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Impact of PRRSV strains of different in vivo virulence on the macrophage population of the thymus

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Abstract

The emergence of "highly pathogenic" isolates of porcine reproductive and respiratory syndrome virus (HP-PRRSV) has raised new concerns about PRRS control. Cells from the porcine monocyte-macrophage lineage represent the target for this virus, which replicates mainly in the lung, and especially in HP-PRRSV strains, also in lymphoid organs, such as the thymus. This study aimed at evaluating the impact of two PRRSV strains of different virulence on thymic macrophages as well as after heterologous vaccination. After experimental infection with PR11 and PR40 PRRSV1 subtype 1 strains (low and high virulent, respectively) samples from thymus were analysed by histopathology and immunohistochemistry for PRRSV N protein, TUNEL, CD172a, CD163, CD107a and BA4D5 expression. Mortality was similar in both infected groups, but lung lesions and thymus atrophy were more intense in PR40 group. Animals died at 10-14 dpi after PR11 or PR40 infection showed the most severe histopathological lesions, with a strong inflammatory response of the stroma and extensive cell death phenomena in the cortex. These animals presented an increase in the number of N protein, CD172a, CD163 and BA4D5 positive cells in the stroma and the cortex together with a decrease in the number of CD107a positive cells. Our results highlight the recruitment of macrophages in the thymus, the increase in the expression of CD163 and the regulation of the host cytotoxic activity by macrophages. However, no marked differences were observed between PR11- and PR40-infected animals. Heterologous vaccination restrained virus spread and lesions extent in the thymus of PR40-infected animals.

Keywords	virulence; PRRSV; macrophages; thymus; cell death			
Manuscript category	Viruses			
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Dear Dr. V. von Messling Editor in Chief Veterinary Microbiology 25th March, 2019 Córdoba, Spain

Dear Editor,

Enclosed you will find the revised version of our original manuscript entitled: "Impact of PRRSV strains of different *in vivo* virulence on the macrophage population of the thymus", to be considered for its publication in Veterinary Microbiology.

We believe that our manuscript has been now substantially improved taking into account the reviewer's comments. Thus, we hope that the new version of the manuscript is suitable for publication in Veterinary Microbiology. All the authors have seen and approved the revised version of the manuscript.

Yours sincerely,

Jaime Gómez-Laguna, DVM, MSc, PhD

Ref: VETMIC_2019_45

Title: Impact of PRRSV strains of different in vivo virulence on the macrophage population of the thymus Journal: Veterinary Microbiology

Answers to reviewer's comments

The taxonomy of PRRSV has been changed again according to the latest ratification of the ICTV committee in October 2018. https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186087 The current classification refers to PRRSV-1 as Betaarterivirus suid 1 (Nidovirales - Arnidovirineae - Arteriviridae - Variarterivirinae - Betaarterivirus - Europbartevirus and Ampobarterivirus), change the text accordingly, to reflect the latest nomanclature, at least the Betaarterivirus genus

There are recent, comprehensive papers available from 2018 regarding PRRSV-1 phylogeny, please refer to those.

The taxonomy of PRRSV has been updated within the manuscript according to the information given by the reviewer. In addition, a new reference has been included to support the diversity in PRRSV1 phylogeny as suggested.

When describing HP strains of PRRSV an entire paragraph could be added regarding HP PRRSV-1 strains and history, including those of subtype 2 (LENA and SU-1bel) and even those of subtype 1 (13V092 in Belgium, Frydas et al 2013; and ACRO in Austria Sinn et al. 2016).

We have added a new phrase with information about the identification of virulent strains within the different subtypes from PRRSV1 (Lines 59-63). In addition, a sentence regarding the clinical signs determined by virulent PRRVS strains has been included in Lines 82-84. We would like to highlight, that with these new changes the length of the Introduction is a little bit over the limit prefixed in the "Guide for the authors" for this journal (not exceed from 2 manuscript pages).

In lines 86-87 (now, lines 96-97), when describing the role of CD163, I suggest to refer first to Calvert et al 2007 (J. Virol), as they were the first group to highlight the role of CD163, then include the groundbreaking papers of Whithworth et al 2015, and Burkard et al 2017 describing the importance of full KO or SRCRD5 deleted CD163 in PRRSV infection, respectively.

The new references have been included as well as a short sentence with the main findings from Whithworth et al., 2015, and Burkard et al., 2017.

In the materials and methods part it would be useful to get more information about the INIA in house mABs in order to increase the reproducibility of the paper. A few sentences about the method of their generation. These mAbs have been extensively used and reported in the literature. Accordingly, a brief paragraph has been added to explain how were the hybridomas produced and the corresponding references have been also included (Lines 176-181).

Lines 179-180. If the authors have gross picture regarding the "complete atrophy" of the cervical part of the thymus please include it along with a control organ, as it would look very informative. Regrettably, we do not have any representative gross picture of the almost complete thymus atrophy in the PR0 group to be included in the manuscript.

Line 182. Rephrase the sentence as in its present form it sounds like the animals were infected with both viruses at the same time.

This sentence has been accordingly modified to avoid misunderstanding.

Line 190. Perivascular area should be used instead of "level". This change has been accomplished as suggested by the reviewer's comment.

Lines 207-208 and lines 308-309. "TUNEL positive cells were mainly found in tingible body macrophages" A bit of explanation is needed or rephrase this sentence in the discussion. Does this mean that the tingible body macrophages are apoptotic themselves or they contain TUNEL positive fragments that are not yet fully degraded?

Both sentences regarding the TUNEL labelling have been modified to clarify that the staining was observed both in cellular fragments phagocytised by tingible body macrophages as well as in apoptotic bodies.

Line 338. *Refer to CD163 knock out animals as indicated for the introduction.* **The reference has been accordingly included.**

Line 340. Philippidis et al., 2004 has a different font, not TNR. **The font of the reference has been modified.**

Figures:

Fig 2B – maybe an asterisk could mark the Hassal's corpuscule similarly to C According to reviewer's comment two arrows have been included in Fig. 2B to better identify the Hassal's corpuscule

Fig 4 - In case of C and maybe even D the level of magnification is not sufficient to observe the positive cells, I suggest to use the same magnification as for 2 B and C New figures at higher magnifications have been included for Fig. 4C and 4D to allow an easier identification of positive cells.

Fig 5 - D is hardly visible maybe a single brown dot can be seen nothing else, increase magnification, maybe along with C making all three IHC figures look more uniform

According to reviewer's comment new figures at higher magnifications have been included for Fig. 5C, 5D and 5E.

1	Impact of PRRSV strains of different in vivo virulence on the macrophage population of the
2	thymus
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21 Abstract

22 The emergence of "highly pathogenic" isolates of porcine reproductive and respiratory syndrome 23 virus (HP-PRRSV) has raised new concerns about PRRS control. Cells from the porcine monocyte-24 macrophage lineage represent the target for this virus, which replicates mainly in the lung, and 25 especially in HP-PRRSV strains, also in lymphoid organs, such as the thymus. This study aimed at 26 evaluating the impact of two PRRSV strains of different virulence on thymic macrophages as well 27 as after heterologous vaccination. After experimental infection with PR11 and PR40 PRRSV1 28 subtype 1 strains (low and high virulent, respectively) samples from thymus were analysed by 29 histopathology and immunohistochemistry for PRRSV N protein, TUNEL, CD172a, CD163, 30 CD107a and BA4D5 expression. Mortality was similar in both infected groups, but lung lesions and 31 thymus atrophy were more intense in PR40 group. Animals died at 10-14 dpi after PR11 or PR40 32 infection showed the most severe histopathological lesions, with a strong inflammatory response of 33 the stroma and extensive cell death phenomena in the cortex. These animals presented an increase 34 in the number of N protein, CD172a, CD163 and BA4D5 positive cells in the stroma and the cortex 35 together with a decrease in the number of CD107a positive cells. Our results highlight the 36 recruitment of macrophages in the thymus, the increase in the expression of CD163 and the regulation of the host cytotoxic activity by macrophages. However, no marked differences were 37 38 observed between PR11- and PR40-infected animals. Heterologous vaccination restrained virus 39 spread and lesions extent in the thymus of PR40-infected animals.

- 40
- 41 *Keywords:* virulence; PRRSV; macrophages; thymus; cell death.

42 **1. Introduction**

43 Porcine reproductive and respiratory syndrome virus (PRRSV) is a major swine pathogen that 44 induces severe respiratory symptoms in growing and finishing pigs and reproductive failure in gilts 45 and sows, causing considerable economic losses worldwide. The genome, of approximately 15 kb 46 in length, consists of a positive-stranded RNA and contains 11 open reading frames (ORFs), coding 47 for structural and non-structural proteins, which are subject to insertions and deletions determining 48 the genetic diversity of the virus (Murtaugh et al., 2010). Recently, the two genotypes of the virus, 49 type 1 or PRRSV1 (European) and type 2 or PRRSV2 (North American), have been included as 50 different viral species within the genus **Betaarterivirus**, particularly **Betaartevirus** suid 1 species for 51 PRRSV1 and Betaarterivirus suid 2 species for the PRRSV2, respectively (Gorbalenya et al., 52 2018). Porartevirus (Adams et al., 2017). Both viruses present high internal variability, with 53 PRRSV1 being divided into at least four subtypes (pan-European subtype 1, encompassing different 54 lineages, and East European subtypes 2, 3 and 4) and PRRSV2 into at least nine lineages (Nelsen et 55 al., 1999; Stadejek, et al. 2006, 2013; Balka et al., 2018). During the last decade, virulent variants of 56 the virus, referred to as highly pathogenic (HP), have emerged within both PRRSV1 and PRRSV2 57 (Lunney et al., 2010). These virulent strains often result in severe clinical signs, higher mortality 58 rates and higher tropism and viral load in blood and tissues than low virulent PRRSV strains (Tian 59 et al., 2007; Karnychuk et al., 2010; Canelli et al., 2017). Although virulent PRRSV1 strains have 60 been traditionally associated to subtype 3 strains (Lena and SU1-bel strains), strains with similar 61 characteristics have been identified within subtypes 1 (13V091, AUT15-33 and PR40/2014 strain) 62 and 2 (BOR59 strain) (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 63 2016; Canelli et al., 2017; Stadejek et al., 2017).

64

The efficacy of modified live virus (MLV) vaccines (PRRSV1 and PRRSV2) have been recently
tested in challenge trials with virulent isolates (Trus et al., 2014; Do et al., 2015; Bonckaert et al.,
2016; Canelli et al., 2018). Partial cross-protection of these vaccines against virulent strains has

been reported under experimental conditions with a reduction of the viremia, the severity of clinical
signs and lesions, and the duration of the clinical phase. Nevertheless, none of the tested vaccines
was able to prevent the transplacental transmission or the respiratory infection.

71

72 The main cell target for PRRSV replication is the pulmonary alveolar macrophage (PAM) but viral 73 replication has been also widely reported in other macrophage subpopulations from lungs as well as 74 from lymphoid organs of infected animals (Duan et al., 1997; Gómez-Laguna et al., 2010; Barranco 75 et al., 2012). Among lymphoid organs, the thymus particularly plays a central role in the 76 development of the immune system through the differentiation and maturation of T cells (Pearse et 77 al., 2006b). PRRSV infection is characterised by an immunosuppression state associated with, 78 among other factors, atrophy of the thymus and a major decrease in the number of thymocytes in 79 the cortex with marked differences according to the virulence of the PRRSV strain (Amarilla et al., 80 2016). Thus, so-called HP-PRRSV strains cause more severe clinical signs, long-lasting viremia, 81 higher virus level in blood and tissues, and higher frequency of mortality (Lunney et al., 2010); 82 moreover, these strains predispose piglets to weak cellular immunity together with thymus atrophy, 83 T cell depletion and impairment of the development of naïve T cells (Han et al., 2017).

84

85 In a general context, macrophages perform three main functions: antigen presentation, phagocytosis 86 and synthesis and secretion of cytokines (Geissman et al., 2010). However, the whole range of 87 functions of thymic macrophages is still nowadays unclear. The macrophage population in the 88 thymus is evenly distributed in the cortex and in the medulla and is particularly designated, at least, 89 to phagocytose and remove apoptotic bodies and self-reactive lymphocytes as well as to release 90 mediators involved in thymocytes maturation (Pearse et al., 2006a). As a myeloid cell, the main 91 macrophage marker extensively used is CD172a, which is strongly expressed from the early stages of differentiation onwards (Summerfield et al., 1997). A restricted marker to monocyte and 92 93 macrophages is CD163, a member of the family of proteins with scavenger receptor cysteine-rich

94 domains (Law et al., 1993). Particularly, CD163 has been identified to be the major receptor for 95 PRRSV uncoating and genome release (Calvert et al., 2007; Van Breedam et al., 2010), with well 96 described effects of the deletion of its SRCR5 domain on PRRSV infection (Whithworth et al., 97 2016; Burkard et al., 2017). CD107a, or lysosomal-associated membrane protein 1 (LAMP-1), 98 despite not being restricted to macrophages, has been demonstrated to be useful for identifying 99 macrophages populations in tissue sections, especially, tingible body macrophages in lymphoid 100 organs as well as macrophages from the thymus cortex and medulla (Bullido et al., 1997; 101 Domenech et al., 2003). An interesting marker with a restricted expression for the macrophage 102 linage is the antigen recognized by the monoclonal antibody BA4D5, which shows features that 103 resemble those of CD68. Thus, this molecule/antigen presents a predominant intracellular location 104 in phagolysosomes with a low expression on the cell surface and has been detected on macrophages 105 from the thymus cortex as well as on other macrophages from spleen and lymph nodes (Ezquerra et 106 al., 2009).

107

Considering the role of macrophages in PRRSV replication and on the onset of the host immune response, the impact of two Italian subtype 1 PRRSV1 strains (PR40/2014 and PR11/2014), with different *in vivo* virulence (Canelli et al., 2017), was evaluated in this study. In addition, the effect of a heterologous vaccination on histopathological lesions as well as on macrophages populations of the thymus of HP-PRRSV infected animals was examined.

113

114 **2. Materials and Methods**

115 2.1. Animals and experimental infection

The *in vivo* study is part of a large project carried out to investigate the pathogenesis and control of PRRSV1 strains of differing virulence; materials analysed in the present study were collected from experiments published elsewhere (Canelli et al., 2017, 2018). Briefly, a total of twelve 4-week-old conventional pigs were assigned to three different experimental groups, as described in Canelli et

120 al. (2017): (i) PR40 group (PR40), with 5 pigs inoculated intra-nasally (IN) with 2 ml, containing 10⁵ TCD₅₀ of PRRSV1 PR40/2014, per pig; (ii) PR11 group (PR11), with 4 piglets inoculated IN 121 122 with 2 ml, containing 10⁵ TCD₅₀ of PRRSV1 PR11/2014, per pig; and, (iii) Control group (C), with 123 3 animals inoculated IN with sterile medium (mock/negative control). In addition, two different 124 vaccinated groups, described in Canelli et al. (2018), were included: (i) VAC-C: 2 pigs were IM-125 vaccinated against PRRSV at 4 weeks of age (Porcilis® PRRS, MSD Animal Health; DV strain; 126 vaccine batch A208AD01) and left uninfected; (ii) VAC-PR40: 6 pigs were IM-vaccinated against 127 PRRSV and IN infected with PR40 at 35 days post-vaccination (dpv) (day 0 post-inoculation, dpi) 128 (Fig. 1).

129

130 No relevant pathogens (PRRSV, SIV, PCV2) were detected in the animals before the beginning of 131 the studies. Animals suffering from severe clinical signs with a fatal prognosis were humanely 132 euthanized according to standard protocols. All the survivors were humanely euthanized at 35 dpi 133 (end of the experiment). At necropsy gross pathology was recorded and thymus samples were 134 collected and fixed in buffered-formalin pH 7.4 for histopathology and immunohistochemical 135 studies. The experimental design and all the procedures were fully in agreement and approved by 136 the Ethical Committee and by the Ministry of Health in Italy according to European and National 137 rules on experimental infection studies and animal welfare.

138

139 2.2. Histopathology and grading of thymus

Four μ m 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 Amarilla et al., 2016): (i) Grade 0, the cortex:medulla ratio (C/M) is about 2:1 with typical histological characteristics of the thymus; (ii) Grade I, diffuse cortical reduction with focal cortical disappearance, 5–9 tingible body macrophages/mm² within the thymic cortex, typical medulla and stroma; (iii) Grade II, focal or multifocal decrease of C/M (<2:1), decrease of cortical layer with slight proportional increase of the stroma and 10–15 tingible body macrophages/mm² within the thymic cortex; (iv) Grade III, focal to multifocal blurring of normal corticomedullary demarcation, increase of the stroma, occasional increase in the number of lymphocytes, mast and plasma cells and \geq 16 tingible body macrophages/mm², with a "starry sky" appearance of the tissue; and, (v) Grade IV, extensive cell death of cortical thymocytes with complete disappearance of corticomedullary boundary demarcation and increase of the stroma.

152

Manual quantification of tingible body macrophages in thymic cortex was assessed in 25 nonoverlapping, consecutively selected high magnification fields of 0.2 mm². Results were expressed as number of cells per mm².

156

157 2.3. Immunohistochemistry

158 The Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was used for the immunolabelling of PRRSV antigen and the different macrophages markers. 159 160 Terminal dUTP Nick End-Labeling (TUNEL) was carried out by using a commercial kit (In Situ 161 Cell Death Detection Kit, POD, Roche, Germany) following manufacturer's instructions. Briefly, 4 162 um tissue sections were dewaxed and rehydrated in a gradient of ethanol, followed by endogenous peroxidase inhibition with 3 % H₂O₂ solution in methanol for 30 minutes (min). After treatment 163 164 with different antigen retrieval methods (Table 1), the slides were washed with PBS (pH 7.4) and 165 incubated for 30 min at room temperature with 100 µl of blocking solution in a humid chamber. 166 Primary antibodies were incubated overnight at 4 °C in a humid chamber (see dilutions in Table 1 for each antibody), while for the negative controls the primary antibody was replaced by either an 167 168 isotype control or by blocking solution. Biotinylated secondary antibody was incubated for 30 min 169 at room temperature. An avidin-biotin-peroxidase complex (Vector Laboratories) was applied for 170 1 hour at room temperature in the darkness. Labelling was visualized by application of the 171 NovaREDTM substrate kit (Vector Laboratories). Sections were counterstained with Harris's
172 haematoxylin, dehydrated and mounted.

173

Hybridomas secreting monoclonal antibodies (mAbs) to porcine CD107a (4E9/11, IgG1), CD163
(2A10/11, IgG1), CD172a (BA1C11, IgG1) and BA4D5 (IgG2b) were derived from fusion of
myeloma cells with spleen cells from Balb/c mice immunized with pulmonary alveolar
macrophages. The characterization of these mAbs has been described elsewhere (Bullido et al.,
1997; Sánchez et al., 1999; Álvarez et al., 2000; Ezquerra et al., 2009). MAbs were used in the
assays as hybridoma supernatants.

180

Labelled cells were analysed in 25 non-overlapping and consecutive high magnification fields of 0.2 mm². The expression of all markers was manually counted and the results were expressed as the number of cells per mm².

184

185 **3. Results**

186 *3.1. Thymus from PR11- and PR40-infected pigs at 10-14 dpi showed strong inflammatory response*

187 of the stroma and extensive cell death phenomena in the cortex

188 The clinical signs and gross lesions have been previously described elsewhere (Canelli et al., 2017, 189 2018). Mortality rate was similar in the two infected groups, with two and three pigs euthanized due 190 to welfare conditions in PR40 and PR11 groups, respectively, between 10 and 14 dpi. Lung lesions 191 were more severe in the PR40 group compared to the PR11 group and consisted of interstitial 192 pneumonia with multifocal, mottled, tanned appearance of the lungs accompanied, in some cases, 193 by bronchopneumonia associated to secondary bacterial infections. Atrophy of the thymus was 194 detected in both infected groups, with an almost complete atrophy of the cervical part of the thymus 195 in the PR40 group. Control animals did not exhibit significant gross or microscopic lesions.

197 The thymus of the infected animals groups with any of both viruses (either PR11 or PR40) was 198 characterised by diffuse cortical reduction, disappearance of the corticomedullary boundary, and, in 199 some cases, a consistent inflammation of the stroma. The most intense changes were observed in 200 the thymus from PR11- and PR40-infected pigs that died at 10-14 dpi, which presented extensive 201 cell death phenomena in the cortex with a strong disappearance of the corticomedullary boundary 202 (Table 2) (Fig. 2A-2B). In most of these animals, a marked interstitial inflammatory infiltrate of the 203 stroma by abundant neutrophils and mononuclear cells (macrophages, lymphocytes and plasma 204 cells in a lesser extent) together with oedema of the connective tissue was also observed (Fig. 2C). 205 This infiltrate was particularly intense at perivascular area and was associated with intravascular 206 trafficking of these immune cells (Fig. 2D).

207

3.2. PRRSV N protein positive cells were increased in PR40- and PR11-infected animals at 10-14 dpi mainly associated to the inflammatory foci in the stroma

PRRSV N protein was not detected in the thymus of control animals (groups C and VAC-C). PRRSV antigen was observed in the cytoplasm of macrophages from the thymic cortex and the stroma, and in a lesser extent in macrophages from the medulla of the thymus of PR40 and PR11 infected animals at 35 dpi (Fig. 3A). Interestingly, PR40 and PR11 infected animals that died between 10-14 dpi, presented a marked increase in the number of PRRSV positive cells, mainly associated to a marked infiltrate of PRRSV positive cells within the inflammatory reaction observed in the stroma of these animals (Fig. 3B-3C).

217

In case of vaccinated PR40-inoculated animals (VAC-PR40), only 3 out of 5 animals presented PRRSV positive cells with a similar frequency and distribution than non-vaccinated PR40inoculated animals at 35 dpi.

221

3.3. TUNEL labelling was increased in association to an intense increase of cell death in the cortex

TUNEL labelling was mainly observed within tingible body macrophages in phagocytised non-fully degraded cellular fragments and occasionally in free apoptotic bodies (Fig. 3D). TUNEL staining was mostly observed in the cortex and, to a lesser extent, in the medulla of the thymus of all piglets. No differences were observed either between infected animals and controls or among infected groups at 35 dpi (Table 2). However, a marked increase of TUNEL labelling was observed in the cortex of PR11 and PR40 infected animals at 10-14 dpi which showed a diffuse labelling associated to an intense increase of cell death which occupied most of the cortex (Fig. 3E).

230

3.4. CD172a positive cells were increased in the thymic cortex and stroma of PR11- and PR40infected pigs at 10-14 dpi

Labelling against CD172a was mainly observed in the cell surface and cytoplasm of monocytes and 233 234 macrophages as well as, in a lesser extent, in granulocytes and occasionally in dendritic-like cells 235 (Fig. 4D). Tingible body macrophages did not stain for this marker. CD172a positive cells were 236 more numerous in the thymic medulla than in the cortex and stroma of control animals. The thymus 237 of the animals infected with PR11 and PR40 and killed at 35 dpi showed a similar distribution of 238 CD172a positive cells than the control group (Fig. 4A). Interestingly, the expression of CD172a in 239 PR11- and PR40-infected pigs that died at 10-14 dpi was dramatically different; specifically, these 240 animals presented a major increase of positive cells in the stroma and minor in the cortex, together 241 with a decrease of CD172a positive cells in the medulla (Fig. 4A). These changes were more 242 pronounced in PR40 infected animals which presented a stunning increase in the number of 243 CD172a positive cells in the stroma (Fig. 4A-4D). In addition, a marked increase in the number of 244 intravascular CD172a positive cells was observed within blood vessels of the cortex, medulla and stroma from both PR11 and PR40 infected animals dead at 10-14 dpi and from PR40 infected 245 246 animals killed at 35 dpi (data not shown).

Both vaccinated groups (VAC-C and VAC-PR40) showed a similar distribution of CD172a positive cells than control animals (CON), with a mild increase in the cortex and medulla of VAC-PR40 animals (Fig. 4A).

251

3.5. A general increase of CD163 positive cells in cortex, medulla and stroma as well as at
intravascular level was observed in the PR40 group (10-14 dpi)

254 CD163 positive immunolabelling was visualized in the cytoplasm and cell surface of positive 255 macrophages. Tingible body macrophages from the cortex were also stained against with CD163 antibody (Fig. 4F). The highest number of cells expressing CD163 was found in the thymic cortex 256 257 for all groups. In the control group, the expression of CD163 was also detected in the medulla and, secondly, in the stroma. A general increase in the number of CD163 positive cells was observed in 258 259 the cortex and in the stroma of infected animals; particularly, in PR40-infected pigs that died at 10-260 14 dpi, which showed an overall enhancement in the three compartments (cortex, medulla and 261 stroma) together with a moderate increase in the frequency of intravascular CD163 positive cells in 262 the cortex and the medulla (Fig. 4B-4F).

263

No changes were observed in the distribution of CD163 positive cells in the thymus of VAC-C and
VAC-PR40 animals (Fig. 4B).

266

267 3.6. The number of CD107a positive cells was decreased in all infected animals

The staining for CD107a was mainly observed in the cytoplasm of macrophages, being also observed in tingible body macrophages from the cortex (Fig. 5C). The number of CD107a positive cells in all experimental groups, except for the control group (CON), was lower than the one detected for CD172a and CD163 positive cells (Fig. 5A). In control animals, the expression of CD107a was mainly found in the thymic cortex, with lower expression in the medulla and only few positive cells in the stroma. A general decrease in the number of CD107a positive cells was observed in all infected animals with only a moderate increase being observed in the stroma of
PR11- and PR40-infected animals at 10-14 dpi (Fig. 5A-5D).

276

Interestingly, vaccinated groups (VAC-C and VAC-PR40) presented a similar trend among them
showing the lowest number of CD107a positive cells (Fig. 5A).

279

3.7. A mild increase of BA4D5 positive cells was observed in PR11- and PR40-infected pigs at 1014 dpi and in vaccinated groups

The staining for BA4D5 was very low in all experimental groups being observed in the cytoplasm 282 283 of macrophages of cortex, medulla and stroma of the thymus (Fig. 5B). Perivascular positive cells 284 were found in the thymic medulla of some animals, whereas intravascular positive cells were 285 scattered (Fig. 5E-5F). No changes were observed in PR11- and PR40-infected animals at 35 dpi 286 when compared with control animals. The animals infected with PR11 and PR40 that died at 10-14 287 dpi showed an increase of BA4D5 positive cells in the cortex and in a lesser extent in the stroma 288 (Fig. 5B). Vaccinated groups displayed an increase in the number of positive cells to this marker in 289 the medulla and in the stroma, being more pronounced in the VAC-C group (Fig. 5B).

290

4. Discussion

292 Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the main viral diseases in pig 293 production, causing huge economic losses to the industry. A high genetic variability has been 294 reported for PRRSV, leading to the current recognition of two independent viral species (PRRSV1 295 and PRRSV2) (Adams et al., 2017) with also a marked intraspecies variability (Stadejek et al., 296 2008; Stadejek et al., 2013). During the last decade, several PRRS outbreaks characterised by 297 severe clinical signs as well as high morbidity and mortality rates have been reported in many countries from Europe and Southeast Asia (Lunney et al., 2010). Thus, Canelli and co-authors 298 299 (2017) characterised the strain PR40/2014, an Italian variant of the so-called HP-PRRSV1 subtype 300 1. According to the severe clinical signs and lesions observed in HP-PRRSV outbreaks as well as 301 the partial cross-protection conferred by commercial MLV vaccines, the study of the host-pathogen 302 interaction, with special emphasis on the role of target and primary lymphoid organs, such as the 303 thymus, is imperative. Therefore, the present study describes the impact of the infection with 304 PRRSV1 strains of different virulence, namely PR11/2014 and PR40/2014, on the macrophages 305 population of the thymus. Furthermore, the effect of a heterologous vaccination in the thymus of 306 animals challenged with the virulent strain PR40 was examined.

307

308 Thymic atrophy was observed in both PR11- and PR40-infected animals with more intense changes 309 in animals that died at 10-14 dpi. Microscopically, no differences were observed among both 310 infected groups, which presented disappearance of the corticomedullary boundary, extensive cell 311 death phenomena in the cortex and a stunning oedema and interstitial infiltration of the stroma at 312 10-14 dpi. However, the highest number of PRRSV positive cells was observed in a PR40-infected 313 animal dead at 10 dpi. Our results agree with previous reports that describe a trend for highly 314 pathogenic strains of the virus to highly replicate in the thymus (Butler et al., 2014), but contrast 315 with the thymus atrophy, cortical T cell depletion and consequent dysfunction of host immune 316 regulation associated to the virulence of the PRRSV strain (Amarilla et al., 2016; Han et al., 2017). 317 These discrepancies may be associated to the intrinsic differences between each experimental 318 setting as well as to the criteria for classifying a PRRSV strain as a virulent strain. Thus, exhaustive 319 criteria need to be established to categorize the virulence of PRRSV strains.

320

321 PRRSV is well known by its ability to induce cell death, being TUNEL labelling widely used for 322 the assessment of this goal (He et al., 2012; Gómez-Laguna et al., 2013; Amarilla et al., 2016). In 323 the present study, TUNEL <u>stainingstainingpositive cells were was</u> mainly found in <u>cellular</u> 324 <u>fragments phagocytised by</u> tingible body macrophages and apoptotic bodies from the thymic cortex 325 at 35 dpi. Noteworthy, pigs infected with PR11 and PR40 strains that died at 10-14 dpi presented marked cell death phenomena in the cortex (Grade IV) with an intense and diffuse TUNEL labelling. These animals also showed a higher number of PRRSV positive cells compared with infected animals at 35 dpi. The severity of cell death phenomena in these animals together with the number and location of PRRSV positive cells support the role of both direct and indirect induction of cell death by PRRSV (Rodríguez-Gómez et al., 2013).

331

The remarkable inflammatory reaction observed in the stroma of the thymus at 10-14 dpi was associated with a high number of PRRSV positive cells both in the cortex and in the stroma of the thymus. These findings suggest that during the acute phase of the disease, PRRSV may be able to actively replicate and disseminate, reaching other organs besides lungs, such as the thymus, through haematogenous dissemination. This hypothesis is also supported by the peak of viremia at 10 dpi (PR11 group) and 7 dpi (PR40 group) detected in a parallel study by Canelli and co-authors (2017).

338

PR40-vaccinated animals (VAC-PR40) presented minimal histopathological lesions in the thymus when compared with vaccinated control animals (VAC-C). In addition, a low number of PRRSV positive cells was detected in the thymus of vaccinated and challenged animals. These results agree with the partial protection conferred by MLV vaccines previously reported by other authors (Trus et al., 2014; Do et al., 2015; Bonckaert et al., 2016; Canelli et al., 2018) and highlight the role of heterologous vaccination in controlling the extension of the lesions and the spread of the virus in animals infected with a virulent PRRSV strain.

346

Macrophages are a central myeloid component of the innate immune system. They are not only activators but also one of the main regulators of the inflammation, being implicated in its resolution and in triggering off the reparative process. Herein, the macrophage population of the thymus was examined by CD172a, CD163, CD107a and BA4D5 immunolabelling. These markers have been previously used in many studies to characterise porcine tissue macrophages (Bullido et al., 1997; Domenech et al., 2003; Pérez et al., 2008). CD172a is one of the markers most commonly used and identifies myeloid cells from precursor stages until cellular differentiation (Summerfield et al., 1997). CD163, recognised as a major receptor for PRRSV (Calvert et al., 2007)(Van Breedam et al., 2010), play also a role as a scavenger receptor and in the induction of the anti-inflammatory mediators haptoglobin and IL-10 (Philippidis et al., 2004). Moreover, CD107a is directly related to the cytotoxic activity and has been demonstrated to be a useful marker of macrophage populations in tissues (Bullido et al., 1997; Aktas et al., 2008).

359

Our results showed no differences in CD172a immunolabelling between control group, infected 360 361 animals at 35 dpi and vaccinated animals. However, an enhancement in the number of CD172a 362 positive cells was observed in the cortex and especially in the stroma of infected animals at 10-14 363 dpi. These changes were more pronounced in PR40-infected pigs which also presented positive 364 cells within the blood vessels. The number of CD163 positive cells was increased in infected 365 animals throughout the study, and particularly in PR40-infected pigs at 10-14 dpi which displayed a 366 general increase of CD163 labelling in all the compartments (cortex, medulla and stroma) with 367 abundant intravascular CD163 positive cells. The increase in the number of CD172a and CD163 positive cells observed in both infected groups at 10-14 dpi was associated with the marked 368 369 inflammatory infiltrate of the stroma of the thymus as well as with the extensive cell death of 370 cortical thymocytes. Thus, monocytes/macrophages may be migrating from the bloodstream and 371 other tissues to the thymus through chemotaxis from inflammatory foci as well as from a high 372 demand of phagocytosis of cell death debris in the thymic cortex. The identification of intravascular 373 CD172a and CD163 positive cells observed in the present study supports this hypothesis. 374 Furthermore, the increase in the number of CD163 positive cells may also get along with the 375 induction of this surface molecule in resident tissue macrophages from the thymus. Interestingly, 376 the induction of CD163 has been proved in CD163 negative monocytes from bone marrow after in 377 vitro PRRSV infection (Fernández-Caballero et al., 2018) The higher frequency of CD163 positive

cells observed in both infected groups along our study may answer to different strategies of the virus: (1) to increase the number of susceptible cells to virus replication (Patton et al. 2009); (2) to allow PRRSV persistence in the thymus (Patton et al. 2009); (3) to lead to the modulation of the inflammatory and immune response through the induction of haptoglobin and IL-10 (Philippidis et al., 2004) or (4) to increase the phagocytic activity of macrophages through the binding of the scavenger receptor to Gram-positive and Gram-negative bacteria (Fabriek et al., 2009).

384

385 CD107a immunolabelling was mainly found in the thymic cortex in the control group, while a generalised decrease in the frequency of positive cells was observed in infected animals, with the 386 387 only exception of infected animals at 10-14 dpi, which presented a mild increase of CD107a 388 positive cells in the stroma. Compared with the other markers, the number of BA4D5 positive cells 389 was much lower, with an enhancement in the number of positive cells mainly in the cortex of both 390 infected groups at 10-14 dpi and in a lesser extent in the thymic medulla of vaccinated animals. The 391 decrease in the number of CD107a positive cells together with the increase in the number of 392 BA4D5 positive cells highlight different mechanisms of regulation of the cytotoxic activity not only 393 in infected pigs but also in vaccinated animals, which may be potentially involved in the 394 modulation of the host immune response. BA4D5 antibody is thought to be specific for porcine 395 CD68, which is mainly expressed by cells from the monocyte lineage, by circulating macrophages 396 and by tissue macrophages (Taylor et al., 2005). Among other functions CD68 plays a role in the 397 cytotoxic activity, with a predominant intracellular location in phagolysosomes (Kurushima et al., 398 2000); phagocytic activity, associated to the scavenger receptor family and promoting cellular 399 debris clearance (Taylor et al., 2005) and mediating the recruitment and activation of macrophages 400 through binding to specific lectins and selectins (Song et al., 2011). In our study, the increase in the 401 number of perivascular and intravascular BA4D5 positive cells observed in animals from both 402 infected groups at 10-14 dpi as well as in vaccinated animals support the potential role of this 403 molecule in macrophages recruitment observed mainly in infected pigs at 10-14 dpi.

405 The main evidence observed in the present work was the presence of severe histopathological 406 lesions in the thymus of the animals infected with PR11 and PR40 PRRSV strains that died at 10-14 407 dpi, and the increase in the number of macrophages in the different compartments of the thymus. 408 The different markers used in this study allow us identifying the recruitment of macrophages 409 associated to the strong and early inflammatory response in the stroma of the thymus, the increase in the expression of the major receptor of PRRSV and the regulation of the host cytotoxic activity 410 411 by macrophages. Interestingly, no marked differences were observed between the low virulent 412 PR11 and the virulent PR40 strains used in this study. Our results give some light to the 413 dysregulation of the host immune response by PRRSV and how the infection of the macrophage 414 population during the early phases of the disease may influence the decrease of the T cell population, already demonstrated in other studies (Canelli et al., 2017). Finally, our results point out 415 416 that heterologous vaccination is a useful strategy to restrain virus spread as well as the extent of the 417 lesions observed in animals infected with virulent strains of PRRSV.

418

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620 Figure captions

621 Fig. 1. Experimental design

622 Fig. 2. Representative photomicrographs of the thymus from a control pig (A; Haematoxylin-eosin, 623 HE; Bar, 100 µm), a PR11-infected pig dead at 10-14 dpi with a strong disappearance of the 624 corticomedullary boundary (B; HE; Bar, 100µm; Hassall's corpuscles are identified with two black 625 arrows), and a PR40-infected pig dead at 10-14 dpi with a marked interstitial inflammatory infiltrate 626 of the stroma by abundant neutrophils and mononuclear cells (macrophages, lymphocytes and 627 plasma cells in a lesser extent) particularly intense at perivascular level is showed (C; HE; Bar, 100µm; a Hassall's corpuscle is identified with an asterisk). A higher magnification of the 628 629 perivascular infiltrate, highlighted with a black dashed line in C, is showed (D; HE; Bar, 50µm).

Fig. 3. (A) Counts for PRRSV N protein positive cells in the thymic cortex (blue column), medulla 630 631 (red column), stroma (green column) and total (the empty circles represent individual values; the 632 mean is showed as a black solid line). (B) N protein positive cells (arrows) in the thymic cortex of a 633 PR11-infected pig that died at 10-14 dpi (IHC, Bar, 50µm). (C) High number of N protein positive 634 cells in the stroma and in the thymic cortex of a PR40-infected pig that died at 10-14 dpi (IHC, Bar, 635 50µm). Inset, detail of the cytoplasmic staining against PRRSV N protein in a macrophage from the 636 stroma of a PR40-infected pig that died at 10-14 dpi (IHC, Bar, 20µm). (D) TUNEL labelling of 637 tingible body macrophages in the cortex of the thymus of a control animal (TUNEL, Bar, 50µm). 638 (E) Marked increase of TUNEL labelling in the cortex of a PR11-infected animal at 10-14 dpi, with 639 a diffuse labelling associated to an intense increase of cell death (TUNEL, Bar, 50µm).

Fig. 4. Counts for CD172a (A) and CD163 (B) positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (C) CD172a positive cells in the thymic medulla of a PR40-infected animal and killed at 35 dpi (IHC, Bar, 5100μ m). (D) An increased number of CD172a positive cells in the stroma and the cortex of the thymus from a PR11-infected animal that died at 10-14 dpi (IHC, Bar, 5100μ m). *Inset*, detail of the cytoplasmic staining against CD172a in several macrophages from the stroma of a PR11-infected pig that died at 10-14 dpi (IHC, Bar,
20μm). (E) Scattered CD163 positive cells in the cortex and medulla of the thymus from a PR11infected animal that died at 10-14 dpi (IHC, Bar, 50μm). (F) Numerous macrophages and tingible
body macrophages within the thymic cortex and stroma of a PR40-infected pig at 10-14 dpi (IHC,
Bar, 50μm). *Inset*, detail of the cytoplasmic staining against CD163 in macrophages from the
stroma of a PR40-infected pig at 10-14 dpi (IHC, Bar, 20μm).

652 Fig. 5. Counts for CD107a (A) and BA4D5 (B) positive cells in the thymic cortex (blue column), 653 medulla (red column), stroma (green column) and total (the empty circles represent individual 654 values; the mean is showed as a black solid line). (C) Numerous tingible body macrophages 655 immunolabelled against CD107a in the thymic cortex of a control animal at 35 dpi (IHC, Bar, 656 2100µm). Inset, detail of the cytoplasmic staining against CD107a in a macrophage with 657 cytoplasmic prolongations in the thymic cortex from the same animal (IHC, Bar, 20um). (D) 658 Scattered CD107a positive cells in the cortex and medulla of the thymus from a PR40-infected 659 animal that died at 10-14 dpi (IHC, Bar, 2100µm). (E) BA4D5 positive cells in the medulla and at 660 perivascular level in the thymus from a VAC-PR40 animal at the end of the study (IHC, Bar, 661 250µm). (F) Higher magnification of another field of the thymus from the same animal with a 662 marked perivascular infiltrate by BA4D5 positive cells (IHC, Bar, 20µm).

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Specifiticy (clone)	Type of antibody	Commercial origin	Fixative	Blocking solution	Dilution	Antigen retrieval
Anti-PRRSV (clone SDOW17)	mAb	Rural Technologies Inc., Brookings, SD, USA	Formalin	BSA 1%	1:500	Protease Type XIV ^a
TUNEL	N.A.	Roche Diagnostics, Indianapolis, USA	Formalin	N.A.	N.A.	Proteinase K ^b
Anti-CD172a (BA1C11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
Anti-CD163 (2A10/11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
Anti-CD107a (4E9/11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
Anti-BA4D5 (BA4D5)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2

Table 1. Clones, sources and dilutions of the primary antibodies used for the immunohistochemical detection of macrophages markers.

666 N.A.: Not applicable; ^aProtease Type XIV (Sigma-Aldrich): 8 min at 37 °C in water bath; ^bProteinase K (Roche): 15 min at 37 °C in heat incubator.

Table 2. Histopathology grading of the thymus of piglets from each experimental group and average number of tingible body macrophages and

		CON (35 dpi)	PR11 (35 dpi)	PR40 (35 dpi)	PR11 (10-14 dpi)	PR40 (10-14 dpi)	VAC-C	VAC-PR40
Grades								
	0	2/3	-	1/3	-	-	-	3/5
	Ι	-	-	-	-	-	-	1/5
	II	1/3	1/1	2/3	-	-	2/2	1/5
	III	-	-	-	-	-	-	-
	IV	-	-	-	3/3	2/2	-	-
Tingible body macrophages		9.87 ± 1.79	7	8.87 ± 6.82	ND^*	ND^*	13.1 ± 8.63	10.4 ± 1.79
TUNEL positive cells		54.97 ± 63.40	67.91	43.53 ± 31.07	ND^*	ND^*	53.82 ± 40.20	53.18 ± 34.59

TUNEL positive cells (expressed as the mean \pm SD).

ND*: Not determined due to extensive cell death of thymocytes in the cortex.

1 Ingingino

- The impact of PRRSV strains of different virulence on thymic macrophages was
 examined
- Animals died 10-14 days after PR40 or PR11 infection showed the most severe
 lesions
- The number of N-protein⁺, CD172a⁺, CD163⁺ and BA4D5⁺ cells increased at 10-
- 7 14dpi
- No marked differences were observed between the PR11 and PR40 strains in this
 study
- Vaccination restrained virus spread and lesions in thymus of PR40-infected animals

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3 4	1	Impact of PRRSV strains of different <i>in vivo</i> virulence on the macrophage population of the
5 6	2	thymus
7 8	3	
9 10	4	Giulia Ogno ^a , Irene M. Rodríguez-Gómez ^b , Elena Canelli ^a , Inés Ruedas-Torres ^b , Belén Álvarez ^c ,
11 12	5	Javier Domínguez ^c , Paolo Borghetti ^a , Paolo Martelli ^{a,1} , Jaime Gómez-Laguna ^{b,1,*}
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Abstract

The emergence of "highly pathogenic" isolates of porcine reproductive and respiratory syndrome virus (HP-PRRSV) has raised new concerns about PRRS control. Cells from the porcine monocyte-macrophage lineage represent the target for this virus, which replicates mainly in the lung, and especially in HP-PRRSV strains, also in lymphoid organs, such as the thymus. This study aimed at evaluating the impact of two PRRSV strains of different virulence on thymic macrophages as well as after heterologous vaccination. After experimental infection with PR11 and PR40 PRRSV1 subtype 1 strains (low and high virulent, respectively) samples from thymus were analysed by histopathology and immunohistochemistry for PRRSV N protein, TUNEL, CD172a, CD163, CD107a and BA4D5 expression. Mortality was similar in both infected groups, but lung lesions and thymus atrophy were more intense in PR40 group. Animals died at 10-14 dpi after PR11 or PR40 infection showed the most severe histopathological lesions, with a strong inflammatory response of the stroma and extensive cell death phenomena in the cortex. These animals presented an increase in the number of N protein, CD172a, CD163 and BA4D5 positive cells in the stroma and the cortex together with a decrease in the number of CD107a positive cells. Our results highlight the recruitment of macrophages in the thymus, the increase in the expression of CD163 and the regulation of the host cytotoxic activity by macrophages. However, no marked differences were observed between PR11- and PR40-infected animals. Heterologous vaccination restrained virus spread and lesions extent in the thymus of PR40-infected animals.

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Keywords: virulence; PRRSV; macrophages; thymus; cell death.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a major swine pathogen that induces severe respiratory symptoms in growing and finishing pigs and reproductive failure in gilts and sows, causing considerable economic losses worldwide. The genome, of approximately 15 kb in length, consists of a positive-stranded RNA and contains 11 open reading frames (ORFs), coding for structural and non-structural proteins, which are subject to insertions and deletions determining the genetic diversity of the virus (Murtaugh et al., 2010). Recently, the two genotypes of the virus, type 1 or PRRSV1 (European) and type 2 or PRRSV2 (North American), have been included as different viral species within the genus Betaarterivirus, particularly Betaartevirus suid 1 species for PRRSV1 and Betaarterivirus suid 2 species for the PRRSV2, respectively (Gorbalenya et al., 2018). Both viruses present high internal variability, with PRRSV1 being divided into at least four subtypes (pan-European subtype 1, encompassing different lineages, and East European subtypes 2, 3 and 4) and PRRSV2 into at least nine lineages (Nelsen et al., 1999; Stadejek, et al. 2006, 2013; Balka et al., 2018). During the last decade, virulent variants of the virus, referred to as highly pathogenic (HP), have emerged within both PRRSV1 and PRRSV2 (Lunney et al., 2010). These virulent strains often result in severe clinical signs, higher mortality rates and higher tropism and viral load in blood and tissues than low virulent PRRSV strains (Tian et al., 2007; Karnychuk et al., 2010; Canelli et al., 2017). Although virulent PRRSV1 strains have been traditionally associated to subtype 3 strains (Lena and SU1-bel strains), strains with similar characteristics have been identified within subtypes 1 (13V091, AUT15-33 and PR40/2014 strain) and 2 (BOR59 strain) 166 62 (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017). 168 63

172 65 The efficacy of modified live virus (MLV) vaccines (PRRSV1 and PRRSV2) have been recently tested in challenge trials with virulent isolates (Trus et al., 2014; Do et al., 2015; Bonckaert et al., 2016; Canelli et al., 2018). Partial cross-protection of these vaccines against virulent strains has

been reported under experimental conditions with a reduction of the viremia, the severity of clinical signs and lesions, and the duration of the clinical phase. Nevertheless, none of the tested vaccines was able to prevent the transplacental transmission or the respiratory infection.

The main cell target for PRRSV replication is the pulmonary alveolar macrophage (PAM) but viral replication has been also widely reported in other macrophage subpopulations from lungs as well as from lymphoid organs of infected animals (Duan et al., 1997; Gómez-Laguna et al., 2010; Barranco et al., 2012). Among lymphoid organs, the thymus particularly plays a central role in the development of the immune system through the differentiation and maturation of T cells (Pearse et al., 2006b). PRRSV infection is characterised by an immunosuppression state associated with, among other factors, atrophy of the thymus and a major decrease in the number of thymocytes in the cortex with marked differences according to the virulence of the PRRSV strain (Amarilla et al., 2016). Thus, so-called HP-PRRSV strains cause more severe clinical signs, long-lasting viremia, higher virus level in blood and tissues, and higher frequency of mortality (Lunney et al., 2010); moreover, these strains predispose piglets to weak cellular immunity together with thymus atrophy, T cell depletion and impairment of the development of naïve T cells (Han et al., 2017).

In a general context, macrophages perform three main functions: antigen presentation, phagocytosis and synthesis and secretion of cytokines (Geissman et al., 2010). However, the whole range of functions of thymic macrophages is still nowadays unclear. The macrophage population in the thymus is evenly distributed in the cortex and in the medulla and is particularly designated, at least, to phagocytose and remove apoptotic bodies and self-reactive lymphocytes as well as to release mediators involved in thymocytes maturation (Pearse et al., 2006a). As a myeloid cell, the main macrophage marker extensively used is CD172a, which is strongly expressed from the early stages of differentiation onwards (Summerfield et al., 1997). A restricted marker to monocyte and macrophages is CD163, a member of the family of proteins with scavenger receptor cysteine-rich

242 243 domains (Law et al., 1993). Particularly, CD163 has been identified to be the major receptor for 94 244 245 95 PRRSV uncoating and genome release (Calvert et al., 2007; Van Breedam et al., 2010), with well 246 247 described effects of the deletion of its SRCR5 domain on PRRSV infection (Whithworth et al., 96 248 249 2016; Burkard et al., 2017). CD107a, or lysosomal-associated membrane protein 1 (LAMP-1), 97 250 251 252 98 despite not being restricted to macrophages, has been demonstrated to be useful for identifying 253 macrophages populations in tissue sections, especially, tingible body macrophages in lymphoid 254 99 255 organs as well as macrophages from the thymus cortex and medulla (Bullido et al., 1997; 256100 257 ²⁵⁸101 Domenech et al., 2003). An interesting marker with a restricted expression for the macrophage 259 ²⁶⁰₂₆₁102 linage is the antigen recognized by the monoclonal antibody BA4D5, which shows features that ²⁶²₂₆₃103 resemble those of CD68. Thus, this molecule/antigen presents a predominant intracellular location 264 265104 in phagolysosomes with a low expression on the cell surface and has been detected on macrophages 266 267105 from the thymus cortex as well as on other macrophages from spleen and lymph nodes (Ezquerra et 268 al., 2009). 269106 270 271107 272 273108 Considering the role of macrophages in PRRSV replication and on the onset of the host immune 274 275109 response, the impact of two Italian subtype 1 PRRSV1 strains (PR40/2014 and PR11/2014), with 276 ²⁷⁷₂₇₈110 different in vivo virulence (Canelli et al., 2017), was evaluated in this study. In addition, the effect ²⁷⁹ 280¹¹¹ of a heterologous vaccination on histopathological lesions as well as on macrophages populations of 281 ₂₈₂112 the thymus of HP-PRRSV infected animals was examined. 283 284113

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286114 2. Materials and Methods

288115 *2.1. Animals and experimental infection* 289

The *in vivo* study is part of a large project carried out to investigate the pathogenesis and control of PRRSV1 strains of differing virulence; materials analysed in the present study were collected from experiments published elsewhere (Canelli et al., 2017, 2018). Briefly, a total of twelve 4-week-old conventional pigs were assigned to three different experimental groups, as described in Canelli et

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³⁰³120 al. (2017): (i) PR40 group (PR40), with 5 pigs inoculated intra-nasally (IN) with 2 ml, containing 304 305 10⁵ TCD₅₀ of PRRSV1 PR40/2014, per pig; (ii) PR11 group (PR11), with 4 piglets inoculated IN 121 306 307 ₃₀₈122 with 2 ml, containing 10⁵ TCD₅₀ of PRRSV1 PR11/2014, per pig; and, (iii) Control group (C), with 309 3 animals inoculated IN with sterile medium (mock/negative control). In addition, two different 310123 311 vaccinated groups, described in Canelli et al. (2018), were included: (i) VAC-C: 2 pigs were IM-312124 313 vaccinated against PRRSV at 4 weeks of age (Porcilis® PRRS, MSD Animal Health; DV strain; 314125 315 316126 vaccine batch A208AD01) and left uninfected; (ii) VAC-PR40: 6 pigs were IM-vaccinated against 317 ³¹⁸127 PRRSV and IN infected with PR40 at 35 days post-vaccination (dpv) (day 0 post-inoculation, dpi) 319 ³²⁰ 321</sub>128 (Fig. 1).

324 ₃₂₅130 No relevant pathogens (PRRSV, SIV, PCV2) were detected in the animals before the beginning of 326 the studies. Animals suffering from severe clinical signs with a fatal prognosis were humanely 327131 328 euthanized according to standard protocols. All the survivors were humanely euthanized at 35 dpi 329132 330 (end of the experiment). At necropsy gross pathology was recorded and thymus samples were 331133 332 collected and fixed in buffered-formalin pH 7.4 for histopathology and immunohistochemical 333134 334 ³³⁵135 studies. The experimental design and all the procedures were fully in agreement and approved by 336 337 the Ethical Committee and by the Ministry of Health in Italy according to European and National 338 ³³⁹₃₄₀137 rules on experimental infection studies and animal welfare.

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2.2. Histopathology and grading of thymus

₃₄₆140 Four µm tissue sections were stained with haematoxylin and eosin (H&E). The severity of the 347 lesions in thymus was scored as follows (adapted and modified from Amarilla et al., 2016): (i) 348141 349 Grade 0, the cortex:medulla ratio (C/M) is about 2:1 with typical histological characteristics of the 350142 351 thymus; (ii) Grade I, diffuse cortical reduction with focal cortical disappearance, 5–9 tingible body 352143 353 354144 macrophages/mm² within the thymic cortex, typical medulla and stroma; (iii) Grade II, focal or 355 ³⁵⁶145 multifocal decrease of C/M (<2:1), decrease of cortical layer with slight proportional increase of the 357

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stroma and 10–15 tingible body macrophages/mm² within the thymic cortex; (iv) Grade III, focal to multifocal blurring of normal corticomedullary demarcation, increase of the stroma, occasional increase in the number of lymphocytes, mast and plasma cells and \geq 16 tingible body macrophages/mm², with a "starry sky" appearance of the tissue; and, (v) Grade IV, extensive cell death of cortical thymocytes with complete disappearance of corticomedullary boundary demarcation and increase of the stroma.

Manual quantification of tingible body macrophages in thymic cortex was assessed in 25 nonoverlapping, consecutively selected high magnification fields of 0.2 mm². Results were expressed as number of cells per mm².

57 2.3. Immunohistochemistry

The Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was used for the immunolabelling of PRRSV antigen and the different macrophages markers. Terminal dUTP Nick End-Labeling (TUNEL) was carried out by using a commercial kit (In Situ Cell Death Detection Kit, POD, Roche, Germany) following manufacturer's instructions. Briefly, 4 µm tissue sections were dewaxed and rehydrated in a gradient of ethanol, followed by endogenous peroxidase inhibition with 3 % H₂O₂ solution in methanol for 30 minutes (min). After treatment with different antigen retrieval methods (Table 1), the slides were washed with PBS (pH 7.4) and incubated for 30 min at room temperature with 100 µl of blocking solution in a humid chamber. Primary antibodies were incubated overnight at 4 °C in a humid chamber (see dilutions in Table 1 for each antibody), while for the negative controls the primary antibody was replaced by either an isotype control or by blocking solution. Biotinylated secondary antibody was incubated for 30 min at room temperature. An avidin-biotin-peroxidase complex (Vector Laboratories) was applied for 1 hour at room temperature in the darkness. Labelling was visualized by application of the

NovaRED[™] substrate kit (Vector Laboratories). Sections were counterstained with Harris's haematoxylin, dehydrated and mounted.

Hybridomas secreting monoclonal antibodies (mAbs) to porcine CD107a (4E9/11, IgG1), CD163 (2A10/11, IgG1), CD172a (BA1C11, IgG1) and BA4D5 (IgG2b) were derived from fusion of myeloma cells with spleen cells from Balb/c mice immunized with pulmonary alveolar macrophages. The characterization of these mAbs has been described elsewhere (Bullido et al., 1997; Sánchez et al., 1999; Álvarez et al., 2000; Ezquerra et al., 2009). MAbs were used in the assays as hybridoma supernatants.

Labelled cells were analysed in 25 non-overlapping and consecutive high magnification fields of 0.2 mm². The expression of all markers was manually counted and the results were expressed as the number of cells per mm².

³185 **3. Results**

3.1. Thymus from PR11- and PR40-infected pigs at 10-14 dpi showed strong inflammatory response
of the stroma and extensive cell death phenomena in the cortex

The clinical signs and gross lesions have been previously described elsewhere (Canelli et al., 2017, 2018). Mortality rate was similar in the two infected groups, with two and three pigs euthanized due to welfare conditions in PR40 and PR11 groups, respectively, between 10 and 14 dpi. Lung lesions were more severe in the PR40 group compared to the PR11 group and consisted of interstitial pneumonia with multifocal, mottled, tanned appearance of the lungs accompanied, in some cases, by bronchopneumonia associated to secondary bacterial infections. Atrophy of the thymus was detected in both infected groups, with an almost complete atrophy of the cervical part of the thymus in the PR40 group. Control animals did not exhibit significant gross or microscopic lesions.

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The thymus of the infected animals with any of both viruses (either PR11 or PR40) was characterised by diffuse cortical reduction, disappearance of the corticomedullary boundary, and, in some cases, a consistent inflammation of the stroma. The most intense changes were observed in the thymus from PR11- and PR40-infected pigs that died at 10-14 dpi, which presented extensive cell death phenomena in the cortex with a strong disappearance of the corticomedullary boundary (Table 2) (Fig. 2A-2B). In most of these animals, a marked interstitial inflammatory infiltrate of the stroma by abundant neutrophils and mononuclear cells (macrophages, lymphocytes and plasma cells in a lesser extent) together with oedema of the connective tissue was also observed (Fig. 2C). This infiltrate was particularly intense at perivascular area and was associated with intravascular trafficking of these immune cells (Fig. 2D).

3.2. PRRSV N protein positive cells were increased in PR40- and PR11-infected animals at 10-14 dpi mainly associated to the inflammatory foci in the stroma

PRRSV N protein was not detected in the thymus of control animals (groups C and VAC-C). PRRSV antigen was observed in the cytoplasm of macrophages from the thymic cortex and the stroma, and in a lesser extent in macrophages from the medulla of the thymus of PR40 and PR11 infected animals at 35 dpi (Fig. 3A). Interestingly, PR40 and PR11 infected animals that died between 10-14 dpi, presented a marked increase in the number of PRRSV positive cells, mainly associated to a marked infiltrate of PRRSV positive cells within the inflammatory reaction observed in the stroma of these animals (Fig. 3B-3C).

In case of vaccinated PR40-inoculated animals (VAC-PR40), only 3 out of 5 animals presented PRRSV positive cells with a similar frequency and distribution than non-vaccinated PR40inoculated animals at 35 dpi.

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TUNEL labelling was mainly observed within tingible body macrophages in phagocytised non-fully degraded cellular fragments and occasionally in free apoptotic bodies (Fig. 3D). TUNEL staining was mostly observed in the cortex and, to a lesser extent, in the medulla of the thymus of all piglets. No differences were observed either between infected animals and controls or among infected groups at 35 dpi (Table 2). However, a marked increase of TUNEL labelling was observed in the cortex of PR11 and PR40 infected animals at 10-14 dpi which showed a diffuse labelling associated to an intense increase of cell death which occupied most of the cortex (Fig. 3E).

3.4. CD172a positive cells were increased in the thymic cortex and stroma of PR11- and PR40infected pigs at 10-14 dpi

Labelling against CD172a was mainly observed in the cell surface and cytoplasm of monocytes and macrophages as well as, in a lesser extent, in granulocytes and occasionally in dendritic-like cells (Fig. 4D). Tingible body macrophages did not stain for this marker. CD172a positive cells were more numerous in the thymic medulla than in the cortex and stroma of control animals. The thymus of the animals infected with PR11 and PR40 and killed at 35 dpi showed a similar distribution of CD172a positive cells than the control group (Fig. 4A). Interestingly, the expression of CD172a in PR11- and PR40-infected pigs that died at 10-14 dpi was dramatically different; specifically, these animals presented a major increase of positive cells in the stroma and minor in the cortex, together with a decrease of CD172a positive cells in the medulla (Fig. 4A). These changes were more pronounced in PR40 infected animals which presented a stunning increase in the number of CD172a positive cells in the stroma (Fig. 4A-4D). In addition, a marked increase in the number of intravascular CD172a positive cells was observed within blood vessels of the cortex, medulla and stroma from both PR11 and PR40 infected animals dead at 10-14 dpi and from PR40 infected animals killed at 35 dpi (data not shown).

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Both vaccinated groups (VAC-C and VAC-PR40) showed a similar distribution of CD172a positive cells than control animals (CON), with a mild increase in the cortex and medulla of VAC-PR40 animals (Fig. 4A).

252 3.5. A general increase of CD163 positive cells in cortex, medulla and stroma as well as at 253 intravascular level was observed in the PR40 group (10-14 dpi)

CD163 positive immunolabelling was visualized in the cytoplasm and cell surface of positive macrophages. Tingible body macrophages from the cortex were also stained with CD163 antibody (Fig. 4F). The highest number of cells expressing CD163 was found in the thymic cortex for all groups. In the control group, the expression of CD163 was also detected in the medulla and, secondly, in the stroma. A general increase in the number of CD163 positive cells was observed in the cortex and in the stroma of infected animals; particularly, in PR40-infected pigs that died at 10-14 dpi, which showed an overall enhancement in the three compartments (cortex, medulla and stroma) together with a moderate increase in the frequency of intravascular CD163 positive cells in the cortex and the medulla (Fig. 4B-4F).

No changes were observed in the distribution of CD163 positive cells in the thymus of VAC-C and
VAC-PR40 animals (Fig. 4B).

3.6. The number of CD107a positive cells was decreased in all infected animals

The staining for CD107a was mainly observed in the cytoplasm of macrophages, being also observed in tingible body macrophages from the cortex (Fig. 5C). The number of CD107a positive cells in all experimental groups, except for the control group (CON), was lower than the one detected for CD172a and CD163 positive cells (Fig. 5A). In control animals, the expression of CD107a was mainly found in the thymic cortex, with lower expression in the medulla and only few positive cells in the stroma. A general decrease in the number of CD107a positive cells was observed in all infected animals with only a moderate increase being observed in the stroma of PR11- and PR40-infected animals at 10-14 dpi (Fig. 5A-5D).

Interestingly, vaccinated groups (VAC-C and VAC-PR40) presented a similar trend among them showing the lowest number of CD107a positive cells (Fig. 5A).

3.7. A mild increase of BA4D5 positive cells was observed in PR11- and PR40-infected pigs at 1014 dpi and in vaccinated groups

The staining for BA4D5 was very low in all experimental groups being observed in the cytoplasm of macrophages of cortex, medulla and stroma of the thymus (Fig. 5B). Perivascular positive cells were found in the thymic medulla of some animals, whereas intravascular positive cells were scattered (Fig. 5E-5F). No changes were observed in PR11- and PR40-infected animals at 35 dpi when compared with control animals. The animals infected with PR11 and PR40 that died at 10-14 dpi showed an increase of BA4D5 positive cells in the cortex and in a lesser extent in the stroma (Fig. 5B). Vaccinated groups displayed an increase in the number of positive cells to this marker in the medulla and in the stroma, being more pronounced in the VAC-C group (Fig. 5B).

91 4. Discussion

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the main viral diseases in pig production, causing huge economic losses to the industry. A high genetic variability has been reported for PRRSV, leading to the current recognition of two independent viral species (PRRSV1 and PRRSV2) (Adams et al., 2017) with also a marked intraspecies variability (Stadejek et al., 2008; Stadejek et al., 2013). During the last decade, several PRRS outbreaks characterised by severe clinical signs as well as high morbidity and mortality rates have been reported in many countries from Europe and Southeast Asia (Lunney et al., 2010). Thus, Canelli and co-authors (2017) characterised the strain PR40/2014, an Italian variant of the so-called HP-PRRSV1 subtype

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1. According to the severe clinical signs and lesions observed in HP-PRRSV outbreaks as well as the partial cross-protection conferred by commercial MLV vaccines, the study of the host-pathogen interaction, with special emphasis on the role of target and primary lymphoid organs, such as the thymus, is imperative. Therefore, the present study describes the impact of the infection with PRRSV1 strains of different virulence, namely PR11/2014 and PR40/2014, on the macrophages population of the thymus. Furthermore, the effect of a heterologous vaccination in the thymus of animals challenged with the virulent strain PR40 was examined.

Thymic atrophy was observed in both PR11- and PR40-infected animals with more intense changes in animals that died at 10-14 dpi. Microscopically, no differences were observed among both infected groups, which presented disappearance of the corticomedullary boundary, extensive cell death phenomena in the cortex and a stunning oedema and interstitial infiltration of the stroma at 10-14 dpi. However, the highest number of PRRSV positive cells was observed in a PR40-infected animal dead at 10 dpi. Our results agree with previous reports that describe a trend for highly pathogenic strains of the virus to highly replicate in the thymus (Butler et al., 2014), but contrast with the thymus atrophy, cortical T cell depletion and consequent dysfunction of host immune regulation associated to the virulence of the PRRSV strain (Amarilla et al., 2016; Han et al., 2017). These discrepancies may be associated to the intrinsic differences between each experimental setting as well as to the criteria for classifying a PRRSV strain as a virulent strain. Thus, exhaustive criteria need to be established to categorize the virulence of PRRSV strains.

PRRSV is well known by its ability to induce cell death, being TUNEL labelling widely used for the assessment of this goal (He et al., 2012; Gómez-Laguna et al., 2013; Amarilla et al., 2016). In the present study, TUNEL staining was mainly found in cellular fragments phagocytised by tingible body macrophages and apoptotic bodies from the thymic cortex at 35 dpi. Noteworthy, pigs infected with PR11 and PR40 strains that died at 10-14 dpi presented marked cell death phenomena in the cortex (Grade IV) with an intense and diffuse TUNEL labelling. These animals also showed a higher number of PRRSV positive cells compared with infected animals at 35 dpi. The severity of cell death phenomena in these animals together with the number and location of PRRSV positive cells support the role of both direct and indirect induction of cell death by PRRSV (Rodríguez-Gómez et al., 2013).

The remarkable inflammatory reaction observed in the stroma of the thymus at 10-14 dpi was associated with a high number of PRRSV positive cells both in the cortex and in the stroma of the thymus. These findings suggest that during the acute phase of the disease, PRRSV may be able to actively replicate and disseminate, reaching other organs besides lungs, such as the thymus, through haematogenous dissemination. This hypothesis is also supported by the peak of viremia at 10 dpi (PR11 group) and 7 dpi (PR40 group) detected in a parallel study by Canelli and co-authors (2017).

PR40-vaccinated animals (VAC-PR40) presented minimal histopathological lesions in the thymus when compared with vaccinated control animals (VAC-C). In addition, a low number of PRRSV positive cells was detected in the thymus of vaccinated and challenged animals. These results agree with the partial protection conferred by MLV vaccines previously reported by other authors (Trus et al., 2014; Do et al., 2015; Bonckaert et al., 2016; Canelli et al., 2018) and highlight the role of heterologous vaccination in controlling the extension of the lesions and the spread of the virus in animals infected with a virulent PRRSV strain.

Macrophages are a central myeloid component of the innate immune system. They are not only activators but also one of the main regulators of the inflammation, being implicated in its resolution and in triggering off the reparative process. Herein, the macrophage population of the thymus was examined by CD172a, CD163, CD107a and BA4D5 immunolabelling. These markers have been previously used in many studies to characterise porcine tissue macrophages (Bullido et al., 1997;

899 900 Domenech et al., 2003; Pérez et al., 2008). CD172a is one of the markers most commonly used and identifies myeloid cells from precursor stages until cellular differentiation (Summerfield et al., 1997). CD163, recognised as a major receptor for PRRSV (Calvert et al., 2007), play also a role as a scavenger receptor and in the induction of the anti-inflammatory mediators haptoglobin and IL-10 (Philippidis et al., 2004). Moreover, CD107a is directly related to the cytotoxic activity and has been demonstrated to be a useful marker of macrophage populations in tissues (Bullido et al., 1997; Aktas et al., 2008).

Our results showed no differences in CD172a immunolabelling between control group, infected animals at 35 dpi and vaccinated animals. However, an enhancement in the number of CD172a positive cells was observed in the cortex and especially in the stroma of infected animals at 10-14 dpi. These changes were more pronounced in PR40-infected pigs which also presented positive cells within the blood vessels. The number of CD163 positive cells was increased in infected animals throughout the study, and particularly in PR40-infected pigs at 10-14 dpi which displayed a general increase of CD163 labelling in all the compartments (cortex, medulla and stroma) with abundant intravascular CD163 positive cells. The increase in the number of CD172a and CD163 positive cells observed in both infected groups at 10-14 dpi was associated with the marked inflammatory infiltrate of the stroma of the thymus as well as with the extensive cell death of cortical thymocytes. Thus, monocytes/macrophages may be migrating from the bloodstream and other tissues to the thymus through chemotaxis from inflammatory foci as well as from a high demand of phagocytosis of cell death debris in the thymic cortex. The identification of intravascular CD172a and CD163 positive cells observed in the present study supports this hypothesis. Furthermore, the increase in the number of CD163 positive cells may also get along with the induction of this surface molecule in resident tissue macrophages from the thymus. Interestingly, the induction of CD163 has been proved in CD163 negative monocytes from bone marrow after in vitro PRRSV infection (Fernández-Caballero et al., 2018) The higher frequency of CD163 positive 15

 cells observed in both infected groups along our study may answer to different strategies of the virus: (1) to increase the number of susceptible cells to virus replication (Patton et al. 2009); (2) to allow PRRSV persistence in the thymus (Patton et al. 2009); (3) to lead to the modulation of the inflammatory and immune response through the induction of haptoglobin and IL-10 (Philippidis et al., 2004) or (4) to increase the phagocytic activity of macrophages through the binding of the scavenger receptor to Gram-positive and Gram-negative bacteria (Fabriek et al., 2009).

CD107a immunolabelling was mainly found in the thymic cortex in the control group, while a generalised decrease in the frequency of positive cells was observed in infected animals, with the only exception of infected animals at 10-14 dpi, which presented a mild increase of CD107a positive cells in the stroma. Compared with the other markers, the number of BA4D5 positive cells was much lower, with an enhancement in the number of positive cells mainly in the cortex of both infected groups at 10-14 dpi and in a lesser extent in the thymic medulla of vaccinated animals. The decrease in the number of CD107a positive cells together with the increase in the number of BA4D5 positive cells highlight different mechanisms of regulation of the cytotoxic activity not only in infected pigs but also in vaccinated animals, which may be potentially involved in the modulation of the host immune response. BA4D5 antibody is thought to be specific for porcine CD68, which is mainly expressed by cells from the monocyte lineage, by circulating macrophages and by tissue macrophages (Taylor et al., 2005). Among other functions CD68 plays a role in the cytotoxic activity, with a predominant intracellular location in phagolysosomes (Kurushima et al., 2000); phagocytic activity, associated to the scavenger receptor family and promoting cellular debris clearance (Taylor et al., 2005) and mediating the recruitment and activation of macrophages through binding to specific lectins and selectins (Song et al., 2011). In our study, the increase in the number of perivascular and intravascular BA4D5 positive cells observed in animals from both infected groups at 10-14 dpi as well as in vaccinated animals support the potential role of this molecule in macrophages recruitment observed mainly in infected pigs at 10-14 dpi.

The main evidence observed in the present work was the presence of severe histopathological lesions in the thymus of the animals infected with PR11 and PR40 PRRSV strains that died at 10-14 dpi, and the increase in the number of macrophages in the different compartments of the thymus. The different markers used in this study allow us identifying the recruitment of macrophages associated to the strong and early inflammatory response in the stroma of the thymus, the increase in the expression of the major receptor of PRRSV and the regulation of the host cytotoxic activity by macrophages. Interestingly, no marked differences were observed between the low virulent PR11 and the virulent PR40 strains used in this study. Our results give some light to the dysregulation of the host immune response by PRRSV and how the infection of the macrophage population during the early phases of the disease may influence the decrease of the T cell population, already demonstrated in other studies (Canelli et al., 2017). Finally, our results point out that heterologous vaccination is a useful strategy to restrain virus spread as well as the extent of the lesions observed in animals infected with virulent strains of PRRSV.

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Figure captions

Fig. 1. Experimental design

Fig. 2. Representative photomicrographs of the thymus from a control pig (A; Haematoxylin-eosin, HE; Bar, 100 μ m), a PR11-infected pig dead at 10-14 dpi with a strong disappearance of the corticomedullary boundary (B; HE; Bar, 100 μ m; Hassall's corpuscles are identified with two black arrows), and a PR40-infected pig dead at 10-14 dpi with a marked interstitial inflammatory infiltrate of the stroma by abundant neutrophils and mononuclear cells (macrophages, lymphocytes and plasma cells in a lesser extent) particularly intense at perivascular level is showed (C; HE; Bar, 100 μ m; a Hassall's corpuscle is identified with an asterisk). A higher magnification of the perivascular infiltrate, highlighted with a black dashed line in C, is showed (D; HE; Bar, 50 μ m).

Fig. 3. (A) Counts for PRRSV N protein positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (B) N protein positive cells (arrows) in the thymic cortex of a PR11-infected pig that died at 10-14 dpi (IHC, Bar, 50µm). (C) High number of N protein positive cells in the stroma and in the thymic cortex of a PR40-infected pig that died at 10-14 dpi (IHC, Bar, 50µm). *Inset*, detail of the cytoplasmic staining against PRRSV N protein in a macrophage from the stroma of a PR40-infected pig that died at 10-14 dpi (IHC, Bar, 20µm). (D) TUNEL labelling of tingible body macrophages in the cortex of the thymus of a control animal (TUNEL, Bar, 50µm). (E) Marked increase of TUNEL labelling in the cortex of a PR11-infected animal at 10-14 dpi, with a diffuse labelling associated to an intense increase of cell death (TUNEL, Bar, 50µm).

Fig. 4. Counts for CD172a (A) and CD163 (B) positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (C) CD172a positive cells in the thymic medulla of a PR40-infected animal and killed at 35 dpi (IHC, Bar, 50μm). (D) An increased number of CD172a positive cells in the stroma and the cortex of the thymus from a PR11-infected animal that died at 10-14 dpi (IHC, Bar, 50μm). *Inset*, detail of the cytoplasmic staining against CD172a in

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 $^{1563}_{1564}_{1564}_{46}$ several macrophages from the stroma of a PR11-infected pig that died at 10-14 dpi (IHC, Bar, $^{1565}_{1566}$ 20µm). (E) Scattered CD163 positive cells in the cortex and medulla of the thymus from a PR11-infected animal that died at 10-14 dpi (IHC, Bar, 50µm). (F) Numerous macrophages and tingible body macrophages within the thymic cortex and stroma of a PR40-infected pig at 10-14 dpi (IHC, Bar, 50µm). Inset, detail of the cytoplasmic staining against CD163 in macrophages from the **Ø**50 stroma of a PR40-infected pig at 10-14 dpi (IHC, Bar, 20um).

Fig. 5. Counts for CD107a (A) and BA4D5 (B) positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual $^{1580}_{1581}_{1581}_{1581}$ values; the mean is showed as a black solid line). (C) Numerous tingible body macrophages 1583 1583 immunolabelled against CD107a in the thymic cortex of a control animal at 35 dpi (IHC, Bar, 20µm). Inset, detail of the cytoplasmic staining against CD107a in a macrophage with cytoplasmic prolongations in the thymic cortex from the same animal (IHC, Bar, 20um). (D) Scattered CD107a 158\$57 positive cells in the cortex and medulla of the thymus from a PR40-infected animal that died at 10-14 dpi (IHC, Bar, 20µm). (E) BA4D5 positive cells in the medulla and at perivascular level in the thymus from a VAC-PR40 animal at the end of the study (IHC, Bar, 20µm). (F) Higher **8**61 magnification of another field of the thymus from the same animal with a marked perivascular ¹⁵⁹/₆₂ infiltrate by BA4D5 positive cells (IHC, Bar, 20µm).

Table 1. Clones, sources and dilutions of the primary antibodies used for the immunohistochemical detection of macrophages markers.

1624 1625 1626	Specifiticy (clone)	Type of antibody	Commercial origin	Fixative	Blocking solution	Dilution	Antigen retrieval
1627 1628 1629	Anti-PRRSV (clone SDOW17)	mAb	Rural Technologies Inc., Brookings, SD, USA	Formalin	BSA 1%	1:500	Protease Type XIV ^a
1630 1631	TUNEL	N.A.	Roche Diagnostics, Indianapolis, USA	Formalin	N.A.	N.A.	Proteinase K ^b
1632 1633	Anti-CD172a (BA1C11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
1634 1635	Anti-CD163 (2A10/11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
1636 1637	Anti-CD107a (4E9/11)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
1638 1639	Anti-BA4D5 (BA4D5)	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
1641 164 867 1643 1644 1645	N.A.: Not applicable, "Protease	Type XIV (Sign	na-Aldrich): 8 min at 57 °C in water bath, °Pro	iternase K (Ko	ene). 15 min at s	or "C in neat i	ncubator.
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Table 2. Histopathology grading of the thymus of piglets from each experimental group and average number of tingible body macrophages and

TUNEL positive cells	(expressed as the mean \pm SD).
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		CON (35 dpi)	PR11 (35 dpi)	PR40 (35 dpi)	PR11 (10-14 dpi)	PR40 (10-14 dpi)	VAC-C	VAC-PR40
Grades								
	0	2/3	-	1/3	-	-	-	3/5
	Ι	-	-	-	-	-	-	1/5
	II	1/3	1/1	2/3	-	-	2/2	1/5
	III	-	-	-	-	-	-	-
	IV	-	-	-	3/3	2/2	-	-
Tingible body macrophages		9.87 ± 1.79	7	8.87 ± 6.82	ND^*	ND^*	13.1 ± 8.63	10.4 ± 1.79
TUNEL positive cells		54.97 ± 63.40	67.91	43.53 ± 31.07	ND^*	ND^*	53.82 ± 40.20	53.18 ± 34.59

ND*: Not determined due to extensive cell death of thymocytes in the cortex.



*humanely killing of animals due to animal welfare issues







