Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Virus Research 154 (2010) 141–149

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Review

Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV)

Y.W. Huang, X.J. Meng*

Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

ARTICLE INFO

Article history:
Available online 23 July 2010

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Reverse genetics system
Vaccine development

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important swine pathogen. Since its discovery in the early 1990s, tremendous progresses have been made in understanding the molecular biology and pathogenesis of PRRSV. Although modified live-attenuated vaccines (MLVs) and inactivated vaccines against PRRSV have been available for more than a decade, the disease remains difficult to control. The efficacies of these vaccines especially against heterologous strains remain questionable: the MLVs were generally effective against homologous strains but variable in success against heterologous strains, and the outcomes of inactivated vaccines in the field are not very promising. With the development of PRRSV reverse genetics systems and the acquisition of new understanding on anti-PRRSV immunity, rational design of the next generation of PRRSV vaccines can now be explored. In this review, we discussed the recent advances in anti-PRRSV immunity and vaccinology, the recent progresses in PRRSV vaccine development particularly the reverse genetics system-based vaccine development, and provided a perspective on potential novel strategies and approaches that may be applicable to the development of the next generation of PRRSV vaccines.

© 2010 Elsevier B.V. All rights reserved.

Contents

1. The disease, virus and current vaccines 141
2. Key issues relevant to anti-PRRSV immunity 143
   2.1. Correlation of clearance of PRRSV infection and the level of host immunity 143
   2.2. The roles of dendritic cells and regulatory T cells in PRRSV infection 143
   2.3. Other major obstacles for the development of a more effective PRRSV vaccine 143
3. Recent advances in reverse genetics system-based PRRSV vaccine development 144
   3.1. PRRSV subunit vaccines and reverse genetics system-based MLVs 144
   3.2. PRRSV reverse genetics systems 144
   3.3. Chimeric infectious cDNA clones for attenuation of PRRSV 144
   3.4. Development of PRRSV gene-deletion marker vaccines 145
4. Potential novel strategies and approaches for developing the next generation of PRRSV vaccines 145
   4.1. Vaccines targeting porcine dendritic cells 145
   4.2. Vaccines suppressing regulatory T cell induction 146
   4.3. Vaccines inducing type I IFN response 146
   Acknowledgements 146
   References 146

1. The disease, virus and current vaccines

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease characterized by reproductive failure in sows and respiratory disease in pigs of all ages (Botner, 1997; Done and Paton, 1995). Pigs suffered from the disease first manifest acute illness including anorexia and lethargy, and sometimes pyrexia, dyspnea, and cyanosis of extremities. Reproductive failure in sows, including stillbirths, mummifications,

Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; PAM, porcine alveolar macrophages; DCs, dendritic cells; Tregs, regulatory T cells; MLVs, modified live-attenuated vaccines; IFN, interferon; GFP, green fluorescent protein.

* Corresponding author at: Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1981 Kraft Drive, Room 2036, Blacksburg, VA 24061-0913, USA.
Tel.: +1 540 231 6912; fax: +1 540 231 3426.
E-mail address: xjmeng@vt.edu (X.J. Meng).

0168-1702/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2010.07.020

weak born piglets and high preweaning mortality, occurs concurrently or soon after the acute illness (Mengeling et al., 1998; Meulenberg, 2000; Rowland, 2007). The causative agent of PRRS, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped, single-stranded, positive-sense RNA virus (Collins et al., 1992; Dea et al., 2000; Meulenberg, 2000; Wensvoort et al., 1991).

PRRSV belongs to the family *Arteriviridae* in the order of the *Nidovirales* that include members of the *Coronaviridae* and *Roniviridae* families (Gorbalenya et al., 2006). The family *Arteriviridae* also comprises three other viruses: equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV). Members of the *Arteriviridae* family have distinct host range for each virus: pigs for PRRSV, horses and donkeys for EAV, monkeys for SHFV, and mice for LDV (Snijder and Meulenberg, 1998). The four viruses all target macrophages of the respective host during infection. Two receptors on porcine alveolar macrophage (PAM), porcine sialoadhesin (pCD169) and pCD163, have been identified for PRRSV entry and uncoating, respectively (Calvert et al., 2007; Van Gorp et al., 2008; Vanderheijden et al., 2003). PRRSV isolates are divided into two distinct genotypes: the European (Type I) and North American (Type II) (Meng et al., 1995). The two genotypes of PRRSV cause the same disease symptoms but are antigenically different (Kapur et al., 1996; Meng et al., 1995; Meng, 2000; Meulenberg, 2000; Nelsen et al., 1999; Ropp et al., 2004; Stadejek et al., 2006).

The PRRSV genome is approximately 15 kb in length and consists of a 5′-untranslated region (UTR), nine open reading frames (ORFs), ORF1a, ORF1b, ORF2a, ORF2b and ORF3 to ORF7, followed by a 3′-UTR and a poly(A) tail (Dea et al., 2000; Meng et al., 1994; Meng, 2000). The ORF1a and ORF1b located in the 5′-proximal part consist of approximately 75% of the genome and encode 13 putative non-structural proteins (nsp), nsp1α, nsp1β, and nsp2 to nsp12 that are cleaved by proteolytic processing (Meulenberg et al., 1997; Snijder and Meulenberg, 1998; Ziebuhr et al., 2000). Nsp4 is the main proteinase (3C-like serine proteinase, 3CLpro) mediating most of the nsp processing (Ziebuhr et al., 2000). Cleavages of the nsp3/nsp4, nsp4/nsp5, and nsp11/nsp12 junctions by the nsp4 were confirmed (Tian et al., 2009). Nsp1α, nsp1β, and nsp2 are three accessory cysteine proteinases responsible for autocatalytic processing of a single cleaved site (den Boon et al., 1995; Ziebuhr et al., 2000). Nsp2 contains several immunodominant B-cell epitopes that are dispensable for virus replication but somehow modulate the host immune response (Chen et al., 2010b; Oleksiewicz et al., 2001). The ORF1b-encoded nsps 9–11 comprises the putative domains of viral RNA-dependent RNA polymerase (RdRp), helicase (Bautista et al., 2002) and uridylate-specific endoribonuclease (NendoU), respectively (Nedialkova et al., 2009; Snijder and Meulenberg, 1998). The functional activities of helicase in nsp10 and NendoU in nsp11 of PRRSV have been experimentally verified (Bautista et al., 2002; Nedialkova et al., 2009). Recently, nsp1α, nsp1β, nsp2, nsp4 and nsp11 were shown to inhibit the activation of interferon beta (IFN-β) promoter with different intensity, indicating that some PRRSV nsps are able to subvert host innate immunity by attenuating type I IFN response (Beura et al., 2010; Chen et al., 2010a).

The 3′-proximal part of the genome encodes seven PRRSV structural proteins that are translated from a 3′-coterminal nested set of six subgenomic mRNAs (Meng et al., 1996; Meulenberg et al., 1995). ORF2a and ORFs3–5 encode four membrane-associated N-glycosylated proteins (GP2a, GP3, GP4 and GP5) whereas ORF2b and ORF6 encode two non-glycosylated membrane proteins (E and M) (Dea et al., 2000; Meulenberg et al., 1995). The last ORF (ORF7) encodes for a nucleocapsid protein (N) encapsidating the viral RNA genome (Spilman et al., 2009). The GP5 protein is the most abundant enveloped glycoprotein containing major neutralizating epitopes (Ostrowski et al., 2002; Pirzadeh and Dea, 1997; Plagemann, 2004a,b; Plagemann et al., 2002). The GP5 and M proteins form a disulfide-linked heterodimer that serves as a ligand for the PRRSV internalization receptor, pCD169, on alveolar macrophages (Van Breedam et al., 2010). Recently, interactions of the other three minor envelope glycoproteins (GP2a, GP3, GP4) with GP5 were also detected by co-immunoprecipitation assay (Das et al., 2010). GP2a, GP3 and GP4 form a heterotrimer complex that is required for assembly of infectious PRRSV virions (Wissink et al., 2005), but only GP2a and GP4 were found to interact with pCD163 PRRSV receptor (Das et al., 2010). Overall, the recent findings on PRRSV non-structural and structural proteins provide new insights on PRRSV biology and vaccine development.

Since its recognition in the late 1980s, PRRS has devastated the swine industry worldwide. PRRS outbreaks frequently occur worldwide and cause tremendous economic losses. A recent example is the emergence of a variant strain of PRRSV associated with porcine high fever syndrome (PHFS) in China (Tian et al., 2007), which resulted in significant economic losses in China and Vietnam. In the United States alone, it is estimated that annual losses associated with PRRSV can reach up to half a billion dollars (Neumann et al., 2005). Two types of commercial vaccines are currently available against PRRSV, modified live-attenuated vaccines (MLVs) and inactivated vaccines (Kimman et al., 2009). MLVs against PRRSV became available in 1994 and were generally effective against homologous strains but variable in success (less effective or sometimes ineffective) against heterologous strains. The current MLVs have been associated with numerous problems such as shedding of vaccine virus, persistent infection, incomplete protection, and reversion to virulence. For example, acute PRRS outbreak occurred in Danish herds vaccinated with an MLV, and the outbreak was linked to reversion of the vaccine virus to a pathogenic phenotype (Botner et al., 1997; Storgaard et al., 1999). Vaccine-derived PRRSV strains were isolated from non-symptomatic persistently-infected pigs (Key et al., 2001, 2003; Zimmerman et al., 2006), and a MLV vaccine-derived isolate was found to cause disease in experimentally-infected pigs (Opriessnig et al., 2002). MLV derived from a single PRRSV vaccine strain do not fully protect pigs against heterologous PRRSV infection (Kimman et al., 2009; Labarque et al., 2004). Commercial inactivated vaccines, except for farm-specific autogenous products, are not available in the United States. The outcomes of the use of inactivated vaccines in other countries are not promising. Given the degree of genetic diversity observed among PRRSV strains, it is unlikely that a vaccine based on a single strain will confer effective protection against the antigenically and genetically diversified field strains currently circulating in swine herds worldwide. The effectiveness of a vaccine against heterologous strains will largely depend on the antigenic and genetic relatedness of the virus strain to which the vaccinated animals were exposed. The observed genetic diversity among field isolates will continue to be the major obstacle for PRRS control. Therefore, the design of future vaccines must take the antigenic and genetic diversity of PRRSV into consideration or PRRS will remain difficult to control.

In this article, we will first review the recent advances in anti-PRRSV immunity and vaccinology. In depth reviews of PRRSV biology and pathogenesis can be found elsewhere (Kimman et al., 2009; Lopez and Osorio, 2004; Mateu and Diaz, 2008; Murtaugh et al., 2002). We will then discuss the recent progresses in PRRSV vaccine development, particularly the use of reverse genetics system for PRRSV vaccine development. Finally, we will provide a perspective on potential novel strategies and approaches that may be applicable to the development of the next generation of PRRSV vaccines.

2. Key issues relevant to anti-PRRSV immunity

2.1. Correlation of clearance of PRRSV infection and the level of host immunity

PRRSV-infected pigs survived from the acute stage of infection that last up to 1 month usually develop persistent infection with low level of viral loads. The time of complete PRRSV clearance was estimated to be at least 150 days that is beyond the market lifetime of a pig (Allende et al., 2000). Although the mechanisms underlying the failure to promptly clear PRRSV infection are poorly understood, it appears that a major reason is the inability of pigs to develop effective protective immune responses (Lunney et al., 2010; Mateu and Diaz, 2008).

It has been demonstrated that PRRSV neutralizing antibodies play a critical role in clearance of virus and are able to completely protect pigs against PRRSV re-infection (Lopez and Osorio, 2004; Murtaugh et al., 2002; Osorio et al., 2002). However, neutralizing antibodies against the major structural component, GP5, appear later than non-neutralizing antibodies induced by other structural proteins and nsp2 after PRRSV infection. As a consequence, infected pigs do not efficiently resolve virus infection at the early phase (Lopez and Osorio, 2004). There are three possible explanations for the delayed appearance of neutralizing antibodies. First, GP5 contains an immunodominant epitope (epitope A or "decoy" epitope) in its ectodomain inducing an early and strong non-neutralizing immune response that diminish or mask the immune responses elicited by a downstream nearby neutralizing epitope (epitope B) (Osorio et al., 2002; Ostrowski et al., 2002; Yang et al., 2000). Secondly, there exist high mannose glycans at the N-linked glycosylation sites flanking epitope B on GP5 that diminish the sensitivity of PRRSV virions to neutralizing antibodies and thus impair the immunogenicity of the nearby epitope B (Ansari et al., 2006; Faaberg et al., 2006; Plagemann et al., 2002). Thirdly, PRRSV employ various strategies to inhibit the initial innate immune responses, in particular, the production of type I IFN, which subsequently dampens the host adaptive immune responses including the humoral immune response (Mateu and Diaz, 2008; Murtaugh et al., 2002; Royaee et al., 2004). Therefore, an ideal PRRSV vaccine should be able to induce more rapid and robust responses of neutralizing antibodies without suppressing the anti-PRRSV innate immunity.

2.2. The roles of dendritic cells and regulatory T cells in PRRSV infection

Dendritic cells (DCs) are professional antigen-presenting cells (APC) located throughout the peripheral immune system and play a critical role in linking innate detection and capture of virus to induction of adaptive immune responses (Palucka and Banchereau, 1999). Invading foreign pathogens trigger the migration of immature DCs from the blood into tissues where they detect and capture the pathogens. Activated DCs uptake pathogen antigens, process into immunogenic peptides through MHC molecules and further present to T cells (Banchereau and Steinman, 1998). Many viruses that cause persistent infection, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), exploit or eliminate DC function to evade innate and adaptive immunity (Liu et al., 2009). For examples, DCs serves as the "Trojan horses" that efficiently sequester HIV and then transfer HIV to target CD4+ T cells, thus facilitating persistent virus transmission (Wu and KewalRamani, 2006). This process is mediated by the interaction of HIV gp120 protein with a C-type lectin, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), on DCs (van Kooyk and Geijtenbeek, 2003).

Whether PRRSV targets porcine DCs to exercise its immune evasion strategies may have been understudied. Current researches on PRRSV and DCs interactions mainly focused on whether the production of interferons and specific inflammatory cytokines in DCs that may activate and prime differentiation of distinct types of T cells are down- or up-regulated in response to PRRSV exposure. Plasmacytoid DCs (pDCs) are considered as key effector DCs that express high level of type I IFN in the early antiviral innate immunity (Gilliet et al., 2008). Most recently, using novel fluorescent-activated cell sorting procedures, porcine pDCs characterized by $CD4^{high}CD172^{low}$ phenotype have been isolated from porcine peripheral blood mononuclear cells (PBMC) (Calzada-Nova et al., 2009). It was shown that porcine pDCs exposed to PRRSV *in vitro* did not secret IFN-α, IL-6, IL-8, IL-12, IFN-γ, or TNF-α (Calzada-Nova et al., 2009). PRRSV also appeared to be capable of infecting porcine myeloid DCs (mDCs) including bone marrow-derived DCs (BMDCs) and monocyte-derived DCs (MDDCs) *in vitro* and altering the expression level of various cytokines and CD markers, which has been speculated to contribute to the suppression of anti-PRRSV protective immune responses (Chang et al., 2008; Charerntantanakul et al., 2006; Flores-Mendoza et al., 2008; Loving et al., 2007; Wang et al., 2007). In addition, the porcine DC-SIGN (pDC-SIGN) gene was recently cloned from *in vitro* cultured MDDCs and found to enhance the *in vitro* transmission of PRRSV to target cells (Huang et al., 2009a). It will be interesting to see whether pDC-SIGN functions as the attachment receptor on specific subsets of porcine DCs to disseminate PRRSV in the course of infection *in vivo*. To aid in designing novel strategies and vaccines that can efficiently modulate the pig immune system against PRRSV infection, future studies are warranted to identify and dissect the distinct signaling pathways and molecules as well as relevant viral components involved in the interplay between DCs and PRRSV, and to investigate the role of DCs in facilitating PRRSV transmission.

Regulatory T cells (Tregs) characterized by CD4+CD25+Foxp3+ phenotype are also crucial players in balancing innate and adaptive immune responses (Sakaguchi et al., 2009). Adaptive (inducible) Tregs that are induced by DCs-presenting microbial antigens secret high amounts of IL-10 or TGF-β to suppress excessive inflammatory responses and thus reduce damage to host tissues (Sakaguchi et al., 2009). However, many pathogens including HIV, HCV and HBV also exploit inducible Tregs to subvert protective immune responses, allowing the establishment of persistent infection (Belkaid, 2007). Recently, two studies independently showed that porcine inducible Tregs were indeed induced when co-cultured with porcine MDDCs exposed to type II PRRSV *in vitro*, as indicated by increased numbers of CD4+CD25+Foxp3+ cells and enhanced expression of Foxp3+ mRNA (Silva-Campa et al., 2009; Wongyanin et al., 2010). These inducible Tregs displayed a suppressive activity on PHA-stimulated PBMC. Moreover, the expression of both mRNA and protein of TGF-β, but not IL-10, was up-regulated in co-cultured cells infected with PRRSV, indicating that the PRRSV-induced Tregs belong to T helper 3 (Th3) subset (Silva-Campa et al., 2009). Interestingly, using the same experimental protocols, no CD25+Foxp3+ Tregs and TGF-β were induced by type I PRRSV-infected MDDCs, suggesting that PRRSV-induced Th3 response may be type- or strain-dependent, at least *in vitro* (Silva-Campa et al., 2010). These findings may provide the basis for a potential strategy that improves PRRSV vaccine efficacy by inhibiting PRRSV-induced Tregs activity. However, whether PRRSV infection primes Tregs activation, leading to immunosuppression of pigs *in vivo*, remains to be determined in future studies.

2.3. Other major obstacles for the development of a more effective PRRSV vaccine

PRRSV utilized different means to subvert the host immune responses during the later stage of infection. Due to the low fidelity of viral RdRp, mutations are introduced into the PRRSV genome, resulting in the existence of antigenic heterogeneity on epitopes

that are recognized by neutralizing antibodies and T cells. This leads to the escape of host immune surveillance and makes it difficult for the host to generate protective immune responses to eliminate the virus, especially heterologous strains (Meng, 2000). In addition, PRRSV-infected PAM may sequester the virus so that there are no viral proteins expressed on the plasma membrane, which makes the viral information become invisible to the host immune system. As a consequence, the infected cells are refractory to antibody-dependent and complement-mediated lysis (Costers et al., 2006). It is reasonable to hypothesize that, as discussed above, other host non- or semi-permissive cells such as pDCs and mDCs likely have a similar property to help PRRSV remain in a persistent state.

One of the unusual characteristics of PRRSV-inducing immunity is that PRRSV-specific antibodies of maternal origin, or conferred by vaccination, can facilitate the entry of virus into target cells, leading to increased infectivity (Yoon et al., 1996, 1997). This phenomenon, known as antibody-dependent enhancement (ADE) of viral infection, is also considered as an obstacle to PRRSV vaccine development, although the underlying mechanism remains elusive (Christianson et al., 1993; Shibata et al., 1998; Tirado and Yoon, 2003). Recent studies of ADE mechanisms from other RNA viruses have suggested that, in addition to enhancing viral uptake, ADE may suppress the innate antiviral response, especially type I IFN system of the host (Mahalingam and Lidbury, 2002). Moreover, ADE enables the viruses to benefit from the anti-inflammatory and immunosuppressive environment created by autocrine and paracrine IL-10 production (Berlato et al., 2002; Ito et al., 1999; Mahalingam and Lidbury, 2002). Since production of IL-10 were significantly increased in bronchoalveolar lavage fluids of PRRSV-infected pigs (Thanawongnuwech et al., 2004), it is speculated that, in the course of ADE of PRRSV infection, stronger up-regulation of IL-10 would occur in PAM, which may contribute to down-regulating the production of IFN-α/β and other inflammatory cytokines. Therefore, attenuating or eliminating the negative effects of ADE that may be induced by vaccination should be considered as a key factor for novel PRRSV vaccine development.

3. Recent advances in reverse genetics system-based PRRSV vaccine development

3.1. PRRSV subunit vaccines and reverse genetics system-based MLVs

Experimental subunit vaccine systems based upon plasmid DNA, bacteria, baculovirus, adenovirus, fowlpox virus and pseudorabies virus, carrying several PRRSV antigens including GP5, M, and/or GP3, have been developed and evaluated against PRRSV (Jiang et al., 2004, 2006, 2007, 2008; Shen et al., 2007). In some of these cases, animals immunized with GP5 together with M or GP3 antigen developed higher titers of neutralizing antibodies to PRRSV, and produced stronger lymphocyte proliferation responses compared to animals immunized with GP5, M or GP3 alone. It remains to be seen whether such subunit vaccines will be better than the existing MLVs and killed products.

Reverse genetics system is defined as the generation of viruses possessing a genome derived from cloned cDNAs (infectious clone) (Boyer and Haenni, 1994). It not only provides a powerful tool for dissecting the role of viral proteins in virus life cycle and pathogenicity, but also opens the door to develop live-attenuated virus vaccines by introducing attenuating mutations on viral genome. Both efficient humoral and cellular immunities against the engineered virus are induced, which increases the likelihood of protection from viral disease (Neumann et al., 2002). Such vaccines can be designed to be more stable and safer than conventional live-attenuated viruses generated by continuous blind cell passages.

A major limitation for developing effective subunit vaccines and reverse genetics-based MLVs is the heterogeneic nature of the PRRSV strains worldwide. The current experimental subunit vaccines and reverse genetics-based MLVs are mostly based upon a single strain of PRRSV. It remains doubtful that such a vaccine will provide sufficient level of heterologous protection in the field.

3.2. PRRSV reverse genetics systems

The first reverse genetics system for both European genotype (Lelystad virus) and the North American genotype (strain VR2332) of PRRSV was RNA-launched, using a low-copy-number DNA plasmid as the cloning vector and template for generation of *in vitro* RNA transcripts that are directly transfected into susceptible cells to recover the virus (Meulenberg et al., 1998; Nielsen et al., 2003). T7 or SP6 promoter was inserted immediately upstream the full-length viral genomic cDNA to drive the *in vitro* transcription reaction (Choi et al., 2006; Nielsen et al., 2003). The RNA-launched system has been reported for recovering both genotypes of PRRSV of different geographic origins, and has been widely used for functional studies of viral genes (Fang et al., 2006; Lv et al., 2008; Truong et al., 2004; Yoo et al., 2004; Zhou et al., 2009).

A DNA-launched reverse genetics system was reported for recovering a type II PRRSV strain P129 by direct transfection of cells with the full-length viral genomic cDNA clone under the cytomegalovirus (CMV) promoter recognized by the cellular RNA polymerases II (Lee et al., 2005). The DNA-launched system could be improved by introduction of self-cleaving ribozyme elements at both termini of the viral genomic cDNA, which was expected to retain the authentic terminal nucleotide sequences of the viral genome (Huang et al., 2009b). The rescue efficacy of a type I PRRSV (strain SD01-08) expressing GFP with this system was approximately 10- to 50-fold higher than the RNA-launched system and the traditional DNA-launched system without the engineered ribozyme elements, as determined by reporter GFP level in transfected cells and the peak titer of the recovery virus (Huang et al., 2009b). This improved reverse genetics system has also been applied to rescuing a type II PRRSV strain VR2385 with higher efficacy (Ni et al., unpublished data). With the availability of the improved DNA-launched system, future production of the "seed virus stock" for reverse genetics-based MLV will have a reduced cost and time by bypassing the need of an RNA *in-vitro*-transcription step.

3.3. Chimeric infectious cDNA clones for attenuation of PRRSV

Understanding the molecular basis of PRRSV virulence is critical for the generation of attenuated PRRSV vaccines. A series of PRRSV infectious cDNA clones were constructed where specific genomic regions of an attenuated type II PRRSV vaccine strain, Prime Pac (PP18) (Kwon et al., 2006), were systematically swapped with their counterparts in the backbone of a highly virulent infectious cDNA clone of PRRSV, FL12 (Kwon et al., 2008; Truong et al., 2004). Transfection of the respective chimeric constructs into cells resulted in rescue of viable viruses. Pregnant sows were inoculated with the chimeric viruses, along with PP18 and FL12, at 90 days of gestation and the viability of their offspring at birth and weaning was evaluated. The results showed that the virulence determinants possibly locate in all nsps (except nsp9) and structural ORFs, confirming the multigenic character of PRRSV virulence as deduced from sequence comparisons. In particular, nsp3–8 and ORF5 appeared to comprise major virulence determinants, whereas other virulence determinants may be resided in nsp1–3, nsp10–12 and ORF2 (Kwon et al., 2008). In addition, when chimeric viruses were used to infect PAM *in vitro* to assess the genetic determinants affecting the host

immune responses, nsp1–8 in ORF1a was identified to play a regulatory role (Gudmundsdottir and Risatti, 2009).

A similar study was also conducted using chimeric PRRSV infectious cDNA clones derived from two distinct type II PRRSV strains, the attenuated Ingelvac PRRS MLV and the virulent strain MN184 (Wang et al., 2008). Two reciprocal chimeric cDNA clones with the region of 5′-UTR/ORF1 from one genome fused to ORF2–7/3′-UTR from the other genome displayed similar antibody responses to the two parent viruses, but had much less severe pathogenicity when compared to the MN184 group, indicating that the virulent determinants were presented throughout the viral genome (Wang et al., 2008). The two reciprocal chimeric viruses as well as a chimera with the ORF5–6 (encoding GP5 and M) of strain MN184 cloned in the backbone of Ingelvac PRRS MLV were able to protect pigs against challenge with two heterologous strains, SDSU73 and JA-142, respectively (Ellingson et al., 2010; Wang et al., 2008). The results suggest that MLVs based on the backbone of a traditionally cell culture-attenuated vaccine strain can be developed in the future using chimeric infectious cDNA clones. Moreover, it appears that a simple exchange of the ORF5–6 region from a field strain could provide an improved vaccine efficacy for heterologous protection. It will be interesting to see whether chimeric constructs between type I and type II PRRSV can rescue the recombinant viruses.

3.4. Development of PRRSV gene-deletion marker vaccines

A disadvantage of current PRRSV MLVs is that pigs vaccinated with MLVs cannot be distinguished from those naturally-infected with field strains of PRRSV by serological assays. Generation of a marker or DIVA (differentiating infected from vaccinated animals) PRRSV vaccine based on a deletion marker (i.e., an immunogenic marker absent from the vaccine strain but present in field strains) on the viral genome by reverse genetics system will allow for differentiation and be of great value for the control and eventual elimination of PRRSV. The candidate gene used for generating a deletion marker is nsp2 since it appears to tolerate large deletions associated with several immunodominant B-cell epitopes in both type I and type II PRRSV genomes (Chen et al., 2010b; Kim et al., 2009; Oleksiewicz et al., 2001). de Lima et al. demonstrated that a PRRSV mutant FLdNsp2/44 lacking an immunodominant B-cell linear epitope within the nsp2 gene derived from a type II PRRSV was viable *in vitro* and infected pigs (de Lima et al., 2006, 2008). The mutant also displayed an efficient growth both *in vitro* and *in vivo*, and induced N protein-specific serum antibodies but not deletion epitope-specific antibodies, as measured by an epitope peptide-based ELISA in all fifteen inoculated pregnant sows. Both the mutant virus and parental wild-type virus FL12 produced a similar virulence phenotype in the pregnant sow model that was assessed by PRRSV challenge of pregnant sows at gestation and measuring the viability of the offspring at birth and upon weaning at 15 days of age. The results indicated that deletion of nsp2 marker epitope did not contribute to attenuation of the mutant virus (de Lima et al., 2008).

In another proof-of-principle study, a recombinant type I PRRSV (strain SD01-08) expressing GFP with a deletion of an immunogenic epitope (ES4) within the nsp2 gene was used as the marker vaccine candidate in a nursery pig disease model (Fang et al., 2008). The anti-N protein antibody response of pigs inoculated with the mutant virus reached similar levels to the parental recombinant virus after 21 days post-immunization as measured by a commercial ELISA kit. However, compared to the parental virus, the mutant virus expressing GFP appeared to be attenuated, as evidenced by the presence of a lower level of viremia. Using GFP- and ES4 epitope-specific ELISAs, it was shown that pigs immunized with the mutant virus did not develop antibodies against the deleted epitope, but did develop a high-level antibody response to GFP by 14 days post-infection. The stability of an introduced marker in the nsp2 region is a potential concern (Kim et al., 2007). It has been demonstrated that, when the enhanced green fluorescent protein (EGFP) was inserted into the nsp2 region, the EGFP-containing genomes were properly expressed and produced virus, although the EGFP fluorescence was lost during passage of recombinant EGFP viruses in culture, indicating that the introduced marker in the nsp2 region is not genetically stable. Nevertheless, the results suggested that it is possible to develop a double-marker MLV containing both the positive (GFP) and negative (deleted ES4 epitope) markers, allowing serological differentiation between vaccinated pigs and those infected with field strains alone or those co-infected with MLV and field strains (Fang et al., 2008).

Amino acid sequence analysis of the ES4 epitope region revealed that it contains seven small B-cell epitopes, two of which are highly conserved among all the type I PRRSV strains with known nsp2 sequence. Accordingly, the anti-ES4 antibodies could be detected in serum samples of pigs infected with four representative field strains of type I PRRSV, as measured by the ES4 epitope-specific ELISA (Fang et al., 2008), suggesting that the ES4 epitope-deleted mutant could be useful as a PRRSV marker vaccine to distinguish type I PRRSV strains. However, since the ES4 epitope is not conserved between type I and type II PRRSV, it did not react with serum samples of pigs infected with type II isolates. Indeed, a comparison of the identified B-cell epitopes in the nsp2 revealed that none of these epitopes were conserved between type I and type II PRRSV isolates (de Lima et al., 2006; Oleksiewicz et al., 2001). Therefore, it appears that the type I or type II marker virus based on partial nsp2 gene deletion cannot be used as a "universal" vaccine candidate for differentiating both genotypes. Nevertheless, these two proof-of-principle studies (de Lima et al., 2008; Fang et al., 2008) exemplified the potential for rational design of PRRSV marker vaccines that may be developed in the future.

4. Potential novel strategies and approaches for developing the next generation of PRRSV vaccines

4.1. Vaccines targeting porcine dendritic cells

The central role of DCs in host immunity indicates that efficacious vaccinations rely on how efficient the vaccine interacts with DCs. DCs are now being considered as the target in the design of novel vaccines by enhancing the immunogenicity of vaccines (McCullough and Summerfield, 2009; Steinman and Banchereau, 2007). Development of vaccine delivery systems capable of targeting the vaccine antigen and the agonist to DCs are recognized as the research priority ("Advances in immunology and vaccine discovery": Report of the United States-European Union Commission Workshop; http://www.theaavi.org/EU-US_Report.doc). DC-targeting immunization approaches have been validated in both primate and murine models (Steinman and Banchereau, 2007). For examples, Tacken et al. demonstrated that antibody-mediated targeting of antigen to human DCs via DC-SIGN effectively induces antigen-specific naive and memory T-cell responses. The antigen KLH cross-linked to a humanized DC-SIGN antibody (hD1) specifically bound to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. The targeting efficiency of hD1-KLH to DCs is 100-fold higher than that of KLH alone (Tacken et al., 2005).

Recently, Revilla et al. demonstrated that this new avenue could be applied to pigs by using pCD169 (sialoadhesin) as the antigen-delivery target on porcine MDDCs (Revilla et al., 2009). Using T cells from pigs immunized with antigen (mouse Ig) as responder cells, and MDDCs with or without the expression of pCD169, they com-

pared the T cell proliferative response induced by an anti-pCD169 Mab with that of a non-targeting control Mab. The anti-pCD169 Mab bound to pCD169 is internalized rapidly and induced recall T cell proliferative responses at concentrations 100-fold lower than the control Mab *in vitro*, indicating that targeting antigen to pCD169 is an effective strategy to increase the immunogenicity of antigen (Revilla et al., 2009). Since pCD169 has been identified as the entry receptor for PRRSV through interaction with GP5-M heterodimers (Van Breedam et al., 2010), it will be very interesting to see whether targeting the PRRSV antigens directly to pCD169 will increase the vaccine efficacy. Other potential DC receptors used for targeting PRRSV antigens may include several porcine Toll-like receptors (TLRs), such as TLR3 and TLR7 that were involved in PRRSV infection (Chaung et al., 2010; Sang et al., 2008), and C-type lectin receptors such as pDC-SIGN (Huang et al., 2009a), pLangerin (Nfon et al., 2008) and pCLEC4G (Huang and Meng, 2009).

4.2. Vaccines suppressing regulatory T cell induction

As mentioned above, inducible CD25+Foxp3+ Tregs appeared to be involved in inhibiting T-cell activity during PRRSV persistent infection and thus may be an obstacle for vaccination with PRRSV MLV. If this phenomenon is confirmed *in vivo*, suppressing Tregs induction to enhance vaccine efficacy will be important for developing the next generation of PRRSV vaccines based on attenuated virus. In human and mouse models, many Tregs-depleting strategies have been investigated. For examples, it has been reported that depletion of natural Tregs using anti-CD25 Mab before vaccination with a vaccine against herpes simplex virus type 1 enhanced $CD8^+$ T cell response to the virus (Toka et al., 2004). Administration of anti-IL-10R antibody before immunization with a murine leishmaniasis vaccine can improve vaccine efficacy (Stober et al., 2005). However, these approaches may be cost-prohibitive for porcine vaccines. Future studies are needed to identify the viral components associated with Tregs induction, and then design recombinant MLVs by reverse genetics approaches that can abolish the potential risks associated with Tregs induction.

4.3. Vaccines inducing type I IFN response

MLVs can be generated by deletion of essential factors associated with pathogenicity using reverse genetics techniques. In some coronaviruses, the deletion of nsp genes has been shown to confer attenuation in their natural hosts (de Haan et al., 2002; Haijema et al., 2004; Ortego et al., 2003). The major role of nsp1 in mouse hepatitis virus (MHV) and SARS-coronavirus (SARS-CoV) is to counter the antiviral type I IFN response by inhibiting IFN-dependent signaling or the downstream events (Wathelet et al., 2007; Zust et al., 2007). A mutant MHV containing a deletion in nsp1 gene was shown to grow normally *in vitro*, but was strongly attenuated in mice. The nsp1 mutant of MHV replicated in conventional DCs and macrophages, and induced robust type I IFN in pDCs. Inoculation with low doses of nsp1 mutant of MHV resulted in eliciting strong cytotoxic T cell responses and protected mice against homologous and heterologous virus challenge (Zust et al., 2007). Similarly, by introducing mutations into the viral NS1 gene through reverse genetics, attenuated influenza virus mutants were generated and shown attenuation in different animal models due to induction of a strong type I interferon response that limits their replication (Richt and Garcia-Sastre, 2009).

As described previously, several PRRSV nsps (nsp1α, nsp1β, nsp2, nsp4 and nsp11) are involved in suppressing antiviral type I IFN response (Beura et al., 2010). Among them, nsp1β appeared to have a strong inhibitory activity (Beura et al., 2010; Chen et al., 2010a). However, Beura et al. suggested that, since nsp1β is a multifunctional viral protein, it may not be feasible to structurally separate proteinase function from its IFN antagonistic function, and therefore an nsp1β mutant virus lacking anti-IFN activity may not be rescued (Beura et al., 2010). Therefore, selection of other nsps as deletion targets may be more appropriate for searching a suitable MLV candidate. Nevertheless, genetic manipulations of viruses to restore type I IFN response may provide a novel strategy for the development of the next generation of PRRSV vaccines.

Acknowledgements

The authors' work on PRRSV is supported in part by a grant from USDA PRRS-CAP NIFA 2008-55620-19132 and from Fort Dodge Animal Health Inc (now Pfizer Animal Health Inc). Due to limited scope of this review, we apologize to those whose important PRRSV publications are not cited here.

References

Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A., 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J. Virol. 74 (22), 10834–10837.

Ansari, I.H., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J. Virol. 80 (8), 3994–4004.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392 (6673), 245–252.

Bautista, E.M., Faaberg, K.S., Mickelson, D., McGruder, E.D., 2002. Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology 298 (2), 258–270.

Belkaid, Y., 2007. Regulatory T cells and infection: a dangerous necessity. Nat. Rev. Immunol. 7 (11), 875–888.

Berlato, C., Cassatella, M.A., Kinjyo, I., Gatto, L., Yoshimura, A., Bazzoni, F., 2002. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J. Immunol. 168 (12), 6404–6411.

Beura, L.K., Sarkar, S.N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A.K., Osorio, F.A., 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J. Virol. 84 (3), 1574–1584.

Botner, A., 1997. Diagnosis of PRRS. Vet. Microbiol. 55 (1–4), 295–301.

Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S., Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet. Rec. 141 (19), 497–499.

Boyer, J.C., Haenni, A.L., 1994. Infectious transcripts and cDNA clones of RNA viruses. Virology 198 (2), 415–426.

Calvert, J.G., Slade, D.E., Shields, S.L., Jolie, R., Mannan, R.M., Ankenbauer, R.G., Welch, S.K., 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. J. Virol. 81 (14), 7371–7379.

Calzada-Nova, G., Schnitzlein, W., Husmann, R., Zuckermann, F.A., 2009. Characterization of the cytokine and maturation responses of pure populations of porcine plasmacytoid dendritic cells to porcine viruses and toll-like receptor agonists. Vet. Immunol. Immunopathol..

Chang, H.C., Peng, Y.T., Chang, H.L., Chaung, H.C., Chung, W.B., 2008. Phenotypic and functional modulation of bone marrow-derived dendritic cells by porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 129 (3–4), 281–293.

Charerntantanakul, W., Platt, R., Roth, J.A., 2006. Effects of porcine reproductive and respiratory syndrome virus-infected antigen-presenting cells on T cell activation and antiviral cytokine production. Viral Immunol. 19 (4), 646–661.

Chaung, H.C., Chen, C.W., Hsieh, B.L., Chung, W.B., 2010. Toll-Like Receptor expressions in porcine alveolar macrophages and Dendritic Cells in responding to poly IC stimulation and porcine reproductive and respiratory syndrome virus (PRRSV) infection. Comp. Immunol. Microbiol. Infect. Dis. 33 (3), 197–213.

Chen, Z., Lawson, S., Sun, Z., Zhou, X., Guan, X., Christopher-Hennings, J., Nelson, E.A., Fang, Y., 2010a. Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology 398 (1), 87–97.

Chen, Z., Zhou, X., Lunney, J.K., Lawson, S., Sun, Z., Brown, E., Christopher-Hennings, J., Knudsen, D., Nelson, E., Fang, Y., 2010b. Immunodominant epitopes in nsp2 of porcine reproductive and respiratory syndrome virus are dispensable for replication, but play an important role in modulation of the host immune response. J. Gen. Virol. 91 (Pt 4), 1047–1057.

Choi, Y.J., Yun, S.I., Kang, S.Y., Lee, Y.M., 2006. Identification of 5′ and 3′ cis-acting elements of the porcine reproductive and respiratory syndrome virus: acquisition of novel 5′ AU-rich sequences restored replication of a 5′-proximal 7-nucleotide deletion mutant. J. Virol. 80 (2), 723–736.

Christianson, W.T., Choi, C.S., Collins, J.E., Molitor, T.W., Morrison, R.B., Joo, H.S., 1993. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. Can. J. Vet. Res. 57 (4), 262–268.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., et al., 1992. Isolation of

swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4 (2), 117–126.
Costers, S., Delputte, P.L., Nauwynck, H.J., 2006. Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody-dependent, complement-mediated cell lysis. J. Gen. Virol. 87 (Pt 8), 2341–2351.
Das, P.B., Dinh, P.X., Ansari, I.H., de Lima, M., Osorio, F.A., Pattnaik, A.K., 2010. The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. J. Virol. 84 (4), 1731–1740.
de Haan, C.A., Masters, P.S., Shen, X., Weiss, S., Rottier, P.J., 2002. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. Virology 296 (1), 177–189.
de Lima, M., Kwon, B., Ansari, I.H., Pattnaik, A.K., Flores, E.F., Osorio, F.A., 2008. Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes. Vaccine 26 (29–30), 3594–3600.
de Lima, M., Pattnaik, A.K., Flores, E.F., Osorio, F.A., 2006. Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus. Virology 353 (2), 410–421.
Dea, S., Gagnon, C.A., Mardassi, H., Pirzadeh, B., Rogan, D., 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145 (4), 659–688.
den Boon, J.A., Faaberg, K.S., Meulenberg, J.J., Wassenaar, A.L., Plagemann, P.G., Gorbalenya, A.E., Snijder, E.J., 1995. Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases. J. Virol. 69 (7), 4500–4505.
Done, S.H., Paton, D.J., 1995. Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet. Rec. 136 (2), 32–35.
Ellingson, J.S., Wang, Y., Layton, S., Ciacci-Zanella, J., Roof, M.B., Faaberg, K.S., 2010. Vaccine efficacy of porcine reproductive and respiratory syndrome virus chimeras. Vaccine 28 (14), 2679–2686.
Faaberg, K.S., Hocker, J.D., Erdman, M.M., Harris, D.L., Nelson, E.A., Torremorell, M., Plagemann, P.G., 2006. Neutralizing antibody responses of pigs infected with natural GP5 N-glycan mutants of porcine reproductive and respiratory syndrome virus. Viral Immunol. 19 (2), 294–304.
Fang, Y., Christopher-Hennings, J., Brown, E., Liu, H., Chen, Z., Lawson, S.R., Breen, R., Clement, T., Gao, X., Bao, J., Knudsen, D., Daly, R., Nelson, E., 2008. Development of genetic markers in the non-structural protein 2 region of a US type 1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. J. Gen. Virol. 89 (Pt 12), 3086–3096.
Fang, Y., Rowland, R.R., Roof, M., Lunney, J.K., Christopher-Hennings, J., Nelson, E.A., 2006. A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region. J. Virol. 80 (23), 11447–11455.
Flores-Mendoza, L., Silva-Campa, E., Resendiz, M., Osorio, F.A., Hernandez, J., 2008. Porcine reproductive and respiratory syndrome virus infects mature porcine dendritic cells and up-regulates interleukin-10 production. Clin. Vaccine Immunol. 15 (4), 720–725.
Gilliet, M., Cao, W., Liu, Y.J., 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat. Rev. Immunol. 8 (8), 594–606.
Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., Snijder, E.J., 2006. Nidovirales: evolving the largest RNA virus genome. Virus Res. 117 (1), 17–37.
Gudmundsdottir, I., Risatti, G.R., 2009. Infection of porcine alveolar macrophages with recombinant chimeric porcine reproductive and respiratory syndrome virus: effects on cellular gene transcription and virus growth. Virus Res. 145 (1), 145–150.
Haijema, B.J., Volders, H., Rottier, P.J., 2004. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J. Virol. 78 (8), 3863–3871.
Huang, Y.W., Dryman, B.A., Li, W., Meng, X.J., 2009a. Porcine DC-SIGN: molecular cloning, gene structure, tissue distribution and binding characteristics. Dev. Comp. Immunol. 33 (4), 464–480.
Huang, Y.W., Fang, Y., Meng, X.J., 2009b. Identification and characterization of a porcine monocytic cell line supporting porcine reproductive and respiratory syndrome virus (PRRSV) replication and progeny virion production by using an improved DNA-launched PRRSV reverse genetics system. Virus Res. 145 (1), 1–8.
Huang, Y.W., Meng, X.J., 2009. Identification of a porcine DC-SIGN-related C-type lectin, porcine CLEC4G (LSECtin), and its order of intron removal during splicing: comparative genomic analyses of the cluster of genes CD23/CLEC4G/DC-SIGN among mammalian species. Dev. Comp. Immunol. 33 (6), 747–760.
Ito, S., Ansari, P., Sakatsume, M., Dickensheets, H., Vazquez, N., Donnelly, R.P., Larner, A.C., Finbloom, D.S., 1999. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma-induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 93 (5), 1456–1463.
Jiang, P., Jiang, W., Li, Y., Wu, S., Xu, J., 2004. Humoral immune response induced by oral administration of S. typhimurium containing a DNA vaccine against porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 102 (3), 321–328.
Jiang, W., Jiang, P., Wang, X., Li, Y., Du, Y., 2008. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus Res. 136 (1–2), 50–57.
Jiang, Y., Fang, L., Xiao, S., Zhang, H., Pan, Y., Luo, R., Li, B., Chen, H., 2007. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine 25 (3), 547–560.
Jiang, Y., Xiao, S., Fang, L., Yu, X., Song, Y., Niu, C., Chen, H., 2006. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine 24 (15), 2869–2879.
Kapur, V., Elam, M.R., Pawlovich, T.M., Murtaugh, M.P., 1996. Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J. Gen. Virol. 77 (Pt 6), 1271–1276.
Key, K.F., Haqshenas, G., Guenette, D.K., Swenson, S.L., Toth, T.E., Meng, X.J., 2001. Genetic variation and phylogenetic analyses of the ORF5 gene of acute porcine reproductive and respiratory syndrome virus isolates. Vet. Microbiol. 83 (3), 249–263.
Key, K.F., Guenette, D.K., Yoon, K.J., Halbur, P.G., Toth, T.E., Meng, X.J., 2003. Development of a heteroduplex mobility assay to identify field isolates of porcine reproductive and respiratory syndrome virus with nucleotide sequences closely related to those of modified live-attenuated vaccines. J. Clin. Microbiol. 41 (6), 2433–2439.
Kim, D.Y., Calvert, J.G., Chang, K.O., Horlen, K., Kerrigan, M., Rowland, R.R., 2007. Expression and stability of foreign tags inserted into nsp2 of porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 128 (1–2), 106–114.
Kim, D.Y., Kaiser, T.J., Horlen, K., Keith, M.L., Taylor, L.P., Jolie, R., Calvert, J.G., Rowland, R.R., 2009. Insertion and deletion in a non-essential region of the nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome (PRRS) virus: effects on virulence and immunogenicity. Virus Genes 38 (1), 118–128.
Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M., Stockhofe-Zurwieden, N., 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27 (28), 3704–3718.
Kwon, B., Ansari, I.H., Osorio, F.A., Pattnaik, A.K., 2006. Infectious clone-derived viruses from virulent and vaccine strains of porcine reproductive and respiratory syndrome virus mimic biological properties of their parental viruses in a pregnant sow model. Vaccine 24 (49–50), 7071–7080.
Kwon, B., Ansari, I.H., Pattnaik, A.K., Osorio, F.A., 2008. Identification of virulence determinants of porcine reproductive and respiratory syndrome virus through construction of chimeric clones. Virology 380 (2), 371–378.
Labarque, G., Reeth, K.V., Nauwynck, H., Drexler, C., Van Gucht, S., Pensaert, M., 2004. Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22 (31–32), 4183–4190.
Lee, C., Calvert, J.G., Welch, S.K., Yoo, D., 2005. A DNA-launched reverse genetics system for porcine reproductive and respiratory syndrome virus reveals that homodimerization of the nucleocapsid protein is essential for virus infectivity. Virology 331 (1), 47–62.
Liu, B., Woltman, A.M., Janssen, H.L., Boonstra, A., 2009. Modulation of dendritic cell function by persistent viruses. J. Leukoc. Biol. 85 (2), 205–214.
Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Vet. Immunol. Immunopathol. 102 (3), 155–163.
Loving, C.L., Brockmeier, S.L., Sacco, R.E., 2007. Differential type I interferon activation and susceptibility of dendritic cell populations to porcine arterivirus. Immunology 120 (2), 217–229.
Lunney, J.K., Fritz, E.R., Reecy, J.M., Kuhar, D., Prucnal, E., Molina, R., Christopher-Hennings, J., Zimmerman, J., Rowland, R.R., 2010. Interleukin-8, interleukin-1beta, and interferon-gamma levels are linked to PRRS virus clearance. Viral Immunol. 23 (2), 127–134.
Lv, J., Zhang, J., Sun, Z., Liu, W., Yuan, S., 2008. An infectious cDNA clone of a highly pathogenic porcine reproductive and respiratory syndrome virus variant associated with porcine high fever syndrome. J. Gen. Virol. 89 (Pt 9), 2075–2079.
Mahalingam, S., Lidbury, B.A., 2002. Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NF-kappa B) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. Proc. Natl. Acad. Sci. U.S.A. 99 (21), 13819–13824.
Mateu, E., Diaz, I., 2008. The challenge of PRRS immunology. Vet. J. 177 (3), 345–351.
McCullough, K.C., Summerfield, A., 2009. Targeting the porcine immune system—particulate vaccines in the 21st century. Dev. Comp. Immunol. 33 (3), 394–409.
Meng, X.J., Paul, P.S., Halbur, P.G., 1994. Molecular cloning and nucleotide sequencing of the 3′-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75 (Pt 7), 1795–1801.
Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch. Virol. 140 (4), 745–755.
Meng, X.J., Paul, P.S., Morozov, I., Halbur, P.G., 1996. A nested set of six or seven subgenomic mRNAs is formed in cells infected with different isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 77 (Pt 6), 1265–1270.
Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74 (4), 309–329.
Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. Am. J. Vet. Res. 59 (12), 1540–1544.
Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31 (1), 11–21.
Meulenberg, J.J., Bos-de Ruijter, J.N., van de Graaf, R., Wensvoort, G., Moormann, R.J., 1998. Infectious transcripts from cloned genome-length cDNA of

porcine reproductive and respiratory syndrome virus. J. Virol. 72 (1), 380–387.

Meulenberg, J.J., Petersen den Besten, A., de Kluyver, E., van Nieuwstadt, A., Wensvoort, G., Moormann, R.J., 1997. Molecular characterization of Lelystad virus. Vet. Microbiol. 55 (1–4), 197–202.

Meulenberg, J.J., Petersen-den Besten, A., De Kluyver, E.P., Moormann, R.J., Schaaper, W.M., Wensvoort, G., 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology 206 (1), 155–163.

Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol. 15 (4), 533–547.

Nedialkova, D.D., Ulferts, R., van den Born, E., Lauber, C., Gorbalenya, A.E., Ziebuhr, J., Snijder, E.J., 2009. Biochemical characterization of arterivirus nonstructural protein 11 reveals the nidovirus-wide conservation of a replicative endoribonuclease. J. Virol. 83 (11), 5671–5682.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73 (1), 270–280.

Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J., 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc. 227 (3), 385–392.

Neumann, G., Whitt, M.A., Kawaoka, Y., 2002. A decade after the generation of a negative-sense RNA virus from cloned cDNA—what have we learned? J. Gen. Virol. 83 (Pt 11), 2635–2662.

Nfon, C.K., Dawson, H., Toka, F.N., Golde, W.T., 2008. Langerhans cells in porcine skin. Vet. Immunol. Immunopathol. 126 (3–4), 236–247.

Nielsen, H.S., Liu, G., Nielsen, J., Oleksiewicz, M.B., Botner, A., Storgaard, T., Faaberg, K.S., 2003. Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. J. Virol. 77 (6), 3702–3711.

Oleksiewicz, M.B., Botner, A., Toft, P., Normann, P., Storgaard, T., 2001. Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. J. Virol. 75 (7), 3277–3290.

Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J. Virol. 76 (23), 11837–11844.

Ortego, J., Sola, I., Almazan, F., Ceriani, J.E., Riquelme, C., Balasch, M., Plana, J., Enjuanes, L., 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences *in vivo* virus replication and virulence. Virology 308 (1), 13–22.

Osorio, F.A., Galeota, J.A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F., Laegreid, W.W., 2002. Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology 302 (1), 9–20.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76 (9), 4241–4250.

Palucka, K., Banchereau, J., 1999. Dendritic cells: a link between innate and adaptive immunity. J. Clin. Immunol. 19 (1), 12–25.

Pirzadeh, B., Dea, S., 1997. Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J. Gen. Virol. 78 (Pt 8), 1867–1873.

Plagemann, P.G., 2004a. GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. Virus Res. 102 (2), 225–230.

Plagemann, P.G., 2004b. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 102 (3), 263–275.

Plagemann, P.G., Rowland, R.R., Faaberg, K.S., 2002. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147 (12), 2327–2347.

Revilla, C., Poderoso, T., Martinez, P., Alvarez, B., Lopez-Fuertes, L., Alonso, F., Ezquerra, A., Dominguez, J., 2009. Targeting to porcine sialoadhesin receptor improves antigen presentation to T cells. Vet. Res. 40 (3), 14.

Richt, J.A., Garcia-Sastre, A., 2009. Attenuated influenza virus vaccines with modified NS1 proteins. Curr. Top. Microbiol. Immunol. 333, 177–195.

Ropp, S.L., Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg, K.S., 2004. Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. J. Virol. 78 (7), 3684–3703.

Rowland, R.R., 2007. The stealthy nature of PRRSV infection: the dangers posed by that ever-changing mystery swine disease. Vet. J. 174 (3), 451.

Royaee, A.R., Husmann, R.J., Dawson, H.D., Calzada-Nova, G., Schnitzlein, W.M., Zuckermann, F.A., Lunney, J.K., 2004. Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. Vet. Immunol. Immunopathol. 102 (3), 199–216.

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., 2009. Regulatory T cells: how do they suppress immune responses? Int. Immunol. 21 (10), 1105–1111.

Sang, Y., Ross, C.R., Rowland, R.R., Blecha, F., 2008. Toll-like receptor 3 activation decreases porcine arterivirus infection. Viral Immunol. 21 (3), 303–313.

Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., Lu, H., Zhu, G., Jin, H., Jin, M., Huo, X., Qin, X., Yin, R., Li, C., Li, H., Li, Y., Han, Z., Chen, Y., 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine 25 (21), 4193–4202.

Shibata, I., Mori, M., Uruno, K., 1998. Experimental infection of maternally immune pigs with porcine reproductive and respiratory syndrome (PRRS) virus. J. Vet. Med. Sci. 60 (12), 1285–1291.

Silva-Campa, E., Cordoba, L., Fraile, L., Flores-Mendoza, L., Montoya, M., Hernandez, J., 2010. European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology 396 (2), 264–271.

Silva-Campa, E., Flores-Mendoza, L., Resendiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W., Hernandez, J., 2009. Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. Virology 387 (2), 373–379.

Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79 (Pt 5), 961–979.

Spilman, M.S., Welbon, C., Nelson, E., Dokland, T., 2009. Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. J. Gen. Virol. 90 (Pt 3), 527–535.

Stadejek, T., Oleksiewicz, M.B., Potapchuk, D., Podgorska, K., 2006. Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. J. Gen. Virol. 87 (Pt 7), 1835–1841.

Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449 (7161), 419–426.

Stober, C.B., Lange, U.G., Roberts, M.T., Alcami, A., Blackwell, J.M., 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine Leishmania major infection. J. Immunol. 175 (4), 2517–2524.

Storgaard, T., Oleksiewicz, M., Botner, A., 1999. Examination of the selective pressures on a live PRRS vaccine virus. Arch. Virol. 144 (12), 2389–2401.

Tacken, P.J., de Vries, I.J., Gijzen, K., Joosten, B., Wu, D., Rother, R.P., Faas, S.J., Punt, C.J., Torensma, R., Adema, G.J., Figdor, C.G., 2005. Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. Blood 106 (4), 1278–1285.

Thanawongnuwech, R., Thacker, B., Halbur, P., Thacker, E.L., 2004. Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Clin. Diagn. Lab. Immunol. 11 (5), 901–908.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One 2 (6), e526.

Tian, X., Lu, G., Gao, F., Peng, H., Feng, Y., Ma, G., Bartlam, M., Tian, K., Yan, J., Hilgenfeld, R., Gao, G.F., 2009. Structure and cleavage specificity of the chymotrypsin-like serine protease (3CLSP/nsp4) of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). J. Mol. Biol. 392 (4), 977–993.

Tirado, S.M., Yoon, K.J., 2003. Antibody-dependent enhancement of virus infection and disease. Viral Immunol. 16 (1), 69–86.

Toka, F.N., Suvas, S., Rouse, B.T., 2004. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. J. Virol. 78 (23), 13082–13089.

Truong, H.M., Lu, Z., Kutish, G.F., Galeota, J., Osorio, F.A., Pattnaik, A.K., 2004. A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the *in vivo* virulence and transmissibility properties of the parental virus. Virology 325 (2), 308–319.

Van Breedam, W., Van Gorp, H., Zhang, J.Q., Crocker, P.R., Delputte, P.L., Nauwynck, H.J., 2010. The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. PLoS Pathog. 6 (1), e1000730.

Van Gorp, H., Van Breedam, W., Delputte, P.L., Nauwynck, H.J., 2008. Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 89 (Pt 12), 2943–2953.

van Kooyk, Y., Geijtenbeek, T.B., 2003. DC-SIGN: escape mechanism for pathogens. Nat. Rev. Immunol. 3 (9), 697–709.

Vanderheijden, N., Delputte, P.L., Favoreel, H.W., Vandekerckhove, J., Van Damme, J., van Woensel, P.A., Nauwynck, H.J., 2003. Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. J. Virol. 77 (15), 8207–8215.

Wang, X., Eaton, M., Mayer, M., Li, H., He, D., Nelson, E., Christopher-Hennings, J., 2007. Porcine reproductive and respiratory syndrome virus productively infects monocyte-derived dendritic cells and compromises their antigen-presenting ability. Arch. Virol. 152 (2), 289–303.

Wang, Y., Liang, Y., Han, J., Burkhart, K.M., Vaughn, E.M., Roof, M.B., Faaberg, K.S., 2008. Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence. Virology 371 (2), 418–429.

Wathelet, M.G., Orr, M., Frieman, M.B., Baric, R.S., 2007. Severe acute respiratory syndrome coronavirus evades antiviral signaling: role of nsp1 and rational design of an attenuated strain. J. Virol. 81 (21), 11620–11633.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., et al., 1991. Mystery

swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13 (3), 121–130.

Wissink, E.H., Kroese, M.V., van Wijk, H.A., Rijsewijk, F.A., Meulenberg, J.J., Rottier, P.J., 2005. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J. Virol. 79 (19), 12495–12506.

Wongyanin, P., Buranapraditkun, S., Chokeshai-Usaha, K., Thanawonguwech, R., Suradhat, S., 2010. Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Immunol. Immunopathol. 133 (2–4), 170–182.

Wu, L., KewalRamani, V.N., 2006. Dendritic-cell interactions with HIV: infection and viral dissemination. Nat. Rev. Immunol. 6 (11), 859–868.

Yang, L., Frey, M.L., Yoon, K.J., Zimmerman, J.J., Platt, K.B., 2000. Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. Arch. Virol. 145 (8), 1599–1619.

Yoo, D., Welch, S.K., Lee, C., Calvert, J.G., 2004. Infectious cDNA clones of porcine reproductive and respiratory syndrome virus and their potential as vaccine vectors. Vet. Immunol. Immunopathol. 102 (3), 143–154.

Yoon, K.J., Wu, L.L., Zimmerman, J.J., Hill, H.T., Platt, K.B., 1996. Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol. 9 (1), 51–63.

Yoon, K.J., Wu, L.L., Zimmerman, J.J., Platt, K.B., 1997. Field isolates of porcine reproductive and respiratory syndrome virus (PRRSV) vary in their susceptibility to antibody dependent enhancement (ADE) of infection. Vet. Microbiol. 55 (1–4), 277–287.

Zhou, L., Zhang, J., Zeng, J., Yin, S., Li, Y., Zheng, L., Guo, X., Ge, X., Yang, H., 2009. The 30-amino-acid deletion in the Nsp2 of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China is not related to its virulence. J. Virol. 83 (10), 5156–5167.

Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81 (Pt 4), 853–879.

Zimmerman, J.J., Benfield, D.A., Murtaugh, M.P., Osorio, F., Stevenson, G.W., Torremorell, 2006. Porcine reproductive and respiratory syndrome virus (porcine arterivirus). In: Straw, B.J., et al. (Eds.), Diseases of Swine, 9th edition. Blackwell Publishing Ltd, Oxford, pp. 387–418.

Zust, R., Cervantes-Barragan, L., Kuri, T., Blakqori, G., Weber, F., Ludewig, B., Thiel, V., 2007. Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the rational design of coronavirus vaccines. PLoS Pathog. 3 (8), e109.