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Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains

Shyrley Paola Amarilla[a,*], Jaime Gómez-Laguna[b], Librado Carrasco[a], Irene M. Rodríguez-Gómez[a], José M. Caridad y Ocerín[c], Simon P. Graham[d,e], Jean-Pierre Frossard[d], Falko Steinbach[d,e], Francisco J. Salguero[e]

[a] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, Campus Universitario de Rabanales, 'International Excellence Agrifood Campus, CeiA3', 14071 Córdoba, Spain
[b] CICAP—Food Research Centre, 14400 Pozoblanco, Córdoba, Spain
[c] Department of Statistics, Econometrics, Operations Research, Business Organization and Applied Economics, Faculty of Law and Economics, 14071 Córdoba, Spain
[d] Department of Virology, Animal and Plant Health Agency, Addlestone, Surrey, KT15 3NB, United Kingdom
[e] Department of Pathology and Infectious Diseases, School of Veterinary Medicine, University of Surrey, Guildford GU2 7TE, United Kingdom

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) exists as two distinct viruses, type 1 (PRRSV-1) and type 2 (PRRSV-2). Atrophy of the thymus in PRRSV-2 infected piglets has been associated with a loss of thymocytes. The present study aimed to evaluate the impact of PRRSV-1 strains of differing virulence on the thymus of infected piglets by analysing the histomorphometry, the presence of apoptotic cells and cells producing cytokines. Thymic samples were taken from animals experimentally infected (with LV, SU1-bel, and 215-06 strains) or mock inoculated animals at 3, 7 and 35 days post-infection (dpi) and processed for histopathological and immunohistochemical analyses. PRRSV antigen was detected in the thymus from 3dpi until the end of the study in all virus-infected animals with the highest numbers of infected cells detected in SU1-bel group. The histomorphometry analysis and counts of $CD3^+$ thymocytes in the thymic cortex displayed significant differences between strains at different time-points ($p \leq 0.011$), with SU1-bel group showing the most severe changes at 7dpi. Cell death displayed statistically significant increase in the cortex of all infected animals, with SU1-bel group showing the highest rate at 3 and 7dpi. The number of cells immunostained against IL-1α, TNF-α and IL-10 were predominantly detected in the medulla ($p \leq 0.01$). An increase in the number of TNF-α and IL-10 positive cells was observed in LV and SU-1bel groups. Our results demonstrate that different PRRSV-1 strains induced depletion of the thymic cortex due to apoptosis of thymocytes and that the most severe depletion was associated with the highly virulent SU1-bel strain.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single stranded positive sense RNA virus belonging to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Meulenberg, 2000; Snijder et al., 2013). PRRSV is divided into two distinct viruses, type 1 (formerly European; PRRSV-1) and type 2 (formerly North American; PRRSV-2), with significant genetic differences between both genotypes (Salguero et al., 2015). Sequence analysis of different PRRSV-1 strains led to the definition of three distinct subtypes, namely pan-European subtype 1 and East European subtypes 2 and 3, and recently a fourth subtype has been proposed (Stadejek et al., 2013). Highly pathogenic (HP) PRRSV strains firstly emerged in China and were classified within the PRRSV-2 genotype; these strains caused high morbidity and mortality, high fever and multifocal skin haemorrhages in pigs of all ages (Tong et al., 2007). More recently, HP-PRRSV strains were also identified in Eastern Europe (Karniychuk et al., 2010). The emergence of HP-PRRSV in Asia and Eastern Europe have revived the interest in

* Corresponding author.
E-mail address: ashyrleypaola@yahoo.com (S.P. Amarilla).

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understanding the immunobiology of PRRSV strains of differing virulence (Gómez-Laguna et al., 2013; Salguero et al., 2015).

Differences in the virulence among PRRSV-1 strains have been associated with either an enhanced pro-inflammatory immune response, mainly associated to an increased pulmonary expression of IL-1α/β, or to higher levels of virus replication (Gómez-Laguna et al., 2010; Karniychuk et al., 2010; Morgan et al., 2013, 2014; Weesendorp et al., 2013; Amarilla et al., 2015; Salguero et al., 2015). However, the exact mechanisms by which PRRSV-1 exert its virulence are unknown.

The thymus is the lymphoid organ responsible for T lymphocyte differentiation and maturation and it is essential for the normal development and function of the immune system (Pearse, 2006b). A decrease in the relative thymus weight, associated to a decreased cellularity in the cortex and less commonly in the medulla, is a sensible indicator for immunosuppresion (Elmore, 2006; Pearse, 2006a, 2006b). Cortical involution of the thymus with a poor demarcation of cortico-medullary boundary and apoptosis of cortical thymocytes have been reported in PRRSV-2 infected piglets but data for PRRSV-1 strains are very scarce (Feng et al., 2002; Wang et al., 2011; He et al., 2012; Li et al., 2014).

Apoptosis of immune cells can be involved in the immunopathogenesis of viral diseases and has been suggested to play a significant role in PRRSV infection, with increased apoptotic cells widely distributed within both PRRSV-1 and PRRSV-2 infected tissues (Feng et al., 2002; Labarque et al., 2003; Wang et al., 2011; Gómez-Laguna et al., 2012; He et al., 2012; Li et al., 2014; Morgan et al., 2014; Rodríguez-Gómez et al., 2014). PRRSV replicates in thymic macrophages and dendritic cells (Halbur et al., 1996). The infected cells may interact with thymocytes delivering pro-apoptotic signals (He et al., 2012; Li et al., 2014). Several cytokines including TNF-α and IL-1α/β, promote apoptosis of T cell lines (Feng et al., 2002; Salguero et al., 2005). In addition, during the course of PRRSV, infected macrophages and, to a lesser extent neutrophils and lymphocytes, have been shown to up-regulate the expression of IL-1α, IL-6 and TNF-α (Labarque et al., 2003; Gómez-Laguna et al., 2010; Barranco et al., 2012).

Due to the limited available data on the effects of PRRSV-1 infection on the thymus, the present study aimed to evaluate the impact of PRRSV-1 strains of different virulence in the thymus of piglets by analysing the histomorphometry, the presence of apoptotic phenomena and the local expression of cytokines by analysing the number of immunostained cells with immunohistochemistry.

2. Materials and methods

2.1. Viruses

Three PRRSV-1 strains were used in this study, as previously reported (Morgan et al., 2013): Lelystad virus-Ter Huurne (LV), the prototype PRRSV-1 strain; strain 215-06; and strain SU1-bel. Both SU1-bel and 215-06 strains were used at the 4th passage and the LV strain was used at the 8th passage.

2.2. Animals and experimental design

The animal experiment protocol was described in detail by Morgan et al. (2013). Briefly, fifty four 5-weeks old male, Yorkshire cross Dutch Landrace piglets were obtained from an isolated, specific-pathogen-free pig farm in The Netherlands. All pigs were free of PRRSV, porcine circovirus type 2 (PCV-2) and *Mycoplasma hyopneumoniae* by ELISA and PCR. Animals were statistically blocked by weight and allocated into four groups and housed in separate pens of a containment facility at the Animal and Plant Health Agency (APHA) as follows (Table 1). The piglets were intranasally inoculated with 10^5 $TCID_{50}$ of the respective virus (LV, 215-06 or SU1-bel) in 1.5 ml of cRPMI. Controls were mock inoculated with 1.5 ml of naïve PAM cryolysate diluted in cRPMI.

Table 1
Distribution of all pigs experimentally infected within each group, namely control and three different PRRSV-1 strains: LV, 215-06 and SU1-bel. Animals were euthanized at 3, 7 and 35 dpi.

DPI	Control	LV[a]	215–06[b]	SU1-bel[c]
3	3	4	5	5
7	4	5	5	5
35	3	5	5	5[d]

[a] LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain.
[b] 215-06: British field strain.
[c] SU1-bel: Highly pathogenic strain.
[d] Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi.

Clinical signs associated with PRRSV infection and rectal temperatures were recorded daily from 3 days before experimental inoculation to 35 days post-infection (dpi) (Morgan et al., 2013; Weesendorp et al., 2013). At 3, 7 and 35 dpi, three to four pigs from the control group and four to five pigs from each infected group were euthanised by administration of an intravenous lethal dose of pentobarbitone, followed by exsanguination. At the necropsy, thymus samples were fixed in 10% neutral buffered formalin (Fisher Scientific Ltd., Loughborough, UK) and in Bouin's solution (Fisher Scientific Ltd.). This experiment was approved by the APHA Ethical Review Committee, and all procedures were carried out under the Animals (Scientific Procedures) Act, 1986, UK.

2.3. Histopathology and histomorphometry analysis of thymus

Four micron tissue sections were stained with haematoxylin and eosin (H&E). The severity of the lesions in thymus was scored as follows (adapted and modified from Contreiras et al., 2004):

Grade 0: The cortex:medulla ratio (C/M) is about 2:1 with typical histological characteristics of the thymus.

Grade I: Diffuse cortical reduction with focal cortical disappearance, 5–9 tingible-body macrophages/mm^2 within the thymic cortex, typical medulla and extraparenchymal compartment, which includes capsule, interlobular connective tissue septa and perivascular spaces (PVS).

Grade II: Focal or multifocal decrease of C/M (<2:1), decrease of cortical layer with slight proportional increase of the extraparenchymal compartment and 10–15 tingible-body macrophages/mm^2 within the thymic cortex.

Grade III: Focal to multifocal disappearance of cortico-medullary boundary distinction, increase of the extraparenchymal compartment, mainly PVS, occasional increase in the number of lymphocytes, mast and plasma cells and ≥16 tingible-body macrophages/mm^2, which give the tissue a "starry sky" appearance.

Histomorphometry was conducted using H&E-stained sections of the thymus subjected to digital image analysis by ImageJ 1.45 and NIS Elements BR (4.20.00 Build 967, 64 bits, University of Surrey, Guildford, UK) software. The percentage of the parenchyma (thymic cortex and thymic medulla) and stroma (capsule, interlobular connective tissue septa, PVS and adipose tissues) was calculated in a total area of 400 mm^2 for each slide. Differentiation of the parenchyma and stroma was performed according to the typical histological features of the thymus (Pearse, 2006b). Automatic and manual quantification of thymocytes and tingible-body macrophages in thymic cortex were assessed in 25