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Characterization of immune responses following homologous reinfection of pigs with European subtype 1 and 3 porcine reproductive and respiratory syndrome virus strains that differ in virulence

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes significant economic losses to the pork industry worldwide. Vaccination results often in limited protection. Understanding host immune responses elicited by different PRRSV strains could help to develop more efficacious vaccines. In the current study we characterized immunological responses and viral kinetics in pigs after primo infection and homologous challenge of the highly virulent European subtype 3 strain Lena, and the moderate to low virulent subtype 1 strain LV. Eighteen pigs were infected per strain, and 18 non-infected pigs served as control. Post mortem analysis was performed at days 7, 46 and 60 p.i. At day 46, pigs were challenged with the homologous strain. After the first inoculation, pigs infected with Lena developed fever and clinical symptoms, while this was not observed in pigs infected with LV. Virus titres in serum were about 100-fold higher in pigs infected with Lena than in pigs infected with LV. An inflammatory response was observed in pigs after primo infection with Lena with significantly higher levels of IL-12, IL-1β and TNF-α in the bronchoalveolar lavage. IFN-γ ELISPOT assay showed comparable responses between Lena and LV. Neutralizing antibodies were detected earlier in serum of pigs infected with Lena than in pigs infected with LV. After the challenge, a boost in antibody levels in both groups was observed. Challenge infection resulted in both groups in complete protection and sterile immunity, with no viraemia, clinical symptoms or viral RNA in tissues. In conclusion, although there were clear differences in immunological, clinical and virological responses to the primo infection, there were no differences observed in protection against homologous challenge.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single stranded RNA virus of the Arteriviridae family (Meulenberg et al., 1994). This virus is widespread throughout the world and causes disease characterized by abortions and stillbirth, increased pre-weaning mortality and respiratory disorders. PRRSV infections are one of the most significant causes of economic losses in the swine industry (Neumann et al., 2005), not only because of a direct effect of the virus infection, but also because of secondary bacterial infections that exacerbate clinical symptoms in growing pigs (reviewed by Drew, 2000; Gómez-Laguna et al., 2013).

There are two genotypes of PRRSV described, represented by two prototypes: Lelystad virus (European type or genotype I) and VR-2332 (American type or genotype II) (Nelsen et al., 1999). The European type strains can be further divided into at least three subtypes: Pan-European subtype 1, and Eastern European subtypes 2 and 3 (Stadejek et al., 2006, 2008). There are significant antigenic and pathogenic differences between and within genotypes. Within the European genotype, the Eastern European subtype 3 strains are considered to be more virulent, as determined by clinical manifestations in infected pigs under field and experimental conditions (Karniychuk et al., 2010; Morgan et al., 2013; Weesendorp et al., 2013).

After infection with PRRSV, the adaptive immune response is often weak and delayed, resulting in pigs that are not fully protected against re-infection. After re-introduction of a PRRSV strain, or introduction of a new PRRSV strain in a herd, the

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recovered pigs can become re-infected and excrete virus, which can subsequently infect new pigs. Experimental studies have shown that challenge with homologous or heterologous strains can result in new infections and virus excretion (Díaz et al., 2012; Shibata et al., 2000). As with natural infections, vaccination does not always result in sterile immunity, and vaccinated pigs can excrete virus after vaccination and infection. None of the currently used PRRSV vaccines, that all contain subtype 1 strains, can claim full protection (Diaz et al., 2006; Zuckermann et al., 2007; reviewed by Darwich et al., 2010).

There is only limited understanding of the mechanisms involved in protection. Most studies used the development of neutralizing antibodies (NA) or virus-specific IFN-γ secreting cells (IFN-y-SC) as the main correlates of protection, but often did not include challenges to confirm this. There are also contradictory results about the predictive value of these assays. To aid the development of more efficacious vaccines, an improved knowledge of the immune response against PRRSV is necessary.

In previous experiments conducted by the authors (Morgan et al., 2013; Weesendorp et al., 2013), the pathogenicity and immune responses after infection with European type PRRSV strains were compared. These studies included the Pan-European subtype 1 and Eastern-European 3 strains. It was shown that with highly virulent subtype 3 strains, a stronger early inflammatory response was induced and more rapid virus clearance was observed compared to low virulent subtype 1 strains. No neutralizing antibodies were detected at day 35 p.i. for both strains. However, strains differed in the induction of IFN-γ-SC. In conclusion, in these previous studies, infection with different PRRSV strains resulted in different virological and immunological outcomes. Because no challenge was included, it is not known how this related to protection. To aid the development of more efficacious vaccines, it is important to know what type of immune responses induces protection. Therefore, we analysed some immunological parameters and followed the virus kinetics after primo inoculation and homologous challenge of two strains that give distinct clinical differences and host responses, the subtype 3 strain Lena, and the subtype 1 strain LV. We hypothesised that the stronger inflammatory response after primo infection in pigs infected with European genotype subtype 3 strains will result in better protection compared to the weaker response, which is elicited by European genotype subtype 1 strains.

2. Materials and methods

2.1. Viruses

Strain Lena is a recently isolated Eastern European subtype 3 strain which was used at the fourth passage on porcine alveolar macrophages (PAM). Lena was isolated from a Belarusian farm with reproductive and respiratory failure (Karniychuk et al., 2010). Strain LV-*Ter Huurne* (LV) is an European subtype 1 strain. This strain was isolated during the 1991 epizootic from a clinical case of PRRS in the Netherlands (Wensvoort et al., 1991). This strain was used at the seventh passage on PAM.

2.2. Animals and housing

Fifty-four six week old male cross-breed pigs (pig breeding line TOPIGS 20), were obtained from a PRRSV-free farm in the Netherlands with a high health status. All pigs were confirmed negative for PCV2 by PCR one week prior to transport. PCV2 was analyzed because PRRSV and PCV2 co-infection can increase the severity of disease and cause post-weaning multisystemic wasting syndrome (Drew, 2000). Pigs were evenly distributed between three groups. Each group, containing eighteen pigs, was housed in a different room of an isolation unit. Between rooms, clothing, footwear and gloves were changed and materials needed for sampling and rectal temperature monitoring were provided separately for each room. Standard feed for finishing pigs was provided once a day, and the pigs had unlimited access to water.

2.3. Experimental protocol

After one week of acclimatization, the pigs were inoculated intranasally with 1.5 ml containing 10^5 50% tissue culture infectious dose ($TCID_{50}$) of either European subtype 3 strain Lena, European subtype 1 strain LV or an equal volume of PBS (control group). Six pigs per group were euthanized at day 7 p.i., and four pigs at day 46 p.i. All eight remaining pigs were challenged with 1.5 ml containing 10^5 $TCID_{50}$ of the homologous virus strain at day 46 p.i. These pigs were euthanized at the termination of the experiment at day 60 p.i. All inocula were back titrated to confirm the dose administered. The experiment was approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR.

2.4. Clinical signs and body temperature

Rectal temperature and clinical signs were recorded daily. Fever was defined as body temperature higher than 40 °C for two consecutive days. A list of eleven PRRSV-relevant criteria was used for quantitative assessment of the severity of disease (Weesendorp et al., 2013). For each criterion, a score was recorded as either normal (score 0), or symptoms associated with the PRRSV infection (scores 1–3 representing increasing severity). The scores of all criteria were added up to obtain a total score per pig per day.

2.5. Blood sample collection and pre-treatment

Serum samples were collected at days 0, 3, 5, 7, 10, 14, 21, 28, 35, 45, 49, 51, 53 and 59 p.i. to determine virus titres, antibody levels and cytokine levels. These samples were stored directly at −70 °C until testing. Heparin stabilized blood samples of eight pigs per group (only the pigs that were kept in the experiment until day 60 p.i.) were collected at days 0, 14, 28, 42, 49 and 56 p.i. Peripheral blood mononuclear cells (PBMC) were isolated from these blood samples and used for IFN-γ ELISPOT assay. Isolation of PBMC was performed by density gradient centrifugation using 50 ml Leucosep® tubes (Greiner Bio-One). In brief, the heparinized whole-blood samples were diluted with equal volumes of PBS, and 30 ml of the diluted blood was added to a Leucosep® tube. The cell separation tubes were centrifuged at room temperature for 20 min at 1380 × *g* without braking. The cells were then washed twice in PBS (centrifugation for 15 min at 640 × *g*) and resuspended in 1 ml PBS for counting with the Z2Coulter Counter (Beckman Coulter).

2.6. (Gross) pathology examination, tissue and bronchoalveolar lavage fluid (BALF) collection and pre-treatment of samples

After euthanasia, pigs were weighted and a gross examination was performed. Lungs were removed from the body and weighted to calculate the relative lung weight as potential indicator of inflammation of the lungs. Gross pathology examination of the lungs was performed by one examiner, who was blinded in regard to treatment. Areas of macroscopically altered lung tissue (colour, consistency) were assessed on the ventral and dorsal view of the lungs and true to scale drawings recorded on one lung sketch. From this sketch the proportion of affected lung was estimated. Tissue samples were collected from the right lung, the tracheobronchial lymph node (from now on referred to as 'lymph node'), and spleen.