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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.

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,

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AUT15-33 and PR40/2014 strain) and 2 (BOR59 strain) (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017).

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

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.

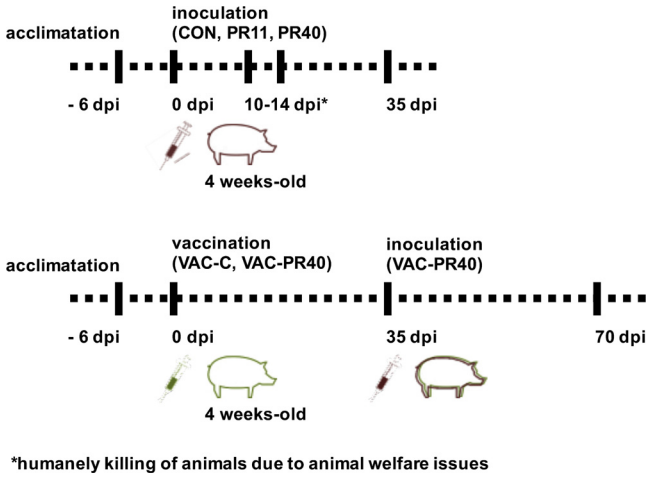

Fig. 1. Experimental design.

2. Materials and methods

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 al. (2017): (i) PR40 group (PR40), with 5 pigs inoculated intra-nasally (IN) with 2 ml, containing 10^5 TCD_{50} of PRRSV1 PR40/2014, per pig; (ii) PR11 group (PR11), with 4 piglets inoculated IN with 2 ml, containing 10^5 TCD_{50} of PRRSV1 PR11/2014, per pig; and, (iii) Control group (C), with 3 animals inoculated IN with sterile medium (mock/negative control). In addition, two different vaccinated groups, described in Canelli et al. (2018), were included: (i) VAC-C: 2 pigs were IM-vaccinated against PRRSV at 4 weeks of age (Porcilis® PRRS, MSD Animal Health; DV strain; vaccine batch A208AD01) and left uninfected; (ii) VAC-PR40: 6 pigs were IM-vaccinated against PRRSV and IN infected with PR40 at 35 days post-vaccination (dpv) (day 0 post-inoculation, dpi) (Fig. 1).

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

2.2. Histopathology and grading of thymus

Four μm 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/mm^2 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/mm^2 within the thymic cortex; (iv) Grade III, focal to multifocal blurring of normal corticomedullary demarcation, increase

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

Specifitiy (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

N.A.: Not applicable.
[a] Protease Type XIV (Sigma-Aldrich): 8 min at 37 °C in water bath.
[b] Proteinase K (Roche): 15 min at 37 °C in heat incubator.

of the stroma, occasional increase in the number of lymphocytes, mast and plasma cells and ≥ 16 tingible body macrophages/mm^2, 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 non-overlapping, consecutively selected high magnification fields of 0.2 mm^2. Results were expressed as number of cells per mm^2.

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% H_2O_2 solution in methanol for 30 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 h 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 mm^2. The expression of all markers was manually counted and the results were expressed as the number of cells per mm^2.

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.

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 and B). 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

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 ± SD).

	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
I	–	–	–	–	–	–	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.

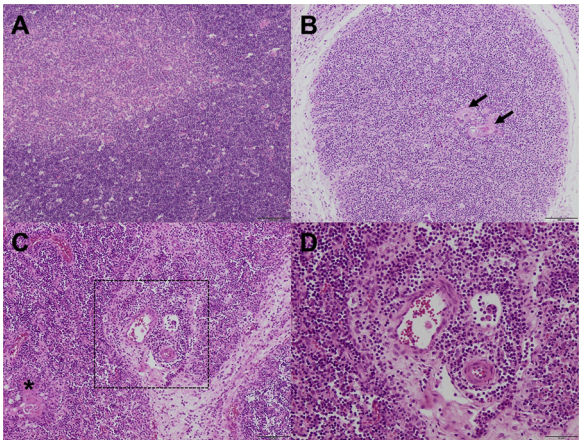

Fig. 2. Representative photomicrographs of the thymus from a control pig (A; Haematoxylin-eosin, HE; Bar, 100 µm), a PR11-infected pig dead at 10–14 dpi with a strong disappearance of the corticomedullary boundary (B; HE; Bar, 100 µm; Hassall's corpuscles are identified with two black arrows), and a 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 µm; 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 µm).

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 and C).

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 PR40-inoculated animals at 35 dpi.

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).

3.4. CD172a positive cells were increased in the thymic cortex and stroma of PR11- and PR40-infected 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 and D). 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).

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).

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)

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-4 F).

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 and D).

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 10–14 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

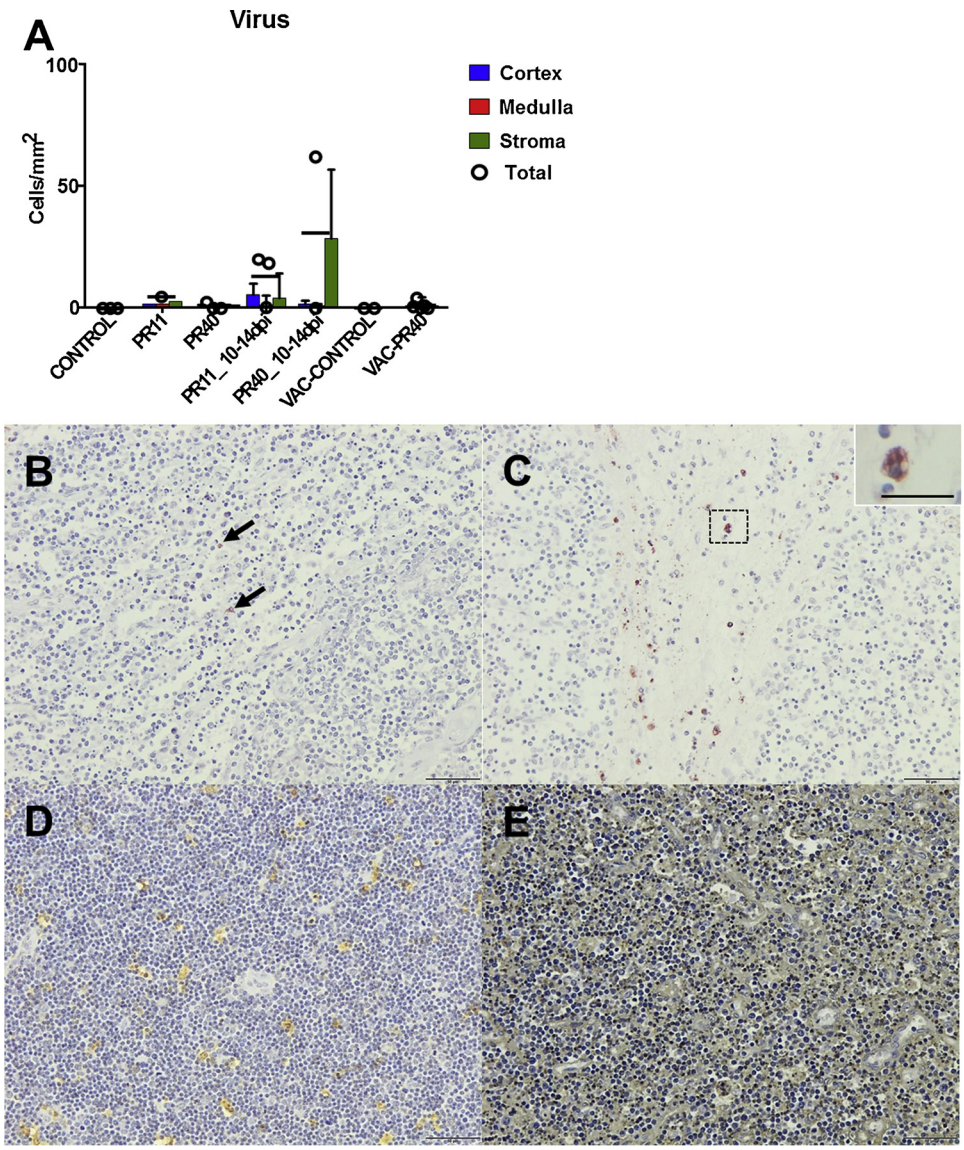

Fig. 3. (A) Counts for PRRSV N protein positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (B) N protein positive cells (arrows) in the thymic cortex of a PR11-infected pig that died at 10–14 dpi (IHC, Bar, 50 μm). (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, 50 μm). *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, 20 μm). (D) TUNEL labelling of tingible body macrophages in the cortex of the thymus of a control animal (TUNEL, Bar, 50 μm). (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, 50 μm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cells were scattered (Fig. 5E and F). 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).

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, 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 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

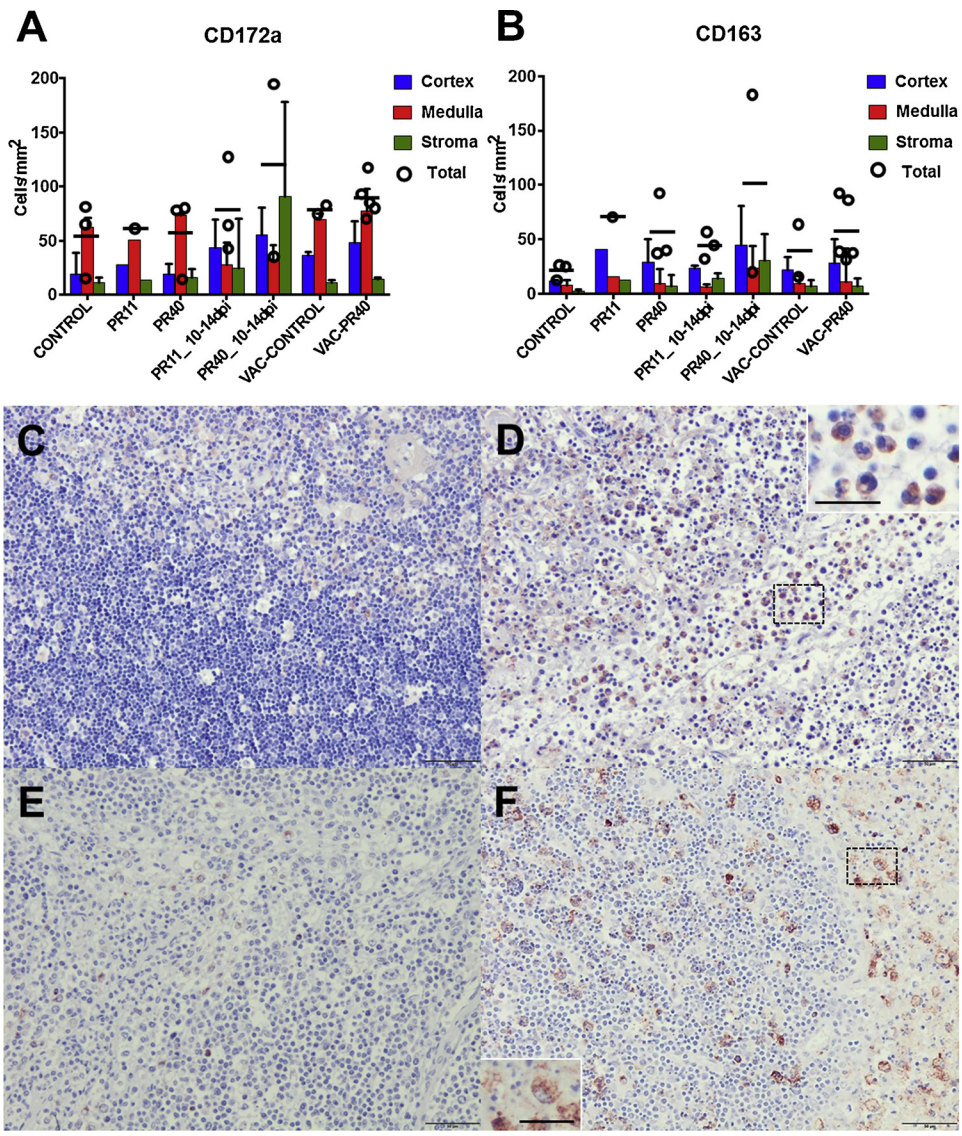

Fig. 4. Counts for CD172a (A) and CD163 (B) positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (C) CD172a positive cells in the thymic medulla of a PR40-infected animal and killed at 35 dpi (IHC, Bar, 50 μm). (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, 50 μm). *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, 20 μm). (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, 50 μm). (F) Numerous macrophages and tingible body macrophages within the thymic cortex and stroma of a PR40-infected pig at 10–14 dpi (IHC, Bar, 50 μm). *Inset*, detail of the cytoplasmic staining against CD163 in macrophages from the stroma of a PR40-infected pig at 10–14 dpi (IHC, Bar, 20 μm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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; Domenech et al., 2003; Perez et al., 2008). CD172a is one of the markers most commonly used and identifies myeloid cells from precursor stages until cellular differentiation (Summerfield and McCullough, 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

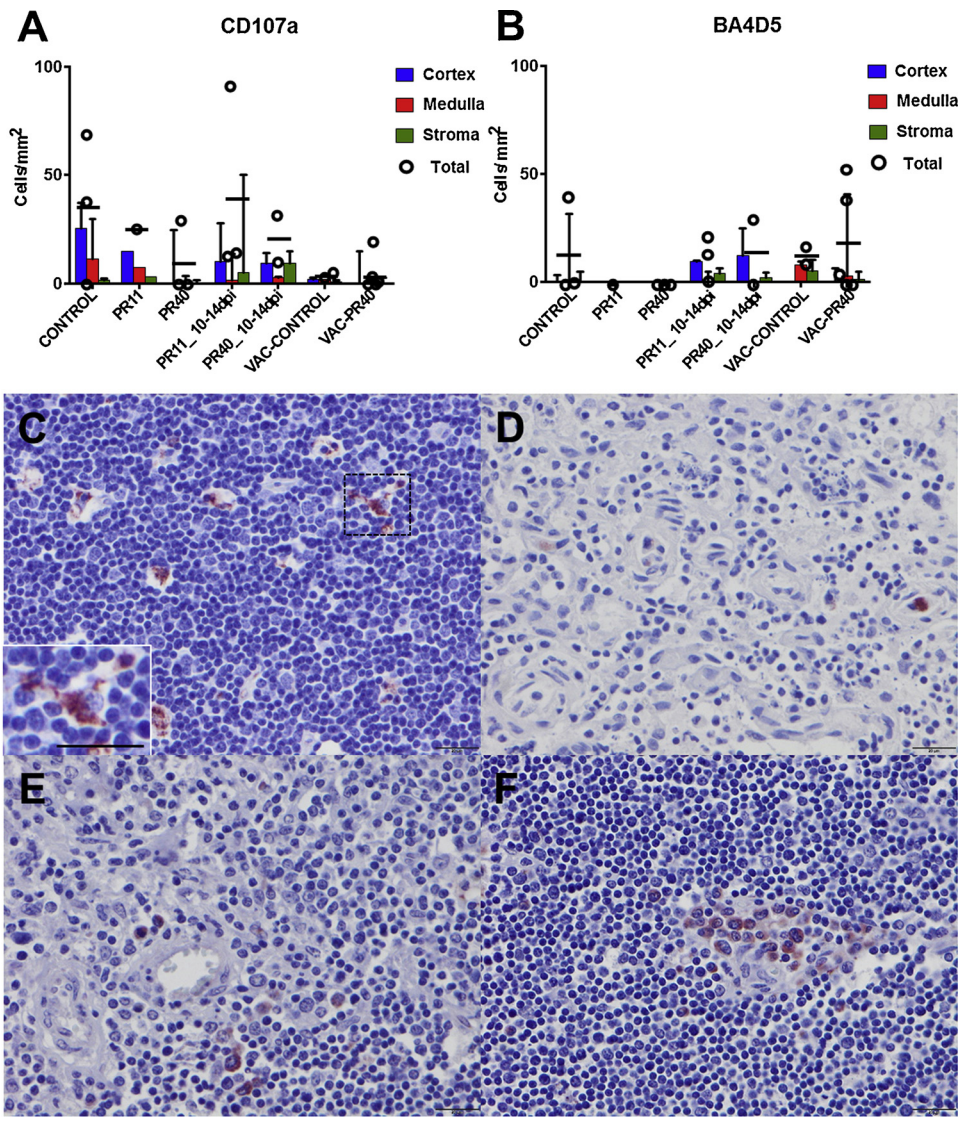

Fig. 5. Counts for CD107a (A) and BA4D5 (B) positive cells in the thymic cortex (blue column), medulla (red column), stroma (green column) and total (the empty circles represent individual values; the mean is showed as a black solid line). (C) Numerous tingible body macrophages immunolabelled against CD107a in the thymic cortex of a control animal at 35 dpi (IHC, Bar, 20 µm). *Inset*, detail of the cytoplasmic staining against CD107a in a macrophage with cytoplasmic prolongations in the thymic cortex from the same animal (IHC, Bar, 20 µm). (D) Scattered CD107a positive cells in the cortex and medulla of the thymus from a PR40-infected animal that died at 10–14 dpi (IHC, Bar, 20 µm). (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, 20 µm). (F) Higher magnification of another field of the thymus from the same animal with a marked perivascular infiltrate by BA4D5 positive cells (IHC, Bar, 20 µm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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 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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