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

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Highlights:

- We evaluate the impact of PRRSV-1 strains on the thymus of infected piglets
- We demonstrate that different PRRSV-1 strains induced depletion of the thymic cortex
- The most severe depletion was associated with infection with the SU1-bel strain

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-1 α , 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.

Keywords: PRRSV-1, virulence, thymus, depletion, apoptosis, cytokine.

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* (Meulenbergh, 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 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-1 α / β , or to higher levels of virus replication (Gómez-Laguna et al., 2010; Karniyuchuk 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 immunosuppression (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-1 α / 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-1 α , 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 TCID₅₀ 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.

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/mm² 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/mm² 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/mm², 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 mm² 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 non-overlapping, consecutively selected high magnification fields of 0.2 mm². Results were expressed in number of cells per mm².

2.4. Immunohistochemistry

The Chemmate Dako Envision detection kit (Dako, Burlingame, CA, USA) was used for the detection of PRRSV nucleocapsid protein (Morgan et al., 2014) and the Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA)

was used for the detection of CD3, MAC387 and cytokines (IL-1 α , TNF- α and IL-10) (Gómez-Laguna et al., 2010; Barranco et al., 2011). Cleaved Caspase 3 (cCasp3) immunolabelling and Terminal dUTP Nick End-Labeling (TUNEL) were carried out with commercial kits (SignalStain® Apoptosis Cleaved Caspase-3 IHC Detection Kit, Cell Signaling, USA; and, In Situ Cell Death Detection Kit, POD, Roche, Germany, respectively) following the manufacturer's instructions. Briefly, 4- μ m sections were dewaxed and rehydrated, followed by endogenous peroxidase inhibition with 3% H₂O₂ solution in methanol for 45 minutes. The slides were exposed to different antigen retrieval methods or left untreated as summarized in Table 2. Sections were washed with PBS (pH 7.4, 0.01 M) and Tris-buffered saline (pH 7.6, 0.005 M) (Sigma–Aldrich, USA) with 0.01% Tween20 (TBST) and incubated for 30 minutes at room temperature with 100 μ l of blocking solution in a humid chamber. All primary antibodies against cytokines and cCasp3 were incubated overnight at 4 °C in a humid chamber. Primary anti-PRRSV antibody was incubated for 2 hours at room temperature. Anti-CD3 and anti-MAC387 antibodies and TUNEL were incubated for 1 hour at room temperature. In each case, the corresponding biotinylated secondary antibody (Vector Laboratories) was incubated for 30 minutes at room temperature. Chemmate Dako Envision detection kit was applied for 30 minutes at room temperature with the addition of 5% normal swine serum (Life Technologies) (PRRSV) and avidin-biotin-peroxidase complex was applied for 30 minutes (CD3 and MAC387) and for 1 hour (cytokines) at room temperature. Immunostaining was 'visualized' by application of the NovaRED™ substrate kit (Vector Laboratories) and 3,30-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Harris's haematoxylin, dehydrated and mounted. For negative controls, the primary antibody was replaced by an isotype control. Antibody specificity was verified by substituting the primary antibody by

isotype matched reagents of irrelevant specificity. Labelled cells were analysed in 25 non-overlapping and consecutively selected high magnification fields of 0.2 mm². The assessment in the expression of CD3 was calibrated according to the intensity of immunollabeling with threshold colour adjustment and expressed in percentage. While the expression of MAC387, cytokines and apoptosis cells (cCasp3 and TUNEL) were manually counted and expressed as the number of cells per mm². Immunolabelled cells were identified and counted, and morphologically differentiated as macrophages, thymocytes or neutrophils.

2.5. Statistical analysis

Several univariate models were estimated for different response variables, and also a multivariate analysis of variance, for the measuring of the histomorphometry, C/M ratio, the number of tingible-body macrophages and the expression of the PRRSV antigen, MAC387, CD3, cCasp3, TUNEL and the number of immunostained positive cells against cytokines (IL-1 α , TNF- α and IL-10), depending on the factors that may be the cause of variation: time-point and strain. All infected groups were compared to each other and with the control group and the differences were assessed by Dunnet and Mann Whitney tests, according to the parameters under study. A bidimensional contingency table and a log-linear model for the analysis of the interaction between different strains and histopathology score were performed. Correlation between PRRSV antigen labelled cells with the histomorphometry measurements, the score of the histopathology, the expression of cCasp 3, TUNEL and the number of immunostained positive cells against cytokines (IL-1 α , TNF- α and IL-10) were assessed by the Pearson and Spearman tests. Differences with p -values ≤ 0.05 were considered to be statistically significant. All

statistical analyses were performed using SPSS 22.0 (IBM SPSS Statistics 22, Chicago, USA).

3. Results

3.1. Clinical signs

The clinical signs have been previously described elsewhere (Morgan et al., 2013, 2014). Briefly, SU1-bel infected group had the highest percentage of animals with clinical signs and hyperthermia (above 40°C) and the mean temperatures/clinical signs of the LV and 215-06 groups were not different from controls. Two animals in the SU1-bel group displayed a prolonged hyperthermia along with high clinical scores and were euthanised for welfare reasons at 12 and 13 dpi.

3.2. Histopathological score and histomorphometric analysis of thymus

Control animals did not show significant microscopic changes in the thymus throughout the study. In the infected animals, the microscopic lesions were characterised by a diffuse cortical reduction with focal cortical disappearance, slight increase of the extraparenchymal compartment and focal to multifocal cortico-medullary inversion and, occasionally variable number of macrophages, mast cells and eosinophils were observed (Figs. 1A, C, E and G). Table 3 summarises the histopathological scores of the thymus for each group and time-point. Two animals infected with the SU1-bel strain and one animal infected with the LV strain displayed focal to multifocal disappearance of cortico-medullary boundary with an increase of the extraparenchymal compartment, mainly PVS, at 7 dpi. A significant increase in the number of tingible-body macrophages in the thymic cortex was detected in infected groups ($p < 0.001$), with

215-06 and SU1-bel groups differing significantly from the control group ($p \leq 0.003$) (Figs. 1B, D, F and H). Furthermore, at 3 and 7 dpi, SU1-bel group displayed significant differences when compared with LV and 215-06 groups ($p \leq 0.03$) and 215-06 group displayed significant differences when compared to LV group ($p < 0.009$) (Table 4). Statistically significant association was detected between different strains and the histopathology score ($p < 0.001$), mainly at 7dpi; besides, SU1-bel group displayed more significant differences in the histopathology score when compared with 215-06 group ($p < 0.03$).

The histomorphometric analysis of the thymus displayed significant differences in the percentage of parenchyma and stroma of the thymus between different strains and the control group ($p < 0.001$) (Table 4). In addition, statistically significant differences were observed in the percentage of thymic stroma at different time-points ($p < 0.005$). The percentage of the thymic cortex showed significant differences between different strains and control group ($p \leq 0.002$) (Table 4). Moreover C/M ratio in LV and SU1-bel infected animals showed the lowest ratio at 7 dpi ($p < 0.05$) (Table 3).

The number of thymocytes in the thymic cortex showed statistically significant differences at different time-points ($p = 0.001$) and between different strains and control group ($p \leq 0.011$). SU1-bel group displayed higher statistically significant differences when compared with the other PRRSV-1 strains at 7 and 35 dpi ($p < 0.0001$) (Table 4).

3.3. Evidence of PRRSV replication in the thymus

PRRSV antigen was detected at all time-points in all virus-infected animals and not detected in control group. The highest number of PRRSV antigen immunostained cells

was observed in SU1-bel group and secondly in LV group, whereas 215-06 group displayed the lowest number of immunostained cells. Statistical analysis of the expression of PRRSV antigen evidenced significant differences between different strains ($p = 0.003$) (Fig. 2).

3.4. Tissue distribution of macrophages and T cells in the thymus

The expression of MAC387 as a marker of macrophages was evidenced in a higher number of cells in the thymic medulla than in the cortex ($p \leq 0.01$) being mainly observed in the cytoplasm of macrophages and monocytes around the Hassall's corpuscles, PVS in the thymic medulla and in a lesser extent in PVS, subcapsular region and areas of decreased cellular density in the cortex (Figs. 3A, C, E and G). Tingible-body macrophages did not stain for MAC387. A statistically significant interaction between different strains and time-points was observed for the expression of MAC387 in the medulla of the thymus ($p = 0.005$), whereas statistically significant differences were only observed between different strains in the cortex ($p < 0.035$). Specifically, SU1-bel group showed a significant increase in the number of MAC387 positive cells in both cortex and medulla of the thymus when compared with the control group ($p = 0.018$) (Table 5).

CD3 as a marker for T cells was observed mainly in the thymic cortex and only in some scattered cells in the medulla (Figs. 3B, D, F and H). The SU1-bel group showed the lowest percentage of the expression of CD3 in the thymus cortex, presenting statistically significant differences when compared to LV and 215-06 groups ($p < 0.001$) at 7 and 35 dpi. Besides, the expression of CD3 in the thymic cortex was lower in all PRRSV-1 strains in comparison to control animals at the different time-points ($p < 0.0001$) (Table 5).

3.5. Apoptosis in the thymus of PRRSV-1 infected pigs

cCasp3 staining was mainly found in the cortex of the thymus ($p \leq 0.02$), within the nuclei of cells and secondly in free apoptotic bodies (Figs. 4B, D, F, H, and insets). The expression of cCasp3 yielded statistically significant differences both in the thymic cortex and medulla between different time-points ($p < 0.001$) and between the infected and control groups ($p \leq 0.051$). The expression of cCasp3 showed a significant increase in the thymic medulla of SU1-bel group at 3 dpi when compared to 215-06 group ($p < 0.03$) (Table 5).

TUNEL labelling, was mostly observed in the cortex and to a lesser extent in the medulla of the thymus of all piglets including the control group (Figs. 4A, C, E and G). TUNEL labelling was mainly observed in apoptotic bodies inside macrophages and occasionally in free apoptotic bodies (Fig. 4C inset). Statistically significant differences in the expression of TUNEL in thymic cortex were observed between different time-points and between the infected and control groups ($p < 0.001$). In the medulla of the thymus, statistically significant differences were only detected at different time-points ($p = 0.025$). SU1-bel group showed a significant increase in apoptosis cells in both the thymic cortex and medulla at 3 and 7 dpi when compared with 215-06 group ($p < 0.03$) and at 7 dpi in the thymic cortex when compared with LV group ($p < 0.01$) (Table 5).

3.6. Immunostained cells against cytokines in the thymus

The immunostaining of IL-1 α , TNF- α and IL-10 was mainly detected in the cytoplasm of macrophages and to a lesser extent in the cytoplasm of neutrophils and lymphocytes in the thymic medulla ($p \leq 0.01$) (Figs. 5A, C and E). The number of IL-1 α

immunostained cells did not display statistically significant changes between different strains and time-points (Figs. 5B). At 3 dpi, the LV group displayed a significant increase in the number of TNF- α immunostained cells when compared to the control group ($p = 0.016$) and 215-06 group ($p = 0.032$) (Fig. 5D). A significant increase in the number of IL-10 immunostained cells was observed in LV and SU1-bel groups at 3 dpi when compared with the control group ($p = 0.006$ and $p = 0.004$, respectively) (Fig. 5F).

3.7. Correlation of apoptosis and the number of immunostained cells against cytokine with PRRSV-1 infection

In the thymic cortex, a significant negative correlation was found between the expression of cCasp3 and the percentage of parenchyma in LV and SU1-bel groups at different time-points ($r \geq -0.70$; $p \leq 0.007$). To the other hand, a significant negative correlation was observed between the expression of cCasp3 in the cortex and the expression of CD3 in the 215-06 group ($r = -0.74$; $p < 0.001$). In the SU1-bel group at 3 dpi, a significant positive correlation was observed between the expression of TUNEL and PRRSV antigen in the thymic medulla ($r = 0.90$; $p = 0.037$), and in the thymic cortex between cCasp3 and PRRSV antigen ($r \geq 0.90$; $p \leq 0.037$); and between TUNEL and PRRSV antigen in the thymic cortex in the LV group ($r = 0.979$; $p = 0.021$). In addition, the expression of TUNEL was negatively correlated with the number of thymocytes in the cortex of the thymus of SU1-bel infected animals at 7 dpi ($r = -1.00$; $p < 0.001$). A significant positive correlation was detected between the number of TNF- α and IL-1 α immunostained cells in LV and 215-06 strains ($r \geq 0.70$; $p \leq 0.004$) and between the number of TNF- α and IL-10 immunostained cells in LV strain ($r = 0.83$; $p < 0.001$) in the thymic cortex at different time-points. A significant positive correlation between the three cytokines was only detected in the thymic medulla of the SU1-bel

group at 7 dpi ($r = 0.90$; $p = 0.037$). Finally, the number of IL-1 α immunostained cells showed a significant positive correlation with the number of MAC387 positive cells in the thymic cortex in LV group at different time-points ($r = 0.71$; $p < 0.004$) and in 215-06 and SU1-bel infected pigs at 3 dpi ($r \geq 0.90$; $p \leq 0.037$).

4. Discussion

PRRSV is characterised by a marked genetic variability and the impairment of the host immune response (Stadejek et al., 2013). Atrophy and depletion of the thymus in piglets infected with PRRSV-2 strains of different virulence have been associated with a loss of T cells in the cortex of the thymus and direct or indirect induction of apoptotic phenomena (Feng et al., 2002; He et al., 2012; Li et al., 2014). In our study all infected animals, independently of the PRRSV-1 strain used, showed changes in the histomorphometry of the thymus and significant amounts of apoptosis was detected. Piglets infected with the more pathogenic PRRSV-1 strain SU1-bel, showed the most severe changes when compared with two less virulent strains belonging to the pan European subtype 1 (LV and 215-06 strains).

All infected groups displayed a similar kinetics of the histopathological scores with a peak of severity at 7 dpi. The average score throughout the study was grade II for all the strains, although microscopic lesions observed in one animal from LV group and two animals from SU1-bel group were of grade III. A decrease in the percentage of parenchyma and thymic cortex was observed in all infected animals from 3 dpi onwards when compared to control group leading to a reduction of the C/M ratio. These changes were associated with a decrease in the cellularity of the cortex, specifically in the number of thymocytes and the expression of CD3, which were significantly more severe

in the animals infected with the SU1-bel strain. Some of these parameters were negatively correlated with the expression of cCasp3 in PRRSV-1 strain infected animals. Decreased cellularity of the thymus is frequently associated with cell death and an increased number of tingible-body macrophages (Elmore, 2006). In our study, the infection with any PRRSV-1 strain showed an increase in the number of tingible-body macrophages compared to the control group, with SU1-bel infected animals presenting the highest counts, giving the tissue a "starry sky" appearance. These findings support the fact that PRRSV-1-infection suppresses the immune system in piglets and provides additional explanation regarding the pathogenesis of PRRSV-1 strains.

Several authors have previously measured PRRSV induced apoptosis in the thymus of infected animals using TUNEL method which was associated with severe thymus atrophy of thymocytes (Feng et al., 2002; He et al., 2012). Interestingly, despite the differences in the experimental design among these studies, the results were similar and showing a moderate to severe atrophy of thymus accompanied by significant increase of apoptotic cells in the thymic cortex. Caution should be given when evaluating apoptosis solely by TUNEL labelling since this technique may not differentiate between apoptosis and necrosis (Charriaut-Marlangue and Ben-Ari, 1995), and thus may rather be considered as marker for cell death. Therefore, in order to confirm apoptosis the use of a second assay based on a different principle is recommended (Watanabe et al., 2002). Our assessment of thymocyte death by means of TUNEL and cCasp3 labelling displayed a similar distribution, but the expression of TUNEL was higher than the expression of cCasp3. This finding could be attributed to several factors: (1) TUNEL assay is a sensible technique to detect cell death but may yield false positive results, from necrotic cells and cells in the process of DNA repair and gene transcription,

(Charriaut-Marlangue and Ben-Ari, 1995; Watanabe et al., 2002; Elmore, 2007); (2) TUNEL labels a later stage of the apoptotic pathway, whereas cCasp3 labelling shows an earlier event in the apoptotic cascade (Watanabe et al., 2002; Resendes et al., 2004); (3) Apoptosis may be also triggered by Casp3-independent pathways (Rodríguez-Gómez et al., 2014), which may justify the lower expression of cCasp3 than TUNEL.

The results of the present study show that different PRRSV-1 strains induced changes in the histopathology and histomorphometry in the thymus, mainly in the cortex, associated to apoptosis of thymocytes with the highest change observed in the thymus of piglets infected with the highly pathogenic SU1-bel strain. Indeed, in the animals infected with SU1-bel strain the expression of PRRSV antigen was positively correlated with both TUNEL and cCasp3 labelling. A significant positive correlation was also detected between TUNEL and the expression of PRRSV antigen in LV-infected animals.

Macrophages play a pivotal role in the induction of the host immune response through the secretion of mediators such as cytokines, the removal of dead cells and cellular debris, and the presentation of antigens to T-cells for the induction of an effective adaptive immune response (Elmore, 2006; Pearse, 2006a; Rodríguez-Gómez et al., 2013). Macrophages may be classified attending to their polarization into classically (M1) or alternatively (M2) activated macrophages. M2 macrophages have been reported to be more susceptible than M1 macrophages to PRRSV infection and to induce higher levels of anti-inflammatory cytokines (e.g. IL-10), enhanced phagocytic activity but reduced antigen presenting functions (Mosser, 2003; García-Nicolás et al., 2014). In the present study, SU1-bel infected piglets showed the highest increase in the number of macrophages both in the cortex and in the medulla of the thymus. Interestingly, the

number of IL-10 immunostained cells was also higher in SU1-bel infected animals compared to the other PRRSV-1 strains at 7 dpi, coinciding also with a higher count of tingible-body macrophages and an increase of apoptotic phenomena detected by TUNEL and cCasp3. These results suggest the presence of alternatively activated M2 macrophages and reflect the requirement for enhanced phagocytosis after the increased cell death (tissue remodelling). The reduced generation of T cells could result in an impaired T cell response, as previously suggested (Rodríguez-Gómez et al., 2013; Salguero et al., 2015).

IL-1 α and TNF- α are considered as pro-apoptotic cytokines, while IL-10 plays a moderating role in pro-apoptotic effects (Choi et al., 2002, Goetz et al., 2004; Salguero et al., 2004). In previous studies by our group, animals infected with SU1-bel strain showed a higher expression of IL-1 α in the lung than piglets infected with LV or 215-06 strains (Amarilla et al., 2015). In this study, the number of IL-1 α immunostained cells in the thymus of infected animals was variable. However, the number of TNF- α and IL-10 immunostained cells showed an increase in LV and SU1-bel groups. Previous studies have reported an association between apoptosis in the lungs, lymphoid tissues and the expression of several cytokines, such as IL-1, IL-10, IL-6, TNF- α and TGF- β , suggesting a mechanism for cytokine induced apoptosis in PRRSV infection (Choi et al., 2002; Labarque et al., 2003; Rodríguez-Gómez et al., 2014). The results herein with the higher counts for apoptotic cells rather than PRRSV-positive cells point towards a strain-dependent behaviour in the induction of apoptosis and reinforce the hypotheses of both PRRSV-induced apoptosis and indirect mechanisms involved in the induction of apoptosis during PRRSV infection (Li et al., 2014).

Conclusion

Our results demonstrate that different PRRSV-1 strains induce changes mainly in the cortex of the thymus due to the apoptosis of thymocytes. Highly pathogenic PRRSV-1 (SU1-bel) showed the most severe depletion of the thymus with the highest expression of PRRSV antigen compared to the two low virulent strains of the pan European subtype 1 (LV and 215-06 strains). In addition, apoptosis of thymocytes was preceded in our study by a peak in the local number of TNF- α and/or IL-10 immunostained cells mainly in medulla of all infected animals.

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References

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Morgan, S. B., Graham, S. P., Frossard, J. P., Drew, T. W., Salguero, F. J., 2015. A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: Upregulation of IL-1 α

- in highly pathogenic strain induced lesions. *Vet. Immunol. Immunopathol.* 164, 137-47.
- Barranco, I., Gómez-Laguna, J., Rodríguez-Gómez, I. M., Salguero, F. J., Pallarés, F. J., Bernabé, A., Carrasco, L., 2011. Immunohistochemical detection of extrinsic and intrinsic mediators of apoptosis in porcine paraffin-embedded tissues. *Vet. Immunol. Immunopathol.* 139, 210-6.
- Barranco, I., Gómez-Laguna, J., Rodríguez-Gómez, I. M., Quereda, J. J., Salguero, F. J., Pallarés, F. J., Carrasco, L., 2012. Immunohistochemical expression of IL-12, IL-10, IFN-alpha and IFN-gamma in lymphoid organs of porcine reproductive and respiratory syndrome virus-infected pigs. *Vet. Immunol. Immunopathol.* 149, 262-71.
- Contreiras, E. C., Lenzi, H. L., Meirelles, M. N., Caputo, L. F., Calado, T. J., Villa-Verde, D. M., Savino, W., 2004. The equine thymus microenvironment: a morphological and immunohistochemical analysis. *Dev. Comp. Immunol.* 28, 251-64.
- Charriaut-Marlangue, C., Ben-Ari, Y., 1995. A cautionary note on the use of the TUNEL stain to determine apoptosis. *Neuroreport.* 7, 61-4.
- Choi, C., Cho, W. S., Kim, B., Chae, C., 2002. Expression of Interferon-gamma and tumour necrosis factor-alpha in pigs experimentally infected with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). *J. Comp. Pathol.* 127, 106-13.
- Elmore, S. A., 2006. Enhanced histopathology of the thymus. *Toxicol. Pathol.* 34, 656-65.
- Elmore, S., 2007. Apoptosis: A Review of Programmed Cell Death. *Toxicol. Pathol.* 35, 495-516.

- Feng, W. H., Tompkins, M. B., Xu, J. S., Brown, T. T., Laster, S. M., Zhang, H. X., McCaw, M. B., 2002. Thymocyte and peripheral blood T lymphocyte subpopulation changes in piglets following in utero infection with porcine reproductive and respiratory syndrome virus. *Virology* 302, 363-72.
- García-Nicolás, O., Baumann, A., Vielle, N. J., Gómez-Laguna, J., Quereda, J. J., Pallarés, F. J., Ramis, G., Carrasco, L., Summerfield, A., 2014. Virulence and genotype-associated infectivity of interferon-treated macrophages by porcine reproductive and respiratory syndrome viruses. *Virus Res.* 179, 204-11.
- Goetz, F. W., Planas, J. V., MacKenzie, S., 2004. Tumor necrosis factors. *Dev. Comp. Immunol.* 28, 487-97.
- Gómez-Laguna, J., Salguero, F. J., Barranco, I., Pallarés, F. J., Rodríguez-Gómez, I. M., Bernabé, A., Carrasco, L., 2010. Cytokine expression by macrophages in the lung of pigs infected with the porcine reproductive and respiratory syndrome virus. *J. Comp. Pathol.* 142, 51-60.
- Gómez-Laguna, J., Salguero, F. J., Fernández de Marco, M., Barranco, I., Rodríguez-Gómez, I. M., Quezada, M., Carrasco, L., 2012. Type 2 Porcine Reproductive and Respiratory Syndrome Virus Infection Mediated Apoptosis in B- and T-Cell Areas in Lymphoid Organs of Experimentally Infected Pigs. *Transbound. Emerg. Dis.* 60, 273-278
- Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., Carrasco, L., 2013. Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. *Vet. J.* 195, 148-155
- Halbur, P. G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J., Rathje, J. A., 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory

- syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *J. Vet. Diagn. Invest.* 8, 11-20.
- He, Y., Wang, G., Liu, Y., Shi, W., Han, Z., Wu, J., Jiang, C., Wang, S., Hu, S., Wen, H., Dong, J., Liu, H., Cai, X., 2012. Characterization of thymus atrophy in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. *Vet. Microbiol.* 160, 455-62.
- Karniychuk, U. U., Geldhof, M., Vanhee, M. , Van Doorselaere, J., Saveleva, T. A., Nauwynck, H. J., 2010. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet. Res.* 6, 30.
- Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., Pensaert, M., 2003. Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. *Vet. Res.* 34, 249-60.
- Li, Y., Wang, G., Liu, Y., Tu, Y., He, Y., Wang, Z., Han, Z., Li, L., Li, A., Tao, Y., Cai, X., 2014. Identification of apoptotic cells in the thymus of piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. *Virus Res.* 189, 29-33.
- Meulenbergh, J. J., 2000. PRRSV, the virus. *Vet. Res.* 31, 11-21.
- Morgan, S. B., Frossard, J. P., Pallarés, F. J., Gough, J., Stadejek, T., Graham, S. P., Steinbach, F., Drew, T. W., Salguero, F. J., 2014. Pathology and Virus Distribution in the Lung and Lymphoid Tissues of Pigs Experimentally Inoculated with Three Distinct Type 1 PRRS Virus Isolates of Varying Pathogenicity. *Transbound. Emerg. Dis.* In press, DOI: 10.1111/tbed.12272.

- Morgan, S. B., Graham, S. P., Salguero, F. J., Sánchez Cordán, P. J., Mokhtar, H., Rebel, J. M., Weesendorp, E., Bodman-Smith, K. B., Steinbach, F., Frossard, J. P., 2013. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. *Vet. Microbiol.* 163, 13-22.
- Mosser, D. M., 2003. The many faces of macrophage activation. *J. Leukoc. Biol.* 73, 209-12.
- Pearse, G., 2006a. Histopathology of the thymus. *Toxicol. Pathol.* 34, 515-47.
- Pearse, G., 2006b. Normal structure, function and histology of the thymus. *Toxicol. Pathol.* 34, 504-14.
- Resendes, A. R., Majo, N., Segalés, J., Espadamala, J., Mateu, E., Chianini, F., Nofrarias, M., Domingo, M., 2004. Apoptosis in normal lymphoid organs from healthy normal, conventional pigs at different ages detected by TUNEL and cleaved caspase-3 immunohistochemistry in paraffin-embedded tissues. *Vet. Immunol. Immunopathol.* 99, 203-13.
- Rodríguez-Gómez, I. M., Gómez-Laguna, J., Carrasco, L., 2013. Impact of PRRSV on activation and viability of antigen presenting cells. *World J. Virol.* 2, 146-51.
- Rodríguez-Gómez, I. M., Barranco, I., Amarilla, S. P., García-Nicolás, O., Salguero, F. J., Carrasco, L., Gómez-Laguna, J., 2014. Activation of extrinsic- and Daxx-mediated pathways in lymphoid tissue of PRRSV-infected pigs. *Vet. Microbiol.* 172, 186-94.
- Salguero, F. J., Sánchez-Cordón, P. J., Núñez, A., Fernández de Marco, M., Gómez-Villamandos, J. C., 2005. Proinflammatory cytokines induce lymphocyte apoptosis in acute African swine fever infection. *J. Comp. Pathol.* 132, 289-302.

- Salguero, F. J., Sánchez-Cordón, P. J., Sierra, M. A., Jover, A., Núñez, A., Gómez-Villamandos, J. C., 2004. Apoptosis of thymocytes in experimental African Swine Fever virus infection. *Histol. and Histopathol.* 19, 77-84.
- Salguero, F. J., Frossard, J. P., Rebel, J. M., Stadejek, T., Morgan, S. B., Graham, S. P., Steinbach, F., 2015. Host-pathogen interactions during porcine reproductive and respiratory syndrome virus 1 infection of piglets. *Virus Res.* In press, DOI: 10.1016/j.virusres.2014.12.026.
- Snijder, E. J., Kikkert, M., Fang, Y., 2013. Arterivirus molecular biology and pathogenesis. *J. Gen. Virol.* 94, 2141-63.
- Stadejek, T., Stankevicius, A., Murtaugh, M. P., Oleksiewicz, M. B., 2013. Molecular evolution of PRRSV in Europe: current state of play. *Vet. Microbiol.* 165, 21-8.
- Tong, G. Z., Zhou, Y. J., Hao, X. F., Tian, Z. J., An, T. Q., Qiu, H. J., 2007. Highly pathogenic porcine reproductive and respiratory syndrome, China. *Emerg. Infect. Dis.* 13, 1434-6.
- Wang, G., Song, T., Yu, Y., Liu, Y., Shi, W., Wang, S., Rong, F., Dong, J., Liu, H., Cai, X., Zhou, E. M., 2011. Immune responses in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. *Vet. Immunol. and Immunopathol.* 142, 170-8.
- Watanabe, M., Hitomi, M., van der Wee, K., Rothenberg, F., Fisher, S. A., Zucker, R., Svoboda, K. K., Goldsmith, E. C., Heiskanen, K. M., Nieminen, A. L., 2002. The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. *Microsc. Microanal.* 8, 375-91.
- Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Popma-De Graaf, D. J., Graham, S. P., Rebel, J. M., 2013. Comparative analysis of immune responses

following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. *Vet. Microbiol.* 163, 1-12.

Figure Legends

Fig. 1. Representative photomicrographs of the thymus from a pig from control group and from pigs inoculated with different PRRSV-1 strains and euthanised at 7 days post-infection. H&E stained thymus sections a pig from (A-B) control group and from pigs infected with (C-D) SU1-bel, (E-F) LV and (G-H) 215-06 strains are presented. Bar, 500 μm (A,C,E and G) and 50 μm (B,D,F and H). The SU1-bel infected animal thymus presented a grade III histopathology score, whereas the LV and 215-06 infected animals presented a grade II. Focal to multifocal disappearance of cortico-medullary boundary distinctions are shown by white arrows and tingible-body macrophages by black arrows.

Fig. 2. Graph represent the number of immunostained PRRSV-antigen-positive cells per mm^2 in the thymus from all the groups at all time-points. Counts for PRRSV-antigen-positive cells in the thymus of control animals and animals infected with the three different PRRSV-1 strains included in the study. Animals were euthanised at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains and control group, whereas the letter "b" indicates significant differences between different time-points and "c" indicates significant differences with low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at $p\text{-values} \leq 0.05$.

Fig. 3. Representative photomicrographs of MAC387 and CD3 immunolabeling in the thymus from a pig from control group and from pigs inoculated with different PRRSV-1

strains and euthanised at 7 dpi. IHQ stained thymus sections from (A-B) a control pig and from pigs infected with (C-D) SU1-bel, (E-F) LV and (G-H) 215-06 strains are presented. Bar, 100 μ m and 50 μ m. The SU1-bel group showed an increase in the number of MAC387 positive cells in both cortex and medulla of the thymus and the lowest labelled of CD3 in the thymic cortex when compared with the control group and other PRRSV-1 strains.

Fig. 4. Representative photomicrographs of TUNEL and cCasp3 labelling in the thymus from a pig from control group and from pigs inoculated with different PRRSV-1 strains and euthanised at 7 dpi. TUNEL labelling of tingible-body macrophages and apoptotic bodies in the thymic cortex of (A) a control animal, (C) SU1-bel, (E) LV and (G) 215-06 infected animal. TUNEL. Bar, 50 μ m. C, *inset*: detail of tingible-body macrophages labelled with TUNEL method. TUNEL. Bar, 10 μ m. cCasp3 labelling of lymphocytes and macrophages in the thymus of (B) a control animal, (D) SU1-bel, (F) LV and (H) 215-06 infected animal. IHQ. Bar, 50 μ m. D and F, *insets*: detail of a lymphocyte (D) and a macrophage (F) immunolabelled against cCasp3. IHQ. Bar, 10 μ m.

Fig. 5. Representative photomicrographs of the thymus from pigs infected with (A, C) SU1-bel and (E) LV strains at 3 dpi. Macrophages in the thymus, mainly in the thymic medulla expressing IL-1 α of a pig from LV group and TNF- α and IL-10 of a pig from SU1-bel groups euthanised at 3 dpi. IHQ. Bar, 50 μ m. (B, D, F). Counts for IL-1 α , TNF- α , and IL-10 positive cells in the thymus from control animals and pigs infected with PRRSV-1 strains varying in virulence. Animals were euthanised at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers

show maximum and minimum values. The letter "a" indicates significant differences between different strains and control group, whereas the letter "b" indicates significant differences between the different time-points and "c" indicates significant differences with low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p -values ≤ 0.05 .

PRRSV_a

Table Legends

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 euthanised 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*

a LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain; **b** 215-06: British field strain; **c** SU1-bel: Highly pathogenic strain; * 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.

Table 2. Summary of immunohistochemical methodology.

Specificity	Type of antibody	Commercial Origin	Fixative	Blocking solution	Dilution	Antigen retrieval
Anti-PRRSV (clone SDOW17)	mAb	Rural Technologies, Brookings, SD,USA	Formalin	BSA	1 in 70	Protease
Anti-human CD3 (clone F7.2.38)	mAb	Dako	Formalin	10% NGS	1 in 1000	Protease
Anti-human MAC-387 (clone MAC387)	mAb	Serotec	Formalin	10% NGS	1 in 100	Protease
Anti-pig IL-1 α	pAb	Endogen, Woburn, USA	Bouin	BSA	1 in 100	Tween 20 0.01%
Anti-human TNF- α (clone 68B6A3 L1)	mAb	Invitrogen, Camarillo, USA	Bouin	BSA	1 in 75	Tween 20 0.01%
Anti-pig IL-10	pAb	R&D Systems, Abingdon, UK	Bouin	10% NGS	1 in 20	Tween 20 0.01%

mAb, monoclonal antibody; pAb, polyclonal antibody; Tween 20 diluted 0.01% in phosphate-buffered saline for 10 min; BSA, Bovine Serum Albumin; NGS, Normal Goat Serum; Protease, enzymatic digestion with trypsin (0.5%) / chymotrypsin (0.5%) at 37°C for 10 minutes; PRRSV, Porcine reproductive and respiratory syndrome virus.

Table 3. Cortex:Medulla ratio and histopathology score in thymus of piglets from each group at 3, 7 and 35 dpi in relation to the total number of animals per group, classified according to the ratio and scores obtained.

DPI	Ratio	1:1	2:1	Histopathology Score	Control	LV	215-06	SU1-bel
	Control		3/3	Grade 0	3/3	2/4	2/5	1/5
	LV		4/4	Grade I		2/4	2/5	2/5
3	215-06	1/5	4/5	Grade II			1/5	2/5
	SU1-bel		5/5	Grade III				
DPI	Ratio	1:1	2:1	Histopathology Score	Control	LV ^a	215-06 ^a	SU1-bel ^{a,c}
	Control		4/4	Grade 0	4/4			
	LV ^a	2/5	3/5	Grade I		3/5	2/5	
7	215-06		5/5	Grade II		1/5	3/5	3/5
	SU1-bel ^a	2/5	3/5	Grade III		1/5		2/5
DPI	Ratio	1:1	2:1	Histopathology Score	Control	LV	215-06	SU1-bel
	Control		3/3	Grade 0	3/3	3/5	2/5	
	LV		5/5	Grade I		1/5	2/5	2/3
35	215-06		5/5	Grade II		1/5	1/5	
	SU1-bel		3/3	Grade III				1/3

Normal Cortex:Medulla ratio: 2:1. Grade 0: normal histology. Grade I: Diffuse cortical reduction with focal cortical disappearance, about 5 to 9 tingible-body macrophages by mm^2 within the thymic cortex. Grade II: Focal or multifocal decrease of C/M ($< 2:1$), decrease of cortical layer with slight proportional increase of the extraparenchymal compartment and about 10 to 15 of tingible-body macrophages by mm^2 within the thymic cortex. Grade III: Focal to multifocal disappearance of cortico-medullary boundary distinction, increase of the extraparenchymal compartment and higher number of tingible-body macrophages ($\geq 16 / \text{mm}^2$), which give the tissue a “starry sky” appearance. Different letters indicate statistically significant differences ($p \leq 0.05$): "a" Statistically significant differences between different strains and control group; "b" Statistically significant differences between different time-points; "c" Statistically significant differences with low virulent strains (LV and/or 215-06 strains).

Table 4. The percentage of the tissue in the thymus, number of tingible-body macrophages and thymocytes in the cortex of the thymus at all time points in all virus-infected animals and control group. Animals were euthanised at 3, 7 and 35 dpi.

	STRAINS	3 DPI Median±SD	7 DPI Median±SD.	35 DPI Median±SD.
PARENCHYMA	CONTROL	82,47 ±8,58	80,60 ±7,53	82,63 ±8,27
	LV	69,70 ±7,35 ^a	56,06 ±3,44 ^a	71,71 ±4,45 ^a
Percentage of tissues	215-06	62,06 ±0,98 ^a	59,91 ±7,66 ^a	59,39 ±17,55 ^a
	SU1-bel	68,43 ±3,95 ^a	49,26 ±11,25 ^a	60,27 ±9,88 ^a
CORTEX	CONTROL	60,15 ±12,75	61,00 ±8,43	63,77 ±3,82
	LV	49,61 ±12,46 ^a	40,52 ±5,89 ^a	55,62 ±4,29 ^a
Percentage of tissues	215-06	40,41 ±1,67 ^a	42,46 ±8,09 ^a	44,57 ±14,00 ^a
	SU1-bel	49,30 ±7,66 ^a	27,65 ±8,69 ^a	44,97 ±13,61 ^a
MEDULLA	CONTROL	21,82 ±3,46	19,60 ±4,38	18,53 ±7,85
	LV	20,08 ±7,04	15,53 ±3,13	16,69 ±2,54
Percentage of tissues	215-06	21,65 ±1,39	17,44 ±3,73	14,62 ±6,18
	SU1-bel	19,13 ±4,67	21,61 ±8,28	15,29 ±3,96
STROMA	CONTROL	17,90 ±8,81	19,57 ±7,65	17,72 ±8,76
	LV	30,29 ±7,35 ^{a,b}	43,93 ±3,44 ^{a,b}	27,64 ±3,79 ^{a,b}
Percentage of	215-06	37,93 ±0,98 ^{a,b}	40,08 ±7,66 ^{a,b}	40,84 ±17,64 ^{a,b}
tissues	SU1-bel	31,56 ±3,95 ^{a,b}	50,73 ±11,25 ^{a,b}	39,72 ±9,88 ^{a,b}
THYMOCYTES	CONTROL	68,00 ±2,82	65,00 ±4,89	64,67 ±5,13
	LV	56,50 ±3,10 ^{a,b}	59,80 ±2,16 ^{a,b}	61,40 ±3,13 ^{a,b}
1000 cells / mm ²	215-06	58,4 ±6,020 ^{a,b}	60,40 ±2,79 ^{a,b}	60,00 ±1,87 ^{a,b}
	SU1-bel	55,50 ±1,29 ^{a,b,c}	57,60 ±1,94 ^{a,b,c}	58,67 ±4,04 ^{a,b}

TINGIBLES-BODY MACROPHAGES	CONTROL	2,20 ±2,23	2,44 ±4,88	2,67 ±4,62
	LV	4,68 ±9,36 ^a	6,10 ±13,65 ^a	13,56 ±2,11 ^a
cells / mm ²	215-06	16,93 ±5,34 ^a	10,39 ±6,50 ^a	11,33 ±2,77 ^a
	SU1-bel	19,88 ±,974 ^{a,c}	28,76 ±8,17 ^{a,c}	26,73 ±3,67 ^{a,c}

Data are represented as median and standard deviation ($\pm$ SD). The letter "a" indicates significant differences between different strains and the control group, whereas the letter "b" indicates significant differences between different time-points and "c" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p-values ≤ 0.05 .

Table 5. The percentage of the expression of CD3, the number of immunostained MAC387 and apoptosis-positive cells per mm² in the thymus from all the groups at all time-points. Animals were euthanised at 3, 7 and 35 dpi.

	STRAINS	3 DPI Median±SD.	7 DPI Median±SD.	35 DPI Median±SD.
CD3	CONTROL	54,14 ±2,12	53,10 ±3,40	58,50 ±4,41
Percentage	LV	50,39 ±3,01 ^{a,b}	44,13 ±2,67 ^{a,b}	53,29 ±5,53 ^{a,b}
	215-06	51,74 ±1,74 ^{a,b}	43,21 ±2,29 ^{a,b}	51,47 ±3,83 ^{a,b}
	SU1-bel	51,17 ±2,64 ^{a,b}	33,90 ±6,41 ^{a,b,c}	35,54 ±0,318 ^{a,b,c}
MAC387	CONTROL	2,54 ±0,947	1,71 ±1,51	2,45 ±1,54
	LV	6,14 ±4,18 ^a	2,30 ±1,90 ^a	4,79 ±5,00 ^a
CORTEX	215-06	2,49 ±2,18 ^a	2,80 ±1,81 ^a	4,87 ±2,96 ^a
cells/mm ²	SU1-bel	6,37 ±5,99 ^a	7,67 ±3,35 ^a	2,77 ±1,49 ^a
MAC387	CONTROL	7,79 ±1,30	5,74 ±1,53	6,17 ±2,61
	LV	11,15 ±3,43	7,10 ±2,49	9,63 ±3,17
MEDULLA*	215-06	4,49 ±2,55	9,40 ±2,09	10,74 ±7,44
cells/mm ²	SU1-bel	15,87 ±4,72 ^a	12,13 ±5,90 ^a	4,55 ±3,45
cCasp3	CONTROL	3,75 ±4,72	9,76 ±3,63	4,66 ±2,57
	LV	8,32 ±3,34 ^{a,b}	49,74 ±28,71 ^{a,b}	9,60 ±3,79 ^{a,b}
CORTEX*	215-06	10,46 ±7,15 ^{a,b}	43,00 ±13,44 ^{a,b}	9,24 ±6,98 ^{a,b}
cells/mm ²	SU1-bel	18,60 ±11,97 ^{a,b}	46,15 ±44,56 ^{a,b}	5,86 ±6,56 ^{a,b}

cCasp3	CONTROL	2,46 ±2,73	6,08 ±2,46	4,33 ±2,75
	LV	7,47 ±2,94 ^{a,b}	19,86 ±10,34 ^{a,b}	4,33 ±2,33 ^{a,b}
MEDULLA	215-06	5,66 ±3,71 ^{a,b}	15,76 ±5,62 ^{a,b}	3,73 ±2,18 ^{a,b}
cells/mm ²	SU1-bel	10,75 ±2,06 ^{a,b}	17,61 ±8,27 ^{a,b}	4,07 ±2,16 ^{a,b}
TUNEL *	CONTROL	60,43 ±15,90	107,30 ±35,71	90,33 ±26,10
	LV	182,87 ±91,74 ^{a,b}	223,64 ±38,46 ^{a,b}	150,32 ±34,69 ^{a,b}
CORTEX	215-06	129,80 ±19,35 ^{a,b}	234,28 ±43,42 ^{a,b}	164,32 ±31,45 ^{a,b}
cells/mm ²	SU1-bel	216,76 ±57,92 ^{a,b,c}	327,3 ±59,810 ^{a,b,c}	145,60 ±2,64 ^{a,b}
TUNEL	CONTROL	25,50 ±0,45	46,10 ±7,40	36,19 ±32,56
	LV	50,30 ±19,29 ^b	95,80 ±16,84 ^b	46,08 ±20,14 ^b
MEDULLA	215-06	31,52 ±8,35 ^b	90,47 ±27,37 ^b	53,40 ±19,83 ^b
cells/mm ²	SU1-bel	57,68 ±24,59 ^{b,c}	190,39 ±228,04 ^{b,c}	53,47 ±9,39 ^b

Data are represented as median and standard deviation (±SD). The letter "a" indicates significant differences between different strains and control group, whereas the letter "b" indicates significant differences between different time-points and "c" indicates significant differences with low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p-values ≤ 0.05.