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1 **Interferon gamma induction correlates with protection by DNA vaccine expressing E2 glycoprotein**
2 **against classical swine fever virus infection in domestic pigs**

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22 **Keywords:** Classical swine fever virus, DNA vaccines, interferon gamma, protection.

23

24 **Abstract**

25

26 Classical swine fever is a highly contagious viral infection affecting domestic and wild pigs. For
27 CSFV, immunization with plasmids expressing different versions of glycoprotein E2 has proven an effective
28 way to induce protection. Previously, we have also shown that immunization with DNA vaccine expressing
29 glycoprotein E2 (DNA-E2) induced specific T helper cell responses in the absence of neutralizing
30 antibodies. However, the role of T-cell responses in protection against CSFV is largely unknown.

31 Here we have extended these studies to deeply characterize the role of T-cell responses by a DNA-
32 E2 and their correlation with protection against CSFV infection. Thus, pigs vaccinated with the DNA
33 vaccine induced a strong cellular immune response, characterized by the specific induction IFN-gamma
34 expressing T cells after vaccination without any detectable levels of CSFV neutralizing antibodies.
35 Constant levels of CSFV-specific IFN gamma producing cells observed from the beginning of the
36 infection until 7 days after challenge in vaccinated animals might contribute to early control of CSFV
37 replication, at least until neutralizing antibodies are developed.

38 Severe clinical signs of the disease, including high titers of viremia, pyrexia and virus spread to
39 different organs, were recorded in the non vaccinated challenge animals, in comparison to the vaccinated
40 animals where only one animal showed mild clinical signs and a short pick of viremia. Lack of complete
41 protection in this animal correlated with a delay on the induction of neutralizing antibodies, detectable
42 only from day 11 post-CSFV challenge. Conversely, the rest of the pigs within the group developed
43 neutralizing antibodies as early as at day two post-challenge, correlating with sterile protection. Finally,
44 an inverse correlation seemed to exist between early induction of IFN-alpha and the protection observed,
45 while IL-10 seemed to be differentially regulated in vaccinated and non-vaccinated animals.

46 Our results support the relevance of the induction of a strong T cellular response to confer a
47 solid protection upon DNA vaccination against CSFV. Further experiments are needed to be done in
48 order to clarify the key cytokines playing a role in CSFV-protection and to obtain emergency vaccines
49 capable to confer robust and fast protection.

50

51

52 1. Introduction

53

54 Classical swine fever (CSF), a highly contagious viral infection of domestic and wild pigs, is one
55 of the more devastating porcine diseases worldwide (Moennig et al., 2003). The disease is endemic in Asia
56 and prevails in many Central and South American countries, as well as in Eastern Europe. Despite the
57 stringent controls adopted in the EU from the early 1990s, CSFV has been periodically reintroduced in the
58 EU, either via wild pigs or through external imports, with outbreaks in the 1990s in Belgium, Germany,
59 Holland, Spain and Italy, and in the 2000s in UK, Spain and Germany (Dong and Chen, 2006).

60 The etiological agent, classical swine fever virus (CSFV), is an icosahedral RNA virus with (+)
61 polarity and a lipid envelope that integrates the genera Pestivirus along with bovine diarrhoea virus (BVDV)
62 and border disease virus (BDV), all closely related at both genomic and antigenic levels.

63 Clinical presentation and severity of CSF are diverse, depending largely on the virulence of the
64 strain and the immunological status of the host. In its natural host, CSFV infection results in haemorrhage,
65 leukocytopenia and immunosuppression (Susa et al., 1992). The virus displays tropism towards immune
66 cells such as dendritic and monocyte/macrophage lineage cells (Carrasco et al., 2004), and infection of
67 such cells is assumed to play a key role in immunosuppression, dissemination and/or viral persistence,
68 through hitherto unknown mechanisms. Thus, reductions in circulating CD4⁺ and CD8⁺ T cells subset and
69 granulocytes have been reported, the latter being replaced by immature precursors. The
70 immunomodulation induced by a highly virulent strain is detected in the first day post-infection, much
71 earlier than viremia, clinical signs or specific antibodies appear (Torlone et al., 1965; Summerfield et al.,
72 1998). Thus, besides its basic interest, the study of the early immunomodulation induced by CSFV can
73 lead to the future development of early diagnostic and preventive strategies (Ganges et al., 2008).

74 Glycoprotein E2 is regarded as the most immunogenic of CSFV proteins. It is mainly responsible
75 for the induction of neutralizing antibodies and it is the only viral protein that can elicit them and it can
76 confer protection when given alone (van Zijl et al., 1991; Hulst et al., 1993; König et al., 1995). For these
77 reasons glycoprotein E2 is an excellent antigen and potentially an ideal candidate which is using currently
78 for the development of a different strategies of recombinant vaccines against CSFV (Reviewed in Dong
79 and Chen, 2007; Ganges et al., 2008). Although considerable efforts towards the development of a marker
80 vaccine, new strategies and better vaccines against CSFV are urgently needed that can confer protection at
81 very early time after single administration, even when no neutralizing antibodies are detected. That prevent

82 vertical and horizontal virus spreading, permit differentiation of infected from vaccinated animals (DIVA)
83 and fulfil safety requirement (Reviewed in Dong and Chen, 2007; Ganges et al., 2008).

84 Despite the correlation between the induction of neutralizing antibodies by vaccination and
85 protection against CSFV (Terpstra and Wensvoort, 1988; Ganges et al., 2008), there are occasions in
86 which this protection was conferred in the absence of detectable anti-CSFV antibodies, suggesting that
87 other immune mechanisms, such as cellular responses against CSF antigens, might be involved in the
88 protection observed (Rumenapf et al., 1991; Hulst et al., 1993; Suradhat et al., 2001; Ganges et al., 2005).

89 In spite of the potential involvement of T cellular response in protection against CSFV in
90 absence of neutralizing antibodies, its role in CSFV protection is not well defined. In particular, the role
91 of different cytoquine such as interferon (IFN) gamma (pro-inflammatory) and IL-10 (anti-inflammatory)
92 in elimination and pathogenesis of the virus is poorly understood.

93 Previously, we have also shown that immunization with DNA-E2 induced specific T helper cell
94 responses in the absence of neutralizing antibodies (Ganges et al., 2005). Interestingly, T cell response
95 elicited can prime an efficient B cell response, since immunized animals developed an accelerated
96 neutralizing antibody response immediately after challenge and they were fully protected upon a severe
97 viral challenge against CSFV (Ganges et al., 2005).

98 Our aim is to unravel the possible immunological mechanisms involved in DNA-E2 protection
99 but not the efficacy of our experimental DNA vaccine, as it has been previously proven (Ganges et al.,
100 2005). The ultimate objective of this work was to use a DNA -E2 (Ganges et al., 2005) as a model to
101 study the cellular immune response focusing on the effective role of different cytoquine as interferon
102 (IFN) gamma (pro-inflammatory) and IL-10 (anti-inflammatory) associated with protection and CSFV
103 infection.

104

105 **2. Materials and Methods**

106

107 **2.1. Cells and Viruses**

108 PK-15 cell line was cultured in complete DMEM media supplemented with 10% fetal bovine
109 serum (FBS) at 37°C in 5% CO₂. Cells were infected with 0.1 TCID₅₀/cell in 2% FBS and virus was
110 harvested 48 h later. Viral stocks were titrated by using peroxidase-linked assay (PLA) (Wensvoort et al.,
111 1986), following the statistical method described by Reed and Muench (Reed and Muench, 1938). The
112 virulent strain Margarita used in this study was isolated in Havana in 1958, and has been used since 1965
113 for vaccine potency tests in Cuba (Ganges et al., 2007).

114

115 **2.2. DNA immunization of pigs**

116 To evaluate immune response induced by DNA-E2 (Ganges et al., 2005), 5 pigs (Landrace x
117 Large white, 8 weeks old; numbered from 1 to 5) were used. As control, 5 additional pigs were inoculated
118 with pcDNA3.1+ (numbered 6 to 10). Three doses of 400 µg of DNA were administered at 14-day
119 intervals. In all cases, one third of the total amount of DNA was intramuscularly (i.m) injected in the
120 femoral quadriceps, one third in the neck muscle and the last third was subcutaneously injected in the ear.

121

122 **2.3. CSFV challenge**

123 45 days after the first injection all pigs were challenged with 10⁵ TCID₅₀ of CSFV (strain
124 Margarita) by i.m injection in the neck. This viral dose often causes the death of injected animals after 10
125 to 15 days (Ganges et al., 2005; Ganges et al., 2007). Both rectal temperature and external clinical signs
126 of disease were scored daily (1 point: pyrexia; 2 point: pyrexia + mild clinical signs; 3 point: severe
127 clinical signs and 4: death). After euthanized, animals were subjected to an exhaustive necropsy in which
128 the presence of pathological signs in different organs and tissues was evaluated.

129

130 **2.4 Isolation of porcine PBMC and Elispot assay for detection CSFV-specific IFN gamma and IL- 131 10 producing cells**

132

133 Pigs were bled 45 days after the first DNA immunization (Pre- challenge) and later at 7 and 14
134 days post challenge (p.c.). Blood was collected with 5mM EDTA and used to obtain periphery blood

135 mononuclear cells (PBMC) by density-gradient centrifugation with Histopaque 1077 (Sigma). The total
136 number of live PBMC recovered was estimated by trypan-blue staining. PBMC were cultivated in RPMI-
137 10% FBS, 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-
138 Mercaptoethanol (Sigma), 50 000 UI penicillin l^{-1} (Invitrogen), 50 mg streptomycin l^{-1} (Invitrogen) and
139 50 mg gentamicin l^{-1} (Sigma).

140 Elispot assay for detection CSFV-specific IFN gamma and IL-10 producing cells was performed
141 as described (Diaz and Mateu, 2005). Briefly, for IFN gamma ELISA plates (Costar 3590, Corning) were
142 coated overnight with 5 μ g / ml of capture antibody (P2G10, Pharmigen). For detecting we use
143 biotinylated detection antibody (P2C11, Pharmigen). In both cases (for IL-10 and IFN gamma producing
144 cells), 5×10^5 live PBMC/well was plated in triplicates at 0.02 multiplicity of infection of CSFV Margarita
145 strain. As controls, triplicate of cells were incubated in absence of virus (negative control), or with
146 phytohaemagglutinin (PHA) (10 μ g/ml). To calculate the antigen-specific frequencies of IFN gamma and
147 IL-10 producing cells, count of spots in the media for non stimulated wells were subtracted from counts
148 of the media for CSFV stimulates wells. Frequencies of cytokine- producing cells were expressed as
149 responding cells in 5×10^5 PBMC.

150

151 **2.5. Antibody detection**

152 Pigs were bled weekly to follow CSFV specific neutralizing antibody induction. Serum samples
153 were tested by neutralization peroxidase-linked assay (NPLA) (Terpstra et al., 1984) and titers were
154 expressed as the reciprocal dilution of serum that neutralized 100 TCID₅₀ of strain Margarita in 50% of
155 the culture replicates. E2 specific antibodies were detected using an ELISA (CEDITEST; Lelystad),
156 following manufacturer recommendations.

157

158 **2.6. RT-PCR and Virus isolation**

159 The presence of CSFV RNA in different tissues and organs was analyzed using the RT-PCR
160 assay previously described (Diaz de Arce et al., 1998), which allows detection of 2 TCID₅₀ in samples
161 from CSFV infected animals. Serum samples, nasal swabs collected at 2 and 7 days post challenge (p.c)
162 and samples (1g) from different organs collected at necropsy were used to isolate virus after three
163 consecutive passages in susceptible PK-15 cells (Ganges et al., 2005).

164

165 **2.7. ELISA for IFN alpha detection from serum samples**

166 Anti-IFN alpha monoclonal antibodies (K9 and K17) and an IFN alpha recombinant protein

167 (PBL Biomedical laboratories, Piscataway, New Jersey) were used in ELISA as described (Guzylack-

168 Piriou et al., 2004). TMB (3,3',5,5'tetramethylbenzidine) was from Sigma.

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171 **3. Results**

172 **3.1. DNA immunization of pigs induces IFN gamma producing cells in absence of detectable anti-**
173 **CSFV antibodies.**

174 Previously, we have shown that immunization with DNA-E2 induced specific helper T cell
175 responses. In order to investigate whether immunization also induced IFN gamma production, an Elispot
176 assay was performed to examine number of IFN gamma producing cells after three doses of the same
177 vaccination design (45 days p.i). Increased number of specific CSFV IFN gamma producing cells was
178 observed in animals vaccinated with DNA-E2, whereas control non vaccinated animals (6 to 10) showed
179 no responses to the virus (Fig. 1). Similar to our previous work, no detectable levels of neutralizing
180 antibodies were found in sera collected prior to the second DNA boost (day 28 p.i) and at subsequent time
181 intervals (days 35 and 45 p.i). The absence of antibodies was further confirmed using an ELISA specific
182 for E2 protein (data not shown).

183

184 **3.2. DNA vaccine confers protection against lethal challenge with CSFV**

185 All vaccinated and non-vaccinated animals were challenged with a lethal dose (10^5 TCID₅₀) of
186 Margarita strain 15 days after the third dose of plasmid DNA-E2 (45 days p.i). As expected, control non
187 vaccinated animals (pigs 6 to 10) showed pyrexia (rectal temperature above 40°C) that appeared, in
188 average, at day 5 p.c, with peaks reaching above 41°C. Additional clinical signs of the disease were
189 developed from day 5 p.c in control non vaccinated pigs, such as anorexia, conjunctivitis, constipation,
190 fibrin accumulation in feces, abdominal petechiae, nervous disturbers and prostration (Fig. 2). Control
191 non vaccinated animals were euthanized at day 14 p.c to avoid suffering. Post-mortem analysis of these
192 pigs showed pathological lesions typical of CSFV infection such as: marginal spleen infarcts,
193 hemorrhagic mesenteric and mediastinic lymph nodes, enteritis, kidney petechiae, pulmonary edema and
194 hydrothorax (data not shown).

195 As we have previously shown, none of the vaccinated animal (pigs 1 to 5) developed any of the
196 clinical signs observed in control non vaccinated pigs. These animals were euthanized at day 17 p.c and
197 no pathological changes were observed upon post-mortem analysis. A mild clinical symptom was
198 observed only in one of the vaccinated animals (pig 3) with transient pyrexia (with a peak of 41°C) at
199 days 5 to 6 p.c. This animal (pig 3) was euthanized at day 14 p.c for analysis revealing a mild congestion
200 in mesenteric lymph nodes (data not shown).

201 **3.3. Protection against CSFV is associated with induction of cellular and humoral responses upon**
202 **challenge**

203 To further understand the mechanisms underlying protection against CSFV viral challenge
204 elicited after vaccination with DNA-E2, CSFV neutralizing antibody titers were determined for both
205 groups of animals at different days p.c. As shown in Figure 3, no specific antibodies were detected in
206 control non vaccinated animals (pigs 6 to 10). Conversely, elevated levels of E2 specific neutralizing
207 antibodies were determined at 7 days post infection in vaccinated animals, reaching the highest values at
208 day 17 p.c. One animal (pig 3) which showed transient pyrexia upon challenge, elicited low titers of
209 neutralizing antibodies, with a peak of 1:80 at day 14 p.c (Fig. 3).

210 After CSFV challenge, numbers of specific CSFV IFN gamma producing cells were analyzed.
211 Levels of IFN gamma producing cells in vaccinated animals (Fig. 4) remained in the same range than
212 unchallenged vaccinated animals (Fig. 1). On the other hand, control non vaccinated animals showed no
213 significant numbers of IFN gamma producing cells in response to virus challenge (Fig. 4).

214

215 **3.4. Absence of CSFV in animals protected by DNA vaccine**

216 To correlate the lack of clinical and pathological symptoms after viral challenge with virus
217 clearance RT-PCR was used, in serum, nasal swabs and organs. CSFV RNA was amplified from serum
218 and nasal swabs samples of 5 non vaccinated control pigs at day 7 p.c. (Table 1). Viral nucleic acid was
219 detected in non vaccinated control animals when RNA extracted at necropsy from diverse organs such as
220 spleen, lymph nodes and kidney were used as template for the assay (data not shown).

221 Consistent with the protection of pigs immunized with DNA-E2, negative results were obtained
222 in the attempts to amplify viral RNA from serum of these animals at different days p.c (Table 1). Lack of
223 amplification was also observed in samples from organs collected at day 17 p.c, when vaccinated pigs
224 were euthanized. Pig 3 showed positive amplification at day 7 p.c; however no CSFV RNA was amplified
225 from any of the organ samples collected at day 14 p.c, when this animal was euthanized (Table 1).

226 Detection of infectious CSFV by viral isolation in tissue culture in samples collected after animal
227 necropsy confirmed the results obtained by RT-PCR. CSFV was isolated in all organ samples from non-
228 immunized control pigs after a single passage in PK-15 cells. In contrast, no virus was recovered from
229 immunized pigs even after three serial passages in this cell line (data not shown).

230

231 **3.5. Augmented levels of IFN alpha associated with CSFV infection**

232 Presence of elevated levels of IFN alpha in serum have been reported as a response of natural
233 interferon producing cells (NIPC) to the presence of virus (Summerfield et al., 2006). Systemic
234 replication of virulent CSFV in vivo during the acute phase of infection induces type I IFN (Torlone et
235 al., 1965; Summerfield et al., 2006). Therefore, levels of IFN alpha in serum samples obtained from
236 vaccinated and non vaccinated animals at 2 and 7 days post challenge were measured. Only infected non
237 vaccinated animals (6 to 10) show high amount of IFN alpha as early as day 2 until day 7 post infection
238 (Fig. 5). In the vaccinated group, only one animal (pig number 3) showed high amounts of IFN alpha at 7
239 days post challenge. This response correlated very well with viral detection in serum at 7 days post
240 infection and the mild clinical signs observed in this pig (Table 1).

241

242 **3.6. Increased numbers of IL-10 producing cells after CSFV challenge**

243 Finding high amounts of gamma IFN producing cells (pro-inflammatory cytokine) in protecting
244 animals lead us to investigate whether the levels of other important anti-inflammatory cytokine, IL-10,
245 were altered. After vaccination (45 p.i.), both groups (vaccinated and non vaccinated infected animals)
246 showed similar levels of IL-10 producing cells. Surprisingly, higher amount of IL-10 producing cells
247 specific to CSFV was observed in both groups at 7 days post infection (Fig. 6). However, vaccinated
248 animals showed almost double levels of IL-10 producing cells specific to CSFV when compared to levels
249 in non vaccinated infected animals, suggesting that vaccination with our DNA-E2 construct was able to
250 modulate immune responses in those animals after viral infection. Moreover, IL-10 producing cells
251 specific to CSFV disappeared at 10 days post infection from non-vaccinated infected animals whereas
252 vaccinated animals recovered pre-challenge levels (fig. 6).

253

254 **4. Discussion**

255 DNA immunization is a relatively new method of vaccination with promising future and several
256 advantages over more conventional vaccines (Rajcani et al. 2005). During the last years, this methodology
257 has shown successful results against various pathogens and tumor models, and many strategies have been
258 developed to enhance its immunogenicity (Rodríguez et al. 2000; Belakova et al. 2007). Nowadays, there
259 are several DNA vaccines included in clinical trials, and four have already been licensed for animal use
260 (Kutzler & Weiner, 2008). For CSFV, immunization with plasmids expressing different versions of
261 glycoprotein E2 has proven an effective way to induce protection (Andrew et al., 2000; Ganges et al., 2005).

262 We have reported that immunization of domestic pigs with a DNA-E2 of CSFV induced specific
263 CD4⁺ T cell responses against CSFV without any detectable antibodies to CSFV (Ganges et al., 2005). The
264 elicited T cell response after DNA vaccination seemed to efficiently prime B cell response, since
265 immunized animals developed significant titers of neutralizing antibodies and they were fully protected
266 upon a severe viral challenge (Ganges et al., 2005). Thus, our previous data paved the way for further
267 understanding the involvement in protection of the T cell responses elicited after administration of our
268 DNA-E2. In this report, we analyzed the Th1 and Th2 cytokine profile induced by our vaccine, in
269 particular IFN gamma and IL-10. Additionally, the induction of a key component of the innate immunity,
270 IFN alpha, was also followed after vaccination and viral CSFV infection.

271 Our results clearly showed that the DNA vaccine triggered the induction of CSFV -specific IFN
272 gamma producing cells in vaccinated animals prior to challenge with CSFV, consistently detected also
273 after CSFV infection. In concordance with our previous results using same DNA vaccine prototype
274 (Ganges et al., 2005), no neutralizing antibodies were detectable prior to challenge. Early upon challenge
275 with a severe viral dose in the range indicated by the OIE Manual, vaccinated animals induced detectable
276 neutralizing antibody levels and were fully protected from the clinical signs of CSFV infection. Despite
277 being clinically protected, one vaccinated challenged animal (pig 3 immunized with DNA vaccine)
278 developed a mild and transient peak of pyrexia (Fig. 2). Although transient viremia was detected in serum
279 collected at day 7 p.c from this animal (Table 1) neither signs of disease were observed nor was virus
280 recovered from the tissue samples analyzed upon necropsy.

281 These results pointed towards the specific IFN gamma producing T-cells, playing a role in the
282 elicited protection against CSFV. Our findings is in agreement with previous reports showing full
283 protection and elevated number of IFN gamma producing cells as early as 6 days after vaccination with

284 life CSFV vaccines (Suradhat et al., 2001; Suradhat et al., 2005). The presence of constant levels of
285 CSFV-specific IFN gamma producing cells from the beginning of the infection until 7 days after challenge
286 in vaccinated animals, might contribute to the early control of CSFV replication, at least until neutralizing
287 antibodies are developed, as it has been previously shown for other viral infectious (Neveu et al., 2008;
288 Savarin et al., 2008). Conversely, CSFV-specific IFN gamma producing cells in non vaccinated animals
289 was below the detection level after CSFV infection, (almost zero) consistent with the fact that these
290 animals were unprotected and so, immune-suppressed (see Table 1 and Fig.2).

291 Besides IFN gamma, our results also depicted IFN alpha involvement in the resistance against
292 CSFV. Lower level of IFN alpha was detected in the serum of DNA vaccinated challenged animals at 2
293 and 7 days after challenge compared to non-vaccinated challenged animals. The DNA-E2 was efficient in
294 limiting the viremia as no virus (or viral RNA) could be detected in clinical and tissue samples obtained
295 from protected animals. Therefore, elevated levels of serum IFN alpha in infected pigs might correlate
296 with leucopenia caused by CSFV infection and is suggestive of a certain level of virus replication or
297 persistence (Rau et al., 2006; Summerfield et al., 2006). As consequence of virus replication detected in
298 all non vaccinated infected animals, higher levels of IFN alpha was detecting in the serum of these
299 animals at 2 and 7 days after challenge.

300 The lack of complete inhibition of virus spread in vaccinated challenged animal (pig 3
301 immunized with DNA-E2) correlated with high levels of IFN alpha detection at 7 days after challenge.
302 Anyhow, finding elevated levels of serum IFN alpha can be a good marker of the disease progression.

303 Our observation also supports the relevant role that neutralizing antibodies play in protection
304 against CSFV (Tepstra and Wensvoort, 1988; van Rijn et al., 1996; Bouma et al., 1999; Moormann et al.,
305 2000), since the only pig vaccinated with the DNA-E2 that did not result fully protected (pig 3), showed a
306 clear delay in the induction of neutralizing antibodies, (see Fig 2 and Fig 3).

307 The fact that both IFN gamma and IL-10 are concomitantly expressed upon CSFV challenge in
308 protected animals suggests a possible modulation in the immune response balance (Th1 vs. Th2) might be
309 relevant to obtain protection against CSFV. It has been suggested that emergency vaccination for foot and
310 mouth disease virus with classical vaccines induces protecting immune responses with early expression of
311 simultaneous presence of Th1 and Th2 like-cytokines, including IFN gamma and IL-10 (Barnard et al.,
312 2005; Eble et al., 2006). IL-10 is a pleiotropic cytokine involved in many different events having a
313 complex role leading to different functions in the immune system (Li and Flavell, 2008), including

314 enhancement of B cell survival, proliferation and antibody production, (Reviewed in Mosser and Zhang
315 2008). Taking into account IL-10 function on B cell survival, it is plausible to think that this cytokine
316 could be involved in keeping B cell homeostasis after vaccination, by comparison with CSFV infected
317 animals suffering from leucopenia (Susa et al., 1992; Summerfield et al., 2000). However, in CSFV
318 unvaccinated infected animals, we have described and increased number of IL-10 producing cells after
319 challenge (Figure 6), suggesting a major role for this cytokine in CSFV infection. Additionally, it has
320 been reported that CSFV modulates T cells for cytokines secretion, such as IL-10, which is probably a
321 key cytokine in the immunosuppression observed after CSFV infection (Suradhat et al, 2005). Thus, we
322 assume that the amount of IL-10 produced in the animals regulates the fine tuned balance between the
323 protection induced and the IL-10 low secretion pattern in non-vaccinated challenged animals. IL-10
324 involvement in CSFV infection will be the topic of further experiments.

325

326 **5. Conclusion**

327 DNA immunization with a plasmid encoding full-length E2 induced a strong cellular immune
328 response characterized by specific induction of CSFV-specific IFN gamma expressing T cells. Constant
329 levels of CSFV-specific IFN gamma producing cells observed from the beginning of the infection until 7
330 days after challenge in vaccinated animals might contribute to early control of CSFV replication, at least
331 until neutralizing antibodies are developed. The immunological profile observed in protecting animals
332 against CSFV also was associated with high levels of specific CSFV IL-10 producing cells and low levels
333 of IFN alpha in serum at 2 and 7 days post challenge.

334 Further studies are necessary for continuing with the characterization of cytokine profile after
335 different strategies of vaccination and after infection with CSFV. Understanding the immune mechanisms
336 operating during CSFV infection and the key components mediating the immunoprotection conferred by
337 our experimental vaccines can be useful to develop new marker vaccines and more importantly
338 innovative new diagnostic tools against CSFV in the future.

339

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347

348 **7. Conflict of interest**

349 None

350

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351 **8. References**

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- 453

454 **9. Tables**

455 **Table 1:** Detection of CSFV RNA for RT-PCR in serum and nasal swabs

456

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457 **10. Figure captions**

458 **Figure 1: Elispot of specific CSFV IFN gamma producing cells after 3 dose of DNA vaccine (Pre-**
459 **challenge).** Pigs numbered 1 to 5 were immunized three times with DNA-E2 (black bars). Pigs numbered
460 6 to 10 were inoculated three times with an empty plasmid, pCDNA3.1+ (grey bars). Specific CSFV IFN
461 gamma producing cells were expressed as spot number per 5×10^5 PBMC.

462

463 **Figure 2: Clinical signs associated with CSFV.** Both rectal temperature and external clinical signs of
464 disease were scored daily (1 point: pyrexia; 2 point: pyrexia + mild clinical signs; 3 point: severe clinical
465 signs and 4: death). Pigs numbered 1 to 5 were immunized DNA-E2 (black bars). Pigs numbered 6 to 10
466 were inoculated three times with an empty plasmid, pCDNA3.1+ (grey bars). Both groups were later
467 challenged with virulent CSFV Margarita strain.

468

469 **Figure 3: Induction of neutralized antibodies after challenge with virulent Margarita strain of**
470 **CSFV.** Pigs numbered 1 to 5 were immunized DNA-E2. Pigs numbered 6 to 10 were inoculated three
471 times with an empty plasmid, pCDNA3.1+. Both groups were later challenged with virulent Margarita
472 strain of CSFV. Blood sampling were collected at 2, 7, 11, 14 and 17 days post challenge. Pig number 3
473 (vaccinated animal) and pig 6-10 were euthanized at 14 days post challenge.

474

475 **Figure 4: Induction of specific CSFV IFN gamma producing cells in vaccinated and non vaccinated**
476 **animals at 7 days post challenge.** Pigs numbered 1 to 5 were immunized DNA-E2 (black bars). Pigs
477 numbered 6 to 10 were inoculated three times with an empty plasmid, pCDNA3.1+ (grey bars). Both
478 groups were later challenged with virulent CSFV Margarita strain. Specific-CSFV IFN gamma producing
479 cells were expressed as spot number per 5×10^5 PBMC.

480

481

482 **Figure 5: IFN alpha levels in serum at 2 and 7 days post challenge with CSFV.** Pigs numbered 1 to 5
483 were immunized DNA-E2. Pigs numbered 6 to 10 were inoculated three times with an empty plasmid,
484 pCDNA3.1+. Both groups were later challenged with virulent CSFV Margarita strain. Black bars indicate
485 IFN alpha levels at 2 days after challenge and grey bars indicate levels at day 7 post challenge.

486

487 **Figure 6: Kinetic of detection of specific CSFV IL-10 producing cells after challenge in vaccinated**
488 **and none vaccinated animals.** Average values of IL-10 producing cells from pigs immunized or non
489 immunized with DNA-E2. Specific CSFV gamma IFN producing cells were expressed as spot number
490 per 5×10^5 PBMC. Values of IL 10 producing cells are shown before challenge (light grey bars), 7 days
491 (black bars) and 11 days (dark grey bars) after challenge. Standard deviations were not above 20% of
492 each value.

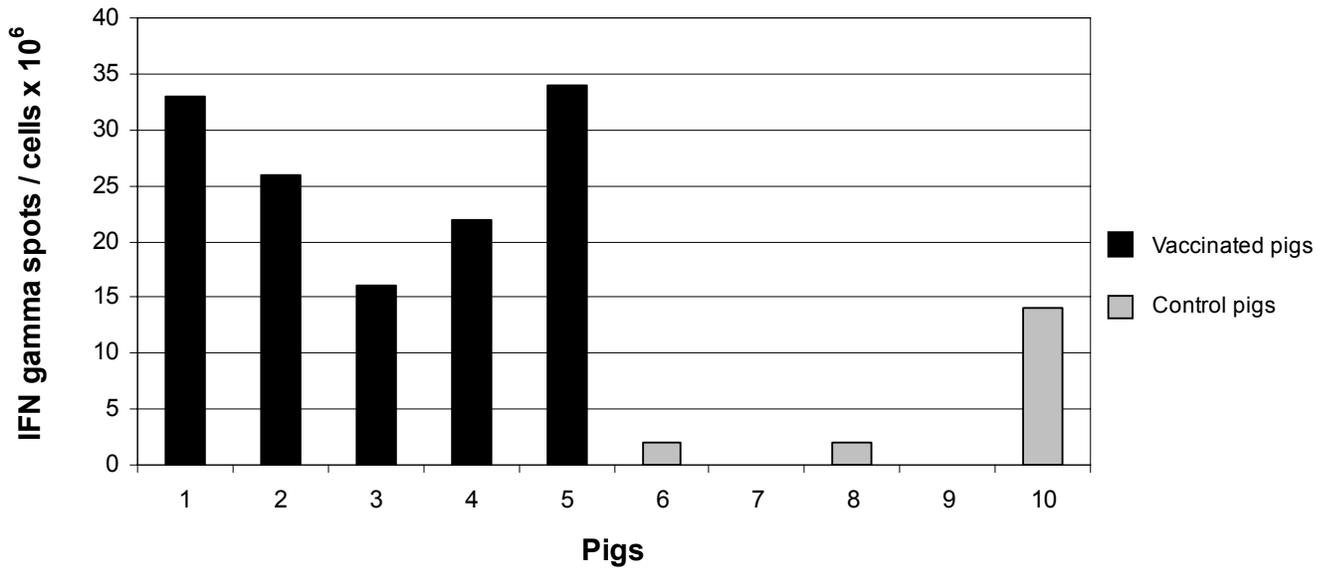
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Table 1:

Pigs	Serum		Nasal swab	
	2 p.c.	7 p.c.	2 p.c.	7 p.c.
1	Negative	Negative	Negative	Negative
2	Negative	Negative	Negative	Negative
3	Negative	Positive	Negative	Negative
4	Negative	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative
6	Negative	Positive	Negative	Positive
7	Negative	Positive	Negative	Positive
8	Negative	Positive	Negative	Positive
9	Negative	Positive	Negative	Positive
10	Negative	Positive	Negative	Positive

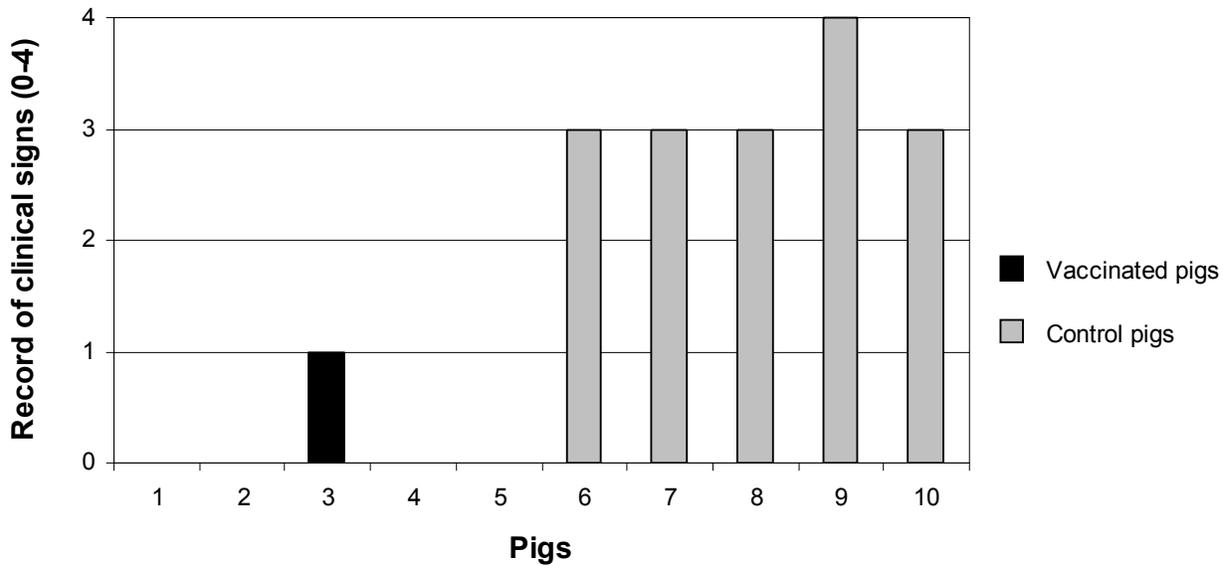
Pigs 1-5 vaccinated, pigs 6-10 non-vaccinated

Figure 1:



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Figure 2:



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Figure 3:

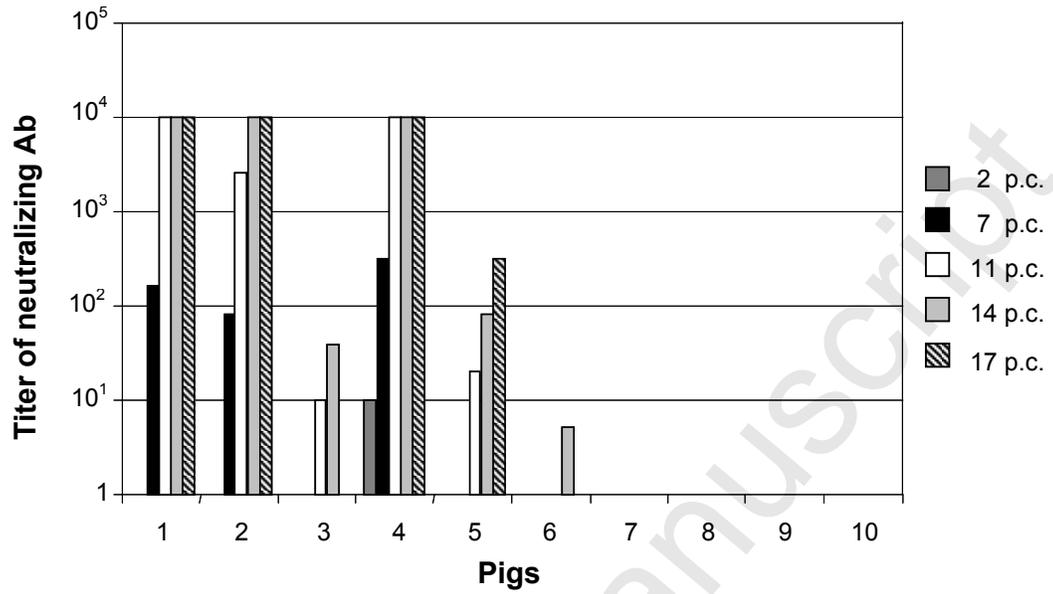
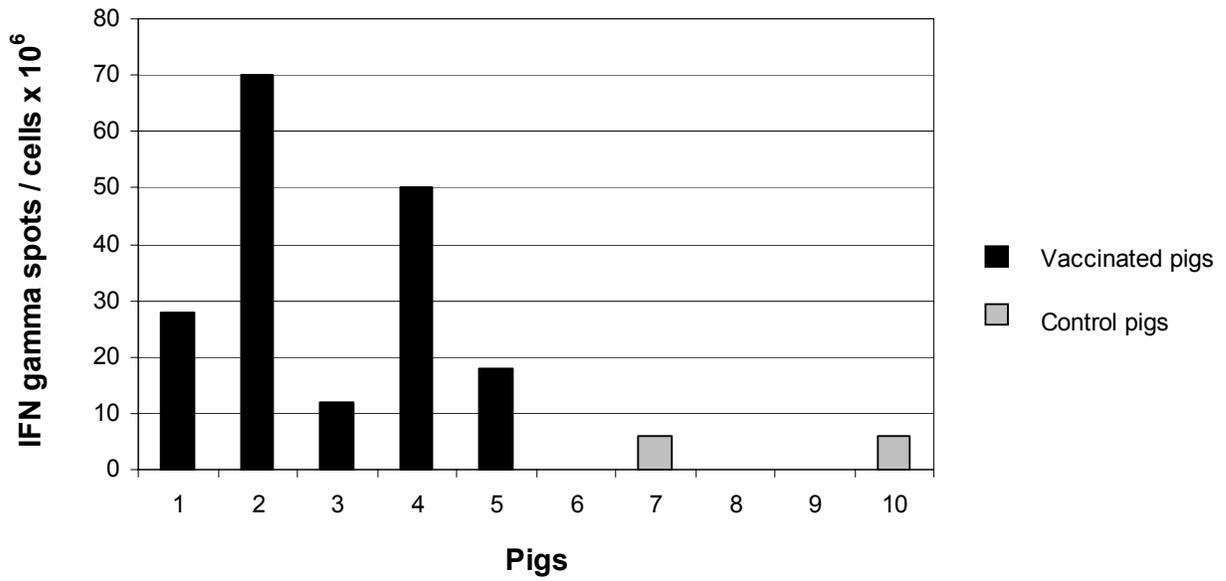


Figure 4:



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Figure 5:

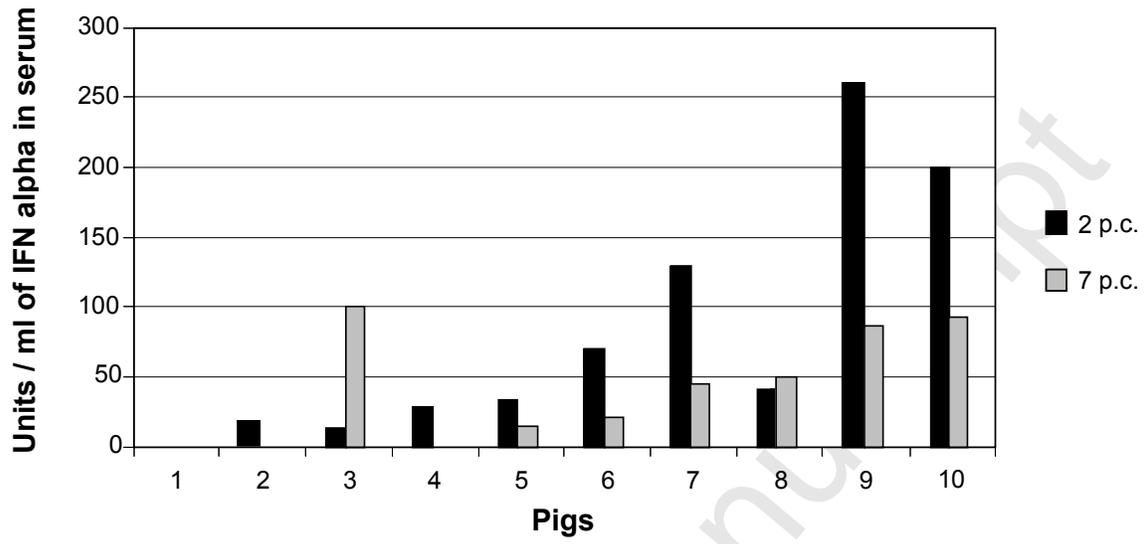
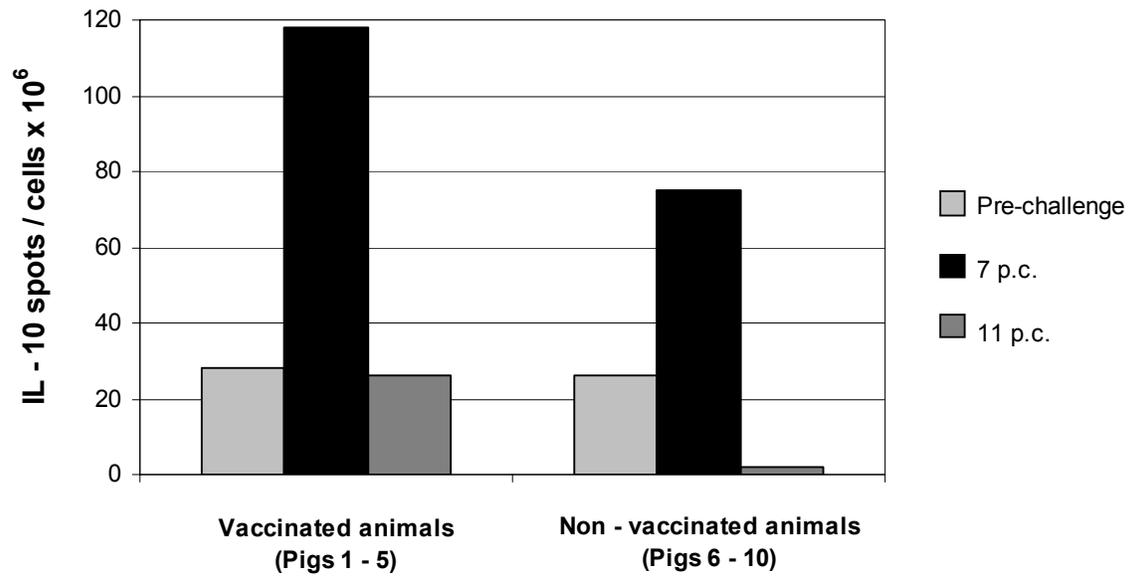


Figure 6:



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