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The impact of the CSFV virulence strain on the immune response to control an infection

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Short title: CSFV infection and immune response

ABSTRACT

In the present work, we studied the infection caused by the low virulence strain Cat01 in domestic pigs, which is responsible for the 2001- 2002 CSFV outbreak in the Catalonia region in Spain, in comparison with the infection caused by a CSFV virulent strain responsible for inducing the acute form of CSF in the field. We focused on assessing the impact of CSFV virulence on the kinetics of different cytokines, such as IFN-α (innate immunity) and IFN-γ (adaptive immune response), during the first weeks after infection. In addition, we evaluated the impact of CSFV virulence on the induction of the humoral immune response and its relation to the course of infection and the RNA CSFV viral load. IFN-α levels in the serum samples from pigs infected with the low virulent strain were lower than those detected in pigs infected with the virulent one. After the infection with the low virulent strain, some IFN-γ response against CSFV was detected. Likewise, the humoral response was detected only in pigs infected with the low virulent strain. Noteworthy, the lowest load of CSFV RNA was detected in the serum samples of these animals. Similarly, the lowest viral load levels were detected in the tonsils of these pigs. Both the T cells and the humoral response generated in most of the pigs infected with strain Cat01 could be related to the protection in the symptom progression of CSF against this CSFV strain. However, despite the levels of protection observed, this strain still impaired the adaptive immunity against CSFV, as not all animals developed a detectable T-cell response and adequate levels of neutralising antibodies that are capable of neutralising the virus at both the serum and the tissue levels. Thus, RNA viral detection was observed in the tonsils from all Cat01-infected pigs. This fact can encourage the spread of the virus and its persistence after infection with CSFV low viral strains. These types of results explain in part why CSFV are capable of producing an

outbreak that goes unnoticed during the first months of infection and how difficult its resulting eradication is in endemic countries today.

INTRODUCTION

Classical swine fever (CSF) is a highly contagious and deadly viral disease caused by a small enveloped single-stranded RNA virus that belongs to the genus Pestivirus within the family *Flaviviridae* (Heinz et al., 2004). Today, CSF represents one of the leading threats to the pig industry worldwide (Wang et al., 2008). The virus is known to have tropism for vascular endothelial and immune system cells, mainly those derived from the monocyte-macrophage lineage (Summerfield et al., 1998, Summerfield et al., 2001). It is assumed that the infection of these cells plays an important role in the disease epidemiology and pathogenesis, for instance for the spread and the persistence of this virus, as well as for immunosuppression.

Depending on the virulence of the strain and the immunological status of the infected animals, pigs can have as much as 90% of their total T cells depleted in the final stages of the disease (Pauly et al., 1998). This effect can be observed as early as one day after infection, even before viraemia has been established (Summerfield et al., 1998). Immunosuppression can be detected much earlier than seroconversion and the presence of clinical signs, which is relevant for early diagnosis and for the study of viral pathogenesis (Ganges et al., 2008, Pauly et al., 1998).

Type-I interferon (IFN) has antiviral and immunomodulatory effects and is a component of the innate immune response against viruses. It is attributed a beneficial effect in viral infections, restricting the viral dissemination and promoting immunopathological events when released at high levels over longer periods (Summerfield et al., 2006). However, CSFV exacerbates the IFN-α response that is detected in the serum of infected pigs, and this has been suggested to relate to disease

75 severity rather than to protective immune responses (Summerfield et al., 2006; Tarradas
76 et al., 2010; Summerfield, 2011).

77 Depending on the virulence of the strain, varying degrees of disease severity can
78 be observed, ranging from acute to subclinical or chronic forms (van Oirschot 1988;
79 2004). Interestingly, whereas infections with virulent strains result in an acute
80 hemorrhagic disease, the infection caused by less virulent isolates can become chronic.
81 Due to the severity of the disease, the acute form of the infection has been widely
82 studied, whereas there is very little information about the pathogenesis of these types of
83 CSFV strains and their relation with the disease progress. Curiously, a trend toward
84 milder, chronic presentations of CSF has been observed as a consequence of years of
85 several CSF outbreaks in different endemic countries (Pérez et al., 2012; Shen et al.,
86 2011). In addition, pigs infected with low-virulence strains are known to shed virus
87 continuously or intermittently for months, representing a major threat not only for
88 countries endemically infected but also worldwide (Pérez et al., 2012, Weesendorp et al.,
89 2011).

90 In spite of the potential involvement of the T cellular response against CSFV vaccines,
91 the principal role of IFN-γ is to control the infection (Tarradas et al., 2010; Graham et
92 al., 2012). The immune response against CSFV infection with strains of various levels of
93 virulence is poorly understood.

94 In the present work, we studied the infection caused by the low virulent strain
95 Cat01 in domestic pigs, which was responsible for the 2001- 2002 CSFV outbreak in the
96 Catalonia region in Spain, in comparison with the infection caused by a CSFV virulent
97 strain responsible for inducing the acute form of CSF in the field. We focused on
98 assessing the impact of CSFV virulence on the kinetics of various cytokines, such as IFN-
99 α (inmate immunity) and IFN-γ (adaptive immune response), during the first weeks after

infection. In addition, we evaluate the impact of CSFV virulence on the induction of humoral immune response and its relation to the course of infection and the viral load of CSFV RNA.

MATERIALS AND METHODS

Cells and viruses

The porcine kidney PK15 cell line was used to grow and titre the viral stocks. The cells were cultured in DMEM media (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) at 37ºC in 5% CO_2. PK15 were infected with a 0.1 $TCID_{50}$/cell using DMEM media with 2% FBS, and the virus was harvested at 48 h post-infection. Stock titration was performed using the peroxidase-linked assay (PLA) (Wensvoort et al., 1986), following the statistical method previously described (Reed and Muench, 1938). The Catalunya 01 (Cat01) viral strain used in this study was isolated from CSF Spanish epizootic in 2000-2001. This isolate was included by sequence analysis into the CSFV 2.3 genogroup (Pérez et al., 2012). The course of the infection at the farm, where this virus was isolated, was found to be mild (Allepuz et al., 2007). The virulent Margarita strain belongs to the 1.4 genotype, and has been used in Cuba since 1965 for a vaccine potency test (Díaz de Arce et al., 1999; Ganges et al., 2005).

Experimental design

To evaluate the pathogenesis induced by both strains, we used three groups of domestic pigs. Each group included six Landrace x Large White healthy pigs of ten weeks of age, free of porcine circovirus type 2, porcine reproductive respiratory syndrome virus and Pestivirus. The groups were: group 1 or the Margarita group (numbered 1-6), group 2 or the Cat01group (7-12), and group 3 or the control, non-infected group (13-18). The animals were experimentally infected by i.m. injection in the neck with 10^5 $TCID_{50}$ of

CSFV Margarita, Cat01strains, and PBS, respectively. After infection, serum samples and nasal swabs were collected at 0, 7, 13 and 17 days post-infection (DPI); whole blood samples at 0, 7 and 13 DPI and tonsils samples were taken at the time of necropsy (for Margarita group) or at 18 DPI (for Cat01 and control group). A trained veterinarian recorded the rectal temperature and the clinical signs of disease daily in a blind manner. Clinical signs compatible with a CSFV infection were anorexia, conjunctivitis, diarrhoea, constipation, abdominal petechiae, and prostration. In addition, the presence or absence of nervous symptoms was recorded independently. The clinical status of the animals were scored from 0 to 7, rating fever, clinical signs (not including nervous symptoms) and nervous symptoms as follows: 0 - no signs; 1 - mild pyrexia; 2 - pyrexia plus mild clinical signs; 3 - mild-moderate clinical signs but absence of nervous disorders; 4 - slight nervous disorders and moderate rest of clinical signs; 5 - moderate nervous disorders and moderate–severe rest of clinical signs; 6 - severe clinical signs (including nervous disorders); 7 - death. For ethical reasons, the animals were euthanised either when the clinical score reached 5 or higher or when showing prostration and moderate–severe nervous disorders. After euthanasia, an exhaustive necropsy was conducted in which the presence of pathological signs in different organs and tissues was evaluated. The experiments were approved by the Ethics Committee for Animal Experiments of the University Autonomous of Barcelona (UAB) according to existing national and European regulations.

Detection of CSFV RNA

The samples from sera, nasals swabs and tissue homogenates from nasal swabs and tissues were processed following the methodology described previously (Díaz de Arce et al., 1998; Pérez et al., 2011). The RNA was extracted from sera, nasals swabs and tissue homogenates using the RNA viral isolation kit Nucleospin II according to the

manufacturer's instructions (Macherey-Nagel). In all cases, an initial volume of 150 µL was used to obtain a final volume of 50 µL of RNA, which was stored at −80 °C. The real-time RT-PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany). Specific CSFV detection was performed in serum**,** nasals swabs and tonsil samples by using primer pairs targeting a region corresponding to the NS5B protein (Díaz de Arce et al., 1998). The serum and nasals swab samples were collected at 0, 7, 13 and 17 DPI. The improved primer set concentration and thermocycling conditions have been recently described (Pérez et al., 2011). The limits of detection used were 1, 10 and 10^2 gene copies/µl for nuclease-free water, serum and tissue homogenate, respectively.

Antibody detection

To evaluate the humoral response, we followed the methodology as described previously (Ganges et al., 2005; Tarradas et al., 2010; Tarradas et al., 2011). Serum samples were collected from all animals at 0, 7, 13 and 17 DPI. The serums were tested for neutralising antibodies using a neutralisation peroxidase-linked assay (NPLA) (Terpstra et al., 1984), and titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of Catalunya and Margarita strains, respectively, in 50% of the culture replicates. Detection of E2 specific antibodies was performed using a commercial ELISA (CEDITEST; Lelystad); samples were considered to be positive when blocking percentages ≥40%, following the manufacturer's recommendations.

ELISA for IFN-α and IFN-γ detection in serum samples

Anti-IFN-α monoclonal antibodies (K9 and K17) and IFNα recombinant protein (PBL Biomedical Laboratories, Piscataway, New Jersey) were used in the ELISA assay (Nowacki and Charley et al., 1993; Guzylack-Piriou et al., 2004). For IFN-γ detection, mAbs P2G10, P2C11 and IFN-γ recombinant protein SD066 were used (BD Biosciences),

(De Antonio et al., 1998). The 3,3',5,5'- tetramethylbenzidine (TMB) was from Sigma. For each ELISA test, the cut-off value was calculated as the average of the optical density of negative controls (blank and negative serums before CSFV infections) plus three standard deviations. Cytokine concentrations in the serum were determined using a regression line consisting of the optical densities of the cytokine standards used in each test.

PBMCs and Elispot assay for CSFV-specific IFN-γ detection

Peripheral blood mononuclear cells (PBMCs) were obtained from total blood collected at 0, 7 and 13 DPI and were separated by density-gradient centrifugation with Histopaque 1077 (Sigma). The PBMC number and the viability of the cells were obtained by staining with Trypan Blue (Ganges et al., 2005). The Elispot assay to detect CSFV-specific IFNγ cells was performed as previously described (Tarradas et al., 2010). In brief, plates (Costar 3590, Corning) were coated overnight with 5 μg/ml of capture antibody (P2G10, Pharmigen). Detection was performed using a biotinylated antibobody (P2C11, Pharmigin). A number of samples with $5x10^5$ PBMC/well were plated in triplicate at 0.1 multiplicity of infection (MOI) of the CSFV Cat01 and Margarita strains, respectively. Controls were incubated in the absence of virus or with phytohaemagglutinin (PHA) (10 μg/ml). The counts of spots in the media for mock-stimulated wells were considered to be the baseline for the calculation of antigen-specific frequencies of IFN-γ producing cells.

Statistical Analysis

All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) using "pig" as the experimental unit. The significance level (α) was set at 0.05, with statistical tendencies reported when $P<0.10$. Throughout the trial, a non-parametric test (Mann–Whitney) was chosen to compare values obtained from the immunological and

the clinical parameters between groups. This non-parametric analysis was chosen due to the number of animals used in each experimental group.

RESULTS

Development of clinical signs

The clinical score results showed that no pigs infected with the Cat01 strain reached level 3 in the clinical score value (grading from 0 to 7), and moreover, level 2 (pyrexia plus mild clinical signs) was not observed until days 11 to 15 post-infection (Fig. 1 and 2). Animals infected with the Margarita strain developed pyrexia (rectal temperature above 40ºC) after day 2 days post-infection, with peaks reaching 42º C. From day 3 until the end of the trial, the rectal temperature was always lower in the Cat01 group than in the Margarita group ($p<0.05$) (Fig. 1). In Margarita-strain infected pigs, moderate and severe clinical signs ($\geq$ 4 points in score value), such as anorexia, conjunctivitis, diarrhoea, constipation, abdominal petechiae, severe nervous signs, and prostration were observed from day 5 up to day 12 -13 post-infection, when animals were euthanised for ethical reasons after reaching level 5 or more in the clinical score valuation (Figure 1 and 2). The difference in the clinical score values for the Cat01 and the Margarita strains were statistically significant from day 3 until the end of the trial ($p < 0.05$) (Fig. 2).

Virus detection in serum, nasals swabs and tonsils

Viral RNA in the serum and the nasals swabs was detected from day 7 post-infection in all infected animals (Fig 3a, 3b). The RNA viral load was higher for the Margarita-strain infected pigs in the serum and the nasals swabs than for the Cat01-strain infected pigs in all samples evaluated ($p<0.05$). Three out of the six animals infected with the Margarita strain were tested at 13 DPI and showed similar RNA loads to the ones previously determined at 7 DPI. The other three animals were either dead or were euthanised before

13 DPI since they reached a clinical score of 5 (Fig 2). At day 17 PI, only pigs infected with the Cat01 strain could be evaluated. Interestingly, only one pig (number 11) could clear the RNA virus in the serum and the nasals swabs (Fig. 3a, 3b). However, all Cat01-infected pigs were positive to RNA viral detection in the tonsil samples. Nonetheless, the RNA viral load was significantly higher for the Margarita-strain infected pigs than for those infected with Cat01 ($p<0.0005$) (Fig 3c).

E2-specific antibodies and neutralising activity at 13 and 17 DPI detected in pigs infected with Cat01 CSFV

The serum Samples from the infected animals and the control pigs were analysed for the presence of E2-specific antibodies (ELISA) and for neutralising antibodies (NPLA); no antibodies were detected before 13 DPI (data not shown). Interestingly, the presence of E2-specific antibodies detected by ELISA was observed in almost all infected animals with the Cat01 strain from day 13 to 17 PI (Fig 4). This observation could not be made for pigs infected with the Margarita strain since these animals died or were euthanised at approximately 13 DPI, but at the time of death, there were no detectable antibody levels. Neutralising antibodies were detected in two pigs infected with the Cat01 strain at 13 DPI and in four pigs at 17 DPI (Table 1), particularly in animals number eight and eleven, which had high neutralising antibody titrations (>32).

Detection of levels of IFN-α and IFN-γ in the serum samples of Cat01-infected pigs

The IFN-α cytokine levels in the serum samples obtained from Cat01- and Margarita-strain infected animals were compared. The results showed significantly lower IFN-α levels in all Cat01-infected pigs at day 3 ($p<0.003$), and at day 7 post-infection ($p=0.01$) (see Fig 5a) than those observed in animals infected with the Margarita strain. Levels of IFN-γ in the serum samples were determined by the ELISA technique (Fig 5 b). No

cytokines were detected for the Margarita-strain infected pigs (pig 1 to 6) or for the control pigs. Interestingly, for the Cat01 strain, significant amounts of the cytokine were detected in all samples. The ELISA results for IFN-γ were positive for five animals at day 7 PI and for one animal at day 13 PI. The IFN-γ serum concentration was significantly higher for Cat01- ($p=0.01$) than for Margarita-strain infected pigs (no detectable levels) at day 7 post-infection (Fig. 5b).

CSFV-specific IFN-γ producing cells after infection

The Elispot results showed that the CSFV-specific IFN-γ producing cells response was not detectable in pigs infected with the Margarita strain. Therefore, the response detected in pigs infected with the Cat01 strain was statistically significant ($p=0.0025$) at 7 and 13 DPI (Fig. 5c).

DISCUSSION

The severity of the acute form of CSF responsible for the high mortality rate, the auto-limiting nature of the virulent virus strains, and the pathogenesis of these forms have been the subject of many studies (Hüsser et al., 2012; Summerfield et al., 2009; Ganges et al., 2005; Sánchez-Cordon et al., 2005; Knoetig et al., 1999). Nevertheless, some animals are likely to develop a chronic, or worse, unapparent form of the disease, which can be misdiagnosed because of the confusing pathological picture and can be the source of virus dissemination for an extended period of time (Weesendorp et al., 2010; Pérez et al., 2012, Rout et al., 2012).

In most cases in the outbreak of early 2000 in Spain, the animals presented nonspecific clinical signs consisting only of fever and apathy (Allepuz et al., 2007). In the present work, we studied the infection caused by the Cat01 strain in domestic pigs, responsible for the 2001- 2002 CSFV outbreak in the Catalonia region in Spain in comparison with the

infection caused by the Margarita strain, responsible for inducing the acute form of CSF in the field (Diaz de Arce et al., 1999; Ganges et al., 2005). We focused on assessing the impact of CSFV virulence on the kinetics of various cytokines involved in the innate and the adaptive immune responses to infection as well as the humoral response generated and its relation to the course of infection and the RNA CSFV load during the first weeks after CSFV infection. In accordance with the data previously collected in the field (Allepuz et al., 2007), the Cat01 strain caused a very mild form of the disease with almost unapparent CSF clinical signs in the experimentally infected pigs (Fig. 1 and 2). Nevertheless, the viral RNA load was detected in the majority of the samples tested, including in the tonsils of Cat01-infected pigs at 18 DPI, although the detected RNA loads were significantly lower than those detected in pigs infected with the virulent strain (Fig. 3). The results of this study are in agreement with those suggesting that the RNA CSFV load detected in the serum, the nasal swabs and the tonsils has a direct correlation between strain virulence and the clinical signs developed (Donahue et al., 2012, Uttenthal et al., 2003, Weesendorp et al., 2010).

Type I IFN responses are usually associated with the antiviral defences of the host. They are induced by viral infection or double-stranded RNA (dsRNA), a by-product of viral replication, and lead to the production of a broad range of antiviral proteins and immunoactive cytokines (Haller et al., 2006). However, in accordance with previous reports, in our in vivo experiment, we detected high levels of IFN-α in the sera of pigs infected with the CSFV virulent strain after infection (Tarradas et al., 2010; Renson et al., 2010; Rosen et al., 2013). In contrast, pigs infected with the Cat01 strain, which developed a very mild or unapparent CSF disease, expressed delayed and lower levels of IFN-α (Fig. 5a), associating the IFN-α levels with the severity of the disease after infection. Similar findings were obtained for the influenza virus strains, where low pathogenic strains could

294 be related to low levels of type-I IFN (Moulin et al., 2011). Our results support the
295 previously reported correlation between the exacerbation of the type-I IFN production in
296 the host by virulent CSFV strain, the degree of severity of the illness, and the level of
297 replication and detection of the CSFV (Fig. 1, 2 and 5a) (Renson et al., 2010).
298 Additionally, type-I IFN is known to promote apoptosis in infected and non-infected cells
299 (Sivaraman et al., 2011, Tanaka et al., 1998, Thyrell et al., 2002). It has been reported that
300 there is a very strong correlation between high IFN-α levels in pigs with the acute form of
301 CSF and lymphopenia caused by bystander apoptosis (Renson et al., 2010, Summerfield et
302 al., 2009, Sun et al., 2010). Likewise, the surviving animals infected with the Margarita
303 strain showed very severe clinical signs and leucopoenia at 13 DPI (Figure 1, 2 and Table
304 2). Conversely, pigs infected with the less virulent strain (CAT01) showed a lower degree
305 of impact on the number of PBMC after infection (Table 2). The severe lymphocyte
306 depletion observed in the acute CSF reflects the failure of the adaptive response in pigs
307 infected with the Margarita strain (Ganges et al., 2005, Renson et al., 2010, Summerfield
308 et al., 2006).

309 In general, for highly virulent hemorrhagic fever viruses, the failure of the adaptive
310 immune responses can play a crucial role in its lethality (Bray et al., 2005). For example,
311 data from Ebola outbreaks indicate that failure to develop specific antibodies by the
312 second week of illness is predictive of death (Ksiazek et al., 1999, Sanchez et al., 2004).
313 Our results confirm the ineffectiveness of highly virulent CSFV strains to induce
314 neutralising antibodies after 13 days post infection (Ganges et al., 2005, Tarradas et al.,
315 2010).

316 It is noteworthy that previous studies indicate the role of specific IFN-γ
317 producing cells, mainly the $CD4^{+}CD8\alpha^{low}$ double-positive T cells (memory), in the
318 elicited protection against CSFV (Suradhat et al., 2001; Tarradas et al., 2010; Graham et

al., 2012; Franzoni et al., 2013). After the infection with the low virulent strain Cat01 at 7 and 13 DPI, and despite the slight cell depletion detectable in PBMC (Table 2), the PBMC in pigs infected with the low virulent Cat01strain were capable of being activated and produced some levels of IFN-γ response against CSFV (Fig. 4). Alongside the T-cell response, the humoral response was detected in some of the low virulent infected pigs (Cat01) among the 13 and 17 DPI, mainly in pigs 8, 10 and 11 (Figure 3 and Table 1). Curiously, in the serums samples of these animals were detected the lowest load of CSFV RNA, achieving clearance of the virus in one of them at 17 DPI. Similarly, in the tonsils of two of these animals (pigs 10 and 11) the lowest viral load levels were detected. Both the T cells and the humoral response generated in most of the infected pigs with strain Cat01 could be related to the protection in the symptom progression of CSF against this CSFV strain (Fig 1 and 2).

However, despite of the levels of protection that were observed, the Cat01 strain still impaired the adaptive immunity against CSFV because not all animals developed a detectable T-cell response and, in parallel, the adequate levels of antibodies that are capable of neutralising the virus in both the serum and the tissues. Thus, RNA viral detection was observed in the tonsils from all Cat01infected pigs, corroborating the role of this lymphoid organ in the persistence of CSFV (Ganges et al., 2008), principally in pigs infected with a low virulence strain. Thus, pigs with nearly unapparent CSF symptoms, and even negative for CSFV RNA in the serum that are positive in lymphoid tissues, and with a chance of excreting the virus (even though in low levels), can hamper the diagnosis and the control of this viral infection. This fact can encourage the spread of the virus and its persistence in endemic countries. These types of results explain in part why CSFV of low virulence strains are capable of producing an outbreak that goes

unnoticed during the first months of infection (Allepuz et al., 2007) and how difficult its resulting eradication is in endemic countries today.

Author Disclosure Statement

None

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TABLES

Tarradas et al.,

TABLE 1. Detection of neutralising antibodies titres after infection with the Cat01 strain of CSFV

TABLE 2. Total number of live PBMCs obtained per ml of blood at days 0 (T0) and 13 (T13) post CSFV infection (DPI)

Tarradas et al., Figure captions

FIGURE 1. Clinical signs after CSFV infection. (a) Individual rectal temperature values detected during the experiment. Pigs infected with the Margarita strain were euthanised between 7 and 13 DPI. Pigs infected with the Cat01 strain or the control (uninfected) pigs lived until 18 DPI, which was the end of the experiment. A temperature above 40°C was considered fever.

(b) Mean and standard deviation of the rectal temperature recorded after CSFV infection with the Margarita and the Cat01 strains. An asterisk indicates statistical significance between the two groups from day 3 until day 12 ($p < 0.05$), and the Margarita strain score is significantly higher.

FIGURE 2. Clinical signs after CSFV infection. Mean of the clinical scores after CSFV infection with the Margarita and Cat01 strains. An asterisk indicates statistical significance between the two groups from day 3 until day 13 ($p < 0.05$), and the Margarita strain score is significantly higher. The symbol † indicates euthanasia for human reasons or death. Scores are defined in the material and methods section 2.

FIGURE 3. Detection of CSFV RNA through real time RT-PCR in serum, nasal swabs and tonsils obtained from pigs infected with the CSFV Margarita and Cat01 strains. (a) Shows the serum viral loads from pigs 1-6 infected with the Margarita strain at day 7 PI, for all animals, and at day 13 for 3 animals because the other three were either euthanised or dead. Serum viral loads from pigs 7-12 were analysed at days 7, 13 and 17pi and were found to be significantly lower than those obtained in Margarita infected pigs ($p<0.05$).
(b) Shows the nasal swab viral loads from pigs 1-6 infected with the Margarita strain at day 7 PI, for all animals, and at day 13 for 3 animals because the other 3 were either euthanised or dead. Nasal swabs viral loads from pigs 7-12 were analysed at days 7, 13 and 17pi and were found to be significantly lower than those obtained in Margarita infected pigs ($p<0.05$). (c) Depicts the tonsils viral loads of Margarita- and Cat01-infected animals and highlights the differences between both groups ($p<0.0005$).

FIGURE 4. Antibody response against E2 glycoprotein detected by ELISA (in blocking %) from pigs infected with the Cat01 strain at 13 and 17 DPI. Values above 40% were considered positive.

FIGURE 5. Serum levels of IFN-α, and IFN-γ and induction of CSFV-specific-IFN-γ producing cells from pigs infected with the CSFV Margarita and Cat01 strains. (a) IFN-α levels at days 0, 3, 7 and 13 PI. These levels are significantly higher in animals 1-6 (Margarita group) than in 7-12 (Cat01 group) at days 3 and 7 PI ($p=0.003$ and $p=0.01$, respectively). (b) IFN-γ levels in serum can only be detected in pigs from the Cat01 group ($p=0.01$ at day 7 PI). (c) Lack of response to IFN-γ in the Elispot assay in pigs infected with the Margarita strain and the detection of a response in some of the pigs infected with the Cat01 strain at 7 and 13 DPI ($p<0.0025$).

Table 1:

Neutralizing antibodies vs Cat 01 strain		
Pig	**13 DPI**	**17 DPI**
7	1/5	1/10
8	1/160	1/160
9	1/5	N
10	1/5	1/20
11	1/80	>1/640
12	N	1/20

Table 2:

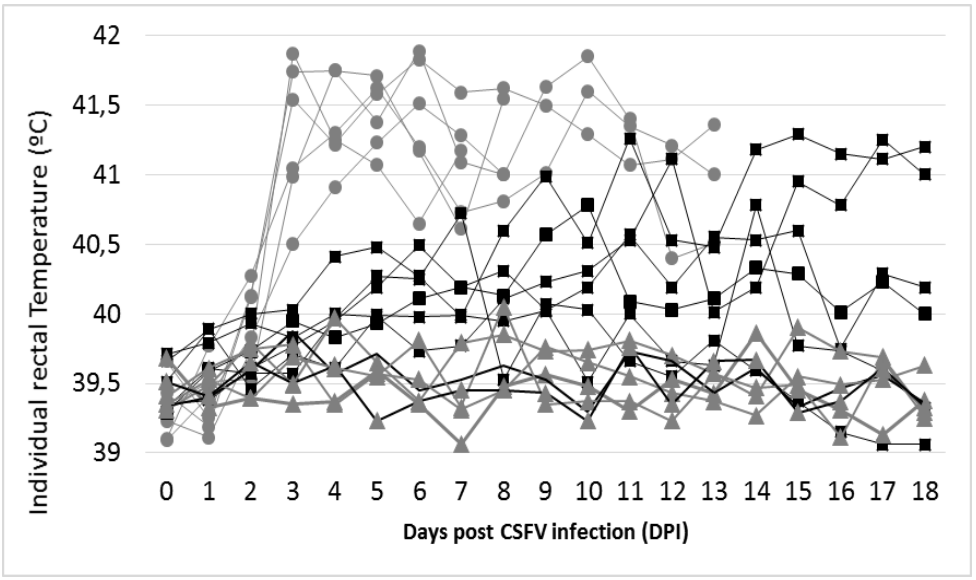
Pig	Live cells [a]	
	T0	T13
1	3.5	0.12
2	4.8	0.18
3	6.5	0.15
4	1.8	†
5	5.5	†
6	4.1	†
7	6.5	2.8
8	5.5	3.5
9	6.1	1.7
10	2.5	1.6
11	1.7	2.0
12	6.4	3.1
13	4.5	5.5
14	6.0	5.4

[a] Total number of live PBMCs ($x10^6$) obtained per ml of blood at days 0 (T0) and 13 (T13) pi (DPI)
Pigs 1 to 6 infected with Margarita strain.
Pigs 7 to 12 infected with Cat01 strain.
Pigs 13 and 14 belong to the control group.

Figure(s)

Figure 1:

a



b

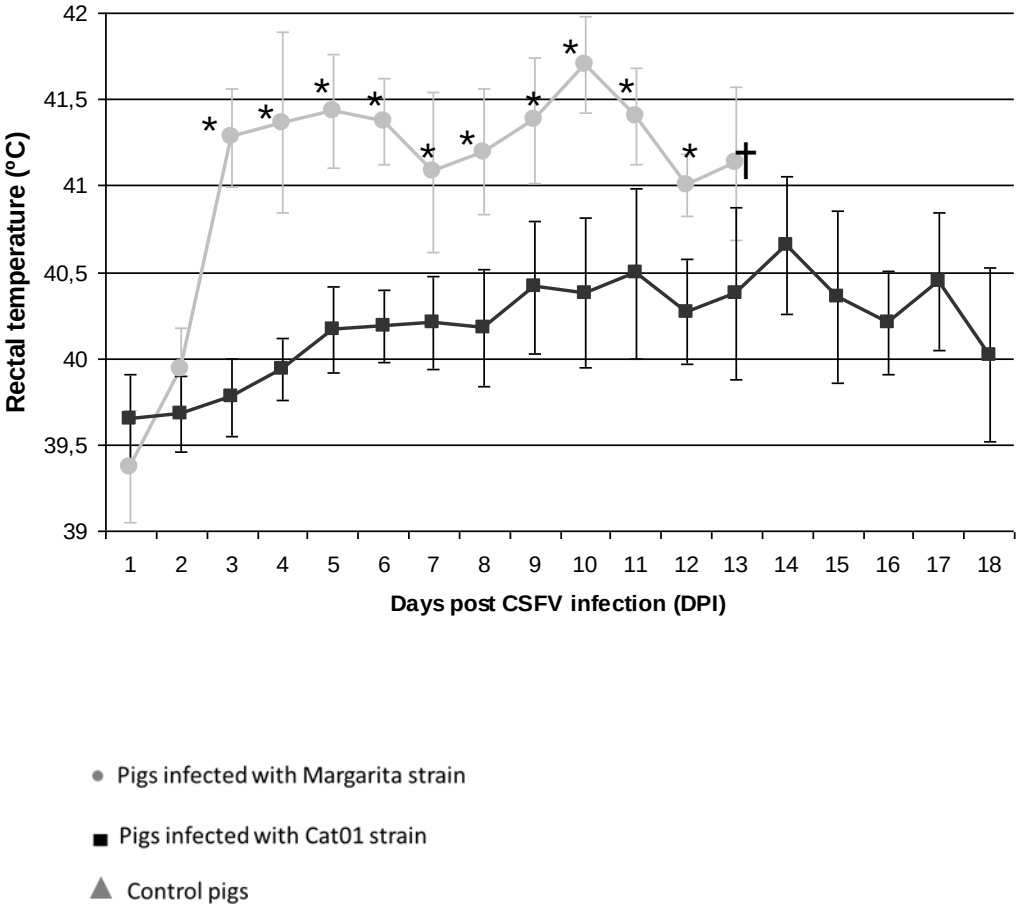


Figure 2:

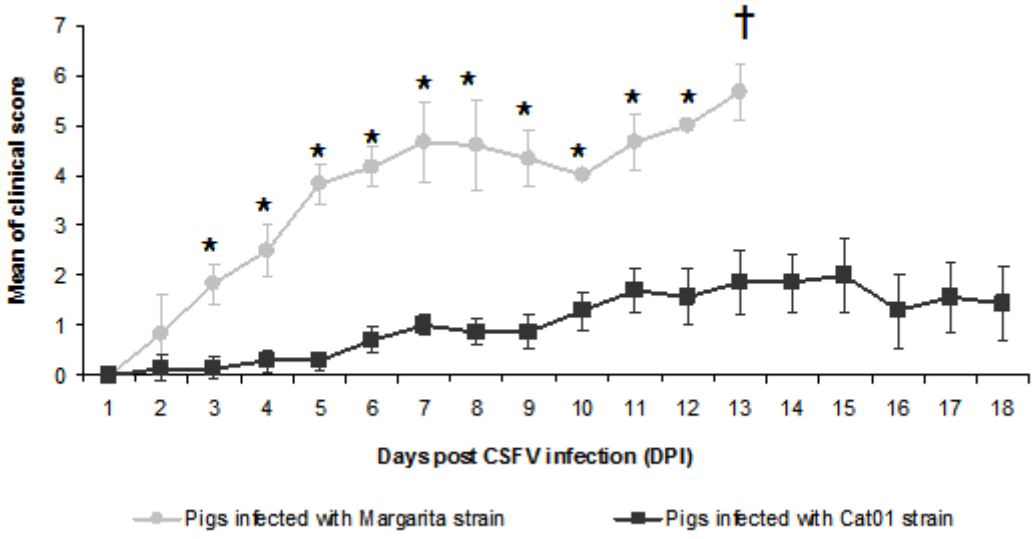
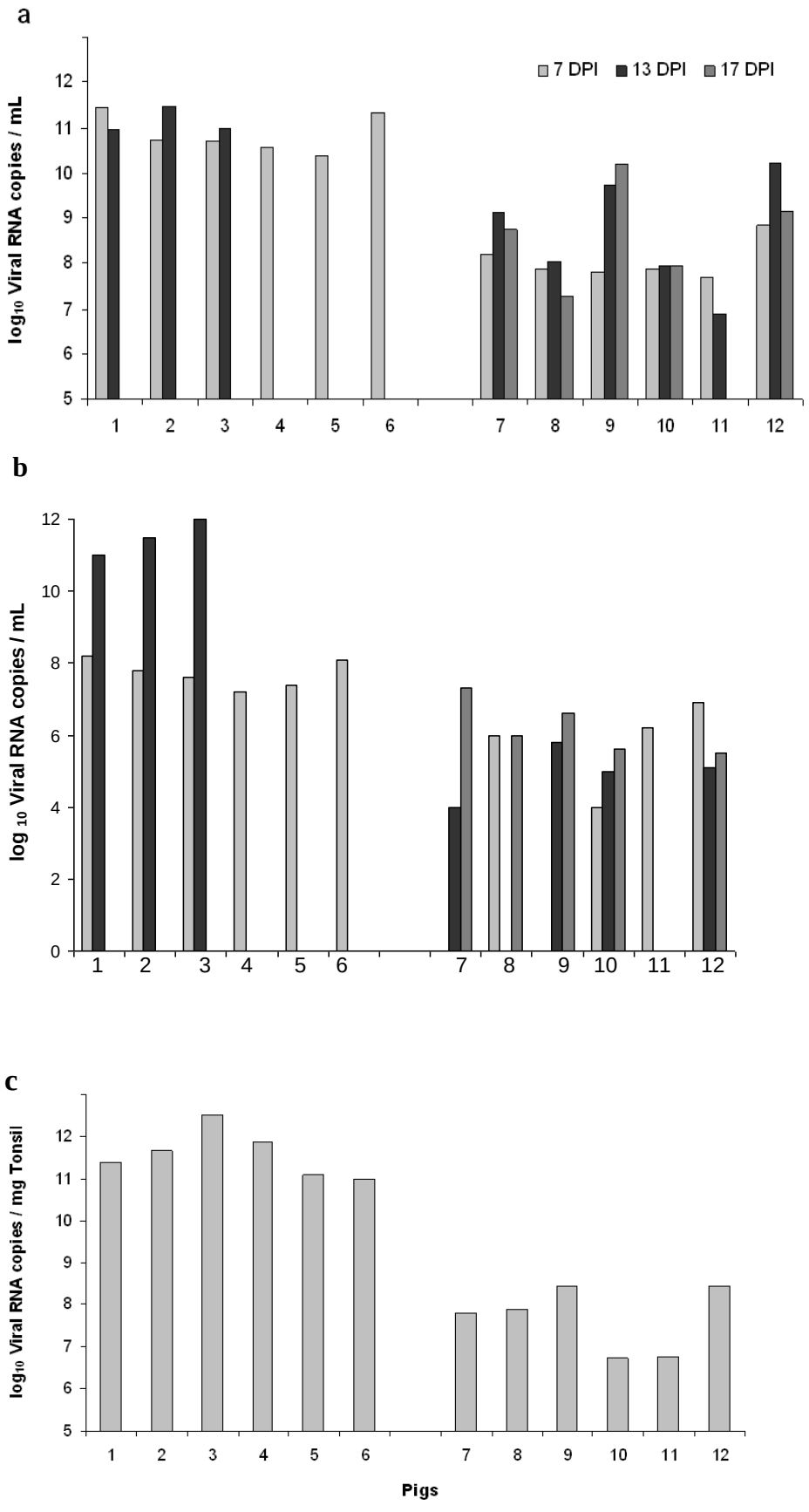
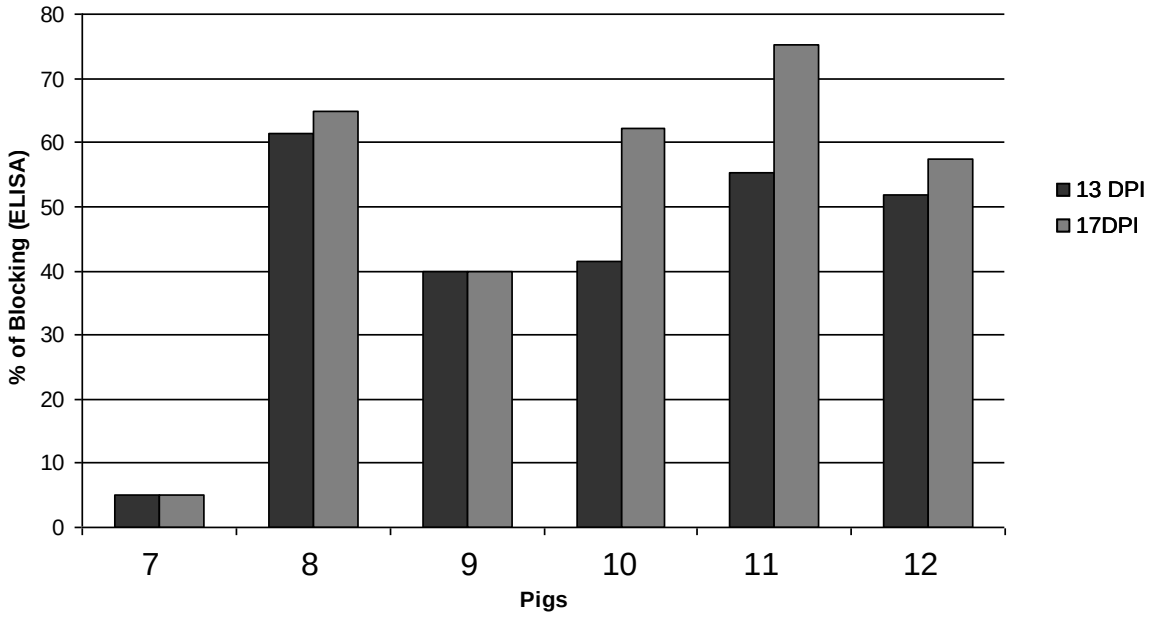

Mean of clinical score
Days post CSFV infection (DPI)
Pigs infected with Margarita strain
Pigs infected with Cat01 strain

Figure 3:



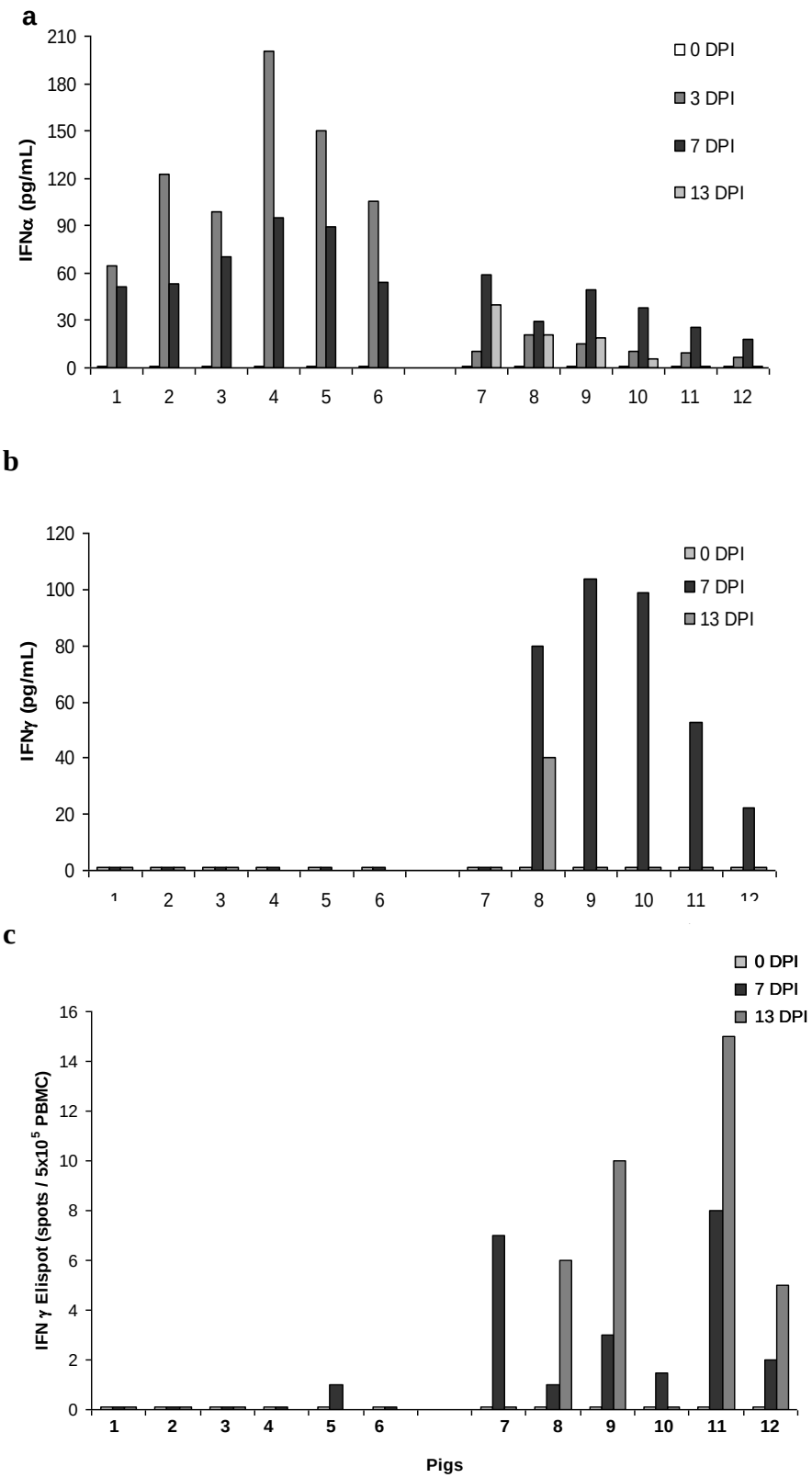
(Pigs 1 to 6: infected with Margarita strain / Pigs 7 to 12: infected with Cat01 strain)

Figure 4:



(Pigs 7 to 12: infected with Cat01 strain)

Figure 5:



(Pigs 1 to 6: infected with Margarita strain
Pigs 7 to 12: infected with Cat01 strain)