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PII: S0378-1135(17)31398-6
DOI: https://doi.org/10.1016/j.vetmic.2018.02.001
Reference: VETMIC 7864

To appear in: *VETMIC*

Received date: 30-11-2017
Revised date: 1-2-2018
Accepted date: 1-2-2018

Please cite this article as: Ferrari L, Canelli E, De Angelis E, Catella A, Ferrarini G, Ogno G, Bonati L, Nardini R, Borghetti P, Martelli P, A highly pathogenic porcine reproductive and respiratory syndrome virus type 1 (PRRSV-1) strongly modulates cellular innate and adaptive immune subsets upon experimental infection, *Veterinary Microbiology* (2010), https://doi.org/10.1016/j.vetmic.2018.02.001

A highly pathogenic porcine reproductive and respiratory syndrome virus type 1 (PRRSV-1) strongly modulates cellular innate and adaptive immune subsets upon experimental infection

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Highlights

- HP PRRSV PR40 infection induced recruitment of CD14+CD16+ and CD14+CD163+ monocytes
- The CD3+CD16+ NKT cell increase may sustain inflammation or the anti-viral response
- PR40 infection showed depletion/altered recruitment of Th and CD8α+/- γ/δ T cells
- PR40 induced early CD4+CD25+ and Treg lymphocyte depletion
- PR40 induced a strong CD21+ B cell depletion, as feature of immune dysregulation

Abstract

Highly pathogenic (HP) PRRSV isolates have been discovered within both PRRSV-1 and PRRSV-2 genotypes and investigated in recent years especially for their ability to cause extremely severe disease in conventional pig herds. The exacerbation of general and respiratory clinical signs has been attributed not only to an efficient replication (virulence) but also to the ability to dysregulate viral recognition and induce mechanisms of immune evasion or immune enhancement of humoral and cellular anti-viral responses differently from non-HP PRRSV isolates in terms of intensity and temporal onset. Thus, the understanding of the immunopathogenesis of HP PRRSV is a major concern for the study of virus biology and development of efficacious vaccines. The present study aims at addressing the modulation of relevant immune cell subsets by flow cytometry in the blood of 4-week-old pigs experimentally infected with the recently discovered PR40/2014 HP PRRSV-1.1 strain phenotypically characterized in Canelli et al. (2017) compared to pigs infected with a non-HP PRRSV isolate (PR11/2014) and uninfected controls. PR40 infected animals showed an early and marked reduction of pro-inflammatory CD172α+ CD14+CD16+ and CD14+CD163+ monocytes and TCRγδ+ CD8α+/CD8α- lymphocytes when pigs were most infected, possibly due to a recruitment sustaining an acute inflammatory response in target tissues. The prolonged increased CD3+CD16+ NKT cell levels may sustain peripheral inflammation and/or the anti-viral response. The late reduction (potential depletion) of γ/δ T lymphocytes and CD3+CD4+CD8α- naïve Th

lymphocytes paralleled with the delayed increase of CD3+CD4+CD8α+ memory and CD3+CD4-CD8α/β+ cytotoxic T lymphocytes. In addition, PR40 infection showed an early depletion of activated CD4+CD25+ T lymphocytes and Tregs together with an intense and lasting depletion of CD21+ B lymphocytes. Overall, these features demonstrate that the more severe clinical signs observed upon infection with the HP PR40 strain are sustained by remarkable changes in the peripheral blood distribution of immune cells and provide further insights into the immune regulation/immunopathogenesis induced by PRRSV-1 subtype 1 European isolates.

Keywords: highly pathogenic (HP) PRRSV; experimental infection; CD14+CD16+ and CD14+CD163+ monocytes; natural killer T (NKT) cells; gamma-delta (γ/δ) T lymphocytes; adaptive T and B immune cell subsets; T regulatory lymphocytes (Tregs).

Introduction

It is well known that porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most widespread and severely impairing virus in pig farms worldwide. Besides two highly heterologous genotypes of the virus represented by the PRRSV type 1 (European, LV prototype) and the PRRSV type 2 (North American, VR2332 prototype), high genetic variation has been observed within the same genotype. Therefore, within the PRRSV-1 genotype, 4 subtypes have been classified, namely Pan-European subtype 1 and Eastern European subtypes 2, 3 and 4 (Stadejek et al., 2017). The PRRSV-2 genotype has had a distribution far from North America becoming widely spread especially in China and South Asia. Furthermore, different clinical and virological outcomes were recorded within both genotypes, suggesting the emergence of highly pathogenic (HP) PRRSV isolates. It has been demonstrated that among the PRRSV-1 subtype 3, a strain named Lena is more virulent than other strains. PRRSV-2 strains, which spread in Asia, evolved so that high virulence features were acquired (Wang et al., 2016). Although the replication rate in susceptible cells and tissues, the tissue damage and the dysregulation of the innate and adaptive immune responses all represent potential factors that can sustain the high virulence of the

virus, the mechanisms of augmented virulence and the consequences on the immune responses are scarcely understood. The immune dysregulation was observed towards early immune activation in terms of downregulation of antigen presenting cell (APC) markers such as swine leukocyte antigen-I (SLA-I), SLA-II, CD80/86, CD14, CD172α and CD163 by Lena virus as well as immune cells (Weesendorp et al., 2013a,b). However, the knowledge on the innate and adaptive immunity during HP PRRSV infection is not thorough (Wang et al., 2011, 2016; Frydas et al., 2013; Morgan et al., 2013; Weesendorp et al., 2013a,b; Fan et al., 2015; Li et al., 2017).

The present study aims at gaining insights into the modulation of the peripheral immune cell compartment upon HP PRRSV infection by evaluating the time-related changes of relevant monocyte and lymphocyte subsets in the blood of pigs experimentally challenged with an Italian HP PRRSV-1.1 (namely PR40/2014) compared to pigs infected with a non-HP PRRSV-1.1 (namely PR11/2014) (Canelli et al., 2017) and uninfected controls. To the authors' knowledge, this is the first paper showing the changes in relevant innate and adaptive immune cell subsets in pigs infected with a HP PRRSV type 1 subtype 1 strain.

Materials and methods

Animals and experimental infection

The study was approved by the Ethical Committee and by the Ministry of Health in Italy (171/2016-PR), according to European and National rules on experimental infection studies and animal welfare. The study was conducted in a biosafety level 2 (BSL-2) facility. No relevant pathogens (PRRSV; swine influenza virus, SIV; porcine circovirus type 2, PCV2) were detected in the animals.

Seventeen 4 week-old conventional pigs from a PRRSV-negative herd were randomly assigned to three groups and after 6 days of acclimation treated as follows:

1) PR40 group: 7 animals intranasally (IN) infected with the highly pathogenic (HP) Italian PRRSV-1.1_PR40/2014 (hereafter PR40) (GenBank accession number: MF346695);

2) PR11 group: 7 animals IN infected with the Italian PRRSV-1.1_PR11/2014 (hereafter PR11);

3) C group: 3 animals IN inoculated with medium only (mock-infected/negative control).

For both infected groups, namely PR40 and PR11, 10^5 tissue infectious dose 50% ($TCID_{50}$) PRRSV/pig in 2 ml (1 ml/nostril) was IN administered. The main genetic, clinical, virological and pathological features of the HP PR40 strain are described by Canelli and coll. (2017). Blood samples were collected at 0, 7, 10, 14, 17, 21, 28 and 35 days post-infection (PI) in order to determine the immune modulation upon infection with this HP PRRSV by quantification of relevant immune cell subsets by flow cytometry (FCM) in peripheral blood mononuclear cells (PBMC).

Viremia

PRRSV titre quantification in serum is reported in Canelli et al. (2017). Briefly, RNA was extracted by Trizol™ LS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and PRRSV copy number was determined by quantitative PCR according to Martelli and coll. (2013). The proportion of PRRSV-infected animals out of the number of animals in each group at each time point and the virus titres are reported in the present study in order to provide complete information on the course of infection.

Isolation of porcine PBMC

PBMC were isolated using Histopaque-1077® density gradient (Sigma-Aldrich, St. Louis, Missouri, USA) as previously described (Ferrari et al., 2013). Specifically, after isolation, the cells were washed twice with phosphate buffered solution (PBS) + 1% heat-inactivated (hi) foetal bovine serum (FBS), suspended in complete RPMI-1640 medium (cRPMI-1640) + 10% hi FBS (Gibco, Carlsbad, CA, USA), quantified and checked for viability by Trypan blue (Sigma-Aldrich) by using a TC20™ automated cell counter (BioRad, Hercules, CA, USA) before staining for FCM immune cell analysis.

Quantification of immune cell subsets in PBMC by flow cytometry

Quantification of relevant immune cell subsets within PBMC by FCM was performed according to previous reports (Gerner et al., 2009; Martelli et al., 2009; Juul-Madsen et al., 2010; Fairbairn et al., 2013; Ferrari et al., 2014). The phenotype, marker combinations and main biological functions for the immune subsets considered for FCM analysis are reported in table 1, while the details relative to the antibodies used are reported in table 2. In quadruple CD3/CD4/CD8α/CD8β staining, cells were first stained for CD8β and then for the other antigens after optimization of single and multiple staining combinations; in triple CD172α/CD14/CD163 staining, cells were first stained for CD163 and then for CD172α and CD14; in double TCRγδ/CD8α staining, cells were first stained for TCRγδ and then for CD8α. For identification of T regulatory lymphocytes (Tregs) by triple CD4/CD25/FoxP3 staining, cells were surface stained for CD25 and then for CD4, fixed for 30 min., washed and then permeabilized/intracellularly stained for FoxP3 for 30 min. in the dark at 4°C using the fixation and permeabilization solutions of the FoxP3 staining set (cat. 72-5775; Affymetrix eBioscience), according to the manufacturer's recommendations.

If not differently specified, each staining step for both unlabeled primary antibodies and primary and secondary fluorochrome-conjugated antibodies was carried out in the dark for 15 min. Unstained cells and cells incubated with secondary antibodies were used as negative controls (non-specific binding); single staining and fluorescence-minus-one (FMO) controls were used to set proper compensations. For all staining procedures, all washing steps were performed with 1 ml and 2 ml of PBS + 1% FBS before and after incubating cells with primary fluorochrome-labelled or unlabelled antibodies and after secondary-labelled antibodies, respectively. The analysis was performed using a dual-laser (argon 488 nm and red solid state 631 nm) equipped Cytomics FC500 MCL flow cytometer and CXP software (Beckman Coulter, Indianapolis, IN, USA) based on lymphocyte and monocyte gating (forward scatter vs. side scatter) after acquisition of at least 50,000 cell events for each gate. Quantification of monocyte subsets was performed from CD172α+ gated monocytes; T lymphocytes were quantified from CD3+ gated lymphocytes and NK cells were quantified from CD3- gated lymphocytes. Analysis of Tregs was performed by quantification of

CD25+FoxP3+ cells from gated CD4+ lymphocytes. The absolute levels (cells/μL) of each subset were determined based on the leukocyte counts (white blood cells: WBC) calculated using an automated Cell Dyn 3500 plus hematology analyzer (Abbott Diagnostics, Lake Forest, IL, USA) and lymphocyte and monocyte percentages on whole blood samples.

Statistical analysis

Cell levels as determined by FCM were submitted to statistical analysis by using ANOVA (analysis of variance). Kruskal-Wallis test and Dunn's multiple comparisons test were used in order to highlight differences between treatment groups. Friedman test and Dunn's test were used for the evaluation of changes over time within the same group throughout the experimental period.
P values < 0.05 (probability of Type I Error) were considered significant throughout the study. Statistical analysis was carried out using the GraphPad Prism v.7 software.

Results

Viremia was more pronounced in pigs experimentally infected with the PR40 PRRSV strain.

PRRSV-positive pigs and PRRSV titres in serum were quantified in order to monitor PR40 and PR11 experimental infections. The results relative to the levels of PRRSV in serum and re-isolation of PR40 and PR11 from infected pigs are reported in Canelli and coll. (2017). Briefly, viremia peaked at 7 days PI in PR40 pigs and at 10 days PI in PR11 pigs. Overall, the infection was more severe and lasted longer in PR40 infected pigs compared to PR11 pigs ($p<0.05$), while uninfected control animals remained negative throughout the study (Table 3). The virulence of the PR40 strain resulted to be more pronounced and supports the high pathogenicity feature of the virus.

The CD172α+ CD14+CD16+ and CD14+CD163+ monocyte subsets were subjected to an early and lasting modulation after PR40 PRRSV experimental infection.

Lymphocyte and monocyte subsets were enumerated from isolated PBMC by FCM in order to detect relevant changes following PR40 and PR11 experimental infection in both innate and adaptive immune cell subsets. The levels of CD172α+ monocytes significantly diminished in the PR40 group at 7 days PI ($p<0.05$) and thereafter, PR40 pigs showed lower values compared to PR11 pigs and controls throughout the study ($p<0.05$) (Fig. 1). The PR11 group showed a course comparable with controls. After infection, the CD172α+(CD14+)CD16+ monocytes proved to be the fraction responsible for the early and lasting decrease of CD172α+ cells ($p<0.05$) in the PR40 group, which showed the lowest levels throughout the study compared to controls and PR11 pigs ($p<0.05$). Conversely, PR11 infected animals did not show any significant decrease, resulting comparable with the higher levels of controls (Fig. 1). The levels of CD172α+CD14+CD163+ monocytes expressing the CD163 receptor, used by PRRSV for cell infection, showed the same early reduction in the PR40 group only ($p<0.05$), reverted to pre-infection levels from 14 days PI, and further decreased at the later time points. The course of this subset in PR11-infected pigs showed a significant decrease at 28-35 days PI ($p<0.05$) (Fig. 1). The fraction of CD172α+CD14+CD163- monocytes did not show differences among groups PI, despite lower mean levels were observed in PR40 pigs at 14 and 28 days PI (data not shown).

Subsets of innate and partially specific lymphocytes were investigated in order to have insights into the modulation of potentially early responsive cells upon PRRSV infection. Natural killer (NK) cells identified as CD3-CD4-CD8α+/β- and CD3-CD16+ cells, expressing the CD16 ADCC receptor, did not show significantly different levels between groups post-infection, with mean values ranging between 4% and 10% (data not shown). Taking into account T lymphocyte subsets, CD3+ T lymphocytes significantly decreased only in the PR40 group at 10 days PI and showed a peak modulation at 21 days PI ($p<0.05$). Both PR40 and PR11 groups had comparably lower cell levels compared to controls at 28-35 days PI ($p<0.05$) (data not shown). Interestingly, PR40 pigs showed significantly higher NKT cell levels (CD3+CD16+) from 10 to 21 days PI, while the PR11 group showed a delayed marked peak response at 17 days PI ($p<0.05$) (Fig. 2). Noteworthy,

TCRγδ+ cells showed a transient early decrease in both PR40 and PR11 groups ($p<0.05$) due to the decrease of the TCRγδ+CD8α- and TCRγδ+CD8α+ subsets, which resulted significantly lower also at some subsequent time points ($p<0.05$) (Fig. 2). Overall, NKT cell levels showed a differential modulation between PR40 and PR11 pigs while γ/δ T lymphocytes showed a comparable course between the two groups.

Quadruple CD3/CD4/CD8α/CD8β FCM staining allowed simultaneously identifying major T lymphocyte subsets involved in adaptive immune responses upon PRRSV infection in order to obtain information on the onset and development of primary and secondary memory immune modulation. The levels of CD4+ lymphocytes in PR40 pigs showed a significant decrease at 7-10 days PI ($p<0.05$) (data not shown), while CD8α+ and CD8β+ cells showed a statistically significant peak at 21 days PI compared to the PR11 group and controls. PR11 pigs showed comparable values with controls throughout the study (data not shown). Specifically within T cell subsets, naïve T helper CD3+CD4+CD8α- lymphocytes had a significant early decrease in PR40 pigs ($p<0.05$) compared to controls and PR11 and a following increase up to 21 days PI. Their levels significantly decreased during the last two weeks of the experimental period similarly to PR11 infected pigs; at 35 days PI, these lower levels were significantly different when compared to controls ($p<0.05$) (Fig. 3). Circulating memory T helper CD3+CD4+CD8α+ cells in the PR40 group showed a significant increase from 21 days PI onwards ($p<0.05$) while the PR11 group did not show any significant modulation after infection, like controls (Fig. 3). Similarly, the fraction of CD3+CD4-CD8α/β+ cytotoxic T lymphocytes (CTL) in the PR40 group was significantly increased from 21 days PI onwards ($p<0.05$) whereas the levels in the PR11 group were not different from controls and were not subjected to any significant positive modulation (Fig. 3).

The levels of activated CD4+CD25+ cells were evaluated together with the levels of CD4+CD25+FoxP3+ Tregs in order to provide insights into the activation and immunosuppression of T lymphocytes. The levels of CD4+CD25+ cells, expressing the activation marker CD25, showed a significant reduction within the first 2 weeks PI in both PR40 and PR11 infected animals

compared to controls ($p<0.05$) and a further increase in PR40 pigs ($p<0.05$) (Fig. 4). The levels of Tregs decreased only in PR40 animals during the first 3 weeks ($p<0.05$) while significantly higher levels were detected in PR11 pigs at 21 days PI compared to controls; no significant changes were observed in controls over time (Fig. 4). The results about T cell subsets further testify that pigs subjected to PR40 experimental infection had a strong modulation of the major T cell subsets in the blood, including cells involved in innate/partially specific antigen recognition (NKT cells and γ/δ T lymphocytes) as well as adaptive T cell subsets involved in a regulatory/cytotoxic activity and Tregs.

The levels of CD21+ B lymphocytes were subjected to a very intense early reduction in PR40 infected animals which lasted for the entire study period ($p<0.05$), while PR11 pigs showed a less marked decrease ($p<0.05$), which resulted significantly different from PR40 pigs ($p<0.05$). Both infected groups displayed significantly much lower levels compared to controls throughout the study ($p<0.05$) (Fig. 4). The course of CD21+ B lymphocytes demonstrates that both the T and B cell compartment were subjected to a prolonged modulation after PR40 infection.

Discussion

The cellular immune modulation upon experimental infection with the HP PR40 PRRSV was evaluated in comparison with a non-HP PRRSV and uninfected pigs. In PR40 pigs, the early CD172α+CD14+CD163+ monocyte decrease was attributed to a recruitment into inflamed/infected tissues such as lungs and lymph nodes and reduction from circulation as observed under field and experimental conditions (Dwivedi et al., 2012; Ondrackova et al., 2013). In fact, in a study focused on PR40 clinical features (Canelli et al., 2017), we observed severe dyspnea, lung atelectasia, alveolar congestion, interlobular edema and alveolar septa thickening due to lymphocyte and macrophage infiltration. In pig, CD14+CD163+ monocytes are mainly involved in the inflammatory response also in the lungs and lymph nodes, highly expressing chemokine receptors (Ondrackova et al., 2013) and CD163 acts as a co-receptor in PRRSV-susceptible macrophages and

sialoadhesin-positive (Sn+) pulmonary alveolar macrophages (PAMs), sustaining PRRSV persistence in infected cells and evasion of immune recognition by T cells and antibody-mediated responses. The delayed modulation in PR11 pigs suggests a different involvement of this monocyte subset in a non-HP PRRSV infection. CD172α+CD16+ monocytes, all expressing CD14 in pig (CD172α+CD14+CD16+), potentially displays a more remarkable role in antigen processing and presentation by increased SLA-II (Fairbairn et al., 2013); therefore, in PR40 pigs, this subset may have been recruited early after infection and consequently maintained at lower levels in the blood because of more active virus replication and thus antigen presence in infected tissues and lymph nodes. Moreover, the long lasting lower levels were associated with extended viremia; this can support an altered viral recognition and clearance as shown in Canelli et al. (2017). The reduction of CD172α+ monocytes was timely comparable with what observed upon infection with Lena-like viruses, which proved to be highly pathogenic (Morgan et al., 2013). NK cells, also expressing the Fc receptor CD16 (CD3-CD16+) were not subjected to a significant modulation in infected groups, while the early increase of NKT cells in PR40 pigs, concomitantly with the viremia peak, supports a major function of this subset commonly involved in the communication between innate and adaptive immunity (Denyer et al., 2006; Artiaga et al., 2014). Fu and coll. (2012) demonstrated that high IL-15 levels were found in PAM, macrophages and monocyte-derived dendritic cells (mDC) upon infection by a HP PRRSV; this cytokine plays an important role in the immediate response to infection, regulating the activation/proliferation of NK and NKT cells, and the influx of CD8+ CTL in the lungs. In our study, the increase of NKT cells temporally associated with early decreased CD14+CD16+ and CD14+CD163+ monocytes can further suggest a strong and early inflammatory recruitment in the infection sites. The concerted modulation of NKT and CD14+CD16+ and CD14+CD163+ monocytes in the blood, together with the severe clinical signs observed (Canelli et al., 2017), can suggest a pathogenetic feature of the HP PR40 strain. The NKT cell increase in PR11 pigs highlights that this subset may be involved also in non-HP PRRSV infection. The early reduction of CD4+CD8α- Th naïve lymphocytes in PR40 pigs can be attributed to a depletion or a

recruitment into the infection sites, which however was not associated with the onset of a CD4+CD8α+ memory T cell positive modulation observed only after several weeks, when Th cells may have been recruited and subjected to an effective antigen activation, thus sustaining the Th1 response by CD8+ CTL, as shown in Ferrari et al. (2013). In fact, the increase of blood T cells able to produce IFN-γ at the later time points of PR40 infection is in accordance with the concomitant increase of PRRSV-specific IFN-γ secreting cells (Ferrari, unpublished data). Our data on CD4+CD8- Th cells are in agreement with results in piglets IN-challenged with the HP PRRSV-2 HuN4 isolated in China (Wang et al. 2016) and in gilts infected with the PRRSV-2 isolate NVSL 97-7895 (Ladinig et al., 2014). Regarding the onset of a memory T cell response upon HP PRRSV infection, previous works showed that CD4+CD8α+ cells can be detected early after infection (Wang et al., 2016) or later (Weesendorp et al., 2013a). In our study, the delayed increase of memory cells and CTL is in accordance with studies with HP PRRSV-1 subtype 3 viruses (Morgan et al., 2013; Weesendorp et al., 2013a). The early decrease of TCRγδ+CD8α+ γ/δ T lymphocytes might testify that this subset was involved in virus recognition in both PR40 and PR11 pigs when virus-neutralizing (VN) antibodies had not been induced yet (Canelli et al., 2017). This subset can display cytotoxicity and IFN-γ secretion upon activation (Meier et al., 2003; Olin et al., 2005), however, as for Th cells, the early decrease of γ/δ T lymphocytes may be due to the PRRSV apoptotic/necrotic effect on lymphocyte subsets in secondary immune organs (Wang et al., 2014). Together with the NKT cell course, the reduction of TCRγδ+CD8α- cells in both infected groups may testify the involvement in an early anti-viral immune regulation; in fact, in accordance with Meier and coll. (2003), heterologous challenge with a field isolate can stimulate CD8α-IFNγ+ cells (Ferrari et al., 2013). However, the lasting low levels even after the course of viremia may be due to a PR40 increased immunosuppressive potential, similarly to the Lena strain (Weesendorp et al., 2016). The reduction of Th and γ/δ CD8α+/- T lymphocytes in both PR40 and PR11 groups during the later phase can be a consequence of the CD3+ T cell lymphocytopenia/altered T cell distribution observed in bronchial lymph-nodes and thymus (Canelli et al., 2017); however, the course of Th

lymphocytes and memory T cells can suggest the development of the memory response. The concomitant reduction and long-lasting low levels of TCRγδ+CD8α+ lymphocytes and CD172α+CD14+CD16+ monocytes can be related to the high PR40 pathogenicity. The induction of Tregs by PRRSV is controversial: this was especially shown with PRRSV-2 strains early after infection, whereas less or no Treg activity was observed upon PRRSV-1 infection (Fan et al., 2015; Rodríguez-Gómez et al., 2015; Silva-Campa et al., 2010, 2012; Wang et al., 2011; Wongyanin et al., 2010, 2012). Regarding HP PRRSV infection, a study co-culturing PBMC with MoDC infected with a Chinese HP PRRSV demonstrated Treg induction (Fan et al., 2015), whereas no modulation was observed upon SU1-bel virus infection (Morgan et al., 2013). In our study, the reduction of activated CD4+CD25+ Th lymphocytes in infected groups can be interpreted as a depletion due to total T cell depletion or a peripheral organ homing due to inflammation. The later increase is associated with the increase of memory cells and CTL, thus accounting for a concerted activation/positive modulation. However, in partial accordance with LeRoith and coll. (2011) and Suradhat and coll. (2015), in PR40 pigs only, a fraction of these cells was constituted of Tregs which may have been also depleted or involved in immunosuppressive viral evasion mechanisms so that the Th1 response was delayed. The early low Treg levels may have been also responsible for poorly regulated inflammation by monocyte recruitment. Tregs may have dampened an effective immune regulation when PR40 was highly replicating, thus impairing/delaying the clearance from permissive cells and blood. This may be a further hallmark of more severe pathogenicity. Regarding PR11, the late increased levels are in agreement with our previous results of FoxP3 up-regulation in infected pigs (Ferrarini et al., 2015). The early marked lymphopenia of the CD21+ B cell fraction in PR40 pigs, with a very poor/absent recovery, was also observed by Ladinig and coll. in gilts (2014) and by Morgan and coll. (2013) and Weesendorp and coll. (2016) in piglets IN-infected with the HP Lena-like subtype 3 SU1-bel PRRSV and Lena virus, respectively. The mature CD21+ B cell reduction also observed in PR11 pigs, was a clear feature of the PRRSV infection. In PR40 pigs, however, the reduction of CD21+ cells in the blood run together with a marked depletion of B cells

in lymph nodes at necropsy (Canelli et al., 2017). In spite of that, such decrease may have been also due to tissue distribution of differentiated B cells secreting non-VN antibodies during early infection. In fact, the results are in agreement with a study in germ-free piglets IN-infected with the PRRSV-2 VR-2332, in which the reduction of primed CD2-CD21+ B cells was associated with the increase of effector/antibody secreting CD2+CD21- cells (Sinkora et al., 2014).

Conclusions

In summary, it was demonstrated that the early cellular modulation in PBMC to the PR40 strain (7-10 days PI) when viremia was more pronounced, was characterized by a strong recruitment of pro-inflammatory CD172α+ CD14+CD16+ and CD14+CD163+ monocytes possibly sustaining an early acute inflammatory response in target tissues (Canelli et al., 2017). The marked increase of CD3+CD16+ NKT cells early upon infection is more difficult to interpret as it can be related to the overall inflammatory status as well as evaluated in its potential to trigger the anti-viral response. The involvement of NKT cells, able to play a pivotal role between innate and acquired immunity, in association γ/δ T lymphocytes and pro-inflammatory cytokine patterns, surely needs further studies in pig since it can be also responsible for protective mechanisms against viruses (Franzoni et al., 2014; Artiaga et al., 2016). The delayed onset of CD4+CD8α+ memory and CD4-CD8α/β+ cytotoxic T cell responses together with the reduction of Th and γ/δ T lymphocytes is in line with the effects that the Lena HP PRRSV can exert (Weesendorp et al., 2013) and can account for the severe thymus and lymph node alteration observed (Canelli et al., 2017). PR40 infection also induced a decrease of CD4+CD25 cells, Tregs and a strong CD21+ B cell depletion, a scenario which supports the detrimental effects/dysregulation also at a cellular level of the HP PR40 strain.

Conflict of interest statement

All authors declare that they do not have any competing interests.

Acknowledgements

This study was funded by the Department of Veterinary Science (Martelli Symposium) with the income of the 6th European Symposium of Porcine Health Management held in Sorrento, Italy, in 2014. The PhD studies of Dr. Alessia Catella and Dr. Giulia Ogno are funded by a pre-doctoral grant of the Department of Veterinary Science, University of Parma, Italy (PhD Course in Veterinary Science). Dr. Elena Canelli is funded by grants of the Department of Veterinary Science, University of Parma.

References

1. Artiaga, B.L., Whitener, R.L., Staples, C.R., Driver, J.P., 2014. Adjuvant effects of therapeutic glycolipids administered to a cohort of NKT cell-diverse pigs. Vet. Immunol. Immunopathol. 162, 1-13. doi: 10.1016/j.vetimm.2014.09.006.
2. Artiaga, B.L., Yang, G., Hutchinson, T.E., Loeb, J.C., Richt, J.A., Lednicky, J.A., Salek-Ardakani, S., Driver, J.P., 2016. Rapid control of pandemic H1N1 influenza by targeting NKT-cells. Sci. Rep. 6, 37999. doi: 10.1038/srep37999.
3. Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F.C., Guazzetti, S., Martelli, P., 2017. Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Vet Microbiol. 210, 124-133. doi: 10.1016/j.vetmic.2017.09.002.
4. Denyer, M.S., Wileman, T.E., Stirling, C.M., Zuber, B., Takamatsu, H.H., 2006. Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing phenotypic and functional analysis of natural killer, cytotoxic T, natural killer T and MHC un-restricted cytotoxic T-cells. Vet. Immunol. Immunopathol. 110, 279–292. doi: 10.1016/j.vetimm.2005.10.005

5. Dwivedi, V., Manickam, C., Binjawadagi, B., Linhares, D., Murtaugh, M.P., Renukaradhya, G.J., 2012. Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. Virol. J. 9, 45. doi: 10.1186/1743-422X-9-45.
6. Fairbairn, L., Kapetanovic, R., Beraldi, D., Sester, D.P., Tuggle, C.K., Archibald, A.L., Hume, D.A., 2013. Comparative analysis of monocyte subsets in the pig. J. Immunol. 190, 6389-6396. doi: 10.4049/jimmunol.1300365.
7. Fan, B., Liu, X., Bai, J., Li, Y., Zhang, Q., Jiang, P., 2015. The 15N and 46R Residues of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein Enhance Regulatory T Lymphocytes Proliferation. PLoS One. 10, e0138772. doi: 10.1371/journal.pone.0138772.
8. Ferrari, L., Martelli, P., Saleri, R., De Angelis, E., Cavalli, V., Bresaola, M., Benetti, M., Borghetti, P., 2013. Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. Vet. Immunol. Immunopathol. 151, 193-206. doi: 10.1016/j.vetimm.2012.11.006.
9. Ferrari, L., Borghetti, P., De Angelis, E., Martelli, P., 2014. Memory T cell proliferative responses and IFN-γ productivity sustain long-lasting efficacy of a Cap-based PCV2 vaccine upon PCV2 natural infection and associated disease. Vet. Res. 45, 44. doi: 10.1186/1297-9716-45-44.
10. Ferrarini, G., Borghetti, P., De Angelis, E., Ferrari, L., Canelli, E., Catella, A., Di Lecce, R., Martelli, P., 2015. Immunoregulatory signal FoxP3, cytokine gene expression and IFN-γ cell responsiveness upon porcine reproductive and respiratory syndrome virus (PRRSV) natural infection. Res. Vet. Sci. 103, 96-102. doi: 10.1016/j.rvsc.2015.09.018.

11. Franzoni, G., Edwards, J.C., Kurkure, N.V., Edgar, D.S., Sanchez-Cordon, P.J., Haines, F.J., Salguero, F.J., Everett, H.E., Bodman-Smith, K.B., Crooke, H.R., Graham, S.P., 2014. Partial Activation of natural killer and γδ T cells by classical swine fever viruses is associated with type I interferon elicited from plasmacytoid dendritic cells. Clin. Vaccine Immunol. 21, 1410-1420. doi: 10.1128/CVI.00382-14.

12. Frydas, I.S., Verbeeck, M., Cao, J., Nauwynck, H.J., 2013. Replication characteristics of porcine reproductive and respiratory syndrome virus (PRRSV) European subtype 1 (Lelystad) and subtype 3 (Lena) strains in nasal mucosa and cells of the monocytic lineage: indications for the use of new receptors of PRRSV (Lena). Vet. Res. 44, 73. doi: 10.1186/1297-9716-44-73.

13. Fu, Y., Quan, R., Zhang, H., Hou, J., Tang, J., Feng, W.H., 2012. Porcine reproductive and respiratory syndrome virus induces interleukin-15 through the NF-κB signaling pathway. J. Virol. 86, 7625-7636. doi: 10.1128/JVI.00177-12.

14. Gerner, W., Käser, T., Saalmüller, A., 2009. Porcine T lymphocytes and NK cells – an update. Dev. Comp. Immunol. 33, 310-320. doi: 10.1016/j.dci.2008.06.003.

15. Gerner, W., Talker, S.C., Koinig, H.C., Sedlak, C., Mair, K.H., Saalmüller, A., 2015. Phenotypic and functional differentiation of porcine αβ T cells: current knowledge and available tools. Mol. Immunol. 66, 3-13. doi: 10.1016/j.molimm.2014.10.025.

16. Juul-Madsen, H.R., Jensen, K.H., Nielsen, J., Damgaard, B.M., 2010. Ontogeny and characterization of blood leukocyte subsets and serum proteins in piglets before and after weaning. Vet. Immunol. Immunopathol. 133, 95-108. doi: 10.1016/j.vetimm.2009.07.006.

17. Ladinig, A., Gerner, W., Saalmüller, A., Lunney, J.K., Ashley, C., Harding, J.C., 2014. Changes in leukocyte subsets of pregnant gilts experimentally infected with porcine reproductive and respiratory syndrome virus and relationships with viral load and fetal outcome. Vet. Res. 45, 128. doi: 10.1186/s13567-014-0128-1.

18. LeRoith, T., Hammond, S., Todd, S.M., Ni, Y., Cecere, T., Pelzer, K.D., 2011. A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with Mycoplasma hyopneumoniae. Vet. Immunol. Immunopathol. 140, 312-316. doi: 10.1016/j.vetimm.2010.12.011.

19. Li, X., Galliher-Beckley, A., Wang, L., Nietfeld, J., Feng, W., Shi, J., 2017. Comparison of Immune Responses in Pigs Infected with Chinese Highly Pathogenic PRRS Virus Strain HV and North American Strain NADC-20. Open Virol. J. 11, 73-82. doi: 10.2174/1874357901711010073.

20. Mair, K.H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A., Gerdts, V., Wilson, H.L., Meurens, F., 2014. The porcine innate immune system: an update. Dev. Comp. Immunol. 45, 321-343. doi: 10.1016/j.dci.2014.03.022.

21. Martelli, P., Gozio, S., Ferrari, L., Rosina, S., De Angelis, E., Quintavalla, C., Bottarelli, E., Borghetti, P., 2009. Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: clinical protection and cell-mediated immunity. Vaccine 27, 3788-3799. doi: 10.1016/j.vaccine.2009.03.028.

22. Martelli, P., Ardigò, P., Ferrari, L., Morganti, M., De Angelis, E., Bonilauri, P., Luppi, A., Guazzetti S., Caleffi A., Borghetti P., 2013. Concurrent vaccinations against PCV2 and PRRSV: study on the specific immunity and clinical protection in naturally infected pigs. Vet. Microbiol. 162, 558-571. doi: 10.1016/j.vetmic.2012.11.016.

23. Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology. 309, 18-31.

24. Morgan, S.B., Graham, S.P., Salguero, F.J., Sánchez Cordón, P.J., Mokhtar, H., Rebel, J.M., Weesendorp, E., Bodman-Smith, K.B., Steinbach, F., Frossard, J.P., 2013. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated

with enhanced adaptive responses and viral clearance. Vet. Microbiol. 163, 13-22. doi: 10.1016/j.vetmic.2012.11.024.

25. Olin, M.R., Batista, L., Xiao, Z., Dee, S.A., Murtaugh, M.P., Pijoan, C.C. Molitor, T.W., 2005. Gammadelta lymphocyte response to porcine reproductive and respiratory syndrome virus. Viral Immunol. 18, 490-499.
26. Ondrackova, P., Leva, L., Kucerova, Z., Vicenova, M., Mensikova, M., Faldyna, M., 2013. Distribution of porcine monocytes in different lymphoid tissues and the lungs during experimental Actinobacillus pleuropneumoniae infection and the role of chemokines. Vet. Res. 44, 98. doi: 10.1186/1297-9716-44-98.
27. Renukaradhya, G.J., Manickam, C., Khatri, M., Rauf, A., Li, X., Tsuji, M., Rajashekara, G., Dwivedi, V., 2011. Functional invariant NKT cells in pig lungs regulate the airway hyperreactivity: a potential animal model. J. Clin. Immunol. 31, 228-239. doi: 10.1007/s10875-010-9476-4.
28. Rodríguez-Gómez, I.M., Käser, T., Gómez-Laguna, J., Lamp, B., Sinn, L., Rümenapf, T., Carrasco, L., Saalmüller, A., Gerner, W., 2015. PRRSV-infected monocyte-derived dendritic cells express high levels of SLA-DR and CD80/86 but do not stimulate PRRSV-naïve regulatory T cells to proliferate. Vet. Res. 46, 54. doi: 10.1186/s13567-015-0186-z.
29. Silva-Campa, E., Cordoba, L., Fraile, L., Flores-Mendoza, L., Montoya, M., Hernández, J., 2010. European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology. 396, 264-271. doi: 10.1016/j.virol.2009.10.024.
30. Silva-Campa, E., Mata-Haro, V., Mateu, E., Hernández, J., 2012. Porcine reproductive and respiratory syndrome virus induces CD4+CD8+CD25+Foxp3+ regulatory T cells (Tregs). Virology. 430, 73-80. doi: 10.1016/j.virol.2012.04.009.

31. Sinkora, M., Butler, J.E., Lager, K.M., Potockova, H., Sinkorova, J., 2014. The comparative profile of lymphoid cells and the T and B cell spectratype of germ-free piglets infected with viruses SIV, PRRSV or PCV2. Vet. Res. 45, 91. doi: 10.1186/s13567-014-0091-x.

32. Stadejek, T., Larsen, L.E., Podgórska, K., Bøtner, A., Botti, S., Dolka, I., Fabisiak, M., Heegaard, P.M.H., Hjulsager, C.K., Hućˊ, T., Kvisgaard, L.K., Sapierzyński, R., Nielsen J., 2017. Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. Vet. Microbiol. 209, 13-19. doi: 10.1016/j.vetmic.2017.05.011.

33. Suradhat, S., Wongyanin, P., Kesdangsakonwut, S., Teankum, K., Lumyai, M., Triyarach, S., Thanawongnuwech, R., 2015. A novel DNA vaccine for reduction of PRRSV-induced negative immunomodulatory effects: A proof of concept. Vaccine. 33, 3997-4003. doi: 10.1016/j.vaccine.2015.06.020.

34. Takamatsu, H.H., Denyer, M.S., Stirling, C., Cox, S., Aggarwal, N., Dash, P., Wileman, T.E., Barnett, P.V., 2006. Porcine gammadelta T cells: possible roles on the innate and adaptive immune responses following virus infection. Vet. Immunol. Immunopathol. 112, 49-61.

35. Wang, G., Song, T., Yu, Y., Liu, Y., Shi, W., Wang, S., Rong, F., Dong, J., Liu, H., Cai, X., Zhou, E.M., 2011. Immune responses in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 142, 170-178. doi: 10.1016/j.vetimm.2011.05.004.

36. Wang, G., He, Y., Tu, Y., Liu, Y., Zhou, E.M., Han, Z., Jiang, C., Wang, S., Shi, W., Cai, X., 2014. Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virol. J. 11, 2. doi: 10.1186/1743-422X-11-2.

37. Wang, G., Li, L., Yu, Y., Tu,Y., Tong, J., Zhang, C., Liu, Y., Li, Y., Han, Z., Jiang, C., Wang, S., Zhou, E.M., He, X., Cai, X., 2016. Highly pathogenic porcine reproductive and

respiratory syndrome virus infection and induction of apoptosis in bone marrow cells of infected piglets. J. Gen. Virol. 97, 1356-1361. doi: 10.1099/jgv.0.000454.

38. Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Popma-De Graaf, D.J., Graham, S.P., Rebel, J.M., 2013a. Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Vet. Microbiol. 163, 1-12. doi: 10.1016/j.vetmic.2012.09.013.
39. Weesendorp, E., Stockhofe-Zurwieden, N., Popma-De Graaf, D.J., Fijten, H., Rebel, J.M., 2013b. Phenotypic modulation and cytokine profiles of antigen presenting cells by European subtype 1 and 3 porcine reproductive and respiratory syndrome virus strains in vitro and in vivo. Vet. Microbiol. 167, 638-650. doi: 10.1016/j.vetmic.2013.09.021.
40. Wongyanin, P., Buranapraditkun, S., Chokeshai-Usaha, K., Thanawonguwech, R., Suradhat, S., 2010. Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Immunol. Immunopathol. 133, 170-182. doi: 10.1016/j.vetimm.2009.07.012.
41. Wongyanin, P., Buranapraditkul, S., Yoo, D., Thanawongnuwech, R., Roth, J.A., Suradhat, S., 2012. Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (Treg). J. Gen. Virol. 93, 1236-1246. doi: 10.1099/vir.0.040287-0.

Figure Captions

Fig. 1: **Levels of total CD172α+, CD172α+(CD14+)CD16+ and CD172α+CD14+CD163+ monocytes in the blood of PR40 infected (PR40) pigs, PR11 infected pigs (PR11) and uninfected control (C) pigs.** Corresponding groups were intranasally (IN) inoculated with 10^5 $TCID_{50}$/pig of PR40 and PR11 PRRSV, respectively. Negative controls were IN-inoculated with medium only (mock infected). The analysis was performed after acquisition of at least 50,000 cell events based on SSC vs. FSC monocyte gating. PI: post-infection. Different superscript letters (a, b) refer to significant differences between groups while no letters at a time point indicates no statistical differences among the three groups. Statistical differences within a group over time are indicated with an asterisk (*). The absolute levels (cells/μL) were determined based on WBC counts and monocyte percentages on whole blood samples.

Fig. 2: **Levels of CD3+CD16+ NKT cells, and TCRγδ+CD8α- and TCRγδ+CD8α+ γ/δ T lymphocytes in the blood of PR40 infected (PR40) pigs, PR11 infected pigs (PR11) and uninfected control (C) pigs.** Corresponding groups were intranasally (IN) inoculated with 10^5 $TCID_{50}$/pig of the PR40 and PR11 PRRSV, respectively. Negative controls were IN-inoculated with medium only (mock infected). The FCM analysis was performed after acquisition of at least 50,000 cell events based on SSC vs. FSC lymphocyte gating. PI: post-infection. Different superscript letters (a, b) refer to significant differences between groups while no letters at a time point indicates no statistical differences among the three groups. Statistical differences within a group over time are indicated with an asterisk (*). The absolute levels (cells/μL) were determined based on WBC counts and lymphocyte percentages on whole blood samples.

Fig. 3: **Levels of naïve T helper CD3+CD4+CD8α- lymphocytes, circulating memory T helper CD3+CD4+CD8α+ cells and CD3+CD4-CD8α/β+ cytotoxic T lymphocytes (CTL) in the blood of PR40 infected (PR40) pigs, PR11 infected pigs (PR11) and uninfected control (C) pigs.** Corresponding groups were intranasally (IN) inoculated with 10^5 $TCID_{50}$/pig of the PR40 and PR11 PRRSV, respectively. Negative controls were IN-inoculated with medium only (mock infected). The FCM analysis was performed after acquisition of at least 50,000 cell events based on SSC vs. FSC lymphocyte gating. PI: post-infection. Different superscript letters (a, b) refer to significant differences between groups while no letters at a time point indicates no statistical differences among the three groups. Statistical differences within a group over time are indicated with an asterisk (*). The absolute levels (cells/μL) were determined based on WBC counts and lymphocyte percentages on whole blood samples.

Fig. 4: **Levels of CD4+CD25+ cells (expressing the activation marker CD25), CD4+CD25+FoxP3+ Tregs and CD21+ B lymphocytes in the blood of PR40 infected (PR40) pigs, PR11 infected pigs (PR11) and uninfected control (C) pigs.** Corresponding groups were intranasally (IN) inoculated with 10^5 $TCID_{50}$/pig of the PR40 and PR11 PRRSV, respectively. Negative controls were IN-inoculated with medium only (mock infected). The FCM analysis was performed after acquisition of at least 50,000 cell events based on SSC vs. FSC lymphocyte gating. PI: post-infection. Different superscript letters (a, b) refer to significant differences between groups while no letters at a time point indicates no statistical differences among the three groups. Statistical differences within a group over time are indicated with an asterisk (*). The absolute levels (cells/μL) were determined based on WBC counts and lymphocyte percentages on whole blood samples.

Fig 1

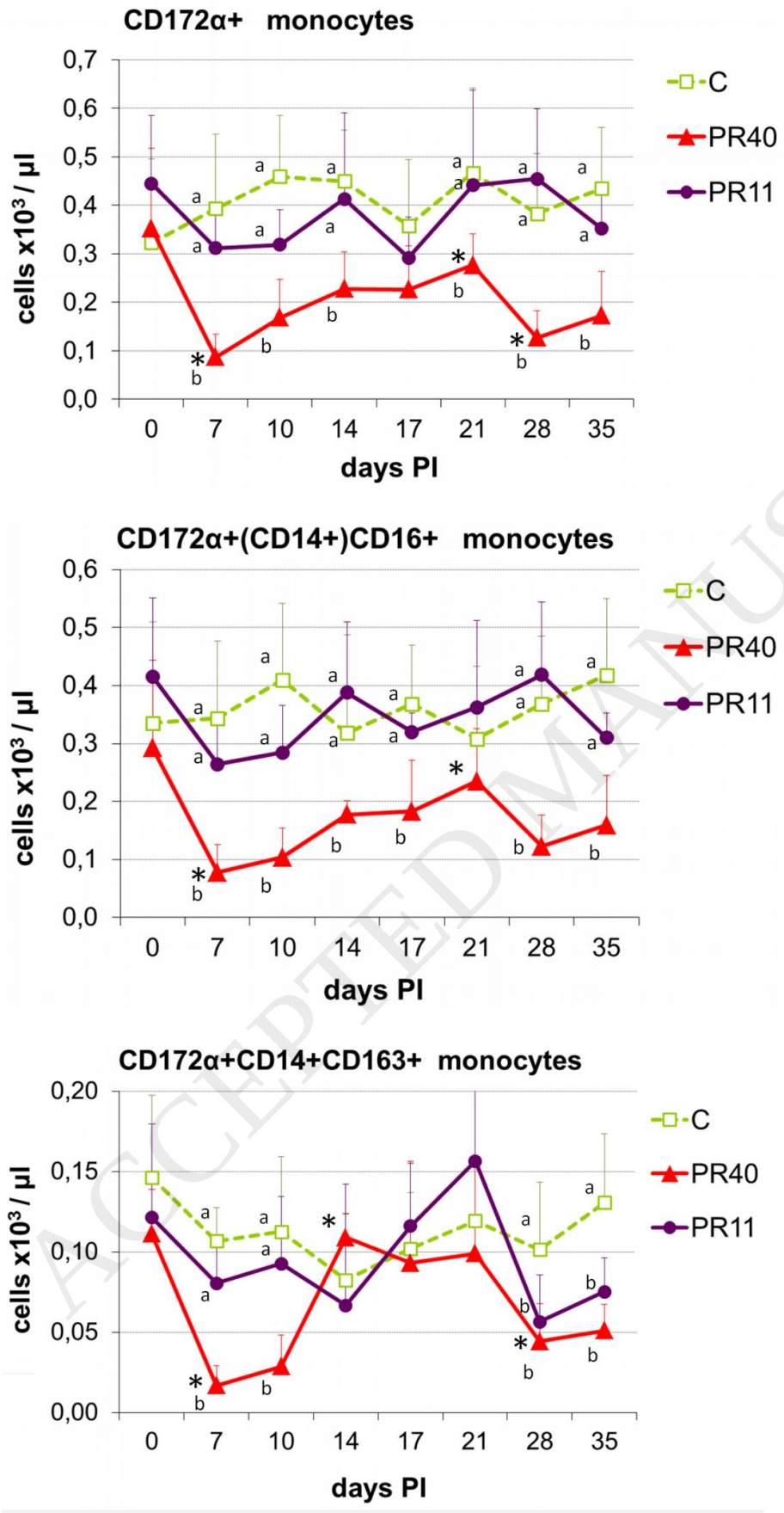

Fig 2

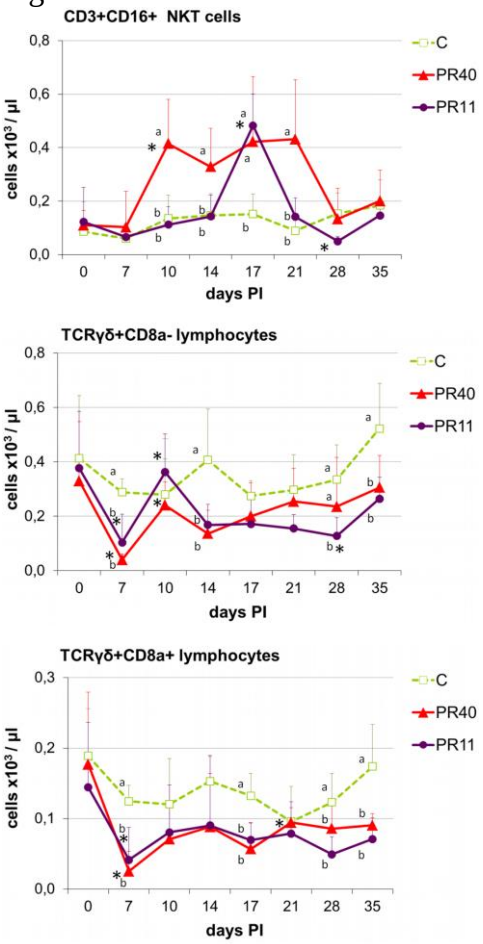

Fig 3

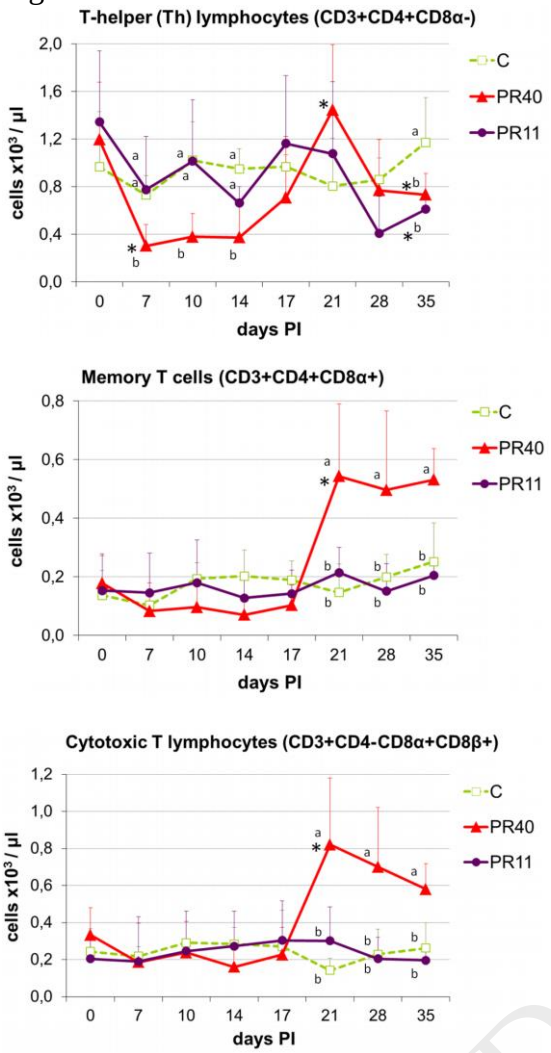

Fig 4

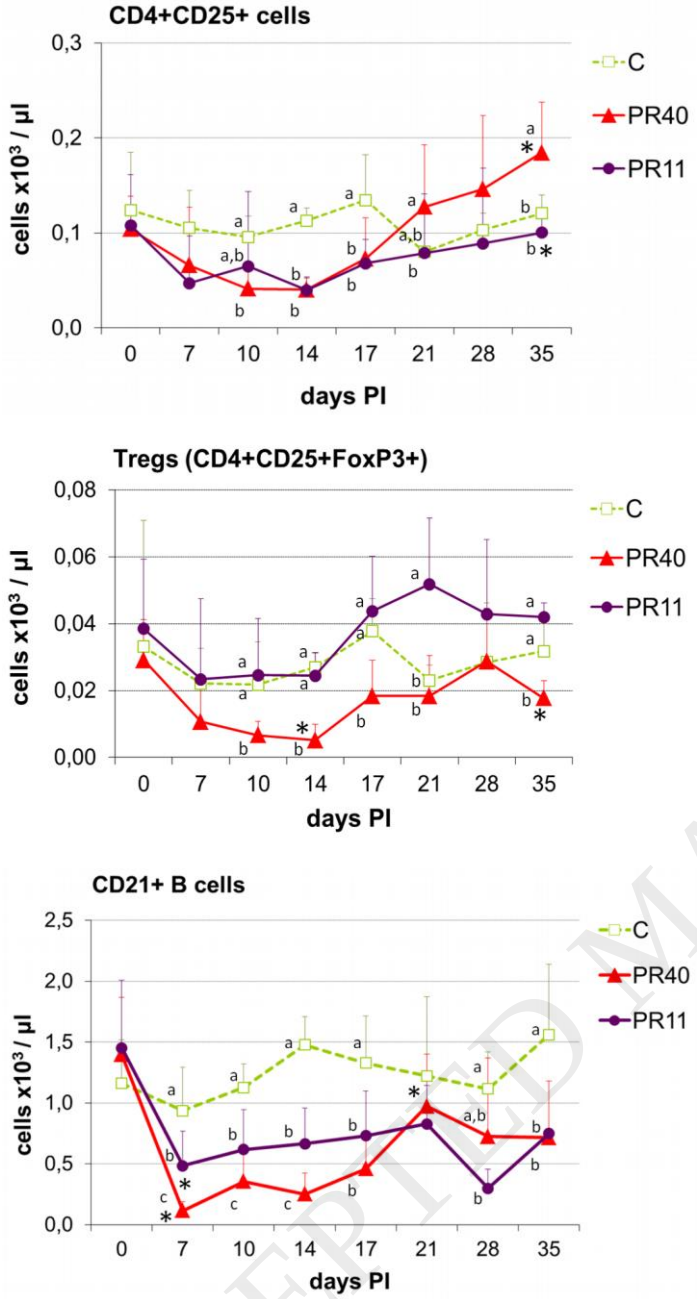

Table 1. Phenotypes and main functions of the immune cell subsets analyzed by flow cytometry. The details for each staining procedure and relative analysis are provided in the materials and methods section and table 2.

Immune subset	**Phenotype/marker combination**	**Main functions**
Monocytes	CD172α+CD14+CD163+	Inflammatory response: recruitment into inflamed/infected tissues such as lungs and lymph nodes CD163 acts as a co-receptor for PRRSV in PAMs APC function (Dwivedi et al., 2012; Ondrackova et al., 2013)
	CD172α+CD14+CD163-	Inflammatory response APC function (Fairbairn et al., 2013)
	CD172α+(CD14+)CD16+	Remarkable role in antigen processing and presentation by increased SLA-II expression, APC function (Fairbairn et al., 2013)
Natural killer (NK) cells	CD3-CD4-CD8α+/β- CD3-CD16+	Innate immune responses (antibody dependent cell cytotoxicity: ADCC via CD16; IFN-γ secretion) (Denyer et al., 2006; Mair et al., 2014)
Natural killer T (NKT) cells	CD3+CD16+	Strengthening and regulation of innate-to-adaptive immune response intercommunication (innate antigen recognition mechanisms; IFN-γ and pro-inflammatory cytokine secretion) (Denyer et al., 2006; Artiaga et al., 2014)
T lymphocytes	CD3+CD4+CD8α-	Naïve T helper lymphocytes (regulatory functions) (Gerner et al., 2015)
	CD3+CD4+CD8α+	T helper memory cells (regulatory functions and memory responses, IFN-γ secretion) (Gerner et al., 2015)
	CD3+CD4-CD8α/β+	Cytotoxic T lymphocytes (cytotoxicity/cytolysis and adaptive immune responses, IFN-γ secretion) (Denyer et al., 2006; Gerner et al., 2015)
γ/δ T lymphocytes	TCRγδ+CD8α+	Lipid antigen presentation via CD1 (Renukaradhya et al., 2011) Cytotoxicity/cytolytic activity and IFN-γ secretion (Meier et al., 2003; Olin et al., 2005; Takamatsu et al., 2006).
	TCRγδ+CD8α-	Lipid antigen presentation via CD1 (Takamatsu et al., 2006; Renukaradhya et al., 2011)
Activated T lymphocyes	CD4+CD25+	Responsiveness to IL-2 induced proliferation upon antigen encounter (a fraction of this subset is composed of

		Tregs) (Fan et al., 2015; Suradhat et al., 2015)
Regulatory T lymphocytes (Tregs)	CD4+CD25+FoxP3+	Regulation and dampening of antigen-specific and unspecific immune responses via IL-10 and TGF-β secretion (Silva-Campa et al., 2009; LeRoith et al., 2011; Suradhat et al., 2015)
B lymphocytes	CD21+	Humoral response by primed/mature cells: antibody secretion APC function at tissue level (Sinkora et al., 2014)

CD: cluster of differentiation; CD172α: CD172alpha**;** CD8α: CD8alpha; CD8β: CD8beta; TCRγ/δ: T cell receptor gamma-delta; FoxP3: Forkhead box P3.

Table 2. Details of the antibodies used for immunophenotyping by flow cytometry. Antigen, clone/cell line and isotype refer to the reactivity and characterization of the primary fluorochrome-labelled or unlabelled antibody.

Antigen	**Clone/cell line**	**Isotype**	**Source**	**Fluorochrome**	**Secondary antibody**
CD172α	74-22-15	$IgG_1\kappa$	Southern Biotech	PE	
CD14	MIL2	IgG_{2b}	AbD Serotec	FITC	
CD16 (FcRIII)	G7	IgG_1	AbD Serotec	FITC	
CD163	2A10/11	IgG_1	AbD Serotec	unlabelled	goat anti-mouse Ig-APC (cat. 550826, BD Pharmingen)
CD3ε	PPT3	$IgG_1\kappa$	Southern Biotech	PE	
CD4	74-12-4	$IgG_{2b}\kappa$	Southern Biotech	PE/Cy5 (SPRD)	
CD8α	76-2-11	$IgG_{2a}\kappa$	Southern Biotech	FITC	
CD8β	PG164A	IgG_{2a}	Kingfisher Biotech	unlabelled	goat anti-mouse $IgG_{2a}\gamma$-PE/Cy7 (cat. 1080-17, Southern Biotech)
TCR1-N4 (δ chain)	PGBL22A	IgG_1	VMRD		goat anti-mouse Ig-APC (cat. 550826, BD

				Pharmingen)	
CD21	BB6-11C9.6	$IgG_1\kappa$	Southern Biotech	PE	
CD25 (IL-2R α-chain)	K231.3B2	IgG_1	AbD Serotec	unlabelled	goat anti-mouse $IgG_1\gamma$-FITC (cat. 1070-02, Southern Biotech)
FoxP3	FJK-16s	IgG_{2a}	Affymetrix eBioscience	PE	

PE: phycoerythrin; FITC: fluorescein isothiocyanate; PE/Cy5: phycoerythrin/cyanin 5; SPRD: Spectral Red™; APC: allophycocyanin; FcRIII: fragment constant receptor 3; IL-2R: interleukin-2 receptor; PE/Cy7: phycoerythrin/cyanin 7; Ig: immunoglobulin; Southern Biotech, Birmingham, AL, USA; AbD Serotec, Raleigh, NC, USA; Kingfisher Biotech Inc., St. Paul, MN, USA; VMRD, Pullman, WA, USA; Affymetrix eBioscience Inc., San Diego, USA; BD Pharmingen, Franklin Lakes, NJ, USA.

Table 3. Proportion of infected pigs and virus titres (mean PRRSV cDNA log_{10} copies/µl ±SD) in the HP PR40 inoculated group, PR11 inoculated group and in mock-inoculated group. The animals were intranasally (IN) inoculated with 10^5 $TCID_{50}$/pig of the PR40 or PR11 PRRSV. Negative controls were IN-inoculated with medium only (mock infected).

	days PI							
treatment group	0	7	10	14	17	21	28	35
PR40								
% infected/total	0%	100%	100%	100%	100%	100%	100%	67%
titre	0.00	6.03	5.54	4.17	4.13	2.66	2.13	0.65
SD	0.00	0.65	0.75	1.06	1.33	1.19	0.87	0.61
PR11								
% infected/total	0%	86%	100%	100%	100%	100%	100%	0%
titre	0.00	4.33	5.65	5.02	3.92	3.45	2.70	0.00
SD	0.00	1.77	0.81	0.80	0.85	0.75	0.71	0.00
C								
% infected/total	0%	0%	0%	0%	0%	0%	0%	0%
titre	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

PR40: pigs experimentally infected with the highly pathogenic HP Italian PR40/2014 PRRSV; PR11: pigs experimentally infected with the Italian PR11/2014 PRRSV; C: pigs inoculated with medium only (mock infection). PI: post-infection.