Veterinary Microbiology 235 (2019) 101–109

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells

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ARTICLE INFO

Keywords:
PRRSV-1
Lena
CD163
BALF
PAMs

ABSTRACT

Highly virulent porcine reproductive and respiratory syndrome virus (PRRSV) strains have increasingly overwhelmed Asia and Europe in recent years. This study aims to compare the clinical signs, gross and microscopic findings as well as the expression of CD163 within live pulmonary alveolar macrophages (PAMs) from bronchoalveolar lavage fluid (BALF) of pigs experimentally infected with two PRRSV strains of different virulence. Pigs were infected with either a subtype 1 PRRSV-1 3249 strain or a subtype 3 PRRSV-1 Lena strain and consecutively euthanized at 1, 3, 6, 8 and 13 days post-inoculation. Clinical signs were reported daily and BALF and lung tissue samples were collected at the different time-points and accordingly processed for their analysis. Pigs infected with Lena strain exhibited greater clinical signs as well as gross and microscopic lung scores compared to 3249-infected pigs. A decreased frequency of PAMs from BALF was observed early in pigs infected with Lena strain. Moreover, the frequency and median fluorescence intensity (MFI) of CD163 within PAMs were much lower in Lena-infected pigs than in 3249-infected pigs. This downregulation in CD163 was also observed in lung sections after the assessment of macrophages expressing CD163 by means of immunohistochemistry. This outcome may result from the effect of PRRSV replication, PRRSV-induced inflammation, the influx of immature macrophages to restore lung homeostasis and/or the evidence of $CD163^{low}$ cells after $CD163^{+}$ cells decrease in BALF.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is still a significant problem for the global swine industry. Twenty years after the first description of PRRS virus (PRRSV) (Wensvoort et al., 1991; Benfield et al., 1992; Collins et al., 1992), a collection of severe outbreaks, by the so-called highly pathogenic-PRRSV (HP-PRRSV) strains, overwhelmed China and Southeast Asia in 2006 (Tian et al., 2007; Tong et al., 2007; Feng et al., 2008; Zhou et al., 2008). Shortly afterwards, PRRSV strains of increased virulence, although not comparable with the ones reported in Asia, emerged in Belarus, Belgium, Austria and Italy between 2007 and 2015 (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017).

Because of the high genetic and antigenic differences, PRRSV-1 and PRRSV-2 species have been recently renamed as *Betaarterivirus suid 1* (formerly genotype 1) and *Betaarterivirus suid 2* (formerly genotype 2) with different subtypes or lineages within them, respectively (Shi et al., 2010; Stoian and Rowland, 2019). In case of PRRSV-1, the phylogenetic diversity is quite broad, especially in Central Eastern Europe, where the transboundary movement of infected animals has contributed to the wide viral diversity (Balka et al., 2018). Up to now, four subtypes are described: Pan-European subtype 1, Eastern European subtypes 2 and 3 (Stadejek et al., 2006, 2008) and subtype 4 (Stadejek et al., 2013). Traditionally, virulent PRRSV-1 strains were restricted to subtype 3 strains (Lena strain -prototype- [Karniychuk et al., 2010] and SU1-Bel strain [Morgan et al., 2013]); however, strains with similar characteristics have been identified within subtypes 1 (13V091 strain [Frydas et al., 2015], AUT15-33 strain [Sinn et al., 2016] and PR40/2014 [Canelli et al., 2017]) and 2 (BOR59 strain [Stadejek et al., 2017]) in recent years, being the hallmark between them a discontinuous deletion of amino acids in nonstructural protein 2 (Van Doorsselaere et al.,

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https://doi.org/10.1016/j.vetmic.2019.06.011
Received 1 May 2019; Received in revised form 6 June 2019; Accepted 12 June 2019

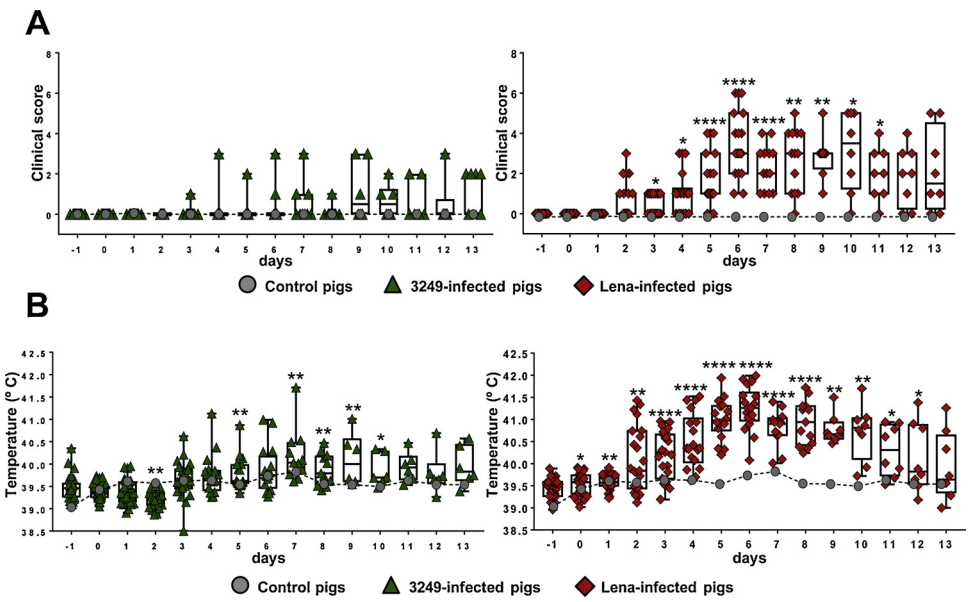

Fig. 1. Clinical findings. Pigs were monitored daily from 1 day prior to infection until 13 days PI for the appearance of clinical signs and pyrexia. Box plots show clinical score (A) and rectal temperature (B) for 3249-infected pigs (green triangles) and Lena-infected pigs (red diamonds). Gray circles indicate the mean average of pigs from the control group. Significant differences between 3249 vs. control group and Lena vs. control group are indicated (* = $P <$ 0.05; ** = $P <$ 0.01; **** = $P <$ 0.0001).

2012; Frydas et al., 2015; Canelli et al., 2017).

Virulent PRRSV-1 strains can induce high fever and cause increased mortality in both field and experimental conditions. Moreover, co-infections with bacterial pathogens which exacerbate clinical symptoms, especially in growing pigs, have been also reported (Karniychuk et al., 2010; Sinn et al., 2016; Canelli et al., 2017; Renson et al., 2017).

Previous studies have showed a stronger inflammatory response in lungs in the early phases of infection for virulent PRRSV-1 compared to animals infected with low-to-moderate virulent isolates (Amarilla et al., 2015). Overall, higher viral loads in serum, lower frequencies of IFN-gamma secreting cells as well as PRRSV neutralizing antibodies have been reported, even though results were contradictory among studies (Karniychuk et al., 2010; Morgan et al., 2013; Weesendorp et al., 2013a, 2014; Frydas et al., 2015; Canelli et al., 2017; Renson et al., 2017; Stadejek et al., 2017). However, a minor proportion of studies have evaluated the impact of the infection with PRRSV strains of high virulence on its target cell and related molecules, such as CD163.

In vivo, PRRSV productively replicates in differentiated macrophages, being porcine alveolar macrophages (PAMs) the major target cells (Duan et al., 1997). CD163 has been identified as the essential receptor for PRRSV infection, playing an important role in viral uncoating and genome release (Van Gorp et al., 2008; Whitworth et al., 2016; Burkard et al., 2018). After infection with highly virulent PRRSV, a decrease in the frequency of mature macrophages from bronchoalveolar lavage fluid (BALF) and its phagocytic activity have been reported (Weesendorp et al., 2013b; Renson et al., 2017).

This study sets out to analyze the differences between the virulent PRRSV-1 subtype 3 Lena strain and the low virulent PRRSV-1 subtype 1 3249 strain, focusing on the clinical signs, lesional pattern as well as CD163 expression in live cells isolated from BALF.

2. Materials and methods

2.1. Animals and experimental design

A total of 70, male and female, four-week-old Landrace x Large White piglets were randomly distributed in three different groups and housed in three separate pens at the Biosafety Level 3 containment facilities of Centre de Recerca en Sanitat Animal (CReSA-IRTA, Cerdanyola del Vallès, Barcelona, Spain). Pigs were obtained from a high health historically PRRSV-negative farm and confirmed as negative for PRRSV, porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (IDEXX PRRS X3 Ab Test, IDEXX Laboratorios, S.L., Barcelona, Spain; in-house PCR for porcine circovirus type 2 and *Mycoplasma hyopneumoniae* detection based on previous publications - Mattsson et al., 1995; Sibila et al., 2004). After a 7-day acclimation period, pigs were intranasally inoculated as follows: (i) 3249 group, 26 pigs were inoculated with 2 ml (1.0 ml/nostril making use of MAD Nasal™ Intranasal Mucosal Atomization Device, Teleflex, Alcalá de Henares, Madrid, Spain) of 1×10^5 $TCID_{50}$ of the PRRSV-1 subtype 1 3249 strain (Gimeno et al., 2011); (ii) Lena group, 28 pigs were inoculated at same conditions with the PRRSV-1 subtype 3 Lena strain (Karniychuk et al., 2010); and (iii) control group, in which 16 pigs were inoculated with porcine alveolar macrophages cryolysate diluted in RPMI at same conditions. Three control pigs and 5 infected-pigs from each group were euthanized on days 1, 3, 6 and 8 post-inoculation (PI). At 13 days PI, 4 control pigs, 6 pigs from the 3249 group and 8 pigs from the Lena group were humanely killed. This experiment was performed according to the guidelines of the European Union (Directive 2010/63/EU) and approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

2.2. Clinical signs, gross pathology and histopathology of the lung

Pigs were monitored daily from one day prior to inoculation until the end of the study. Monitoring included measurement of rectal temperatures and development of clinical signs, such as abnormal behavior, anorexia, dyspnea and cough.

At necropsy, gross lung lesions were recorded by the same pathologist as described elsewhere (Halbur et al., 1998). Briefly, a percentage reflecting the approximate volume frequency of affected lung parenchyma with respect to the entire lung was assigned to each lung lobe. The sum of all frequencies was an estimation of the percentage of affected lung in a 0–100 scale. After that, the left lung was used to perform bronchoalveolar lavages. Thus, after the complete occlusion of the right primary bronchus by a mosquito forceps, the left lung was flushed with 100 ml of sterile PBS (ThermoFisher Scientific, Waltham, Massachusetts, USA). BALF was spun down (350 x g, 10 minutes [min], 4 °C), cell pellets were washed twice with PBS at same conditions, counted and finally resuspended in PBS containing 3% of fetal bovine serum (FBS). Samples from all lung lobes of the right lung were collected and fixed in 10% neutral buffered formalin for histopathological and immunohistochemical investigation.

For the histopathological examination, four-micron tissue sections were cut and stained with hematoxylin and eosin and blindly evaluated by two pathologists as previously described by Halbur et al. (1998) for

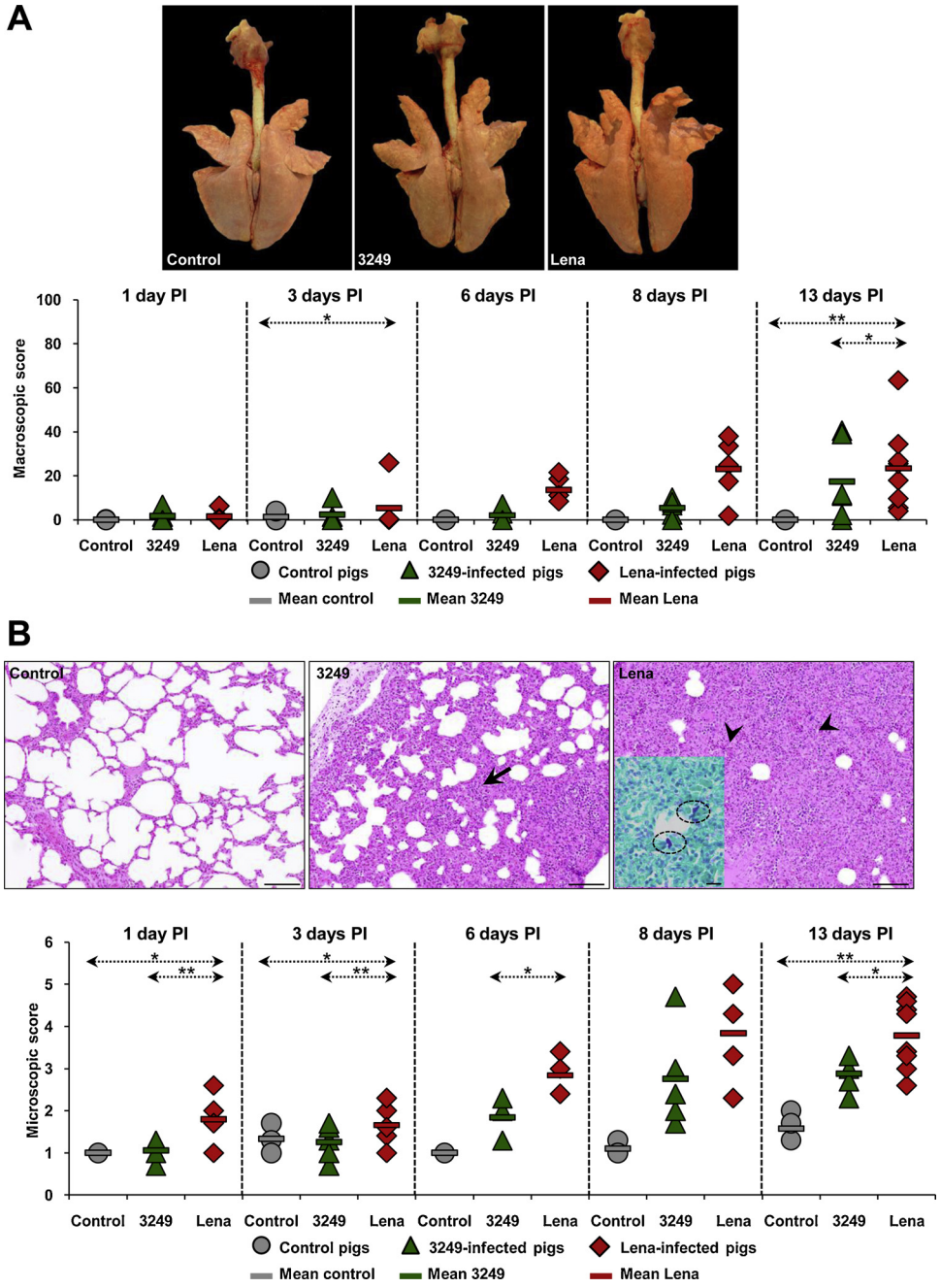

Fig. 2. Gross and microscopic lung findings. At necropsy, gross lung lesions were recorded and lung samples were collected and routinely processed for histopathology examination. (A) Pictures show the macroscopic lung appearance of a representative animal from the control (left), 3249 (middle) and Lena (right) group at 6 days PI. The scatter diagram show the macroscopic score (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. (B) Representative microscopic pictures of the lung of a control (left), 3249-infected (middle) and Lena-infected pig (right) at 6 days PI (hematoxylin and eosin; bars, 100 µm). Black arrow indicates the thickening of the alveolar septa. Arrow heads point to neutrophils and cell debris filling the alveoli. The inset shows clumps of chromatin stained red-purple for Feulgen technique (black dashed circles; bar, 20 µm). The scatter diagram displays the microscopic score (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. (A + B) Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated (* = $P < 0.05$; ** = $P < 0.01$).

the diagnosis of interstitial pneumonia. Accordingly: 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia. In addition, following the same pattern, a similar score was developed considering the diagnosis of suppurative bronchopneumonia. Hence: 0, no microscopic lesions; 1, mild bronchopneumonia; 2, moderate multifocal bronchopneumonia; 3, moderate diffuse bronchopneumonia; and 4, severe bronchopneumonia. The final score comprised the sum of both, the interstitial pneumonia score and the bronchopneumonia score, being 8 points the maximum possible score. The Feulgen technique was also performed to demonstrate the presence of clumps of free chromatin within the alveoli (Feulgen and Rossenbeck, 1924).

2.3. Flow cytometry (FCM) staining and analysis

Freshly isolated cells from BALF were adjusted to 1.5×10^6 cells per sample in a final volume of 200 µl. Staining was performed in 96-well round-bottom plates. Firstly, cells were stained for CD163 (clone 2A10/11, IgG1, 10 µg/ml; Bio-Rad Laboratories, S.A., Alcobendas, Madrid, Spain). Thereafter, cells were washed twice with 200 µl of PBS and primary antibody was followed by a second incubation step with a fluorochrome-labelled isotype-specific secondary antibody (Alexa Fluor™ 647 goat anti-mouse IgG1, 7 µg/ml; Invitrogen™, Carlsbad, CA, USA) in combination with Live/Dead™ Fixable Aqua Dead Cell Stain (Invitrogen™). Following surface labeling, cells were fixed and permeabilized with methanol overnight at -20 °C (VWR® International, Llinars del Vallès, Barcelona, Spain). Then, cells were incubated with anti-PRRSV-*N*-protein monoclonal antibody (clone 1CH5, IgG2b, 10 µg/ml; INGENASA, Madrid, Spain) and, after two washing steps, a secondary antibody was included (Alexa Fluor™ 488 goat anti-mouse IgG2b, 5 µg/ml; Invitrogen™). All incubation steps, except specified, took place for 30 min in the fridge. For all stainings, isotype-matched control samples were prepared.

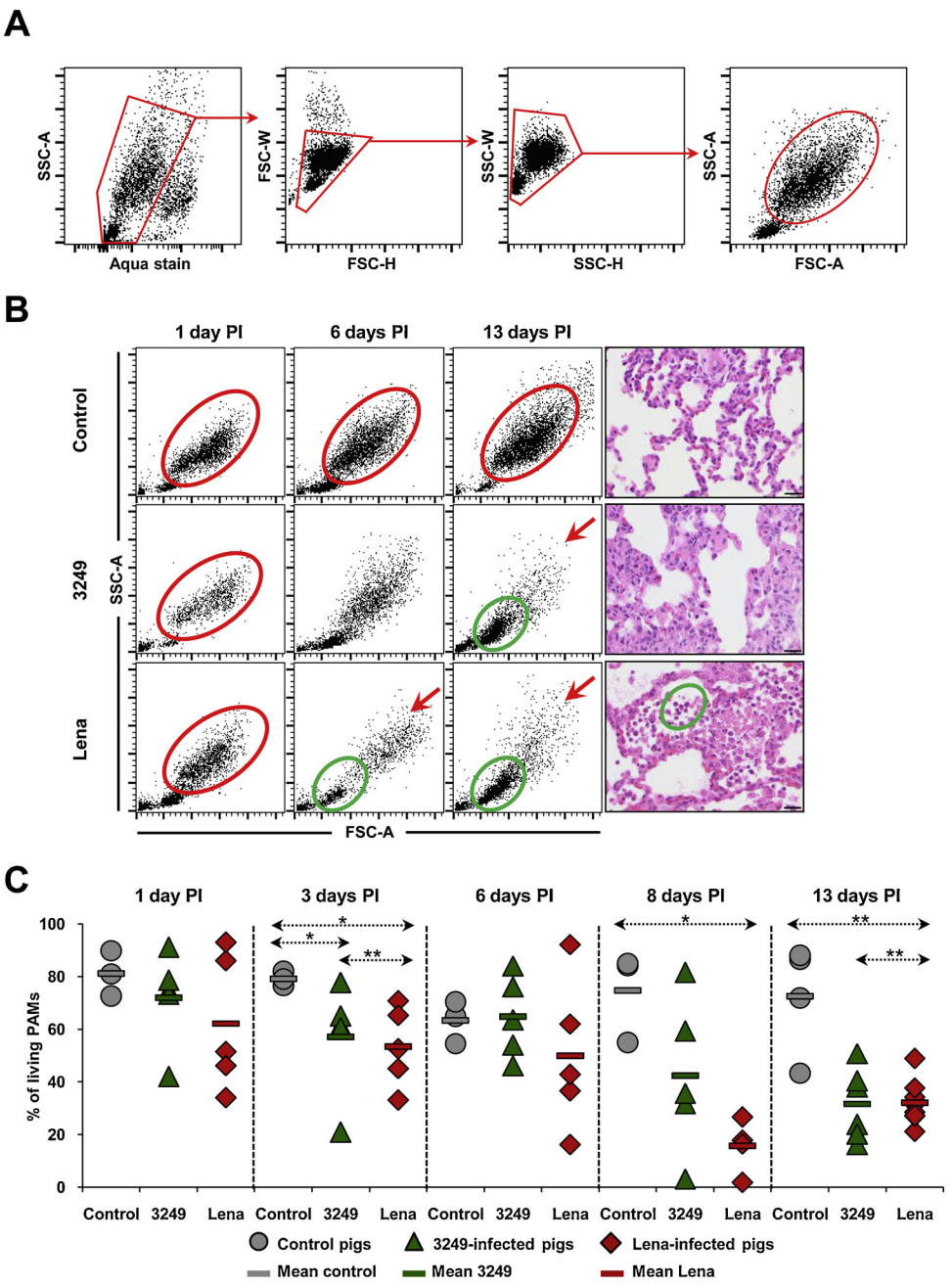

Fig. 3. Gating strategy and frequency of live PAMs. BALF cells were stained to determine the viability of freshly isolated PAMs by FCM. (A) Gating strategy followed to analyze potential PAMs. Sequentially, exclusion of dead cells (Aqua stain vs. SSC-A) and doublets (FSC-H vs. FSC-W; SSC-H vs. SSC-W) were performed to identify living PAMs (red circle). (B) Black dot plots (FSC-A vs. SSC-A) from a representative pig of the control, 3249 and Lena-infected group at 1, 6 and 13 days PI are showed. Red circles indicate living potential PAMs according to light scatter properties (size and granularity). Red arrows refer to the decrease of the above mentioned subset in 3249-infected and Lena-infected pigs. Green circles indicate a mixture of neutrophils, monocytes and, in a lesser extent, lymphocytes, according to light scatter properties. Microscopic pictures of hematoxylin and eosin for each representative animal at 13 days PI are included to further support FCM findings. Bars, 20 µm. (C) Scatter diagram shows the frequency of living potential PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are highlighted (* = $P < 0.05$; ** = $P < 0.01$).

FCM analysis was performed on a FACSCanto II (BD Biosciences, New Jersey, USA) flow cytometer equipped with three lasers (405, 488 and 633 nm). Between 5×10^5 and 1×10^6 cells per sample were recorded. By making use of FlowJo software version 10 (FLOWJO LLC, Ashland, Oregon, USA), cells were gated according to light scatter properties (FSC-A versus SSC-A) and subjected to doublet (FSC-H versus FSC-W and SSC-H versus SSC-W) and dead cell discrimination and further analyzed for the expression of CD163 and PRRSV-*N*-protein.

2.4. Immunohistochemistry in lung sections

Four µm formalin-fixed sections from lung were dewaxed in xylene for 30 min and rehydrated in descending grades of alcohol followed by endogenous peroxidase inhibition with 3% H_2O_2 solution in methanol for 30 min. For antigen retrieval, enzymatic digestion with type XIV protease (Sigma-Aldrich, USA) at 37 °C for 8 min in water bath was used for PRRSV, in case of CD163, high temperature citrate buffer pH 3.2 (microwave 420 W, 10 min) was used. Thereafter, sections were washed with PBS (pH 7.4, 0.01 M) and incubated for 1 h at room temperature with 100 µl of bovine serum albumin 2% (BSA) (Sigma-Aldrich, USA) as blocking solution in humid chamber. Monoclonal primary antibodies against PRRSV (diluted 1 in 500 in BSA; clone SDOW17; Rural Technologies, Brookings, SD, USA) and CD163 (undiluted; clone 2A10/11; kindly provided by Dr. J. Domínguez, INIA, Madrid, Spain) were incubated overnight at 4 °C in a humid chamber. Biotinylated secondary antibody was incubated for 30 min at room temperature. Then, avidin-biotin-peroxidase complex (ABC Vector Elite, Vector laboratories, USA) was applied for 1 h at room temperature. Labeling was revealed by application of NovaRED™ substrate kit (Vector Laboratories, USA). Revealed sections were counterstained with Harris's haematoxylin, dehydrated in graded ascending ethylic alcohol and xylene and, finally, mounted. Antibody specificity was verified by substituting the primary antibody by isotype matched reagents of irrelevant specificity. Negative controls consisting on replacement of primary antibody by BSA

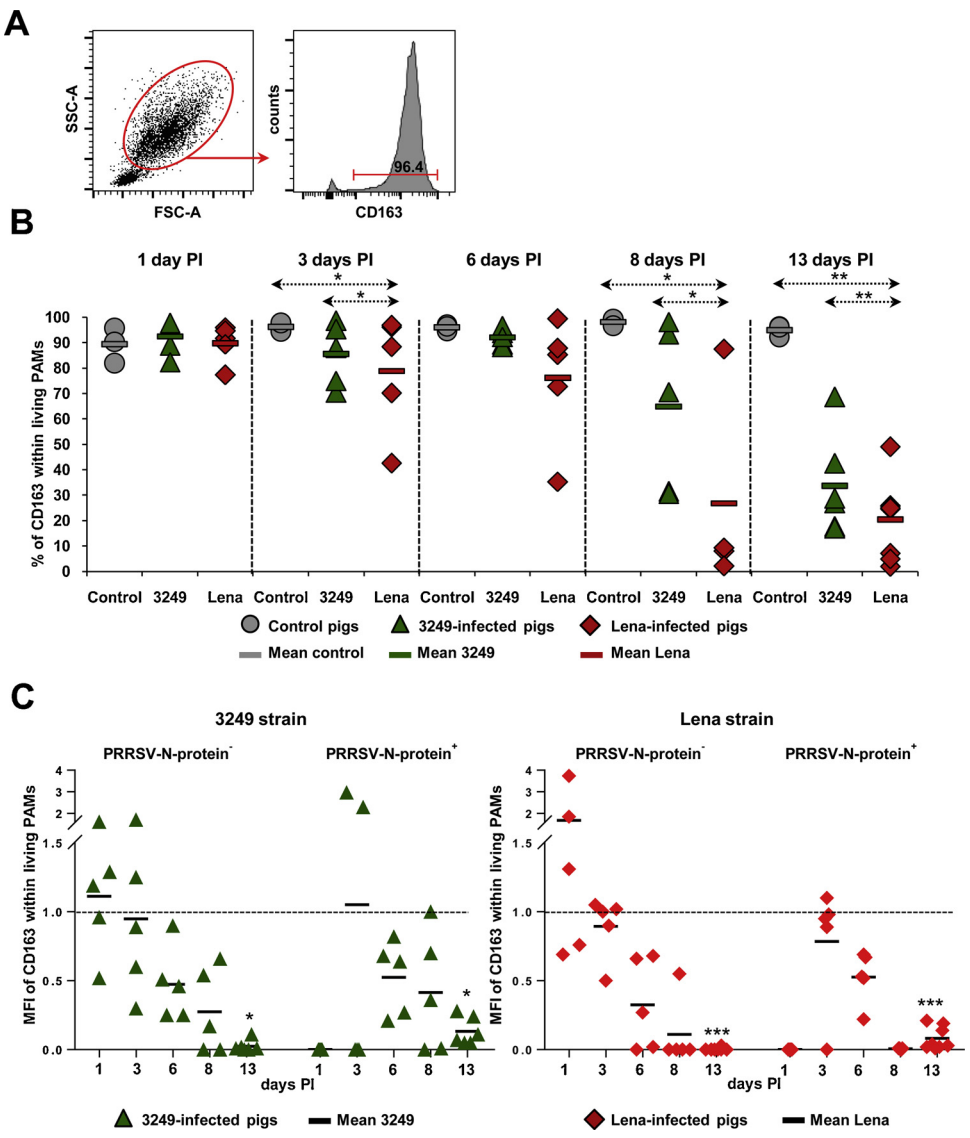

Fig. 4. Frequency and MFI of live CD163$^+$ PAMs. Freshly isolated BALF cells from control and infected pigs were stained and analyzed for the expression of CD163 by FCM. (A) CD163 expression within living PAMs (red circle) was analyzed as indicated in sub Fig. 4A. (B) The scatter diagram shows the frequency of CD163 living PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated (* = $P <$ 0.05; ** = $P <$ 0.01). (C) Scatter diagrams show the ratio of the MFI of CD163 (y-axis) within PRRSV-*N*-protein$^-$ and PRRSV-*N*-protein$^+$ live PAMs of 3249-infected (green triangles) and Lena-infected pigs (red diamonds) for each day PI (x-axis). MFI of control animals were set to 1 and represented as black dashed line. Statistical differences between PRRSV-*N*-protein$^-$ and PRRSV-*N*-protein$^+$ live PAMs are indicated (* = $P <$ 0.05; *** = $P <$ 0.001).

blocking solution were included in each assay to confirm the lack of non-specific bindings. Labeled cells were counted on 25 non-overlapping consecutive high-magnification power fields of 0.2 mm^2 (Olympus BX51, Olympus Iberia SAU, L'Hospitalet de Llobregat, Barcelona, Spain) and expressed as the number of cells per mm^2. Positive cells were identified as PAMs, interstitial macrophages or intravascular macrophages.

2.5. Statistical analysis

Statistical analyses were performed using SPSS Statistics version 17.0.1 (IBM, Armonk, NY, USA). Data was evaluated for normality distribution by the Shapiro-Wilk test. Subsequently, data sets were analyzed using the Mann-Whitney *U* non-parametric test. Accordingly, *P* values below 0.05 were considered statistically significant and in accordance indicated (* $P <$ 0.05; ** $P <$ 0.01; *** = $P <$ 0.001; **** = $P <$ 0.0001).

3. Results

3.1. PRRSV-infected pigs exhibited respiratory disease consisting of interstitial pneumonia and bronchopneumonia

PRRSV-infected pigs showed respiratory signs; however, differences between the infected groups were patent. Animals infected with strain 3249 exhibited mild dyspnea, especially visible from 9 days PI until the end of the study (5 pigs out of 26); in contrast, most animals infected with Lena strain (22 pigs out of 28) displayed severe dyspnea with tachypnea, apathy, lethargy, even with prostration, and anorexia from the beginning of the study (2 days PI) (Fig. 1A). As shown in Fig. 1B, these clinical signs were accompanied by pyrexia in both infected groups, but the difference was particularly noteworthy in pigs from the Lena group, with highest values (> 40.5 °C) from 2 to 13 days PI.

Grossly, lungs from infected pigs showed tan-mottled areas, atelectasis, rubbery consistency and areas of consolidation (Fig. 2A). Macroscopic scores of 3249-infected pigs gradually increased alongside the study, with the highest score at 13 days PI due to the presence of consolidation, although remarkable individual differences between pigs took place. Lena-infected pigs reached severe macroscopic scores, mainly due to the presence of consolidation as well; however, the presentation of the lesions was earlier (from 6 days PI) and stronger than in 3249-infected pigs (Fig. 2A, scatter diagram).

Histopathological lesions consisted of mild to moderate interstitial pneumonia characterized by thickening of the alveolar walls by infiltrating lymphocytes and macrophages in a lesser extent (Fig. 2B). Syncytial cells were sporadically observed. Of note, lung sections from pigs at 13 days PI for 3249 group and from 6 to 13 days PI for Lena group, showed neutrophils, cell debris and mucus filling the bronchial,

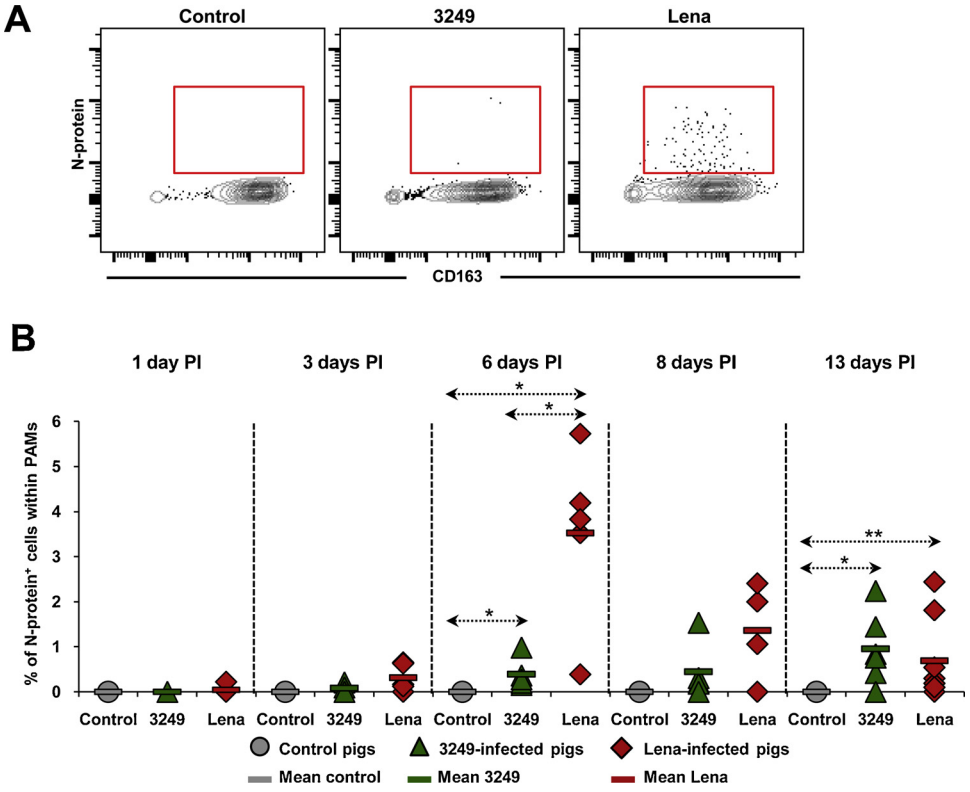

Fig. 5. Frequency of PRRSV-infected PAMs. BALF cells were fixed and permeabilized with methanol and stained for the identification of PRRSV$^+$ cells. (A) Contour plots from a representative pig of the control (left), 3249-infected (middle) and Lena-infected (right) group at 6 days PI are showed. (B) Scatter diagram exhibits the frequency of PRRSV-*N*-protein$^+$ cells within PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated (* = $P < 0.05$; ** = $P < 0.01$).

bronchiolar and alveolar spaces, accompanied by secondary atelectasis. Edema of the interlobular septa as well as dilation of lymphatic blood vessels was also observed, confirming microscopically the co-existence of suppurative bronchopneumonia. In rare cases, the pleura was also affected and covered by fibrinous material. Additionally, clumps of free chromatin, demonstrated by the Feulgen technique, within the alveoli were identified (Fig. 2B, inset). Histopathology scores of individual pigs are showed in Fig. 2B. Findings were similar to that reported for gross pathology. Control animals did not show signs of disease throughout the study.

3.2. Downregulation and decrease of CD163$^+$ cells within live PAMs from BALF of PRRSV-infected pigs

CD163 expression was studied by FCM in cells isolated from BALF of control and PRRSV-infected pigs. In order to accurately analyze this molecule, only live cells from BALF were considered. Thereafter, live cells were subjected to doublet discrimination and further analyzed according to light and scatter properties. Data of one representative animal is showed in Fig. 3A. A homogeneous and stable subset of cells, compatible with PAMs because of size and granularity properties, was identified in all control animals alongside the study (Fig. 3B, red circle). In contrast, in both infected groups, this subset proportionately decreased throughout the study (Fig. 3B, red arrows and C). In the case of cells isolated from Lena-infected pigs, this drop occurred earlier (3 days PI vs. 8 days PI in 3249 group) and was more pronounced, reaching on average only ˜16% of live PAMs. Additionally, another population compatible with a mixture of neutrophils, monocytes and, to a lesser extent, lymphocytes was identified (Fig. 3B, green circle). This population of cells was clearly observed in cells isolated from BALF at 13 days PI in 3249-infected pigs and from 6 days PI onwards in Lena-infected animals.

Next, as shown in Fig. 4A, the frequency of CD163$^+$ cells within live PAMs was analyzed by FCM. CD163$^+$ cells remained stable in control animals (average > 90%) throughout the experiment (Fig. 4B). Contrarily, a decrease in the proportion of CD163$^+$ cells took place from 8 days PI onwards in 3249-infected pigs, even though a high degree of variation was observed between individuals. The onset of this drop appeared earlier (from 3 days PI) and stronger in Lena-infected pigs, where four out of five animals had less than 10% of live CD163$^+$ PAMs at 8 days PI (Fig. 4B). Moreover, as illustrated in Fig. 4C, live PAMs for each individual animal were divided into PRRSV-*N*-protein$^-$ and PRRSV-*N*-protein$^+$ and the Median Fluorescence Intensity (MFI) of CD163 was analyzed for each subset of cells. MFI of control animals was set to 1 and fold-increase or fold-decrease were compared. In general, the average of the MFI of CD163 in infected pigs, independently of the strain, was lower than in control animals from 3 days PI onwards, reaching zero values at 13 days PI in most of the animals. At the end of the study, PRRSV-*N*-protein$^+$ PAMs showed a higher MFI than PRRSV-*N*-protein$^-$ of the same animal for both strains.

3.3. PRRSV-N-protein$^+$ cells were detected in BALF cells of infected pigs by FCM

The expression of PRRSV-*N*-protein was analyzed in live PAMs from BALF of control and PRRSV-infected pigs at the different time-points by means of FCM (Fig. 5A). The frequency of PRRSV-*N*-protein$^+$ cells in pigs of the 3249-group increased alongside the study, showing the highest values at 13 days PI (Fig. 5B). In the case of pigs from Lena group, infected-cells increased with time, reaching a peak at 6 days PI and gradually decreasing until the end of the study.

3.4. CD163$^+$ macrophages decreased in the lung of PRRSV-infected pigs whereas the number of PRRSV-N-protein$^+$ cells increased

Expression of scavenger receptor CD163 and PRRSV antigen were analyzed at strategic time points in the lung of control and infected pigs by immunohistochemistry, in order to match above described results for BALF. CD163 was mainly observed in the cell membrane and cytoplasm of PAMs and interstitial macrophages (Figs. 6A, 1–3), and occasionally labelling intravascular macrophages as well. Infected groups showed a decrease in the number of CD163$^+$ cells at 6 and 8 days PI

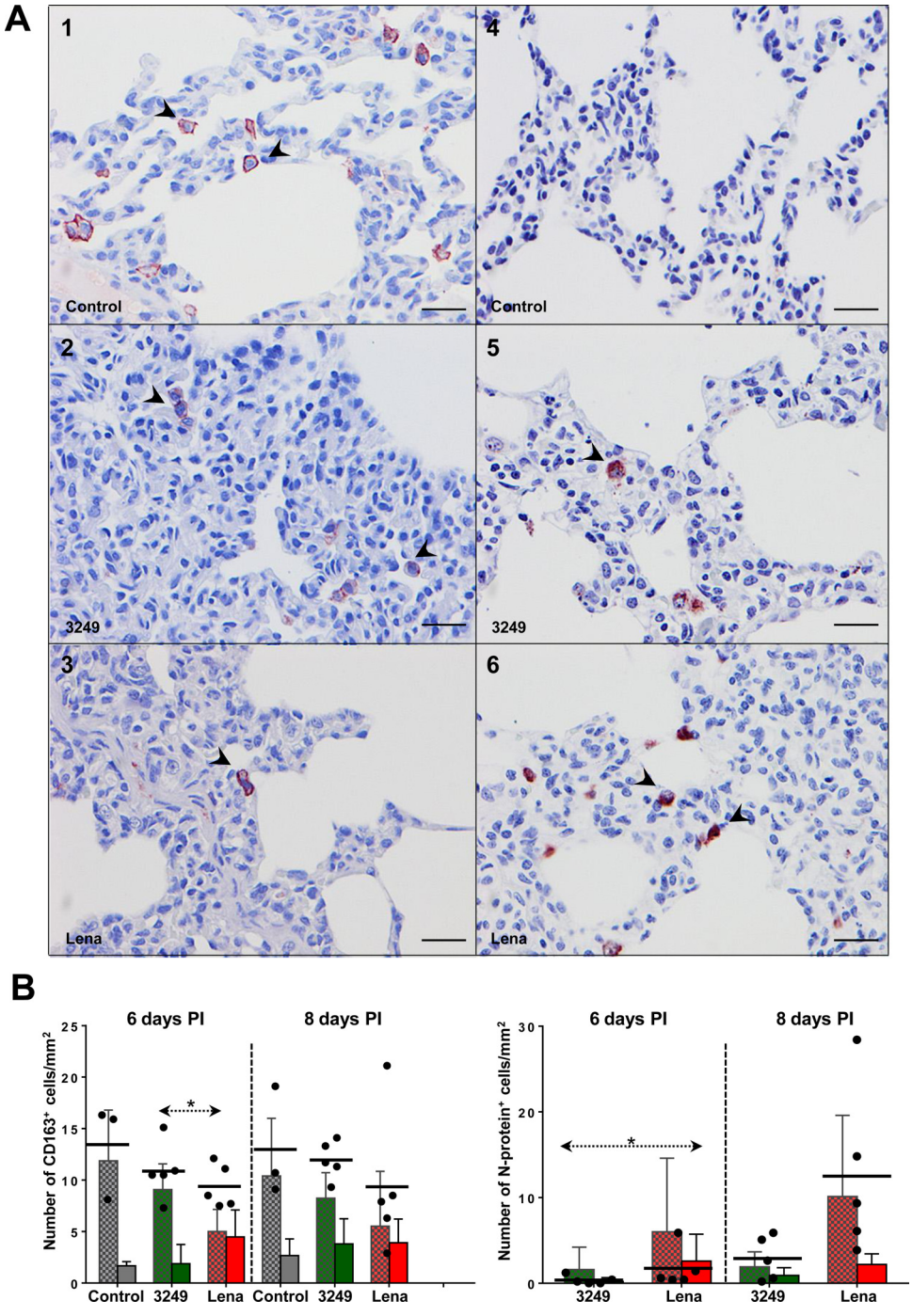

Fig. 6. Immunohistochemical expression of CD163 and PRRSV-*N*-protein in lung tissue at 6 and 8 days PI. Lung tissue sections were accordingly processed and immunolabeled against CD163 and PRRSV antigen. (A) Pictures numbered as 1, 2 and 3 show the expression of CD163 molecule in a control, 3249-infected and Lena-infected representative pig at 6 days PI, respectively. Pictures 4, 5 and 6 exhibit the expression of PRRSV-*N*-protein in a control, 3249-infected and Lena-infected representative pig at 6 days PI, respectively. Arrow heads indicate immunolabelled PAMs or septal macrophages (Bars, 20 µm). (B) Bar charts display the number of CD163$^+$ cells per mm^2 or the number of PRRSV-*N*-protein$^+$ cells per mm^2 at 6 and 8 days PI. Tiled light colored bars refer to the number of positive PAMs, whereas dark colored bars comprise the number of positive interstitial and intravascular macrophages. Black dots represent the total number of macrophages for each pig and black lines the average of the different subsets of macrophages. Statistical differences between groups are indicated (* = $P < 0.05$).

with respect to control group (Fig. 6B). Lena-infected piglets displayed a pronounced drop at both time points compared to piglets from control and 3249 groups. CD163$^+$ intravascular and interstitial macrophages slightly increased in both infected groups with respect to control animals.

With regard to the expression of PRRSV antigen in lungs, positive cells were detected in all piglets from both infected groups at 6 and 8 days PI. No PRRSV-*N*-protein labelling was found in the control group. Staining was mainly observed in alveolar macrophages and in interstitial and intravascular macrophages in a lesser extent (Fig. 6A, 4–6). Of note, clusters of PRRSV-*N*-protein$^+$ macrophages were observed in bronchopneumonic foci from Lena-infected piglets at 6 and 8 days PI. The number of PRRSV-*N*-protein$^+$ macrophages was higher at 8 days PI in 3249-infected pigs; this increase was stronger and earlier (6 days PI) in pigs belonging to the Lena group.

4. Discussion

The appearance of sudden and severe outbreaks caused by PRRSV strains of high virulence have increased in the last decade in China, Southeast Asia and Europe (Tian et al., 2007; Zhou et al., 2008; Karniychuk et al., 2010; Sinn et al., 2016). This fact has led to increase the consistent and continuous efforts of the scientific community to decipher the immunopathogenesis of PRRSV, especially by comparing the behavior of strains of different virulence (Morgan et al., 2013; Weesendorp et al., 2013a; Amarilla et al., 2015; Frydas et al., 2015; Canelli et al., 2017; Renson et al., 2017; Stadejek et al., 2017).

During the time course of our study, piglets infected with strain

Lena showed severe clinical signs and developed pyrexia. Gross lung lesions were observed in both PRRSV-infected groups; however, the highest lung scores were found in pigs belonging to Lena group, due to an earlier onset of the typical interstitial pneumonia as well as the complication with suppurative bronchopneumonia, as microscopically confirmed. Our results reproduce the key clinical and lesional features described in the literature for pigs infected with the Lena strain (Karniychuk et al., 2010; Weesendorp et al., 2014; Renson et al., 2017) and other virulent PRRSV strains (Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017). In all cases, as soon as 2–3 days PI, severe general and respiratory clinical signs together with pyrexia were reported. Moreover, secondary findings such as catarrhal to suppurative bronchopneumonia, fibrinous pleuropneumonia and fibrinous pericarditis, among others, were also described (Karniychuk et al., 2010; Sinn et al., 2016; Canelli et al., 2017; Renson et al., 2017).

Subsequently, we analyzed the quantity of live PAMs within the cellular fraction of BALF from control and infected pigs. The frequency of PAMs decreased alongside the study in both PRRSV-infected groups. However, this decrease was much more evident in Lena-infected pigs. Besides this population, another subset of cells compatible with a mixture of mainly neutrophils and some monocytes, according to light scatter properties and histopathology observations, became obvious at 6 and 13 days PI in Lena- and 3249-infected pigs, respectively. This precisely coincides with the onset of the bronchopneumonia for the different infected groups and would be consistent with the development of secondary bacterial infections.

In addition, we analyzed the expression of the surface receptor CD163 within live PAMs from BALF of PRRSV-infected pigs. This receptor is widely known for being the key player in PRRSV uncoating and genome release (Calvert et al., 2007; Van Gorp et al., 2008; Van Breedam et al., 2010). The frequency of CD163 in live PAMs decreased in both infected groups, but more evidently in Lena-infected compared to 3249-infected piglets at the end of the study. This data matched with the expression of CD163 in the lung at 6 and 8 days PI by immunohistochemistry. Together, there was a decreased proportion of live PAMs and a decrease in the frequency of $CD163^+$ cells within live PAMs in the BALF and lung section of infected animals compared to controls. Weesendorp et al. (2013b) reported similar results with a lower frequency of bone marrow-derived dendritic cells expressing CD163 *in vitro* and $CD163^+$ PAMs *in vivo* after the infection with the strain Lena at 7 days PI. Likewise, Renson et al. (2017) described a reduced frequency of mature macrophages of phenotype $SWC3^+SWC7^+SWC8^-$ in the BALF of piglets infected with Lena strain. The replication of PRRSV may be one of the causes for the decrease of this subset, although more determinants such as induction of cell death, already reported in the lung and lymphoid organs of pigs infected with the virulent PRRSV-1 SU1-Bel strain (Morgan et al., 2016), could contribute to this fall.

Moreover, we wanted to analyze not only the frequency but also the MFI of CD163 within live PRRSV-*N*-protein$^-$ and PRRSV-*N*-protein$^+$ PAMs for each infected animal. PRRSV-*N*-protein$^+$ PAMs showed high levels of CD163 at the end of the study, although, in general, the MFI of CD163 within PRRSV-*N*-protein$^-$ and PRRSV-*N*-protein$^+$ PAMs decreased through the study with respect to control group. Apart from virus replication, this decreased frequency as well as expression of CD163 could be explained by different scenarios: (i) a downregulation of CD163 in the context of PRRSV-induced inflammation. Guo et al. (2014) demonstrated that the metalloprotease ADAM17 is directly activated during PRRSV infection of PAMs and MARC-145 cells *in vitro* decreasing the level of CD163. Thus, following inflammatory stimuli, such as cytokine production or LPS presence, metalloproteases like ADAM17 can be stimulated and cleaved CD163 (Etzerodt et al., 2010; Guo et al., 2014). (ii) The result of the influx of immature macrophages reaching the alveoli to restore the normal lung function. Due to the loss of $CD163^+$ PAMs, it is expected that new macrophages, still expressing low levels of CD163, replenish the lung to replace this subset. In this context, a clear increase of $CD14^+$ cells in the interstitium of PRRSV-infected pigs, as a source for macrophages, has been previously described (Van Gucht et al., 2005). And, (iii) since the alveoli are devoid of $CD163^+$ PAMs, the frequency of $CD163^{low}$ cells becomes proportionally more noticeable in BALF cells.

5. Conclusion

In summary, the present study shows a decreased frequency and expression of the scavenger receptor CD163 in live PAMs isolated from the BALF of PRRSV-1 infected piglets, being this fact earlier and stronger when the viral strain was a virulent PRRSV-1 strain. This outcome may be due to the direct effect of the virus as well as other indirect mechanisms.

Conflict of interest

The authors declare that they have no competing interest.

Acknowledgements

We express our appreciation to Dr. Javier Domínguez for the kindly supply of the monoclonal antibody CD163 used in the immunohistochemical study. We also thank Gema Muñoz, Alberto Alcántara and Esmeralda Cano for their technical assistance and Dr. Hans Nauwynck for providing us the PRRSV-1 subtype 3 Lena strain. This work was supported by the Spanish Ministry of Economy and Competitiveness (AGL2016-76111-R). I.M. Rodríguez-Gómez was supported by a post-doctoral contract from the University of Córdoba (XXI Programa Propio UCO, modalidad 5.2.). J. Gómez-Laguna is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Economy and Competitiveness (RyC-2014-16735).

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