

## Assessment of pulmonary tissue responses in pigs challenged with PRRSV Lena strain shows better protection after immunization with field than vaccine strains

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1	Assessment of pulmonary tissue responses in pigs challenged with PRRSV Lena strain
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27 The porcine reproductive and respiratory syndrome virus (PRRSV) is plaguing porcine production. Previously piglets were immunized with a PRRSV-1 commercial modified live 28 29 virus vaccine (MLV1), a PRRSV-2 MLV (MLV2) or a Western European strain (Finistere: 30 Fini) to assess and compare the protection brought by these strains upon challenge with 31 virulent Lena strain. Lena viremia was reduced in all the immunized groups with a slightly higher level of protection following immunization with Fini. Using lung samples collected 32 33 from the same experiment, tissue response to Lena challenge was assessed at the acute and chronic stages of infection. A pre-immunization with any one of the three PRRSV strains 34 35 globally exacerbated microscopic lung lesions. Ten days post-challenge (DPC), MLV1 group score was higher than unimmunized group score and 42 DPC, MLV2 group score was higher 36 37 than in unimmunized group. Lowest lung Lena viral loads were measured in Fini group. Using principal component analysis, we showed 10 DPC that the lesion score was positively 38 39 correlated with chemokine receptors and negatively correlated with viral load. Forty-two 40 DPC, variables for lesion score, IL6, IL8, and CCL20 transcripts were positively correlated together and negatively correlated with CCL28, CXCL6, and CXCR4 transcripts suggesting a 41 42 role for the latter ones in the tissue recovery process. In conclusions, our study shows a 43 significant impact of the three immunizations on pulmonary tissue with the best protection 44 against Lena challenge conferred by Fini strain. Furthermore, it gives insight into the interactions between vaccine and Fini strains and the lung upon Lena challenge. 45

46

47 **1. Introduction** 

The porcine reproductive and respiratory syndrome viruses (PRRSV-1 and PRRSV-2) 49 50 - both members of the Arteriviridae family and Nidovirales order like equine arteritis virus, 51 simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus - have a huge impact on the porcine production worldwide (Lunney et al., 2016; Snijder et al., 2013). These 52 53 viruses are able to counteract the innate immune response of the porcine host and induce a 54 disease characterized by respiratory and genital disorders (for a review see (Du et al., 2017; 55 Lunney et al., 2016)). Historically, two genotypes of PRRSV were identified in the late 1980s: genotype 1 in Europe and genotype 2 in Asia and North America (Keffaber, 1989; Lunney et 56 57 al., 2016; Wensvoort et al., 1991). These viruses are now classified as two different species. Moreover, different subtypes have been described among PRRSV-1. Subtype 1 strains are 58 59 mostly low pathogenic strains and circulate primarily in Western Europe even if also present 60 in Asia and North America. Conversely, subtype 3 strains are described as clearly more virulent than subtype 1 strains and have already been identified in Eastern Europe 61 (Weesendorp et al., 2013). 62

63 Previously, and to prepare for the possible emergence of a subtype 3 strain in Western Europe, piglets were immunized with a PRRSV-1 commercial modified live virus (MLV) 64 65 vaccine (MLV1), a PRRSV-2 commercial MLV vaccine (MLV2), and a Western European 66 PRRSV strain (Finistere: Fini), to assess and compare the protection brought by these strains 67 upon challenge with the virulent subtype 3 Lena strain four weeks later (Renson et al., 2017a). 68 Basically, it was observed that the levels of Lena viremia were diminished for all the 69 immunized groups and that the immunization with Fini, MLV1, and MLV2 strains shortened 70 the hyperthermia induced by Lena strain (Renson et al., 2017a). Moreover, in the Fini group, 71 they also observed an improvement in growth performance possibly related to a better cell-72 mediated immune response as suggested by the level of Lena-specific IFN $\gamma$ -secreting cells. 73 Thus, it appeared that commercial PRRSV-1 and PRRSV-2 MLV vaccines as well as the field

74 strain Finistere could provide partial clinical and virological protection against a challenge 75 with the virulent Lena strain. However, the study focused primarily on clinical and 76 zootechnical parameters, blood viral load, systemic humoral and cellular responses (ELISPOT 77 using peripheral blood mononuclear cells - PBMC). Because PRRSV multiplies mostly in 78 pulmonary alveolar macrophages and to further our understanding of previous results, we 79 performed the following study. Using lung samples collected from the experiment performed 80 by Renson and collaborators (Renson et al., 2017a), we carried out a molecular and histo-81 pathological analysis of the immune response of the lung tissues in the different groups of 82 non-immunized and immunized pigs. This lung tissue analysis completes and broadens the previous report. 83

- 84
- 85 2. Materials and methods
- 86

#### 87 2.1. Vaccine and virus strains

Two MLV vaccines were used in the *in vivo* experiment as described by Renson and 88 collaborators (Renson et al., 2017a): the PRRSV-1 Ingelvac PRRSFLEX<sup>®</sup> EU vaccine 89 (Boehringer Ingelheim France, Paris, France, 94881 strain, GenBank accession no. 90 KT988004) (MLV1) and the PRRSV-2 Ingelvac® PRRS MLV vaccine (SCS Boehringer 91 Ingelheim Comm, Brussels, Belgium, USA ATCC VR2332 strain, GenBank accession no. 92 93 EF484033) (MLV2). The PRRSV-1 subtype 1 (1.1) Finistere PRRSV strain (PRRS-FR-2005-29-24-1, GenBank accession no. KY366411) was isolated in France in 2005 from a herd with 94 95 reproductive failures in sows. In specific pathogen-free (SPF) pigs, Finistere infection induces a mild clinical expression (Rose et al., 2015). The PRRSV-1 subtype 3 (1.3) Lena PRRSV 96 97 strain (GenBank accession no. JF802085) was kindly provided by Dr. Hans Nauwynck (University of Ghent, Belgium). The Lena strain was isolated in Belarus in 2007 from a herd 98

with mortality, reproductive failures and respiratory disorders (Karniychuk et al., 2012). The
Finistere and the Lena strains were propagated and titrated on pulmonary alveolar
macrophages as previously described (Renson et al., 2017a).

102

#### 103 2.2. Experimental setting

104 The experimental setting has been described previously (Renson et al., 2017a). Briefly, 105 41 four-week-old pure Large White piglets coming from a specific pathogens free nucleus herd were housed in biosecurity level-3 air-filtered animal facilities. The 41 piglets were 106 randomly distributed according to their origin, weight and gender and assigned to five groups 107 housed in separate rooms (Table I). At 6 weeks of age (D-27), 9 piglets were vaccinated 108 intramuscularly with either the MLV1 (minimum dose 10<sup>4.4</sup> TCID50/piglet) or the MLV2 109 vaccine (minimum dose 10<sup>4.9</sup> TCID50/piglet) (MLV1 and MLV2 group). At the same time, 7 110 piglets were inoculated intranasally with the PRRSV Finistere strain (5  $\times$  10<sup>5</sup> 50% tissue 111 culture infectious dose (TCID50) per piglet) (Fini group). Intranasal inoculation was 112 performed by direct instillation of the virus suspension in each nostril, using a 5 ml syringe 113 114 without needle. At 10 weeks of age (D0), all the piglets from the Fini, MLV1, and MLV2 groups were challenged intranasally with the Lena strain (genotype 1.3,  $5 \times 10^5$ 115 116 TCID50/piglet). At the same time, 8 non-immunized piglets were also inoculated with the 117 Lena strain (non-immunized, NI group) and 8 non-immunized piglets were mock inoculated 118 (control group).

All the animal experiments were authorized by the French Ministry for Research (authorization no. 2015060113297443\_v1) and approved by the national ethics committee (authorization no. 07/07/15-3).

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#### 123 **2.3.** Sampling/necropsy and histopathological observations

Half of the pigs in each group were euthanized and necropsied at the acute phase of 124 Lena infection (10±1 days post-challenge, DPC) and the remaining pigs at the end of the 125 126 follow-up ( $42\pm1$  DPC). Then, pieces from the dorsal diaphragmatic lobe of the lung were collected for each pig and frozen at -80°C or formalin-fixed. Fixed samples were then 127 128 paraffin-embedded, and sectioned (5 µm thick) before staining with a routine hematoxylin-129 eosin-saffron (HES) stain (Riva et al., 2014). The microscopic observation was performed by 130 a trained veterinary pathologist who was blind to the experimental setting. A previously published histopathological lung lesion scoring method was used (Jung et al., 2007) and an 131 132 adapted lesion scoring method including subacute lesions was added. Briefly, in the adapted lesions scoring method the following criteria were scored (i) thickening of alveolar septa 133 (ranging from 0 to 6, where 0 is normal appearance, 1 is mild focal or multifocal, 2 is mild 134 diffuse, 3 is moderate focal or multifocal, 4 is moderate diffuse, 5 is severe focal or 135 136 multifocal, and 6 is severe diffuse interstitial pneumonia); (ii) accumulation of material in respiratory airways (ranging from 0 to 3, where 0 is normal appearance and 3 is severe); (iii) 137 peribronchiolar or perivascular inflammatory cells cuffing (ranging from 0 to 3, where 0 is 138 normal appearance and 3 is severe); (iv) proliferative subacute bronchiolitis (ranging from 0 139 to 3, where 0 is normal appearance, 1 is diffuse thickening, 2 is partially occluding and 3 is 140 141 obliterative); (v) alveolar emphysema (ranging from 0 to 3, where 0 is normal appearance, 1 is 142 focal or multifocal, 2 is focally extensive and 3 is diffuse) and (vi) BALT hyperplasia 143 (ranging from 0 to 3, where 0 is normal appearance and 3 is severe hyperplasia). The total histopathological lung lesion scores ranged from 0 (no abnormalities) to 21 (most severe 144 bronchointerstitial pneumonia) and was expressed as a percentage of the maximal score. 145

146

147 2.4. Immune gene expression analysis and virus detection in the pulmonary tissue using
148 quantitative real-time PCR

To detect immuno-related transcripts (cytokines, chemokines, and chemokine 149 receptors) and PRRSV strains, real-time PCR primers were previously developed (Meurens et 150 151 al., 2009; Nygard et al., 2007) or designed and optimized using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Eurogentec (Liège, 152 153 Belgium) (see Table II for primer list). Pulmonary tissue samples (two pieces of 1 mm<sup>3</sup>) were 154 suspended in Trizol reagent (Invitrogen, Cergy Pontoise, France) with ceramic beads 155 (BioSpec Products, OK, USA) and total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Courtaboeuf, France). The absence of genomic DNA contamination was verified 156 157 using prepared RNA as a template for quantitative real-time PCR (qPCR). RNA concentration was determined by measuring optical density at 260 nm (OD260) and the RNA quality was 158 assessed by calculating OD260/OD280 ratio and by capillary electrophoresis (Agilent 2100 159 160 Bioanalyzer, Agilent Technologies Inc., Santa-Clara, USA). cDNA was generated from 100-161 200 ng of RNA per reaction following a previously described method (Meurens et al., 2007). The generated cDNA was stored at -80 °C. qPCR was performed using cDNA synthesized 162 163 with Moloney Murine Leukemia Virus reverse transcriptase (Eurogentec). Diluted cDNA (2X) was combined with primer sets (0.2  $\mu$ L of 10 mM primer stock) and MESA GREEN 164 qPCR MasterMix (Eurogentec) according to the manufacturer's recommendations. The real 165 166 time PCR was run on CFX96 Bio-Rad Connect (Bio-Rad, Hercules, CA, USA). The cycling 167 conditions were 1 cycle of denaturation 95°C 5 minutes (min), followed by 40 cycles of 168 amplification (95°C/15 seconds (s), 58°C-67°C/40 s depending on the selected gene, see Table II). The fluorescence was automatically measured during the PCR assay. Software CFX 169 170 manager 3.1 (Bio-Rad) was used to determine the Cycle quantification (Cq) in each reaction. A melting curve was elaborated for each primer pair to verify the presence of one gene 171 specific peak. qPCR assays were carried out as previously described using the three most 172 stable reference genes (Delgado-Ortega et al., 2011; Delgado-Ortega et al., 2014; Dobrescu et 173

al., 2014). Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the Cq from the samples for the different transcripts.

177 Regarding the Taqman PCR assay, the mix and the conditions were different. Two  $\mu$ L 178 of 2X diluted cDNA were combined with 5  $\mu$ L of Takyon No Rox Probe MasterMix dTTP 179 blue 2x (Eurogentec), 0.3  $\mu$ L of each primer (10  $\mu$ M), 0.25  $\mu$ L of probe (10  $\mu$ M), and water 180 for a final volume of 10  $\mu$ L. The qPCR conditions were 95°C for 3 min followed by 40 cycles 181 with denaturation at 95°C for 6 s and annealing/elongation for 15 s at 60°C. The Taq-man 182 PCR was also run on a CFX96 Bio-Rad Connect (Bio-Rad).

183

#### 184 **2.5. Statistical analysis**

185 Comparison of histological lesions, immune responses and Lena strain viral load between
186 experimental groups

Data for the comparison of differences in relative mRNA expression between experimental groups were expressed as relative values. One-Way ANOVA was used to detect differences in relative mRNA expression and composite score between experimental groups. To account for the non-normal distribution of the data, all data were sorted by rank status prior to ANOVA statistical analysis. Tukey's test was used to compare the means of the ranks among the groups. *P*-values less than 0.05 were considered significant. All statistical analyses were done using computer software Prism 6 for Windows (version 6.02; GraphPad Software,

194 San Diego, CA, USA).

195 Relationships between immunological, histological and virological descriptors

Associations between the relative mRNA expressions of different genes involved in the immune response, the histological Jung score and the Lena strain viral load in the lungs of MLV1, MLV2, Fini and Lena groups were investigated by principal component analysis (PCA) at 10 DPC (R free software, available from: http://www.R-project.org). Two outliers (one pig from MLV1 and one from MLV2 group) with the highest values on the level of expression of chemokines receptors transcripts and having a singular immune response quite different from the other pigs (confirmed by a clustering analysis, data not shown) were discarded from the analysis to better described the associations between the variables.

Due to the absence of detection of Lena strain 42 DPC in all groups, the associations between the set of immune response descriptors and the histological score were assessed by PCA at this time point.

207 The main objective in PCA is to detect the associations within a set of continuous variables in a small number of dimensions and to provide a low-dimensional (often two-208 dimensional) graphical representation of these associations (Joliffe, 2002). Each variable is 209 represented by an arrow inside a correlation circle, the higher the length of the arrow, the 210 211 higher the variable contribution to the inertia. The angle between arrows indicates the degree of correlation between the variables, the smaller the angle the higher the correlation. An angle 212 of 90° indicates that the two variables are independent and an angle of  $180^{\circ}$  shows a negative 213 correlation. 214

215

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3.1. Viral replication/load in the lung tissue was lower in the Fini group than in the other
immunized groups

At 10 DPC, except in control group, the PRRSV genome was detected without any significant difference between groups (p > 0.05). Because PRRSV universal primers could not differentiate the strains used for immunization and challenge, we had to use primers specifically designed for the detection of Lena challenge strain to assess the impact of immunization on Lena virus load. With these primers, the Lena viral load was found to be significantly lower in the Fini group than in the other immunized and non-immunized control groups (p < 0.05, Figure 1). Using PRRSV universal primers, as well as Lena-specific primers, no PRRSV genome was detected for Lena-infected groups 42 DPC (Figure 1).

228

# 3.2. Histologic lesions: Pre-immunization with MLV strains showed higher composite scores than with Fini strain

231 To evaluate the tissular impact of the Lena strain challenge after immunization with 232 another PRRSV strain, lung samples were observed histologically and lesions were scored using a previously published score grid (Jung et al., 2007) adapted to take into account sub-233 234 acute lesions including proliferative bronchiolitis and alveolar emphysema (see Table III). Most control animals (7 out of 8) displayed mild interstitial pneumonia (scored 28.7±7.8 % 235 and  $27.5\pm4.3$  % 10 and 42 DPC, respectively) mainly represented by a slight thickening of 236 alveolar walls due to infiltration by some inflammatory cells (Figures 2 and 3). These findings 237 238 indicate a low background of inflammation which may be associated with dust aspiration.

In contrast, all animals (8 out of 8) inoculated with Lena displayed severe and extended lesions of interstitial bronchopneumonia resulting from the thickening of alveolar septa by a mixed population of inflammatory cells and from respiratory airway alterations including the presence of intra-luminal necrotic material, epithelium hyperplasia and peribronchiolar inflammatory cell infiltration.

A pre-immunization with one of the three studied PRRSV strains globally exacerbated microscopic lung lesions: The MLV1-immunized group composite score was higher than the unimmunized group 10 DPC (p < 0.05) and the MLV2-immunized group score was higher than the unimmunized group 42 DPC (p < 0.01) (Figures 2A and 3). MLV1, MLV2 and Finiimmunized animals displayed prominent alveolar wall thickening and respiratory airway obstruction by intraluminal materials. In MLV1-immunized animals, we also observed a decreasing trend in the histopathological score between the studied time-points (mean score 70.2±9.7 % and 52.2±12.7 % 10 and 42 DPC, respectively) corresponding mainly to a clearance of the airway debris. Specifically, in MLV1- and MLV2-immunized animals, some foci of necrotic cells admixed with degenerated neutrophils were scattered in lung parenchyma 10 DPC (Figure 2B), and a prominent BALT hyperplasia was identified 42 DPC.

255

#### **3.3.** Analysis of the lung immune response in the different groups of pigs

257 In order to analyze in more detail, the lung response to Lena strain challenge in preimmunized pigs, we assessed the expression of various immuno-related transcripts in the lung 258 259 tissue (see Table II and Figure 4). Regarding transcripts associated with CCR6, IL10, IFN $\gamma$ , 260 and TNF $\alpha$  no statistically significant differences were observed between groups (p > 0.05, data not shown). On the contrary, transcripts associated with chemokines IL8/CXCL8, 261 CCL20, and CXCL6 were more expressed in MLV1, MLV2, and NI groups 10 DPC than in 262 263 Fini and control groups (Figure 4A). Most of the time, the observed differences were 264 statistically significant (p < 0.05). Because we observed an induction of chemokine genes, we next assessed the expression of various CXC chemokine receptors (CXCR1-7) transcripts 265 266 (Figure 5 and data not shown for CXCR7). Differences in transcript expression were 267 observed. For CXCR2, CXCR5, and CXCR6 transcripts 42 DPC there were trends (p > 0.05)268 for a higher expression in MLV1 and MLV2 groups than in other groups (Figure 5). We then evaluated the transcript expression of the mucosal chemokine CCL28 and its two main 269 receptors, CCR3 and CCR10 (Figure 4B). Again, a trend for a higher expression of some 270 transcripts (CCL28, CCR3, and CCR10) in MLV1 and MLV2 groups 42 DPC than in other 271 groups was observed but the difference was not statistically significant (p > 0.05). Moreover, 272

the highest level of expression of CCL28 transcripts was observed in the MLV1 group 10
DPC (Figure 4B).

275 Relationships between immunological, histological and virological descriptors in pre276 immunized and/or challenged groups

277 The PCA revealed three groups of associations between variables describing the levels 278 of immunological, histological and virological lung responses 10 DPC (Figure 6A). One 279 group of positively correlated variables (top left corner of the map) comprised the level of expression of chemokines (CCL20, CCL28, CXCL6, IL8/CXCL8) and chemokine receptor 280 281 transcript CXCR1. A second group of positively correlated variables, located in the right part of the map, was mainly independent from the first group and was related to the level of 282 283 expression of the transcripts of all other chemokine receptors. To a lesser degree the 284 histological Jung score was positively correlated with this second group of variables. The 285 second group of variables was negatively correlated with the Lena strain viral load in the lung. Forty-two DPC, the histological score, the levels of expression of IL6, and of 286 287 chemokine CCL20 were positively correlated (Figure 6B). This group of descriptors was negatively correlated with the level of expression of the CCL28 transcripts. The level of 288 expression of CCL28 transcripts was positively correlated with the level of CXCL6 and 289 290 CXCR4 transcripts. A third group of positively correlated parameters included the level of

expression of transcripts of CXC chemokine receptors 1-6, CCR10 and CCR3.

292

#### 293 **4. Discussion**

294

In a previous study aimed at assessing and comparing the immune protection conferred by a Western European PRRSV strain (genotype 1.1) and genotypes 1 and 2 commercial MLV (MLV1 & MLV2) vaccines against challenge with the virulent Lena strain, it was

298 observed that the level of Lena viremia and clinical signs were reduced in all the immunized 299 groups (Renson et al., 2017a). However, compared to vaccine strains, a slightly higher level 300 of protection following immunization with Finistere strain was observed and attributed to a better cellular immune response. Thus, this previous study showed that cross-protection upon 301 302 challenge with PRRSV Lena strain was possible with two vaccine strains (genotypes 1 and 2, 303 respectively) and one circulating wild-type strain (Finistere) and that the protection was not 304 related with the level of genetic similarity (Renson et al., 2017a). To reach these conclusions the previous study focused on clinical and zootechnical parameters, blood viral load, systemic 305 306 humoral and cellular responses. However, it did not take into account the lung immune response even though in the first steps of infection PRRSV multiplies mostly in pulmonary 307 308 macrophages. Thus, we performed this complementary investigation.

309 In this study, microscopic pulmonary lesions were observed in all groups of animals 310 and histological scores were significantly higher in all infected groups both 10 and 42 DPC 311 than in non-infected. This observation is not surprising considering that, 10 DPC, both blood 312 and lung viral loads remained high in most of the groups except to some extent in Fini and 313 that fever was mostly detected in NI and MLV2 where lesions were well identified (Renson et al., 2017a). Moreover, it is well-known that infections with virulent type 1 subtype 3 Lena 314 315 strain result in more severe diseases than with other type 1 (Lelystad, Finistere or Belgium A 316 for instance) strains (Renson et al., 2017b; Rose et al., 2015; Weesendorp et al., 2013). 317 Regarding viral loads in the lung, results were similar to what was reported previously with blood viral loads (Renson et al., 2017a) and the lung viral load in the Fini group was 318 significantly lower than in other groups 10 DPC. As previously suggested, the higher 319 320 protection conferred by Finistere strain immunization compared to other immunization groups (MLV1 and MLV2) could be explained by a better cellular response as deduced from 321 PRRSV-specific IFNy response detected by ELISPOT. This observation could also be linked 322

323 to the attenuation of vaccine strains *versus* non-attenuated wild type Finistere strain and/or 324 route of administration (intranasal versus intramuscular) (Wu et al., 1997). Surprisingly, most 325 severe lesions 10 DPC were observed in pigs immunized with MLV1 ( $70.2\pm9.8$  %) and to a lower extent MLV2 (63±9%) and foci of necrotic cells admixed with degenerated neutrophils 326 327 were scattered in the lung parenchyma of the pigs from these groups. However, as previously 328 reported, clinical signs such as rectal temperatures were already reduced 10 DPC in the MLV1 329 group and 11 DPC in the MLV2 group (Renson et al., 2017a) showing the protective effect of immunization with the two vaccines. 330

331 Because of the presence of foci of necrotic cells and degenerated neutrophils in the lung tissues of the MLV1 and MLV2 groups, we then assessed the expression of some 332 transcripts related to inflammation (IL6 and  $TNF\alpha$ ) and coding for some chemokines mostly 333 involved in neutrophil recruitment (Kulkarni et al., 2017; Zlotnik and Yoshie, 2012). 334 335 Interestingly, the highest expression of not only CXCL6 and IL8/CXCL8 but also CCL20 transcripts was identified in the MLV1 and MLV2 groups with statistically significant 336 337 differences. Moreover, using PCA we identified positive correlation between CXCL8/CCL20 and the lesion score 42 DPC, and the induction of CCL20 was the highest in the MLV1 group 338 10 DPC where the lesion score was also the highest (similar observations were made for the 339 340 transcripts of CXCR1, receptor of CXCL8). To further verify our hypothesis, we looked at the 341 specific role of the selected chemokines and their receptors. CXCL6, also known as 342 granulocyte chemotactic protein 2 (GCP-2), is produced by macrophages, epithelial and mesenchymal cells during inflammation, is chemoattractant for neutrophilic granulocytes 343 (Proost et al., 1993; Zlotnik and Yoshie, 2012), has antimicrobial properties (Linge et al., 344 2008), and interacts with two receptors: CXCR1 and CXCR2 (Wuyts et al., 1997). 345 IL8/CXCL8, a CXC chemokine known as neutrophil chemotactic factor, is produced by 346 347 epithelial cells and macrophages and interacts with CXCR1 and CXCR2 (Harada et al., 1994;

IUIS/WHO Subcommittee on Chemokine Nomenclature, 2003), which are expressed by 348 eosinophils (Petering et al., 1999), neutrophils (Zlotnik and Yoshie, 2012), mast cells (Lippert 349 350 et al., 1998), and some macrophages (Williams et al., 2000). CCL20, also known as Macrophage Inflammatory Protein-3 (MIP3A), belongs to the CC chemokine family and is 351 352 strongly chemotactic for lymphocytes while it weakly attracts neutrophils (Hieshima et al., 353 1997). Thus, our qPCR data support more pronounced inflammatory processes in the MLV1 354 and MLV2 groups 10 DPC than in the Fini group confirming histo-pathological analyses. We then looked at various chemokine receptor transcripts, especially the ones involved in 355 356 neutrophil recruitment, to see if chemokine transcript expression had visible consequences on receptor transcript expression. For that purpose, RT-qPCR assays targeting all the CXC 357 358 chemokine receptors were developed for the pig species. In most cases, no significant differences but trends were observed between groups 42 DPC, probably because of a transient 359 360 overexpression of receptor transcripts. For instance, it has been shown in mice that IgA antibody-secreting cells express high levels of the receptor CCR9 in lymphoid tissues 361 associated with the gut but down-regulate the expression of the receptor once located in the 362 363 lamina propria (Pabst et al., 2004).

364 To determine which variables were associated together, we carried out principal 365 component analyses 10 and 42 DPC. Ten DPC, chemokine and CXCR1 variables were all 366 positively correlated probably because the lung was facing Lena assaults and recruited 367 inflammatory cells, particularly in MLV groups. Variables describing other chemokine receptors were all positively correlated together and negatively correlated to Lena lung viral 368 369 load, illustrating most probably the importance of establishing the cellular arm of the immune 370 response for Lena control. Indeed, CXCR3, CXCR4, CXCR5, CXCR6, and CCR10 are all involved in lymphocyte trafficking (Bonini and Steiner, 1997; Dobner et al., 1992; Groom and 371 Luster, 2011; Loetscher et al., 1997; Moriuchi et al., 1997; Oin et al., 1998; Zlotnik and 372

Yoshie, 2012). Moreover, the variables describing all these chemokines receptors were 373 positively correlated to Jung score 10 DPC. Forty-two DPC, the situation was different. This 374 375 is not surprising when we consider that the lung is recovering from PRRSV infection after the clearance of most of the virus particles. At this time, the lesion score and the levels of 376 377 expression of IL6, IL8, and CCL20 transcripts were all positively correlated while they were 378 negatively correlated with the levels of expression of CCL28, CXCL6, and CXCR4 379 transcripts. Similarly, according to what was observed 10 DPC, other chemokine receptor variables were all positively correlated. Thus, it appears that the picture is less clear long after 380 challenge than immediately after it. However, a positive correlation between major 381 inflammation actors such as IL6 and IL8/CXCL8 and the lesion score can still be observed. 382 383 Lena strain induces a stronger inflammatory response than Western European PRRSV strains (Weesendorp et al., 2013) and even if it can contribute to a faster viral clearance it could take 384 385 a longer time for the tissue to fully recover from the induced lesions. CXCL6, CXCR4, and CCL28 variables were negatively correlated to the first group of variables, which mostly 386 387 included inflammatory mediators and histological score. Interestingly, an alternative name for CXCL6 is Alveolar Macrophage Chemotactic Factor 2 (Hunninghake et al., 1980; Proost et 388 al., 1993), CXCR4 is associated with lymphocytes and its signaling regulates the expression 389 390 of CD20 on B cells (Moriuchi et al., 1997; Pavlasova et al., 2016), and CCL28 is a strong 391 chemoattractant of antibody secreting cells (Berri et al., 2008; Feng et al., 2006; Kunkel et al., 392 2003; Lazarus et al., 2003; Meurens et al., 2006; Wilson and Butcher, 2004). CCL28, also known as mucosae-associated epithelial chemokine (MEC), has antimicrobial properties and 393 394 is expressed by lung mucosa where it drives homing of T and B lymphocytes expressing CCR10 and eosinophils, macrophages, and T lymphocytes expressing CCR3 (Danilova et al., 395 2015; Humbles et al., 2002; John et al., 2005; Kulkarni et al., 2017; Kunkel et al., 2003; 396 Mantovani et al., 2004; Zlotnik and Yoshie, 2012). Thus, we could hypothesize a role for 397

these chemokines and chemokine receptor, all involved in macrophage and T cell trafficking, in the recovery process. Regarding potential links between biological variables and immunization groups, we did not observe any correlation, probably because of the low numbers of animals per groups and the relatively small differences between groups.

402 In conclusions, our study complements and further valorizes valuable samples 403 collected from pigs immunized with two commercial vaccine strains and the Fini wild-type 404 strain before Lena challenge. It shows a significant impact of the three types of immunizations on pulmonary tissue with the best protection against Lena challenge conferred by Fini strain, 405 406 which induced less the expression of some inflammatory cytokines and chemokines. Furthermore, it gives insight into the interactions between vaccine and Fini strains of PRRSV 407 408 and the lung upon Lena challenge. The study also provides a more global analysis of the 409 immune lung response following PRRSV challenge giving clues for further studies aiming at 410 better understanding the complex immuno-patho-physiology of PRRSV infections.

411

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413

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#### 423 Conflict of interest statement

424

425 The authors declare that they have no conflict of interests.

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427 **References** 

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#### 608 Figure captions

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Figure 1: Viral replication - Relative quantification of viral load in lung tissue 10 or 42 days post-challenge (DPC). In each group n=3 to 5 (median is indicated by the small bar). Dot plots within each graph with no common superscripts are significantly different (p < 0.05). MLV1 group: pigs immunized with the MLV1 vaccine; MLV2 group: pigs immunized with the MLV2 vaccine; Fini group: pigs immunized with the Finistere strain of PRRSV; NI group: non-immunized pigs.

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**Figure 2:** (A) Lung histopathology - Representative pictures of the histopathological lesions observed in control, MLV1, MLV2 or Finistere PRRSV (Fini) strain-inoculated pigs before Lena strain challenge or Lena strain alone (NI), 10 (upper panel) and 42 (lower panel) days post-challenge (DPC). Compared to control animals, lungs of PRRSV inoculated/immunized and challenged animals displayed severe bronchopneumonia characterized by alveolar wall thickening by inflammatory cells, presence of necrotic and cellular debris in respiratory 623 airways ((open) arrowhead) and peri-bronchiolar and peri-vascular cuffing by inflammatory cells (arrow). Note, 42 DPC, hyperplasia of Bronchi-Associated Lymphoid Tissues (\*). 624 625 Hematoxylin-eosin-saffron stain, bar =  $100 \ \mu m$ . (B) High magnification of lung parenchyma. Compared to control animals, all Lena-infected animals displayed thickening of 626 627 alveolar septa by some inflammatory cells, mainly mononucleated ones (black arrowhead). 10 628 days post-challenge (DPC), in MLV1 and MLV2 groups, some neutrophils (arrow) were 629 regularly observed around small foci of necrotic debris accumulation (open arrowhead). Meanwhile, these foci and neutrophils were almost absent in Fini and NI animals. 630 631 Hematoxylin-eosin-saffron stain, bar =  $10 \mu m$ .

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Figure 3: Composite scores in the different groups 10 and 42 DPC. In each group n=3 to 5 (median is indicated by the small bar). Dot plots within each graph with no common superscripts are significantly different (p < 0.05). MLV1 group: pigs immunized with the MLV1 vaccine; MLV2 group: pigs immunized with the MLV2 vaccine; Fini group: pigs immunized with the Finistere strain of PRRSV; NI group: non-immunized pigs.

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**Figure 4: (A) Innate immune response** - Relative expression of transcripts in lung tissues 10 or 42 days post Lena challenge (DPC). **(B) CCL28 chemokine and its receptors** - Relative expression of transcripts in lung tissues 10 or 42 days post-challenge (DPC). In each group n=3 to 5 (median is indicated by the small bar). Dot plots within each graph with no common superscripts are significantly different (p < 0.05). MLV1 group: pigs immunized with the MLV1 vaccine; MLV2 group: pigs immunized with the MLV2 vaccine; Fini group: pigs immunized with the Finistere strain of PRRSV; NI group: non-immunized pigs.

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**Figure 5: CXCR chemokines** - Relative expression of transcripts in lung tissues 10 or 42

days post-challenge (DPC). In each group n=3 to 5 (median is indicated by the small bar). Dot plots within each graph with no common superscripts are significantly different (p < 0.05). MLV1 group: pigs immunized with the MLV1 vaccine; MLV2 group: pigs immunized with the MLV2 vaccine; Fini group: pigs immunized with the Finistere strain of PRRSV; NI group: non-immunized pigs.

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Figure 6: Results of the principal component analysis describing, 10 days (A) and 42 654 655 days (B) after a Lena strain challenge, the associations between viral, histologic and 656 immune descriptors of pigs, pre-immunized or not, before the Lena strain challenge (16 pigs in A and 18 in B) - IL6\_10: Level of expression of the IL6; IL8\_10: Level of expression 657 of the chemokine ligand 8; CCL20\_10: Level of expression of the chemokine ligand 20; 658 659 CXCL6 10: Level of expression of the chemokine ligand 6; CXCR1 10: Level of expression 660 of transcript of the chemokine receptor 1; CXCR2\_10: Level of expression of transcript of the chemokine receptor 2; CXCR3\_10: Level of expression of transcript of the chemokine 661 receptor 3; CXCR4 10: Level of expression of transcript of the chemokine receptor4; 662 CXCR5\_10: Level of expression of transcript of the chemokine receptor 5; CXCR6\_10: Level 663 of expression of transcript of the chemokine receptor 6; CCL28\_10: CXCL6\_10: Level of 664 665 expression of the chemokine ligand 28; CCR3 10: Level of expression of transcript of the chemokine receptor 3; CCR10\_10: Level of expression of transcript of the chemokine 666 667 receptor 10; RTPCR\_Lena\_10: Viral Lena strain load in the lung; IL6\_42: Level of expression of the IL6; IL8\_42: Level of expression of the chemokine ligand 8; CCL20\_42: 668 669 Level of expression of the chemokine ligand 20; CXCL6\_42: Level of expression of the 670 chemokine ligand 6; CXCR1\_42: Level of expression of transcript of the chemokine receptor 1; CXCR2\_42: Level of expression of transcript of the chemokine receptor 2; CXCR3\_42: 671 Level of expression of transcript of the chemokine receptor 3; CXCR4\_42: Level of 672

expression of transcript of the chemokine receptor4; CXCR5\_42: Level of expression of transcript of the chemokine receptor 5; CXCR6\_42: Level of expression of transcript of the chemokine receptor 6; CCL28\_42: CXCL6\_42: Level of expression of the chemokine ligand 28; CCR3\_42: Level of expression of transcript of the chemokine receptor 3; CCR10\_42: Level of expression of transcript of the chemokine receptor 10; Jung score: Histologic Jung score.

# **PRRSV** replication





10 DPC





68.6±10.4%

68.3±12.7%

NI

50.0±6.1%

42 DPC

10 DPC











**B** Control



MLV2

Fini

NI













Figure 3



Figure 4



Figure 5



Figure 6

Groups	Immunization (D -27)	Challenge (D0)	Number of pigs necropsied 10 DPC	Number of pigs necropsied 42 DPC	
Control	/	/	4	4	
<b>MLV1</b> Genotype 1.1	Ingelvac PRRSVFLEX EU, IM	Lena, IN	4	5	
MLV2 Genotype 2	Ingelvac PRRSV MLV, IM	Lena, IN	4	5	
<b>Finistere (Fini)</b> Genotype 1.1	Finistere strain, IN	Lena, IN	3	4	
Lena (NI) Genotype 1.3	/	Lena, IN	4	4	

Table I: Experimental design

IN: intranasal ; IM: intramuscular ; DPC: day post challenge; NI: non-immunized

Primer abbreviation and full name	Primer sequences: sense (S) & anti-sense (AS)	Amplicon size (bp)	Annealing temperature (°C)	Efficiency (%)	Accession number or reference
<u>1) PRRSV</u>					
PRRSV Universal	(S) ATGGCCAGCCAGTCAATCAG (AS) GGAACGTTCAGTTCCGGTGA	nd	60	95	NC_001961.1 JF802085.1 KJ127878.1 AY366525.1
PRRSV Lena	(AS) TCTTTTTCGCCTGTCCTCCC (P) (6FAM)- AAACACAGCTCCAATGGGGAATGGC-(TAM)	135	60	97	Renson et al., 2017b
2) REFERENCE GENES					
<b>B2MI</b> Beta-2-microgobulin	(S) CAAGATAGTTAAGTGGGATCGAGAC (AS) TGGTAACATCAATACGATTTCTGA	161	58	101	Nygard et al., 2007
<b>HPRT1</b> Hypoxanthine phosphoribosyltransferase 1	(S) GGACTTGAATCATGTTTGTG (AS) CAGATGTTTCCAAACTCAAC	91	60	99	Nygard et al., 2007
<b>RPL19</b> Ribosomal protein L19	(S) AACTCCCGTCAGCAGATCC (AS) AGTACCCTTCCGCTTACCG	147	60	96	Meurens et al., 2009
3) INTERFERON AND CYTOKI	NES				
<b>IFNγ</b> Interferon gamma (Type II)	(S) GCTCTGGGAAACTGAATGAC (AS) TCTCTGGCCTTGGAACATAG	167	60	97	Meurens et al., 2009
<b>IL6</b> Interleukin 6	(S) ATCAGGAGACCTGCTTGATG (AS) TGGTGGCTTTGTCTGGATTC	177	60	106	Meurens et al., 2009
<b>IL10</b> Interleukin 10	(S) ACCAGATGGGCGACTTGTTG (AS) TCTCTGCCTTCGGCATTACG	123	65	110	Meurens et al., 2009
<b>TNFα</b> Tumor Necrosis Factor alpha	(S) CCAATGGCAGAGTGGGTATG (AS) TGAAGAGGACCTGGGAGTAG	116	60	94	Meurens et al., 2009
4) CHEMOKINES					
<b>CCL20</b> Chemokine (C-C motif) ligand 20	(S) GCTCCTGGCTGCTTTGATGTC (AS) CATTGGCGAGCTGCTGTGTG	146	65	90	Meurens et al., 2009
<b>CCL28</b> Chemokine (C-C motif) ligand 28	(S) GCTGCTGCACTGAGGTTTC (AS) TGAGGGCTGACACAGATTC	144	63	106	Meurens et al., 2009
<b>CXCL6</b> Chemokine (C-X-C motif) ligand 6	(S) TTGCCAGCGCTAGTCCTATC (AS) TTCAGGGTGGCTATCACTTC	166	62	107	NM_213876
CXCL8/IL8 Chemokine (C-X-C motif) ligand 8	(S) TCCTGCTTTCTGCAGCTCTC (AS) GGGTGGAAAGGTGTGGAATG	100	62	92	Meurens et al., 2009
5) CHEMOKINE RECEPTORS					
<b>CCR3</b> Chemokine (CC motif) receptor 3	(S) TCCTATTCACGCTGCCATTC (AS) TGCAGACCACATCTCCAAAC	303	64	100	XM_013981570
<b>CCR6</b> Chemokine (CC motif) receptor 6	(S) GGCAGAAGTTCCGGAGCTAC (AS) TGGTGAAGGAGGACGGATTG	165	63	92	XM_021086056
<b>CCR10</b> Chemokine (CC motif) receptor 10	(S) TCCTGCTTTCTGCAGCTCTC (AS) GGGTGGAAAGGTGTGGAATG	444	60.5	94	DQ157761
<b>CXCR1</b> Chemokine (CXC motif) receptor 1	(S) ATGGCTGGTGATTCAGATCG (AS) ACCAGGGCATAGATGACAAC	185	64	109	XM_003133655.4
<b>CXCR2</b> Chemokine (CXC motif) receptor 2	(S) GATATCTCGGGTTTCCAACG (AS) GGGCAGAGTCTGGTAGAATC	176	64	97	XM_021075282.1
<b>CXCR3</b> Chemokine (CXC motif) receptor 3	(S) TATCGGCCCACCCTGATGAG (AS) GGATGCGGGGCGTAGCAATAG	147	62	98	XM_003135179
<b>CXCR4</b> Chemokine (CXC motif) receptor 4	(S) CCTGGCCTTCATCAGTCTGG (AS) GCGGTCACAGATGTACCTCC	187	64	96	DQ124104
<b>CXCR5</b> Chemokine (CXC motif) receptor 5	(S) CTCTGCAAGACTGTGATAGC (AS) TGGTTGCACAGGTGATATGG	157	60	100	XM_003129915
<b>CXCR6</b> Chemokine (CXC motif) receptor 6	(S) GCTTCATTGCAGTGGTTAGG (AS) ATGATCTGTGGCAAGGAGAC	124	58.5	109	NM_001001623
<b>CXCR7</b> Chemokine (CXC motif) receptor 7	(S) CAGCCTCGTGCAGCATAACC (AS) TGGACGTGTGGGGCGAAGTAG	155	65	104	XM_003133759

### Table II: List of primers used in the study

			Peri-					
			bronchiolar and					
	Alveolar septa		-vascular	Alveolar	Proliferative			Composite
Group	thickening	Airway material	inflammation	emphysema	bronchitis	BALT	Jung score	score
Control 10 DPC	3	1	1	1	0	0	42%	29%
Control 10 DPC	0	1	2	1	0	0	25%	19%
Control 10 DPC	3	1	0	2	0	0	33%	29%
Control 10 DPC	3	2	2	1	0	0	58%	38%
Control 42 DPC	2	0	2	1	0	0	33%	24%
Control 42 DPC	1	1	1	2	0	0	25%	24%
Control 42 DPC	1	2	1	2	0	0	33%	29%
Control 42 DPC	2	1	2	2	0	0	42%	33%
MLV1 10 DPC	6	3	3	3	1	0	100%	76%
MLV1 10 DPC	6	3	3	3	2	0	100%	81%
MLV1 10 DPC	5	2	3	3	0	0	83%	62%
MLV1 10 DPC	5	2	2	3	0	1	75%	62%
MLV1 42 DPC	5	1	1	2	2	1	58%	57%
MLV1 42 DPC	4	2	3	2	1	1	75%	62%
MLV1 42 DPC	5	1	2	2	0	1	67%	52%
MLV1 42 DPC	3	1	1	1	0	1	42%	33%
MLV1 42 DPC	5	1	1	2	3	0	58%	57%
MLV2 10 DPC	4	2	3	1	2	1	75%	62%
MLV2 10 DPC	6	1	3	2	0	0	83%	57%
MLV2 10 DPC	5	2	3	2	0	0	83%	57%
MLV2 10 DPC	6	3	3	2	2	0	100%	76%
MLV2 42 DPC	5	3	2	3	2	0	83%	71%
MLV2 42 DPC	4	3	3	3	2	2	83%	81%
MLV2 42 DPC	4	2	2	3	2	2	67%	71%
MLV2 42 DPC	5	2	2	3	1	1	75%	67%
MLV2 42 DPC	5	1	1	2	1	1	58%	52%
Fini 10 DPC	5	1	2	3	0	0	67%	52%
Fini 10 DPC	5	0	2	3	0	0	58%	48%
Fini 10 DPC	6	2	2	3	1	0	83%	67%
Fini 10 DPC	4	2	2	3	1	0	67%	57%
Fini 42 DPC	6	3	3	3	0	0	100%	71%
Fini 42 DPC	5	3	3	3	0	0	92%	67%
Fini 42 DPC	6	2	3	3	0	0	92%	67%
NI 10 DPC	3	2	3	2	0	0	67%	48%
NI 10 DPC	3	2	3	3	0	0	67%	52%
NI 10 DPC	2	2	3	2	0	0	58%	43%
NI 10 DPC	5	3	2	2	0	0	83%	57%
NI 42 DPC	4	1	2	3	0	0	58%	48%
NI 42 DPC	4	1	2	2	0	0	58%	43%
NI 42 DPC	5	1	2	2	0	0	67%	48%
NI 42 DPC	4	1	2	2	0	0	58%	43%