Received: 27 July 2018 | Revised: 9 October 2018 | Accepted: 10 November 2018

DOI: 10.1111/tbed.13080

ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Low CD4/CD8 ratio in classical swine fever postnatal persistent infection generated at 3 weeks after birth

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Funding information
Ministerio de Ciencia, Innovación y Universidades, Spain, Grant/Award Number: AGL2015–66907; China Scholarship Council

Abstract

Classical swine fever virus (CSFV) is one of the most important pathogens affecting swine. After infection with a moderate virulence strain at 8 hours after birth, CSFV is able to induce viral persistence. These animals may appear clinically healthy or showed unspecific clinical signs despite the permanent viremia and high viral shedding, in absence of immune response to the virus. Given the role played by this infection in disease control, we aimed to evaluate the capacity of CSFV to induce postnatal persistent infection at 3 weeks after birth. Nine pigs were CSFV infected and sampled weekly during 6 weeks and viral, clinical, pathological and immunological tests were carried out. Also, the CD4/CD8 ratio was calculated with the purpose to relate this marker with the CSFV persistent infection. The IFN-α response was detected mainly 1 week after infection, being similar in all the infected animals. However, 44.4% of animals were CSFV persistently infected, 33.3% died and 22.2% developed specific antibody response. Interestingly, in persistently infected pigs, the T-CD8 population was increased, the T-CD4 subset was decreased and lower CD4/CD8 ratios were detected. This is the first report of CSFV capacity to confer postnatal persistent infection in pigs infected at 3 weeks after birth, an age in which the weaning could be carried out in some swine production systems. This type of infected animals shed high amounts of virus and are difficult to evaluate from the clinical and anatomopathological point of view. Therefore, the detection of this type of infection and its elimination in endemic areas will be relevant for global CSF eradication. Finally, the low CD4/CD8 ratios found in persistently infected animals may be implicated in maintaining high CSFV replication during persistence and further studies will be performed to decipher the role of these cells in CSFV immunopathogenesis.

KEYWORDS
CD4/CD8 ratio, CSFV, CSFV RNA load, pathogenesis, viral persistence

1 | INTRODUCTION

Classical swine fever (CSF) continues to be one of the most important diseases affecting swine. Due to its high social impact in some affected regions, the disease has been considered as a problem for the food security of the population. Currently, CSF is endemic in some countries of Asia, Eastern Europe and Latin America (Ganges et al., 2008; Pérez et al., 2012).

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The aetiological agent, CSF virus (CSFV), belongs to the *Pestivirus* genus, within the Flaviviridae family (Schweizer & Peterhans, 2014). The virus targets different cell types of the immune system, such as those that are derived from the monocyte-macrophage lineage (Summerfield, Hofmann, & McCullough, 1998; Zingle, Summerfield, McCullough, & Inumaru, 2001). Therefore, the infection in this type of cells plays an important role in viral pathogenesis, viral persistence and spread. It has been established that the balance between the nature of the virus and different host conditions, for instance age or immunological status, plays a role in the severity of disease, which can vary from acute to subclinical or chronic forms (Blome, Staubach, Henke, Carlson, & Beer, 2017). However, these underlying interactions are not completely known.

A significant trait of the *Pestivirus* genus is its ability to induce congenital persistent infection of the foetus by viral transplacental transmission, being known for over 40 years that congenital persistent infection is the most important cause by which CSFV is perpetuated in swine population (Liess, 1984). Nevertheless, little is known about the mechanisms involved. Persistently infected animals may appear to be healthy for some weeks, and develop runting-like signs later in life, despite replicating and excreting significantly high viral load (Liess, 1984; van Oirschot, 1979a,b).

Recently, it has been established that persistent infection can also occur after early postnatal infection, in pigs infected within the first 24 hr after birth, with a moderately virulent CSFV strain (Muñoz-González, Ruggli, et al., 2015). These animals remained apparently healthy for several weeks, or showed clinical signs different from those previously described for CSF (Liess, 1984; Muñoz-González, Ruggli, et al., 2015). Notably, these infected animals showed a high and persistent viral load in blood and body secretions for several weeks, as well as inability to generate specific cellular and humoral response to the virus. In addition to the adaptive immune response, recent studies have also shown that the innate immune response to the virus, as measured by type I IFN-α in the serum, was impaired in pigs with a CSFV postnatal persistent infection, therefore, an immunological anergy has been observed in these animals (Cabezón et al., 2017; Muñoz-González, Ruggli, et al., 2015).

Previous studies have shown that a low CD4/CD8 ratio can be interpreted as a measure of dysregulation of a patient's immune system (Serrano-Villar, Moreno, et al., 2014; Serrano-Villar, Sainz, et al., 2014), which has proven to be very useful marker in human immunodeficiency virus (HIV) therapy. Lower CD4/CD8 ratios have been detected in humans suffering persistent and chronic infections with HIV and hepatitis C virus (HCV) (Dustin, 2017). In this regard, a lower CD4/CD8 ratio has also been found in patients with persistently higher HIV-1 viral load (Gandhi et al., 2017).

Bearing in mind the proven CSFV ability to generate postnatal persistent infection in newborn piglets, the aim of the present study was to evaluate the capacity of CSFV to induce postnatal persistent infection at a later time point than previously reported. To this end, pigs were CSFV infected at 21 days after birth, and the characteristics of the immunological and virological response related to viral persistence were studied during a 6-week period. In addition, the phenotypic profile of peripheral blood mononuclear cells (PBMC) was evaluated for lymphocytic and myeloid lineages. Finally, the CD4/CD8 ratio was determined with the purpose to relate this marker with the CSFV persistent infection.

2 | MATERIALS AND METHODS

2.1 | Cells and viruses

PK-15 cells (ATCC CCL 33) and SK6 cells (Kasza, Shadduck, & Christofinis, 1972) were cultured in DMEM medium, supplemented with 10% foetal bovine serum (FBS) pestivirus-free at 37°C in 5% CO2. The Catalonia 01 (Cat01) strain used in this study belongs to the CSFV 2.3 genogroup (Pérez et al., 2012). This strain has been characterized as a moderately virulent strain (Tarradas et al., 2014). Alfort 187 strain was used in the neutralisation peroxidase-linked assay (NPLA). Viral stocks were produced using PK-15 cells that were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 72 hr later. For virus isolation, both cell lines (PK-15 and SK6) were used. Peroxidase-linked assay (PLA) (Wensvoort, Terpstra, Boonstra, Bloemraad, & Zaane, 1986) was used for viral titration following the statistical methods described by Reed and Muench (Reed & Muench, 1938).

2.2 | Experimental design

One pregnant sow (Landrace), pestivirus-free, of 108 days into gestation was housed in the BSL3 animal facility at IRTA-CReSA (Barcelona, Spain). Delivery was at 115 days of gestation and nine piglets were born. Twenty-one days after birth, piglets were numbered from 1 to 9 and were inoculated intranasally with 2.5×10^4 TCID of Cat01 strain. The inoculation of the piglets was conducted separately from their mother and the sow was kept with the piglets until 9 days after inoculation (30 days after birth). The piglets were fed (StartRite, Cargill, Spain) from week 4 onwards. Serum and rectal swab samples were collected every week after infection during the 6 weeks of the trial. Whole blood samples for the isolation of PBMCs were obtained at 4 weeks post infection. The tonsils were collected at necropsy and were used to quantify CSFV RNA and in virus titration assay. After macroscopic examination, a portion of the tonsil from pigs numbers 3, 5, 6 and 7 was placed into 10% phosphate buffered formalin in order to conduct histopathological evaluation. Fixed samples were sliced and embedded in paraffin wax after dehydration through increasing alcohol concentrations and xylene. Four micrometre sections were mounted on glass microscope slides which were stained with haematoxylin and eosin for routine morphological evaluation. Whole blood and tonsil samples from two non-infected pigs from the same origin as the sow and with the same age of the infected animals (7 and 9 weeks respectively) were also collected (numbered 10 and 11). These samples were used as negative controls.

The procedure for the euthanasia of the animals was based on an accepted method included in European Directive 2010/63/EU,

using an anaesthetic overdose of 60–100 mg of pentobarbital per kilogram of weight, administered via the vena cava.

A trained veterinarian recorded the temperature and clinical signs daily in a blinded manner. The experiments were approved by the Ethics Committee for Animal Experiments of the Autonomous University of Barcelona (UAB) under number 8642, according to existing national and European regulations.

2.3 | CSFV RNA detection

RNA extraction was performed using the NucleoSpin RNA isolation kit (Macherey-Nagel). For all the analysed samples, an initial volume of 150 μl was used in order to obtain 50 μl of RNA, which was stored at −80°C. The presence of CSFV RNA in sera, rectal swabs and tonsil was analysed by real time (qRT-PCR) (Hoffmann, Beer, Schelp, Schirrmeier, & Depner, 2005). Cycle threshold (Ct) values equal or less than 42 were considered as positive. Samples in which fluorescence was undetectable were considered negative.

2.4 | Humoral response detection by ELISA and NPLA

The presence of E2-specific antibodies in serum was evaluated using a commercial ELISA kit (IDEXX), the samples were considered as positive when the blocking percentage value was ≥40%. Serum samples were also tested by NPLA (Terpstra, Bloemraad, & Gielkens, 1984) against homologous and heterologous CSFV strains, Cat01 and Alfort-187 respectively. Neutralising antibody titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the CSFV strains in 50% of the culture replicates.

2.5 | ELISA for IFN-α detection in serum samples

IFN-α concentration in serum was evaluated by ELISA at 0, 7 and 14 days post infection (dpi) (Muñoz-González, Perez-Simó, et al., 2015; Muñoz-González, Ruggli, et al., 2015) using monoclonal antibodies (K9 and K17) and IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, NJ, USA). Cytokine concentrations (units/ml) in sera were determined using a regression line built with the optical densities of the cytokine standards used in the test

2.6 | PBMC collection and flow cytometry analysis

Considering the virological and immunological profile previously evaluated, whole blood sample was collected from animals 3, 5, 6 and 7 at 4 weeks post infection in order to obtain PBMC. Cells were separated by density-gradient centrifugation with Histopaque 1077 (Sigma) and afterwards were subjected to osmotic shock in order to eliminate the remaining erythrocytes. The number and viability of the PBMCs were determined by staining with Trypan Blue. In addition, PBMCs from non-infected pigs, served as control (samples from pigs 10 and 11).

Flow cytometry was used in order to phenotype the PBMCs from infected and naïve pigs at 4 weeks after infection (7 weeks of age). Hybridoma supernatant (kindly provided by Dr. J. Dominguez (INIA, Madrid, Spain)) was used for staining CD172a (BA1C11, IgG1), with an anti-Mouse IgG1 antibody labelled with Alexa Fluor 647 (thermofisher scientific, produced in goat) used as a secondary antibody to detect the primary anti-CD172a antibody. Moreover, conjugated mAbs detecting porcine T-CD4 (Alexa Fluor® 647 Mouse Anti-Pig CD4a 74-12-4, IgG2b, BD Pharmingen), and T-CD8-α (FITC Mouse Anti-Pig CD8a 76-2-11, IgG2a, BD Pharmingen) were also used.

Briefly, 5×10^5 cells were plated in each well and the cell culture medium was removed after centrifugation. For single-colour staining, cells were incubated with either hybridoma supernatant or conjugated mAbs for 20 min at 4°C. After washing with PBS + 2% FBS, the secondary antibody was added to cells that had been incubated with hybridoma supernatant. Meanwhile, for two-colour staining, cells were incubated simultaneously with both primary mAbs (mouse anti-pig T-CD4 and T-CD8) for 20 min at 4°C. Finally, a viability control (propidium iodide, 1 μg/ml) was added and twenty thousand live cell events were recorded for each sample in the cytometer (FACSAria IIu, BD Biosciences).

The cells were analysed by FACSDiva software, version 6.1.2 and the results were expressed as the percentage of positive cells obtained for each staining, using irrelevant isotype-matched mAbs as staining controls. In order to corroborate the reproducibility of the results, flow cytometry assays were performed twice for the infected animals and three times for the naïve animals. Afterwards, the mean value and standard deviation of each staining were calculated for every animal. Following the flow cytometry analysis, the CD4/CD8 ratio in PBMCs from pigs numbers 3, 5, 6, 7, 10 and 11 was determined using the mean value obtained from each marker.

2.7 | Sorting of T-CD8$^+$ cells

The T-CD8$^+$ cell subsets were sorted using a live sterile cell sorting system (FACSAria, Beckton Dickinson; San Jose, California, USA). 20×10^6 PBMC from pig number 5 were incubated with T-CD8 conjugated monoclonal antibody (FITC Mouse Anti-Pig CD8a 76-2-11, IgG2a, BD Pharmingen) for 30 min on ice and washed with PBS containing 2% FBS. Finally, the viability control was added and single cell sorting was performed in using the yield mode, with a 70 μm nozzle. The fluorescence reading was performed upon excitation with a 488 nm argon laser. Recovered cells after sorting were resuspended in RPMI-1640 Medium (Lonza) at final concentration of 2×10^6 cells/ml. The presence of CSFV RNA in T-CD8$^+$ and T-CD8$^-$ sorted cells was quantified by qRT-PCR (Hoffmann et al., 2005) and viral isolation in PK-15 cells was performed as explained above.

3 | RESULTS

3.1 | Clinical manifestations during 6 weeks of CSFV infection

Twenty-four hours post infection, an increase in the rectal temperature value was registered in all the infected pigs. Likewise, from 4 to

9 dpi, the temperature values increased above 40°C in all the infected animals, with five of nine pigs showing temperatures above 41°C reaching 42°C in some of them. Subsequently, at 10 dpi, a decrease in rectal temperature was recorded in six of nine infected animals (Figure 1a). Values of rectal temperature generally below 40°C were recorded again in six animals from the study after this time. These values were maintained until the end of the experiment and in few cases individual peaks of fever were registered in some of them during the trial, never surpassing the 40.5°C. On the other hand, two animals (numbers 8 and 9) showed high fever peaks from 11 to 18 dpi, which maintained high temperatures values until 17 and 21 dpi (Figure 1a).

Besides an increase in body temperature, the majority of the pigs did not show any clinical signs during the first week post infection, with only one animal showing mild apathy starting at 5 dpi. At the beginning of the second week of the trial, four of nine animals developed mild diarrhoea and/or mild apathy during 3 days. At 10 dpi, two animals, pigs 2 and 8 showed moderate apathy and weakness of the hindquarters respectively. Pig 2 was found dead in the pen at 11 dpi, whereas, pig 8 developed diarrhoea, tremors, cyanosis in the ears and severe apathy and was euthanized at 17 dpi (Figure 1b). Entering into the third week post infection, five pigs remained with a clinically healthy status, meanwhile the other three showed from mild to severe clinical manifestations, such as, moderate skin lesions (mainly vesicles and desquamation in the skin) which began to appear in pigs 1 and 9 at 19 and 16 dpi respectively. Pig number 9 went on to develop ulcerations in the abdomen, as well as severe dyspnoea at 20 dpi and thus had to be euthanized at 21 dpi (Figure 1b). Thus, during the second and third weeks post infection, the most severe clinical outcome was observed, with three animals that were either euthanized or found dead (33.3%).

From week 4 to the end of the trial (week six), six pigs were maintained, two of them showed mild diarrhoea, some vesicles and skin desquamation (pigs 1 and 5). Finally, the four remaining animals were apparently clinical healthy during this period (Figure 1b).

3.2 | Macroscopic and histopathological findings in tonsils after 6 weeks post CSFV infection

No macroscopic changes were found in the tonsils from the analysed animals after infection, when compared to the control pigs. In addition, after histopathological examination, no significant

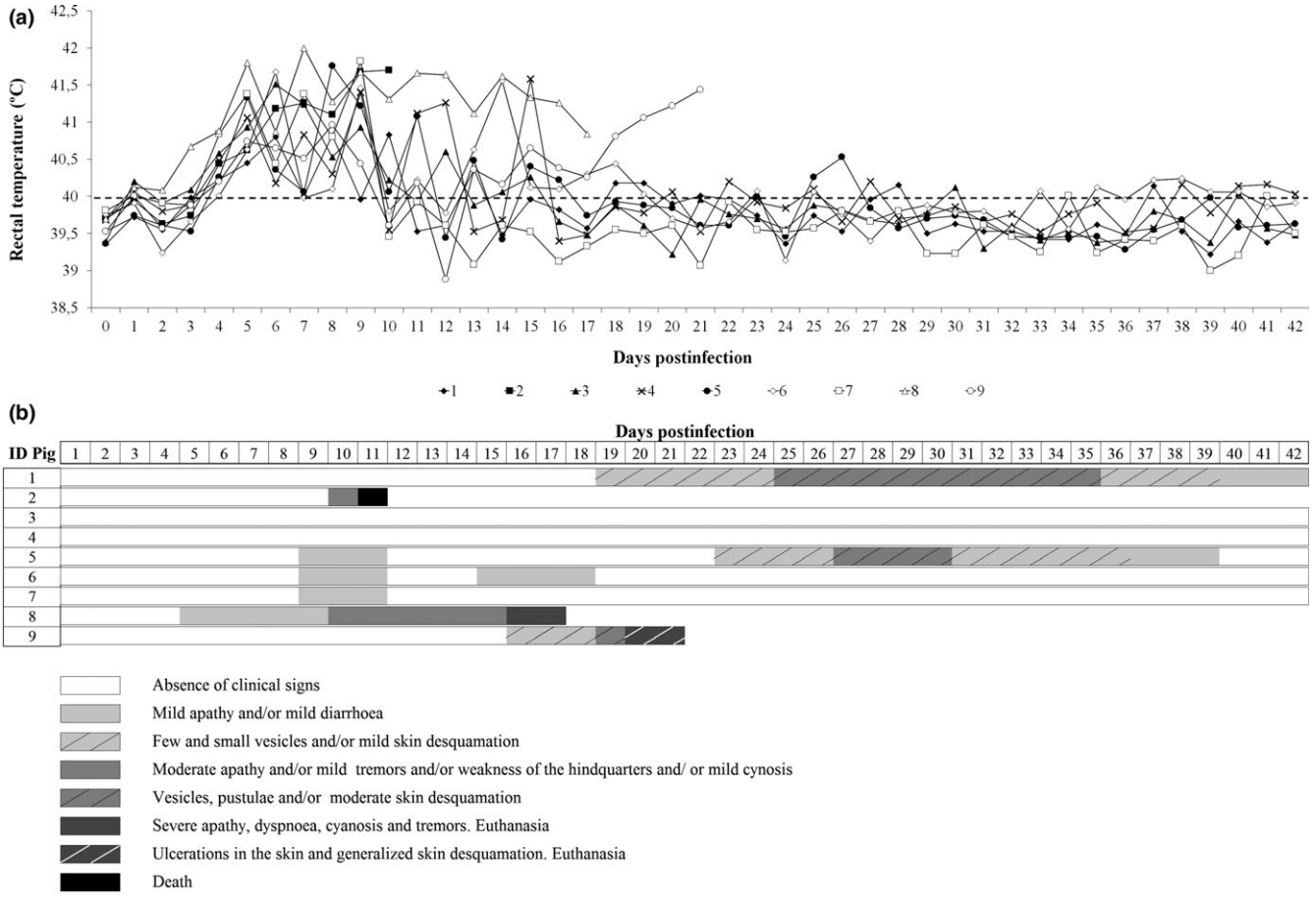

FIGURE 1 Clinical signs developed after CSFV postnatal infection at 21 days of age. (a) Daily rectal temperature measured in infected animals. Values greater than 40°C (dotted line) were considered to indicate fever. (b) Individual clinical signs in piglets after postnatal infections with CSFV Cat01 strain at 21 days of age. The piglets were monitored daily during the 6 weeks of the study. The severity of the clinical signs is represented by the colour scale, from white (absence of clinical signs) to black (death)

abnormalities were observed in the tonsils from the infected pigs. A mild increase in the amount of tingible body macrophages in the lymphoid follicles was evidenced only in pigs numbers 3 and 7, when compared to the tonsils from pigs 5 and 6 as well as, the non-infected (Figure 2).

3.3 | Most of the infected animals showed permanent CSFV load in sera and rectal swabs

During the first week of the study, similar viral RNA load was detected in all the CSFV infected animals, with Ct values between 30.5 and 33.3. In the second week of the study, two profiles of CSFV RNA load in sera were detected, with six animals, numbers 1, 4, 5, 6, 8 and 9, showing low Ct values between 21.6 and 23.9, and two animals (numbers 3 and 7) with high Ct values (32.7 and 34.8). Subsequently, no viral RNA was detected in sera from pigs 3 and 7 at 3 and 4 weeks post infection. On the other hand, CSFV RNA was detected in the remaining animals (44.4%) with similar Ct values to those found in the second week of the trial, being the RNA levels maintained or even increased during weeks 5 and 6 (Ct values between 17.4 and 22). Alternatively, during these 2 weeks, one pig was negative (pig 7) and the other showed high Ct values in the fifth and sixth weeks (Figure 3a).

In rectal swabs, CSFV RNA was detected with similar Ct values in three of nine infected pigs (numbers 2, 5 and 7) in the first week after infection. At week 2 post infection, most of the animals were positive with moderate Ct values (between 27 and 35.3), with the exception of pig 7 that was negative until the end of the study (Figure 3b). During the third week post infection, pig 3 showed a Ct value of 33.5. Whereas in the remaining animals (numbers 1, 4, 5, 6, 8 and 9), lower Ct values (between 24.5 and 28.7) were observed. These Ct values were similar for the surviving animals (1, 4, 5 and 6) during the fourth and fifth weeks and in some of them were decreased at week 6 (from 23.5 to 25). Meanwhile, pig number 3 was negative at weeks 4 and 6 after infection, however, it was positive with high Ct value (35.2) at week 5 of the study (Figure 3b).

All of the analysed tonsil samples were positive, animals 1, 4, 5 and 6 showed low Ct values, while animals 3 and 7 showed higher Ct values (from 26.3 to 29.5 respectively) (Figure 3c). Only tonsil samples from pigs numbers 1, 4, 5 and 6 were positive by viral isolation test, the viral load in samples from pigs 1, 4 and 5 was about $10^{6.6}$ $TCID_{50}$/ml, whereas the viral load in the tonsil from pig number 6 was $10^{5.6}$ $TCID_{50}$/ml.

3.4 | Only two infected pigs were able to seroconvert

Seven of the nine infected animals did not develop a humoral response detectable neither by ELISA nor neutralization test during the 6 weeks of the trial. By contrast, the remaining two animals, numbers 3 and 7 (22.2%) developed specific anti-E2 antibody response, detected by ELISA test, starting at 3 weeks post infection (Figure 3d). The results were confirmed by NPLA test and the neutralizing antibody response against the heterologous CSFV strain (Alfort-187) was detected also at 3 week post infection with a low antibody titre (1:10). The titres were increased between weeks 5 and 6 post infection with a titre of 1:60 in both pigs. Meanwhile, neutralizing antibody titres against the homologous CSFV strain (Cat01) were detected 1 week earlier in animal 7 with higher antibody titres (1:120) to those found against the heterologous strain. The titres were increased at 3 weeks post infection until the end of the trial in both animals (from 1:80 to 1:1280).

3.5 | IFN-α levels detected mainly 1 week after infection

At time of viral infection, all the pigs showed low baseline levels (between 10 and 20 units/ml) of IFN-α in serum sample. An increase in IFN-α values was recorded for all the animals at 1 week after infection with the CSFV Cat01 strain, with values ranging from 20 to 42 units/ml. During the second week post infection, IFN-α concentration had severely decreased in all the animals, showing concentrations between 4.44 and 8.65 units/ml (Figure 4).

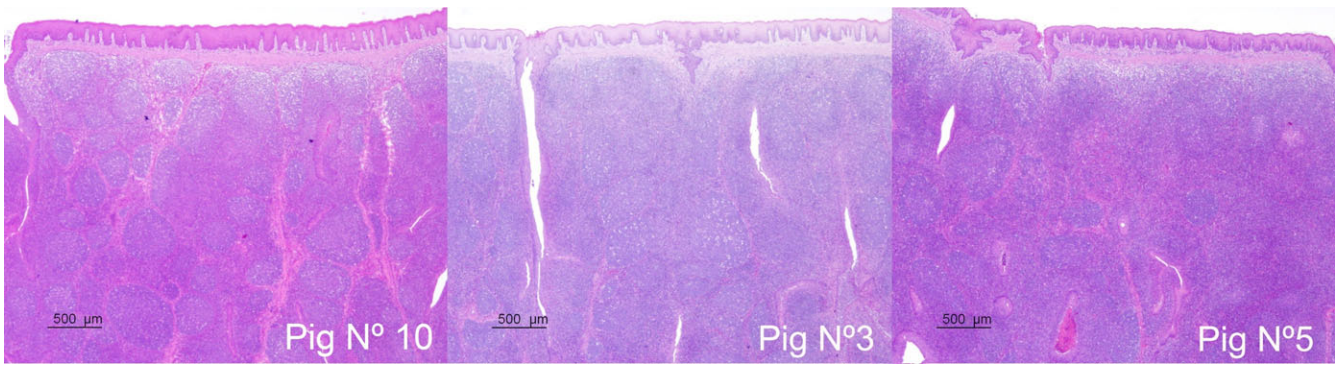

FIGURE 2 Histopathological evaluation of tonsil samples from CSFV infected pigs at 6 weeks post infection. Haematoxylin and eosin staining of tonsil microdissections from an uninfected animal (pig 10), one CSFV infected pig that seroconverted (pig 3) and a CSFV infected pig that showed permanent viremia (pig 5) [Colour figure can be viewed at wileyonlinelibrary.com]

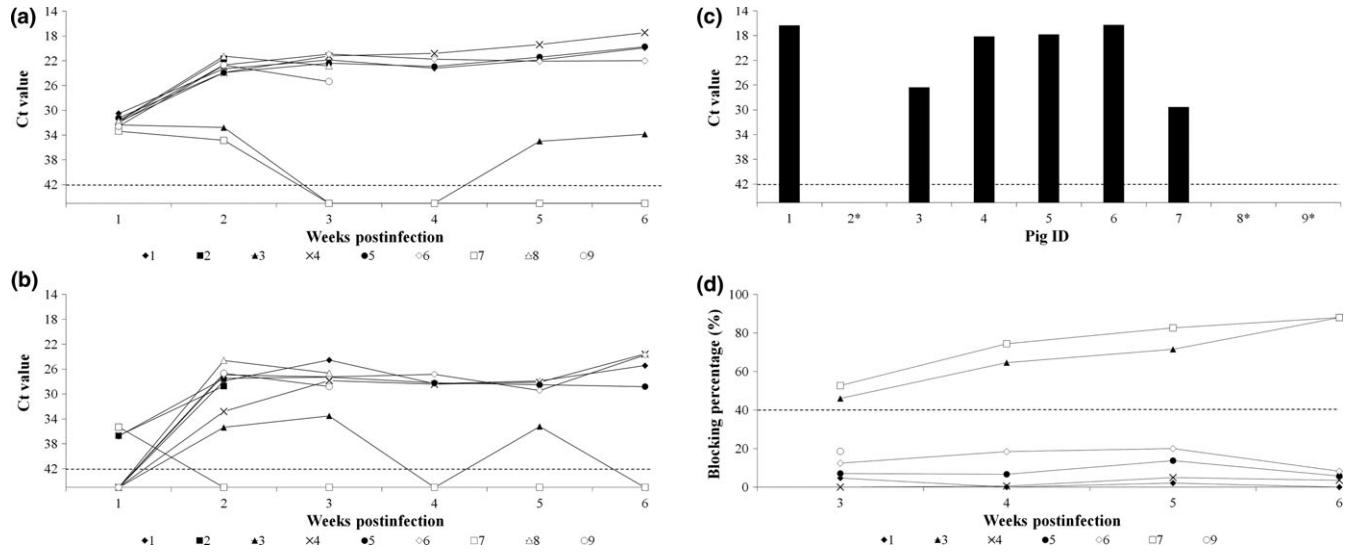

FIGURE 3 Detection of CSFV RNA and specific humoral response during 6 weeks of infection in pigs infected at 21 days of age. CSFV RNA was evaluated weekly in sera (a) and rectal swab (b) samples as well as in tonsil at time of euthanasia (c). Ct values above 42 (dotted line) were considered as negative. Antibodies against the E2 glycoprotein of CSFV were evaluated weekly (d). Blocking percentage values above 40% (dotted line) were considered as positive

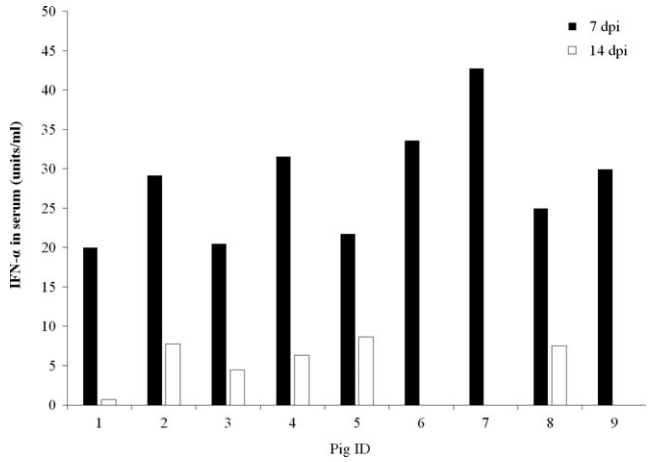

FIGURE 4 IFN-α levels in serum from CSFV infected pigs. IFN-α was evaluated at 7 (black bars) and 14 dpi (white bars) in CSFV infected pigs

3.6 | Increased T-CD8$^+$ subset targeted by CSFV in pigs showing permanent viremia

The percentage of T-CD4$^+$ cells oscillated between 17.05% and 18.3% in samples from pigs 3 and 7, while for pigs 5 and 6 the values were lower, ranging from 10.3% to 11.3%, being similar in the control pigs (10.8% and 12.3%). Additionally, CD4$^+$/CD8$^+$ double positive cells were found to be 0.8% in pigs 3 and 7 and lower values (0.3%) were detected in pigs 5 and 6. In control animals, percentage values of 0.4% and 3.2% were also detected (Figure 5). The CD172a cell population ranged between 3% and 9% in PBMCs from infected pigs, reaching in animals 3 and 7 from 8.3% to 9.05% and in samples from pigs 5 and 6 from 3.1% to 6.9%. This cell population ranged from 6% to 12% in control animals (Figure 5). The T-CD8$^+$ subset oscillated from 16.2% to 32.4% in infected animals. Notably, samples from animals 3 and 7 were between 19.15% and 16.2%. Higher values of 29.3% and 32.4% were found in pigs 5 and 6 respectively. Meanwhile, T-CD8 marker from control samples ranged from 8.2% to 14% (Figure 5).

The T-CD8$^+$ cells were sorted from PBMC of pig number 5 (29.3% of T-CD8$^+$ cells). With a purity over 91%, a total of 1373002 cells were recovered. While, 3350150 T-CD8$^-$ cells were obtained (purity over 98%). CSFV RNA was detected in the T-CD8$^+$ cells with a Ct value of 25.82. In the case of T-CD8$^-$ cells, the Ct value was 24.41. The presence of CSFV was confirmed by viral isolation test in PK-15 cells.

3.7 | Low CD4/CD8 ratio in pigs showing permanent viremia

Finally, the CD4/CD8 ratio was calculated, values ranging from 0.31 to 1.05 resulted in samples for infected animals. The lower ratio was found in pigs 5 and 6 with value about 0.3 in both of them. On the contrary, the CD4/CD8 ratio resulted in values about 1 for pigs 3 and 7. Likewise, the values obtained in PBMC from naïve pigs were 0.95 and 0.88 (Figure 6).

4 | DISCUSSION

Here, we show that persistently infected piglets could also be generated following infection using a previously characterized CSFV strain (Muñoz-González, Ruggli, et al., 2015) at 3 weeks after birth, an age in which the weaning could be carried out in some swine

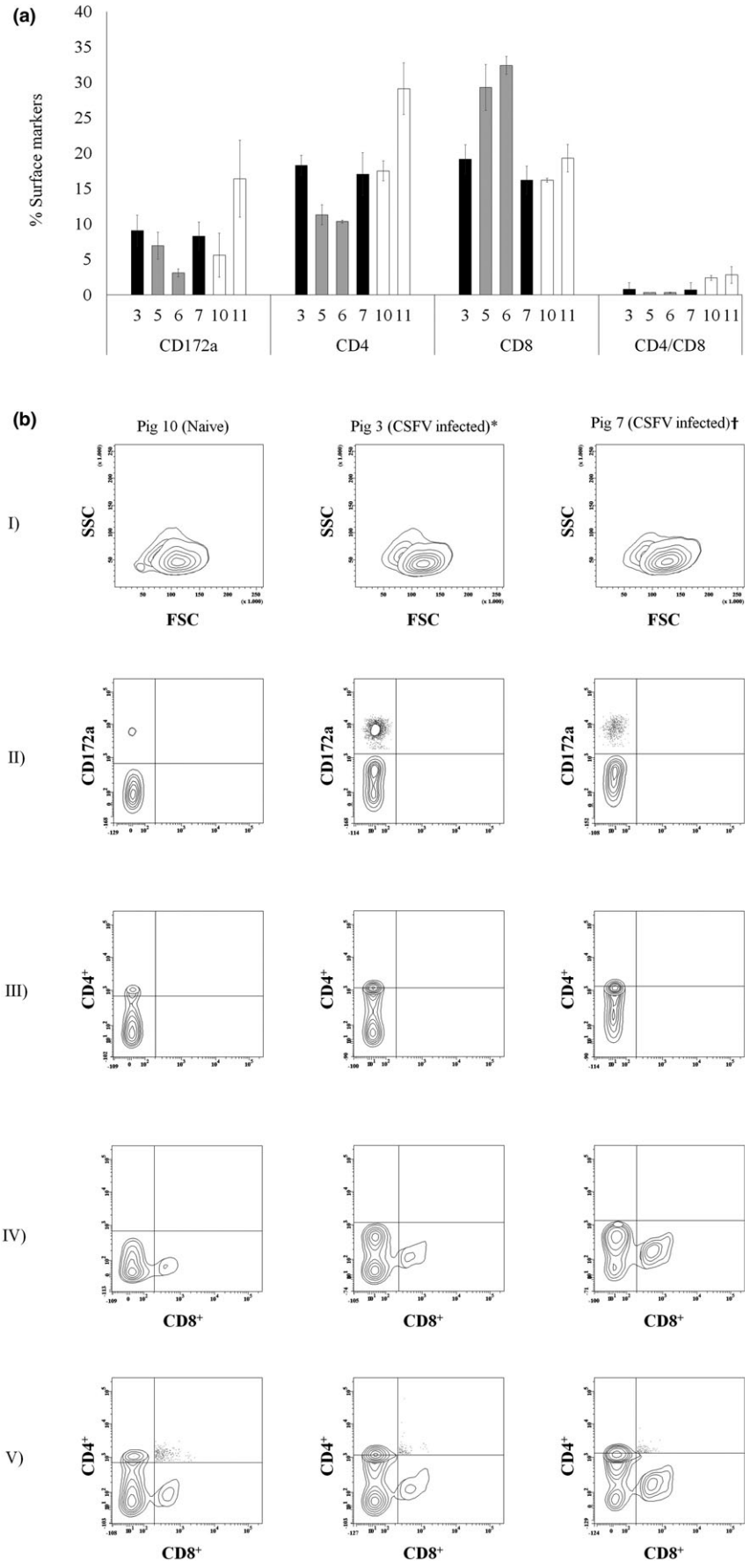

FIGURE 5 Expression of cell surface markers in PBMCs from naïve and CSFV infected pigs at 4 weeks post infection. (a) Phenotypic profile in PBMCs from CSFV seropositive (black bars) seronegative (grey bars) and naïve (white bars) animals. (b) Comparative phenotypes in PBMCs from an animal representative of naïve and CSFV infected pigs. *Indicates an infected pig that seroconverted, †Indicates an infected pig that did not seroconvert. Parameters evaluated included (I) forward scatter (relative cell size, *x*-axis) and side scatter (relative granularity, *y*-axis) and expression of cellular markers CD172a (II, *y*-axis), CD4 (III and V, *y*-axis), CD8 (IV and V, *x*-axis) and CD4/CD8 double positive cells (V). The experiments were repeated twice under the same conditions

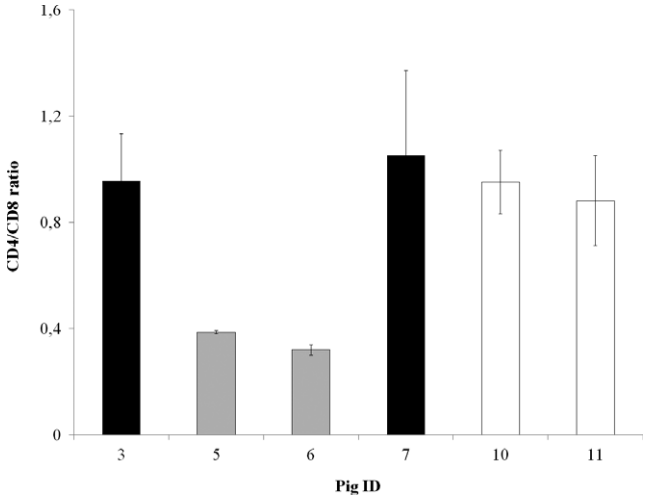

FIGURE 6 CD4/CD8 ratio in naïve and CSFV infected animals at 28 days post infection. The CD4/CD8 ratio was calculated for pigs that seroconverted (black bars), pigs that did not seroconvert (grey bars) and naïve animals (white bars)

production systems (Mahan, Cromwell, Ewan, Hamilton, & Yen, 1998; Park, Ha, Park, & Lee, 2014). After the infection that was monitored during 6 weeks, besides the increase in rectal temperature, very mild or even no clinical signs were observed in some of the infected pigs. Notably, a significant number of these animals (44.4%) showed permanent viremia with high viral load in sera, rectal swabs and tonsils samples in the absence of CSFV specific humoral immune response for the 6-week duration of the experiment. These findings are in agreement with the profile previously described for persistently infected pigs that were infected during the first hours after birth (Muñoz-González, Ruggli, et al., 2015). Considering that, the proportion of animals that may develop CSFV persistent infection decreases with age, being 60% when the infection is carried out during the first 24 hr after birth (Muñoz-González, Ruggli, et al., 2015) to 44.4% when it is performed at 3 weeks of age. Nevertheless, taking into account the important role of persistently infected animals in the CSF control (van Oirschot, 2003; Van Oirschot & Terpstra, 1977), the proportion of persistently infected animals remains significant when the infection takes place at 3 weeks of age.

A previous report also showed that humoral response is not generated after newborn piglets infection with the CSFV Cat01 strain during 6 weeks (Muñoz-González, Ruggli, et al., 2015). By contrast, when animals are infected at 3 weeks old, this viral strain was capable to generate humoral response starting the second and the third weeks post infection, although in a small proportion of infected animals (22.2%). Hence, the age of infection also plays a relevant role in the establishment of an immune response against CSFV. Furthermore, these two pigs were capable to clear the virus or reduce the viral load in serum samples, rectal swabs and tonsils considerably compared to persistently infected animals. However, both animals became CSFV asymptomatic carriers. On the other hand, similar to previous work, some of the infected animals from this trial (33.3%) either died or had to be euthanized due to the severity in the clinical manifestation. Likewise, all of infected animals showed high rectal temperature values during the first 2 weeks after infection, being in agreement with a previous report (Muñoz-González, Ruggli, et al., 2015).

It is noteworthy that some infected pigs in the present work, including some of persistently infected animals, developed vesicular lesions, ulcerations and skin desquamation after 3 weeks post infection. This fact highlights the ability of CSFV in its interaction with the host to generate a wide variety of clinical signs (Blome et al., 2017; Ganges et al., 2008; Tarradas et al., 2014). Therefore, the CSF persistent forms may manifest clinical signs so far never described. It is likely that, given the level of immune suppression (Muñoz-González, Ruggli, et al., 2015), the clinical manifestations that may develop in these animals could be related to the endemic pathogens circulating in each environment in coinfection with CSFV.

One of the most common anatomopathological findings associated after CSFV infection is found in the tonsil, a target tissue for CSFV replication and wherein the virus persists for more than 30 days post infection (Koenig et al., 2007; Vrancken et al., 2009). The most relevant histopathological finding in the tonsil are congestion of the blood vessels along with focal to diffuse areas of haemorrhage (Malswamkima, Rajkhowa, Chandra, & Dutta, 2015). Necrosis and depletion of lymphocytes, haemorrhages and tonsillitis have also been described (Blome et al., 2017; Quezada et al., 2000). Notably, despite the high viral load detected in the tonsils from persistently infected pigs, neither macroscopic nor histopathological lesions were found, a fact that may hinder the detection of this type of infected animals.

IFN-α levels in sera after CSFV infection are indicative of the activation of innate immunity to the virus (Summerfield, Alves, Ruggli, De Bruin, & McCullough, 2006; Tarradas et al., 2014). Remarkably, the levels of IFN-α detected after 7 days post infection were similar to those observed in newborn piglets (Muñoz-González, Ruggli, et al., 2015) and in 10-week-old pigs infected with the same CSFV strain (Cat01) (Tarradas et al., 2014). Thus, we consider that the innate immune response, in terms of IFN-α levels in sera, is similarly induced in the above mentioned ages. Therefore, even though an immunotolerance related with the age of the host might be taking place in the pathogenesis of CSFV persistent infection, further studies will clarify the role of this mechanism.

Likewise, it should be noted that previous studies have shown that a hallmark of acute CSF form is the high IFN-α levels found in the serum early after infection with strains of high degree of virulence, being this cytokine related to the cytokine storm phenomenon previously described in this disease form (Tarradas et al., 2014). So, unlike pigs that suffer from the CSF acute form and in agreement with previous studies, CSFV persistently infected animals from the present study also showed lower IFN-α levels (Muñoz-González, Ruggli, et al., 2015; Tarradas et al., 2014). Hence, the impact of this phenomenon may be decreased or has not been detected thus far, explained in part the difference in the clinical outcome between both types of CSF forms.

To understand the effect of infection in the different immunological profiles found in CSFV infected pigs, we focussed on the main T lymphocyte populations as well as a myelomonocytic marker. An increase in the T-CD8 subset was observed in persistently infected pigs in comparison with pigs that seroconverted and the naive animals. After the evaluation of the viral load in this sorted cell population, we proved that these cells were CSFV infected. Thus, the CSFV infected T-CD8 cells subset may promote the virus dissemination into the host and may aid the virus persistence.

The T-CD4 cell population was found decreased in the two persistently infected animals analysed. Previous reports have underlined the role of porcine T-CD4 cells in activation of an effective anamnestic response against CSFV (Ganges et al., 2005). Considering that, the effect of CSFV infection in T-CD4 cells from persistently infected pigs may aid in understanding the lack of humoral response in this form of infection and subsequently the immunological anergy state. The decrease in the T-CD4 cell population may impair the development of an effective adaptive immune response and also favour the viral persistence (Agrati et al., 2016).

As we explained above, the CD4/CD8 ratio correlates with the effectivity of the immune response against different infection models (Agrati et al., 2016; Serrano-Villar, Moreno et al., 2014; Serrano-Villar, Sainz et al., 2014). The low ratio values have been associated with an immune exhausted state with a strong immunosuppression in infected hosts (Ferrando-Martinez et al., 2011; Serrano-Villar, Moreno et al., 2014; Serrano-Villar, Sainz et al., 2014). Immune exhaustion is a phenomenon in which the constant activation by viral antigens or other mechanisms causes some effector or activated T cells to persist but become functionally unresponsive to further antigen stimulation (Yao & Moorman, 2013). This phenomenon is associated with chronic and persistent infections, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection (Dustin, 2017; Ferrando-Martinez et al., 2011; Serrano-Villar, Moreno et al., 2014; Serrano-Villar, Sainz et al., 2014). Previous reports assert that CD4/CD8 ratio values around 1 correlated with the generation of an effective immune response in swine (Appleyard, Furesz, & Wilkie, 2002; Cordes, Riber, Jensen, & Jungersen, 2012). Accordingly, the CD4/CD8 ratio values in the CSFV seropositive pigs were around 1. By contrast, in CSFV persistently infected pigs, that showed high and constant viral load, we found a lower CD4/CD8 ratio with respect to naïve or CSFV antibody positive pigs. This finding is in agreement with studies in persistent infection in humans, where the low CD4/CD8 ratio correlated with high viral load and an inability of the immune system to clear the virus (Gandhi et al., 2017). Also, it has been established that during HIV persistent infection, the T-cell functions including cytokine secretion and proliferative capacity appear to decrease gradually, being associated with the immune exhaustion phenomenon (Khaitan & Unutmaz, 2011).

Consequently, considering the low CD4/CD8 ratio and the reduced impact of the cytokine storm phenomenon, at least in terms of IFN-α, evidenced in CSFV persistently infected animals after super-infection with CSFV or African swine fever virus (Cabezón et al., 2017; Muñoz-González et al., 2016); we suggest the possible implication of the immune exhaustion mechanism that may favour the constant and high CSFV replication levels during CSFV persistence (Cabezón et al., 2017; Muñoz-González et al., 2016). Nevertheless, further studies will be performed to clarify the role of the immune exhaustion phenomenon and other immunosuppressive disorders in the CSFV pathogenesis.

Taken together, our findings provide the first report of CSFV postnatal persistent infection in pigs at 21 days after birth. This type of infected animals shed high amounts of virus and are difficult to evaluate from the clinical and anatomopathological point of view. Considering that, persistent infected pigs may go unnoticed in the serological methods used for surveillance, as well as being unresponsive to vaccination (Muñoz-González, Perez-Simó, et al., 2015). Therefore, the detection of this type of infection and its elimination in endemic areas is relevant for global CSF control. Finally, our findings support the use of the CSFV postnatal persistent infection as a model in the study of immunological phenomena associated with viral persistence.

ACKNOWLEDGEMENTS

This research was supported by grant AGL2015–66907, Ministerio de Ciencia, Innovación y Universidades from the Spanish government. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from Spanish government. M.W. has a pre-doctoral fellowship CSC scholarship (2017) from Chinese government. The authors thank Ivan Cordón and the animal facility staff for their help during the in vivo part of the experiment as well as Maria Jesus Navas for her assistance in the flow cytometry assay.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Bohórquez JA, Wang M, Pérez-Simó M, Vidal E, Rosell R, Ganges L. Low CD4/CD8 ratio in classical swine fever postnatal persistent infection generated at 3 weeks after birth. *Transbound Emerg Dis*. 2018;00:1–11. https://doi.org/10.1111/tbed.13080