Virology 476 (2015) 11–18

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Foot-and-mouth disease vaccination induces cross-reactive IFN-γ responses in cattle that are dependent on the integrity of the 140S particles

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ARTICLE INFO

Article history:
Received 28 September 2014
Returned to author for revisions
10 November 2014
Accepted 20 November 2014
Available online 10 December 2014

Keywords:
FMDV
FMD vaccines
IFN-γ
Capsid integrity
Cattle

ABSTRACT

Interferon-γ (IFN-γ) recall responses against foot-and-mouth disease virus (FMDV) in FMD vaccinated cattle are utilized to study T-lymphocyte immunity against this virus. Here, a recall IFN-γ assay based on a commercial ELISA was set up using 308 samples from naïve and vaccinated cattle. The assay was used to study cross-reactive responses between different FMDV vaccine strains. Blood samples from cattle immunized with monovalent vaccines containing A24/Cruzeiro/Brazil/55, A/Argentina/2001 or O1/Campos/Brazil/58 strains were tested using purified-inactivated FMDV from homologous and heterologous strains. A24/Cruzeiro was the most efficient IFN-γ inducer in all vaccinated animals, both when included in the vaccine or as stimulating antigen. We demonstrate that this was mainly due to the structural stability of the whole viral particle. These results show that IFN-γ production relies on the presence of 140S particles that can maintain their integrity along the incubation process *in vitro*, and throughout the vaccine´s shelf-life, when used *in vivo*.

Introduction

Foot and mouth disease (FMD) is an acute and highly contagious-febrile disease affecting all ruminants and cloven-footed animals (Alexandersen and Mowat, 2005). Its causative agent, the FMD virus (FMDV), belongs to the Picornaviridae family, being the main member of the genus Aphtovirus (King et al., 2011). The disease has been a major cause of economic losses to livestock industries worldwide: even though fatal cases are usually restricted to young animals and certain FMDV strains (Alexandersen et al., 2003), infected animals significantly reduce productive performance and international trade of animals and animal-derived products is severely restricted between countries with different sanitary status of the disease. The potential of FMD to disrupt normal social and economic function (Perry and Rich, 2007) has been dramatically demonstrated in the last two decades by outbreaks occurred in different countries irrespectively of their economic development (Muroga et al., 2012; Perez et al., 2004; Thompson et al., 2002; Yang et al., 1999).

Several concerns have progressively favored the use of vaccines to control FMD outbreaks (Mackay et al., 2004; Poulin and Christianson, 2006). Vaccination has been successfully applied as the main control measure for FMD in different South American countries, in most cases by means of polyvalent oil vaccines formulated using inactivated virus from strains previously detected in the region (Saraiva and Darsie, 2004). Good quality vaccines may prevent the development and transmission of the disease and decrease the incidence of persistently infected animals (Anderson et al., 1974; Cox et al., 2006; Orsel et al., 2005).

FMDV structural proteins are highly variable and seven antigenically distinct serotypes have been described for this virus (Knowles and Samuel, 2003). Consequently, protection provided by FMD vaccines, which is closely related to the induction of specific antibody responses (Maradei et al., 2008; Pay and Hingley, 1987), is serotype-specific and, in numerous examples, very limited even for strains within the same serotype (Doel, 2003).

Serological assays, such as virus neutralization tests (VNT) and liquid-phase blocking ELISA (LPB-ELISA), were designed to detect levels of antibodies against viral capsid proteins and represent a major tool to predict the immunological and protective status of the animals and susceptible populations against FMDV (Barnett et al., 2003; Goris

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http://dx.doi.org/10.1016/j.virol.2014.11.023
0042-6822/

et al., 2008; Maradei et al., 2008; Pay and Hingley, 1992). Although generation of adaptive humoral responses after FMDV infection in cattle showed to be independent of T-cell collaboration (Juleff et al., 2009; Pega et al., 2013), vaccine-induced antibody responses are dependent on the collaboration of FMDV-specific $CD4^+$ lymphocytes (Carr et al., 2013). However, induction of FMDV-specific cellular immune responses has not usually been considered for the evaluation of vaccine efficacy (Becker, 1994), probably because of the lack of high-throughput cell-mediated immunity tests.

The induction of IFN-γ recall responses from bovine mononuclear cells stimulated with inactivated antigens from infectious pathogens has been largely used as a measure of pre-existing adaptive cellular immunity against protozoa, bacteria and viruses (Brown et al., 1993; Lightbody et al., 1998; Woolums et al., 2003). The *in vitro* assessment of gamma interferon (IFN-γ) production was developed as a high throughput test to detect pathogen-specific cellular immune responses in cattle (Wood and Rothel, 1994) using whole blood, thus avoiding the need of preparing cell-suspensions. Cellular responses against FMDV have also been studied by detection of IFN-γ, showing correlation with protection against homologous virus challenge in sheep (Barnett et al., 2004) and cattle (Oh et al., 2012). In contrast, heterologous anti-FMDV cellular responses in cattle have been exclusively reported in early studies using lymphoproliferation assays (Collen et al., 1998; Collen and Doel, 1990).

Here, we adjusted a protocol and its corresponding acceptance parameters to measure FMDV-specific anamnesic IFN-γ responses, using a large number ($n=308$) of whole blood samples from naive and FMD-vaccinated cattle. Homologous and heterologous responses were evaluated in groups of animals immunized with O1/Campos, A/Arg/01 or A24/Cruzeiro monovalent formulations or tetravalent FMD vaccines comprising all the above mentioned strains. Our results demonstrated the existence of cross-reactive intra- and inter-serotypic cellular responses in FMD-vaccinated cattle. Also, we observed a differential ability among viral strains to elicit FMDV-specific IFN-γresponses, both *in vivo* and *ex-vivo*, and related these observations to the particular stability of the 140S particles. We concluded that the FMDV-specific production of IFN-γ is strongly affected by the integrity of the viral capsids. Although this information should be considered when detecting FMDV-specific production for this cytokine, it may also result relevant to understand the immunogenic capacity of FMD vaccines and develop strategies to improve it, for homologous and heterologous immunity.

Results

Set up of FMDV-specific bovine IFN-γ ELISA

IFN-γ levels measured from whole blood are extremely variable (Schiller et al., 2009), consequently the application of this assessment as a diagnostic tool is based on positive and negative validation parameters and comparative ratios to a non-stimulated aliquot of the sample (or stimulated with an unrelated antigen). An IFN-γassay based on a commercial ELISA was set up with 308 whole blood samples obtained from naïve and vaccinated animals (Table 1) and incubated with PWM or PBS (Fig. 1). Most of the animals presented high levels (> 3,400 pg/ml) of IFN-γproduction after incubation with PWM and no differences were found between samples from näive and vaccinated animals (data not shown). These results corroborated both the viability of the cells and their capacity to secrete this cytokine. On the contrary, most of the blood samples incubated with PBS produced very low or undetectable IFN-γ levels (< 1,000 pg/ml), indicating that there was no significant basal secretion of the cytokine in this assay. Blood samples from non-vaccinated animals ($n=114$), as well as those from FMD-vaccinated cattle ($n=194$), did not produced IFN-γ when neither stimulating antigen nor mitogen were dispensed, showing that vaccinated animals yield no basal responses of this cytokine.

Considering the outcome of the test as positive (PWM activated) or negative (PBS treated), we exercised a ROC analysis to establish the minimum IFN-γ value obtained after PWM stimulation as to consider the cells viable and capable to produce IFN-γ, and the maximum value acceptable for PBS treatment as to exclude non-specific IFN-γ production (data not shown). These limits of cell viability and functionality were fixed in 3,200 pg/mL and lower (specificity) limit was calculated to be 1,450 pg/mL. In these conditions, the assay had a sensitivity of 98.7%, and a specificity of 97.9%. From then on, all samples were run including PBS and PWM controls and results from FMD assessment were considered to be valid when PBS stimulation rendered less than 1,450 pg/mL and PWM more than 3,200 pg/mL.

Using these acceptance parameters, a protocol for FMDV-specific bovine IFN-γ ELISA was standardised by testing whole blood samples from single- or multi-vaccinated animals incubated at 37 °C for 24, 48 or 72 h with PWM, PBS or with 3 different concentrations (10.0, 5.0 and 2.5 μg/ml)of purified inactivated 140S particles of FMDV O1/Campos strain. Responses increased along with the stimulating antigen's concentration (10.0 μg/mL > 5.0 μg/mL > 2.0 μg/ml) and decreased with incubation times; conditions were finally standardized using 10 μg/mL of 140S particles-stimulation for 24 h (Suppl. Fig. 1). With these conditions, blood samples from naïve (pre-immune) animals ($n=64$) showed no reaction to the stimulation with all tested FMDV strains, while steers immunized with a single dose of the commercial tetravalent vaccine (30 dpv, $n=8$) produced very vigorous responses against the viral antigens, in some animals with similar levels to those obtained with PWM (Fig. 2).

Homologous and cross-reactive IFN-γELISA responses in vaccinated cattle

FMDV-driven recall production of IFN-γ was determined in peripheral blood from cattle immunized with FMD monovalent

Table 1
Samples used to determine the acceptance parameters of the IFN-γ assay.

Vaccine regimen[a]	n	dpv	PWM[b]	PBS[b]
Without vaccination	114	–	12,512 ± 6,617	325 ± 707
A24/Cruzeiro Monovalent	10	30	16,697 ± 5,632	390 ± 8
A/Arg/2001 Monovalent	10	30	12,644 ± 6,626	390 ± 11
A24/Cruzeiro + A/Arg/2001 Bivalent	20	30	15,826 ± 5,997	390 ± 7
O1/Campos Monovalent	70	30	9,711 ± 4,968	382 ± 1,137
Tetravalent	50	30/120	17,089 ± 6,756	231 ± 49
Tetravalent (multiple doses)	34	30/120	20,599 ± 6,147	244 ± 187
Total	308		13,866 ± 7,019	322 ± 695

[a] All animals were primo-vaccinated unless otherwise indicated.
[b] PWM= pokeweed mitogen; PBS=phosphate buffer saline. Values are expressed as mean IFN-γ concentrations (pg/mL ± standard deviation) induced for samples incubated for 24 h with PWM (10 μg/mL) or PBS (n=number of samples; dpv=days post vaccination)

vaccines comprising A24/Cruzeiro(10 μg/dose), A/Arg/01(10 μg/dose) or O1/Campos(20 μg/dose)strains. Whole blood samples taken at 30 dpv were stimulated withA24/Cruzeiro, A/Arg/01, C3/Indaial or O1/Campos sucrose-purified inactivated 140S particles (Fig. 3). Homologous stimulation was significantly higher for the A24/Cruzeiro strain than for O1/Campos or A/Arg/01 strains ($p < 0.01$). Also, cellular responses were cross-reactive among the different serotypes, showing a differential pattern of IFN-γ production depending on the strain. Although the A24/Cruzeiro vaccine induced higher IFN-γ levels than the O1/Campos and A/Arg/01 vaccines when stimulated with heterologous strains (A/Arg/01 and C3/Indaial strains, respectively) ($p < 0.05$), the O1/Campos and A/Arg/01 vaccines were only significantly cross-reactive against the A24/Cruzeiro antigen (Figs. 3B and C, respectively).

FMDV-IFN-γ production requires stimulation with whole 140S particles

The data showed above suggested that the virus strain was related to the ability for inducing IFN-γproduction. Based on early reports indicating the differential structural stability among strains, we explored whether this factor may be related or not to the FMDV strains' capacity to stimulate IFN-γ responses.

We first addressed if A24/Cruzeiro and O1/Campos particles have differential stability in physiological conditions. Purified 140S particles were kept at 37 °C and capsid stability was evaluated at different time points by a 15%–45% SDG purification protocol followed by spectrophotometry and ELISA analyses (Fig. 4). Freshly thawed A24/Cruzeiro FMDV aliquots (time "0 h") contained most of the initial viral amount (120 μg) as 140S particles, as indicated by the presence of coincident peaks of viral RNA (Fig. 4A) and capsid proteins (Fig. 4C) in the lower fractions of the gradient (3 to 5 mL) as well as by their absence in the upper range of the gradient (13 to 15 mL). Contrarily, O1/Campos preparations already had a significant fraction of 12S subunits at this same time, as verified by the coincident spectrophotometry and ELISA peaks in the upper fractions of the gradient (13 to 15 mL, Fig. 4B and D). Following a 2 h incubation period at 37 °C, 140S particles from the A24/Cruzeiro strain decreased about 26% with little differences up to 24 h of incubation (Fig. 4E). In contrast, O1/Campos 140S particles were continuously disassembled along the experiment, and RNA and capsid protein peaks corresponding to 12S subunits clearly increased over time (Fig. 4B and D). After 24 h at 37 °C, over 80% of the O1/Campos 140S particles were broken down into 12S subunits (Fig. 4E).

We next studied if whole viral capsids were more efficient than 12S particles stimulating blood samples to elicit specific FMDV-IFN-γ responses. Disrupted capsids were prepared from both 140S purified preparations by heat treatment, and presence of 12S pentamers was confirmed by sucrose-gradient and antigen-specific ELISA (Fig. 5A and B). Protein degradation in these preparations was assessed by SDS-PAGE (Fig. 4C), showing that primary structures of the capsid proteins, and thus T-cell epitopes comprised within them, were not affected by the heat-treatment. Next, IFN-γ specific responses against O1/Campos and A24/Cruzeiro FMDV strains were determined by incubating whole blood from9 multi-vaccinated animals with heat-treated and untreated viral preparations. As it is shown in Fig. 5D, while IFN-γ levels detected in samples incubated with PBS wells were lower than in any of the antigen stimulated wells ($p < 0.01$), stimulations induced by whole 140S particles were significantly above those induced by heat-treated virus for both strains (A24/Cruzeiro, $p < 0.01$; O1/Campos, $p < 0.05$). These results clearly indicated that capsid disruption strongly affected recall IFN-γ responses in FMD vaccinated cattle.

Discussion

Several research lines have investigated the relation between IFN-γ and immunity against FMDV in natural hosts. Different publications have related the production of bovine IFN-γ with innate and antiviral responses to FMDV *in vivo* (Toka et al., 2011) and *in vitro* (Zhang et al., 2002), and antiviral properties of this cytokine together with type I interferon were also shown *in vivo* for swine (Diaz-San Segundo et al., 2010; Moraes et al., 2007). However, in ruminants most of the effort has been focused on its use as indicative of FMDV vaccine- or infection-induced adaptive immunity (Barnett et al., 2003; Carr et al., 2013; Oh et al., 2012; Parida et al., 2006).

Here, we first set up the incubation conditions of bovine blood samples to measure high-throughput FMDV-specific IFN-γ production, using a commercial ELISA originally developed for *Mycobacterium bovis* (Wood and Rothel, 1994). Initial validation parameters of the assay included 308 samples and were associated to the capacity of the blood cells to produce IFN-γ (PWM-stimulated wells) and to the IFN-γ synthesised by cells of the innate immune system in a non-specific manner (PBS-stimulated wells). These parameters permitted to harmonize IFN-γ measurements from different samples by discarding those that do not fit the positive/negative assay parameters, thus allowing to compare groups/treatments rather than individuals. Once set up, the assay applied for FMDV antigens rendered negligible unspecific responses: neither blood samples from non-vaccinated animals stimulated with FMDV antigens, nor samples in PBS-stimulated wells were capable to produce significant levels of IFN-γ. On the other side, blood samples from FMD primo- or multivaccinated cattle effectively produced IFN-γ after incubation with purified inactivated FMDV from homologous strains.

FMDV-specific IFN-γ responses were also evoked in blood samples from cattle vaccinated with monovalent formulations and stimulated with heterologous strains. Only one report measured heterologous IFN-γ production before and suggested a high degree of serotype specificity after FMDV O_1Manisa stimulation in blood samples from A Iran 96 vaccinated cattle, although data was not shown in the publication (Parida et al., 2006). Cross-reactive T-cell responses among FMDV strains have been previously observed by *in vitro* lymphoproliferation assays in cattle (Collen et al., 1998; Collen and Doel, 1990) and swine (Saiz et al., 1992) and were mainly attributed to CD4+ T lymphocytes. T-cell cross-activation among FMDV strains is expectable since T-cell epitopes, as short and linear sequences, are more promiscuous than conformational epitopes. Consequently, stimulating antigens used in most of the FMDV-specific lymphoproliferative assays previously published did not require complex structures (Cartwright et al., 1980; Collen et al., 1998; Collen and Doel, 1990)

Our findings on the existence of cross-stimulation among serotypes are in agreement with the above cited reports. In our hands, however, A24/Cruzeiro strain activated higher heterologous IFN-γ responses than the other strains, when used as an *ex-vivo* stimulator or as a vaccine antigen. Conversely, FMDV O1/Campos and A/Arg/01 strains appeared as less effective antigens in both situations. Interestingly, such differential ability among strains was not reflected in tetravalent formulations, suggesting that cross-reactivity also takes place *in vivo*. Since both papers by Collen et al. (Collen et al., 1998; Collen and Doel, 1990) demonstrated that there were no significant disparities in the homologous and heterologous lymphoproliferative capacity between A24/Cruzeiro and O1/Campos strains, we hypothesised that the differences observed in their ability to induce IFN-γ were not related to their primary protein sequences. Thus, based on the different capsid stability reported among FMDV strains, we explored whether FMDV-specific IFN-γ production might be affected by the antigen structure or not. The stability of whole capsids was higher for

A24/Cruzeiro preparations than for O1/Campos antigens, in accordance with early reports (Doel and Baccarini, 1981). Interestingly, we found that the *ex-vivo* production of IFN-γ in FMD-vaccinated cattle was strongly related to the capsid integrity of the stimulating FMDV antigen, independently of the strain tested. It is then possible to hypothesise that the lower thermal stability of the FMDV O1/Campos capsids compared to the A24/Cruzeiro strain may explain its reduced ability to elicit IFN-γ synthesis in blood from FMD-vaccinated cattle, either as an *ex-vivo* stimulator or as a vaccine antigen.

The mechanisms behind all these observations are not clear yet. Preliminary results from our lab using purified PBMC from FMD-vaccinated cattle in the presence or absence of autologous serum did not show an evident correlation between the IFN-γ production and the existence of antibodies against FMDV in the sample (data not shown). Moreover, FMD cell-mediated assessments stimulating with 140S particles, were similar when measured by lymphoproliferative (using purified PBMC) or IFN-γ responses (using whole blood samples) (Bucafusco et al., unpublished results). Other variables which may differentiate the antigen presentation triggered by 140S and 12S particles are currently under study.

At first, our results have a clear methodological impact for the high-throughput testing of cell-mediated immunity using stimulated plasma, since the *ex-vivo* FMDV-specific IFN-γ evaluation seems to be strongly affected by the structural integrity of the stimulating antigen. The use of disrupted capsid particles in the stimulation phase of the assay may lead to false negative results, especially when using structurally unstable FMDV strains, such as those from the O or SAT serotypes.

Furthermore, the phenomenon here described may also affect the *in vivo* performance of FMD vaccines. Monovalent vaccines from O1/Campos were less efficient IFN-γ inducers than A24/Cruzeiro vaccines or tetravalent formulations. Our observations may be linked to early reports reflecting quantitative differences in the antigenicity of 140S and 12S particles (Cartwright et al., 1982; Doel and Chong, 1982; Rao et al., 1994). The lower neutralizing antibody titres induced by 12S particles may be obviously related to destruction of neutralizing epitopes from the 140S to 12S particles. Furthermore, FMDV-specific IFN-γ production in vaccinated cattle has been associated to the activation of antigen-specific CD4+ T cells (Oh et al., 2012) which are responsible for supporting antibody neutralising responses (Carr et al., 2013). Thus, it is also possible to hypothesise that the reduced ability of the 12S particles to trigger anamnestic FMDV-specific IFN-γ responses may also contribute for the lower immunogenic capacity in FMD vaccines comprising structurally unstable strains. Further for-purpose experiments are required to explore this hypothesis.

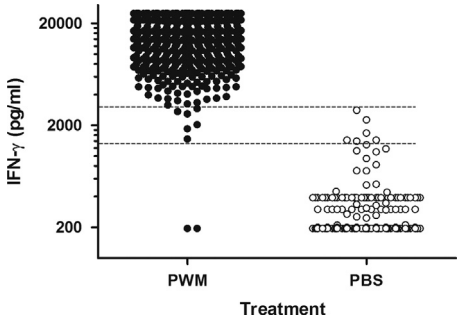

Fig. 1. Assessment of the acceptance parameters of the IFN-γ production assay. Whole blood samples ($n=308$) from FMD-vaccinated and non-vaccinated animals (see vaccine status, Table 1) were incubated in the presence of PWM (10 μg/mL) or PBS for 24 h. Collected plasma samples were tested by ELISA Bovigam®. Results are expressed as individual IFN-γ concentrations (pg/mL) estimated using a standard curve built with recombinant bovine IFN-γ. Horizontal dotted lines indicate cut-off values for sample acceptance as determined with ROC analysis. Limit for PWM stimulation > 3200 pg/mL (upper line); limit for PBS stimulation < 1450 pg/mL (lower line).

Materials and methods

Virus purification

FMDV A24/Cruzeiro/Brazil/55 (A24/Cruzeiro), O1/Campos/Brazil/58 (O1/Campos), A/Argentina/2001 (A/Arg/01) and C3/Indaial/Brazil/71 (C3/Indaial) were kindly provided by a local vaccine producer as inactivated and concentrated preparations from clarified-infected cell cultures. Purified particles were obtained by a 15%–45% sucrose density gradient (SDG) centrifugation method (Barteling and Meloen, 1974) further optimized in our laboratory (Pega et al., 2013).

Animals, vaccination and sampling

Different groups of bovines were utilized for the experiments described in this work, totalling 308samples (Table 1). In all cases, IFN-γ assays were performed over whole blood samples obtained by jugular venipunction using Vacutainer® tubes (BD) containing heparin. Samples were transported to the laboratory at room temperature and processed within 8 h after bleeding. All animal handling procedures were previously approved by the INTA's Animal Welfare Commission (protocol approval No. 025/2011).

Heterologous reactivity was assessed using blood samples from cattle immunized (30 dpv) with one dose of a single-emulsion oil monovalent FMD vaccines were produced by a local vaccine manufacturer containing 10 μg of A24/Cruzeiro ($n=10$), 10 μg of A/Arg/01 ($n=10$), or 20 μg of O1/Campos ($n=30$) inactivated

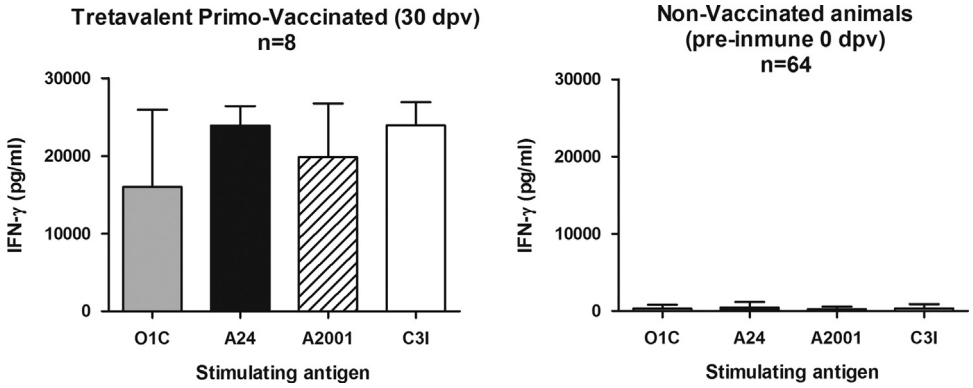

Fig. 2. FMDV-specific IFN-γ responses in *naïve* and primo-vaccinated cattle. Whole blood samples were stimulated with 10 μg/mL of FMDV O1/Campos (O1C), A24/Cruzeiro (A24), A/Arg/2001 (A2001) and C3/Indaial (C3I) for 24 h in tetravalent primo-vaccinated animals (**A**) or *naïve* cattle (**B**). Results are expressed as IFN-γ concentrations (pg/mL, mean ± SEM). Samples included in the graph complied the acceptance parametersestablished for PWM and PBS stimulation.

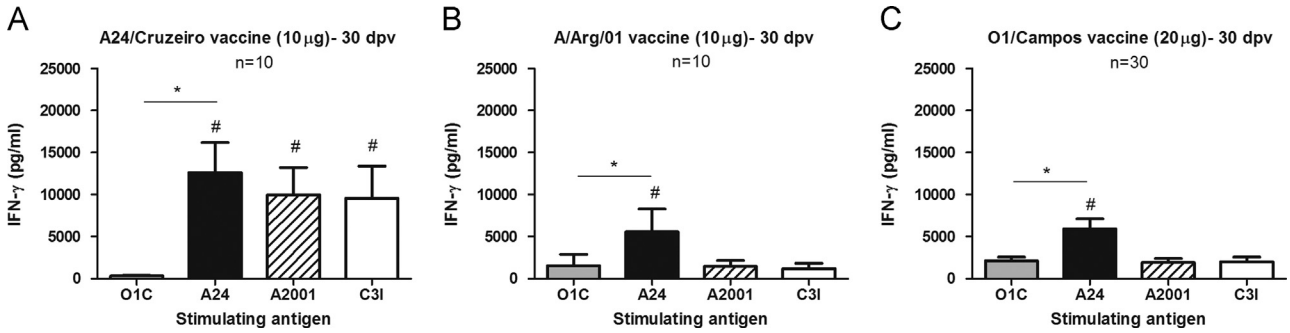

Fig. 3. Heterologous IFN-γ responses in FMD vaccinated cattle. IFN-γ responses to O1/Campos (O1C), A24/Cruzeiro (A24), A/Arg/2001 (A2001) and C3/Indaial (C3I) observed in cattle vaccinated with single-strain formulations 30 days post vaccination. Sample size and composition for each vaccine are indicated in the panel titles. (#) indicates mean values significantly higher than the non-stimulated control (PBS), (∗) indicates mean values significantly different between A24/Cruzeiro versus O1/Campos stimulation ($p < 0.05$, Mann-Whitney).

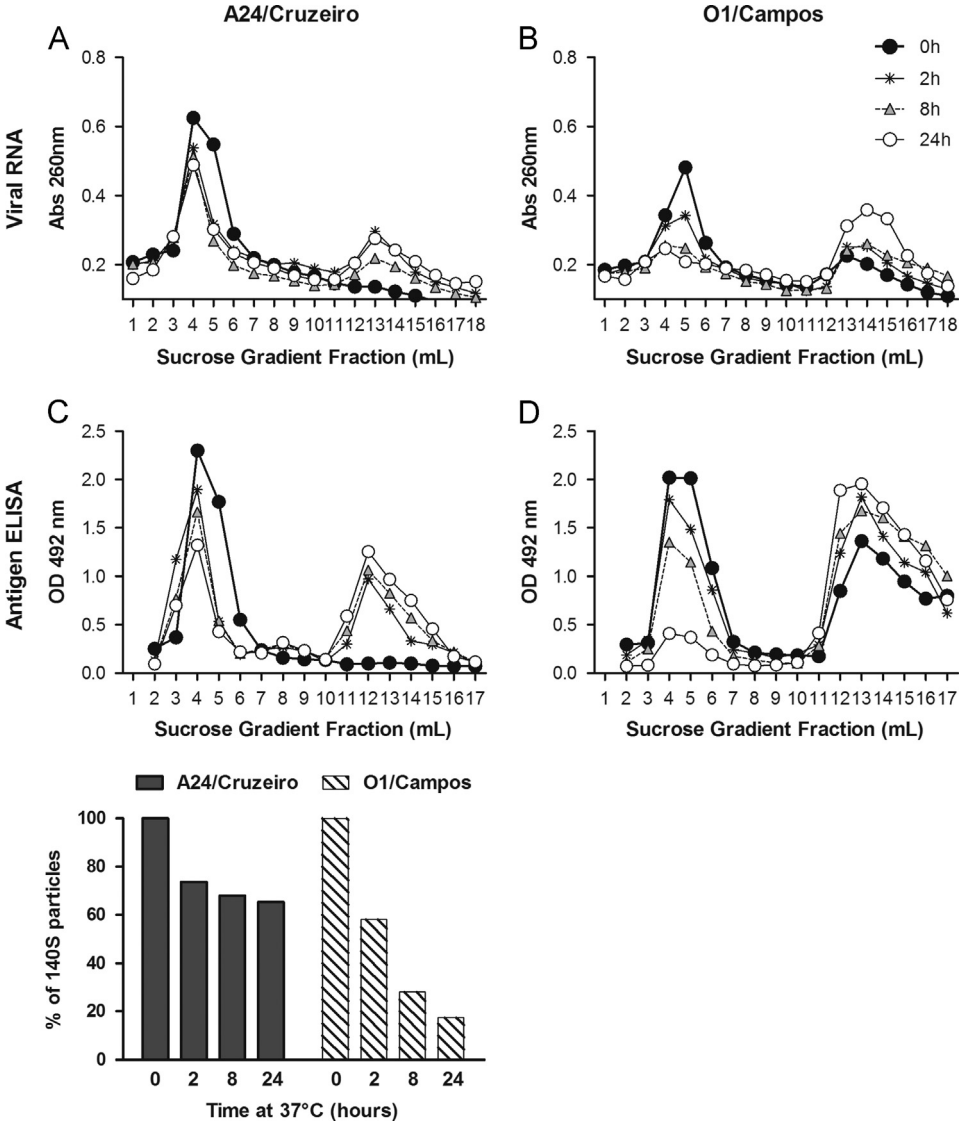

Fig. 4. Stability of purified FMDV A24/Cruzeiro and O1/Campos whole particles. Purified 140S particles from A24/Cruzeiro (A, C) and O1/Campos (B, D)strains were incubated for 0, 2, 8 and 24 h at 37 °C. After incubation, purified FMDV preparations were submitted to sucrose density gradient and resulting fractions were evaluated by spectrophotometry at 260 nm (viral RNA) (A, B) and by antigen-ELISA (structural proteins) (C, D). Areas under curve between fractions 3 and 7 (mL) in charts A and B (representing 140 S particle concentration for O1 /Campos and A24/Cruzeiro, respectively) were determined at time 0 h and 140S concentrations for subsequent time points were expressed as a percentage of this initial value for each virus strain (E).

FMDV. Vaccine payloads were confirmed by disrupting the vaccine emulsions after a 2 h incubation with an equal volume of chloroform at 4 °C under constant stirring, followed by centrifugation at 3000 xg during 15 min at 4 °C. Aqueous phase was then processed by a 15–45% SDG and recovered 140S particles were quantified as described (Barteling and Meloen, 1974). All the remaining vaccinated

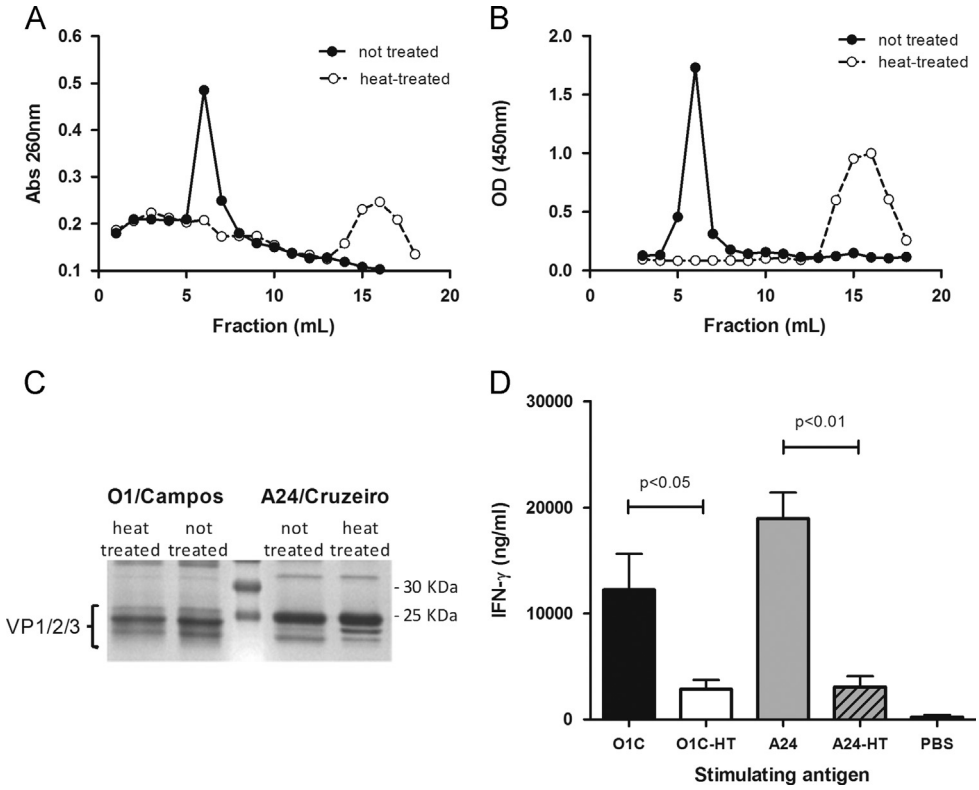

Fig. 5. Capacity of 140S and 12S particles to induce recall IFN-γ responses. SDG fractions of FMDV A24/Cruzeiro were heat treated (60 °C, 20 min.) and measured by (A) spectrophotometry at 260 nm (viral RNA) and (B) by antigen ELISA. Heat treated and non-treated FMDV purified particles were analyzed in a 12% denaturing SDS-PAGE and stained with Coomassie blue (C). IFN-γ responses of multi-vaccinated cattle ($n=9$) after stimulation with 140 S (O1C, A24) or 12S particles (O1C-HT, A24-HT) from both strains (D).

animals were immunized in the frame of the official FMD vaccine campaign using commercially available single oil emulsion tetravalent FMD vaccines including A24/Cruzeiro, O1/Campos, A/Arg/01 and C3/Indaial strains.

Assays to detect FMDV-specific IFN-γ production

Experiments to establish the IFN-γ assay's acceptance parameters were performed over the entire set of samples, using whole blood (1.5 ml)from näive ($n=114$) or FMD-vaccinated cattle ($n=164$) immunized following different vaccine regimes (Table 1). Samples were incubated in 24-well sterile cell culture plates during 24 h at 37 °C with 5% CO_2 (Nunc, USA) using Pokeweed mitogen (PWM, 10 μg/mL) or phosphate buffer saline pH 7.0 (PBS). Plates were centrifuged for plasma collection and samples were stored at −20 °C until processing. IFN-γproduced in each well was analysed using Bovigam® ELISA (Prionics) (Gormley et al., 2006), performed according to manufacturer's specifications. IFN-γ levels were expressed as pg/mL of plasma using a standard curve build with known concentrations of a recombinant bovine IFN-γ (AbD-Serotec) ranging from 195 to 25,000 pg/ml. This IFN-γ detection and quantification protocol from stimulated plasma samples was applied for all subsequent experiments. Experiments aimed to set up the FMD-specific IFN-γ assays were carried out using whole blood samples from näive individuals ($n=2$) or cattle vaccinated with single or multiple ($n>3$) doses ($n=4$ each group) of a commercial tetravalent FMD oil vaccine. Blood samples, taken 28 days after the last vaccination, were incubated with PBS, PWM or purified inactivated FMDV O1Campos at 2.5, 5 or10 μg/mL (final concentration). Cultures were left for 24, 48 and 72 h at 37 °C with 5% $CO_2$and further processed for IFN-γ detection (results shown in Supplementary Figure).

Heterologous IFN-γ production was assessed using blood samples from cattle vaccinated with FMD monovalent formulations (described in the previous section) and cultured for 24 h (37 °C, 5% CO_2) using inactivated purified FMDV 140S particles from A24/Cruzeiro, A/Arg/01, O1/Campos or C3/Indaial strains (10 μg/mL). Finally, assays using whole or disrupted viral particles as stimulating antigens were performed using blood samples from 9 multivaccinated bovines receiving commercial tetravalent FMD oil vaccines from the official FMD vaccination campaigns. Conditions of the assay were as described above.

FMDV 140S particle-stability assessment

Inactivated purified O1/Campos (395 μg/mL) and A24/Cruzeiro (315 μg/mL) 140S particle preparations stored at −80 °C were used for this experiment. Five aliquots (120 μg each) of both virus strains were prepared and frozen at −80°C. Each aliquot was then incubated at 37 °C for 0, 2, 4, 8 and 24 h. After each incubation time, samples were run in a 15%–45% SDG as above. Gradient was collected in 1 mL fractions and further evaluated by spectrophotometry at 260 nm (to detect viral RNA) and by an in house antigen ELISA (to detect FMDV structural proteins). For this ELISA, fractions were diluted 1:600 in PBS 0.05% Tween 20 (PBST) OVA 1% and transferred to 96-well ELISA plates (Maxisorp, Nunc) precoated with anti-A24/Cruzeiro or anti-O1/Campos rabbit hyperimmune sera (1:3000). After 1 h of incubation at 37 °C, plates were washed 3 times with PBST and captured antigen was detected using strain-specific guinea-pig antiserum (1:200) incubated for 1 h at 37 °C. Plates were washed as described above and a peroxidase-labeled sheep polyclonal anti-guinea-pig antibody (1:2500, KPL) was added. Finally, after 5 washes with PBST, the reaction was developed by addition of o-Phenylenediaminedihydrochloride/H_2O_2 in 50 mM phosphate-citrate buffer, pH 5.0. The

reaction was stopped by the addition of 50 µl of 1.25 M H_2SO_4 and OD values were read at 492 nm. Amounts of 140S particles for each strain at each time-point were calculated as the area under the curve for a range of fractions previously identified to comprise FMDV 140S particles.

Disruption of 140S particles

Dissociation of 140S particles into 12S pentameric subunits was performed by heat disruption. One hundred µl of purified A24 Cruzeiro or O1Campos 140S particles suspensions (1.6 ng/mL) were heated at 60 °C during 20 min or left in ice for the same period. Both heat-treated and non-treated samples were diluted in NET buffer (0.1 M NaCl, 0.004 M EDTA, 0.05 M Tris pH 8.0) to a final volume of 4 ml and then 3.4 ml were overlayered in a 15–45% SDG as described for virus purification. Fractions collected from sucrose gradients were analyzed by spectrophotometry at 260 nm and by the antigen-ELISA described above. Fractions of the untreated and heat-treated virus purified preparations (25 µg) were resolved in a 12% denaturing SDS-PAGE and proteins were stained by Coomassie blue to study viral structural proteins integrity.

Statistical analyses

Statistical analyses were carried out using biostatistics, curve fitting and scientific graphing software (Statistix v8.0, Analytical Software and GraphPad Prism v5.03,GraphPad Software). Significance was established within the 95% or 99% confidence interval, as indicated. To compare the tests accuracy in discriminating between positive (PWM activated) and negative (PBS) samples, receiver operator characteristic curves (ROC) were constructed and tested for statistical significance by Mann-Whitney non-parametric test for independent samples. ROC curves were built by plotting the corresponding Se and 1-Sp values at all possible cut-off values to predict a dichotomous outcome (positive or negative), considering the area under the curve (AUC) to determine the overall result of the test performance. The greater the AUC, the better the test at predicting the outcome, considering all possible combination of cut-off values (the null hypothesis was AUC = 0.5). ROC curves, AUC values and confidence interval, as well as cut-off test values for maximization of accuracy in terms of Se and Sp, were computed using ROCR package in R(Sing et al., 2005). The Se and Sp of dichotomized test results was determined by the optimal cut-off value in the ROC curves. Data is presented in a scatter plot of the individual animal values and the protection status between the confidence interval selected as the criteria of acceptance of values obtained by PBS and PWM treatment. Comparison between mean values of two groups (i.e.: mean concentration results against two viruses) was assessed by unpaired non-parametric Mann-Whitney test. Comparison between groups was performed by two way ANOVA followed by Bonferroni (post-ANOVA).

Acknowledgments

The research leading to these results have received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement n° 226556 (FMD-DISCONVAC), the Agencia Nacional de Promoción Científica y Tecnológica Project PID 87-2009-PAE 37.206 and INTA's Project PNSA1115052.We would like to thank Dr. Ricardo D'Aloia (SENASA) for handling of experimental cattle and collection of samples. AVC and MPF are researchers of CONICET, DB and JP have post-doctoral fellowships from CONICET and NC has a post-doctoral scholarship from The Bunge y Born Foundation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.11.023.

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