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Host–pathogen interactions during porcine reproductive and respiratory syndrome virus 1 infection of piglets

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is a major disease affecting pigs worldwide and resulting in considerable economic losses. While PRRS is a global phenomenon, the causative viruses PRRSV-1 (first detected in Europe) and PRRSV-2 (isolated in North America) are genetically and biologically distinct. In addition, the disease outcome is directly linked to co-infections associated with the porcine respiratory disease complex and the host response is variable between different breeds of pigs. It is therefore warranted when studying the pathogenesis of PRRS to consider each viral genotype separately and apply careful consideration to the disease model studied. We here review the respiratory pig model for PRRSV-1, with a focus on a recent set of studies conducted with carefully selected virus strains and pigs, which may serve as both a baseline and benchmark for future investigation.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease affecting pigs worldwide, causing considerable economic losses to producers due to its effect on pigs of various ages. It is considered the most economically devastating disease for the global swine industry (Garner et al., 2001; Neumann et al., 2005; Nieuwenhuis et al., 2012; Rowland et al., 2012), costing an estimated $664 million annually in the United States of America alone (Holtkamp et al., 2011). The first PRRS outbreaks in Europe were reported in 1991 (Lindhaus and Lindhaus, 1991), but the disease emerged almost simultaneously in Western Europe and in North America during the late 1980s, presenting as a respiratory disease in young piglets, and as a reproductive failure in sows, evidenced by abortions and the production of mummified foetuses and weak piglets (Done et al., 1996; Halbur et al., 1996a; Batista et al., 2004). PRRS also predisposes infected piglets to infection by other respiratory viral and bacterial pathogens in the respiratory disease complex, leading to even more severe disease.

The aetiologic agent of PRRS is an enveloped, single-stranded, positive sense RNA virus (Benfield et al., 1992) of the genus *Arterivirus*, family *Arteriviridae* and order *Nidovirales* (Cavanagh, 1997). Other members of the virus family include equine arteritis virus, simian haemorrhagic fever virus, and lactate dehydrogenase elevating virus. Previously considered to be two genotypes of the PRRSV species, PRRSV-1 (European) and PRRSV-2 (North American) are now proposed to be considered two distinct species (Gorbalenya et al., 2014), which despite causing a broadly similar syndrome have a great degree of genetic diversity, sharing only 55–70% nucleotide identity (Forsberg et al., 2002).

In contrast to coronaviruses, arteriviruses are known for their rapid evolution, linked to the lack of a proof-reading enzyme like the exoribonuclease ExoN that Coronaviruses contain and which is vital to their sustainment (Minskaia et al., 2006; Nga et al., 2011). Accordingly, great genetic variation exists within each species of PRRSV. A classification with at least nine lineages has been suggested for the North American PRRSV-2 (Shi et al., 2010). European PRRSV-1 can phylogenetically be divided into at least three, possibly four subtypes: pan-European (and in fact global) subtype 1 and Eastern European subtypes 2 and 3, and possibly subtype 4 from Belarus and Latvia (Stadejek et al., 2013). The subtype 1 strains can be further subdivided into at least 12 clades (Shi et al.,

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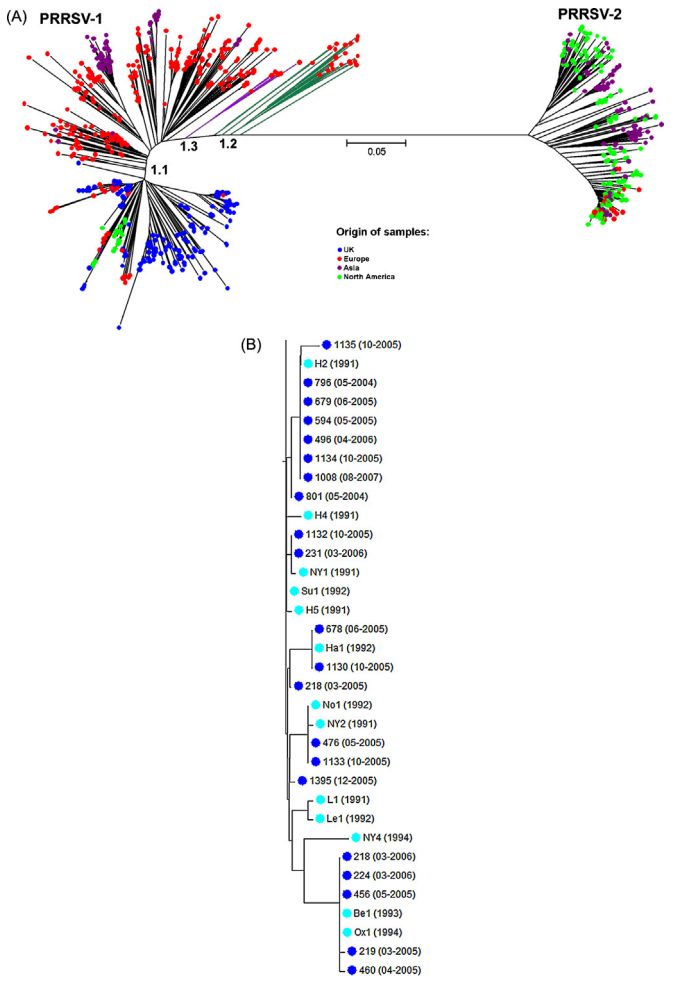

Fig. 1. Phylogenetic analysis of PRRSV ORF5 sequences. The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004). The analyses were conducted in MEGA6 (Tamura et al., 2013). A total of 1154 nucleotide sequences were analysed, including 867 PRRSV-1, 381 of which were from the UK. Panel A shows the relationship between worldwide strains of PRRSV, showing the division between PRRSV-1 and PRRSV-2, and the three subtypes. The geographic origin of each strain is indicated by its colour (UK – blue, rest of Europe – red, Asia – purple, North America – green. Panel B shows the relationship between British viruses similar to the prototype H2 isolate of 1991, demonstrating the continued similarity between isolates circulating in the early 1990s (in light blue) and those still circulating between 2004 and 2007 (in dark blue).

2010). Interestingly, while the genetic and antigenic diversity of PRRSV continues to rapidly expand, strains continue to circulate that resemble those found in the early 1990s (Frossard et al., 2013). This is similar to another important RNA virus of livestock, namely bovine viral diarrhoea virus (BVDV), where almost identical strains were detected in the UK over a period of a decade (Strong et al., 2012). It equally confirms and challenges our understanding of the evolution of such viruses: on the one hand the evolution and diversity is directly correlated to the host pressure and passages *in vivo*, on the other side certain "old" variants (strains) continue to persist (Fig. 1). This feature of RNA viruses poses increasing challenges both for the design of effective vaccination strategies (Mateu and Diaz, 2008), as well as for consistently effective diagnostic methods.

In addition to the familiar endemic and chronic PRRS disease, outbreaks of more severe disease have periodically occurred. Historically they were linked to PRRSV-2, first in the USA (Mengeling et al., 1998) but more recently the emergence of the porcine high fever disease (PHFD), linked to a novel PRRSV-2 strain, observed in China in 2005 (Tian et al., 2007; Zhou et al., 2008) and subsequently in other parts of South East Asia (Tong et al., 2007; Metwally et al., 2010). Despite intensive studies of the PHFD-related viruses, the genetic determinants of this increased pathogenicity remain to be fully elucidated, although a recent study reported that mutations in non-structural proteins-9 and -10 may confer an increase in viral virulence (Li et al., 2014). Not surprisingly, several authors have also described differences in virulence among PRRSV-1 strains, finding Eastern European subtype 3 strains significantly more pathogenic than subtype 1 strains (Karniychuk et al., 2010; Weesendorp et al., 2013; Morgan et al., 2013, 2014; García-Nicolás et al., 2014). Even more recently, the emergence of other virulent PRRSV-1 subtype 1 strains in Western Europe (in Italy and Belgium) (Nauwynck, personal communication) highlights the concerns about the impact and evolution of the disease on this continent.

PRRSV-1 and -2 have now been proposed to represent two species not only through their genetic distance, but also due to the differences in their biology. PRRSV-1 strains for example have to be adapted *in vitro* to grow on cells other than myeloid pig cells (most notably porcine alveolar macrophages (PAM)), whereas many PRRSV-2 strains grow easily on MARC-145 (green monkey kidney) cells. *In vivo*, Halbur et al. (1995) have demonstrated differences in the pathogenicity of PRRSV-1 and PRRSV-2. The respiratory disease following infection with PRRSV-1 appears less severe than that seen with PRRSV-2 (Martinez-Lobo et al., 2011). There is a perception that PRRSV-2 causes more severe clinical disease in North America than PRRSV-1 in Western Europe, but direct comparisons of the disease in the field due to the two viruses are difficult due to the differences in commercial swine breeds used and in farming practices between Western Europe and North America. It is therefore warranted to fully separate studies of PRRSV-1 and PRRSV-2 and avoid generalisations across genotypes unless experimentally thoroughly validated. In the following review, we therefore focus almost exclusively on the respiratory model of PRRSV-1 infection in pigs.

In vitro models of PRRSV-1 infection are not ideal, due to the limited range of cells types that can be infected with the virus. Alveolar macrophages are a natural host cell for the virus *in vivo* that can be isolated effectively. However, their large variability in susceptibility to PRRSV infection *in vitro* is an obstacle to reproducible research activities as is their change during and maintenance in culture. Cell lines modified to express specific receptors, CD163 and sialoadhesin/CD169, are useful to study the molecular biology of the virus during replication (Delrue et al., 2010; Huang et al., 2013), but these cells are sufficiently different to the natural host cells that their use to evaluate host virus interactions beyond certain (important) aspects of molecular virology, such as the virus internalisation is questionable.

To understand the complex nature of syndromic diseases, experimental *in vivo* studies are required to further elucidate the interactions between viruses, such as PRRSV, and the porcine host. Given the nature of the two viruses, the strain diversity and the lack of pathogenicity markers, it is understandable that in experimental infections with PRRSV there is great variation in the clinical signs produced. More so, the comparability of PRRSV studies is generally confounded by two variables: namely the co-infection with other pathogens and the pig breed used. The presence of other pathogens is generally not intended as it becomes impossible to attribute biological changes to one or the other and further increases the spectrum of disease observed. Thus the specific freedom of experimental animals from pathogens, such as other common viruses needs to be carefully checked. This, however, has until recently not been broadly possible and remains expensive. In reality, several studies in Europe and North America have been carried out with pigs concomitantly infected with either viral or bacterial pathogens, such as porcine circovirus 2 (PCV-2) or *Mycoplasma* spp. In some countries it is virtually impossible to obtain "clean"

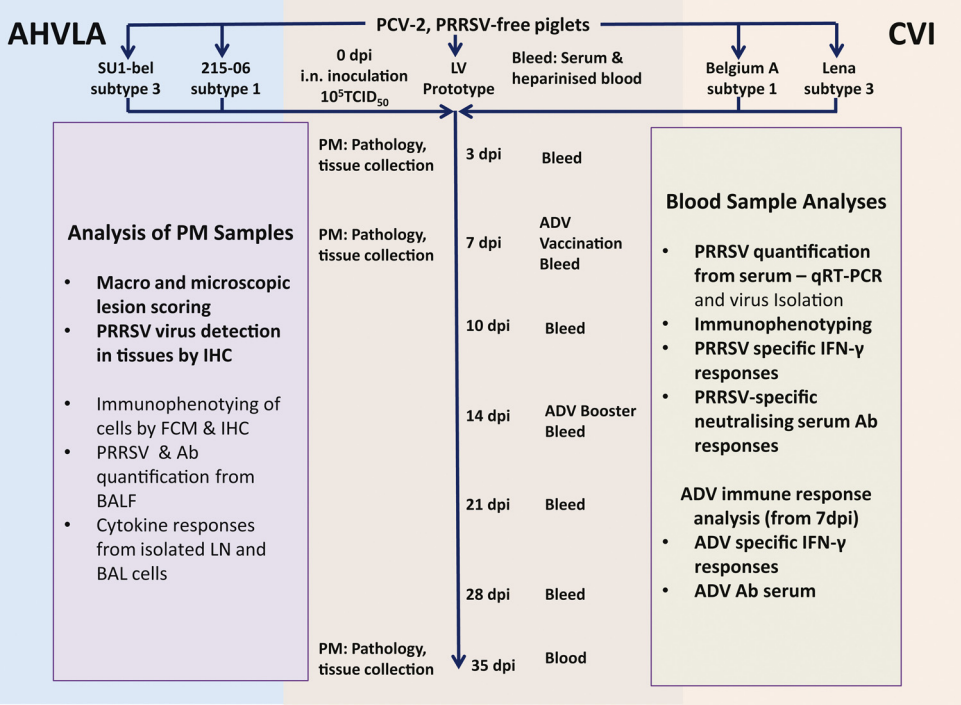

Fig. 2. Schematic representation of the experimental design and timetables for the two coordinated *in vivo* experiments carried out at different institutes.

pigs, which while reflecting the field situation does not provide the basis for sustainable and reproducible research into PRRSV. In addition, variation in the genetic background of the pigs infected also results in considerable differences in the clinical signs seen during disease, as reported in several experimental studies. Lewis et al. (2010) have shown that PRRSV-1 infection affects pregnant gilts differently depending on their breed, while Ait-ali et al. (2007) have demonstrated great differences in the susceptibility of PAMs from pigs of different breeds to infection with PRRSV-1 *in vitro*. Similar differences in host susceptibility to respiratory disease have also been shown for PRRSV-2 (Boddicker et al., 2013). While the reproductive form of PRRS is very important and contributes greatly to the economic cost of the disease, most of the research to date into the pathogenesis of PRRSV-1 infections has been conducted with piglets, using the respiratory disease model. This is largely due to the understandable physical and financial constraints of conducting large *in vivo* studies with pregnant gilts, but does represent a real limitation in the experimental infection data available.

Vaccination is fundamental in the control strategies for most viral diseases including PRRS. However, despite the development of a number of either inactivated or modified-live PRRSV vaccines, none has yet been demonstrated to be fully efficacious on a large scale in controlling the disease or its spread. One of the challenges in developing improved vaccines is the limited understanding of the immune responses of animals to infection with PRRSV. In contrast to PRRSV-2, cellular immune responses appear to be crucial to achieving protection, while the antibody response overall seems to be less useful for PRRSV-1 (Lowe et al., 2005; Zuckermann et al., 2007). The importance of generating virus neutralising antibodies remains very unclear, perhaps in part due to the difficulty in accurately assessing these with current *in vitro* techniques. A better understanding of the common features of these immune responses, at least for each genotype individually, is necessary to inform an intelligent approach to the design of improved vaccines.

The combination of all these variables makes the comparison of different pathogenesis studies very difficult, if not to say impossible, which explains why a more definitive understanding of the pathogenesis of PRRSV-1 infections is still lacking. To address this, a large experimental infection study to investigate the pathogenesis of different PRRSV-1 strains, as well as the host immune response was conducted (Weesendorp et al., 2013; Morgan et al., 2013). For practical reasons, the study was divided among two institutes: the Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridge, UK, and the Central Veterinary Laboratory (CVL), Lelystad, The Netherlands. The outline is summarised in Fig. 2. Each institute worked with a different conventional subtype 1 strain and a divergent Eastern European subtype 3 strain and the prototype Lelystad virus was included in each study as a standard to aid comparison as was the same source of piglets. Here we aim to summarise available knowledge about host–pathogen interactions following infection with PRRSV-1 strains, with a focus on the immune responses in the lung and local lymphoid organs, as delineated from our studies in comparison to pre-existing data.

2. Materials and methods

2.1. Viruses

Five PRRSV-1 strains have been used in these studies: Lelystad virus-Ter Huurne (LV), the prototype PRRSV-1 (subtype 1), which was presumed to cause a mild form of disease (Wensvoort, 1993); Strain 215-06 (subtype 1) was isolated at AHVLA from the serum of a post-weaning piglet showing signs of wasting and poor condition on a UK farm in 2006; Strain Belgium A (07V063; subtype 1) was isolated from a stillborn piglet from a Belgian farm in 2007 (Karniychuk et al., 2010). Strain SU1-Bel was also isolated at AHVLA, from lung tissue homogenate from a 30-day-old piglet from a farm in Belarus in 2010 (Morgan et al., 2013); Strain Lena was isolated from a Belarusian farm with reproductive failure (Karniychuk et al., 2010). Both Lena and SU1-Bel are subtype 3 strains of PRRSV-1 and were presumed to cause severe disease. All of the strains

were propagated in PAMs which were harvested from the lungs of piglets as described previously (Wensvoort et al., 1991).

2.2. Animals, experimental design and sampling

Experiments were approved by the AHVLA Ethical Review Committee and the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR. All procedures at AHVLA were carried out under the Animals (Scientific Procedures) Act, 1986, UK. Full details of the piglets, experimental design and sampling regime have been previously described (Morgan et al., 2013; Weesendorp et al., 2013). In brief, 5-week-old Yorkshire, Dutch Landrace and Large White crossbred male piglets, from a high health herd, negative for PCV-2, were utilised. At seven weeks of age, after acclimatising for 14 days, the piglets were inoculated intranasally with 10^5 $TCID_{50}$ of the respective virus or equivalent volume of 'mock' material as a control. Monitoring of clinical scores and rectal temperatures was performed daily from −3 days post-infection (dpi) until the end of the study, at 35 dpi. Blood samples were taken at 0, 3, 5, 7, 9, 12, 14, 21, 28 and 35 dpi. At both 3 and 7 dpi, pigs from the control group and from each infected group were euthanised. All remaining animals were euthanised at 35 dpi. Humane end-points were set before the onset of the experiment and any animals that met these criteria were euthanised. At post mortem, gross pathology of the lungs was scored, and both bronchoalveolar lavage fluid (BALF) and tissue samples were collected. Buffered fixed samples from a variety of tissues were processed and stained routinely with H&E for histopathology and immunohistochemical detection of PRRSV antigen, cell markers for macrophages (MAC387) and T cells (CD3) and different cytokines and chemical mediators (Morgan et al., 2014; Amarilla et al., 2015; García-Nicolás et al., 2015).

2.3. Assessment of immunological responses in bronchoalveolar lavage fluid

After collection, an aliquot of BALF was analysed directly on a volumetric flow cytometer (MACSQuant Analyzer, Miltenyi Biotec, Bisley, UK) to determine cellularity. The remaining BALF was centrifuged at 640 × *g* for 10 min at 4 °C. The supernatant fluid was removed from the cell pellets and stored at −80 °C for subsequent analyses. Cells were washed with sterile PBS and resuspended in RPMI-1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (All Life Technologies, Paisley, UK) and 10% FBS (Autogen Bioclear, Calne, UK) and IFN-γ expression assessed by ELISpot assay as previously described (Morgan et al., 2013). Culture supernatants of BALF-derived cells were additionally assessed for cytokine content using a multiplex ELISA (Cira porcine cytokine 1 array; Aushon, Billerica, MA, USA). Cell-free BALF was assessed for PRRSV-specific antibody (Ab) using the HerdCheck PRRS X3 kit (IDEXX Laboratories) according to the manufacturer's instructions, with the modification that undiluted BALF was used.

3. Clinicopathological outcomes after PRRSV-1 respiratory infection

The variability of clinical features of PRRSV infection in the field has been reported since the appearance of the first outbreaks (White, 1992), and the presence of subclinical infections are common (Halbur et al., 1994; Done and Paton, 1995; Done et al., 1996; Gómez-Laguna et al., 2010a,b). To reproducibly judge the clinical symptoms, we adapted an extensive scoring system to monitor the clinical signs (Mittelholzer et al., 2000; Weesendorp et al., 2013). Importantly, to compare the studies between two difference centres, we sourced the pigs from the same farm and carried one PRRSV-1 strain (LV) along as reference in both places. The resulting data showed a high degree of similarity for this baseline. While LV has been extensively studied and is well characterised, the origin and passage history (here, both labs used the same stock from passage 8) still needs to be considered.

The main clinical signs observed in SU1-Bel infected piglets were systemic, with depression, anorexia or appearance of rough coat, whereas Lena infected piglets displayed milder respiratory signs. High body temperature was observed in both groups of animals infected with the subtype 3 strains (Lena and SU1-Bel) from 2 dpi, in contrast to animals infected with LV or classical field strains (215-06 and Belgium A) that displayed mild or no hyperthermia (Weesendorp et al., 2013; Morgan et al., 2013). So as expected, the infection with both subtype 3 strains resulted in a clinical rather than subclinical disease. The weight gain in the first 7 days was considerably lower in the SU1-Bel group than in the LV group. Some animals infected with the Su1-Bel strain were clinically deteriorating after days of continued hyperthermia and increasing clinical scores and had to be culled for welfare reasons at 12 and 13 dpi.

Animals were subjected to an extensive post-mortem examination that was focused primarily on the presence of lung lesions that were scored depending on the percentage of the lung surface displaying gross pathology compatible with PRRSV infection, as previously described (Halbur et al., 1996b). The type of lung lesions were similar in all the infected groups, consisting of mottled tan areas with a rubbery aspect and failure to collapse, indicating the presence of interstitial pneumonia characteristic of this syndrome. The highest gross pathology scores were observed at 7 dpi, with the virulent strains Lena and SU1-Bel displaying the higher scores than Lelystad and field viruses. Infected animals displayed gross pathology lung lesions as early as 3 dpi. However, Lelystad virus did not induce significant pathology at this time point. Interestingly, infected animals did not present any significant lung gross pathology at 35 dpi. SU1-Bel infected animals that had to be euthanised at 12 and 13 dpi showed the highest gross pathology scores.

The pathological changes in the respiratory tract of PRRS infected piglets are always suggestive of PRRS but not pathognomonic (Done et al., 1996), and the presence of secondary infections in field cases (van Reeth, 1997; Thacker et al., 1999; Drew, 2000; Díaz et al., 2005; Gómez-Laguna et al., 2013b) makes it more difficult to diagnose based on gross pathology alone. The presence of secondary infections in our *in vivo* animal model was minimised by sourcing animals from a farm free of the two main bacterial co-pathogens in this syndrome, namely *Mycoplasma hyopneumoniae* and *Haemophilus parasuis* (Thacker et al., 1999; Gómez-Laguna et al., 2013b). While a co-infection cannot be ruled out on this basis, all tests for secondary pathogens at the post mortems, including *Mycoplasma hyopneumoniae* and viral agents with the use of a pan-viral microarray as previously described (McGowan et al., 2014) were negative.

The gross pathology was correlated with the presence of interstitial pneumonia characterised by septal thickening of mild to moderate intensity and focal distribution. This histopathological lesion is the hallmark of PRRSV respiratory infections and is caused by a lymphohistiocytic inflammatory infiltration. Using immunohistochemical staining of different cell types, we identified that the majority of the inflammatory cells are monocyte–macrophages (MAC387/calprotectin$^+$) and T lymphocytes (CD3$^+$). The severity of the histopathological lesions was higher in animals infected with the virulent subtype 3 strains and they showed a higher infiltration of both monocyte–macrophages and T lymphocytes in the alveolar septa (Fig. 3).

Apart from the lungs, mild to moderate lesions were also observed in lymphoid tissues (Morgan et al., 2014; García-Nicolás et al., 2015). At the histopathological level, lymph nodes, mainly within the thoracic cavity but also retropharyngeal displayed a

hyperplasia of the germinal centres of lymphoid follicles with an increase of the number of mitotic figures, pyknosis and the presence of lymphoblasts. These lesions were more severe in animals infected with the virulent strains compared to those infected with LV or "conventional" field strains 215-06 and Belgium A (Morgan et al., 2014). Apoptosis phenomena have been described in both lung and lymphoid tissues in PRRSV-1 and -2 infections (Sirinarumitr et al., 1998; Gómez-Laguna et al., 2013c; Rodríguez-Gómez et al., 2014). We detected a higher number of apoptotic cells within the lung lesions and the areas of lymphoid depletion in the tonsils in those animals infected by SU1-Bel in comparison to LV or the field isolate 215-06 (Morgan et al., 2014).

PRRSV shows a marked tropism for myeloid cells, including alveolar macrophages and other tissue macrophages, monocytes and dendritic cells (Nauwynck et al., 1999; Gómez-Laguna et al., 2010a,b; Rodríguez-Gómez et al., 2013; Halbur et al., 1996b; Silva-Campa et al., 2010). The molecules identified or proposed as cellular receptors or mediators for PRRSV entry are heparin sulphate, sialoadhesin (CD169) and the haemoglobin scavenger CD163 (Duan et al., 1998; Van Gorp et al., 2008; Darwich et al., 2010). Whilst viral replication occurs mainly in the lung (Haynes et al., 1997; Ramirez et al., 2008; Gómez-Laguna et al., 2010a,b, 2013b; Morgan et al., 2014), PRRSV can also be found in lymphoid tissues including the mediastinal, sternal and medial retropharyngeal lymph nodes, tonsils, spleen and thymus (Beyer et al., 2000; Labarque et al., 2000; Barranco et al., 2012a,b; Morgan et al., 2014; Gómez-Laguna et al., 2013a; García-Nicolás et al., 2015). The virus can persist for long periods of time in lymphoid tissues (Wills et al., 2003) and whilst virus had been cleared from blood (Morgan et al., 2013) we still detected PRRSV antigen at 35 dpi in lungs, local lymph nodes and the thymus. It has been reported recently that the virulent PRRSV-1 Lena strain may replicate in CD163$^-$ sialoadhesin$^-$ cells (Frydas et al., 2013). Since we observed a higher number of infected cells in tissues in pigs infected with virulent subtype 3 strain SU1-Bel, we speculate that this may be attributable to other highly pathogenic strains of PRRSV-1 subtype 3.

4. Immune response to PRRSV-1 infection

Similar to many other viruses (such as human immunodeficiency virus (HIV), BVDV, and equine arteritis virus (EAV)) in different families (Retroviridae, Flaviviridae, Arteriviridae), myeloid cells are the primary target for PRRSV-1. But in contrast to other viruses, a paradigm of PRRSV infection is the initiation of a weak immune response, which may in part explain its ability to persist in different organs. The importance of neutralising antibodies remains unclear and T cell responses appear to be important in achieving clearance of PRRSV infection and protection against re-infection (Díaz et al., 2005; Lowe et al., 2005; López Fuertes et al., 1999; Zuckermann et al., 2007).

As mentioned, PRRSV-1 shows a marked tropism for cells of the monocyte–macrophage lineage. The main target of PRRSV-1 are alveolar and other tissue macrophages (Lee et al., 2009), and to a lesser extent, monocytes and dendritic cells (DC) (Halbur et al., 1996b; Silva-Campa et al., 2010), which has been attributed to different levels of expression of CD169 and CD163. On peripheral blood monocytes, these – in particular CD163 – are expressed at low levels and on DC these are variable both *in vitro* and *in vivo* (Chamorro et al., 2004; Beitnes et al., 2011; Singleton et al., unpublished). Conversely then, the appearance of PRRSV-1 in DC *in situ* (Rodríguez-Gómez et al., 2013; Morgan et al., 2014), the detection of PRRSV-1 Lena strain in CD163$^-$ sialoadhesin$^-$ cells in an *ex vivo* nasal mucosa explant culture (Frydas et al., 2013) and the infection of CD163 and CD169 negative monocyte-derived DC (MoDC) with a variety of PRRSV-1 strains *in vitro* (Weesendorp et al., 2013, unpublished data) demonstrates that CD169 and CD163 are not the only receptors for PRRSV-1 infection. Platelet endothelial tetraspan antigen-3/CD151 been proposed as an additional receptor for PRRSV-2 (Shanmukhappa et al., 2007) and the expression of CD151 on follicular DC (fDC) in human tonsils has been described (Sincock et al., 1997). To which extent CD151 resembles another receptor for PRRSV-1 requires further investigation. We could detect PRRSV-1 antigen in fDC in lymph nodes but this may add further complexity to the situation. fDC are long known to trap antigens and the persistence of antigen on fDC is critical for the initiation of strong IgG and IgE antibody responses (Helm et al., 1995; Wu et al., 1996). More so viable viruses, such as HIV, can be trapped and maintained there for months (Smith et al., 2001). Indeed antibodies to PRRSV were readily detected by ELISA in our experimental infection model (Weesendorp et al., 2013; Morgan et al., 2013) and are used in the field for diagnostic purposes. However, neutralising antibodies were undetectable to any of the virus strains even at the termination of the experiments (35 dpi; Weesendorp et al., 2013; Morgan et al., 2013). This absence of neutralising antibody cannot be attributed to the infection of myeloid cells (such as DC), or the notion that multiple receptors are used. While there are multiple pathways for endocytosis of viruses, it is generally assumed that all viruses make use of specific receptors on host cells and that endocytosis requires activation *via* surface molecules (Mercer et al., 2010). This implies that, in contrast to the related Arterivirus EAV, which produces high titres of neutralising antibodies, the neutralising epitopes of PRRSV are hidden and only conformationally exposed during the attachment phase.

Analysis of cytokine and PRRSV-specific T cell responses in our experimentally infected pigs showed significant differences between the PRRSV-1 strains (Weesendorp et al., 2013; Morgan et al., 2013). The most significant IFN-γ T cell responses were detected in animals infected with SU1-Bel and Belgium A, which interestingly were the strains that had the lowest level of viraemia (Belgium A) or cleared most efficiently from blood and lungs (SU1-Bel), suggesting a protective role for these T cell responses. In contrast, the responses in the LV and 215-06 infected groups were weak in our hands and delayed according to another study (Darwich et al., 2010).

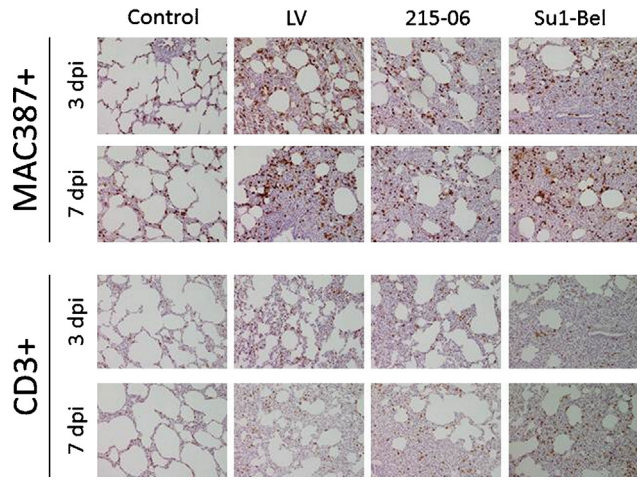

Fig. 3. Inflammatory cell infiltration in the lung of pigs infected with PRRSV-1 strains. The presence of monocyte–macrophage (MAC387$^+$) and T cell (CD3$^+$) infiltration was evaluated by immunohistochemistry at 3 and 7 dpi in pigs infected with the Lelystad virus, a field UK strain (215-06) and the virulent isolate SU1-Bel. All infected groups show a marked thickening of the alveolar septa and hyperplasia of type 2 pneumocytes. There is a marked increase in the number of both MAC387$^+$ and CD3$^+$ cells infiltrating within the alveolar septa in all the infected animals in comparison with the control, particularly in the SU1-Bel infected group.

Assessment of the antigen-specificity of T cell responses from LV and SU1-Bel infected animals was conducted utilising a PRRSV-1 proteome-wide synthetic peptide library pooled to represent viral proteins (Mokhtar et al., 2014). The results showed that despite the genetic distance between these strains, animals showed a remarkably similar profile of antigen-reactivity. The major T cell IFN-γ responses mounted by animals in both groups were to the non-structural proteins nsp1 and nsp2, and the M and GP5 structural proteins. Interestingly all but one of the SU1-Bel infected animals displayed dominant responses to nsp5. nsp5 is a relatively well conserved protein of PRRSV that has previously been identified to carry multiple T cell epitopes (Burgara-Estrella et al., 2013). Further analysis of the antigen has shown it to be an immunodominant target of cytotoxic CD8 T cell responses (Mokhtar et al., unpublished data).

In the CVI component of the study, cytokine responses, as assessed at the level of mRNA expression in blood, showed an upregulation of IL-1β, IFN-α, IL-10, IL-12, and IFN-γ around 7 dpi in both Lena and Belgium A infected animals (Weesendorp et al., 2013). TNF-α was further up-regulated in Lena infected pigs compared to Belgium A, which may have directly contributed to the accompanying fever. In the AHVLA component, cytokine expression analysis conducted using BALF and derived cells. Fig. 4 summarises the results of this analysis for both SU1-Bel and LV-infected animals on 7 dpi. To assess how these immune responses may have contributed to the pathology observed each immune response parameter for an individual pig is plotted against the corresponding gross lung pathology score. In the PRRSV-1 SU1-Bel infected group the magnitude of PRRSV-specific BALF antibody response, the leukocytes in BALF and the secretion of IFN-γ and IL-1β by these cells showed a significant positive correlation with the gross pathology scores. Conversely, the expression of IL-10 by BALF cells showed a significant negative correlation with pathology. However, none of the immunological parameters showed a significant association with the degree of pathology observed in LV-infected lungs.

While it can be summarised that PRRSV-1 strains did induce a significant immune reaction, other studies concluded that the interaction of PRRSV with the immune system would result in an inhibition of the immune system. For this particularly two strands of evidence have been presented. The first one surrounds the interaction of PRRSV-1 with dendritic cells, where it has been concluded that PRRSV-1 inhibits DC. While the plethora of the DC subsets cannot be reviewed here it needs to be kept in mind that two key variants of DC exist, namely myeloid DC (mDC) which are key to antigen presentation and plasmacytoid DC (pDC), which are specialised upon the IFN-α response and thereby switching the immune system on (Merad et al., 2013). Furthermore, within the mDC there are subsets of mDC specialised to cross-present antigen to CD8 T cells, important thus for viral defence. These 'CD8α-like' mDC populations are conserved across mammalian species including pigs (Contreras et al., 2010, Edwards et al., unpublished data). While PRRSV-1 infects mDC, it does not infect pDC (Baumann et al., 2013), and infection of pDC is not required to initiate an IFN-α response such as demonstrated for PRRSV and other viruses (Python et al., 2013). It has been reported that PRRSV-2 fails to initiate an IFN-α response and more so inhibits pDC reaction towards other viruses or a TLR-9 agonist (Calzada-Nova et al., 2011). However, a second study could not confirm these results for PRRSV-1 and showed only a partial inhibition by PRRSV-2 (Baumann et al., 2013). Thus, pDC may be a source of systemic IFN-α responses reported in PRRSV-infected animals including in our studies and an inhibition only occurs with some strains and to a limited extent.

The fact that highly pathogenic PRRSV-1 strains such as Lena and SU1-Bel induce a strong immune response and at the same time are detected in a broader range of target cells *in situ* (Frydas et al., 2013) raises an interesting possibility. It is perhaps not the detrimental

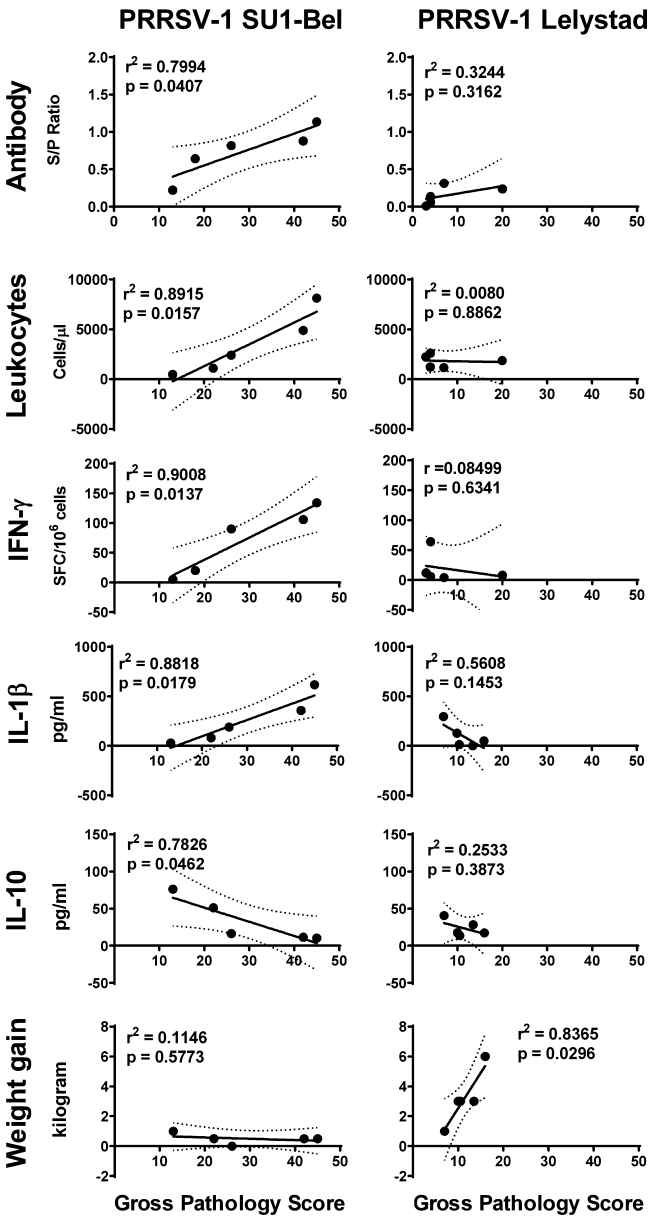

Fig. 4. Lung immune responses associate with pathology induced by virulent PRRSV-1 SU1-Bel but not the prototype Lelystad strain. Seven days post-infection with PRRSV-1 SU1-Bel and Lelystad strains, lungs were isolated post-mortem and scored for gross pathology. Lungs were then lavaged and immune parameters assessed using the BALF. PRRSV-specific antibody was measured by ELISA, leukocytes by volumetric flow cytometry, and cytokine production from isolated cells by IFN-γ ELISpot assay, and multiplex ELISA for IL-1β and IL-10. Weight gain from 0 to 7 dpi is also presented. Data points represent individual pigs from each group ($n = 5$), with the mean best-fit linear regression line and error shown by solid and dotted lines, respectively. r^2 and p values for each response parameter are indicated.

interaction of PRRSV-1 with DC but rather the lack thereof that results in a weaker immune response with less pathogenic strains. It is long known that the uptake of antigen by mDC, their transport to the lymph node and their maintenance there sustains a strong immune response (Moll et al., 1995). With our expanding knowledge of the DC system and the knowledge that only certain mDC types are strong inducers of a T cell response, which in turn is crucial for immunity to PRRSV-1, it might be the case that less pathogenic strains simply avoid the interaction with such mDC directly. Thereby rather than suppressing the immune reaction most PRRSV-1 strains fail to initiate a strong and adequate immune reaction.

Another way to reduce the immune reaction is *via* various forms of suppression, mediated by regulatory (suppressor) T cells and related cytokines, namely IL-10 and TGF-β. Regulatory T cells (Treg) exist in two forms, namely natural Treg (nTreg) which are thymus-derived and antigen-independent *versus* induced Treg (iTreg), which are antigen-specific and the result of a DC–T cell interactions (Josefowicz et al., 2012). The fact that some strains of PRRSV-1 induce IL-10 and TGF-β has paved the way for speculation that Treg are a key factor in the pathogenesis of PRRSV (Cecere et al., 2012; Wongyanin et al., 2012; Gimeno et al., 2011). More so, PRRSV-2 was shown in two studies to generate iTreg responses *in vivo* (Silva-Campa et al., 2012; Wongyanin et al., 2012). However, a previous study failed to demonstrate the induction of iTreg through PRRSV-1 *in vitro* (Silva-Campa et al., 2010) and in our study we were unable to detect increased levels of iTreg cells in blood or BALF (Morgan et al., 2013). It must not be forgotten in this context that both IL-10 and TGF-β were originally not identified as immunosuppressive cytokines and both cytokines have a plethora of functions, most of which are immune-reactive. Likewise it has been suggested that iTregs are the physiological result of a prolonged immune-stimulation (Walker et al., 2005) to avoid chronic inflammations for example. While this is indeed a hallmark of iTreg function, it could also be a piece in the jigsaw of PRRSV-1 pathogenesis, since most strains of PRRSV-1 result in a chronic infection where a sustained immune reaction can be measured.

5. Summary

The studies discussed and reviewed in this paper adopted harmonised procedures and protocols to conduct a multi-centre study of the pathology and immunology of piglet infections with diverse PRRSV-1 strains. These studies highlighted the increased virulence of PRRSV-1 subtype 3 viruses and the risk they pose for the swine industry out-with the Eastern European region. These studies also highlight our limited understanding of the basis of PRRSV virulence and it is hoped that through reverse genetics systems and in-depth analysis of PRRSV-1 strains interactions with immune cell populations that we may begin to unravel and exploit this knowledge to design more effective vaccines and control strategies for PRRS.

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