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Evaluation of the immune response elicited by vaccination with viral vectors encoding FMDV capsid proteins and boosted with inactivated virus

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ABSTRACT

The aim of the present study was to assess the effect of introducing a priming step with replication-defective viral vectors encoding the capsid proteins of FMDV, followed by a boost with killed virus vaccines, using a suitable BALB/c mice model. Additionally, the immune response to other combined vector immunization regimens was studied. For this purpose, we analyzed different prime-boost immunizations with recombinant adenovirus (Ad), herpesvirus amplicons (Hs) and/or killed virus (KV) vaccines. The highest antibody titers were found in the group that received two doses of adjuvanted KV ($P < 0.002$). Antibody titers were higher in those groups receiving a mixed regimen of vectors, compared to immunization with either vector alone ($P < 0.0001$). Priming with any of the viral vectors induced a shift of the cytokine balance toward a Th1 type immune response regardless of the delivery system used for boosting. The highest IgG1 titer was induced by two doses of adjuvanted KV ($P = 0.0002$) and the highest IgG2a titer corresponded to the group primed with Ad and boosted with KV ($P = 0.01$). Re-stimulation of all groups of mice with 0.5 μg of inactivated virus five months later resulted in a fast increase of antibody titers in all the groups tested. After virus stimulation, antibody titers in the groups that received KV alone or Ad prime-KV boost, were indistinguishable ($P = 0.800$). Protection from challenge was similar (75%) in the groups of animals that received Ad prime-Hs boost or Ad prime-KV boost, or two doses of oil-adjuvanted KV. The data presented in this study suggest that sequential immunization with viral vectors-based vaccines combined with protein-based vaccines have the potential to enhance the quality of the immune response against FMDV.

1. Introduction

FMD is a very contagious disease whose consequences are extremely damaging. It causes significant distress and suffering to animals, impacts on the livelihood of the farmers and produces huge economic losses (Doel, 2003). Killed FMDV (KV) vaccines currently in use have been very successful in reducing the number of disease outbreaks in

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many parts of the world where the disease is enzootic (Mattion et al., 2004). Nevertheless, there are a number of concerns and limitations regarding its use and production, particularly in FMDV-free countries, such as elevated costs of the high-containment facilities required for manufacturing, the potential escape of infectious virus to the environment, differentiation between antibodies produced by disease or vaccination, among others. For these and other reasons, systematic vaccination is not applied in many FMD-free countries, which are particularly interested in marker vaccines for emergency use, capable of generating fast early protective responses.

New generation vaccines against foot-and-mouth disease virus (FMDV) have been extensively explored, though they have not equaled the efficacy of the conventional killed virus vaccine in use.

Viral vectors capable of expressing FMDV empty capsids directly in the target animals have been used for experimental immunization, such as live replication-defective human adenovirus (Ad) vectors which resulted in protection from disease (Grubman et al., 1993; Mayr et al., 1999). In previous work, we have shown that HSV-1 amplicon vectors (Hs) provided protection from challenge in mice, with a significant reduction of viremia (D'Antuono et al., 2010). This type of vaccine has the additional advantage that they may be used as marker vaccines.

Over the past decade, studies have suggested that heterologous prime-boost (HetPB) vaccination approaches, in which the same antigen is administrated sequentially by different delivery methods, might be more effective in enhancing cellular and humoral immune responses than two doses of the same type of vaccines (Lu, 2009; Vaine et al., 2010). The rapid progress of novel vaccination strategies, such as DNA vaccines and viral vector-based vaccines, has certainly further expanded the scope of HetPB vaccination (Woodland, 2004; Lu, 2009; Davtyan et al., 2010), including FMDV (Li et al., 2008).

Vaccine design strategies against FMD might benefit from focusing also on the simultaneous stimulation of humoral and cellular immune responses. The contribution of the humoral response to the in vivo protection against FMDV has been clearly established. In particular, a strong correlation was found in convalescent and conventionally vaccinated animals between neutralizing activity in sera and protection against FMDV challenge (Sobrino and Domingo, 2001). However, this correlation was not always found, and protection has been observed also in animals which showed no detectable neutralization antibody titers (Borrego et al., 2006). This phenomenon may be explained by additional immune mechanisms that exist in the host, such as innate immunity (Barnard et al., 2005) or cell-mediated immunity, which might contribute to protection (Sanz-Parra et al., 1999a; Borrego et al., 2011).

In the present study we aimed to assess if the use of priming with replication-defective viral vectors encoding proteins of FMDV, may improve the immune responses to subsequent inoculation of KV vaccines. For this purpose, groups of BALB/c mice were primed and boosted with recombinant Ad, Hs and/or KV vaccines and the immune responses to the different immunization regimens were studied. We show that the priming of the immune system with recombinant Ad contributes to a more balanced immune response to KV vaccines. Moreover, combined immunization regimens including viral vectors might induce similar protection in mice than two doses of oil-adjuvanted inactivated FMD virus. The experiments described were performed using BALB/c mice, a well-studied murine model (Fernandez et al., 1986; Dus Santos et al., 2000; Borrego et al., 2006), to circumvent the problems and costs derived from handling a large number of experiments in FMDV natural hosts.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney (HEK293) cells, transformed with sheared human adenovirus type 5 DNA, were acquired from Invitrogen (California, USA). African green monkey kidney cells expressing the HSV-1 immediate-early essential gene ICP27 (Vero 2-2), were kindly provided by Dr. Cornel Fraefel (University of Zürich). FMDV strain O1/Campos/Brazil/58 (O1/Campos) was supplied by SENASA (origin: PANAFTOSA, Brazil).

2.2. Generation of recombinant HSV-1 amplicons and adenovirus vectors encoding FMDV capsid proteins

The HSV-1 plasmid amplicon pHSV[P12A3C], containing the coding sequences of FMDV (O1/Campos strain) structural proteins, 2A protein and 3C protease, was constructed and packaged into virus particles as previously described (Saeki et al., 2001; D'Antuono et al., 2010), using a helper–free system, where the HSV-1 genome was provided in *trans* by a bacterial artificial chromosome (BAC) containing the HSV-1 genome with deletions in the DNA cleavage/packaging signals and the essential *ICP27* gene (fHSVΔpac-ΔICP27). Briefly, Vero2-2 cells were co-transfected with pHSV[P12A3C] DNA, the fHSVΔpacΔICP27 BAC and the plasmid pEBHICP27 (which provides the HSV-1 ICP27 gene in trans), using Lipofectamine and Plus Reagent (Invitrogen). The amplicon particles generated were designed as Hs[P], where P12A3C was abbreviated as [P] for simplicity. Titers were calculated as described (D'Antuono et al., 2010) and expressed as Transducing Units/ml (TU/ml).

The adenovirus type 5 vector (Ad) carrying the same coding sequences from FMDV (O1/Campos strain) (Ad[P]) was obtained using the Virapower™ Adenoviral System (Invitrogen, California, USA) following the manufacturer's instructions. Briefly, the [P] insert was cloned into the adenovirus shuttle vector pENTR™-4, which was recombined in vitro into the pAd/CMV/V5-DEST™ Adenoviral Gateway® Vector. Titration of Ad[P] stocks was performed by the limiting dilution method, and expressed as plaque forming units (PFU)/ml. An Ad vector expressing the enhanced green fluorescent protein (GFP) reporter gene (Ad[G]) was generated and used as a negative control.

2.3. Experimental animals and immunization protocols

The experiments carried out in mice reported in this manuscript have been performed following internationally recognized guidelines with the approval of the Institutional Committee for Care and Use of Experimental Animals, CICUAE [approval reference CICUAE INTA-CICVyA 30/2010].

Groups of four mice (4–6-week-old male BALB/c mice) were immunized twice following the schedule shown in Table 1. Animals primed with Ad[P], were boosted either with the same vector, with the Hs[P] amplicon vector, or with the conventional KV vaccine (adjuvanted killed FMDV). Homologous prime-boost regimens such as Hs[P]/Hs[P] or KV/KV were also carried out for comparison. Inoculations with recombinant Ad vectors expressing GFP (Ad[G]), were carried out as a negative control.

To work on a limited dose level, 5×10^7 PFU of Ad[P] vector and 5×10^5 TU of Hs[P] amplicons per mouse were used (D'Antuono et al., 2010). Mimicking the classical commercial vaccine, KV was formulated with Marcol/Montanide® (Seppic, France). The virus antigenic mass used per dose was either 1 μg of 146S particles when used alone, or 0.5 μg in mixed prime-boost regimens.

At day 56 post vaccination (pv), duplicated groups of mice were killed and splenocytes were harvested for determination of lymphocyte proliferation and cytokines mRNA. The other groups were re-stimulated with 0.5 μg of KV at day 146 pv. The humoral immune response was analyzed in sera from blood collected at days 0, 28, 56, and 146 days post vaccination (dpv), and at days 153 and 174 (7 and 15 days post re-stimulation with inactivated virus).

For challenge experiments, groups of 4 mice were immunized twice following the schedule of Table 1, and challenged with live FMDV O1/Campos strain (10^4 PFU) at 56 dpv. Duplicate groups were challenged at 146 dpv, after a resting period of 4 months. A schematic representation of the experiments is provided in Fig. 1.

2.4. FMDV-specific antibodies titers

Anti-FMDV antibodies were detected by solid phase ELISA (spELISA), following a protocol modified from Seki et al. (2009). Briefly, ELISA microplates (MaxiSorp™, Nunc, Rochester, USA) were coated with a rabbit antiserum against FMDV O1/Campos strain in carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C. Inactivated virus was then added to the wells and incubated for 1 h at 37 °C. Subsequent steps were performed using blocking buffer (PBS containing 3% horse serum and 0.05% Tween 20). Murine sera to be tested were serially diluted in blocking buffer, and subsequently added to the coated ELISA plate and incubated for 60 min at 37 °C. Peroxidase-labeled anti-mouse IgG, IgG1 or IgG2a antibodies were used to develop the reactions. Antibody titers were expressed as the $\log_{10}$ of the reciprocal of serum dilutions giving at least twice the absorbance at 495 nm recorded in the negative control wells. Isotyping of antibody responses was carried out in sera (dilution 1:200) at 56 dpv.

An ELISA-based antibody avidity assay was performed incorporating a urea elution step, as previously described (Perciani et al., 2007). The avidity of each serum was evaluated through its ability to be displaced from the plates by increasing molar (M) concentrations of urea, and expressed as the urea concentration required to displace 50% of IgG initially bound on the plate.

2.5. Detection of cytokines mRNA

The levels of mRNA accumulation of several representative cytokines were detected, as previously described (Zhang et al., 2011), with minor modifications. Briefly, fresh murine spleen cells were plated in 6-well microtiter plates at a density of 3.3×10^7 cells per well. The lymphocytes were incubated ON, in triplicate wells, with inactivated FMDV O1/Campos antigen (40 μg/ml) or Concanavalina A (Con A, 2.5 μg/ml) in RPMI-1640 containing 10% FCS at 37 °C in a 5% CO_2 incubator. After RNA extraction, RT-PCR was performed using the primers described in Table 2. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalizing control. The PCR products were subjected to electrophoresis on 1.5% agarose gels and photographed under the UV light.

2.6. T lymphocyte proliferation

Splenocytes from immunized mice harvested at sacrifice were incubated in 96-well plates (10^6 cells per well) and stimulated with inactivated FMDV O1/Campos antigen (5 μg/ml) or ConA (2.5 μg/ml) for 2 days at 37 °C, in a 5% CO_2 incubator. The in vitro cell proliferation was measured

Table 1
Schedule of mice immunization and challenge.

Group ($n = 4$)	Immunogen		Adjuvant	Imm. Route	Dose (TU)	Challenge (O1/Campos 10^4 PFU)	
	Prime (Day 0)	Boost (Day 28)				56 dpv	146 dpv
1	Ad[P]	Ad[P]	–	im	5×10^7	yes	yes
2	Hs[P]	Hs[P]	–	im	5×10^5	no	no
3	Ad[P]	Hs[P]	–	im	$5 \times 10^7/5 \times 10^5$	yes	yes
4	Ad[P]	KV	–/MM	im/ip	5×10^7/0.5 μg	yes	no
5	KV	KV	MM/MM	ip	1 μg	yes	no
6	Ad[G]	Ad[G]	–	im	5×10^7	yes	yes

Ad, adenovirus vector; Hs, herpesvirus amplicon; KV, killed FMDV; P, FMDV capsid proteins plus 2A and 3C protease; G, GFP; MM: Marcol–Montanide, Seppic®. TU: transducing units; PFU: plaque forming units.

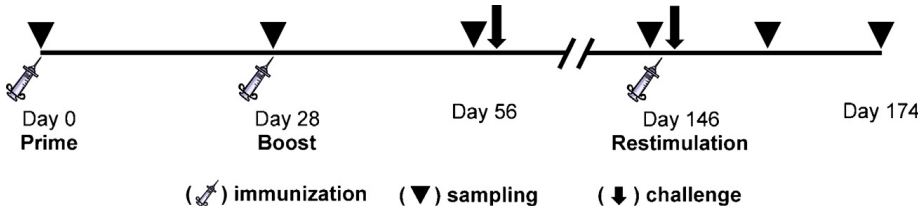

Fig. 1. Schematic representation of the experimental design. Four- to 6-week-old BALB/c mice ($n = 4$) were immunized twice with a 28 days interval using the different prime-boost inoculation regimens shown in Table 1. At day 56 pv, duplicated groups of mice were killed and splenocytes were harvested for the analysis of cellular immune response, or challenged with live FMDV O1/Campos strain at 56 or 146 dpv. After a resting period of 146 days, other similarly vaccinated groups of mice were re-stimulated with 0.5 μg of KV. Blood samples were collected at 0, 28, 56, 146, 153 and 174 dpv.

by incorporation of [3H] thymidine as previously described (D'Antuono et al., 2010). The Stimulation Index (SI) was calculated as the ratio of the average counts per minute (cpm) ± SD (Standard Deviation) from triplicate wells containing antigen-stimulated cells, to the average cpm value of wells without FMDV antigen. Statistical analyses were performed based on the logarithmic transformation of the cpm ratios.

2.7. Viral challenge

Mice were challenged with live FMDV in the facilities located in INTA, Castelar, Buenos Aires, by intraperitoneal (ip) inoculation of 10^4 $TCID_{50}$ of O1/Campos strain per mouse. The groups of mice 1, 3, 4, 5, and 6 described in Table 1 were challenged at 56 dpv in BSL-3A (BSL-4 OIE) facilities and analyzed for the presence of viremia 24 hours post challenge. Other three duplicate groups of mice (vaccinated like groups 1, 3 and 6, Table 1) were challenged at 146 dpv.

Protection against FMDV was assessed as described previously, with minor modifications (Carrillo et al., 1998; Quattrocchi et al., 2011). Twenty-four hours after challenge, the animals were anesthetized and bled by the retro orbital route. Heparinized blood was spread undiluted on BHK-21 cell monolayers and after virus adsorption; monolayers were washed with sterile phosphate-buffered saline (PBS). Fresh DMEM with 2% fetal calf serum was added and the cells were kept for 48 h at 37 °C in a 5% CO_2 incubator. An animal was considered protected if the cell monolayer did not present cytopathic effect (cpe) after two blind passages. Percentages of protection were calculated as $P\%$ = (number of protected mice/number of challenged mice) × 100.

Table 2
Primers used for RT-PCR detection of mRNA of cytokines.

Target gene	Primer sequence	Fragment length
GAPDH	5′ ggCACAgTCAAggCTgAgAAC 3′ CATACTCggCACCAgCATCA	112 bp
IFN-γ	5′ CATTgAAAgCCTAgAAAg 3′ gAAATgCATCCTTTTTCg	263 bp
IL-4	5′ gCCATATCCACggATgCg 3′ TgCAgCTTATCgATgAATCC	285 bp
IL-2	5′ TCCACTTgAAgCTCTACAg 3′ gAgTgAAATCCAgAACATgCC	244 bp

2.8. Statistical analyses

Data analysis was performed with IBM SPSS Statisticis software v17.0 (SPSS Inc., Chicago, IL, USA) and OriginPro v7.5 (OriginLab, Northampton, MA, USA), respectively. Data were analyzed for homogeneity of variances using Levene's test for equality, and for normal distribution using Shapiro–Wilk test. Mean differences between groups were determined using ANOVA with Tukey Honestly Significantly Different post hoc test. *P*-Values of less than 0.05 were considered statistically significant.

3. Results

3.1. Combined prime-boost immunization regimens induced high antibody titers against FMDV in mice

After the first dose (28 dpv), significant differences could not be established in specific antibody titers of the different experimental groups of mice (Fig. 2). However, these titers significantly increased in all groups after a booster immunization (56 dpv, Fig. 2). There was a clear significant difference in the antibody titers of the group vaccinated with two doses of adjuvanted KV ($P < 0.002$) with all the other groups. Interestingly, animals primed with Ad[P] and boosted with either Hs[P] or with 0.5 μg of KV, elicited specific FMDV antibody titers which were significantly higher ($P < 0.0001$) compared with mice that received a prime-boost with the same vector (Ad[P]/Ad[P] or Hs[P]/Hs[P]), suggesting that mixed regimens might be also a powerful tool for generating high and durable titers of anti-FMDV antibodies.

3.2. Stimulation of mice with inactivated virus at 146 dpv resulted in a fast increase of antibody titers

FMD virus-specific antibodies were maintained for at least 5 months in the vaccinated mice. After a new virus encounter, a notable increment in anti-FMDV antibody titers were detected by spELISA as early as 7 days post re-stimulation in all the experimental groups (Fig. 2, 153 dpv), including the groups primed-boosted with the same vectors, but not in mice inoculated with the negative control Ad[G]. The antibody titers further increased at 28 days post re-stimulation (Fig. 2, 174 dpv), showing a clear difference in the antibody titers of the group vaccinated with two doses of KV and the group primed with Ad and

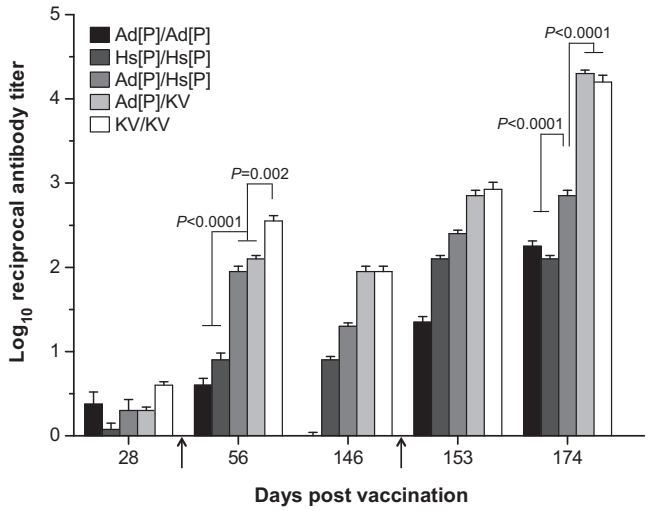

Fig. 2. Specific titers to FMDV induced in mice following different prime-boost vaccination strategies. The specific antibody titers were measured by spELISA. Each bar represents the mean ($n = 4$) of the $\log_{10}$ of the reciprocal antibody titer values ± S.E.M., at 28, 56, 146, 153, and 174 dpv. The background signal obtained with the negative control, Ad[G]/Ad[G] group, have been subtracted from the titers of all the groups. The significance of the differences in antibodies titers between groups at days 156 and 174 dpv are indicated at the top of the bars. The arrows indicate the boost and re-stimulation, respectively. KV: killed FMD virus; [P]: FMDV capsid proteins plus 2A and 3C protease. [G]: GFP.

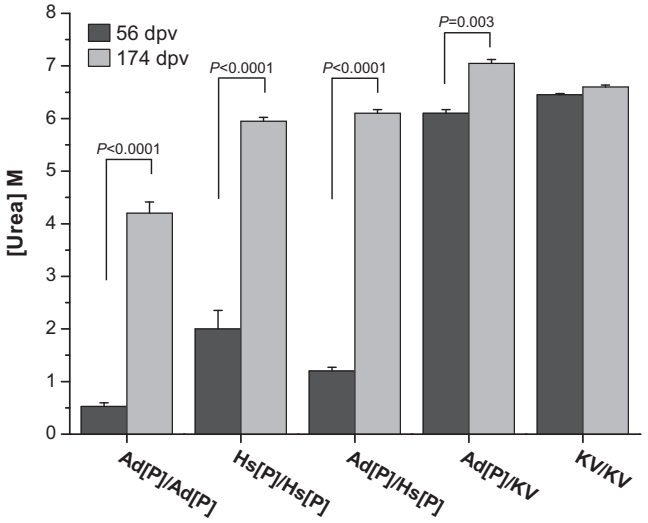

Fig. 3. Evaluation of specific antibody avidity elicited by the different immunization regimens. Binding avidity of the antibodies elicited in mice was analyzed using a urea displacement ELISA at 56 and 174 dpv, after re-stimulation with 0.5 μg of virus. The results are expressed the urea molar concentration ([Urea] M) required to displace 50% of IgG initially bound on the plate. The significance of the increased affinity maturation after re-stimulation is shown for each mice group at the top of the bars. KV: killed FMD virus; [P]: FMDVcapsid proteins plus 2A and 3C protease.

boosted with half dose of KV, compared to all the other groups ($P < 0.0001$). This rise in antibody titers correlated with an increment in the number of FMDV specific antibody secreting cells (ASC, data not shown).

It is important to emphasize that: (i) the immunization with KV/KV and the combined immunization with Ad[P]/KV reached the highest antibody titers, with no significant differences between them ($P = 0.8$); (ii) the vaccination strategy that used a combination of both viral vectors (Ad[P]/Hs[P]) in the absence of KV, elicited very high titers, which were significantly different from the previously mentioned groups and from the homologous vector immunization groups (Ad[P]/Ad[P] and Hs[P]/Hs[P], $P = 0.00004$, Fig. 2).

After prime-boost immunization (56 dpv) the avidity of the elicited antibodies was significantly higher in the groups boosted with KV (Ad[P]/KV and KV/KV) ($P < 0.0001$) and was rather low in the groups immunized with viral vectors alone (Fig. 3). However, antibody avidity increased dramatically in the groups primed with viral vectors after stimulation with virus ($P < 0.0001$), while the increment was not significant in the group that received KV ($P = 0.32$). Statistical significance of the rise in each group primed with vectors is shown in Fig. 3.

3.3. Analysis of T cell responses

T cell stimulation in splenocytes derived from animals primed-boosted with Ad[P]/Hs[P], Ad[P]/KV, or KV/KV, was found significantly higher ($P < 0.01$) than in those of the negative control group (Ad[G]). In contrast, no significant differences in T cell stimulation was found for the group primed-and-boosted with Ad[P] and the negative control, while the group of mice immunized with two doses of Hs[P] showed a SI similar to the heterologous immunization regimens (Table 3).

3.4. Antibody and cytokine profile of the immune responses induced in mice by the different immunization protocols

To further characterize the antibody response resulting from the different immunization approaches, the FMDV-specific IgG1 and IgG2a subclasses were measured. In mice primed with viral vectors, regardless of the boost, a predominance of IgG2a was observed, with lower levels of IgG1 (IgG1/IgG2a = 0.5–0.8), suggesting a bias toward a Th1 type response.

The levels of both IgG1 and IgG2a increased when the animals were primed with Ad[P] and boosted with KV, whereas the IgG subclasses ratio remained similar to the other groups immunized with viral vectors (IgG1/IgG2a = 0.8). As expected, in mice primed-and-boosted with KV, IgG1 was found to be the predominant IgG

Table 3
Lymphoproliferation responses induced by different prime-boost vaccination regimens at 56 dpv.

Vaccine	T cell Stimulation Index	*p*-Values
Ad[P]/Ad[P]	1.0 ± 0.30[a]	0.385
Hs[P]/Hs[P]	2.5 ± 0.50	0.0003[b]
Ad[P]/Hs[P]	2.7 ± 0.50	0.0002[b]
Ad[P]/KV	2.9 ± 0.70	0.0001[b]
KV/KV	3.9 ± 1.1	0.00001[b]
Ad[G]/Ad[G]	0.6 ± 0.35	–

[a] Standard Deviation.
[b] Significant difference compared to the group inoculated with 2 doses of the negative control Ad[G]; KV: killed FMD virus; [P]: FMDV capsid proteins plus 2A and 3C protease; [G]: GFP.

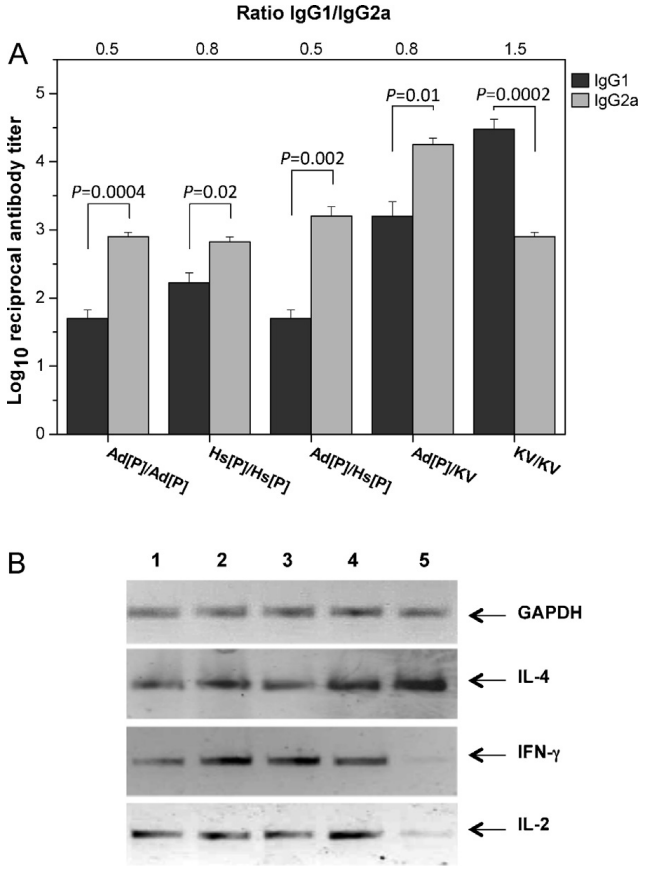

Fig. 4. Type of immune response. (A) Analysis of serum IgG1 and IgG2a specific responses at 56 dpv. ELISA titers of anti-FMDV specific IgG1 or IgG2a are shown. The ratio IgG1/IgG2a is displayed at the top of the graph. The consistency of IgG ratios was assessed through the determination of the significant differences in the titers of both isotypes in each group (shown at the top of the bars). (B) mRNA accumulation of IL-4, INFγ and IL-2 evaluated by RT-PCR in cultured splenocytes obtained from mice sacrificed at 56 dpv and stimulated with O1/Campos virus strain. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used as normalization control. Lane 1: Ad[P]/Ad[P]; lane 2: Hs[P]/Hs[P]; lane 3: Ad[P]/Hs[P]; lane 4: Ad[P]/KV; lane 5: KV/KV. KV: killed FMD virus; [P]: FMDV capsid proteins plus 2A and 3C protease.

subclass (IgG1/IgG2a = 1.5). These results suggested that the system used for priming may be dominant in the induction and activation of the type of response elicited (Fig. 4A).

The highest IgG1 titer was induced by two doses of adjuvanted KV ($P = 0.0002$) and the highest IgG2a titer corresponded to the group primed with Ad[P] and boosted with KV ($P = 0.01$).

Statistical soundness of the IgG ratios was established through the significance of the differences between the levels of IgG1 and IgG2a in each experimental group, indicated in Fig. 4A for each pair of bars.

The results described above were further corroborated by monitoring the mRNA accumulation of representative Th1 (IL-2 and INFγ) and Th2 (IL-4) types of cytokines, by RT-PCR, in cultured splenocytes obtained from mice sacrificed at 56 dpv and stimulated with O1/Campos virus strain. The groups of mice that were primed with viral vectors (Fig. 4B, lanes 1–4) showed a similar pattern of IL-2, IFN-γ and IL-4 mRNA accumulation. In contrast, mice primed and boosted with KV vaccine showed a higher level of accumulation of IL-4 mRNA than the other groups, and only marginal accumulation or mRNA of IL-2 and IFN-γ (Fig. 4B, lane 5). No cytokine mRNAs could be detected when naive control splenocytes were stimulated or when splenocytes were cultured in the absence of inactivated virus (data not shown).

Table 4
Protection of mice after challenge with FMDV O1/Campos strain.

Group	Protection 56 dpv (%)	Protection 146 dpv (%)
Ad[P]/Ad[P]	0/4[a] (0%)	0/4 (0%)
Ad[P]/Hs[P]	3/4 (75%)	4/4 (100%)
Ad[P]/KV	3/4 (75%)	ND
KV/KV	2/3[b] (67%)	ND
Ad[G]/Ad[G]	0/4 (0%)	0/4 (0%)

[a] Number of protected/total number of mice.
[b] One mouse died previous to challenge, by unrelated causes.
ND, not done; Ad, adenovirus vector; Hs, herpesvirus amplicon; KV, killed FMDV; P, FMDV capsid proteins plus 2A and 3C protease; G, GFP.

3.5. Protection from challenge

After the viral challenge performed at 56 dpv, protection was found in 3 out of 4 mice (75%) of the groups primed-boosted with Ad[P]/Hs[P] and Ad[P]/KV, and in 2 out of 3 mice (67%) of the KV primed-and-boosted group. One mouse in the latter group died of unrelated causes before challenge. No protection (0%) was found in the groups 1 and 6, immunized with 2 doses of Ad[P] or Ad[G], respectively (Table 4). Mice in the protected groups developed highly variable levels of neutralizing antibodies, but in all the animals of the non-protected groups the titers were found below the test detection limit (data not shown).

Challenge of other three groups of 4 mice was performed at 146 dpv (Table 4). A group immunized with Ad[P] and boosted with Hs[P] was totally protected from challenge (100%), while one group of mice immunized with 2 doses of Ad[P] and the negative control group Ad[G] were not protected (0/4 protected in both groups). This result highlights that the sequential inoculation of mice with different viral vectors (Ad[P]/Hs[P]) was able to elicit high levels of protection that persisted at least over a period of 4 months after the boost immunization. Similar protection results have been consistently found in this murine model when mice were challenged after vaccination with two doses of KV (Berinstein et al., 1991; Zamorano et al., unpublished).

4. Discussion

The development of different types of recombinant FMDV vaccines has been extensively explored (Sanz-Parra et al., 1999b; Grubman et al., 1993; Grubman, 2005; Borrego et al., 2006; D'Antuono et al., 2010, among others). Although they are not as effective as the conventional inactivated virus vaccine, they may contribute to other valuable properties, mostly related to safety and type of immune response. Heterologous prime-boost vaccination strategies may offer a suitable technology platform to

improve the performance of FMDV conventional or novel vaccines, enhancing the immune responses against the shared antigen.

In the present study, we examined the influence of prime–boost immunization regimens on their ability to promote the development of a protective immune response against FMDV, using either a combination of two viral vectors, or priming with Ad and boosting with the conventional KV vaccine.

A significant finding reported herein is that, combining Ad prime with either Hs or KV boost, it was possible to elicit a sustained and strong response, which was able to protect mice from challenge with FMDV (O1/Campos strain) for at least 5 months after primary immunization (Table 4), similarly than two doses of conventional inactivated vaccine.

Antibody titers after prime-boost were significantly higher in the group that received two doses of adjuvanted KV ($P < 0.002$), followed by the group sequentially vaccinated with different vectors instead of either vector alone ($P < 0.0001$), suggesting that the use of an alternative mode of antigen presentation markedly enhanced the immune response against the shared antigen (here, the P transgene), as it has been previously reported for other pathogens (Sun et al., 2010; Lin et al., 2011; Reyes-Sandoval et al., 2012). Sustained high titers of specific antibodies were maintained up to 146 dpv (Fig. 2).

Analysis of anti-FMDV IgGs isotype profile revealed that both IgG1 and IgG2a were induced in all the immunized groups (Fig. 4A), consistent with a T cell-dependent response against FMDV (Collen et al., 1989). The highest IgG1 titer was induced by two doses of adjuvanted KV ($P = 0.0002$), which is in agreement with previous findings in cattle and pigs (Mulcahy et al., 1990; Cox et al., 2003), while the highest IgG2a titer corresponded to the group primed with Ad[P] and boosted with KV ($P = 0.01$). These data indicate that Ad[P] prime followed by Hs[P] or KV boost promoted a cytokine milieu favoring the switching of IgG isotypes toward IgG2a, thus skewing the immune responses toward a Th1 type. It has been reported for HIV recombinant vaccines, that in prime–boost combinations of Ad and Hs vectors, the initial priming can shape the immune response quality after a boost (Duke et al., 2007). Our results are consistent with this observation, since following the re-stimulation with 0.5 μg of KV at 146 dpv, the groups of animals immunized with either Ad[P]/Hs[P] or Ad[P]/KV increased the total antibody response without changing the IgG1/IgG2a ratio determined at 56 dpv (data not shown). Thus, the initial Ad-priming may play a role in shaping the qualitative nature of the immune response after boosting, as it was previously reported by others (Rodriguez et al., 2008; Lin et al., 2011; Reyes-Sandoval et al., 2012).

Protection against FMDV challenge was observed at 56 and 146 dpv in animals receiving Ad[P] prime – Hs[P] boost, with a variable level of neutralizing antibodies and a predominance of the IgG2a isotype. The immunogen combinations that include at least one viral vector were able to trigger both Th1 and Th2 types of immune responses, which might have acted in concert to induce FMDV-specific antibodies and the proliferation of CD4+ and CD8+ FMDV specific T-cells (Fig. 4 and Table 3). In fact, we observed mRNA accumulation of representative cytokines of both types of immune response (IL-2, IFN-γ and IL-4) in cultured splenocytes from mice receiving the combined immunization, whereas IL-4 but not IFN-γ was present in KV/KV immunized mice.

Upon long-term re-exposure to O1/Campos virus, we observed a strong induction of specific antibodies with high avidity (Figs. 2 and 3), and an augmented population of FMDV-specific splenic ASC (data not shown). This anamnestic response upon exposure to the pathogen might be critical for its rapid elimination before the host is overwhelmed by the infection (Doel, 1999). Interestingly, antibody avidity, which was lower in the experimental groups that include only viral vectors, increased significantly after re-exposure to the virus, showing that these groups can quickly mature their response after a virus encounter (Fig. 3). Heterologous prime-boost vaccination regimens might induce the appearance and persistence of antigen presenting cells, responsible of maintaining the levels of newly synthesized anti-FMDV antibodies, which could be very important in the development of efficient vaccines against the disease (Wigdorovitz et al., 1997).

The use of the in situ antigen expression driven by viral vectors is a relatively recent approach in the FMD vaccinology field. The data presented in this study suggest that the combination of Ad vectors with either Hs or KV in heterologous prime-boost regimens have the potential to enhance the quality and magnitude of the immune response against FMDV, compared to prime-boost with the same viral vectors. In addition, the data generated suggest that an immunization regimen of Ad prime-Hs boost elicited a sustained and strong response, which was able to protect mice from challenge with FMD virus for at least five months after primary immunization. Although these studies were carried out in a mouse model that has shown certain parallelism with protective responses in cattle (Fernandez et al., 1986; Dus Santos et al., 2000), it will be very important to try in the future these combinations in the natural hosts (pigs or cattle). Moreover, this study also supports the rationale for continuing FMDV vaccine studies with combinations of different vectors in a prime-boost regimen since this strategy would allow the discrimination between antibody responses of vaccinated or infected animals.

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