until 10–18 weeks dpi, and the peak titers are usually modest [98, 100]. IgG antibody levels appear to peak at 21–35 dpi in piglets but persist at lower levels thereafter [97]. Some reports indicate that viremia and viral replication can persist even in the presence of VN antibodies [1, 101], and viremia can be resolved before VN antibodies are detected [100, 102, 103]. In the case of PRRSV, LDV, and EAV, Gp5 is considered the most important neutralizing epitope in VN [10, 104–106]. Focus has been on the hydrophilic ectodomain of Gp5 [107]. However, Gp5 has numerous glycosylation sites that might influence the avidity and specificity of antibodies to Gp5. In general and because of the high frequency of mutation in RNA viruses, there is considerable variation in Gp5 among various strains of PRRSV ("PRRS the virus" section). Thus, the concept of the dependence of antibodies to Gp5 for VN is complicated. Using recombinant polypeptides, Li and Murtaugh [107] showed that the titer of antibody to the Gp5 ectodomain did not correlate with the VN antibody titer. Vane et al. [108] used peptide-specific antisera to show that the largest number of antigenic sites was associated with Gp3 and no neutralizing targets were associated with either Gp5 or M. Using chimeric viruses, Lu et al. [109] showed that Gp5 and M were not responsible for tissue tropism. Furthermore, other studies have shown that viremia is resolved before VN antibodies appear [100] (Fig. 3) and animals are protected from the European variant without them [110]. Evidence suggests that recognition may depend on strain variants/types. MAbs to Gp4 recognize the European variant but not the North American variant [111].

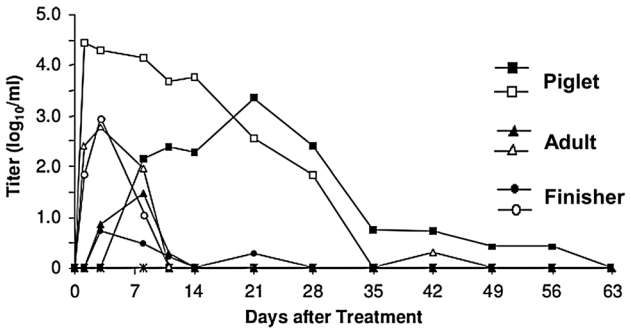

Fig. 2 Viremia in swine infected at different ages. Viremia is persistent when animals are infected as piglets. From Klinge et al. [121]

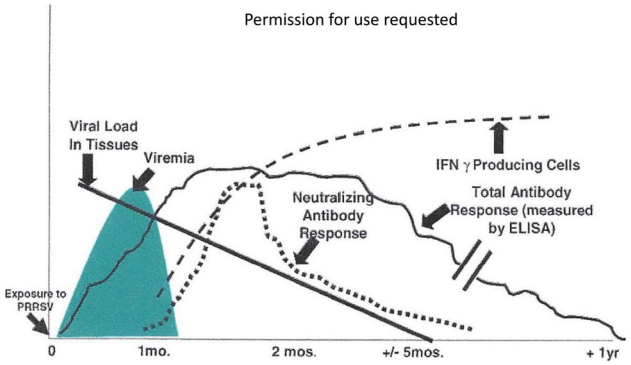

Fig. 3 In piglets, the appearance of neutralizing antibodies is delayed, but other antibodies appear shortly after infection. From Lopez et al. [101]

In spite of these often contradictory reports, the bulk of the evidence supports the view that VN neutralizing antibodies are important for protection [32, 101, 112, 113]. Unfortunately, the mechanism of VN for PRRS has not been researched. As regards VN antibodies to PRRSV, there are some concerns about work already published. One concern is the amount of data available and from what experimental animal group they was obtained. If VN depends on labor intensive culture studies, it is likely that data currently available are from a few time points and a few animals. Whatever viral epitopes or whole virus variants are used, a high throughput microtiter system should be adapted. It would be a shame if the current belief in poor VN activity is a consequence of selected and limited sampling. One can also question the methods used. In most studies, VN is tested using a lab strain virus and MARC 145 cells to which the virus has become adapted in vitro. This is a valid assay for the cell line and the PRRSV strain used but does it test whether neutralization has occurred in vivo in infected animals in which different target cells and virus variants are interacting?

The failure of swine to develop a sterilizing immune response has raised the issue of whether this virus produces

suppression or tolerance [114]. Some have reported the presence of $CD4^+$ cells with a suppressor phenotype ($CD4^+$ $CD25^+$ $Foxp3^+$) after infections with PRRSV [115, 116]. Silva-Campa et al. [117] showed that porcine cells with the Treg phenotype make IL10 and TGFβ, confirming their analogous function to those in mice. It is known that pulmonary dendritic cells can induce tolerance through IL-10 [118]. However, in a three virus study using isolator piglets, an increase in CD4 cells with a suppressor phenotype was not associated with PRRS [119]. Few studies have experimentally tested whether PRRSV is functionally immunosuppressive while many show inhibition of type I interferons by PRRSV ("The innate immune response to PRRSV" section). If Tregs in conventional animals are functional, they appear not to interfere with the antibody response to KLH in PRRSV-infected pigs [97].

The thymic atrophy caused by PRRSV can result in subnormal levels of double-positive thymocytes drives T cell development and loss of peripheral CD4 cells [70, 120]. Some coinfection studies suggest that PRRSV can interfere with protective responses to other viruses ("History and discovery of the causative virus" section), which is supported by extensive field reports of synergy between PRRSV infections and endemic infections within herd. Infections with Asian HP-PRRSV elevate a large number of cytokines associated with both innate and adaptive immunity, both pro-inflammatory and otherwise [16]. This "cytokine storm" suggests that PRRSV affects many pathways leading to innate and adaptive responses or their suppression.

An element in the kinetics of PRRSV infection is the age of the host. Klinge et al. [121] showed that PRRSV antibodies are detected at the same time in infected piglets and adults, yet viremia is immediate and resolved in sows, but develops late and remains persistent in piglets (Fig. 2). The delayed increase in viremia in piglets is correlated with a delay in the infection-induced increase in IL-10; the increase in this suppressive cytokine seems correlated with viral replication, but not the time of infection. The much-cited viral persistence seems to be a feature of piglets since, except for boars, the virus does not persist in swine infected later in life [121] (Fig. 2). Furthermore, the presence of VN antibodies in older pigs is correlated with elimination of the virus [32]. By contrast isolator piglets appear much more susceptible to B cell immune dysregulation ("Response to PRRSV infection in germfree piglets" section) and PRRSV is most immune dysregulatory during the critical window of immunological development before immune homeostasis has been established (Fig. 1).

Figure 2 shows that viremia persists in piglets but not in adults. Figure 3 shows that antibodies detected by ELISA appear early but the appearance of those with VN activity is delayed. This could reflect a difference in sensitivity between ELISA-based assays and VN assays. Resolution of viral infection is normally mediated by cytotoxic T cells (CTLs) although VN antibodies can block/eliminate virions and thereby infection of other cells. This is typical for influenza A and the basis for current vaccination schemes. Early protection to all infections depend on innate immunity which then raises the question of whether persistence of viremia in piglets (Fig. 2) reflects suppression of innate responses in piglets ("The innate immune response to PRRSV" section). While this may initially be critical, there is still too little information to conclude that the adaptive immune response is not impaired. There are reports that the amnestic antibody response to PRRSV is poor or absent [97], yet little is known about T helper and memory cells in response to PRRSV infection. T cell recognition of viral epitopes has been described [122, 123], but a tetramer assay system for these epitopes has not been developed for PRRSV. Despite the fact that so many viruses interfere with Class I presentation, little attention has been given to PRRS. Overall, there is insufficient information as to whether the B cell or the T cell systems are most affected by PRRSV and about the extent to which one or the other is impaired.

The genetic variability of PRRSV ("PRRS the virus" section) could also be a major player in the puzzle that has confounded investigators for >20 years. Hard evidence for escape mutants during infection is lacking but heterologous challenge studies indicate immunity to one strain does not confer immunity to all [124]. In conventional herds, persistence might be due to re-infection with extrinsic variants for which crossprotection is absent. A particularly useful observation comes from so-called herd closure [125, 126]. This essentially involves immunizing adult animals in a virus-free herd and then isolating them from exposure to outside animals. That these animals remain PRRSV-free suggests that: (1) vaccinated adult swine can develop sterilizing immunity if isolated from other animals and (2) escape mutants are unable to establish a re-infection in such herds. However, these experiments have not been performed with Asian HP-PRRSV or with very young piglets whose immune system is just developing (Fig. 1).

More than 20 vaccines have been developed for PRRS, although no single product has been totally successful [17]. These vaccines and their efficacy are the subject of another review (K.M. Lager, submitted).

The cytotoxic T lymphocyte response to PRRSV

The functional, cellular response in adaptive immunity is characterized by the activation and expansion of antigen-specific, MHC-restricted cytotoxic T lymphocytes (CTL). In general, this is the primary effector function and most efficient immunity against viruses in mammalian species as

because CTL kill virus-infected cells and arrest the generation of new viral particles. The role of this aspect of the immune response in PRRSV infection is poorly understood. Costers et al. [127] published that induction of virus-specific CTL in PRRSV-infected swine is very weak and slow to develop. They analyzed this by using PRRSV-infected autologous cells as targets of CTL killing. By comparison, these authors show a strong response of similar pigs infected with pseudo rabies virus (PRV) in CTL assays using PRV-infected target cells. In chronic viral infections, the regulatory element Ppp2r2d plays a significant role in CTL dysfunction [128]. Other in vivo studies have not tested for the predicted PRRSV epitopes that would induce CTL responses [129] and have used non-swine animal models. This complicates interpretation of the small literature available on this subject. Furthermore, analysis of CTL induction is complicated by the nature of this effector function. Experimentally, CTL killing is measured by analysis of these cells killing virus-infected cells in vitro in an antigen-specific, MHC-restricted manner. In most cases, the virus also kills the virus-infected cells. Provided it is allowed by the in vitro system, killing takes days to occur. Thus, new approaches are needed.

The role of γ/δ T cells in PRRS is unclear. Several reports describe that γ/δ cells are affected by PRRSV and other viral infections [44, 130, 131]. The latter shows that γ/δ T cells behave similarly to cytotoxic and NK cells. In isolator piglets, only the subset of $CD2^+$ $CD8^+$ γ/δ T cells was increased, which is the only subset is known to be cytotoxic [119]. The paucity of information at this point is insufficient to construct a meaningful hypotheses regarding the role of γ/δ T cells in PRRS. However, depleting them in vivo using mAbs could determine whether they play a role in either disease resolution or pathology.

PRRSV affects lymphocyte development in thymus

PRRSV infection can cause an acute lymphopenia, thymic atrophy, and lymphadenopathy associated with the presence of PRRSV antigen in the thymus. Thus, development of a protective, adaptive immune response to PRRSV may be impaired because PRRSV infection negatively impacts circulating and developing lymphocyte populations, and reconstitution of the peripheral lymphocyte pool can be impaired. Lymphopenia appears soon after infection [7, 120, 132, 133] and follows an influx of macrophage-like cells in the thymus and secondary lymphoid organs that contain PRRSV [134, 135]. There is also a loss of immature T cells in the thymus [15, 136, 137] accompanied by significant lymphadenopathy [13, 22, 134–136, 138, 139]. It seems important to connect these observations to understand how PRRSV affects the development of PRRSV-specific immunity.

The two mechanisms on which the animal relies to return balance to the circulating T cell pool are thymopoiesis and homeostatic proliferation of peripheral cells [140]. Homeostatic proliferation, or expansion of the existing peripheral T cell pool, is the primary means for reconstitution following peripheral depletion. In mice, both peripheral memory T cells and naïve T cells undergo homeostatic proliferation, though at different rates (fast vs. slow, respectively) and with differing signal requirements (MHC, IL-7, etc.). Naïve T cells undergo slow homeostatic proliferation in secondary lymphoid organs (such as lymph nodes) that is dependent on IL-7 and self-peptide:MHC presentation by an APC [141]. This type of proliferative recovery has been implicated in autoimmunity because of preferential expansion of T cells with greater specificity and stronger avidity for self, which has been observed following administration of lymphodepleting drugs [142]. PRRSV infection has been shown to result in production of autoantibodies [22, 59, 139], which may be related to the expansion of autoreactive T cells and/or the failure of the pre-immune repertoire to diversify ("Response to PRRSV infection in germfree piglets" section). Memory T cells can proliferate outside secondary lymphoid organs, and the signal does not require MHC contact. Collectively, the noted lymphadenopathy associated with PRRSV infection may be the result of homeostatic proliferation of peripheral T cells, and possibly B cells, to repopulate the peripheral pool. If lymphoid hyperplasia is the result of homeostatic proliferation, it requires determining why the cells do not egress from the lymph node.

In addition to proliferation of existing T cells, newly developed thymic emigrants can contribute to restoring the peripheral pool to a normal level following a lymphopenic-inducing event. However, reports indicate a loss of T cells in the thymus following PRRSV infection [8, 15]. Development of T cells in thymus is well described in textbooks, and at a certain stage, $CD4^+$ $CD8^+$cells (double-positive,DP) interact with cortical thymic epithelial cells (cTEC) to scan for positively selecting antigens. Positive selection occurs when the T cell receptor has an intermediate affinity/avidity interaction with self-peptide presented by MHC on the cTEC. Positively selected cells then commit to the CD4 or CD8 lineage (single-positive, SP) and rapidly relocate to the medulla where they sample antigen presented by medullary TECs (mTEC) and/or dendritic cells. These DP cells should not be confused with those DPc cells in the periphery of normal pigs [143]. Medullary TECs are unique in the expression of autoimmune regulator (*Aire*) gene, which controls the expression of tissue-restricted antigens. Tissue-restricted antigens (i.e., self-proteins) are picked up by neighboring thymic medullary dendritic cells for presentation to developing SP T cells, which drives T cell selection. If a high affinity/avidity signal through the T

cell receptor at this stage is received, cells die by negative selection to prevent release of autoreactive cells into the periphery, which is referred to as central tolerance [144]. Mature naïve T cells, presumably those that only recognize foreign antigen, are then released into the periphery.

Various groups have shown a population of macrophage-like cells in the thymus stains for PRRSV antigen by immunohistochemistry [12, 136, 138]. In addition, reports have highlighted the negative impact of PRRSV infection on thymic cellularity [15, 120], primarily as a loss of CD4/CD8 DP cells in the thymus of PRRSV-infected pigs [8]. The loss of developing T cells in the thymus likely affects the number and nature of newly developed T cells exiting the thymus during PRRSV infection. The presentation of PRRSV antigens in the thymus may also induce tolerance (loss of naïve cells that would recognize PRRSV antigen) and provide a mechanism for the reported increase in regulatory T cells after PRRSV infection [117]. These data together give support to the notion that infection of APCs in the thymus has a detrimental effect on the development of naïve T cells, and this likely has a negative impact on the development of a protective immune response to clear the virus from the pig.

Some of the lymphopenia that occurs shortly after birth may reflect the rapidly expanding blood volume but whatever the cause, it is not due to a selective depletion of T cells [119]. In young pigs, PRRSV induces a reduction in circulating lymphocytes early after infection, but not in age-matched controls (C. Loving, pers com). Since the decrease in circulating lymphocytes occurs before obvious phenotypic changes in the thymus, the lymphopenia is: (1) not due to thymus infection by PRRSV, (2) an effect by PRRRV on the peripheral T cell compartment, or (3) a red herring in the quest to understand how PRRSV dysregulates the piglets immune system. It is unclear if the drop in circulating lymphocytes is related to the lymphadenopathy observed later in the infection, but could be a compensatory attempt to repopulate the peripheral lymphocyte pool.

Response to PRRSV infection in germfree piglets

"Isolator piglets" are recovered by Caesarian surgery and reared in germfree isolators [145, 146]. These animals have not encountered gut flora, which drives development of adaptive immunity through stimulation of Toll-like receptors [147, 148] (Fig. 1). Furthermore, they obtain no passive maternal antibody in utero and receive no colostrum that could protect them from pathogens or interfere with immune responsiveness [9]. Finally, isolator piglets have no exposure to other pathogens or to other strains of PRRSV. The response of isolator piglets is intrinsic and not modulated by other pathogens, subclinical infections, maternal antibodies, or exposure to other environmental factors. These piglets provide the best in vivo opportunity to identify the direct in vivo effects of PRRSV on the neonatal immune system. Isolator piglets can also be considered as ex vivo fetal piglets and, therefore, a good model to study PRRSV-infected fetuses.

Since the adaptive immune system is not developed in fetuses, their intrinsic response is either innate or driven by fetal infections that promote development of adaptive immunity (Fig. 1). RNA viruses are often sensed by intracellular by Toll-like receptors which sense either positive or negative single-stranded RNA or double-stranded RNA (a recognized adjuvant) generated as part of viral replication. These molecules can drive development of adaptive immunity as shown with swine influenza [149]. Fetal piglets are immunocompetent as early as 79 days of gestation (DG) [150] and have lymph nodes, an active bone marrow, Ig gene class-switch recombination has occurred, and the ileal Peyer's patches are especially well developed. While some changes are likely to occur between DG 80 and birth (DG 114), these have not been identified. When fetuses are confronted with PRRSV, they respond in the same manner as isolator piglets [151] (see below).

Studies using PRRSV-infected isolator piglets [22, 119, 152, 153] have revealed a number of features about the immune response to PRRSV that may provide clues as to how this virus modulates the host immune system. Immediately obvious is hypergammaglobulinemia, lymphoid adenopathy, and the appearance of autoantibodies [22] (Fig. 4). Polyclonal B cell activation, hypergammaglobulinemia, and the appearance of autoantibodies are also seen in infections by unrelated viruses [23]. Polyclonal B cell activation is also a feature on LDV infection in mice, a related arterivirus that is also persistent [154]. Autoantibodies in PRRSV-infected isolator piglets to Golgi proteins [22] are also a feature of LDV infections [57, 61] and may be in part due to the site of morphogenesis of arteriviruses [60].

In addition to hypergammaglobulinemia and autoimmunity, PRRSV-infected isolator piglets exhibit abnormal antibody repertoire and B cell development. Measured as a repertoire diversification index, the values are in the range of 0.5, not significantly greater than for fetal piglets or sham control isolator piglets but 40–100 fold less than SIV-infected isolator piglets and conventionally reared piglets (PIC; Fig. 5a). Sequence analyses revealed that the CDR3 binding sites of the IG from PRRSV-infected piglets are even more hydrophobic than in newborns and sham controls while those for SIV and PIC are shifted to the hydrophilic region (Fig. 5b) [153]. Hydrophobic binding sites are incompatible with antibodies that recognize glycoproteins and are a feature of the pre-immune antibody repertoire [155]. In these animals, B cell differentiation is extremely rapid and cells representing the activated B cell

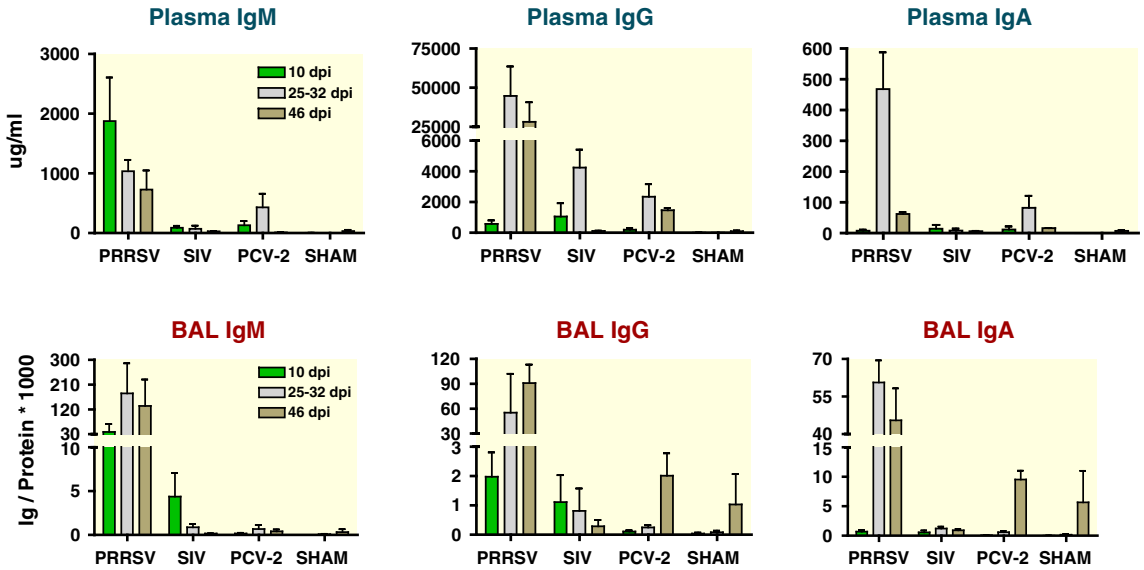

Fig. 4 (*top*) Plasma Ig levels in isolator piglets infected with PRRSV, PCV2, SIV, and sham controls. (*bottom*) Corresponding data from the BAL of these piglets. *Dpi* days post-infection, *SIV* swine influenza, *PCV-2* porcine circovirus type 2. Note split *y*-axis

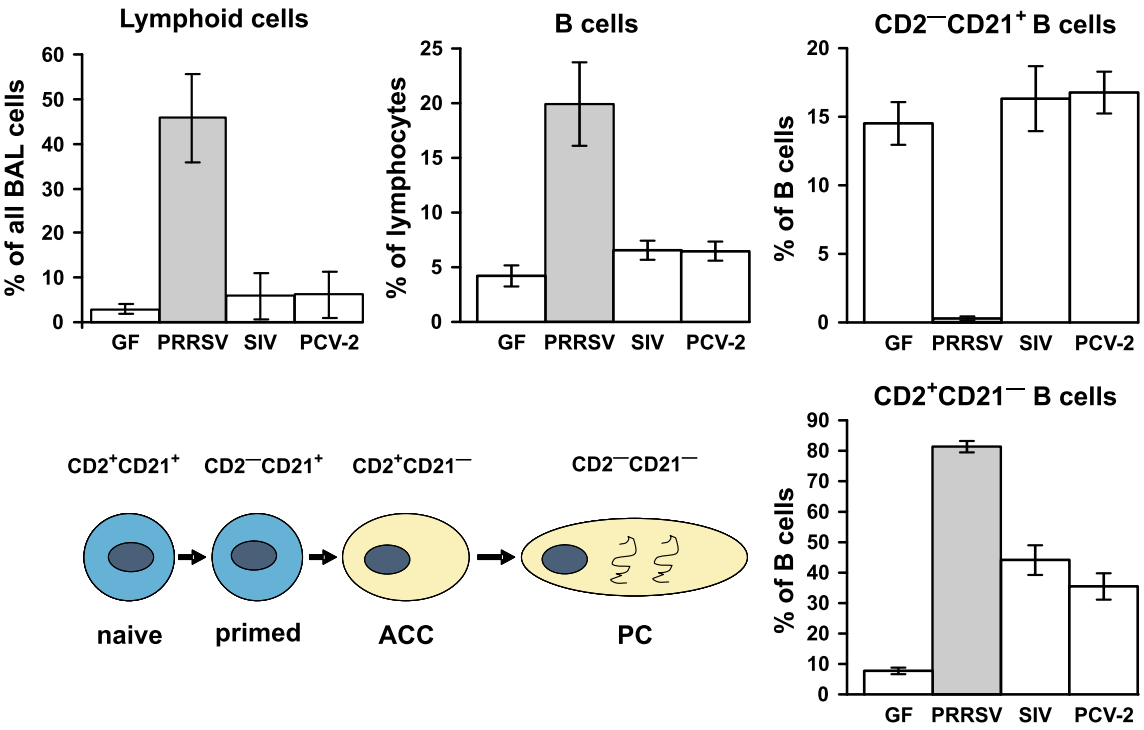

Fig. 5 B lymphocytes and B cell subpopulation in isolator piglets infected with the same three viruses as in Fig. 4. Noteworthy is the apparent loss of the primed B cell subset ($CD2^{-}CD21^{-}$) in PRRSV-infected piglets

stage are nearly undetectable indicating that B cells rapidly become plasma cells [119].

Comparative cellular studies of isolator piglets infected with PRRSV and SIV failed to reveal any evidence of immune suppression, i.e., lack of evidence for elevation of Fox3p $CD4^{+}$, $CD25^{+}$ T cells. However, cells with a suppressor phenotype were observed in parallel studies using PCV2-infected piglets [119] in which functional immune suppression has been reported [156].

Accepting the fact that the effect of a viral, bacterial, or fungal infection in germfree reflects a direct effect of the pathogen, our data suggest that dysregulation of B cell differentiation is one of the principal feature of neonatal infections with PRRSV during the critical window (Fig. 1).

The effect of age, rearing, complement, and the role of mucosal immunity

Much of PRRS research has have been done with young pigs. Emerging themes such as viral persistence, poor VN responses, and delays in viremia and secretion of IL 10 are based on studies with conventional piglets [121] (Figs. 2, 3). By contrast, adult animals make good VN antibodies and eliminate the infection [32]. Some additional support comes from studies using homologous variants [124]. Osorio et al. [112] demonstrated that passively administered Ig-containing VN antibodies obtained from convalescent sows could provide sterilizing immunity in piglets although a follow-up study showed that while viremia was ablated, viral replication persisted in some tissues [101]. In the same studies, passive administration of non-neutralizing anti-PRRSV serum had little effect although the mechanism of VN was not described. It would be wise to know whether active complement was also transferred. Since PRRSV is a respiratory infection, it would also seem important to know whether passive antibodies would have reached the respiratory tract. It is known that parenteral and oral vaccination of the sow generates passive antibodies that are protective against TGEV [157, 158]. These and other studies support the view that effective antibodies were made by adults [32, 112, 121, 125].

TGEV is a gastrointestinal infection, so ingestion of passive maternal antibodies, via milk and colostrum, has access to the site of infection. By analogy to WW II: "You need to stop them on the beaches." The respiratory tract, especially the upper portion, is the domain of the mucosal immune system. Thus, parenterally administered passive antibodies to PRRSV are unlikely to reach mucosal sites. This may explain why follow-up studies by Lopez et al. [101] showed that virus still replicated in some tissues.

The differences among result obtained using isolator versus conventional piglets might provide clues as to the nature of the apparent neonatal immune dysregulation. While lymph node adenopathy and some thymic atrophy are common to both groups, the extraordinary hypergammaglobulinemia of all isotypes and B cell expansion has only been consistently reported for GF isolator piglets (Fig. 4). This may in part be due to the fact that investigators who studied conventional piglets rarely measure Ig levels in serum or BAL. Such measurements in conventional piglets would be difficult to interpret since conventional piglets would have ingested maternal Ig through suckling. Conventional piglets used in these studies would be from PRRSV-free herds, so very little of the ingested and absorbed Ig would be PRRSV specific and therefore not protective. This may explain why the extent of the disease is similar. Both groups of animals make virus-specific antibodies but because of the extraordinary hypergammaglobulinemia seen in isolator piglets, and because absorbed Ig are from PRRS-free sows, only a tiny proportion would be virus specific [22]. However, knowing how many cells are virus-specific relative to other viral infections would be a much more useful parameter for comparing both groups.

The B cell clonal analysis done with isolator piglets showing selected expansion of the pre-immune repertoire has not been performed in studies of conventional piglets. The opposite is true for cytokine studies. However, cytokine studies in conventional piglets might be misleading because of undetected secondary infection or the effect of regulatory elements in colostrum or the impact of gut colonization [159]. While the impact of normal gut flora can impact cytokine levels in conventional animals, investigators typically compare their data to control littermates raised in the same environment, so this should play little role. However, the lack of gut colonization of isolator piglets might be in part responsible for the differences in the degree of hypergammaglobulinemia, since elements received via colostrum could establish immune homeostasis which might dampen polyclonal B cell activation and proliferation [159]. In limited studies, no differences were found between isolator piglets colonized with benign *Escherichia coli* and their colonization-free littermates [22]. However, studies in mice and rabbits indicate that all colonizers are "not created equal" [160], so results obtained using only *E. coli* could be misleading. Difference in the innate immune response in isolator versus conventional piglets has not been reported.

In summary, PRRSV infections that result in fetal abortion, B cell dysregulation in isolator piglets suggests that piglets are more susceptible during the critical window of immunological development (Fig. 1). Since SIV infections are rapidly resolved even in GF piglets, it suggests that age-related neonatal immune incompetence cannot alone explain the persistence of PRRSV. This would appear to shift blame to active immune dysregulation. While SIV is quickly evicted, one must remember it infects primarily epithelial cells, not cells of the hematopoietic/immune system. Thus, SIV infections would theoretically provide less opportunity for immune dysregulation of the developing neonatal immune system. In any case, investigators need to be careful about assuming that what happens in piglets, also happens in adults.

PRRS the virus

The PRRSV genome

As indicated previously, PRRSV is a member of the family *Arteriviridae*, in the order *Nidovirales*, which also includes

the viral families of *Coronaviridae*, inclusive of *Coronavirinae* and *Torovirinae*, and *Roniviridae* [161]. The *Nidovirales* order (Latin: nested set) contains viruses with similar genomic organization and replication strategy. The arterivirion contains a polyadenylated molecule of single-strand, positive-sense RNA (which is itself infectious) that varies in length for PRRSV (14,876–15,520 bp) and EAV (12,704–12,731 bp), but not as yet in complete published genomes for SHFV (15,717 bp) and WPDV (12,093 bp). The particles are roughly spherical with an average virion diameter of 54 nm and consist of a helical nucleocapsid surrounded by a lipid bilayer containing several proteins [6, 162]. All arteriviruses replicate in alveolar macrophages of their respective host, apart from WPDV, for which the host cell type is not known. Except for WPDV, which was only recently genetically characterized [5], each individual arterivirus species consists of many diverse genomes. PRRSV has been most studied in terms of host pathogenesis. There are two recognized PRRSV genotypes: type 1 or European-like (prototype Lelystad) and type 2 or North American-like (prototype VR-2332) [6]. The two main genotypes share approximately 60 % nucleotide identity, but each may vary more than 20 % in nucleotide sequence. The genome length of type 1 (14,876–15,098 bp) not only differs from type 2 (14,968–15,520 bp), but discrete sections of the genomes are different as well.

PRRSV RNA includes a 5′ untranslated region (UTR) of 220–221 (type 1) or 188–191 (type 2) followed a large replicase gene of variable length processed into at least 16 recognized nonstructural proteins (nsp1α, 1β, 2(2TF, 2N), 3–7α, 7β–12) by self-encoded proteases. The proteases include papain-like protease (PLP) 1α and PLP1β in nsp1, PLP2 in nsp2, and a serine protease (SP) in nsp4 [6, 163, 164]. Presently, most of the cleavages have been defined using EAV. PLP1α and 1β, and PLP2 cleave once co-translationally, directly downstream of the respective enzyme. SP completes the remaining cleavages. Nsp9 harbors the core RNA-dependent RNA polymerase (RdRp), nsp10 is a helicase, and nsp11 contains a Mn^{2+}-dependent RNase that cleaves at U stretches (NendoU) and is involved in RNA replication [165]. Downstream of the replicase gene is overlapping open reading frames (ORFs) enumerated as ORF2 encoding for glycoprotein (GP) 2, ORF2b encoding non-glycosylated envelope protein E, ORF3 encoding GP3, ORF4 encoding GP4, ORF5a encoding non-glycosylated protein 5a, ORF5 encoding GP5, ORF6 encoding the non-glycosylated membrane protein M, and ORF7 encoding the nucleocapsid protein N. Since these ORFs overlap, mutations to one coding sequence may affect adjacent ORFs. They are transcribed as a nested set of at least six subgenomic RNAs (sgRNAs) in infected cells. All of the downstream ORFs encode structural proteins [6, 162]. As mentioned above, type 1 PRRSV differs in the length of most structural ORFs when compared to type 2 viruses.

Genetic drift and shift

A remarkable feature of the PRRSV genome has been the rate of mutational diversification. It has been estimated that PRRSV RNA may have evolved at a higher rate (10^{-2}/site/year) than other RNA viruses (10^{-3}–10^{-5}/site/year) [165] although another investigator estimates the rate is similar to other RNA viruses [166]. The frequency of mutation includes not only simple mutation, but also is accounted for by a high rate of recombination [167–170]. It is estimated that there now exist as many as four major subtypes of type 1 PRRSV, based on ORF5 and ORF7 phylogeny [171, 172]. Even more subtypes, as many as nine, have been identified for type 2 PRRSV when based on ORF5. The husbandry of commercial swine, with large numbers of hogs from different source herds and artificial insemination with boar stud semen, is believed to have accelerated the evolution of PRRSV [173]. There is also ample evidence that two or more PRRSV strains may infect an individual pig [174, 175]. The combination of husbandry with genetic mutation and recombination between different viral strains has made the study of PRRSV evolution challenging.

The viral epitopes of PRRSV

The major envelope proteins of PRRSV consist of GP5 and M [100, 176]. GP5 forms a heterodimeric complex with M linked by a disulfide bond [177]. Both GP5 and M are thought to traverse the viral envelope three times and have only a small extravirion domain and a longer intravirion domain, much as was shown for LDV and EAV [178, 179]. GP5 is the most variable structural protein, and the predicted ectodomain after signal sequence cleavage is approximately 32 residues [180, 181]. Within these 32 amino acids, two hypervariable regions surround a quite conserved region, which contains the completely conserved cysteine disulfide-linked to M and two potential N-glycosylation sites [104, 180]. The conserved domain has been shown to harbor a neutralization domain, and the N-terminal sequence has been termed a decoy epitope that is not neutralizing [101, 103, 104, 182–186]. However, since the conserved domain is surrounded by complex oligosaccharides, it is shielded from neutralizing antibodies [184, 186]. The M protein, which is believed to act as glue to bring all virion components together, has also been implicated in neutralization [187–189]. In addition, two of the minor glycoproteins (GP3 and GP4) have also been shown to harbor neutralizing epitopes [108, 176, 190–194]. As shown for EAV, GP2:GP3:GP4 are thought to be disulfide-linked heterotrimers on the extravirion of PRRSV and are

thought to be in very low amounts compared to GP5 [195, 196]. Although the minor glycoproteins may play a role in neutralization of some or all PRRSV strains, there is little else known about the viral functions these proteins perform in PRRSV [197–199].

The phosphorylated N protein encapsidates the RNA genome, probably in a helical conformation [200, 201], and is most likely involved in capsulation and budding from the endoplasmic reticulum as was shown for EAV [202]. The swine host synthesizes the most antibodies to the abundant N protein, which are non-neutralizing [203]. Replicase proteins that have been shown to induce high levels of antibody are nsp1, nsp2, and nsp7 [204]. Nsp2 has also been shown to harbor many B cell epitopes from different PRRSV strains [80, 205–207] and has recently been shown to be incorporated into the virion [208].

Engineered and chimeric PRRSV mutants

Several infectious clones of PRRSV have been produced [16, 209–220]. Most of the clones were developed using type 2 viruses. These infectious clones represent only a fraction of the variability seen in the field, but are extremely useful in probing the genome for dispensable regions [211, 217, 220, 221], insertion of foreign genes to develop DIVA viruses [210, 211, 221], investigation of structure–function relationships [87, 105, 213, 222–225], examination of host virulence [218, 220, 226–228], and/or the probing of host response [222, 229, 230].

There are also several studies using chimeric viruses, either within or between certain arteriviruses. Some chimeric studies have led to the conclusion that the minor glycoproteins, not GP5, are important for tropism in cell culture [109, 231–233] and that the M protein is also not involved [234]. Other investigators have explored combining different regions of type 1 PRRSV with type 2 to examine viability [232, 235] or to explore the effect of N-glycosylation differences between strains [192]. In an attempt to develop broader crossneutralizing antibody, researchers have mixed regions of the PRRSV genome from different strains, creating a panel of chimeric viruses to explore changes in the virus as well as the swine host antibody response [193]. The same investigators used this technique to attenuate a strain of PRRSV [137]. Lastly, researchers have attempted to define regions of the PRRSV genome responsible for attenuation/virulence [219, 227] or to act as vaccines [236]. These studies have led to the knowledge that it appears that attenuation, as well as virulence, is multifactorial, involving two or more regions that can differ based upon the lineage of virus used for study.

The main lesson learned from these studies is that each strain of PRRSV, derived from field isolates or those with defined mutations, harbors individual characteristics that influence the specific pathogenesis seen. These characteristics include viral replication rate, the amount of specific subgenomic messages, the relative ability to process viral replicase proteins, the amount of N-glycans displayed on the virion, the amount of each individual viral protein, the relative interaction rate between viral proteins, and the relative ability of each strain to inhibit type I interferon and to induce humoral and cellular immunity. Added to these viral causes of pathogenic differences under defined clinical conditions are the host response to each individual viral strain, host genetics, climate effects, and herd immunity, among other factors.

The immune dysregulation hypothesis

The need for new experimental tools and approaches

Hypotheses testing and establishment of models

Advances in science have mostly succeeded because the experiments employed were focused on testing a specific hypothesis and because they were designed so that the number of variables was minimized. Naturally, this is much more difficult in biology because of the complexity of living systems and because many variables are unknown when the study begins. The image that emerges from the cumulative literature on PRRS is that many: (a) represent a category that is often derogatorily referred to as fishing expeditions, i.e., exploratory research, (b) are repetitious of other work already done or represents near re-publication of the same work in another journal, and (c) are non-comparative studies. The work appears to be driven by the pressure to produce a vaccine, not to understand how PRRSV modulates the immune system.

The combination of swine and PRRS offers a particular challenge to immunologists. PRRSV does not replicate in mice, there are no practical inbred strains of swine, immunological reagents are limited, and producing stable cells lines has proven to be difficult. Most studies have been done using conventionally reared piglets, which represents a complex model as illustrated in the following hypothetical example. Consider 100 pigs infected with PRRSV and 100 noninfected controls. Since pigs are outbred, difference in responses can be genetic. If they are conventional, each animal in each group has not had the same experience since it may have a different mother, and its passive immune experience could differ in terms of colostral regulatory factors obtained and their dosage. Suckling patterns differ within a litter giving rise to the often used “hind teat” syndrome. If you split the litter, you must then move some piglets to surrogate mothers, which introduces another set of variables. Gut colonization plays

an important role in development of adaptive immunity [147, 148], and colonizers do not have an equal effect [160]. Colonization typically occurs by contamination at the birth canal and thereafter by contact with the mother through suckling or contact with her feces. Assuming that each newborn piglet in each experimental group encounters the same environmental experience is extremely difficult to prove. All of these assumes they have the same living conditions and have no contact with other animals that not part of the study. The "closed herd" studies cited earlier is an example of how this latter aspect can be properly controlled. Conventional animals almost invariably contact other microorganism, some that are pathogens and some that are merely commensals. While experimenters may control for serious pathogens, they typically do not control for subclinical infection or for differences in the make-up and effect of benign colonizers. All of these may affect how a young pig responds to an experimental infection with PRRS or a PRRS vaccine. The literature shows that animals studied differ in age and there appears to be an age factor in their immune responsiveness and in the persistence of the virus ("The effect of age, rearing, complement and the role of mucosal immunity" section).

If the purpose of a study is to understand how a virus affects the immune system, conventional piglets are probably a poor choice. If on the other hand, the goal is only to test a vaccine under farm conditions, then the approach is fine. After all, the Sabin and Sauk vaccines and many successful bacterial vaccine before them prevented the spread of many horrible diseases but it would take decades to understand the etiology of the disease and just why these vaccines worked. The story of PRRS is more like the story of HIV; the old time vaccine recipes do not work, and so, it is now time to understand the etiology of the viral infection and how it interferes with its immune-based eviction.

While there is no mouse model for PRRS, there is a mouse model for LDV. The superficial similarities in outcome are such that one wonders why the LDV model has not been used more for PRRSV given the vast number of immunological reagents that are available for mouse immunology. Assuming that for other reasons, LDV is not a good model, then perhaps the next approach would be to compare how SIV, PRRSV, and FMDV affect the porcine response in a controlled in vivo setting such as the isolator piglet.

An "immunological deficiency" in experimentation

One glance at the literature reveals that compared to their counterparts in mainstream immunology/virology, those in the veterinary field are at a disadvantage. One obvious problem is the lack of reagents for work on the swine immune system. However, the literature also suggests an apparent reluctance to employ some of the 30-year-old technologies already available. Notably, simple assays like quantification of Igs are rarely used, as are immunohistochemical assays that measure Ig-containing cells and ELISPOTs that measure isotypic distributions, antigen-specific B cells, and cytokine secretions. While ELISPOT and PCR assays have been used in PRRS research, neither of these methods provide data on where the cells responsible are located within the geography of the organs studied. Refining these to single cells in situ assays as used in other species would provide more useful information. Single cell sorting and recovery of RNA by micromanipulation are also available.

Given the many studies done in conventional piglets that refer to the lack of VN early in development of PRRSV infection, why there are no assays to determine the mechanism of VN to test if complement is required or if antibody affinity is important is puzzling. Likewise for a disease that affects the respiratory tract, the lack of studies on the mucosal/local immune response to PRRSV is conspicuous.

The role of in vitro studies

While using more controlled in vivo studies can help to understand PRRS, they cannot address questions about what PRRSV does at the cell and molecular level. Without in vitro studies, it will be difficult to understand how PRRSV affects the host immune system. As mentioned above, the lack of stable cell lines presents a real problem. This can partially explain why there are no mixed culture studies to determine whether MHC I is downregulated by PRRSV and how infected macrophages or the virus itself affects T and B cells and their interactions. Even a question still exists as to the exact cell population that can be infected. For example, does PRRSV infect lymphocytes or only macrophages/dendritic cells? If this should occur, lymphocytes are present at all different stages of development, and if a particular viral receptor is needed, it may not be present at all times during lymphocyte differentiation. Since porcine cell lines immortalized at each stage of lymphocyte development are not available, the question is more difficult to answer.

It may also be dangerous to use only laboratory strain for infection studies and only established cell lines to which the strain has been adapted. For example, MARC 145 cells used to propagate PRRSV do not show down-regulation of type 1 IFN, while this is not true for pDC-infected in vivo.

To address whether the remarkable polyclonal B cell proliferation seen in GF isolator piglets is the direct effect of the virus, studies involving T–B cell interactions or contact between B cells and infected macrophages are needed. The

same applies to cytokines: what cells are making which cytokines and where are these cells histologically located since cytokines typically act at short distances? Especially useful for these studies would be engineered PRRSV mutants lacking the ability to make certain gene products. The wealth of information on the PRRSV genome, the many variants, and engineered mutants, provide a rich resource of research material ("PRRS the virus" section). In the last two decades, which covers the same period in which PRRS has been studied, tetramer assays to quantify T cell specificity and involvement have become well established and can now be used with some limitation for cattle and swine. Studies that concern innate immunity are already being conducted in vitro ("The innate immune response to PRRSV" section).

Comparative in vivo studies using isolator piglets

Perhaps the best way to determine how PRRSV modulates or dysregulates the immune system is to start with fetal and neonatal animals since the pandemic nature of PRRS appears developmentally linked. That the effectiveness of neonatal vaccines is age-dependent is no surprise to any immunologist and forms the basis for the timing of childhood vaccination schemes. While for PRRSV and other viruses that cross the placenta, studying the fetal immune response would be wise, but quite impractical. Fortunately, in swine and other Artiodactyls, newborns are essentially ex vivo fetuses since they can be reared in GF isolators in which maternal regulatory factors and the effects of gut colonization are absent [9, 237]. Given the experimental "cleanliness" of using isolator piglets ("Response to PRRSV infection in germfree piglets" section), why they are so seldom used is surprising. First, there is a matter of expense which is not trivial. Second is the rather subjective view that isolator piglets are artifacts because they do not reflect the farm experience and environment. So what is the purpose of PRRS research: to simulate the farm experience and produce a vaccine "in the blind" or to first understand how the virus affects the host? If the former is successful, the latter usually becomes mute. Unfortunately, the latter does not seem to be the case for PRRS since the virus was identified >20 years ago and the disease has not been controlled. One argument favoring isolator piglets is their use as a model for fetal piglets that are aborted after in utero infection. The most compelling argument for the use of isolator piglets to understand how the virus dysregulates the immune system is that it minimizes the number of variables, always a feature of good experimental design. Finally, if PRRS is primarily a persistence problem in neonates, the use of isolator piglets automatically confines studies to the critical window of immunological development (Fig. 1).

All studies in biology must grapple with what is "normal." Eviction of the virus shortly after infection might be considered "normal", while those that are not might be "abnormal." This reasoning is certainly open to discussion. From a practical position, this is a good starting point if the goal is to understand how certain infectious agents affect the immune system. Good experiments cannot be done in a vacuum. A glance of the literature shows that many experimental studies compare virus-infected piglets only with noninfected controls. This overlooks the possibility that the changes observed are common to all viral infections including suppression of NK function, interference with class I presentation, and polyclonal B cell activation. Rather, experiments need to be designed in a manner to identify "PRRS-specific" immune dysregulatory factors. A number of those done in studies on innate immunity have been done comparatively ("The innate immune response to PRRSV" section). Coinfection studies are really relevant. For example, Renukaradhyad et al. [27] showed that while PRCV reduced NK activity by 30 %, dual infection with PRRSV reduced this 80–100 %. In nearly all coinfection studies, there was an increase in disease [29, 238, 239] as might be expected resulting in increased morbidity and mortality. It would be surprising if coinfection did not result in more pathology and perhaps a delayed/depressed immune response. Thus, such studies would seem unreliable in the identification of virulence factors of PRRSV. There are also parallel studies using SIV, PCV2, FMDV, and TGEV to distinguish "normal" versus "abnormal." However, these viruses have different cell tropism. Are there any other porcine virus that infect macrophages and are eliminated in 7–14 days?

There is also the issue of virulence. In the case of PRRSV, one expects the degree of immune dysregulation to parallel the degree of virulence. HP-PRRSV is more virulent because it kills the host in a shorter time or produces more severe clinical symptoms. Does it also cause more severe immune dysregulation? If not, then assuming all events seen with vaccine strains of PRRSV are due to immune dysregulation could lead in the wrong direction.

Hypotheses of immune dysregulation by PRRSV

Individual and global hypotheses

The purpose of this review was to allow individual specialists to review their area of expertise and then to ask each to contribute a subhypothesis. We then assembled these separate views into global hypothesis. Our goal was to especially provide new investigators with a number of testable hypotheses that could explain how PRRSV dysregulates the neonatal porcine immune system.

Individual hypotheses

PRRSV suppresses innate immunity, which delays adaptive immune responses

PRRSV infection in pigs leads to delayed production and low titer of neutralizing antibodies [113] as well as weak cell-mediated immune response [240]. We hypothesize that the suppression of innate immunity can be an important contributing factor to the modulation of host immune responses because type I IFNs promote antigen presentation and natural killer cell functions, enhance antibody production of B cells, and play an important role in the differentiation of both $CD4^+$ and $CD8^+$ T cells. The PRRSV interference with the innate immunity is at multiple levels, from IFN induction, IFN-activated signaling to activity of ISGs. Therefore, viral-mediated suppression of innate immunity not only inhibits early host defense against the infection, but also interrupts the development of adaptive immunity, especially in the young pigs. This may explain why young pigs develop more severe disease and poorer protective immune response during the critical window of development (Fig. 1). Therefore, we would suggest comparative studies using SIV and TGEV to determine at the cytokine/cellular level, if PRRSV-infected PAMs or pDCs alter the signal to T and B cells or even developing thymocytes. Using the IFN-inducing PRRSV strain A2MC2 could add to the value of the model. We further hypothesize that given the divergence of PRRSV strains in sequences and clinical features that experiments utilize various strains and engineered mutants. Since type I IFNs are proinflammatory, the proper amount at the right site and time may be protective, whereas extreme elevation could result in damaging inflammation. A typical example is that HP-PRRSV induces high-level IFN-α, but causes high mortality in pigs [16].

Polyclonal B cell differentiation by-passes germinal center formation resulting in poor affinity maturation and generation of memory cells

Polyclonal B cell activation resulting in hyperplastic lymph nodes packed with Ig-containing cells (IgCC) is a hallmark of PRRSV-infected isolator piglets. This is paralleled by hypergammaglobulinemia in which de novo-synthesized Ig levels can increase as much as 1,000-fold in 3 weeks post-infection although <1 % of these are virus specific [22, 152] (Fig. 4). We assume that the same type of immune dysregulation occurs in conventional piglets, although it may be masked by the high concentration of absorbed passive Ig that increase serum Ig levels to >20 mg/ml. The extraordinary hypergammaglobulinemia simultaneously occurs as B cells rapidly differentiate to plasma cells in a manner in which the intermediate stage of activated B cells ($CD2^+$ $CD21^-$) is virtually absent [119]. Future studies in both conventional and isolator piglets need to confirm or reject the observation that a very small proportion of specific antibodies characterizes the response to PRRSV. If confirmed, it would lend support to the view that rapid B cells differentiation allows little time for diversification of the antibody repertoire. This can be tested after PCR recovery and cloning of the rearranged VDJ from various tissues. Using labeled probes specific for the nonmutated CDR1 and CDR2 regions of the seven porcine VH genes, a repertoire diversification index (RDI) can be calculated as described previously and shown in Fig. 5 [241, 242]. Since the RDI is largely a measure of the degree of somatic hypermutation, it indirectly tests whether GC formation and function have been normal. It would be nice to confirm this in conventional piglets and adult swine, but the data would be uninterpretable since conventional piglets and adult swine have been antigenized through contact with other microorganisms, and changes could not be ascribed to PRRSV.

Suspicion about abnormal GC activity might also explain the findings of Mulupuri et al. [97]. They used in vitro re-stimulation assays to suggest that there is a poor memory B cell response to PRRSV. Work by Raymond and Rowland [12] identified GC in newborn PRRSV-infected piglets using a mAb to CDw75 that has not been validated in swine. The GC and memory cell questions need to be pursued using better reagents and better experimental designs.

The delay in development of VN antibodies in PRRSV-infected piglets while the anti-viral response continue to rise (Fig. 2) might be because early antibodies are: (1) complement dependent for VN, (2) of low affinity, (3) specific for non-neutralizing epitopes, or (4) of the wrong antibody isotype. Alternatively, the differences between IDDEX ELISA titers and VN merely reflect differences in assay sensitivity. In a single study, the addition of fresh serum did not improve VN to LDV, but it did improve the efficiency of VN to EAV in horses suggesting that VN is complement dependent in horses but not in mice [10]. This is a simple assay and should be done with sera from PRRSV-infected swine.

A most likely possibility is that antibody affinity is too low in neonates to perform as effective VN antibodies. In the case of Denge virus, at least 25 % of the neutralizing epitopes must be bound by antibodies for VN to occur [47]. Immunochemists over the last 50 years have developed a plethora of methods to determine antibody affinity. Most of these were developed to study antibody interactions with defined haptens. These studies established a number of very important principles including the observation that avidity, i.e., the staying power of an antibody, was determined by the ratio of the on-rate to the off-rate. Thus, some

"quick and dirty" methods have surfaced based on the principle that antibodies that remain bound in the presence of denaturants like urea or guanidine HCl are used [243], which are of high affinity. Using this procedure, the relative affinity of a non-VN serum could be compared to that from adult swine that has VN capacity.

Should the experiments designed to test the role of complement or antibody affinity give negative results, another approach would be to test the specificity of early antibodies for certain viral epitopes. As reviewed in "Humoral responses of conventional animals" section, VN antibodies to the Lelystad virus preferentially recognize Gp3. Assuming Gp3 is the critical epitope, and affinity has been ruled out; it might suggest that antibodies to Gp3 appear late during infection or that Gp3 is poorly expressed on the virions used in the assay.

Once bound, the fate of the virus-antibody complex can also depend on the isotype of the antibody, which brings us to the fourth possibility. Multivalency such as with pentameric IgM can compensate for intrinsic binding site affinity and, therefore, perform much better than non-polymeric IgG so that early IgM should provide good VN activity. The subclass of the IgG antibody can also play a functional role in the effectiveness of complement-mediated VN. In swine, IgG3 is the most totipotent IgG based on its motifs for complement and FcγR binding [244]. However, actual functional comparisons have not been carried out. IgG3 is expressed very early in fetal and newborn piglets but after antigen exposure, other IgG subclasses, especially IgG1 replace IgG3 [245, 246]. During the period in which VN has been typically measured (Fig. 2), there is at least tenfold more IgG than IgM present, and thus, IgG is most likely the antibody in serum that is being measured in current VN tests. To determine which subclass of IgG is involved would be extremely difficult. First, all commercially available mAbs to swine IgG are more or less pan specific [247]. Even if such reagents were available, those which bind the virus would almost certainly be a mixture, so most probably antibodies of all subclasses involved, albeit probably dominated by IgG1. Perhaps the only way to truly test the effector function of the different IgG subclass antibodies seems at this point unjustifiable. This would require construction of chimeric antibodies for each subclass each with a binding site that recognizes a neutralizing epitope of PRRSV akin to the method we have described for expression and recovery of individual porcine IgG subclass proteins [247].

Confirmation of this subhypotheses might explain the initial ineffectiveness of the humoral response to PRRSV during the critical window, but it does not explain why the extraordinary B cell expansion occurs and what force is driving this event. These require other subhypotheses and experiments to test them.

PRRSV disrupts normal T cell development in the thymus

We hypothesize that PRRSV infects a population of antigen-presenting cells that migrate to or are constituent in the thymus of fetal or newborn animals, e.g., TECs, macrophages, and pDC that are engaged in thymocytes development and compromises proper T cell development. The interaction of thymocytes with these infected APCs might result in cytokine production/transcription and other protein transcription, which is abnormal compared with age-matched controls. Furthermore, the emerging T cell populations could be tested for their ability to recognize peptides derived from PRRSV or a control antigens like ovalbumin. Contrived in vitro systems should be developed to determine whether T cells developed in PRRSV-infected thymi can provide T cell help for antibody responses, activation of macrophages, or can behave as CTLs.

CTL induction is impaired in PRRS

We propose that the role of CTLs in PRRSV infection is fundamentally different in the infection of neonatal pigs compared to adults. We propose that the ability of pigs infected in utero or shortly after birth to mount any CTL response against PRRSV is compromised by the impaired development of CTL precursors due to reduction of thymic selection. Further, T cell selection that does occur could suffer from PRRSV antigens being seen as self-antigen, as a result of infection of thymic cells involved in T cell selection. Contrarily, in animals infected with PRRSV as adults, CTL precursors have developed normally, and even though the infection impairs innate immunity, the presence of virus-infected cells eventually could lead to a protracted development of a moderate CTL response. Further, we propose that the dysregulation of B cell function favors expansion of CD4 helper T cells not those required for induction of CTLs. This could also contribute to or be the sole cause of the protracted development of antiviral CTL responses in adult animals. We describe below techniques to test theses hypotheses.

First, we can use live, virulent virus in the short (hours long) assays to detect CTL killing. Alternatively, avirulent strains of the virus can be used as surrogates, allowing the cell death to be solely a result of CTL killing of the target cell. In other circumstances, viral proteins can be delivered to target cells artificially, by vectors for instance [248]. Since the CTL are from an infected animal and the autologous cells (or MHC matched target cell line) are given the vector expressing viral proteins, the measure of killing is now attributable to the CTL, as there is no live virus.

A dominating concept of the immunopathogenesis of PRRSV infection is the immunosuppression or dysregulation of the adaptive immune response. As with many

livestock studies, there is a body of work describing the antibody response but little analysis of CTLs. The single report of CTL function describes a basic analysis of a single strain of virus and concludes there is a low-level CTL response that is protracted in the kinetics of development [127]. A better understanding of CTL biology in PRRSV infection will require a more sensitive assay for CTL function. Using tools available today, class I MHC tetramers can be designed and tested to track CTL development and function. For instance, CD107a (LAMP1a) is an integral membrane protein that lines the vesicles that contain the granules that mediate killing by NK cells and CTLs. These granules are released by the vesicle membrane fusing with the cell membrane and releasing the contents. As a consequence, CD107a is now detected on the cell surface. So, a tetramer-positive, CD107a expressing cell is a PRRSV-specific CTL that has just killed a virus-infected cell. So, not only is the cell phenotype determines, i.e., PRRSV-specific CD8 T cells but also whether these cells function as CTLs.

Another possibility to explain the decrease in CTLs might be the action of MDSC [50, 52]. These macrophages accumulate at the site of chronic viral infections and tumors and suppress CTLs. Therefore, highly infected sites such as thymus, lung, and certain lymph nodes [8, 13, 136, 138] may harbor these cells. Since PRRSV targets macrophages, could their infection result in differentiation of myeloid cells to MDSC?

With these tools, hypothesis testing can determine whether CTLs are efficiently induced, induced but not functional, develop early but are rapidly downregulated, develop late, etc. Elevation of P3 expressing, $CD4^+$, $CD25^+$ Treg populations reported in PRRSV-infected isolator pigs is controversial ("Humoral responses of conventional animals" section). However, if class II SLA tetramers could be used to focus on the PRRSV reactive cells in that population exclusively, this antigen-specific population may be highly induced, but masked by the present methods of analysis. However, given the evidence available, a more likely hypothesis is that the normal, T cell differentiation is dysregulated as reflected in the apparent dysregulation of helper T cells that promote excessive B cell proliferation while preventing PRRSV-specific CTLs from expanding that become activated to kill virus-infected cells.

Immune evasion is due to specific regions of nsp2

The opportunity to manipulate the PRRSV genome provides the opportunity to test whether certain viral genes/proteins are responsible for immune dysregulation. Nsp2 is the most variable protein in the virus, subject to insertion/deletion(s) compared to the prototype type 2 strain, VR-2332. The fact that the nsp2 protein is an early protein and also a structural component of virions [208] suggests that it may be in contact with host macrophages and DCs, and stimulators derived from those and other host cells. It also possesses that a key protease, PLP2, whose ability to downregulate IFN-α and can act to deubiquinate proteins is well established, has a key role in the viral replication cycle by cleaving the nsp2/3 junction. Lastly, this protein is the largest protein of the virus.

A prior in vivo study has shown that a specific deletion of 87 aa in nsp2 of strain VR-2332 resulted in virus (VR-2332Δ87) with replication kinetics in 4-week-old swine about 1 log lower than the parent strain, while other deletions elsewhere in nsp2 had a more dramatic effect on viral replication ("PRRS the virus" section). It was also shown that swine inoculated with VR-2332Δ87 had no delay in onset of antibodies to the nucleocapsid protein. What was intriguing was that these same animals showed a delay in serum IFN-γ and a significant decrease in lymph node enlargement over that seen with VR-2332. Unfortunately, no comparison was completed on the thymic tissue or any other immune response measurement.

These prior studies must now be examined using more virulent PRRSV strains, and we must delineate the amino acids responsible for immune evasion. Two strains that we will develop deletion mutants for and test our hypothesis are type 2 strains MN-184 and Asian HP-PRRSV. One can begin by deleting the nucleotides of these more virulent viruses that represent the same region as VR2332Δ87. However, other regions of nsp2 may serve to evade immune responses. Only the hypervariable regions (aa 12–24; aa 323–817 of VR-2332) of the respective viruses have been shown to be mutable, so work should concentrate on those areas and make successive deletions based on nsp2 secondary structural predictions in the infectious clones of the parent viruses. Once developed, these mutants will be used in in vivo studies with conventional and isolator piglets and in in vitro studies.

Global Hypothesis for immune dysregulation by PRRSV

Since infected MQ and pDCs fail to secrete IFNα [73, 74], they would also poorly stimulate the antiviral state, so the first event is to compromise the first line of defense (innate immunity), which would allow spread of the virus.

Second, the IFNα-deficient infected MQ may then present to peripheral T cells in lymph nodes and without normal levels of IL-12 from DCs and pDCs, would not favor a Th1 profile and differentiation to CTLs. Thus, a major element in adaptive antiviral immunity is impaired. Rather these events favor a Th2 profile that might cause proliferation of CD4 helper cells at the expenses of Tregs

and CD8 CTLs. The suggestion that infected MQ and pDCs could induce apoptosis of thymocytes might indicate they could have the same effect on the peripheral T cell compartment. This could create a lymphopenic state. The increase in IL-10 suggests suppression that could account for the increase in Tregs [117] and may be derived from MDSC [50]. The elevation of Tregs might be a delayed event, which would have been overlooked by Sinkora et al. [119] who worked only with isolator piglets. It is still difficult to accept that if adaptive immunity is forced to a Th2 profile, it explains the polyclonal B cell activation and runaway B cell proliferation.

The third event is that these infected MQ, cDCs, and pDCs move to the developing thymus as APCs where they interact with DP thymocytes in the medulla that for reasons unknown, resulting in atrophy of DP thymocytes. Together with help from thymic epithelial cells (nurse cells), PRRSV may be therefore recognized as a self-antigen so surviving thymocytes could enter the periphery and recognize PRRSV as self, as reported by the Wieland for anti-Golgi antibodies. In fact, the vasculitis that is a feature or Arterivirus infections may be due to self-antibodies that coat the vascular as shown by Lemke et al. [22].

While the loss of DP thymocytes might lead to the loss of emerging T cells and in T cell lymphopenia, there is little evidence to support this. However, the quality and quantity of emerging CD4, CD8, and Tregs might be altered as described above for the peripheral T cell compartment. Without functional Tregs, activated B cells may initially proliferate out of control as suggested from Sinkora et al. [119]. Could an abundance of self-reactive Th2 cells, some of which may crossreact with PRRSV, be sufficient to drive rapid differentiation to plasma cells or perhaps IL-6 from infected MQ? Alternatively, GC may not form or are abnormal, so there is little selection and the resultant plasma cells show little repertoire diversification (Fig. 6) and therefore poor affinity to viral epitopes so that few which are strongly virus specific.

While PRRSV-specific VN antibodies can control the peripheral spread of the virus, CTLs are needed to eliminate virus-infected cells. In most viral infections, pDCs secrete IL-12 that promotes Th1 cells that can also activate MQ to kill their intracellular parasites/viruses. If chronically infected tissues are infiltrated by MDSC, such T cells may be inhibited [50]. In any case, since events in the thymus might reduce the number of peripheral Th1 helpers, the infection would persist. Perhaps of greatest effect is that if the number of virus-specific peripheral CD8 cells is low, there would be fever potential CTLs to attack the infected MQ. Not trivial is that most scenarios described for CTL involve killing of epithelial cells like in SIV. In the case of PRRSV, it would involve the killing of infected MQ. How easy is that?

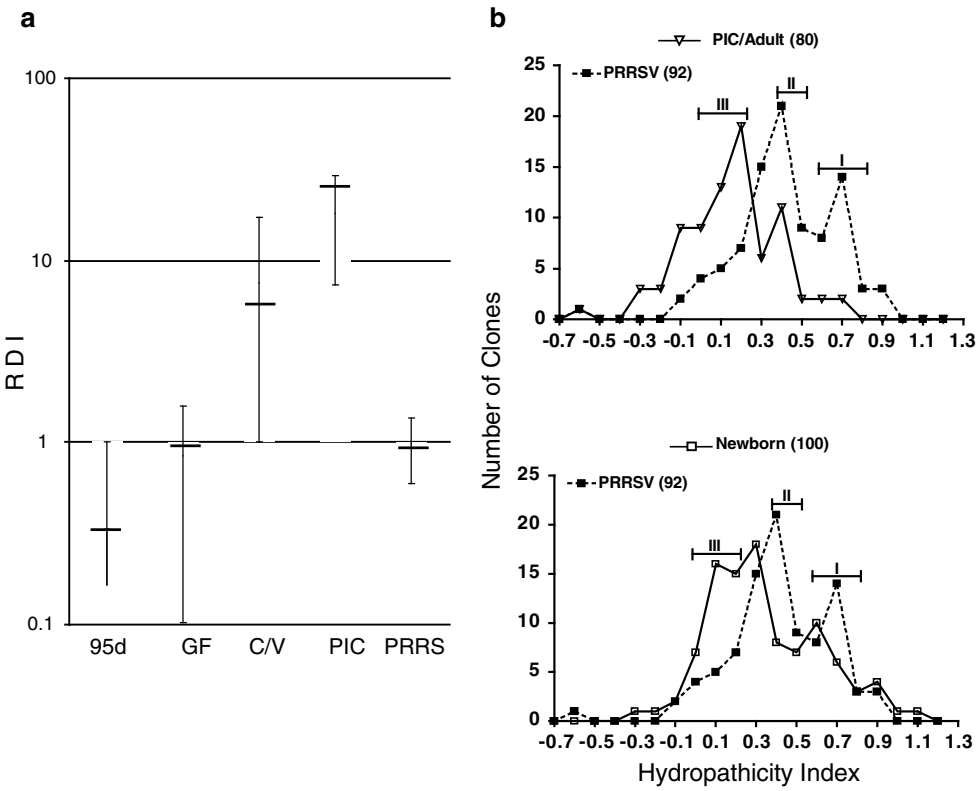

Fig. 6 **a** Antibody repertoire diversification measured as a repertoire diversification index (RDI). PRRS = isolator piglets infected with PRRSV; GF = germfree controls; C/V = isolator piglets colonized with benign *E. coli* or infected with SIV; PIC = young, helminth-infected conventionally reared pigs (PIC). **b** Hydropathicity profiles calculated from sequence analysis of the HCDR3 region of Ig from PRRSV-infected piglets compared to PIC animals (*top*) and compared to newborns (*bottom*). The *numbers in parentheses* indicate the number of sequences examined. Hydrophobic HCDR3 regions I and II are a feature of an undiversified pre-immune repertoire whereas region III is characteristic of a diversified repertoire. From Butler et al. [153]

Adult model

While what we have written above might explain the impact of PRRSV on neonates, the literature we have reviewed suggests that a separate model is required for the situation in adult swine. While we may be dealing with one disease at the cellular/molecular level, we may be dealing with two disease models at the organismal level as regards the immunological perspective: one for adults and one for neonates. For all sorts of reasons, we believe that immune homeostasis is developing during the critical window of immune development (Fig. 1) when most piglets are PRRSV infected. When an adult pig is considered, they have already properly developed their T cell repertoire and compartment. That means they have normal levels of CD8 cells that are potential CTLs. Likewise, they have Th2 cells to form GC and Tregs to prevent uncontrolled B cell expansion. As a result, adult animals mount effective immune responses with VN antibodies and CTLs that resolve the disease, regardless of whether the innate response continues to be compromised since host protection is now heavily dependent on de novo adaptive immunity (Fig. 1). In fetal and newborn piglets, innate immunity probably plays the major role in immune defense but after development of adaptive immunity, it become compensatory, not primary. This most likely explains why studies like those of Robinson et al. [32] show that PRRS is resolved in adults, presumably by both VN antibodies and CTLs. Thus, the host adaptive response override the negative effect of PRRSV on innate immunity in adult animals. This suggests that the principal impact of PRRSV is on the fetus and the neonate during the critical window and is thereafter not a serious threat to adults. From the position of vaccinologists, it would seem wise to supply neonatal vaccinates with the ingredients that would promote immunocompetence as summarized in Fig. 1.

Genetic models

All of the events described for fetal/neonatal and adult animals are relevant to the common vaccine version of PRRSV. However, is the effect of HP-PRRSV merely a quantitative difference or does it have a qualitative effect? Namely, does HP-PRRSV primarily target the thymus so its greatest impact is on T cell cells development? Since HP-PRRSV has a greater effect than vaccine strain, PRRSV on post-natal lymphopenia suggests that HP-PRRSV also acts in the periphery.

As previously described, failure to produce VN antibodies could be epitope dependent, so that differences between animals with and without VN antibodies could be epitope specificity, not a difference in affinity regardless of the mechanism of VN. The beauty of PRRRV genetics is that a large number of variant are available and others can be engineered ("PRRS the virus" section). The availability and expertise of the investigators in this area provide an unusual opportunity for the experimental design of studies to determine how certain viral genes affect immune dysregulation and how epitopes differs in their ability to stimulate protective immune responses.

Testing the global hypothesis

The working hypothesis offers numerous opportunities for testing. Exactly, how each step in the scheme is tested is left to the ingenuity of the investigators. Suffice to say there is a great need to know the cytokine, co-stimulatory molecule expression and signaling features of PRRSV-infected macrophages when acting as APC versus noninfected macrophages both in thymus and in the periphery. Do these IFNα-impaired macrophages preferentially or inappropriately stimulate certain T cell subsets or do they promote differentiation of MDSC? Using engineered mutants, one might determine what genetic features of the virus are responsible for any aberrant signaling. The core protein of HCV promotes MDSC differentiation [51]. Such "defective mutants" might also be the basis for future vaccines. Likewise, it would be wise to know what signaling events are aberrant in thymocytes from PRRSV-infected animals. As the runaway B cell proliferation still lacks an explanation, it would seem important to know whether infected macrophages can explain that part of the puzzle. Testing for germinal center formation, antibody affinity and the complement dependence of VN are relatively straightforward.

The issue of Tregs should be resolved. Are those with a suppressor phenotype functionally suppressive? Could it be the lack of functional Tregs that permits runaway B cell proliferation and differentiation? While tetramer assays could be valuable, they will remain in the distant future given the state of swine genetics. In the meantime, it is reasonable to assume that the same co-stimulatory molecules and signaling pathways that operate in mice also operate in swine so the information gained from studies in pigs can take advantage of the vast resource of information assembled from mouse research. Using simplified systems such as in vitro assays and isolator piglets has the best chance of determining how PRRSV dysregulates the neonatal porcine immune system. If pandemic PRRS is a neonatal phenomenon, developing vaccines that stimulate development and maturation of the adaptive immune system, e.g., probiotic cultures, should also be considered.

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