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Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is difficult to control due to a high mutation rate of the PRRS virus (PRRSV) and the emergence of virulent strains. The objective of this study was to analyse early and late pathological responses in the respiratory tract after infection with the European PRRSV subtype 3 strain Lena in comparison to two European PRRSV subtype 1 strains: Belgium A and Lelystad-Ter Huurne (LV). For each virus strain, groups of twelve pigs were inoculated, and four pigs per group were euthanized at days 3, 7 and 35 post-infection (p.i.) for consecutive examination. Infection with strain Lena resulted in a more severe disease than with the subtype 1 strains, an inflammatory response within the first week of infection with expression of IL-1 α in the lung and lymph node, and an influx of neutrophils and monocytes in bronchoalveolar lavage fluid (BALF). Infection with strain Belgium A or LV resulted in mild or no pathology within the first week of infection, but inflammatory cell influx in the lung interstitium was increased at the end of the experiment at day 35 p.i. At five weeks p.i., all strains induced a higher percentage of cytotoxic T cells and higher levels of IFN- γ producing cells in BALF. This might have contributed to clearance of virus. In general, subtype 3 strain Lena induced a stronger early inflammatory response which led to more severe clinical disease and pathology. On the other hand, this may have supported an enhanced or faster clearance of virus in tissues, compared to subtype 1 strains.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by a single stranded RNA virus belonging to the *Arteriviridae* family (Meulenberg et al., 1994). PRRSV is endemic in most pig producing countries of the world. Infection with PRRSV causes

reproductive disease in sows with abortions and stillbirths at all stages of gestation. In growing pigs, increased pre-weaning mortality, inappetance and respiratory disorders can be observed. PRRSV is considered a primary pathogen, but co-infections with bacterial or viral pathogens commonly occur and exacerbate the clinical symptoms in growing pigs (reviewed by Drew, 2000). As a consequence of both primary and secondary infections with PRRSV, significant economic losses and welfare problems are observed in the swine industry worldwide (Neumann et al., 2005).

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PRRSV is divided into two genotypes, the European PRRSV and the North American PRRSV. These genotypes have a high degree of genetic divergence (Wensvoort et al., 1991; Collins et al., 1992; reviewed by Stadejek et al., 2013). The European genotype strains can be further divided into at least three subtypes: Pan-European subtype 1 and Eastern European subtypes 2 and 3 (Stadejek et al., 2008). The pathogenicity of European genotype subtype 1 strains and North American genotype strains has been studied several times. In general, North American genotype strains show a larger variation in pathogenicity than European genotype subtype 1 strains. In several experimental studies more severe respiratory disease has been observed with North American genotype strains (Halbur et al., 1995, 1996; Van der Linden et al., 2003; Martínez-Lobo et al., 2011; reviewed by Gómez-Laguna et al., 2013; Han et al., 2013a,b). Only limited information is available about European subtype 2 and 3 strains. Subtype 3 strains were described to be more virulent than subtype 1 strains in experimentally infected pigs (Karniychuk et al., 2010; Morgan et al., 2013; Weesendorp et al., 2013a,b). In a previous study (Weesendorp et al., 2013a,b) we analysed the systemic responses of pigs after infection with two European subtype 1 strains, and one subtype 3 strain. The present study focuses specifically on the respiratory tract of these pigs, by characterizing the local immunological and pathological responses. It was shown that the subtype 3 strain Lena induced a stronger early inflammatory response which resulted in more severe clinical disease and lung pathology than observed after infection with subtype 1 strains Belgium A or Lelystad-*Ter Huurne* (LV). Interestingly, although subtype 1 strains did not induce clinical disease, these strains induced more severe long-term microscopic lung lesions to the infection than the subtype 3 strain. On the other hand, the early inflammatory response of strain Lena might have enhanced a faster clearance of virus than observed after infection with subtype 1 strains.

2. Materials and methods

2.1. Viruses

Three different European PRRSV strains were used: the subtype 1 strains, Belgium A virus (Belgium A (07V063) and Lelystad virus-*Ter Huurne* (LV), and a subtype 3 strain, Lena virus. The strain Belgium A was isolated from a stillborn piglet, derived from a Belgium farm during an outbreak of PRRSV associated reproductive disorders (Karniychuk et al., 2010). This strain was used at the third passage on porcine alveolar macrophages (PAM). PRRSV strain LV 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. Strain Lena was isolated from a Belarusian farm with reproductive and respiratory failure (Karniychuk et al., 2010). This strain was used at the third passage on PAM.

2.2. Animals and experimental protocol

The experimental protocol was described in detail by Weesendorp et al. (2013a). In short, sixty-four five-week-old male pigs were obtained from a SPF herd, free of PRRSV, *Mycoplasma hyopneumonia* and *Actinobacillus pleuropneumonia*. Pigs were housed in four groups in different rooms of an isolation unit. Pigs were inoculated intranasally with 1.5 ml inoculum containing 10^5 50% tissue culture infectious dose (TCID₅₀) of either European subtype 1 strain Belgium A, strain LV, European subtype 3 strain Lena, or an equal volume of PBS (group control). In each group, four pigs were vaccinated with an Aujeszky's disease vaccine, but these pigs were excluded from the present study. At days 3, 7 and 35 post-infection (p.i.), four pigs per group were euthanized for post-mortem examination. Rectal temperatures and clinical symptoms were recorded daily. Viraemia was analysed in serum at days 0, 3, 5, 7, 10, 14, 21, 26 and 33 p.i. Results of viraemia were presented in detail by Weesendorp et al. (2013a).

Table 1
Body temperature, clinical symptoms and viraemia.

Virus strain	Days post-inoculation				
	3	7	10	20 ^a	35 ^b
Lena					
No. pigs with fever ^c /total no.	8/12	6/8	4/4	0/4	0/4
No. pigs with clinical symptoms/total no.	6/12	2/8	4/4	0/4	0/4
No. pigs with viraemia/total no.	12/12	8/8	4/4	4/4	0/4
Belgium A					
No. pigs with fever/total no.	0/12	0/8	0/4	0/4	0/4
No. pigs with clinical symptoms/total no.	0/12	0/8	0/4	0/4	0/4
No. pigs with viraemia/total no.	8/12	8/8	4/4	4/4	0/4
Lelystad					
No. pigs with fever/total no.	0/12	0/8	0/3	0/3	0/3
No. pigs with clinical symptoms/total no.	0/12	0/8	0/3	0/3	0/3
No. pigs with viraemia/total no.	10/12	8/8	3/3	3/3	0/3
Non-infected control					
No. pigs with fever/total no.	0/12	1/8	0/4	0/4	0/4
No. pigs with clinical symptoms/total no.	0/12	0/8	0/4	0/4	0/4
No. pigs with viraemia/total no.	0/12	0/8	0/4	0/4	0/4

^a Viraemia was analysed at dpi 21.

^b Viraemia was analysed at dpi 33.

^c Fever was defined as body temperature higher than 40.5 °C for one day, or 40 °C when also the preceding or following day was 40 °C or higher.

A summary of the most important data on rectal temperature, clinical signs and viraemia is presented in [Table 1](#). The experiment was approved by the Animal Experiment Committee of the Central Veterinary Institute of Wageningen UR.

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

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) which are an indication of inflammatory alterations 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 three predefined locations of the right lung, i.e. from the apical lobe, the cardiac lobe and the caudo-ventral diaphragmatic lobe, tracheobronchial lymph node (from now on referred to as 'lymph node'), and spleen. Tissue samples were fixed in 4% formaldehyde by immersion and processed for histological examination. Complementary parts of the lung, lymph node and spleen samples were snap-frozen in liquid nitrogen and stored at -80°C until total RNA was isolated from these samples.

The left part of the lung was directly placed onto ice after gross pathology examination and taken to the laboratory for lavage under sterile conditions, with 250 ml of cold Dulbecco's phosphate buffered saline (PBS) (Gibco). After massaging the lung, the fluid was recovered and centrifuged at $470 \times g$ for 10 min at 4°C . The cells were washed twice with cold PBS and resuspended at 4×10^7 cells/ml in BALF-medium (RPMI 1640 medium with 300 mg/L L-glutamine supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin [all from Gibco, Invitrogen]) with 10% (v/v) dimethyl sulfoxide (DMSO) for preservation in liquid nitrogen. When cells were needed for subsequent analyses, they were thawed and washed twice with BALF-medium. The percentage of viable cells was about 95% as determined by Trypan blue staining.

2.4. Histology and immunohistochemistry

Formalin fixed tissue samples from the three sampled locations of the lungs and lymph nodes were routinely processed, and embedded in paraffin wax. Consecutive 4 μm thick sections were cut and one was stained with haematoxylin and eosin (H&E). Other lung sections were used to determine the presence of PRRSV antigen in tissue and to characterize the infiltration of lymphocytic cells by staining CD3 positive cells. For immunohistochemical (IHC) detection, thin sections were mounted on poly-L-lysine coated slides, deparaffinised and treated with 3% hydrogen peroxidase to block endogenous peroxidase. To detect PRRSV antigen in the tissue slides, the monoclonal antibody SDOW17 (specific for the nucleocapsid antigen of PRRSV, Rural Technologies, Inc.®) was used. To detect CD3 expressing cells (only applied at days 3 and 7 p.i.), an

anti-human CD3 antibody was used. Bound antibody was visualized by using the EnVision™ Detection system followed by incubation with diaminobenzidine (DAB) chromogenic substrate, which results in a dark brown staining of bound primary antibody sites. Slides were counterstained with haematoxylin. A semi-quantitative, pathohistological assessment of the H&E stained slides was performed by one pathologist, who was blinded in regard of treatment. The examination encompassed the extent of pneumonia in each slide, i.e. presence of focal or diffuse alterations with interstitial or catarrhal pneumonia or atelectasis, the extent of infiltration of alveolar septae with mononuclear cells and the extent of infiltration of mononuclear cells in the perivascular/peribronchiolar area. A histological score from 0, i.e. no findings, 1 = mild focal manifestation, 2 = moderate, multifocal manifestation, 3 = moderate diffuse manifestation, 4 = severe diffuse manifestation and 5 = very severe extended manifestation was used to describe the severity of changes. To compare the histological findings between groups, the scores from each slide per lung were added to an overall score, which could add up to a maximum of 15.

For the counting of PRRSV positive cells, 25 microscopic fields at $40\times$ objective magnification, i.e. ca. 5 mm^2 per slide, were examined. The number of positive stained cells or spots were counted. The numbers of CD3 positive cells in the alveolar septae of the lung tissue as well as in the peribronchiolar/perivascular area were counted in tissue slides from three different lung locations in five microscopic fields per slide at a $20\times$ objective magnification (Zeiss® AxioScope); the morphometric analyses were performed by using the image analysis software Image Pro Plus 7 (Media Cybernetics, Silver Spring, MD).

2.5. Immunophenotyping of leucocytes in BALF by flow cytometry

Thawed BALF cells were transferred to microtitre plates (1×10^6 cells in a volume of 100 μl per well) and centrifuged for 3 min at $350 \times g$. Cells were triple stained with mAbs directed to porcine SWC1, CD172a and SWC8 for identification of leukocyte populations or CD3, CD4 and CD8 for identification of T-cell subpopulations (as described by [Weesendorp et al., 2013a,b](#)). The staining with primary antibodies was followed by a combination of APC, FITC and PE labelled secondary antibodies. Primary antibodies used were: mouse anti-porcine-CD3: clone PPT3, IgG₁; mouse anti-porcine-CD4: clone 74-12-4, IgG_{2b}, mouse anti-porcine-CD8 α : clone SL2 (11/295/33) IgG_{2a}, mouse anti-porcine-SWC1a, recently identified as CD52 ([Leitner et al., 2012](#)) clone K263.3D7, IgG₁ (AbD Serotec), mouse anti-porcine-CD172a: clone BL1H7, IgG_{2b} (VMRD), mouse anti-porcine-SWC8: clone MIL3, IgM (AbD Serotec). The primary antibodies were detected by PE, APC and FITC-conjugated anti-mouse isotype specific immunoglobulins (Southern Biotechnology Associates).

Flow cytometry analyses were performed with a CyAn ADP flow cytometer using Kaluza Software (both Beckman Coulter). The leukocyte or alveolar macrophage populations in BALF were gated based on the forward-scatter versus side-scatter diagram as described by [Nielsen et al.](#)

(2003a). Within the leukocyte gate, monocytes were identified as SWC1⁺CD172a⁺SWC8⁻, neutrophils as SWC1⁺CD172a⁺SWC8⁺, and B cells as SWC1⁻CD172a⁻SWC8^{high} (Summerfield et al., 2001). The cytotoxic T cell sub-population was identified as CD3⁺CD4⁻CD8^{high}, T helper cells as CD3⁺CD4⁺ (naive and memory T helper cells), NK cells as CD3⁻CD4⁻CD8⁺ cells, and $\gamma\delta$ T cells as CD3⁺CD4⁻CD8⁻ (Nielsen et al., 2003b; Gerner et al., 2009). Although the majority of $\gamma\delta$ T cells are CD3⁺CD4⁻CD8⁻, they can also exist as CD8⁺ cells (Gerner et al., 2009). We, therefore, refer to this CD3⁺CD4⁻CD8⁻ population as CD8⁻ $\gamma\delta$ T cells. Indicated in the figures are the percentages of cells in the gated area with a specific phenotype.

2.6. BALF cells in IFN- γ ELISPOT assay

The number of antigen-specific IFN- γ -secreting cells per 5×10^5 BALF cells was determined using an ELISPOT assay according to the method described by the manufacturer of the used MultiScreen_{HTS}-IP Filter plates (Millipore). BALF cells from days 7 and 35 p.i. were analysed. Briefly, the plates were pre-coated overnight with 10 $\mu\text{g}/\text{ml}$ of anti-pig IFN- γ mAb (BD). BALF cells (5×10^5 cells in 50 $\mu\text{l}/\text{well}$) were plated in BALF-medium and stimulated for 20–24 h in triplicate wells by addition of homologous PRRSV at a multiplicity of infection (MOI) of 0.01. The virus used was filtered to remove cytokines produced by PAM cell cultures as described by Weesendorp et al. (2013b). Concanavalin A (20 $\mu\text{g}/\text{ml}$ [Sigma]) was used in triplicate wells as a positive control, and non-infected filtered PAM culture medium in triplicate wells as negative control (all 50 $\mu\text{l}/\text{well}$). Plates were incubated with 100 μl of anti-pig IFN- γ biotin-labelled mAb (BD) at a concentration of 0.17 $\mu\text{g}/\text{ml}$. The reaction was revealed by sequential incubation with streptavidin-alkaline phosphatase enzyme conjugate (R&D Systems) and BCIP/NBT substrate solution (R&D systems). The number of specific IFN- γ secreting cells, as determined using an ImmunoSpot[®] S4 Analyzer (Cellular Technology Ltd.), was calculated as the mean number of spots in the BALF cell cultures stimulated with virus, minus the number of spots in negative control wells. The data were expressed as the background corrected number of IFN- γ -secreting cells per 5×10^5 BALF cells.

2.7. RNA isolation from tissues

Approximately 100 mg of frozen lung, lymph node and spleen tissue was homogenized in 1 ml TRIzol[®] reagent (Invitrogen). After homogenization, insoluble material was removed from the homogenate. Further extraction of RNA from these homogenates was performed according to instructions of the TRIzol[®] reagent manufacturer. The yield and purity of the RNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.8. Detection of PRRSV in tissues by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

A one-tube qRT-PCR was performed with the Applied Biosystem 7500 Fast System instrument using the

Quantitect Probe RT-PCR kit from Qiagen. The reaction mixture (25 μl) contained 0.25 μl of kit-supplied enzyme, 12.5 μl of Quantitect Mix, 15 μM of each primer (Fw: 5'-GAT GAC RTC CGG CAY C-3', Rev: 5'-CAG TTC CTG CGC CTT GAT-3') and 10 μM of probe (5'-Fam-TGC AAT CGA TCC AGA CGG CTT-Tamra-3') (Frossard et al., 2012). The RT-PCR was done at 30 min at 50 °C and 15 min at 95 °C. A two-step cycling protocol was used: 94 °C for 20 s, and 55 °C for 45 s for 40 cycles. Analysis was performed with the 7500 Software v2.0.6. The viral RNA concentration (expressed as TCID₅₀ equivalents per g) of each individual sample was calculated using the standard curve. Standard curves were constructed by extracting RNA from five decimal dilutions of medium spiked with known concentrations of infectious virus.

2.9. Quantitative RT-PCR for the detection of cytokine RNA

The concentration of IL-1 β , IL-10, IFN α , and TNF- α mRNA in lung, lymph node and spleen samples was determined by qRT-PCR as described by Weesendorp et al. (2013a). Briefly, 200 ng of total RNA was reverse transcribed using random primers and Superscript III (Promega). An appropriate dilution of cDNA was amplified in a PCR reaction mix (20 μl) containing 5 μM of forward and reverse primer (2.5 μl of each primer), and a concentration of 2 \times SYBR Green Mix (Applied Biosystems). Serial dilutions of pGem-T Easy plasmids containing the PCR fragment of interest were used as internal standards. Ct values for the tested cytokines in each sample were expressed as cDNA quantity (ng) using the internal standards. Subsequently, the cytokine ng levels were normalized with the ng levels of the house keeping gene 18S. The data were expressed as the fold induction compared to the control group.

2.10. Statistical analysis

Statistical analyses to compare flow cytometry data, cytokine induction in tissues and leukocyte or lymphocyte counts in blood between groups were performed at each sampling point using a one-way ANOVA, after verifying homogeneity of variances between groups. Statistical analyses to compare the virus load in tissues, (gross) pathology scores and IFN- γ secreting cells measured by ELISPOT between groups were performed at each sampling point using a non-parametric Kruskal–Wallis test followed by Mann–Whitney *U* tests for multiple comparisons. Calculations were performed with SPSS 19 (SPSS Inc.). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Clinical symptoms, fever and viraemia

Detailed results of clinical signs, fever and viraemia have been presented by Weesendorp et al. (2013a). A summary of the most important results is shown in Table 1. In general, the subtype 3 strain Lena induced fever and clinical symptoms in most pigs between days 2 and

17 p.i. (Weesendorp et al., 2013a,b). Pigs displayed respiratory symptoms and reduced liveliness and appetite. No fever or clinical symptoms were observed in strains Belgium A or LV-infected pigs. In the LV strain infected pigs, one pig died at day 8 p.i. due to an intra-abdominal testicular torsion, thus the mortality was not PRRSV related. Viraemia was induced faster in strain Lena infected pigs than in strain Belgium A or LV-infected pigs, i.e. at day 3 p.i., all strain Lena infected pigs were viraemic, while 8 and 10 of the 12 infected pigs were viraemic with strain Belgium A or LV, respectively. At day 35 p.i., no viraemia was found in any of the pigs. Peak virus levels between days 7 and 10 p.i. were approximately $2 \log_{10} \text{TCID}_{50}/\text{ml}$ higher in pigs infected with strain Lena-infected pigs than in pigs infected with the other strains (Weesendorp et al., 2013a,b).

3.2. Pathology

3.2.1. Gross pathology

At day 3 p.i., in pigs infected with subtype 3 strain Lena a mild decreased post mortem retraction of the lungs ($n=2$), enlarged tracheobronchial lymph nodes ($n=2$), but no circumscribed pneumonic foci were seen. Besides these respiratory tract findings, mild oedematous changes of liver and mesentery ($n=3$) were found. In three pigs infected with strain Belgium A either small foci with a greyish-red discoloration of lung tissue with consolidated parenchyma ($n=2$), indicating areas of pneumonia or a decreased lung retraction, in combination with enlarged tracheobronchial lymph nodes ($n=1$) was found. In two

pigs infected with strain LV mildly enlarged tracheobronchial lymph nodes were found, but no changes in lung tissue. The mean proportion of affected lung per necropsy day is depicted in Fig. 1.

At day 7 p.i., all four pigs infected with strain Lena, one pig infected with strain Belgium A and two pigs infected with strain LV had (multi)focal areas with pneumonia. Lesions were most extended in Lena strain infected pigs, where the affected lung area ranged from 2% to maximum 8%, whereas after infection with subtype1 strains the maximum of affected lung area was 3%. At day 35 p.i., only a decreased retraction of the lungs was observed in pigs infected with subtype 1 strain LV. In one pig infected with strain Belgium A, a small, focal area with pneumonia was seen.

3.2.2. Histopathology

In pigs that developed an interstitial pneumonia in the lungs, the characteristics of pneumonia were similar between the different strains, i.e. an interstitial pneumonia with thickening and mononuclear infiltration of alveolar septae, hyperplasia of type-2 pneumocytes and accumulations with macrophages and cell debris in alveolar spaces. However, the extension of lesions varied between groups. At day 3 p.i. only very mild focal changes were seen, whereas at day 7 p.i. lesions were significantly more extensive and severe in the group infected with strain Lena, where in all pigs a moderate multifocal interstitial pneumonia with focal necrosis of alveolar cells was found (Figs. 1B and Fig. 2). Although, no macroscopic lesions were observed at 35 dpi (with one exception in the pigs infected

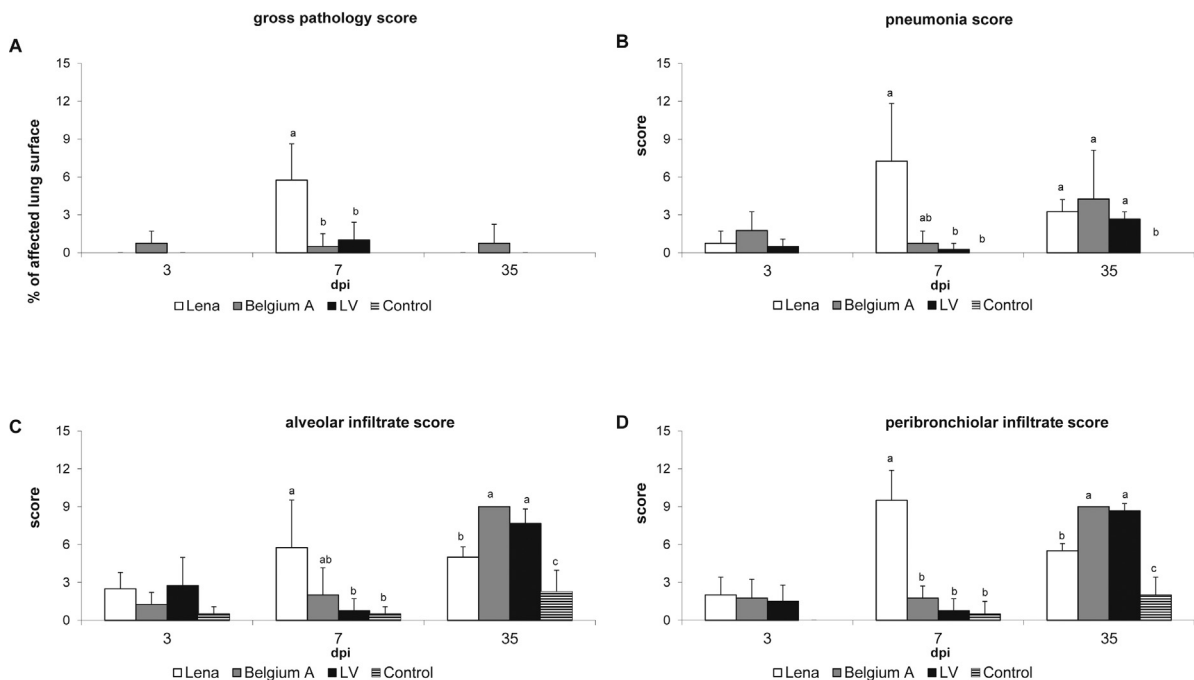


Fig. 1. Gross and histopathology of lungs: extension of lung lesions expressed as proportion of affected lung tissue recorded on a lung sketch and calculated as proportion of affected area of the total lung sketch (A); mean histological scores from three predetermined, standard lung tissue samples of pigs infected with different strains of PRRSV, pneumonia scores (B), leucocyte infiltrate scores in alveolar interstitium (C) and peribronchiolar compartment (D) \pm SD. Bars with no common superscript differ significantly ($p < 0.05$).

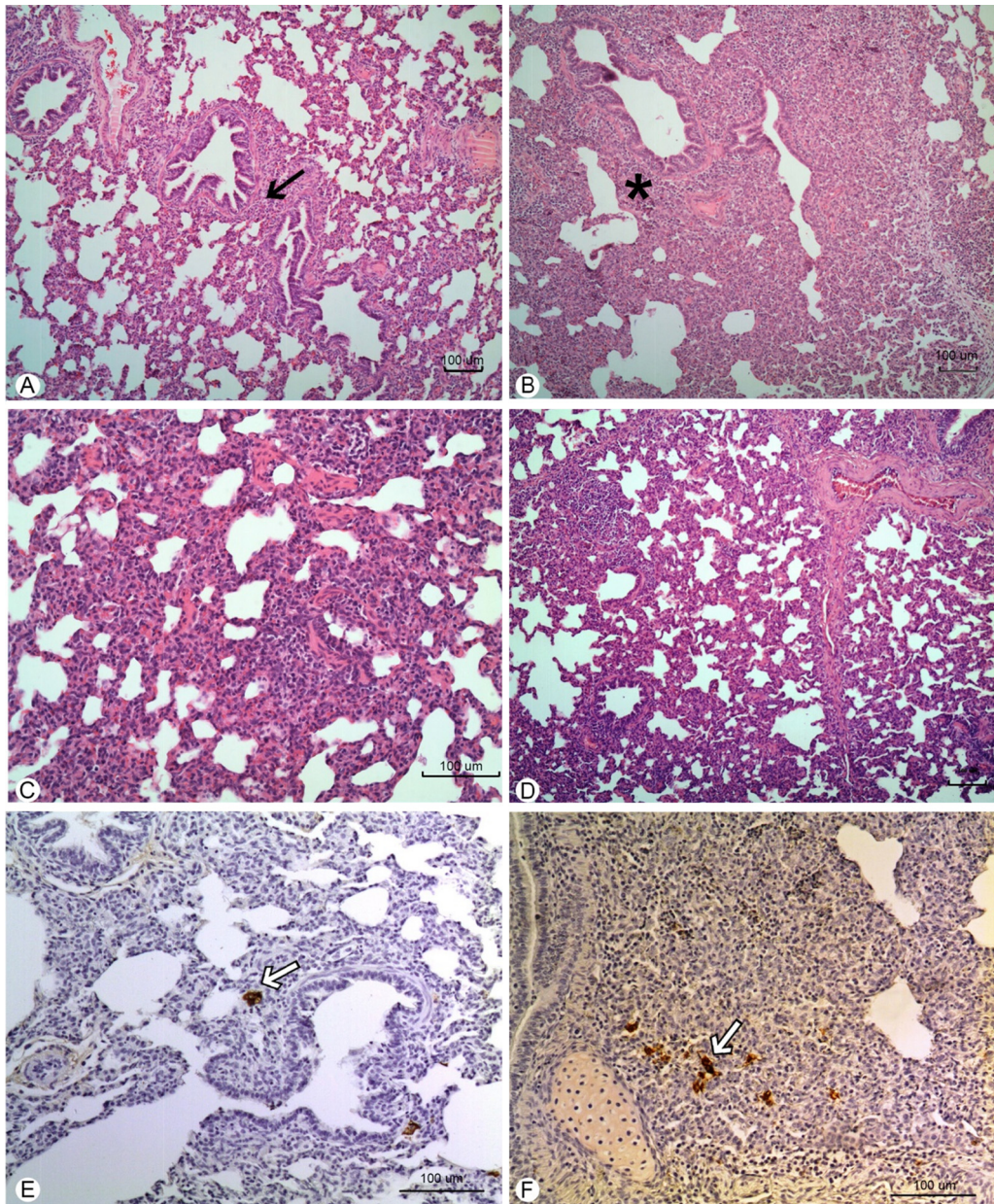


Fig. 2. Histological findings in lungs after infection with PRRSV genotype 1 subtype 1 (left column) or subtype 3 (right column). At 7 days after infection no significant pathological changes with PRRSV LV strain (A), note few mononuclear leucocytes in peribronchiolar area (*dark arrow*) and alveolar interstitium; interstitial pneumonia after infection with subtype 3 strain Lena (B) with extended inflammatory cell infiltrations (*asterisk*); at 35 days post infection diffuse, moderate infiltration in alveolar septae and peribronchiolar compartment with subtype 1 (strain Belgium A) (C) and focal peribronchiolar cell infiltrations after infection with subtype 3 (D). Immunohistological staining of PRRSV shows only few dark-brown-stained positive spots at 7 days post infection with subtype 1 (strain Belgium A) (E) and several PRRSV antigen positive spots in inflamed lung areas after infection with subtype 3 (*open arrows*) (F).

with strain Belgium A), in all groups a mild focal interstitial pneumonia was observed (Figs. 1B and Fig. 2). No pneumonia was seen in any of the control animals.

Next to presence of pneumonic foci, the overall infiltration of leucocytes into alveolar septae and into the peribronchiolar compartments was assessed. Significant differences were observed between groups in the degree of the histological pneumonia score and the extent of interstitial pneumonia based on the alveolar and

peribronchiolar cell infiltrate scores (Fig. 1C and D). At day 7 p.i., strain Lena-infected pigs had the highest pneumonia score, alveolar and peribronchiolar infiltrate scores. However at day 35 p.i., pigs infected with strains Belgium A and LV had higher cell infiltrate scores in alveolar walls and peribronchiolar compartments than the strain Lena-infected or control pigs. In all groups these cells were mostly macrophage-monocytes and the phenotyping of CD3 positive cells in the alveolar interstitium and in the

peribronchiolar compartment outside pneumonic areas did not show significant differences between control and infected groups (data not shown) The pneumonia scores at day 35 p.i. were for all infected groups significantly higher than the control pigs.

3.3. PRRSV load in tissues

Small differences were observed in viral RNA levels in lung, lymph node (Fig. 3) and spleen tissues (spleen data not shown) between the groups. In general, pigs infected with strain LV had at days 3 and 7 p.i the highest levels of viral RNA in the tissues, although mostly not significantly higher than pigs infected with strains Lena and Belgium A. At day 35 p.i., viral RNA was detected only in tissues from pigs infected with strains Belgium A or LV (in total $n = 3$ LV and $n = 3$ Belgium A-infected pigs [one LV-infected pig was only positive in the spleen sample]) and not in tissues from strain Lena-infected pigs.

After immunohistological staining, PRRSV antigen was found mostly in areas with pneumonia and located in the cytoplasm of alveolar macrophages and in cellular debris in alveoli and bronchiolar lumen. The PCR results on the presence of viral nucleic acid in the lungs were, however, not reflected in the quantification of viral antigen by IHC

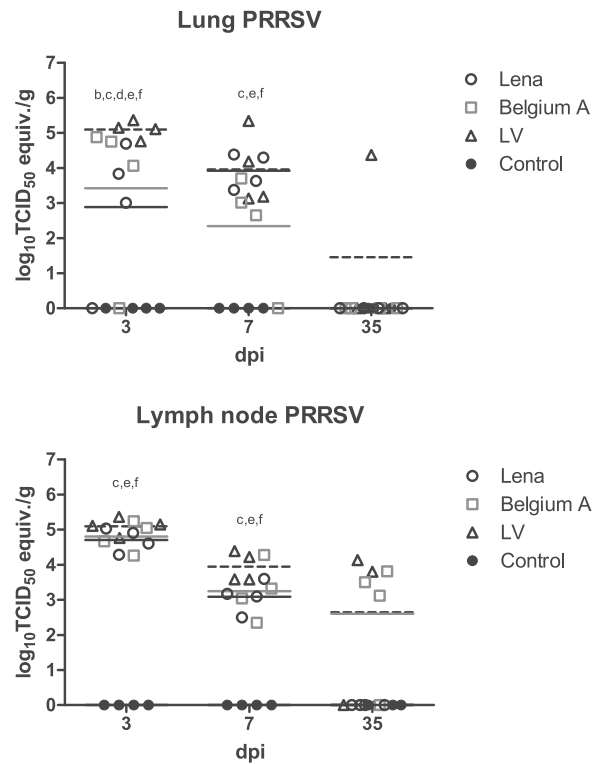


Fig. 3. PRRSV mRNA load \pm SD in the lung and tracheobronchial lymph node of pigs infected with different PRRSV strains. Lines indicate the average of 3 to 4 pigs (—: Lena; —: Belgium A; - - : LV). “a” denotes a significant difference ($p < 0.05$) between Lena and Belgium A-infected pigs; “b” between Lena and LV-infected pigs; “c” between Lena and control pigs; “d” between Belgium A and LV-infected pigs; “e” between Belgium A and control pigs; and “f” between LV and control pigs.

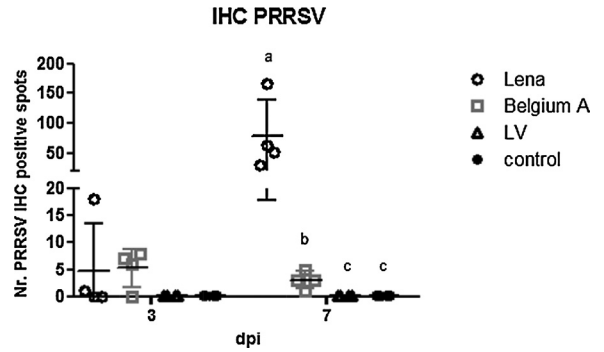


Fig. 4. Immunohistochemical staining of PRRSV antigen in lung tissue. Lines indicate the average of 3 to 4 pigs (—: Lena; —: Belgium A). “a” denotes a significant difference ($p < 0.05$) between Lena and Belgium A-infected pigs; “b” between Lena and LV-infected pigs; “c” between Lena and control pigs; “d” between Belgium A and LV-infected pigs; “e” between Belgium A and control pigs.

(Fig. 4). The numbers of positive spots were generally low. All pigs infected with strain Lena at day 7 p.i. were positive with a significantly higher number of positive stained spots than in lungs of pigs infected with strain Belgium A, but no staining was found in the pigs infected with strain LV at any time point.

3.4. Immunophenotyping of leukocytes in BALF by flow cytometry

With increasing age of the pigs, the proportion of total leucocytes, i.e. monocytes, lymphocytes and granulocytes compared to alveolar macrophages increased in the BALF (Fig. 5). PRRSV infection appeared to enhance this development in the course after infection. Although not significantly different, the proportion of total leucocytes was higher in the infected pigs than in the control pigs at day 35 p.i. The infection with the different virus strains induced strain or subtype specific as well as common changes in leucocyte populations at early and late time points. The following changes were observed in the phenotypes of the leucocytes in the BALF after infection with the different virus strains. At day 3 p.i., a significantly higher percentage of $CD8^- \gamma\delta$ T cells was observed in pigs infected with strains Belgium A and LV than in the control pigs, but not in strain Lena infected pigs. At day 7 p.i., the strain Lena-infected pigs had significantly higher percentages of neutrophils and monocytes, which seemed to have started already at day 3 p.i (not significant). At day 35 p.i., nearly twice as high percentages of cytotoxic T cells were observed in all infected groups compared to the control group, and significantly lower percentages of T helper cells, $CD8^- \gamma\delta$ T cells and B cells.

3.5. IFN- γ production by BALF cells

In the ELISPOT assay, after homologous PRRSV stimulation, no IFN- γ secreting BALF cells were observed at days 7 p.i. in any of the groups. At day 35 p.i., significantly higher numbers of IFN- γ secreting cells were observed in all infected groups, but this was only significantly higher for

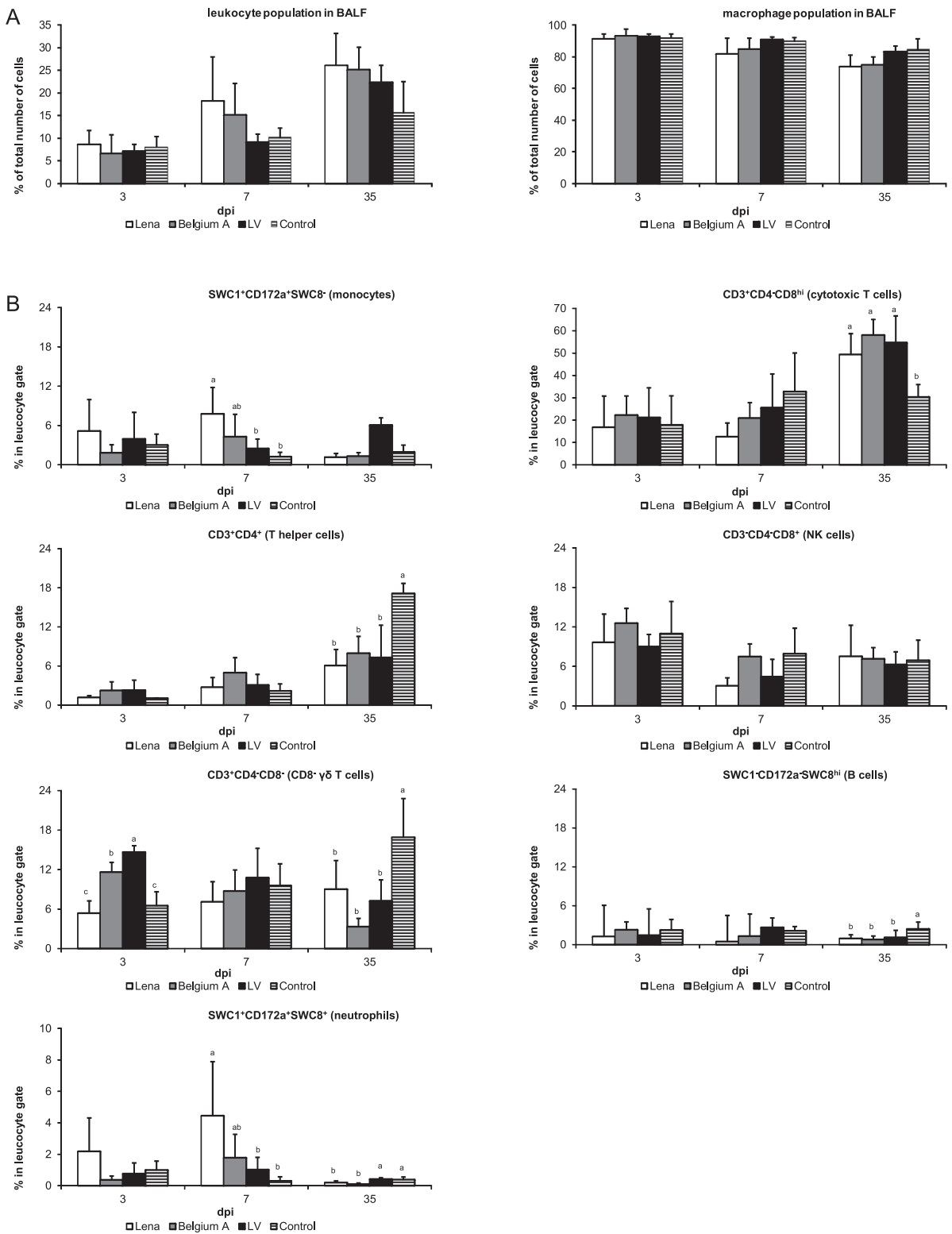


Fig. 5. Proportion of immune cells \pm SD in bronchoalveolar lavage fluid of pigs infected with different PRRSV strains. (A) The proportion of granulocyte or macrophage populations in BALF were gated based on the forward-scatter versus side-scatter diagram. (B) Within the leukocyte gate, the percentages of cells in the gated area with a specific phenotype. Bars with no common superscript differ significantly ($p < 0.05$).

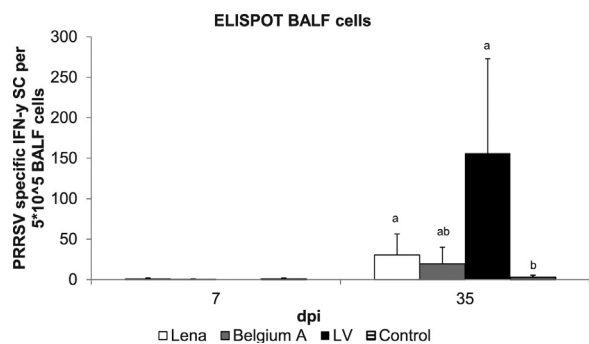


Fig. 6. Virus specific IFN- γ responses \pm SD of pigs infected with different strains of PRRSV. The virus-specific IFN- γ secreting cell responses as determined by ELISPOT assay were background corrected. Bars with no common superscript differ significantly ($p < 0.05$).

pigs infected with the Lena and the LV strain, compared to the control pigs (Fig. 6).

3.6. Cytokine mRNA production in tissues

IL-1 β mRNA upregulation was observed in the lymph nodes of pigs from all infected groups at day 3 p.i. (Fig. 7). At day 7 p.i., the transcriptome expression of this cytokine was significantly higher for strain Lena-infected pigs than for the other groups in the lymph node and lung. An upregulation of TNF- α mRNA was observed in the lymph node at day 3 p.i., and this was significantly higher for strain LV-infected pigs. IFN- α mRNA expression was downregulated in infected groups at days 3 and 35 p.i., with the highest downregulation for strain Lena and Belgium A-infected pigs. In spleen samples, no significant differences were observed between groups (data not shown).

4. Discussion

Significant differences in pathogenicity of the subtype 3 strain, compared to the subtype 1 strains have been observed in the respiratory tract of growing pigs. The subtype 3 strain Lena was more virulent in the early phase of the infection and induced more severe respiratory disease and pneumonia than the subtype 1 strains Belgium A and LV. The lung lesions were most severe for strain Lena-infected pigs in the first week of infection, when also higher levels of the proinflammatory cytokine IL-1 β mRNA and lower levels of the antiviral IFN- α were observed in the lung and lymph node than the control pigs. In this inflammatory phase, the cell population in the BALF shifted to a higher proportion of monocytes and neutrophils. Interestingly, at the end of the experiment at day 35 p.i. microscopic lung lesions were more pronounced after infection with the subtype 1 strains Belgium A and LV, and also viral RNA (detected by PCR) was still present in the lung, lymph node and spleen of these pigs infected with subtype 1 strains, while no viral RNA was detected in these tissues of the strain Lena-infected pigs. Furthermore, all strains induced a higher percentage of cytotoxic T cells in the lung lavage at day 35 p.i. and higher levels of IFN- γ

producing cells (not significant for strain Belgium A) after recall stimulation with virus, which might be involved in (further) clearance of the virus.

Subtype 3 strains appear in general to be more pathogenic than subtype 1 strains. Also other studies (Karniychuk et al., 2010; Morgan et al., 2013) showed a higher incidence of fever, and more severe clinical symptoms and pathology in subtype 3 infected pigs early after infection. In the study of Morgan et al. (2013), the same LV strain and a different subtype 3 strain were used in pigs with a similar genetic background (pigs originated from the same herd as the pigs in the present study). In the study of Morgan et al. (2013), the subtype 1 and 3 strain infected pigs had comparable gross lung pathology at day 7 p.i. as the pigs in the present study, and were also without gross lung pathology at day 35 p.i. Although in the present study no gross pathology was observed at day 35 p.i., pigs had clear histological interstitial changes in the lungs. Interestingly, subtype 1 strains generated at day 35 p.i. more severe microscopic lung lesions to the infection than the subtype 3 strain. Thus, despite the absence of clinical symptoms or manifestations of pneumonia, PRRSV infection has induced long-term interstitial changes, which can contribute to a higher susceptibility to secondary infections, which potentially exacerbates clinical symptoms and pathology as observed in the field (reviewed by Gómez-Laguna et al., 2013).

Besides tissue alterations, PRRSV infections also induced changes in leucocyte populations in blood and the lung, most severely for Lena-infected pigs, and it took more than 5 weeks to return to comparable populations as the control pigs. After infection with subtype 1, as well as subtype 3 strains, an influx of leucocytes, i.e. monocytes, granulocytes and or lymphocytes, indicated by a higher proportion of cells in the leucocyte gate, was observed. Changes in cell populations in the BALF were also observed by Samsom et al. (2000), who showed that during a PRRSV infection, a strong influx of NK cells (CD3⁻, CD6⁻, CD8⁺) and cytotoxic T cells (CD3⁺, CD6⁻, CD8⁺) occurred 2 to 5 weeks after infection. We also found a higher proportion of CD3⁺CD4⁺CD8^{high} cytotoxic T cells at day 35 p.i. This population of cells can be involved in (further) clearance of the virus, in combination with a higher level of PRRSV-specific IFN- γ secreting cells that can directly inhibit PRRSV infection (Rowland et al., 2001).

The detection of PRRSV in tissues in this study was performed by PCR and IHC. The IHC results were quite different from the PCR results. While the PCR detected in general comparable levels of viral RNA between strains within the first week of infection, with IHC no virus was detected in tissues of pigs infected with the LV strain, and significantly higher levels of virus were detected at day 7 p.i. in Lena-infected pigs. The difference between the PCR and IHC results might be caused by differences in sensitivity of the techniques (other authors found IHC less sensitive than assays like virus isolation [Martínez-Lobo et al., 2011]), the target the technique aims to detect (with no good correlation at these sampling moments between viral RNA and viral protein in the tissues), or different parts of the tissues being sampled. Although IHC was less sensitive than PCR, results were more in line with

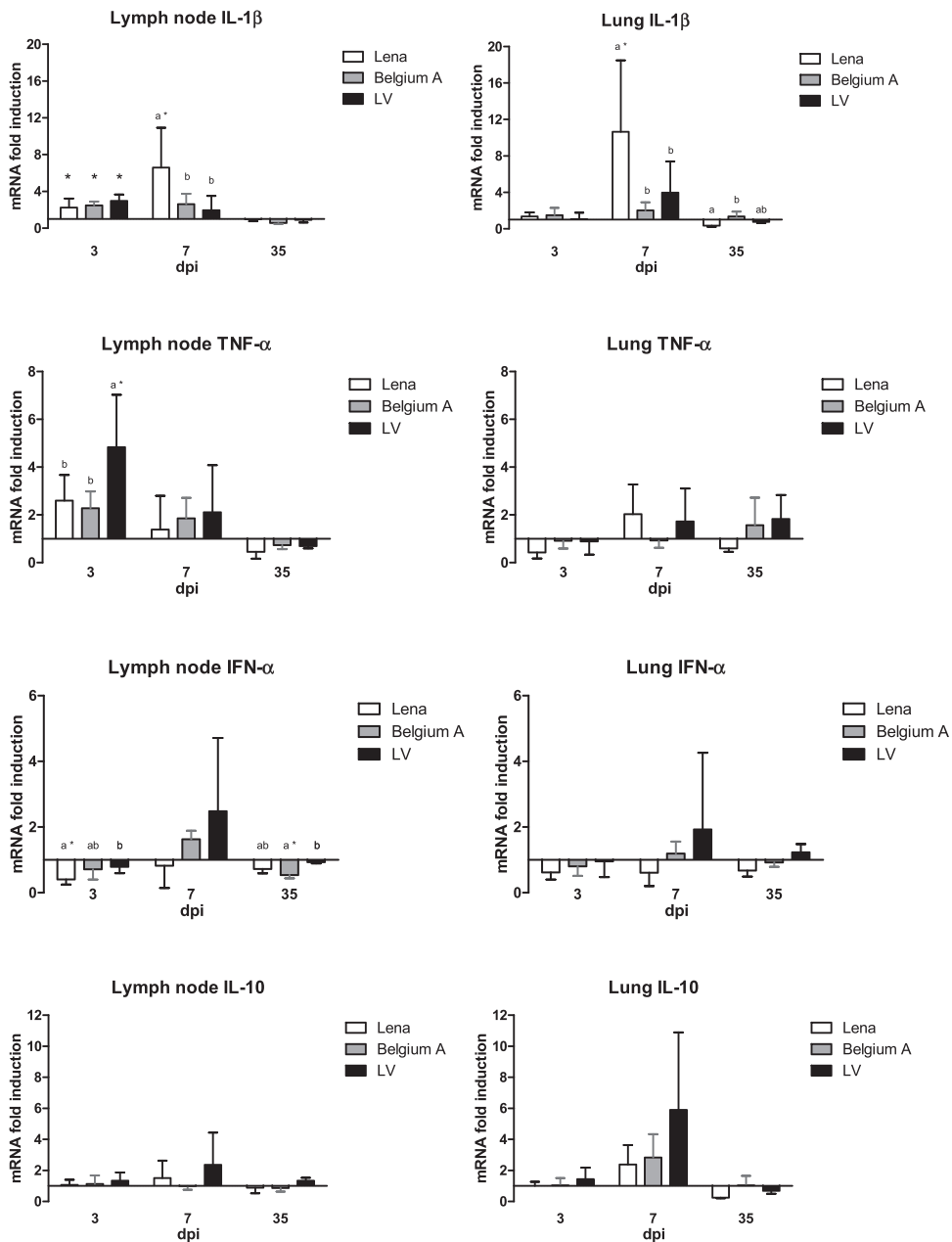


Fig. 7. Cytokine responses \pm SD of pigs infected with different PRRSV strains in the lung and tracheobronchial lymph node. Cytokine gene expression was assessed by qRT-PCR and expressed as fold induction compared to the control group. Bars with no common superscript differ significantly ($p < 0.05$).

the observed gross pathology and pneumonia scores early after infection. The gross pathology and pneumonia scores were in general higher for strain Belgium A at day 3 p.i. (although non-significant), and higher for strain Lena at day 7 p.i. than for the other strains, which is in line with the level of detected viral antigen by IHC.

One would assume that there is in general no relationship between replication ability in tissues in vivo and the pathogenicity of a virus strain, when replication is analysed by PCR. With the same European subtype 1 LV-*Ter Huurne* strain, but another pathogenic European subtype 3 strain SU1-bel, [Morgan et al. \(2013\)](#) found by PCR on

serum and BALF even a higher replication ability of the subtype 1 strain LV than the more pathogenic subtype 3 strain. [Martínez-Lobo et al. \(2011\)](#) observed that infectious virus levels in blood did not differ between pigs infected with strains that differ in lung pathogenicity. In their study, they compared the pathogenicity of different European and North American genotype strains. Their results indicated that the North American genotype strains were more pneumovirulent than the European genotype strains, but that there were no differences in viral load. However, other authors, and also the results of the present study of IHC on lung tissue and viraemia levels

point more to a relationship between pathogenicity and virus levels (Johnson et al., 2004; reviewed by Gómez-Laguna et al., 2013; Han et al., 2013a,b, 2014). In conclusion, it seems that, if there is a relationship, this is strain and tissue dependent and likely also dependent on the method for detection of PRRSV antigen, as shown here by the differences between PCR and IHC.

The increased pathology observed after infection with strain Lena, compared to the subtype 1 strains is possibly not the direct result of higher virus replication in the lung, because of the comparable viral RNA titres between the strains, but more a result of the induced inflammatory cascade. The strain Lena-infected pigs induced higher levels of the proinflammatory IL-1 β and TNF α mRNA in blood cells (Weesendorp et al., 2013a,b) and a higher level of IL-1 β mRNA expression in the lymph node and lung. A correlation between the pathogenicity of the strain and the expression of pro-inflammatory cytokines was also described by other authors (reviewed by Gómez-Laguna et al., 2013; Han et al., 2014). In pigs infected with highly pathogenic PRRSV strains, clinical disease was related to severe interstitial pneumonia and high expression levels of pro-inflammatory cytokines like IL-1 β and TNF- α in the lungs compared to less virulent PRRSV strains. IL-1 β and TNF- α production cause chemotaxis of monocytes and neutrophils (Van Reeth and Nauwynck, 2000; reviewed by Gómez-Laguna et al., 2013), of which we observed a higher percentage in BALF. Release of enzymes, free radicals and inflammatory cytokines from the neutrophils can then also contribute to the lung pathology (Han et al., 2014) that was observed after infection with strain Lena.

Type I IFN (IFN- α/β) is rapidly produced in response to many viral infections such as swine influenza or porcine coronavirus (Van Reeth et al., 1999). IFN- α is the prototypic antiviral cytokine that suppresses initial viral replication and promotes the adaptive immune response (reviewed by Kimman et al., 2009). For PRRSV, it has been shown in vitro that the type I IFN response is suppressed (Ait-Ali et al., 2011), but there are conflicting reports of PRRSV inhibiting or inducing a type I IFN response in vivo (Van Reeth et al., 1999; Gómez-Laguna et al., 2009). In blood (Weesendorp et al., 2013a,b), we observed an induction of IFN- α of the Lena and Belgium A strain at day 7 p.i., when the virus load was at a peak level. Blood cells responded to the virus by an inflammatory response with production of IFN- α and the proinflammatory cytokines IL-1 β and TNF- α . In tissues, however, we observed mostly a downregulation of IFN- α . Downregulation of IFN- α in the tissues might be one of the mechanisms of the virus to facilitate PRRSV replication (Murtaugh et al., 2002).

In general, our data support the conclusion of Morgan et al. (2013) about subtype 3 strains that these strains induce enhanced early immune responses which may lead to more severe clinical disease and pathology, but also support a faster clearance of virus, in contrast to a more delayed or reduced immune response and pathology of subtype 1 strains. On the other hand, besides the longer persistence of virus in tissues, the subtype 1 strains induced long-term microscopic lung lesions, which can

contribute to a higher susceptibility to secondary infections (reviewed by Gómez-Laguna et al., 2013).

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