

Review

Reappraisal of PRRS control strategies: the way forward

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Abstract

The control of Porcine Reproductive and Respiratory Syndrome (PRRS) is still a major issue worldwide in the pig farming sector. Despite extensive research efforts and the practical experience gained so far, the syndrome still heavily affects farmed pigs worldwide and challenges established beliefs in veterinary virology and immunology. The clinical and economic repercussions of PRRS are based on concomitant, additive features of virus pathogenicity, host susceptibility and influence of environmental, microbial and non-microbial stressors. This makes a case for integrated, multi-disciplinary research efforts in which the three types of contributing factors are critically evaluated toward the development of successful disease control strategies. These could be definitely eased by the definition of reliable markers of disease risk and virus pathogenicity. As for the host's susceptibility to PRRSV infection and disease onset, the roles of both innate and adaptive immune responses are still ill-defined. In particular, the overt discrepancy between passive and active immunity and the uncertain role of adaptive immunity vis-à-vis an established PRRSV infection should prompt the scientific community to the development of novel research schemes, in which apparently diverging and contradictory findings could be reconciled, and eventually brought to a satisfactory conceptual framework.

Keywords: Pig; PRRS; PRRS virus; immune response; disease resistance; disease control

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) affects farmed pigs worldwide, and still causes heavy direct and indirect losses [1]. The syndrome emerged in the late 80s, in USA, and later on in Europe, and it eventually became enzootic in most countries among farmed pigs. Late-term reproductive failure in sows with transplacental transmission of the virus, preweaning mortality of piglets, respiratory distress, anorexia, and possible cutaneous hyperemia in weaners and growers are common clinical signs of PRRS [2].

The two swine Arteriviruses sustaining PRRS (PRRSV-1 and PRRSV-2) had been previously identified as European (EU) type I, with the first strain isolated in 1991 and named "Lelystad", and the North American (NA) type II, isolated in 1992 with the acronym ATCC VR-2332 [3]. Whereas PRRS virus (PRRSV) infections are largely prevalent in farmed swine, the repercussions may vary from asymptomatic to very serious clinical courses, often depending on pig age and production phase [2]. On the whole, strong experimental and circumstantial evidence shows that the clinical outcome of PRRSV infection is the product of three components: virus virulence, host susceptibility and environmental stressors [4]. Notably, PRRSV infection gave rise to subclinical courses over several decades, before PRRSV met the very susceptible, lean type, rapid growth pigs reared in western Europe [4].

Eradication of PRRS was shown to be possible on the basis of herd closure and strict biosafety control measures [5]; yet, the underlying costs, the logistics and infrastructure needed have so far prevented the large-scale adoption of this procedure. Accordingly, the control of PRRS is usually based upon a complex of integrated control measures aimed at „stability“, i.e. a condition in which clinical signs of PRRS are absent in the breeding-herd population, and PRRSV is no more transmitted from sows to their offspring [6]. In practice, swine farms aim to co-exist with PRRSV under conditions of minimal clinical fallout and productive losses altogether. In this respect, prevention of PRRSV infection of suckling piglets is a foundation of this control strategy, having in mind the much higher susceptibility of non-adult pigs to PRRSV [7].

The main risk factors underlying serious clinical outcomes of PRRSV infection are depicted in Figure 1. All of them are dealt with in the following sections.

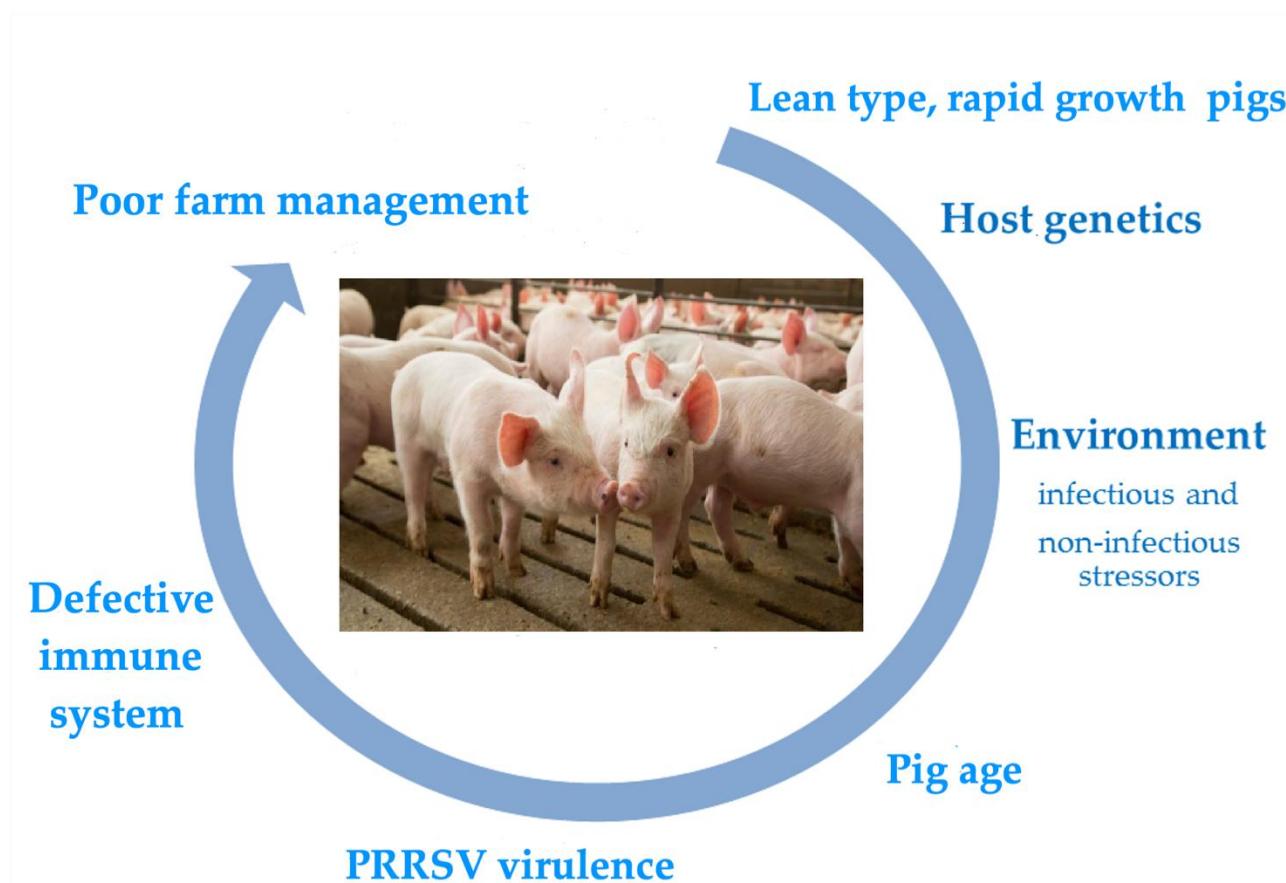


Figure 1. The figure depicts the main risk factors associated with serious clinical outcomes of PRRSV infection in farmed pigs. The rolling circle starts with the lean pig phenotype, which has underlain the clinical history of PRRS since the 80ies. The subsequent risk factors in the figure are not ordered on a time-related or weight basis. Pigs may be actually exposed to multiple risk factors with additive or synergistic final effects.

2. Biosecurity: a foundation of successful disease control strategies

After decades of research and field experience, biosecurity is still the foundation of PRRS control on farm, as detailed e.g. in the guidelines of the American Association of Swine Veterinarians (https://www.aasv.org/aasv/PRRSV_BiosecurityManual.pdf). This implies that farm management procedures aim to reduce PRRSV infectious pressure by a proper combination of “all in – all out” protocols, parity control,

limitation of cross-fostering, strict forward flow, quarantine for replacement sows and gilts, large-scale adoption of multi-site production units where pig groups are channeled throughout distinct production phases, giving rise to the so-called “batch management production systems (BMPS)”. These measures have been conducive to improved animal health standards compared with traditional farrow-to-finish herds, because recirculation of pathogens and microbial infectious pressure can be more easily controlled.

3. Acclimatization as second pillar of successful disease control on farm

In addition to that, PRRS stability demands a successful “acclimatization” of replacement gilts to the PRRSV strains circulating in the farm before the breeding period [8]. Pending the definition of reliable correlates of protection, “acclimatization” should be interpreted as a stepwise process of “adaptation” to field PRRSV strains, in which undefined innate and adaptive immune responses, down-regulation of permissiveness to PRRSV of pig macrophages [9] and, perhaps, “education” of macrophages to a better control of inflammatory responses by epigenetic mechanisms [10] concur to obtain a pig population experiencing PRRSV infection without serious clinical outcomes.

4. Which elements underlie successful disease control?

In hindsight, the above features related to disease control are definitely sobering. They teach us that: (A) the extent of microbial infectious pressure resulting from farm biosafety profiles, and (B) the previous “education” of the immune system are both pivotal to successful disease control.

In a wider perspective, which includes the research efforts made so far, four points seem to be of paramount importance toward an effective control of PRRS:

- The selection of disease-resistant pig phenotypes, differing from the lean type, highly susceptible ones [11]. The high levels of oxidative stress in such pigs [12] are likely to exacerbate the inflammatory responses to infectious and non-infectious stressors and, in particular, the noxious synergism between bacterial LPS and PRRSV infection [13]. This is probably a point of some importance, since LPS can be also inhaled at high concentrations in pig herds [14], and circumstantial evidence on farm showed clinical improvement in PRRSV-infected groups after reduction of the animals’ concentration in outdoor weaning cages. The results of extensive studies on the genetic bases of disease resistance highlighted a single nucleotide polymorphism (SNP) marker that was strongly associated with weight gain and viral load after PRRSV infection, with a possible role of the interferon-induced guanylate-binding protein gene family [15]. Also, editing of the CD163 gene in pig zygotes was shown to be a valuable approach to generate PRRSV-resistant animals [16].
- Strict application of bio-safety measures toward a substantial reduction of microbial infectious pressure and chronic inflammatory responses, as well as outright “herd closure” strategies aimed at eradication [5].
- Higher standards of animal welfare to prevent chronic stress and stress-related immunosuppression [17].
- Active immune control, which may include in turn two distinct aspects: A) Development of innate and adaptive immune responses to PRRSV [18]. B) Reduced permissiveness of macrophages to PRRSV replication as a possible outcome of “trained immunity” [10], and/or of an inflammatory microenvironment affecting maturation of macrophage precursors [9].

On the whole, the first 3 points are commonly accepted, and relevant measures are pursued to varying extents in different parts of the world. Instead, how the immune control of PRRSV takes place is a highly contentious issue, which deserves due attention and, probably, new approaches toward credible translational prospects.

5. PRRSV evasion strategies: impact on vaccine performance

The dubious, inconclusive findings obtained in several studies on the immune response to PRRSV [19] are certainly related to a complex of outright decoy strategies displayed by PRRSV, highlighted in a previous review paper of ours [4]. These decoy strategies are mainly based on glycosylation of structural viral proteins expressing potential neutralizing epitopes [20], and non-structural viral proteins interacting with crucial check points of the innate immune response [21]. Two outcomes of the decoy strategies are of paramount importance: (A) PRRSV infection often leads to poor, late and irregular activation of the innate and adaptive immune response [19] and (B), PRRSV infection does not cause effective induction of cell-mediated immune responses [22,23]. Most important the time-course of PRRSV viremia does not seem to be significantly correlated with the time-course of antibody and cell-mediated immune responses [19]. As for the field data, these often show substantial discrepancies with the findings of experimental infections [24]. In addition to that, the performance of PRRS vaccines on farm may be worse than expected on the basis of experimental findings [25], and concerns about the effectiveness of PRRS vaccines were repeatedly expressed in the past [26]. Interestingly, subsequent “waves” of PRRSV infection can be demonstrated in the same pigs under field conditions, as opposed to what is commonly observed in experimental trials, in which re-infection of pigs with both homologous and heterologous PRRSV strains is quite difficult [27]. This makes a case for dubious reliability of experimental PRRSV infection and vaccination studies in isolation facilities, outside the usual complex of infectious and non-infectious stressors experienced by pigs under field conditions [24].

The regulation of the primary inflammatory response in macrophage precursors, unable to sustain PRRSV replication, is probably a further, fundamental decoy strategy of virulent PRRSV strains. A primary inflammatory response leads to the development of virus-resistant pig macrophages [9]. Accordingly, this kind of response is sustained by attenuated PRRSV strains, whereas it is inhibited to a different extent by the virulent ones [28]. Interestingly, this kind regulation is only observed *in vitro* under non-inflammatory conditions. Instead, in leukocytes previously exposed to inflammatory stimuli, virulent PRRSV strains enhance the inflammasome reaction and the IL-1beta response [28]. This is fully in line with *in vivo* findings of PRRSV infections leading to serious clinical outcomes: these are correlated with enhanced inflammatory cytokine responses, but not with the extent of viral replication [29]. These results hint at a major up-regulation of the inflammatory response following high-titered replication of PRRSV in virus-permissive pig macrophages and at a possible synergism with inflammatory stressors like LPS [13]. Instead, virulent PRRSV strains do not exert this activity in non-permissive cells; this makes sense in order to avoid any subsequent restriction of growth in differentiated pig macrophages [9]. Finally, our *in vitro* findings are in agreement with inflammatory cytokine gene expression in lymphoid tissues of PRRSV-infected pigs: early up-regulation of IL-1, IL-8 and IFN-gamma genes is correlated with successful virus clearance [30].

Interestingly, MicroRNAs (miRNAs) regulate PRRSV replication and infection. In particular, some miRNAs are reported to modulate host antiviral response; thus, miR-26a inhibits and miR-373 promotes the replication of PRRSV by up and down-regulating Type I IFN genes, respectively [31,32]. Also miR-382-5p [33] was found up-regulated in PRRSV infection, with a consequent inhibition of polyI:C-induced Type I IFN production after targeting heat shock protein 60.

The above findings can partly account for the unsatisfactory results of PRRS vaccines sometimes observed on farm. As stressed in our previous review paper [4], the performance of PRRS vaccines is often

unpredictable on farm and cannot be easily interpreted with the current dogmas that highlight nucleotide divergence and amino acid variability of field virus strains as a foundation of failure vs. success of vaccines [34]. Most important, common correlates of protection induced by vaccines have a dubious meaning in the PRRS model [35-37]. Also, despite extensive research in this area, limited translational prospects of next generation vaccines can be foreseen in the near future. In this scenario, interesting field data have been collected about a recently licensed live attenuated vaccine, validated for use in suckling piglets, too [38]. After injection into 1-day old piglets, the vaccine showed some clinical efficacy on farm, despite the presence of maternally-derived antibody and a concomitant infection of vaccinated piglets with a highly virulent field PRRSV strain [39]. This interesting model of “co-existence” of wild type and attenuated PRRSV might imply an outright competition for susceptible macrophages. In this scenario, the vaccine strain could possibly perform successful occupancy of critical macrophages niches and prevent the release of dangerous downstream inflammatory signals after wild type PRRSV infection. The better results obtained in 1-day old piglets compared with the 21-day old ones indirectly confirms the need for an early occupancy of the host’s macrophage compartment [39]. Needless to say, the validation of such a theory could benefit disease control in PRRS-unstable farms, characterized by extensive recirculation of virulent PRRSV strains in both sows and suckling piglets. Finally, the development of mucosal PRRS vaccines [40,41] might be pivotal to circumventing some bottlenecks of current vaccination protocols. In this respect, the possible advantages of a potent, mucosal IgA response for disease control (see chapter 9) could make a case for large-scale investigations into this crucial issue.

Virulence of PRRSV: are there reliable markers ?

In vivo, the early interferon (IFN)-alpha response has been described as an unfavorable prognostic marker in PRRSV-infected sows [42]. Also in our experience, an attenuated PRRSV strain gave rise to an early IFN-gamma response in weaners, as opposed to an early IFN-alpha response induced by a virulent PRRSV strain in the first week after infection [43] (see Figure 2). This feature should be viewed in our opinion in the framework of the so-called “Bad IFN-alpha response” [44], also observed e.g. in Classical Swine Fever cases [45] [46]. Diverse mechanisms (tissue damage, immunopathology, cell death) underlie the detrimental effects of inappropriate, excessive or mistimed Type I IFN responses [47]. Vice versa, effective immunomodulation in sows and piglets can be achieved by oral, low-dose IFN-alpha treatments during PRRS outbreaks [4]. The effector mechanisms of low-dose IFN-alpha were investigated in an *in vitro* model of pig tonsil cells [48]. This outlines once again the crucial roles of cytokine concentration and regional compartment in the clinical outcome of the host’s cytokine responses and cytokine-based treatments.

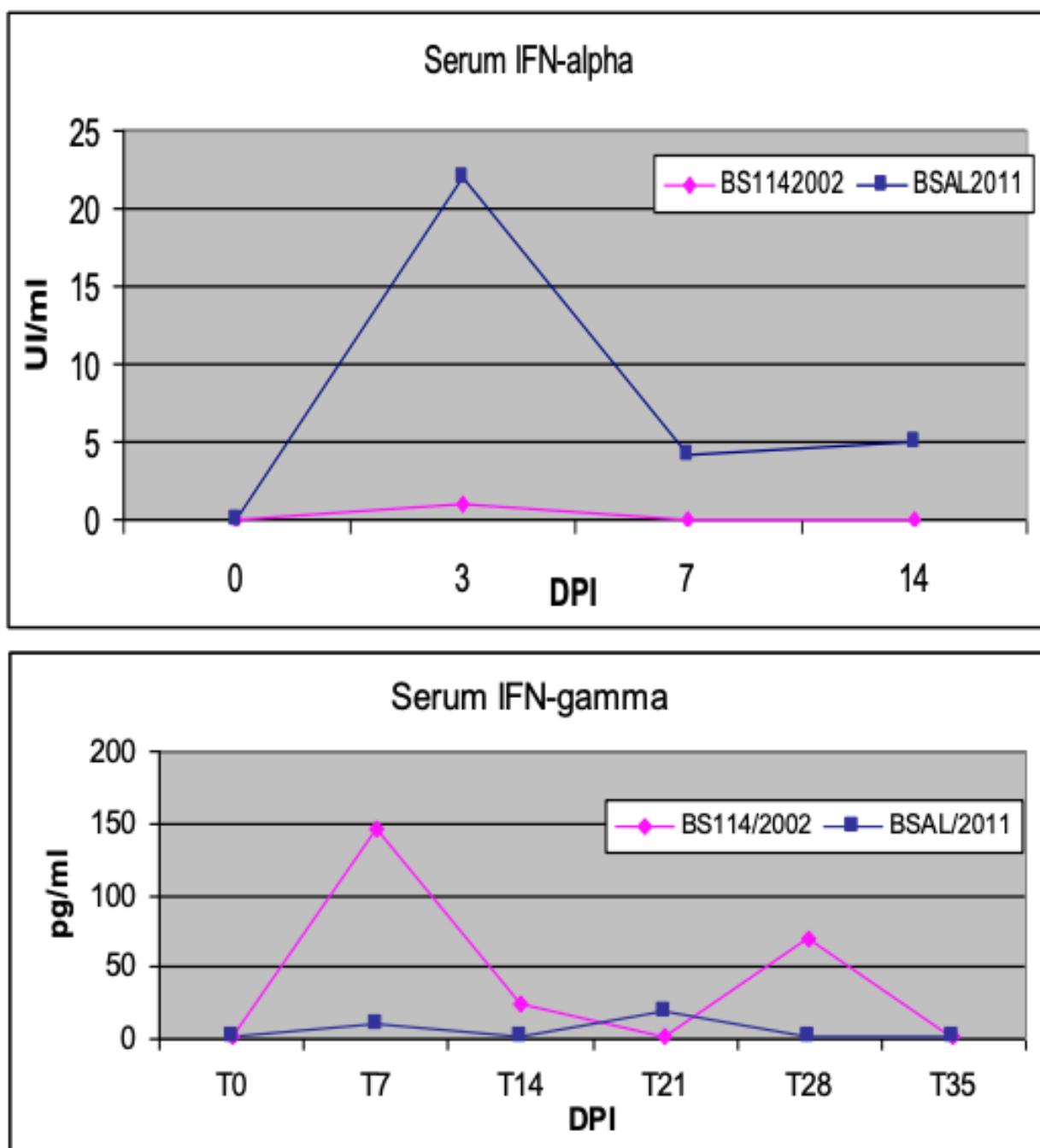


Figure 2. Early cytokine markers of PRRSV infection. Two groups of weaned pigs were intranasally infected with virulent BSAL/2011 and attenuated BS114/2002 PRRSV strains, respectively [43]. Blood serum samples were collected at the indicated days post infection (DPI). Swine IFN- α was measured in serum samples by a cpe-inhibition assay on MDBK cells with Vesicular Stomatitis Virus (VSV); the test was calibrated with a preparation of porcine recombinant IFN- α 1 (PBL Biomedical Laboratories, cat. 17100-1) [48]. Porcine IFN- γ was measured by ELISA with a couple of catcher and biotinylated tracer monoclonal antibodies, as previously described [25].

A second unfavorable marker is the late IL-10 response of PRRSV-infected pigs. In our aforementioned study [43], the plasma IL-10 response in the second week after infection was only observed in two pigs that died few days later. This had been also observed in a previous study of ours on breed-related disease resistance; the more serious clinical outcome of PRRSV infection in Large White pigs was correlated to a persisting, late IL-10 response [49], as opposed to the findings obtained in both Duroc and Landrace pigs. This can be possibly explained in terms of a pro-inflammatory gain of IL-10 within an established inflammatory environment, as previously shown in human models of endotoxemia [50] and Crohn's disease [51]. How can the IL-10 response be reasonably accounted for in the PRRS scenario? In this respect, we have some reasons to postulate a central role of plasmacytoid dendritic cells (pDCs). Also in pigs, these cells can release huge amounts of IFN-alpha following exposure to viral agents [52], including many PRRSV strains [53]. In turn, IFN-alpha can induce high-titered release of IL-10 in LPS-stimulated monocytes and CD4+ T cells [54]. This is probably the mechanism underlying the IL-10 response *in vitro* of swine PBMC to some PRRSV strains [28,55]. Finally, IFN-alpha and IL-10 can promote the differentiation of Type I T regulatory (T reg) cells [56]. The differentiation of T reg cells is promoted by PRRSV-infected dendritic cells, and this was implied as possible cause of virus-driven immunosuppression [57]. This makes a case for new studies into the possible PRRSV / pDCs / IFN alpha / IL-10 loop from *ex vivo* samples of PRRSV-infected pigs.

A recent study [58] suggested to investigate the Wnt/β-catenin signaling pathway to obtain information into virus-host interactions and virus pathogenicity. Indeed PRRSV-infected cells show accumulation of β-catenin in the nucleus; the activation of the Wnt pathway could be caused by PRRSV nonstructural proteins (Nsps) 1α, 1β, 3, 4, 7, 10, and 12. This activation tends to inhibit PRRSV replication by enhancing the NF-κB-dependent innate immune response. Accordingly, PRRSV strains that inhibit the Wnt pathway are probably more pathogenic than those that exalt the same pathway [59].

6. What can we learn from other models of immune response to Arterivirus infection ?

The members of genus Arterivirus include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, simian hemorrhagic fever virus (SHFV) and PRRSV. The infection is strictly species-specific; however, these viruses share many common properties like the ability to establish persistent infections [60].

The dubious results of the studies on the adaptive immune response to PRRSV are consistent with similar findings about other animal Arteriviruses. As recalled in our previous review paper [4], in the murine Arterivirus model there is no difference in terms of viremia between immunocompetent and tolerant mice [61], which substantially detracts from an important role of the adaptive immune response.

Furthermore, neutralizing antibodies to EAV, PRRSV (both North American and European) and LDV are often specific to GP5 major envelope glycoprotein encoded by ORF5. The GP5 proteins of these viruses are similar for size (199–255 amino acids) and location, and major neutralization determinants are included in the N-terminal ectodomain. Like PRRSV, EAV can determine a persistent infection in male reproductive tissues without clinical manifestations. This guarantees shedding in semen and sexual transmission despite the onset of neutralizing antibody responses [62].

The cell-mediated immune response (CMI) to equine arteritis virus has not been well characterized. It is known that specific CTL precursors may persist for at least 1 year after infection and CD8+ T-cell-mediated cytotoxicity is virus strain-specific and genetically restricted. In LDV infection, specific CD4+ and CD8+ T-cell responses do not lead to virus clearance [63].

7. Is there an effective antibody response to PRRSV ?

The role of the antibody response to PRRS virus is highly contentious. The issue of target proteins of neutralizing antibody (NA) and relevant neutralizing epitopes has stimulated several studies. Thus, neutralizing GP5-specific monoclonal Abs have been isolated from sera of hyperimmune sows [64], confirming the presence of neutralizing epitopes on GP5 glycoprotein [65,66]. Neutralizing epitopes has been observed also on the minor surface glycoproteins GP2, GP3, and GP4 [67]. The isolation of specific monoclonal Abs against these proteins will likely uncover additional neutralizing or potentially broadly neutralizing antibodies [64]. It has been observed that also the nsp2 protein contributes to the neutralizing activity of the structural proteins GP5-M *in vitro*, suggesting that there is at least one neutralizing epitope in nsp2 or in the spatial structure formed by nsp2 and structural proteins together [68]. The target of neutralizing antibodies is a relevant field of research. In this regard, an experimental system to enable isolation of PRSSV-specific monoclonal Abs has been recently developed [69]. An important step forward could be the administration of neutralizing monoclonal Abs using the mRNA technology [70]. This technology has been studied toward a possible mRNA-based therapy in swine [71,72], and mRNA-encoded antibody has been successfully investigated as a means of protecting against HIV or rabies virus infections [73,74]. In the future it could be a useful tool for the possible treatment of infectious animal diseases including PRRS, where the virus has developed outright decoy strategies to prevent Ab binding and neutralization [20].

As for the role of antibodies in protective immunity, this was advocated in studies of passive immunization of sows, even though the immune serum did not prevent PRRSV replication in target tissues in young weaned pigs nor transmission to susceptible animals [75,76]. These results on sows were confirmed in another study on intraperitoneal administration of purified, neutralizing, PRRSV-specific antibody in 3-week old piglets; this implied a reduction of viremia levels after challenge infection with both homologous and heterologous PRRSV strains [77]. Yet, no difference in terms of clinical course and average daily weight gain was observed between antibody-treated and control pigs [77]. Also, a commercial inactivated vaccine was shown to evoke a vigorous post-challenge anamnestic NA response and no protection [35], and long-term persistence of PRRSV viremia may be possible in the presence of neutralizing antibodies [37]. Finally, high-titered antibody responses to PRRSV can be even correlated with a worse clinical outcome of PRRS [29], as also reported following administration of DNA-based PRRS vaccines and challenge infection [36]. This is in agreement with previous studies showing a role of some PRRSV-specific IgG antibodies in Antibody-dependent Enhancement (ADE) of infection [78]. On the whole, some protection is afforded by antibody following passive immunization, but there is little if any evidence of Ab-mediated protection following vaccination and PRRSV infection. This is a point of major importance which is difficult to reconcile with current concepts about the adaptive immune response, and which possibly demands a new relevant conceptual framework. Interestingly, a discrepancy between passive and active immunity was also evidenced in case of African Swine Fever Virus (ASFV) infection. Whereas the correlates of protection to an established ASFV infection are still ill-defined [79], a study suggested that colostrum/milk from sows that survived ASFV infection had a protective effect in their offspring in terms of reduced viremia and clinical signs in response to ASFV challenge [80]. Please notice however that absorption of colostrum and milk from sows also implies the passage of leukocytes, including T lymphocytes, and the transfer of relevant effector functions [81]. Thus, protection cannot be unambiguously referred to immunoglobulins in case of colostral immunity.

Beyond the aspects of cell-mediated immunity, passive transfer of immunoglobulins brings about Ig-driven immunoregulatory control actions other than the provision of specific antibody to viral agents. These were clearly evidenced in human transfused patients: immune sera would lead to a control of the inflammatory response based on monomeric IgA. As opposed to IgA immunocomplexes, free monomeric IgA underlie a potent control circuit based on their interaction with Fc α RI (CD89) on myeloid cells; after the

contact, CD89 binds to the ITAM sequence of Fc gamma chain subunit and recruits a tyrosine phosphatase without activating any downstream kinases [82]. The outcome is very clear: free monomeric IgA dampen the response of activated granulocytes and prevent complement deposition; they also inhibit Th17 and IFN-gamma responses while promoting Treg development [83]. "Switch-off" of the Th17 response has been repeatedly observed in human patients after endovenous administration of Ig [84].

On the whole, all the experiments based on passive transfer of immune serum / plasma should be critically evaluated because of the side effects of components with a potential to affect the "cytokine storm" during viral diseases.

8. The IgA puzzle

As opposed to the aforementioned, contradictory findings about the serum antibody response to PRRSV, the peak of IgA mucosal antibody response was clearly associated in our experience to a block of PRRSV shedding in oral fluids (OF) [85]. Can this finding be reconciled with the above properties of IgA? The answer is reasonably affirmative. Mucosal IgA is mostly dimeric. Free dimeric IgA has limited affinity for CD89 [82]. On the contrary, IgA-virus immunocomplexes have a high affinity for CD89 and give rise to full activation of ITAM in the adjoining gamma-chain subunit of Fc gamma receptor, followed by a strong inflammatory response of myeloid cells [82]. Most important, the inflammatory phenotype of macrophages is highly correlated with non-permissiveness for PRRSV replication [9]. This could be reasonably the added value of the mucosal IgA response to PRRSV, possibly more important than the direct antiviral effector functions of IgA.

Therefore, the presence of IgA-PRRSV complexes is conducive to a control of PRRSV infection, whereas IgG-PRRSV complexes may be even associated with antibody-dependent enhancement (ADE) [78]. Accordingly, oral fluids (OF) samples with moderate IgA Ab titers to PRRSV cause yield reduction of PRRSV replication in MO cultures, as opposed to OF samples with little if any IgA antibody to PRRSV [86]. Interestingly, a balance in OF between IgG and IgA antibody response to PRRSV is not observed over several weeks in PRRS-unstable herds experiencing overt clinical cases; once a balance is observed, virus shedding in OF comes to an end despite ongoing viremia [85]. This makes a case for an important role of antiviral Ig isotypes and their molar ratios in shaping an effective control of PRRSV infection in tissues. In this respect, direct antiviral, neutralizing effects of IgA might be even exerted in the intracellular compartment, in agreement with the Influenza virus model [87].

Moreover, local T cell responses, measured in lungs, bronchioalveolar lavages and bronchial lymph nodes, are induced faster than systemic responses and are maintained at significantly higher levels, even after virus clearance in experimentally infected pigs, substantiating a possible role of local immune responses in the clearance of PRRSV from pigs [88].

9. Cell-mediated immunity to PRRSV: what are we measuring?

The studies on PRRSV-specific, cell-mediated immunity (CMI) provided conflicting results. Three points are of paramount importance: A) There is uncertainty as to whether PRRSV proteins are effectively presented to the immune system [22]. B) There is no correlation between the time-course of adaptive immunity and resolution of viremia in PRRSV-infected pigs [19]. C) PRRSV infection causes a strong inhibition of Ag presentation in macrophages [89] and can give rise to a potent induction of suppressor T reg cells [57]. Also, transient depletion of CD8+ T cells does not exacerbate PRRSV infection, and no effect on the ability to clear the virus can be highlighted [90]. Having in mind these fundamental findings, the observed responses should be viewed with some caution. In swine infected by PRRSV, CMI responses are characterized

by IFN gamma-secreting, CD8+ and CD4+/CD8+ double-positive T cells, detectable 2-3 weeks post-infection and showing an erratic behavior [91].

Accordingly, several groups reported on the demonstration of PRRSV-specific, IFN-gamma secreting cells (SCs) by ELISPOT assays using tissue culture-adapted PRRSV as stimulating agent [37,92]. Yet, the correlation between the extent of this response and protection of sows is definitely ill-defined [93]. As a matter of fact, this approach may be affected by concomitant, non-specific IFN-gamma responses to the stress antigens carried by the established cell lines in which PRRSV is grown [94]. This is the reason why we have always employed a control Ag, i.e. a cryo-lysate of uninfected cells processed exactly as raw PRRSV, and subtracted this response from the virus-specific one in both ELISPOT and whole blood cytokine release assays [94]; this way, the PRRSV-specific IFN-gamma response proved transient and low-titered in our experience, following field PRRSV infection [85]. Interestingly, such a response is probably absent in PRRS-unstable herds, except in suckling piglets [85], as a possible activity of maternally-derived immune cells, in agreement with a previous study [81].

10. Natural Killer (NK) cells: a missing link?

The possible role of NK cells in PRRSV infection was highlighted in our previous review paper [4]. Why could NK cells actually play a crucial role? First, NK cells can rapidly recognize virus-induced changes in virus-infected cells as both missing and induced self [95]. Most important, they can mount an early IFN-gamma response, which can suppress TLR-mediated IL-10 production and inhibit expression of CD163 in macrophages [96], thus regulating the susceptibility of cells to PRRSV infection [96]. In this respect, we can surmise that the crucial role of the IFN-gamma response *in vivo* is probably related to a block of PRRSV replication in macrophages. As for NK cells, the early IFN-gamma response to PRRSV infection [97] and the infiltration of CD3-, CD8+, allegedly NK cells in the PRRSV-positive endometrium [98] are in line with an important role of these cells, which undoubtedly deserves further studies *in vitro* and *in vivo*. On the other hand, there is also evidence of impaired NK cell cytotoxicity following PRRSV infection [99], which might bear on the potential role of NK cells in virus clearance.

11. Theoretical strength and weakness of the “trained immunity” model in PRRS

The uncertain role of the adaptive immune response to PRRSV should lead to a major re-appraisal of innate immunity. In particular, within the innate immune response the epigenetic features underlying “trained immunity” [10] could be of crucial importance; they might even lead to a convincing explanation of some contradictory findings in experimental studies and field trials. Some questions do need an answer. For instance, why is vaccine-induced immunity under experimental and field conditions so variable and sometimes disappointing [25,26]? How can a vaccine sometimes induce better protection to a heterologous strain [34] or be effective against a different Arterivirus (other PRRSV genotype) [100]? Which mechanisms underlie “acclimatization” of gilts and sows [85]? Is it a matter of adaptive immune response or sort of “habituation” of macrophages to field PRRSV strains?

Needless to say, one could postulate that both vaccines and exposure to field PRRSV strains induce major epigenetic changes in innate immunity genes of myeloid cells underlying the aforementioned status of “trained immunity”. This is also in line with the possible major role of NK cells, whose activity can be also prone to crucial epigenetic regulation [101]. In this respect, there is evidence of increased cytotoxicity against NK target cells after *in vitro* re-stimulation of PBMC from convalescent pigs with PRRS virus [102].

Interestingly, after infection with a highly virulent PRRSV strain, DNA from PBMC showed strong hypomethylation, as opposed to control pigs and pigs infected with an attenuated PRRSV strain (Amadori M; unpublished results). This makes a case for new studies into the status of chromatin in the promoter regions of genes involved in the innate immune response, with a careful match between macrophages of PRRS-naive, vaccinated and infected pigs. On the whole, the effects of such mechanisms could be two-fold: A) they could underlie non-permissiveness of pig macrophages to PRRSV infection after shift to a M1-like phenotype [9]; B) they could prevent a further major amplification of the inflammatory cytokine response, associated with adverse clinical outcome of PRRSV infection [29]. Although such a theory is in agreement with several experimental findings, it has to be demonstrated on a convincing experimental basis. This is potentially conflicting with the paucity of validated models of epigenetic re-arrangements of innate immunity genes in pigs.

Last, but not least, this area of investigation might be relevant to the crucial link between cell metabolism and PRRSV replication. PRRSV infection is suppressed when *de novo* synthesis of fatty acids is inhibited [103]. Interestingly, free fatty acids are low-affinity ligands of PPAR-g, which dampens the inflammatory response of macrophages [104]. In this respect, “trained immunity” implies a shift of cellular metabolism to anaerobic glycolysis [10], which affects the efficiency of *de novo* synthesis of fatty acids. As a matter of fact, induction of M1 macrophages with inflammatory stimuli leads to a profound shift of energy metabolism without *de novo* fatty acids synthesis [105], in a scenario of little if any susceptibility to PRRSV [9].

12. Crucial areas of investigation into the PRRSV-host relationship

Our studies *in vitro* [28] outlined the importance of the inflammasome response and IL-1beta production during PRRSV infection. In LPS-treated macrophages, PRRSV can enhance the inflammasome response by the small envelope protein E, giving rise to increased release of IL-1beta [106]. Such a regulatory action is counteracted by nsp11 [107]. This is in agreement with the observed kinetics of the inflammasome reaction during PRRSV infection, which shows rapid induction and decay [107]. This highlights the importance to investigate further such a crucial regulation by sequencing protein E and nsp 11 in reputedly virulent and attenuated PRRSV strains. In addition to that, deletions of nsp 2 could also play a role. These were observed e.g. in virulent Chinese PRRSV strains [108]. Interestingly, some nsp2 deletions were shown to reduce the expression of IL-1 beta [99]. This might be relevant to a potentially important decoy strategy of PRRSV, i.e. the inhibition of the primary inflammatory response in macrophage precursors, in order to prevent the differentiation of PRRSV-resistant, mature pig macrophages [28].

Concerning IL-10 and its role in PRRSV pathogenicity, it should be stressed that induction of IL-10 is correlated with the expression of PRRSV protein N [99]. Therefore, it could be worth comparing the N gene sequences of strains inducing or non-inducing IL-10 *in vitro*, to define further virulence markers.

13. Translational prospects of current studies: some open issues

The above sections outline the scope of reappraised PRRS control strategies. Contributions should be channeled into five main areas of discussion, underlying major issues with crucial translational repercussions. The five areas can be depicted as follows:

- **Farm management.** What is really pivotal to PRRS control on farm? Can we nowadays define “minimum requirements” of biosafety toward effective PRRS control? Can eradication be

sometimes cost-effective and sustainable in the long term? Which monitoring actions should be implemented toward effective surveillance? What about the roles of clinical inspection, post mortem examination and laboratory investigations? Can oral fluids and other unconventional organ specimens fully replace blood for PRRS surveillance? [109]

- **Genetic selection.** Can we postulate reasonable translational applications deriving from pig genetics studies? Have we really defined a set of critical genes underlying disease resistance? Does resistance entail clinical or virological protection, or both? Which role is played by the very high, constitutive oxidative stress in rapid growth, lean type pigs [12]? Can we breed against this trait? What about autochthonous pig breeds usually experiencing PRRSV infections without serious clinical outcomes? Can they be a model for fundamental resistance traits lost during genetic selection?
- **Animal welfare.** Which environmental conditions are more strictly related to PRRSV infection prevalence and serious clinical outcomes thereof? How can one effectively prevent chronic stress and stress-related immunosuppression? Which laboratory procedures can best depict immunosuppression in pigs?
- **Immune control.** Can we improve the performance of PRRS vaccines on farm? In case of positive answer, which vaccines are best suited for disease control? Can we think of effective, adaptive immune responses to PRRSV, or need we also think of "habituation" to the virus by means of "trained immunity" mechanisms? Is "acclimatization" of sows and gilts based on a peculiar form of "trained immunity"? On the whole, as reported in a recent study of ours [86], the emerging picture in the PRRS model outlines unusual effector roles of adaptive immunity: both IgA Ab and cell-mediated immune responses (IFN- γ SCs) can concur to a major modulation of macrophage permissiveness to PRRSV, as a foundation of disease control. Finally, immunomodulation by oral, low-dose IFN-alpha treatments has shown some efficacy vis-à-vis field PRRS outbreaks [4]; the peculiarities of this approach have been evaluated in a recent review paper of ours [110] .
- **Markers of risk.** The profile of cytokine responses induced *in vitro* by new PRRSV strains detected on farm can probably define a risk of serious clinical outcomes of PRRSV infection [28,55]; such "immunotypes" might be more important than the usual variants revealed by sequencing of ORF 5 and ORF7 genes. The IgA response to PRRSV in OF as possible marker of effective acclimatization of gilts and sows [85] might also be of some importance.

The above approaches to disease control are summarized in Table 1.

Table 1

Major issues underlying a more effective control of PRRS

	Possible aims	Constraints
Farm management	Biosafety. Reduction of environmental infectious pressure.	Lack of adequate facilities, type of farms (farrow-to finish), lack of validated markers.
Genetic selection	PRRSV-resistant pigs	Limited knowledge of molecular basis, costs, unfavorable pig phenotypes (lean type)
Animal Welfare	Prevention of stress-related immunosuppression	Poor housing and infrastructure, high animal densities, unfavorable pig phenotypes.

Immune control	Adequate innate and adaptive immune responses by means of vaccines and immunomodulators	Effective PRRSV decoy strategies, poor recognition of “danger”.
Markers of risk	Early detection of possibly serious clinical outcomes and pathogenicity of PRRSV isolates	Lack of large-scale validations, costs, lack of recognized sampling protocols and laboratory procedures.

Conclusions

The above issues demand evidence-based opinions of the scientific community. It goes without saying that such issues pertain to different areas of research and practitioners' activity, focusing on improved disease control actions and surveillance. We need multi-disciplinary contributions in the fields of pig farming, clinical sciences, husbandry, genetics, immunology and virology, with very clear translational perspectives. These contributions should hopefully generate highlights for each of the above translational areas.

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