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Pathogenesis of porcine reproductive and respiratory syndrome virus

Ranjni J Chand, Benjamin R Trible and Raymond RR Rowland

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most costly viral pathogen facing a modern pig industry. A unique feature of the virus is the ability to cause severe clinical disease and maintain a life-long subclinical infection. Persistence at the population level poses the biggest challenge for the successful control and elimination of the disease. A mechanistic basis for persistence includes the evasion of innate and adaptive immune responses. Recent advances include the study of how the non-structural proteins (nsp's) inhibit the induction of type 1 interferon genes.

Address
Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS 66506, United States

Corresponding author: Rowland, Raymond RR (browland@vet.k-state.edu)

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is currently the most economically significant disease impacting pig production worldwide. Clinical outcomes following infection include reproductive failure and increased mortality in young pigs as a result of severe respiratory disease and poor growth performance [1]. However, within a production system, PRRSV infection predominantly exists as a subclinical infection, participating as a co-factor in various polymicrobial disease syndromes, such as porcine respiratory disease complex (PRDC) and porcine circovirus associated disease (PCVAD).

The etiological agent, PRRS virus (PRRSV), was identified in Europe in 1991 and termed Lelystad virus [2]. PRRSV was subsequently isolated in the U.S. and assigned the name VR-2332 [3]. Based on nucleotide sequence comparisons of European and North American isolates, PRRSV is divided into type 1 and type 2 genotypes, respectively. Even though type 1 and type 2 viruses appeared simultaneously and produce similar clinical signs, the two groups share only about 70% identity at the nucleotide level [4–6]. PRRSV is an enveloped, positive sense, single-stranded RNA virus. The 15.4 kb genome codes for at least 10 open reading frames (ORFs). The structure and composition of the virion are reviewed elsewhere [7•]. The nucleocapsid (N) protein forms a polymer surrounding the viral genome. The surface of the virion is dominated by glycoprotein (GP) 5 disulfide linked to the matrix (M) protein. Minor surface glycoproteins form a trimer composed of GP2, GP3, and GP4. Additional envelope proteins include E and ORF5a [8,9].

The arteriviruses, which include PRRSV, lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and equine arteritis virus (EAV), possess several novel properties related to viral pathogenesis, including cytopathic replication in macrophages, the capacity to establish a persistent infection, as well as cause severe disease. As a group, the arteriviruses represent the absolute extremes in mammalian pathogenesis. For example, SHFV is nearly 100% fatal in Asian monkeys [1]. In contrast, LDV rapidly reaches levels close to 10^{10} virions per ml in the blood with no apparent clinical signs in mice.

The different outcomes following PRRSV infection are a consequence of a complex set of interactions between the virus and the pig host. The acute phase of viremia covers approximately 28 days and primarily targets alveolar macrophages. The mechanistic basis for acute disease, such as respiratory distress is likely a consequence of the release of inflammatory cytokines in the lung. Following the initial clearance from the blood, viremia periodically re-appears [10••], with lymphoid tissues as the primary site of virus replication. Virus can be isolated from lymph nodes for more than 100 days after infection and virus is easily shed to sentinel pigs during the asymptomatic period. Replication levels gradually decay until the virus eventually becomes extinct [11,12••]. The mechanism for extinction is not clear, but probably relates to the gradual disappearance of permissive cells combined with only a partially effective immune response. By definition, PRRSV is not a 'persistent' virus. However, since the average lifetime of a production pig is approximately 180 days, PRRSV infection is 'life-long' for the vast majority of pigs. The mechanistic basis for persistence is dependent on a combination of factors including; (1) a complex virion structure that possesses a heavily glycosylated surface, (2) re-direction of the humoral response towards non-surface proteins, (3) antigenic and genetic drift, and (4) subversion of interferon gene induction. This review

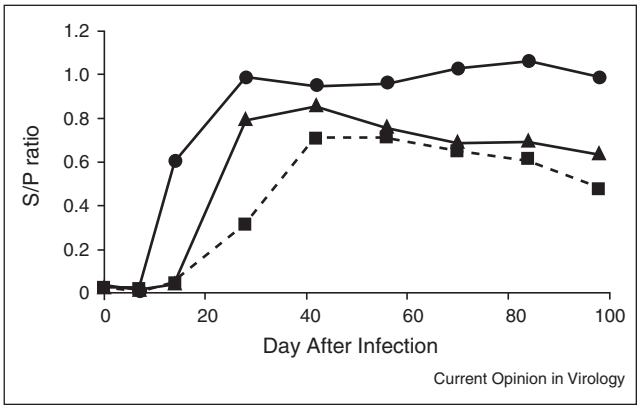
primarily focuses on recent advances, reported during the past ten years, related to understanding the processes that contribute to persistence.

Humoral immune response and the role of glycan shielding

An example of the humoral response to PRRSV structural and non-structural proteins during experimental infection is shown in Figure 1. Following infection, the earliest and strongest antibody response is against the N protein. In contrast, the antibody response against the major surface component, the GP5-M heterodimer, is weak and delayed. In fact, some animals fail to make a detectable antibody response against GP5. The neutralizing antibody response, which is also weak and delayed, follows a similar pattern [13]. Interestingly, a strong antibody response is made against non-structural proteins (nsp), such as nsp2. Nsp2 is not a component of the virion and is found only during the infection of cells. Therefore, the antibody response during infection is primarily directed against viral proteins not associated with virus neutralization.

The mechanism for the weak response against GP5 is linked to the presence of several N-linked glycosylation sites, identified by the peptide sequence N(X)S/T. After removal of the peptide signal sequence, the ectodomain of GP5 is only about 30 amino acids long. The ectodomain possesses two conserved N-glycosylation sites, located at position N44 and N51 in type 2 viruses and N46 and N 53 in type 1 viruses [14,15]. In addition, the distal asparagine/serine-rich domain, located between amino acids 30 and 38, possesses a small region containing a variable number of potential N-glycosylation sites. Depending on the virus isolate, the number of N-sites on the distal end of the ectodomain ranges from 0 to 3 [7,15,16]. The role of the number of N-sites in arterivirus persistence was first demonstrated by changes in the number of N-glycosylation sites in VP-3 following the infection of mice with LDV. VP-3 of LDV possesses a pattern of glycosylation similar to GP5 of PRRSV with conserved sites located at N45 and N52 [17]. The preservation of both sites correlates with resistance to neutralizing antibody and persistent infection. In contrast, certain naturally occurring strains of LDV which are neurotropic, lack the N-terminal and N45 glycosylation sites. These isolates are susceptible to neutralizing antibody and exhibit a low level of viremia. Sequence analysis of VP-3 in the residual circulating viruses showed the reacquisition of both glycosylation sites [18]. Rowland *et al.* [19] followed GP5 ectodomain peptide sequences in pigs exposed to VR-2332 *in utero*. Within a week after birth, a mutant virus appeared that possessed a D to N mutation in GP5 at amino acid position 34, which created an additional N-glycosylation site on the distal end of the ectodomain [19]. However, the mutant virus did not show increased resistance to neutralizing antibody. Costers *et al.* [25] studied the appearance of mutations in GP5 during the infection of pigs with a type 1 PRRSV. The results showed the appearance of a D to N mutation at position 37 of GP5, which created an additional N-glycosylation site.

Figure 1

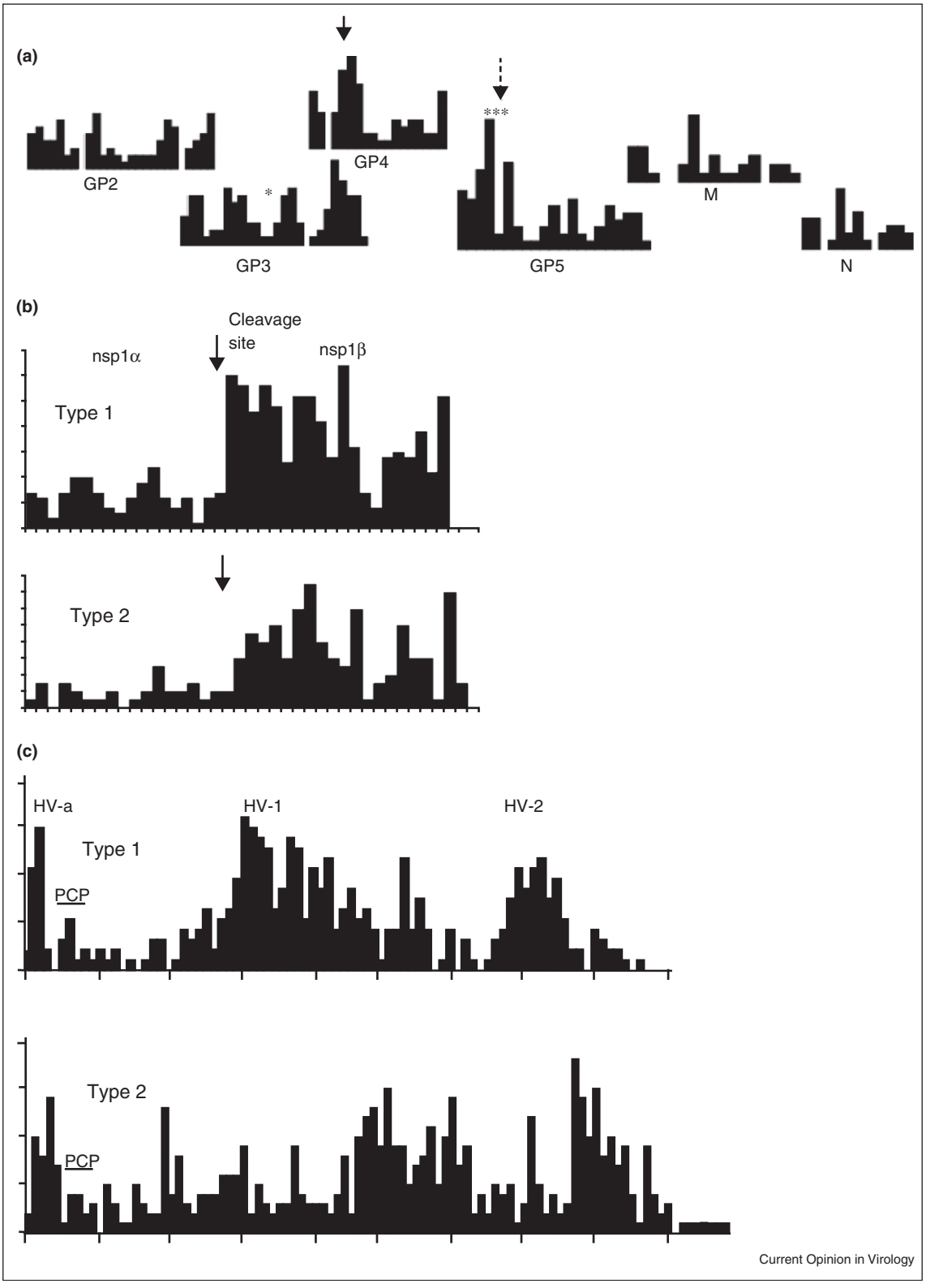


Antibody response to major structural and nonstructural proteins. Ten pigs were experimentally infected with PRRSV and antibody response measured against the N protein (circles), nsp2 (triangles) and the major virion surface components, GP5-M (squares with dotted line). Antibody was measured by ELISA and results shown as the mean of the sample to positive (S/P) ratio.

A specific role for N-glycan shielding in GP5 was demonstrated using reverse genetics of an infectious cDNA clone [20]. A panel of recombinant viruses was constructed with different combinations of mutations at N34, N44, and, N51 in GP5. The results showed that viruses without N44 were non-viable in culture, indicating a requirement of the asparagine or glycans for replication. The elimination of the N34 and N51 glycosylation sites resulted in the increased sensitivity of recombinant viruses to neutralization by antibody from pigs infected with the parent or recombinant viruses. In addition, the infection of pigs with viruses lacking N34 and/or N51 resulted in the production of increased antibody with enhanced neutralization activity against both mutant and parent viruses. The mechanistic basis for the role of glycans in resistance to antibody is linked to the protection of a conserved B cell epitope located between residues 37–45 [21]. A similar epitope was identified in the same location relative to N45 and N52 sites in LDV VP-3 [17].

Using an expression plasmid containing a modified recombinant GP5, which possessed an additional Pan DR T-helper cell epitope (PADRE) combined with the elimination of all glycosylation sites at N30, N34, N35 and N51, an increased anti-GP5 humoral response was detected in mice immunized with the DNA plasmid [22]. PRRSV-specific neutralizing activity in serum was also increased, including the capacity of serum to neutralize a broad range of PRRSV isolates. A similar approach

Figure 2



Peptide sequence variability and hypervariability in structural and nonstructural proteins. Sequence variation is presented as the number of amino acid changes within a 10 amino acid stretch for type 2 structural proteins **(a)**, Type 1 and type 2 nsp1 **(b)**, and type 1 and type 2 nsp2 **(c)**. Within the structural proteins **(a)**, the solid arrow represents the potential location of a neutralizing epitope within GP4 and the dashed arrow identifies the

incorporated the expression of recombinant GP5 by an adenovirus vector [23]. Increased PRRSV neutralizing activity was obtained following the infection of mice with constructs possessing mutations or substitutions at N44, N44/51, N30/44/51, N30/33/44/51 or N30/33.

However, it should be noted that the role of GP5 as the major target for neutralizing antibody is not accepted by all members of the scientific community. Glycan shielding may also play a role in the response to minor glycoproteins. Using a reverse genetics approach, virus neutralization of a recombinant virus lacking N51 in GP5 was further enhanced by the additional removal of the N131 glycosylation site in GP3 [24•]. Viruses recovered from infected pigs showed the restoration of both glycosylation sites. Martínez-Lobo *et al.* [26] placed 39 European type1 PRRS virus isolates into 4 phenotypes based on sensitivity to neutralization; highly sensitive, sensitive, moderately sensitive and neutralization resistant. The authors found no correlation between the pattern of glycosylation in the structural proteins with the neutralization phenotype.

The role of genetic and antigenic drift in persistence

A recent analysis of approximately 8500 ORF5 (GP5) nucleotide sequences indicates that type 2 viruses can be divided into at least nine distinct groups or lineages [27]. The capacity of PRRSV to rapidly change is illustrated in our previous work investigating the emergence of European type 1 isolates in the US [28]. Type 1 isolates of European origin first appeared in North America around 1999 [29–31] and are designated as North American (NA) type 1 PRRSV isolates. Phylogenetic analyses were performed using ORF5 and nsp2 nucleotide sequences from 20 type 1 isolates collected between 1999 and 2004 from 10 states. The NA type 1 viruses were most closely related to the Lelystad virus. Fifteen of the 20 ORF5 sequences fell into one of two major subgroups, designated group A and group B. The two groups had sufficiently diverged to the extent that antibodies derived from pigs infected with a group A isolate failed to neutralize viruses in group B, and vice versa.

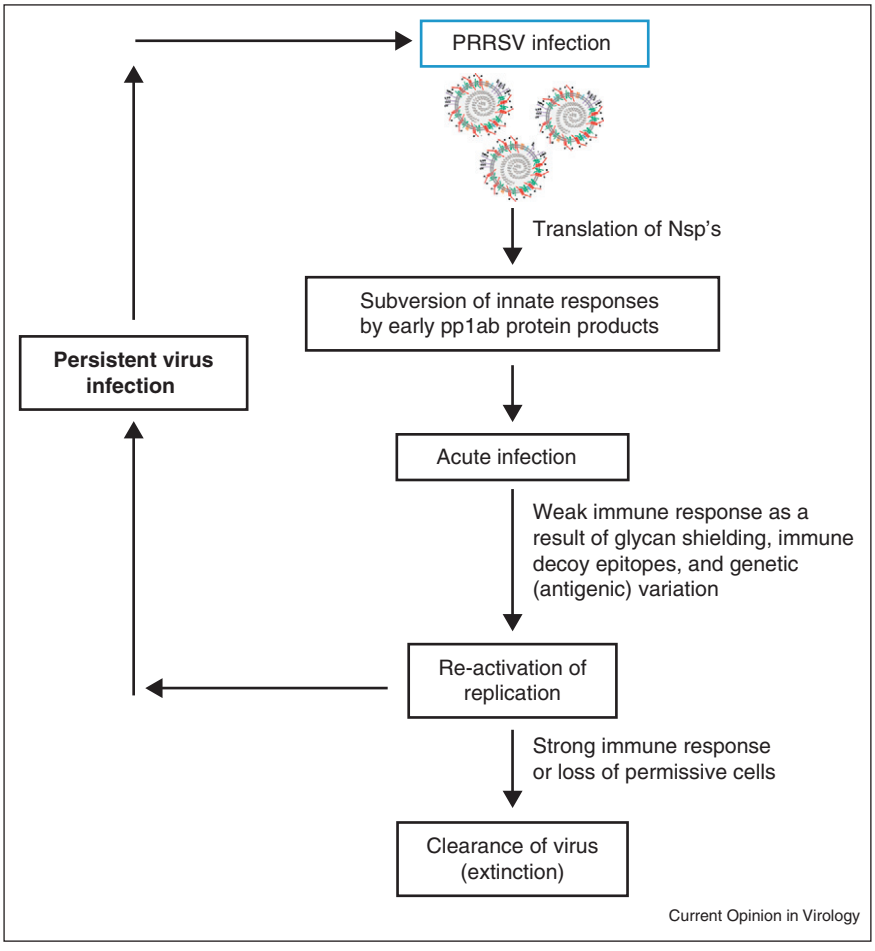
An indication of the origin for the NA type 1 viruses was found in the analysis of nsp2 nucleotide sequence. Nsp2 showed a phylogenetic topology similar to ORF5, including the placement of the same isolates into the same group A and B clades. Furthermore, 18 of the 20 isolates possessed a single 51 nucleotide deletion in nsp2 [30,31]. The deletion does not likely play a role in pathogenesis, but functions as a convenient marker for tracking the origin of the type 1 isolates. The results indicate that the high degree of genetic and biological diversity originated from a single virus introduced into the U.S.

Genetic diversity involves genetic drift through point mutations and genetic shift through recombination. Estimates of the nucleotide substitution rate for PRRSV range from 4.7 to 9.8 $\times 10^{-2}$/site/year, which is the highest rate calculated for any RNA virus to date [32,33]. The resulting peptide sequence variability and hypervariability within the structural proteins are linked to immune selection of B and T cell epitopes. Peptide sequence variability within the structural proteins is represented in Figure 2a. The greatest peptide sequence variation is found in the ectodomain region of GP5, flanking the two conserved N-glycosylation sites. The conserved neutralizing epitope is identified by the dotted arrow. Hypervariability is linked to the presence of a decoy epitope located between amino acids in the region between 27 and 30. However, this epitope may actually lie outside of the ectodomain within the GP5 peptide signal sequence. In type 1 viruses, Wissink *et al.* [34] identified a potential neutralizing epitope between amino acids 29 and 35 of GP5, which is located within a hypervariable region. Another region showing peptide sequence variation in the vicinity of a neutralizing epitope is found in GP4, where the epitope maps to amino acids 51–65 [35,36,25]. Interestingly, the variable epitope region overlaps the open reading frame that encodes GP3. The arteriviruses incorporate overlapping reading frames in the structural genes, as means to maximize the coding capacity. However, the presence of overlapping reading frames does not appear to create any significant constraint on the capacity of the structural proteins to generate peptide sequence variability.

Peptide sequence variability and hypervariability also extends to the non-structural proteins. Examples in Figure 2b and c show the diversity in peptide sequences for nsp1 and nsp2. Within nsp1, the nsp1β polypeptide shows increased peptide sequence variation compared to nsp1α. The protease site marks the separation between the relatively conserved nsp1α and the variable nsp1β. Another example is nsp2, which possesses peptide sequence insertions and deletions combined with peptide sequence hypervariability. Therefore, peptide sequence variation within the nsp's appears to be important, but the mechanism of how peptide sequence variation contributes to fitness is not known. One possibility is that the nsp's contain important T cell epitopes, which undergo antigenic drift. Mutations in nsp1 and nsp2 may contribute to persistence by altering the overall level of virus replication within the cell. Another possibility is the requirement for physical interactions between structural

(Figure 2 Legend Continued) conserved neutralizing epitope within GP5. Asterisks denote glycosylation sites that probably participate in shielding neutralizing epitopes. Within nsp1, solid arrows represent the cleavage site between nsp1α and nsp1β. The location of the papain like cysteine protease (PCP), as well as the three hypervariable regions (HV) are indicated within Nsp2 **(c)**. Peptide sequence information was obtained from GenBank.

Figure 3



Mechanism of PRRSV persistence.

and non-structural proteins during replication. Therefore, a change in the peptide sequence of a structural protein in response to immune selection may require a corresponding change in the partner nsp in order to maintain a stable interaction.

Subversion of the type 1 interferon response by non-structural proteins

A number of studies have shown that pretreatment of cells with type 1 interferon (IFN) inhibits PRRSV replication [37–39,40•]. Therefore, the study of the escape of PRRSV from the innate immune response has primarily focused on the subversion of IFN gene activation. The principal observation is the downregulation of IFN type 1 synthesis during virus infection in cultured primary porcine macrophages and other cell lines. After uncoating and entry of the virus genome in the cytoplasm of the cells, nsp1α and nsp1β are immediately translated and autocleaved from the nascent pp1a and pp1ab polyproteins. This creates the opportunity for nsp1 to effectively block the induction of interferon during the earliest stages of replication. A summary of observations related to the inhibition of IFNβ synthesis by nsp1 and other ORF1 polyprotein fragments is presented in Table 1. The most commonly reported mechanism for the inhibition of IFNβ synthesis is the inhibition of IRF3 phosphorylation, which can be mediated by nsp1, nsp2, and nsp11. (Phosphorylated IRF3 is translocated to the nucleus where it binds to the IFNβ promoter.) However, some laboratories report no inhibition of IRF3 phosphorylation or propose alternative mechanisms for the inhibition of IFNβ gene activation. For example, Kim *et al.* [41] showed that nsp1 induces degradation of CREB-binding protein (CBP), which is a co-activator of phosphorylated IRF3 in the nucleus. An alternative strategy was shown by nsp2, which inhibited the polyubiquitination of IkBα, preventing the activation of NF-kB, a transcription factor for IFN genes [42]. Luo *et al.* [43] observed that PRRSV interferes with the early steps of RIG-1 and TLR3 pathways by blocking the activities of IPS-1 and TRIF respectively.

Table 1

Effects of PRRSV pp1ab non-structural proteins on the activation of IFNβ

Nsp	Cell system	Observation	References
nsp1	MARC-145, HEK-293T	Reduced phosphorylation of IRF3	[44,45]
nsp1	MARC-145 HeLa	Degradation of CREB-binding protein	[41]
nsp1α	HeLa	Reduced IκB phosphorylation and nuclear translocation	[46]
nsp1α, nsp1β	HEK-293T	Decreased IFNβ promoter activation No effect on IRF3 phosphorylation	[47]
nsp2	HEK-293T	Reduced polyubiquitination of IκBα	[42]
nsp2	HEK-293T	Reduced IRF3 phosphorylation	[48]
nsp1α, nsp1β, nsp11	HEK-293, HT1080	Reduced IRF3-mediated gene activation	[44]
nsp1, nsp2, nsp4, nsp11	HeLa	Decreased IFNβ promoter activation	[44]

The differences in results may reflect differences in virus isolate used for infection, the cell line or perhaps, the design of the experiment. One intriguing possibility is that multiple viral proteins possess multiple activities in the inhibition of IFN synthesis.

Conclusion

The persistent nature of PRRSV presents significant challenges for the control and elimination of disease. As summarized in Figure 3, subversion of the innate and humoral responses contribute to PRRSV persistence within a population. The strategies that PRRSV utilizes to evade host defenses have placed similar limitations on the effectiveness of the current modified live virus (MLV) vaccines, such as delayed and weak neutralizing antibody response, persistent infection, and the inability to provide protection against a broad range of field isolates. With this new knowledge, alternative approaches are being incorporated in the design and development of the next generation of vaccines. On the pig side of the virus–host interaction, the improved understanding of the genetics of the response to PRRSV infection and vaccine may lead to a 'vaccine ready' pig [49].

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