Pseudorabies virus glycoprotein B can be used to carry foot and mouth disease antigens in DNA vaccination of pigs.

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PSEUDORABIES VIRUS GLYCOPROTEIN B CAN BE USED TO CARRY FOOT AND MOUTH DISEASE ANTIGENS IN DNA VACCINATION OF SWINE

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Running title: anti-FMDV responses after PrV-gB / FMDV BT injection

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Abstract

To evaluate the feasibility of using Pseudorabies Virus (PrV) glycoprotein B (gB) as a carrier of Foot and Mouth Disease Virus (FMDV) antigens in DNA immunization, FMDV B- and T-cell epitopes were inserted either between the 2 B-cell epitopes of the N-term subunit of PrV gB (BT-PrVgB–N term construct) or within the B-cell epitope of the C-term subunit of PrV gB (BT-PrVgB-C term construct). Two animal experiments were performed, each with 3 injections of plasmids 2 weeks apart, followed by a booster inoculation of peptides corresponding to the FMDV epitopes. Control groups of pigs were injected with plasmids encoding either PrV gB or FMDV BT, or with empty pcDNA3. The results of both assays were combined. Significant titers of FMDV neutralizing antibodies were detected after the peptides boost in groups injected with BT-PrVgB–C term. Insignificant amounts were detected in groups injected with BT-PrVgB-N term and FMDV BT constructs. PBMCs from the BT-PrVgB-N term groups, isolated after the peptide boost injection, produced IFN-γ and IL-4 mRNAs in vitro when stimulated with FMDV peptides. This was not observed with the other groups. These results imply that PrV gB can be used to carry FMDV antigens in a DNA vaccine.

1. Introduction

Foot and Mouth Disease Virus (FMDV) is the etiological agent of an important disease of livestock. FMD is highly contagious and affects cloven-hoofed animals, mostly cattle, swine, sheep and goats. FMDV belongs to the Aphthovirus genus of the Picornaviridae family and is classified into 7 serotypes (Bachrach, 1968; Rodrigo and Dopazo, 1995; Sobrino et al., 2001). One strategy employed to control disease propagation consists of regular vaccination with an inactivated whole virus antigen combined with an adjuvant (Barteling and Vreeswijk, 1991; Grubman, 2005; Saiz et al., 2002) and has resulted in eradication of the disease in some parts of the world (particularly Western Europe) (Sobrino et al., 2001). Pigs were protected against experimental FMDV challenges 4 days after vaccination (Salt et al., 1998) but no antibodies were detected at this time point. A Th1/Th2 balanced immune response was characterized 14 to 21 days after emergency vaccination (Barnard et al., 2005). As these vaccines have certain drawbacks (Cox et al., 2003; Grubman and Baxt, 2004; King et al., 1981), much effort has been made to develop efficient alternative vaccines, such as proteins, peptides, replicating vectors or DNA vaccines (Grubman, 2005). In the latter case, plasmids encoding large fragments of an FMDVgenome-like P1-2A3C3D construct (Cedillo-Barron et al., 2001), VP1 (Li et al., 2007; Park et al., 2006; Xiao et al., 2007) or FMDV B and T cell epitopes (Borrego et al., 2006; Cedillo-Barron et al., 2003; Fan et al., 2007; Wong et al., 2002; Zhang et al., 2003) were tested. Considerable progress has been made to enhance DNA vaccination efficacy but at least 2 to 3 doses of plasmids still need to be injected (Borrego et al., 2006; Cedillo-Barron et al., 2001; Chen and Shao, 2006; Li et al., 2006).

In contrast, a single injection of the Pseudorabies Virus (PrV) specific DNA vaccine induced immune responses against PrV and clinical protection against an experimental lethal PrV infection (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). Among the 3 PrV glycoproteins encoded by the DNA vaccine, PrV gB is of particular interest. This is a 913-

90   amino acid protein which contains a transmembrane domain and a furin cleavage site. This

91   protein is highly immunogenic since 2 B-cell epitope sites (aa 59-126 and 214-279) have been

92   identified in the N-term subunit and one B-cell epitope site (aa 540-734) in the C-term subunit

93   site (Zaripov et al., 1999; Zaripov et al., 1998). Furthermore, sequences allowing PrV-gB

94   endocytosis (aa 884-913), PrV-gB cell to cell spread (aa 824-854) and incorporation of this

95   glycoprotein into virions (aa 854-913) have been well documented (Nixdorf et al., 2000).

96   Thus, as PrV-gB is able to go both inside and outside cells, the opportunities for this

97   glycoprotein to encounter an efficient immune cell may be increased.

98   Therefore, the aim of the present study was to determine the feasibility of using PrV-gB to

99   carry FMDV epitopes in a DNA vaccine. The strategy differs from the one previously

100   published for BHV-1 gB (Keil et al., 2005) in that the carried antigens are not released from

101   gB but benefit from the immune properties of gB by staying attached to this glycoprotein. The

102   FMDV B epitope of VP1 is reported to elicit the production of neutralizing antibodies

103   (Francis et al., 1987). The T cell epitope of VP4 is capable of assisting a B-cell epitope when

104   in tandem (Blanco et al., 2000). These different items i.e. the FMDV type C, isolate C-8Sc1,

105   B-cell epitope (aa 133-156 of VP1) fused to the T-cell epitope (aa 20-34 of VP4), which have

106   been previously evaluated in mice (Borrego et al., 2006), were here inserted into PrV-gB. The

107   method used to insert the FMDV epitopes was therefore based on information in the

108   literature. The protein is known to contain 3 B cell epitopes which means that these sites are

109   recognized by the immune system and are therefore situated on the protein surface. Two

110   constructs were evaluated. The first was obtained by inserting FMDV BT between the 2 B-

111   cell epitopes of the N-term subunit of PrV-gB. The second was obtained by inserting the

112   FMDV epitopes into the B-cell epitope of the C-term subunit of PrV-gB. The immune

113   responses against FMDV obtained with these constructs were compared to those obtained

114   with a plasmid encoding the FMDV B- and T-cell epitopes. As the immune potentials of these

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115   constructs are unknown, the likelihood of measuring subsequent events was maximized by

116   administering 3 injections of plasmids 2 weeks apart followed by a booster inoculation with

117   the corresponding B- and T-cell epitope peptides, 2 weeks after the last plasmids injection.

118   The immune responses against FMDV and PrV were analysed after each injection.

119

2. Materials and methods

2.1 Construction and in vitro characterization of plasmids

The pcDNA3 plasmid encoding PrV glycoprotein gB has already been constructed, characterized and successfully used in our laboratory (Dory et al., 2005a; Dufour et al., 2000). The pGEM-based plasmids encoding the fused FMDV type C, isolate C-8Sc1, B-cell epitope (aa 133-156 of VP1) and T-cell epitope (aa 20-34 of VP4) were kindly provided by Belen Borrego (INIA, Valdeolmos, Spain). The FMDV BT construct was subcloned into the pcDNA3 expression cassette after *Bam*HI and *Not*I digestions. In order to insert the FMDV BT sequence between the 2 B-cell epitopes of the N-term subunit of the PrV gB gene without modifying the reading frame of gB, the BsiWI site (at the level of aa 187-189) was chosen (Fig. 1). BsiWI restriction sites were introduced on the FMDV BT insert by performing PCR on pGEM-FMDV BT with the following primers: 5'CGCATG**CGTACG**GCCGC3' and 5'ATTAGATC**CGTACG**TGGAGTT3' (BsiWI specific site is in bold and underlined). The PCR product and pcDNA3-PrV gB were digested with BsiWI (New England Biolabs) according to the manufacturer's instructions. After dephosphorylation of the digested pcDNA3-PrV gB, the digested PCR product was inserted into the PrV gB gene. The resulting plasmid was called BT-PrVgB–N term. In order to insert the FMDV BT sequence into the B-cell epitope of the C-term subunit of the PrV gB gene without modifying the reading frame of gB, we chose the FspAI restriction site (at the level of aa 682), which generates blunt ends. Appropriate restriction sites generating blunt ends on the FMDV BT insert were produced by performing a PCR on pGEM-FMDV BT with the following primers: 5'TGGAT**CCCGGG**CTACGAC3'containing a *Sma*I restriction site (in bold and underlined) and 5'CTACATGG**AGTACT**GGTACTG3' containing a *Sca*I restriction site (in bold and underlined). The PCR product was first cloned into a TOPO-TA vector (Invitrogen). The resulting vector was then digested with *Sma*I and *Sca*I and the insert transferred into

146 pcDNA3-PrV gB digested with *Fsp*AI. The resulting plasmid was called BT-PrVgB–C term.

147 Each construct was further characterized according to restriction patterns and sequencing (not

148 shown). Porcine kidney cell line 15 (PK15) was transfected with 2 µg of each plasmid by

149 using lipofectamine plus transfection reagent (Invitrogen, Gaithersburg, MD, USA) according

150 to the manufacturer's instructions. Twenty-four hours after transfection, the cells were stained

151 to reveal the expression of either PrV gB or FMDV BT. For PrV gB, the cells were

152 successively incubated with pig PrV-specific hyperimmune antiserum, with HRP-labeled

153 rabbit anti-swine IgG at 1:1000 (Sigma, Saint-Louis, MI, USA) and with the 3-amino-9-

154 ethylcarbazole peroxidase substrate (Serotec Ltd, Oxford, UK) according to the

155 manufacturer's instructions (Fig. 1). For FMDV BT, the cells were successively incubated

156 with the monoclonal GB1 antibody at 1:100, with HRP-labelled rabbit anti-mouse IgG at

157 1:1000 (Dako, Trappes, France) and with the 3-amino-9-ethylcarbazole peroxidase substrate

158 (Serotec Ltd).

159 Each plasmid was individually introduced into the *Escherichia coli* Top10 strain, and

160 amplified and purified using the EndoFree plasmid Mega kit (Qiagen, Hilden, Germany)

161 according to the manufacturer's instructions.

162

163 **2.2 Animal experiments**

164 Specific pathogen-free pigs were housed and treated in accordance with local veterinary

165 office regulations (Direction des Services Vétérinaires des Côtes d'Armor, France). In the

166 first assay, 5 groups of 4 pigs weighing 26.0 ± 0.7 kg at the 1st injection were used. Group 1

167 received the BT-PrVgB–N term-encoding plasmid. Group 2 received the BT-PrVgB–C term-

168 encoding plasmid. Group 3, which received uncarried FMDV BT, was injected with

169 pcDNA3-FMDV BT. Group 4 was injected with pcDNA3-PrV gB in order to evaluate the

170 immune responses against PrV induced by the unmodified PrV gB. Finally, the negative

control group 5 was injected with empty pcDNA3. All plasmids were co-injected with pcDNA3-GM-CSF (previously used in our laboratory (Dufour et al., 2000)) as adjuvant and administered 3 times by both intramuscular (i.m.) and intradermal (i.d.) routes at 2-week intervals. One ml of sterile saline solution containing 150 µg of the plasmids of interest and 50 µg of pcDNA3-GM-CSF were injected by i.m. route in both sides of the neck using 0.8 mm x 40 mm needles. 0.25 ml of saline solution containing 150 µg of plasmids of interest and 50 µg of pcDNA3-GM-CSF were injected by i.d. route into the dorsal surface of both ears using 0.45 mm x 12 mm needles. The i.d. injection was controlled by (i) parallel position of the needle to the ear surface, (ii) high pressure applied to the syringe to inject the solutions and (iii) the transient generation of white spots. The pigs then received 100 µg of the FMDV T cell peptide and 100 µg of the FMDV B cell peptide, 2 weeks after the last plasmids injection. A second assay, with 8 animals per group and 4 animals in the two control groups, was carried out.

The pigs were carefully observed for any adverse reaction after injection. Body temperature was measured daily and 4 hours after each injection. Relative daily weight gains were determined (Stellmann et al., 1989) for each pig. Some pigs were sacrificed during or at the end of the assays for ethical reasons. The injected areas were examined to see whether or not the injection of plasmids or expression of the encoded proteins produced lesions. Other organs were also examined.

For the antibodies determinations, sera were collected before the first plasmid injection, 1 and 2 weeks after each plasmid injection and 1, 2 and 3 weeks after the peptides boost. To isolate PBMCs, total blood samples were collected before the first plasmid injection, 2 weeks after each plasmid injection and 1, 2 and 3 weeks after the peptides boost.

2.3 FMDV neutralizing antibodies (NAb).

196 FMDV neutralizing antibody assays were carried out in 96-well plates as described in the OIE

197 Manual of Standards (OIE, 2000). Serial dilutions of sera were prepared in duplicate and 50µl

198 of each were added for 1 h to 50µl of 100 $TCID_{50}$ of the FMDV C1Noville strain. Cell

199 suspension was then added to each well and the plates were incubated at 37°C for 3 days. The

200 cells were fixed with formalin and stained with methylene blue. Titers were expressed as the

201 last serum dilution that inhibited viral replication in 50% of the wells.

202

203 **2.4 Determination of FMDV-specific serum antibodies.**

204 Anti FMDV type C antibodies were detected by applying a competitive Elisa test as described

205 by Mackay DK *et al* (Mackay et al., 2001). Briefly, 96-well, flat-bottomed plates (Maxisorp;

206 Nunc, Roskilde, Denmark) were coated with a rabbit anti FMDV C1 strain serum diluted in

207 carbonate/bicarbonate buffer pH 9.6 (Sigma, Saint Louis, MO, USA). After 3 washings with

208 PBS, the plates were incubated with FMDV antigen (C 1 Noville) diluted in PBS-tween 20

209 0.05% buffer supplemented with 10% bovine serum and 5% rabbit serum (blocking buffer)

210 for 1 h at 37°C. After washings, samples of pig sera diluted 1/5 in blocking buffer were added

211 in duplicate and a diluted guinea-pig anti type C was also added as a competitor in each well.

212 After washings and incubation with a HRP-conjugated anti guinea-pig serum, the reaction

213 was revealed with an OPD solution (Sigma). Results were expressed as the percentage

214 inhibition of the optical density (OD) obtained with the guinea-pig serum anti type C1 in the

215 antigen control well.

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218 **2.5 PrV neutralizing antibodies (NAb)**

219 After complement inactivation (30 minutes at 56°C), 50 µL of 2-fold dilutions of serum were

220 incubated with 50 µL of 100 $TCID_{50}$ of NIA3 PrV strain in 5% CO_2 in 96-well plates for 1h at

221   37°C. 150 μL of PK15 cells (2.25 x 10^4 cells/150 μL) were then added and incubated in 5%

222   CO_2 for 5 days at 37°C.

223   NAb titers were expressed as the highest serum dilution inhibiting the cytopathic effect in 2

224   out of 4 wells containing the PrV-infected PK15 cell line.

225

226   **2.6 Determination of PrV-specific IgG1 and IgG2 serum antibodies (Ab)**

227   Anti-PrV IgG1 and IgG2 serum Ab titers were determined by indirect ELISA as previously

228   described (Dory et al., 2005b). Briefly, Maxisorb 96-well plates (Nunc, Naperville, IL) were

229   coated with PrV glycoproteins, kindly provided by J.C. Audonnet (Merial, Lyon, France), and

230   successively incubated with serial threefold dilutions of serum, with mouse anti-porcine IgG1,

231   IgG2 or total IgG (Serotec Ltd, Oxford, UK), with HRP-labeled rabbit anti-mouse IgG

232   (Jackson Laboratories, West Grove, Pennsylvania, USA) and finally with the peroxidase

233   substrate tetramethyl benzidine (Pierce, Rockford, IL, USA). The enzyme reaction was then

234   stopped by adding sulfuric acid. The ODs were measured at 450 nm. IgG1 and IgG2 titers

235   ($\log_{10}$) were obtained from the highest dilution that gave a higher OD value than the threefold

236   OD of a control serum from non-vaccinated and non-infected pigs.

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238

239   **2.7 Quantification of porcine IFN-γ and interleukin 4 (IL-4) mRNA produced by**

240   **stimulated Peripheral Blood Mononuclear Cells (PBMC)**

241   PBMC were isolated from blood collected before plasmid injection, 2 weeks after each

242   plasmid injection and 1, 2 and 3 weeks after the peptides boost. PBMC were either incubated

243   *in vitro* for 16 hours with PrV strain NIA3 (multiplicity of infection: 1), with 5 ng of FMDV

244   T cell and B cell peptides, or with the RPMI culture medium alone. PBMC total RNA was

245   isolated using the 96 RNEAsy kit (Qiagen, Hilden, Germany). Porcine IFN-γ and porcine IL-4

246   mRNA expressions were determined by quantitative real-time polymerase chain reaction

247   (PCR) using primers, probes and PCR conditions as previously described (Dory et al., 2005a).

248   Cytokine mRNA and β-actin mRNA threshold cycles (Ct) were determined simultaneously

249   for each sample and the relative quantities determined according to User Bulletin number 2,

250   ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The amount of cytokine

251   mRNA was standardized with the internal β-actin mRNA reference (ΔCt = cytokine Ct - β-

252   actin Ct) and quantified in relation to the non-stimulated sample (ΔΔCt = ΔCt of the

253   stimulated sample - ΔCt of the non-stimulated sample) according to equation $2^{-\Delta\Delta Ct}$.

254

255   **2.8 Statistical analysis**

256   The data were analysed using the nonparametric Mann-Whitney test (Mann and Whitney,

257   1947) included in the Systat 9 software (Systat Software, Inc., Point Richmond, CA, USA).

258   This test was applied because the generated data were few in number, did not present a

259   normal distribution and consisted of unpaired quantitative data.

260   The limit of significance was 0.05 for all comparisons.

261

262

3. Results

3.1 Constructs

Two plasmid constructs encoding PrV-gB/FMDV BT were obtained. Porcine PK15 cells transfected with each construct were stained with a pig PrV hyper-immune serum and an antibody directed against the B cell epitope of the FMDV used here (GB1) (Fig. 1). Each PrV-gB based construct was detected by the PrV hyperimmune serum. Cells transfected with the FMDV BT encoding plasmid were not detected by the GB1 anti-FMDV monoclonal antibody, whereas under the same experimental conditions both PrV gB / FMDV BT chimeric constructs were detected.

3.2 Induction of immune responses against FMDV in pigs

The plasmids were injected by i.m. and i.d. routes 3 times at 2-week intervals. Two weeks after the last injection, individual FMDV B and T cell peptides were injected by the same routes. The different injections were well tolerated. The animals grew normally and gained approximately 1 kg per day in all groups in both assays. No fever peaks were observed 4 hours after any of the injections or during any of the daily measurements, except in 1 pig in the BT-PrVgB-N term group of the 1st assay which had a body temperature of 40.3°C, 72 hours after the 3rd injection of the plasmids. This body temperature had returned to normal by the next day. Several pigs in the second assay had to be euthanized for reasons unrelated to immunization. One pig in the BT-PrVgB-N term group broke a hoof and had to be sacrificed after the 3rd injection. One pig in the FMDV-BT group walked with a limp and was sacrificed after the second injection and another, presenting a rectal prolapse, was euthanized one week after the peptides injection. Finally, one aggressive pig in the group injected with empty-pcDNA3 was euthanized after the peptides injection.

288   Experiments 1 and 2 were combined to determine the induction of immune responses, and the

289   average and standard deviation values were determined for each group. Insignificant amounts

290   of FMDV neutralizing antibodies were first detected 2 weeks after the second injection of

291   plasmids in the BT-PrVgB–C term group (Fig. 2). Statistically significant titers were only

292   observed in this group 1 and 3 weeks after the FMDV B and T peptides boost (p<0.05). NAb

293   were also detected in the BT-PrV gB-C term and FMDV BT groups but the titers, in all cases,

294   were not statistically significant. Furthermore, NAb production in the FMDV BT group was

295   transient and could no longer be detected 3 weeks after the peptides boost, whereas it was still

296   observed in the BT-PrVgB–C term injected group. FMDV-specific antibodies were found at

297   the limit of detection in sera from 3 pigs in the BT-PrVgB–N term and in 2 out of 12 pigs in

298   the BT-PrVgB–C term injected groups. These antibodies were not detected in any of the other

299   groups.

300   IFN-γ has several immunoregulatory roles and effector functions involved in Th1- responses

301   and IL-4 plays a key role in Th2-responses (Finkelman et al., 1988; Wood and Seow, 1996).

302   The PBMCs isolated from all these pigs were restimulated *in vitro* by incubation with FMDV

303   B and T cells peptides. Significant levels of IFN-γ mRNA were detected after the peptides

304   boost in the BT-PrVgB–N term injected group (p<0.05) (Fig. 3). IFN-γ mRNA was not

305   detected in the other groups during the first assay. Significant levels were also found in the

306   BT-PrVgB-N term group one week after the peptides boost (p<0.05), but production was still

307   significantly lower than in the BT-PrVgB-C term group (p<0.05). All the other groups

308   remained negative. Significant amounts of IL-4 mRNA were only detected in the BT-PrVgB-

309   N term group one week after the FMDV B and T peptides boost (p<0.05), but not in any of

310   the other groups or in the second assay (Fig. 3).

311

312   **3.3 Induction of immune responses against PrV in pigs**

313   PrV neutralizing antibodies were produced in significant amounts 1 week after the third

314   injection of pcDNA3 PrV-gB (Fig. 4) and remained at a significant level until the end of the

315   assay. Significant amounts of PrV NAb were produced in the BT-PrVgB- N term and C term

316   groups, 2 and 3 weeks after the third plasmids injection. Nevertheless, except for the BT-PrV

317   gB-N term group 3 weeks after the third injection, the NAb titers were significantly lower

318   than in the PrV gB injected group. No significant NAb production was observed in the two

319   groups that received FMDV BT / PrV gB chimeric constructs, from the fourth week after the

320   last plasmids injection. Significant amounts of PrV-specific IgG1 were first detected 1 week

321   after the second plasmids injection in the groups of pigs injected with PrV gB or BT-PrVgB-

322   N term constructs ($p<0.05$). IgG1 production was then maintained at a significant level until

323   the end of the assay. In contrast, production in the BT-PrV gB-C term group was

324   systematically and significantly lower from the first week after the second injection until the

325   end of the assay ($p<0.01$).

326   IFN-γ and IL-4 mRNAs production was observed in PrV-stimulated PBMCs from pigs

327   injected with PrV gB, BT-PrV gB-N term and BT-PrV gB-C term constructs from week 2

328   after the second injection to week 2 after the third injection of plasmids ($p<0.05$) (Fig. 5).

329   From week 1 to week 3 after the FMDV B and T peptides injection, all 3 groups produced

330   significant amounts of IFN-γ mRNA except for the Bt-PrvV gB-C term group at weeks 2 and

331   3, the BT-PrV gB-N term group at week 2 and the PrV gB group at week 3. The same was

332   true for IL-4 mRNA production, except for the BT-PrV gB-C term group at weeks 1 and 2

333   after the peptides boost, and for the BT-PrV gB-N term group at week 2. No production of

334   IFN-γ and IL-4 RNAs was observed in the other 2 groups throughout the assay.

335

4. Discussion

The production of an efficient DNA vaccine against FMDV represents a challenge for the scientific community. Such a vaccine would provide an attractive alternative to the conventional inactivated FMDV vaccine which often requires 3 injections of plasmids (Bergamin et al., 2007; Cedillo-Barron et al., 2001; Cedillo-Barron et al., 2003; Li et al., 2006). A prime-boost strategy, (Li et al., 2008) using inactivated FMDV as a booster to significantly improve the efficacy of FMDV DNA vaccine, has recently been published. (Li et al., 2008) The results reflect the difficulty of generating a powerful DNA vaccine against FMDV that confers immunity after 1 or 2 injections of plasmids, as with anti-PrV vaccination (Gravier et al., 2007). Our goal in this study was to see if the glycoprotein B of PrV could serve as a carrier of FMDV antigens in a DNA vaccine. This glycoprotein was selected because it is already used in a successful one-shot DNA vaccine combination against PrV-infection in swine (Dory et al., 2005b; Dufour et al., 2000; Gravier et al., 2007). It is also an immunogenic protein (Zaripov et al., 1999; Zaripov et al., 1998) associated with functional domains that enable it to be internalized and to go outside the cell (Nixdorf et al., 2000). It might therefore be possible to take advantage of these characteristics to carry and present foreign antigens. A modified gB of Bovine Herpesvirus 1 (BHV-1) was recently used to transport foreign proteins (Keil et al., 2005). The transport and release of foreign proteins inserted between the furin sites, was in fact facilitated by inserting a second furin cleavage site into this glycoprotein. The concept in the present study is different since gB is expected not only to transport, but also to present foreign antigens. FMDV B and T cell epitopes have already been tested in DNA vaccines or recombinant vaccines to immunize mice or pigs against FMDV. When mice were injected 3 times with a plasmid encoding these epitopes, no neutralizing antibodies were produced and no viremia was present in half of them post-challenge (Borrego et al., 2006). Fusion of these epitopes to a signal peptide produced NAb in

361 1 out of 4 mice. Co-injection of the fungus Agaricus blazei murill (Chen and Shao, 2006) or

362 fusion to swine IgG (Wong et al., 2002) were shown to enhance DNA vaccination against

363 FMDV in mice or pigs, respectively. Finally, the titers of neutralizing antibodies in pigs

364 injected twice with a recombinant adenovirus expressing FMDV B and T cell epitopes were

365 between 4 and 16, and 3 out of 5 pigs were protected (Du et al., 2007). These small FMDV B

366 and T cell epitopes were thus able to induce immunization and/or protection against FMDV,

367 even though the induced humoral immune responses were low or undetectable. In our carrier

368 study, 1 FMDV B-cell epitope (aa 133-156 of VP1) fused with 1 FMDV T-cell epitope (aa

369 20-34 of VP4) (Borrego et al., 2006) was inserted at 2 different sites on PrV-gB. The insertion

370 strategy was based on the knowledge gained from the functional studies of the B-cell epitopes

371 in PrV.

372 The three-dimensional structure of PrV gB has not been determined, but the X-ray structure

373 of gB ectodomain from Herpes simplex virus 1 (HSV-1) is available (Heldwein et al., 2006).

374 PrV and HSV-1 gB share 50% identity, and the high sequence conservation strongly suggests

375 that the PrV gB adopts a structure similar to the one reported for HSV-1. FMDV-BT was

376 inserted in PrV-gB between the two B-cell epitopes located close to the N-terminus of gB

377 (BT-PrV gB-N term), and in the B-cell epitope located in the region preceding C-terminus

378 (BT-PrV gB-C term). The residues in HSV-1 gB, which correspond to the FMDV-BT

379 insertion sites in PrV gB, were identified from the alignment of the PrV and HSV-1 gB

380 sequences. The locations of the PrV insertion sites were then mapped on the HSV-1 gB

381 structure as shown in Figure 6. For the BT-PrV gB-N term construct, the corresponding HSV-

382 1 gB insertion site, residue Y179, is part of the fusion loop of HSV-1 gB. Fusion loops are

383 sequences rich in hydrophobic residues and are typically buried within the protein or in a

384 membrane, suggesting that the N-terminal FMDV-BT epitope may not be fully exposed in

385 PrV-gB. The limited accessibility of the epitope in BT-PrV gB-N term could be one of causes

386 of the inefficient recognition by immune system and low antibody titers. For the BT-PrV gB-

387 C term construct, the homologous insertion site in HSV-1 gB is residue H657, which is

388 located in an exposed beta-strand of domain IV. Based on the HSV-1 gB structure, the C-

389 terminal FMDV-BT epitope would likely localize to the surface of the protein, consistent with

390 its availability for recognition by immune system.

391 The results presented here show that FMDV-BT epitopes could not be detected in vitro by the

392 GB1 monoclonal antibody unless they were carried by PrV-gB. In fact, positively stained

393 cells could be visualised after transfection by either one of the PrV-gB constructs carrying

394 the FMDV-BT epitope. Moreover, PrV-gB was strongly expressed and detected when a pig

395 PrV-hyperimmune serum was used. This implies that multiple epitopes were recognized,

396 whether they were near to the insertion sites or not, suggesting that some of them were not

397 perturbed by the insertion. These findings demonstrate that the concept of PrV-gB as a carrier

398 of FMDV epitopes is feasible, at least in vitro. The immunization potentials of our constructs

399 were evaluated after each of the 3 plasmids injections, as in other studies of DNA vaccination

400 against FMDV. A FMDV peptides boost was then added to observe the production of

401 immune responses against FMDV. The two assays (12 pigs per BT-PrV gB-C term, BT-PrV

402 gB-N term and BT groups or 8 pigs per PrV gB and empty pcDNA3 groups in total) were

403 combined and the averages of the two assays are presented. Significant induction of immune

404 responses against FMDV were observed only after the FMDV B and T peptides boost in the

405 groups primed with plasmids encoding chimeric PrV gB / FMDV BT constructs. This was not

406 observed in the groups that were 3 times injected with empty plasmids or plasmids encoding

407 PrV gB before injection of FMDV B and T peptides. In fact, in these last cases pigs received

408 FMDV antigens only once. FMDV-specific serum antibodies were faintly detected in 5 of the

409 24 pigs that received the FMDV BT / PrV-gB constructs. No specific antibodies were

410 detected in any of the other groups. Significant NAb production against FMDV was only

found in the BT-PrVgB-C term construct groups. Non-significant production was detected in the BT-PrV gB-N term group. Although less NAb was produced than with DNA vaccines containing larger FMDV constructs, such as P1-2A3C3D (Cedillo-Barron et al., 2001), the amounts were similar to previous studies with these small epitopes. Borrego *et al.* found that only 1 out of 31 mice produced NAb after 3 injections of DNA vaccines encoding FMDV B and T cell epitopes (Borrego et al., 2006). Pigs injected twice with rAdV expressing FMDV BT and GM-CSF produced NAb titers between 4 and 16 (Du et al., 2007). It is important to note that the B-cell epitope from the CS8 strain encoded by the vaccine differed in 2 amino acids from the one obtained from the C1 Noville strain used in the neutralizing assay. One of these amino acids is located in an area (aa 146-156) important for NAb production (Francis et al., 1987). It is therefore possible that the NAb produced was less able to inhibit C1 Noville during *in vitro* replication and that the NAb has thus been under-estimated. Furthermore, the conformations of the FMDV B and T cells may be modified by their insertion into PrV gB, and the induction of immune responses may potentially be perturbed and decreased. Another important element in protection against FMDV is the cellular immune response (Borrego et al., 2006; Sobrino et al., 2001). The BT-PrVgB-N term group seemed to favour the production of IFN-γ mRNA (a marker of the cellular immune response) by PBMCs stimulated by FMDV B and T cell epitope peptides. Significant amounts of IL-4 mRNA (a marker of induction of humoral immune responses) were detected in the same group. These results suggest that both cellular and humoral immune responses were induced in this group, as in efficient emergency FMDV vaccination (Barnard et al., 2005). However, the detection of cytokine mRNAs does not necessarily imply that the corresponding proteins are induced. These results should therefore be interpreted with caution. Barnard *et al*. showed that the production of cytokine mRNAs by FMDV-stimulated PBMCs from FMDV vaccinated pigs was correlated, in most cases, with the production of cytokines (Barnard et al., 2005). In some

436 rare cases, the cytokines were not detected, whereas the mRNAs were. We can therefore be

437 relatively confident of the results interpretation. Furthermore, the results presented here are

438 not predictive of the ability of the constructs to protect pigs against FMDV infection. This can

439 only be evaluated experimentally. Previous studies showed that although these FMDV B and

440 T cell epitopes induced no or only low titers of NAb, they were able to protect mice (Borrego

441 et al., 2006) and pigs (Du et al., 2007) against FMDV challenges. Other examples of

442 protection in the presence of low titers of FMDV NAb are reported in the literature (Sobrino

443 et al., 2001).

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445 The immune responses to PrV-gB were equivalent to those previously reported after 3

446 injections of plasmids in pigs (van Rooij et al., 2000). Insertion of the FMDV epitopes into

447 the B-cell epitope of the C-terminal region of PrV-gB, which is described as a strong

448 conformational B-cell epitope with neutralizing activity (Zaripov et al., 1998), greatly reduced

449 the humoral immune response against the PrV studied here. In contrast, insertion of the

450 FMDV epitopes between the 2 B-cell epitopes close to the N-terminus of PrV-gB, described

451 as linear epitopes inducing low antibody responses (Zaripov et al., 1998), abolished or

452 strongly reduced the production of PrV NAb, but not of IgG1 and IgG2. Our constructs could

453 be used to study the influence of modifications of certain important functional sites on the

454 induction of immune responses against FMDV and PrV i.e. the carboxy-terminal part of PrV-

455 gB (Nixdorf et al., 2000) PrV-gB endocytosis (aa 884-913), PrV-gB cell to cell spread (aa

456 824-854) and incorporation of this glycoprotein into virions (aa 854-913).

457 It might be possible to use these constructs to generate a DNA vaccine against FMDV and

458 PrV. The BT-PrVgB–C term construct would probably be unable to protect against PrV

459 infection, due to dramatic attenuation of the immune responses against PrV, but it could be

460 useful when pigs have to be free of PrV-antibodies, as in areas where this virus has been

461   eradicated. The BT-PrVgB–N term construct greatly attenuated the production of PrV specific

462   NAb. Its use in protecting against PrV-infection should be experimentally evaluated as

463   protection against PrV has already been observed in the absence of detectable induction of

464   PrV-specific NAb and with similar levels of IgG1 and IgG2 to those in the present study

465   (Gravier et al., 2007).

466

467

5. Conclusion

In conclusion, the concept of a PrV-gB carrier of FMDV epitopes has been validated in the preliminary studies presented here. The levels of NAb produced against FMDV were similar to those reported in other studies involving immunization with FMDV B and T cell epitopes in which no NAb (Borrego et al., 2006) or titers between 4 and 16 (Du et al., 2007) were obtained. Nevertheless, strategies to improve immunization efficacy, based on co-injection of adjuvants, electroporation or changing the plasmid backbone, need to be evaluated. Other insertion sites on PrV-gB should also be tested, or other combinations of FMDV B- and T-cell epitopes inserted (Borrego et al., 2006). The nature of the immune response against FMDV depends on the site of insertion in PrV-gB. One construct, BT-PrVgB–N term, in which BT is inserted between 2 PrV-gB B cell sites, seems to favor the induction of a balanced cytotoxic and humoral immune response against FMDV (Barnard et al., 2005). The other construct, BT-PrVgB–C term, in which FMDV BT is inserted in a PrV-gB B cell site, favors the induction of FMDV-specific NAb. Finally, and from a more fundamental point of view, it would be interesting to see how the PrV-gB sites involved in endocytosis and cell-to-cell spread (Nixdorf et al., 2000) influence the induction of immune responses with these different constructs.

6. Acknowledgments

Figure legends

Figure 1: constructs Porcine PK15 cells were transfected with pcDNA3 based plasmids encoding PrV-gB, BT-PrV gB N term, BT-PrV gB C term or FMDV BT. Twenty-four hours later, cells were stained with pig PrV hyperimmune serum or anti-FMDV B epitope GD1 monoclonal antibody as described in materials and methods.

Figure 2: Anti-FMDV neutralizing antibodies Anti-FMDV neutralizing antibodies before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box .
*a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups
*b: $p < 0.05$ compared to the PrV gB, Empty pcDNA3 and FMDV BT groups

Figure 3: IFN-γ and IL4 mRNA relative expressions by FMDV B and T cell peptides stimulated PBMCs
The cells were isolated before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were than incubated with FMDV B and T peptides or with culture medium for 16 hours. IFN-γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box .
*a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups
*b: $p < 0.05$ compared to all the other groups
*c: $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups
*d: $p < 0.05$ compared to the PrV gB, BT-PrV gB-N term and Empty pcDNA3 groups

Figure 4: Anti-PrV neutralizing, IgG1 and IgG2 antibodies production.
Sera from pigs in both assays were collected before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box .
*a: $p < 0.05$ compared to all the other groups
*b: $p < 0.05$ compared to the BT-PrV gB-C term, FMDV BT and Empty pcDNA3 groups*c: $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups
**: $p < 0.01$ compared to the PrV gB and BT-PrV gB-N term groups

Figure 5: IFN-γ and IL4 mRNA relative expressions by PrV stimulated PBMCs
The cells were isolated before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were than incubated with live PrV or with culture medium for 16 hours. IFN-γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box.
* $p < 0.05$ compared to the FMDV BT and Empty pcDNA3 groups

Figure 6: Location of the PrV gB FMDV epitope insertion sites mapped on the structure of HSV-1 gB. A) Ectodomains of HSV-1 gB form trimers. Each monomer is colored as blue, green or red (left panel), and a space-filled model of the gB trimeric surface is shown on the right. Y167 and H657 are the HSV-1 residues that correspond to the insertion sites of the

547   FMDV epitopes in PrV gB. HSV-1 gB fusion loops, which are the residues proposed to insert
548   into the target membrane during fusion, are marked. **B, C)** Only domains I and IV,
549   respectively, are shown for clarity, and insertion sites Y167 and H657 are labelled. Residue
550   Y167, which would correspond to the N-term FMDV epitope insertion, seems less accessible
551   than the fully exposed H657, which is analogous to the position where the C-term FMDV
552   epitope was added. This suggests that the latter position might be a better insertion target.
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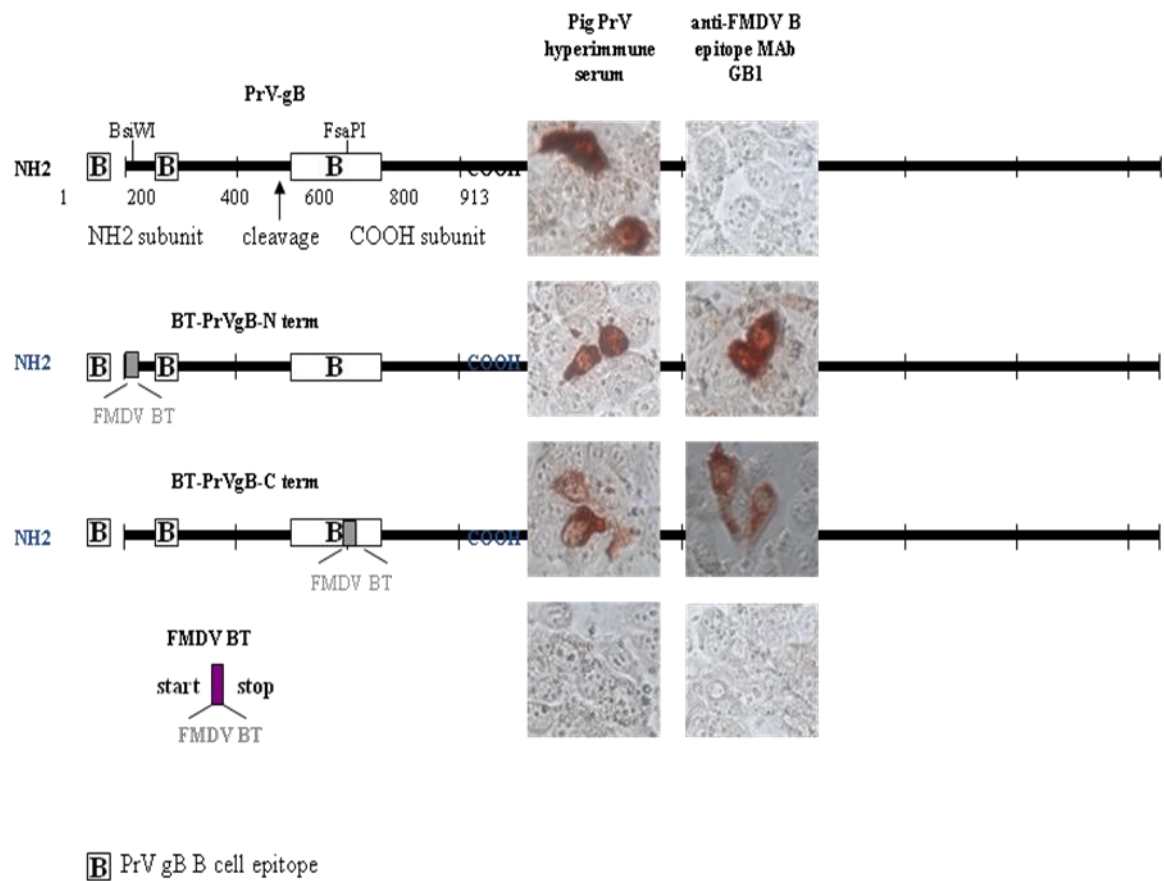686   and-mouth disease virus. Vaccine 21, 4704-4707.
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Figure 1: constructs

Porcine PK15 cells were transfected with pcDNA3 based plasmids encoding PrV-gB, BT-PrV gB N term, BT-PrV gB C term or FMDV BT. Twenty-four hours later, cells were stained with pig PrV hyperimmune serum or anti-FMDV B epitope GB1 monoclonal antibody as described in materials and methods.
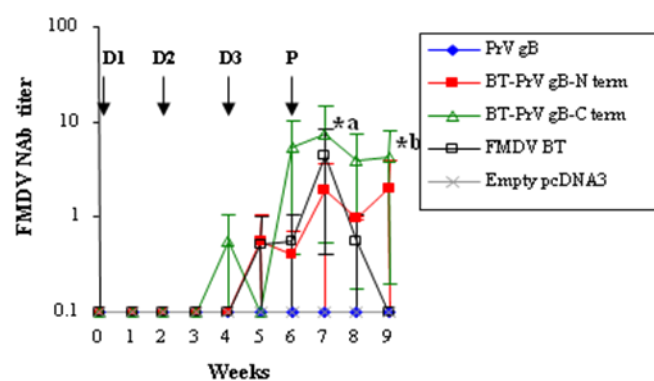
Figure 2: Anti-FMDV neutralizing antibodies
Anti-FMDV neutralizing antibodies before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box .
*a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups
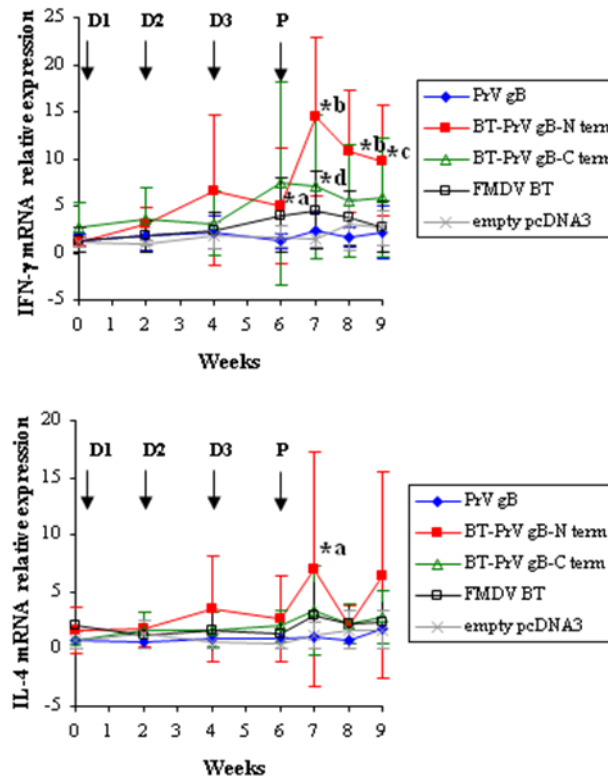*b: $p < 0.05$ compared to the PrV gB, Empty pcDNA3 and FMDV BT groups

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Figure 3: IFN-γ and IL4 mRNA relative expressions by FMDV B and T cell peptides stimulated PBMCs

The cells were isolated before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were than incubated with FMDV B and T peptides or with culture medium for 16 hours. IFN-γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box.

*a: $p < 0.05$ compared to the PrV gB and Empty pcDNA3 groups
*b: $p < 0.05$ compared to all the other groups
*c: $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups
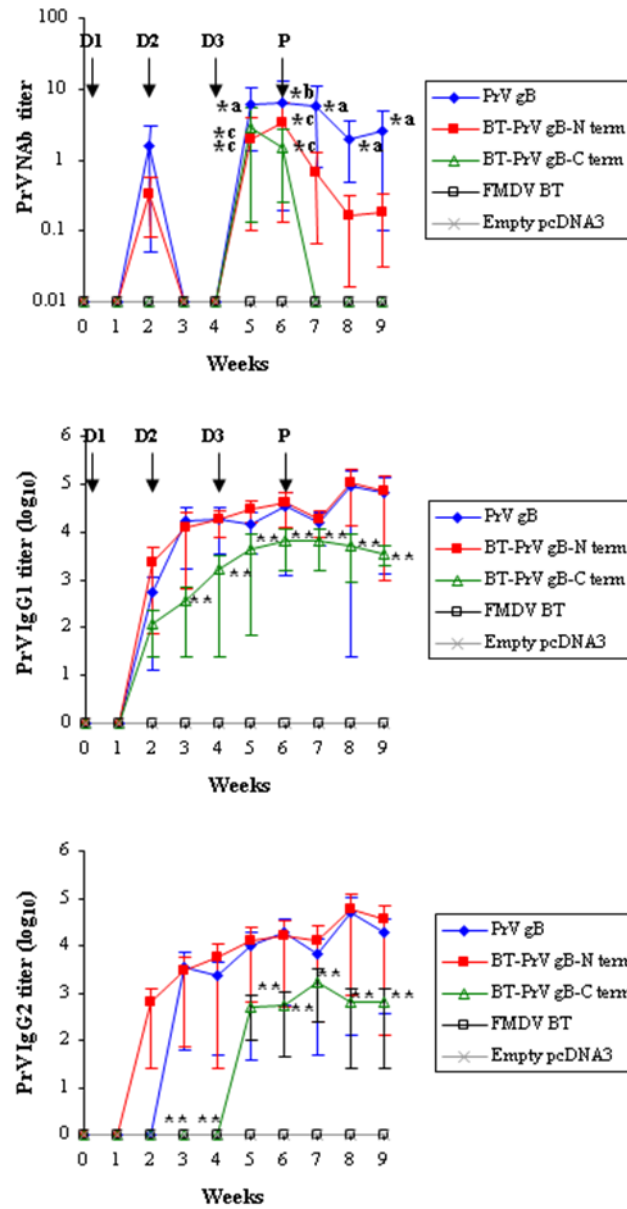*d: $p < 0.05$ compared to the PrV gB, BT-PrV gB-N term and Empty pcDNA3 groups

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Figure 4: Anti-PrV neutralizing, IgG1 and IgG2 antibodies production.
Sera from pigs in both assays were collected before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). Average titers of experiments 1 and 2 $\pm$ SD are shown for each group indicated in the legend box.

*a: $p < 0.05$ compared to all the other groups
*b: $p < 0.05$ compared to the BT-PrV gB-C term, FMDV BT and Empty pcDNA3 groups
*c: $p < 0.05$ compared to the PrV gB, FMDV BT and Empty pcDNA3 groups
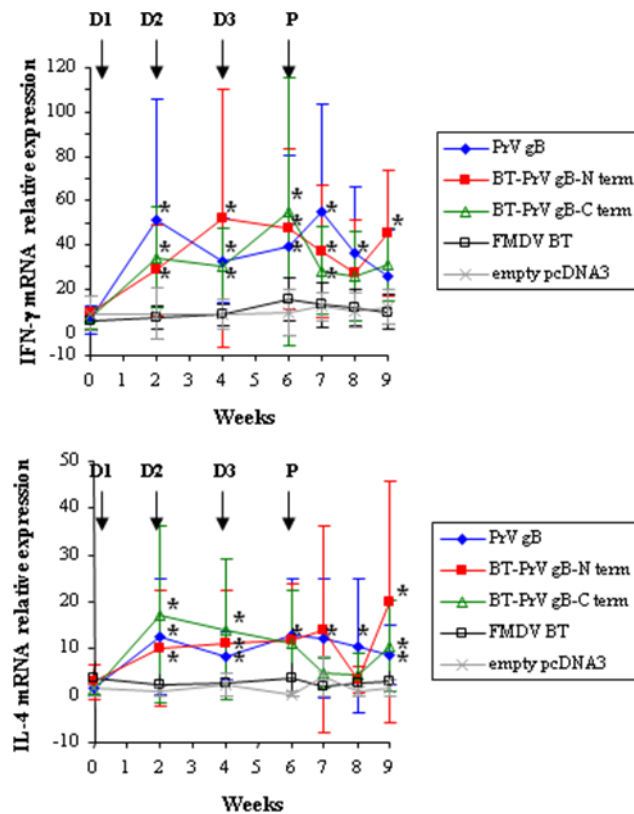**: $p < 0.01$ compared to the PrV gB and BT-PrV gB-N term groups

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Figure 5: IFN-γ and IL4 mRNA relative expressions by PrV stimulated PBMCs
The cells were isolated before the first injection and after each of the 3 injections of plasmids (D1, D2 and D3, indicated with arrows) and after the peptides boost (P, indicated with an arrow). The cells were than incubated with live PrV or with culture medium for 16 hours. IFN-γ and IL4 mRNA relative expressions were determined. Average titers of experiments 1 and 2 ± SD are shown for each group indicated in the legend box.

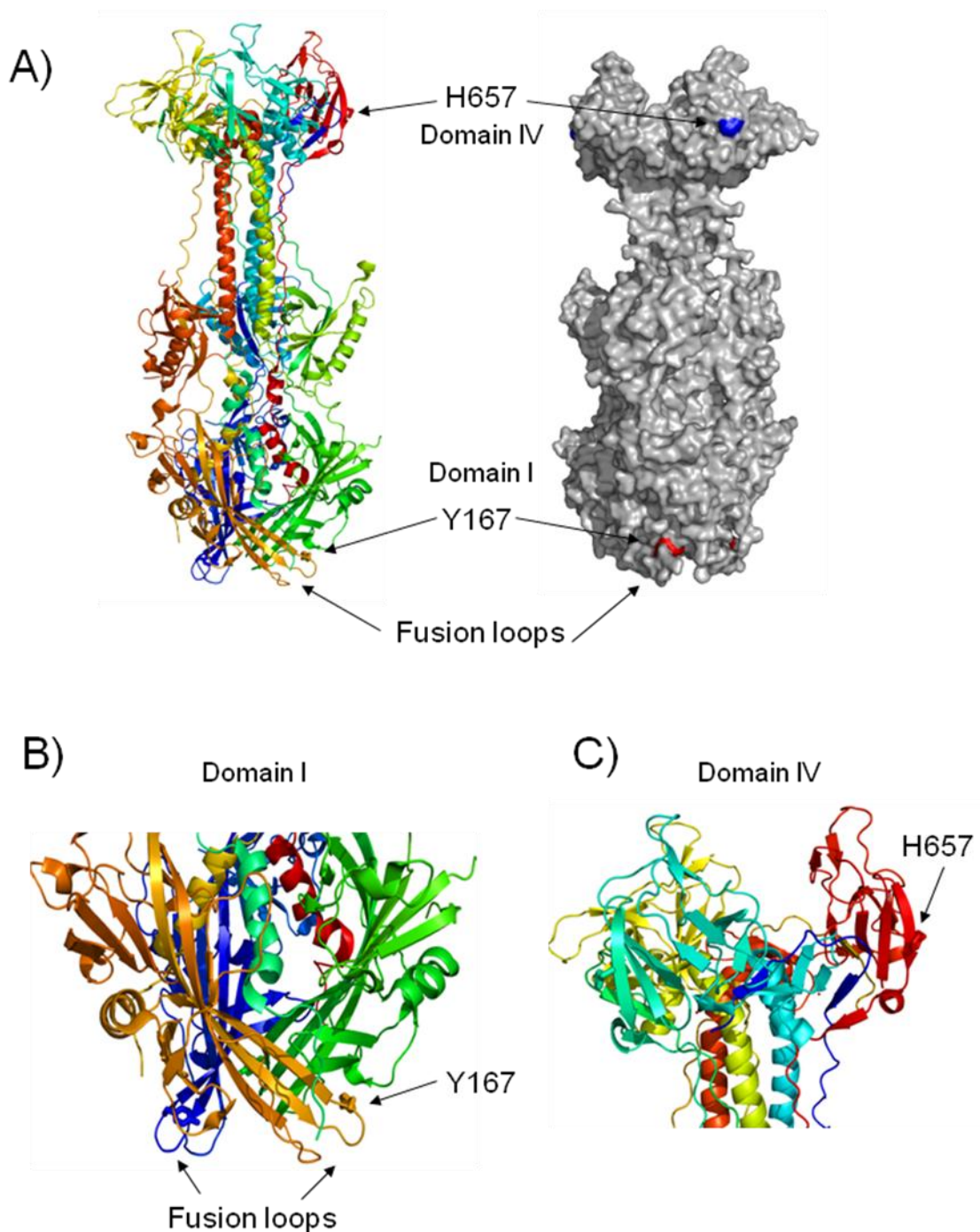*p < 0.05 compared to the FMDV BT and Empty pcDNA3 groups

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Figure 6: Location of the PrV gB FMDV epitope insertion sites mapped on the structure of HSV-1 gB. A) Ectodomains of HSV-1 gB form trimers. Each monomer is colored as blue, green or red (left panel), and a space-filled model of the gB trimeric surface is shown on the right. Y167 and H657 are the HSV-1 residues that correspond to the insertion sites of the FMDV epitopes in PrV gB. HSV-1 gB fusion loops, which are the residues proposed to insert into the target membrane during fusion, are marked. **B, C)** Only domains I and IV, respectively, are shown for clarity, and insertion sites Y167 and H657 are labelled. Residue Y167, which would correspond to the N-term FMDV epitope insertion, seems less accessible than the fully exposed H657, which is analogous to the position where the C-term FMDV epitope was added. This suggests that the latter position might be a better insertion target.

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