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Systemic antibodies administered by passive immunization prevent generalization of the infection by foot-and-mouth disease virus in cattle after oronasal challenge

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ABSTRACT

The role of passively transferred sera in the protection against aerogenous foot-and-mouth disease (FMD) virus infection in cattle was evaluated using vaccine-induced immune serum preparations obtained at 7 and 26 days post-vaccination (dpv). We showed that circulating antibodies were sufficient to prevent disease generalization after oronasal infection in animals passively transferred with 26-dpv serum but not with the 7-dpv serum. Conversely, conventional FMD vaccination provided clinical protection at 7 dpv, promoting fast and robust antibody responses upon challenge and even though antibody titers were similar to those found in animals passively immunized with 7-dpv serum. These results demonstrate that presence of antigen-specific antibodies is critical to prevent the dissemination of the virus within the animal. Conventional FMD vaccination additionally promoted the deployment of rapid, high titer and isotype-switched antibody responses at systemic and mucosal levels after infection, thus conferring protection even in the presence of low pre-challenge antibody titers.

1. Introduction

Foot and mouth disease (FMD) remains a major threat for livestock production and derived industries, affecting biungulate species worldwide. The devastating effects associated with the presence of FMD may be verified at different stages of the production chain: from smallholders, directly affected by the reduced productivity (Knight-Jones et al., 2017), to whole-country economies impacted by the domestic control measures and the severe restrictions imposed on international trade (Thompson et al., 2002). FMD's etiological agent is a small non-enveloped positive-sense single-stranded RNA virus (FMDV) belonging to the *Picornaviridae* family, genus *Aphthovirus*. The FMDV possesses the ability to infect a wide range of domestic and wildlife species (Alexandersen and Mowat, 2005) causing an acute, febrile and vesicular disease with an extremely high morbidity and a variable mortality rate usually restricted to young individuals (Gulbahar et al., 2007). The virus combines its high antigenic variability (Domingo et al., 2002) with an efficient transmission, making it highly contagious among susceptible individuals even from different species (Alexandersen et al., 2003).

Conventional vaccines comprising inactivated whole-FMDV particles as antigens and formulated in aqueous or oil vehicles containing different adjuvants (Doel, 2003) have been successfully used to control the disease in different regions of the world, including Europe (Sutmoller et al., 2003) and large areas of South-America (Mattion et al., 2004; Saraiva and Darsie, 2004). Yet, and in spite of being the first viral disease identified in animals (Brown, 2003), a number of FMD outbreaks have been reported worldwide in the last few years (Brito

Abbreviations: ANOVA, analysis of variance; ASC, antibody secretory cells; AUC, area under the curve; dpi, days post-infection; dpv, days post-vaccination; FMD, foot and mouth disease; FMDV, foot and mouth disease virus; HRP, horseradish peroxidase; LPBE, liquid-phase blocking enzyme-linked immunosorbent assay; MNC, mononuclear cell; MRL, medial retropharyngeal lymph nodes; Nab, neutralizing antibodies; OD, optical density; OIE, World Organisation for Animal Health; PBS, phosphate-buffered saline; PFU, plaque-forming units; RT, room temperature; SD, standard desviation; TBL, tracheobronchial lymph nodes; VNT, virus neutralization

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et al., 2017), not only in endemic settings but also in countries and regions which have been free of the disease for long periods of time (FAO, 2017). This reinforces the need for improved vaccines and control strategies fit-for-purpose according to the particular epidemiological scenarios (Robinson et al., 2016). However, the development of such new prophylactic tools requires a clear understanding of the immune mechanisms involved in protection in susceptible species.

We have previously described the onset of the mucosal and systemic immunity after FMDV aerosol infection in cattle (Pega et al., 2013). We found that mucosal responses begin as soon as 4 days post-infection (dpi), with systemic responses following a similar time-course and isotype profile pattern as the local ones. As a result of these robust and fast responses —mainly mediated by IgM antibodies— FMDV was cleared out of the blood circulation by day 5 after experimental aerosol infection. We also studied the development of mucosal responses in FMD-vaccinated cattle, before and after oronasal infection with a homologous virus strain (Pega et al., 2015). We found that besides systemic antibody responses, FMDV-specific antibody-secreting cells (ASC) could also be detected at lymph nodes draining the respiratory tract as early as 7 days after systemic vaccination by the intramuscular route. Aerosol challenge performed 30 days after vaccination elicited a boosted antibody reaction particularly evident at the local level, resulting in complete protection of the infected animals. These results emphasized the idea that systemic and mucosal responses occur in parallel in both FMDV-infected and vaccinated bovines, regardless of the initial route of contact with viral antigen. However, the relative importance in the generation of protective immunity of the different immune mechanisms involved could not be discerned in those experiments.

Here we initially evaluated the efficacy of passively transferred vaccine-induced circulating anti-FMDV antibodies in preventing the development of FMD in cattle after infection through the oronasal route. We used two different preparations of FMDV-specific immune serum, obtained at 7 and 26 days post-vaccination (dpv), differing in the immunoglobulin isotype composition and in total antibody titers. We compared these results with those of animals vaccinated and challenged at the same times post-vaccination and thus carrying similar titers and isotype profiles as those of the passively immunized steers.

Challenge results demonstrate that circulating antibodies are sufficient to prevent generalization of the disease after oronasal infection in animals passively transferred with 26-dpv serum but not with the 7-dpv serum. Conversely, conventional FMD vaccination afforded clinical protection even at 7 dpv, promoting the generation of fast and robust antibody responses upon aerogenous virus challenge. Thus, the absence of the antigenic priming induced by vaccination might explain the lack of protection observed in animals passively transferred with 7-dpv serum even though their circulating antibody titers were similar to those found in animals at day 7 post-vaccination. These results demonstrate that presence of antigen-specific antibodies is critical to prevent the dissemination of the virus within the animal. Further characterization of the post-challenge responses at both systemic and local levels also revealed that systemic immunization with conventional FMD vaccines promoted the deployment of rapid, high titer and isotype-switched protective antibody responses at systemic and mucosal levels after infection, which might be compatible with the generation of early anti-FMDV memory B-cells as discussed herein.

2. Materials and methods

2.1. Experimental animals

Ten calves (180–220 kg each, 6- to 8-months old) and eight Hereford steers (350–400 kg each, 24-months old) were purchased from a livestock breeder located in the province of Chubut (Patagonia Argentina), an FMDV-free region without vaccination. All animals were checked by liquid-phase blocking enzyme-linked immunosorbent assay (LPBE) for the absence of FMDV-specific antibodies upon their arrival to the experimental field of CICVyA-INTA. Experiments not including infected bovines were carried out at the CICVyA-INTA experimental field while those involving infected animals were performed at the BSL-4 OIE animal boxes facilities also located at the CICVyA. All assays were completed by following biosecurity and animal welfare internal and federal regulations and according to protocol 71/2015 approved by the Institutional Committee for Use and Care of Experimental Animals (CICUAE), CICVyA-INTA.

2.2. Vaccines and vaccinations

All vaccinated animals received one dose of a single-oil-emulsion monovalent vaccine produced by Biogénesis Bagó (Argentina) according to good manufacturing practices (PD_{50} > 6) using inactivated FMDV O1/Campos/Brazil/58 (O1 Campos). Vaccine was controlled and approved by SENASA (Argentine Animal Health Authority) for safety, purity, and potency following local, OIE, and European Pharmacopeia standards.

2.3. Passive immunization

Blood (between 4.5 and 5 L/animal) from steers vaccinated with the monovalent FMD vaccine was collected at 7 dpv (n = 4) and 26 dpv (n = 4) using sterile bags containing anticoagulant solution with citrate, phosphate and dextrose (0.3% citric acid anhydrous, 2.63% sodium citrate dehydrate, 0.22% monosodium phosphate, 3.19% dextrose monohydrate and 0.027% adenine). Sterile serum fractions from each bag were obtained by centrifugation (1600 × *g* for 13 min). Sera from blood taken at 7 or 26 dpv were grouped in separate pools and parenterally transferred (~ 3 L/animal) through an intravenous catheter with an appropriate blood filter to naïve calves (n = 3 for each pool) previously sedated using xylazine 2% (0.075 mg/kg intramuscularly) to reduce stress and aid in catheter placement. The sera was maintained at ~ 37 °C using a warm water bath to avoid hypothermia and the initial rate of transference was ~ 3 mL/kg/h for the first 20 min and then up to 15 mL/kg/h (Balcomb and Foster, 2014). Experimental infections were performed approximately 16 h after passive immunization.

2.4. Aerosol infections and clinical assessment in cattle

The OIE FMD Reference Laboratory at SENASA provided virulent FMDV O1 Campos strain. Experimental infections through the oronasal route were performed with a jet nebulizer attached to an aerosol delivery system (10^7 50% tissue culture infective doses [$TCID_{50}$] in a 2 mL volume per animal) according to the protocols previously described (Pacheco et al., 2010). After the infection, animals were daily monitored for clinical signs of FMD up to 7 dpi. Symptoms included vesiculation in mouth, tongue and feet, lameness, increased salivation, fever (rectal temperature above 39 °C) and loss of appetite. Clinical scores were determined by assigning a score of 0.5 for fever between 39.1 °C and 40.0 °C, 1 for fever > 40.0 °C, 1 for lesions in the oral (dental pad, tongue, gingiva or lips) and nasal cavities and 1 for each foot that developed vesicles, with a maximum clinical score of 6.

2.5. Inactivated FMDV antigens

Concentrated suspensions of inactivated FMDV O1 Campos were provided by Biogénesis-Bagó S.A and 140 S viral particles for *in vitro* experiments were purified using a sucrose density gradient centrifugation method as previously described (Pega et al., 2013).

2.6. Experimental design and sampling

Passively immunized calves were infected 16 h after serum transfer. In addition, vaccinated calves were infected at 7 (n = 1) and 26 dpv

(n = 1), as well as two naïve animals. Clinical assessment was performed in all infected animals (n = 10) daily up to 7 dpi. Serum, whole blood and nasal secretion samples were also collected at the same time-points. One week after infection, all animals were euthanized and necropsied to obtain different lymphoid organs draining the respiratory tract: medial retropharyngeal lymph nodes (MRL) for the upper segment and tracheobronchial lymph nodes (TBL) for the lower fraction of the tract. All these lymphoid tissues were collected aseptically and placed in ice-cold wash buffer (RPMI 1640, 10 Mm HEPES, 10^6 units/mL penicillin G sodium, 700 mg/mL streptomycin, and 500 mg/mL gentamicin) until processing.

2.7. Isolation of mononuclear cells from lymphoid tissues

All tissues were processed to obtain mononuclear cell (MNC) suspensions according to protocols previously set up in our laboratory (Pega et al., 2013).

2.8. ELISPOT assay for FMDV-specific antibody secreting cells (ASC)

Anti-FMDV ASC were detected by means of a virus-specific ASC-enzyme linked immunosorbent spot (ELISPOT) assay previously developed in our laboratory (Pega et al., 2013). Briefly, 96-well nitrocellulose plates (Millipore, MA, USA) were coated overnight (ON) with inactivated purified FMDV O1/Campos (40 μg/mL) at 4 °C and blocked with 4% skim milk for 1 h at room temperature (RT). Mononuclear cells (MNC) were seeded in FMDV-coated plates in 2-fold dilutions (5×10^6 and 2.5×10^6 cell/mL) in triplicate wells and incubated ON at 37 °C with 5% CO_2. All subsequent steps were performed at RT. After five washes with phosphate-buffered saline (PBS), mouse anti-bovine IgG1 or IgG2 monoclonal antibodies (AbD-Serotec, Oxford, United Kingdom) were added (1:500 dilution) and incubated for 1 h. Reactions were revealed with anti-mouse IgG horseradish peroxidase (HRP)-labeled conjugate (1:1000 dilution, KPL, United Kingdom) for 1 h, followed by addition of True Blue peroxidase substrate (KPL, United Kingdom) for 10 min. IgM and IgA ASC were detected with HRP-labeled sheep anti-bovine IgM (AbD-Serotec, Oxford, United Kingdom) and IgA sera (Bethyl, TX, USA) diluted 1:2500 and 1:5000 respectively and revealed as described above. Spots corresponding to ASC were visualized and enumerated manually under a stereomicroscope and results were expressed as the mean number of FMDV-specific ASC per 10^6 total MNC ± standard deviation (SD) of the means.

2.9. Serology assays

FMDV-neutralizing antibodies (Nab) were detected by a microplate virus neutralization (VNT) assay as described (Pega et al., 2013). Virus dilutions were prepared from a 10^6 $TCID_{50}$ FMDV O1 Campos stock suspension, and the concentration was assessed for each test, allowing a variation of ± 0.5 $\log_{10}$ from the expected value. Neutralizing antibody titers were expressed as the $TCID_{50}$ neutralized by the diluted serum sample (1:32) according to the Reed and Muench method (Reed IJ, 1938). FMDV-specific total antibody were determined by a liquid-phase blocking ELISA (LPBE) originally developed by Hamblin et al. (Hamblin et al., 1986) and further modified by Periolo et al. (Periolo et al., 1993). Isotype of FMDV-specific antibodies were determined by three different assays. Anti-FMDV IgG1 and IgG2 ELISA were performed according to Capozzo et al. (Capozzo et al., 1997), except that sheep anti-bovine IgG1 and IgG2 HRP-conjugated antibodies (BioRAD, USA) were used 1:10,000 and 1:2500, respectively. FMDV-specific IgM antibodies were detected using a double sandwich ELISA (Di Giacomo et al., manuscript in preparation), using 96-well plates coated with a sheep anti-bovine IgM serum (BioRAD, USA) diluted 1:1000. Bovine serum samples were then incubated in two-fold serial dilutions, and washed with PBS 0.05% Tween 20 (PBST) before adding an inactivated purified FMDV O1 Campos suspension. FMDV-specific IgM were detected using a guinea-pig hyper immune serum against the O1 Campos strain. Reactions were finally revealed with anti-guinea pig IgG-HRP conjugate (KPL, United Kingdom), followed by addition of o-phenylenediamine (OPD) peroxidase substrate (KPL, United Kingdom) for 15 min and H_2SO_4 2 M to stop color development. Isotype antibody titers were expressed as the highest dilution of the serum reaching an optical density (OD) equal to the mean OD obtained from 4 negative sera ± 2 standard deviations (SD). The avidity of FMDV-specific antibodies was measured using an ELISA test described by Lavoria et al. (2012) with slight modifications. Briefly, 96-well ELISA plates (MICROLON®, Greiner Bio-One, Monroe, NC) were coated with 50 ng/well of inactivated purified FMDV O1/Campos diluted in 50 mM carbonate/bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. After five washes with PBS, plates were incubated for 90 min with a buffer containing PBST 10% equine normal serum (dilution buffer). Two sets of each serum sample (1:50 in dilution buffer) were incubated for 1 h at 37 °C and then washed four times with PBST. One of the sets of sera was incubated for 20 min with PBS, and the other was incubated with the same buffer but containing a chaotropic agent (urea 7 M). All the plates were then washed five times with PBST and FMDV-specific antibodies were detected with HRP-labeled anti-bovine Ig conjugate (Jackson Laboratories) diluted 1:5000 and incubated for 1 h at 37 °C. The colorimetric reaction was revealed with OPD at room temperature, protected from light exposure. Color development was stopped after 15 min by the addition of 50 μl of H_2SO_4 2 M. Absorbances were read at 490 nm (Multiskan® EX, Thermo Scientific, USA) and the resulting avidity index (AI) was calculated as the ratio between the OD of the wells incubated with urea and without urea, as previously described (Lavoria et al., 2012).

2.10. FMDV RNA detection in serum samples

Progression of viremia in each animal was measured by detecting virus RNA in serum samples by RT-qPCR. Specific sense and antisense primers were utilized to amplify a 259-nucleotide fragment of the viral polymerase gene (positions 7079–7338) as previously described (Callahan et al., 2002). Viral RNA was extracted from serum samples (140 μl) using a QIAamp viral RNA minikit (QIAGEN) and reverse transcription was carried out using the antisense primer and reverse transcriptase (from Moloney murine leukemia virus, MML-V, Promega) under standard conditions. The resulting template cDNAs and 10-fold dilutions of a standard plasmid containing the 3D gene were used for PCRs, which were performed with a real-time PCR master mix (Mezcla Real, Biodynamics). The reaction started with a 10-min incubation at 95 °C, followed by 45 amplification cycles (95 °C for 15 s followed by 1 min at 60 °C), and after cycling, a dissociation stage was carried out to detect specific amplification. A standard curve built using standard plasmid dilutions with the specific primers was utilized to correlate threshold cycle (C_T) values obtained from serum samples with the number of FMDV genome copies per mL. Samples and standards were run in duplicate in a Bio-Rad iQ5 thermocycler and analyzed using Bio-Rad iQ5 2.0 Standard Edition Optical System Software. Both primers and the standard plasmid were kindly provided by Dr. Guido König.

2.11. Statistical analyses

Differences in mean antibody titers and ASC numbers at different time points or between treatments were analyzed by two-way analysis of variance (ANOVA), followed by pair comparisons using the Bonferroni method. Total viremia for each animal was calculated using the Graph-Pad 5.0 software to estimate the area under the curve (AUC) from viremia time-courses shown in Fig. 1b. Comparisons of total viremia between animals with or without generalized FMD were performed by a non-parametric *t*-Test (Mann-Whitney). Calculation of the strengths of association between pre-challenge NAb titers and post-challenge maximum clinical score or total viremia, were analyzed using

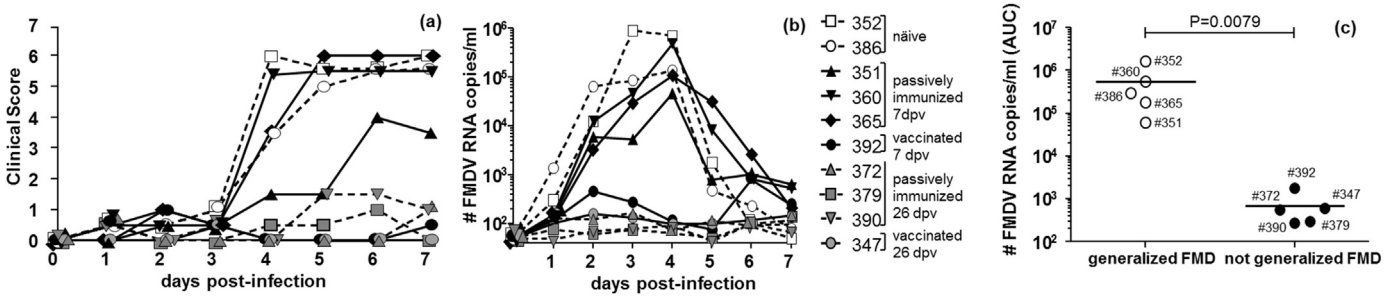

Fig. 1. Clinical scores and viremia after oronasal infection. (a) Scores in the progression of FMD associated symptoms for each infected animal. Values were assigned as described in Materials and Methods. (b) Time course of viral RNA detection in each infected animal. Results are expressed as number of FMDV RNA copies per mL of serum. (c) Comparison of total viremia up to 7 dpi between steers with or without generalization of the infection. Total viremia was estimated as AUC from the corresponding time-courses shown in Fig. 1b. Horizontal bars indicate mean values for each group. Statistical significance is shown at the top of the chart (Mann-Whitney test).

Spearman´s correlation method (Graph-Pad 5.0 software).

3. Results

3.1. Clinical symptoms in passively immunized cattle after virulent challenge

Previous results from our laboratory indicated that conventional FMD vaccination is able to induce not only systemic but also local immunity in cattle (Pega et al., 2015). In order to study the ability of the systemic immunity alone to prevent the dissemination of the FMDV infection after oronasal challenge, a passive transfer immunization model was developed using cattle as a natural host. Two groups of calves (n = 3 each) were transferred with sera from steers immunized with a high payload O1 Campos monovalent FMD vaccine using serum obtained at 7 or 26 dpv. Both groups were infected 16 h after passive immunization using FMDV O1 Campos (10^7 $TCID_{50\%}$/animal) delivered by the oronasal route. Similarly, naïve (n = 2) and vaccinated calves (at 7 dpv and 26 dpv) were aerosol infected using the same dose and strain of virus. Symptoms associated with FMD were daily recorded up to 7 dpi to build clinical score progressions for each animal.

As it is shown in Fig. 1a, by the end of the experiment animals could be grouped into those with generalized infection, showing lesions in the extremities (naïve control groups and calves transferred with serum of 7 dpv), and those without generalization (both vaccinated calves and those transferred with serum from 26 dpv). This same grouping was also observed when analyzing the progression of viremia (detected by the presence of virus RNA in serum) in each animal (Fig. 1b). Naïve control animals and calves passively immunized with 7-dpv serum showed peaks of viremia above 4×10^4 FMDV RNA copies/mL between 3 and 4 days after infection. Conversely, circulating FMDV RNA remained at low levels during the whole week following aerosol infection in vaccinated calves and cattle passively immunized with 26-dpv serum. Moreover, mean total viremia detected for the whole week after infection was significantly lower in animals without FMD generalization than in those showing symptoms of the disease ($P = 0.0079$, Fig. 1c). As a whole, this correspondence directly correlated the severity of symptoms with the concentration of virus circulating in the bloodstream after infection.

3.2. FMDV-specific immunity before oronasal infection

In order to understand the role of the preexisting immunity and its relation with the clinical scores observed after infection, neutralizing antibodies (NAb) were assessed in serum samples obtained immediately before aerogenous infection as well as in serum pools used for passive immunization procedures.

As it is shown in Fig. 2a, neutralizing titers corresponding to 7 dpv in the vaccinated animal as well as in the passively immunized calves were approximately 10-fold lower than those of their corresponding counterparts at 26 dpv. We found an inverse correlation between NAb titers before challenge and maximal clinical scores recorded for each animal ($r = -0.8443$ $P = 0.0011$; Fig. 2b). Similarly, NAb titers were also inversely related to total viremia (measured as the AUC from Fig. 1b) recorded for each animal ($r = -0.8659$ $P = 0.0011$).

Such correlation explains the differences in the severity of symptoms observed between groups passively immunized with serum pools taken at 26 or 7 dpv. However, this is less clear when relating the mild symptoms observed in a vaccinated animal challenged at 7 dpv (# 392, maximum clinical score = 0.5) and its relatively low neutralizing index ($\log_{10} = 1.71$) at the time of challenge, which in fact falls outside the 95% confidence interval (Fig. 2b). These results indicate that, although sufficient titers of circulating antibodies alone may impede FMDV spreading to reach distal tissues, vaccinated animals may develop other protective immunological mechanisms complementing pre-existing low titer neutralizing systemic antibodies.

3.3. FMDV-specific immune responses after oronasal infection

To further analyze the impact of the preexisting immunity in the final outcome of the aerosol infection 7 days later, we studied the progression of the FMDV-specific antibody responses after challenge by different ELISA tests at systemic level and by ASC-ELISPOT in lymph nodes draining the upper and lower respiratory tract.

Naïve animals showed a primary response pattern similar to that we described before (Pega et al., 2013). Systemically, IgM was the predominant isotype from 4 dpi, followed by IgG1 with much lower titers and starting 6 days after infection (Fig. 3a). One week post-infection, isotype profiles at lymph nodes draining the upper and lower respiratory tract (expressed as #ASC/10^6 MNC) correspond to this observation, with IgM levels significantly higher than those of IgG1 in most of the organs and animals tested. Low numbers of FMDV-specific IgA ASC were also detected at this time post-infection in lymph nodes of the upper and lower respiratory tract and no IgG2 ASC responses were observed for this group (Fig. 3b). Cattle passively immunized using the 7-dpv serum pool also corresponded to this description. In these animals, systemic responses started one day later than in naïve animals (at 5 dpi) although final titers for IgM and IgG1 isotypes were similar to naïve cattle (Fig. 3c). Antibody responses measured at the respiratory tract were higher in these animals than in the naïve calves, though preserving a typical primary response pattern at 7 dpi, mostly with IgM ASC counts significantly above than those of IgG1, followed by lower numbers of IgA and IgG2 ASC (Fig. 3d).

Calves passively immunized with the 26-dpv serum pool presented high pre-challenge IgG1 and IgG2 titers. Mean IgG1 and IgG2 titers were above those of the IgM from day 1 to day 6 post-infection. Between 5 and 6 dpi, mean IgM levels sharply increased to reach IgG1 and IgG2 titers, probably indicating the onset of adaptive humoral responses as observed in the 7-dpv passively immunized group (Fig. 3e). Locally, the TBL and MRL lymph nodes showed a primary response

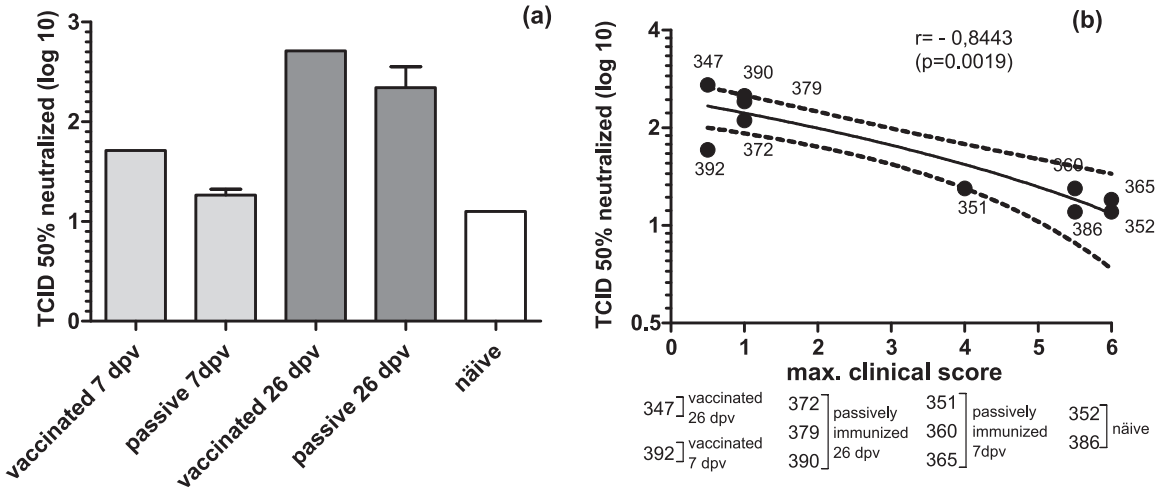

Fig. 2. Neutralizing antibody responses before viral challenge and their correlation with the pathogenesis after oronasal infection. (a) NAb assessed in different serum samples, as depicted on the X axis. Titers are expressed a $\log_{10}$ of the number of $TCID_{50\%}$ neutralized per mL of sample (neutralizing index). Bars from passively immunized calves (7 and 26 dpv) represent a mean value ± SD (n = 3) (b) Correlation analysis between neutralizing titers before challenge and the maximum clinical score recorded for each animal, identified by their corresponding numbers within the chart. Dotted lines indicate the 95% confidence interval and the corresponding Spearman coefficient (r) with its statistical significance (p) are shown in the chart.

composition, with IgM ASC significantly above or equal to IgG1 ASC numbers, except for one animal and organ (#372, MRL). As in the other passively immunized group, IgA counts were significantly below those of IgM and IgG1, and IgG2 ASC were detected in even lower numbers and only in half of the organs analyzed (Fig. 3f). Mean numbers of FMDV-specific ASC in this group were significantly below ($P < 0.001$) those in the group passively immunized with the 7-dpv serum pool. This was observed for all isotypes and in both organs, except for IgA in the MRL lymph node (Fig. 4). This would indicate the pre-existing FMDV-specific antibodies might effectively prevent the virus circulation within the animal, inhibiting the appearance of FMD-related symptoms but also decreasing the development of antigen-specific antibody responses elicited after infection.

Adaptive responses after infection in vaccinated controls were

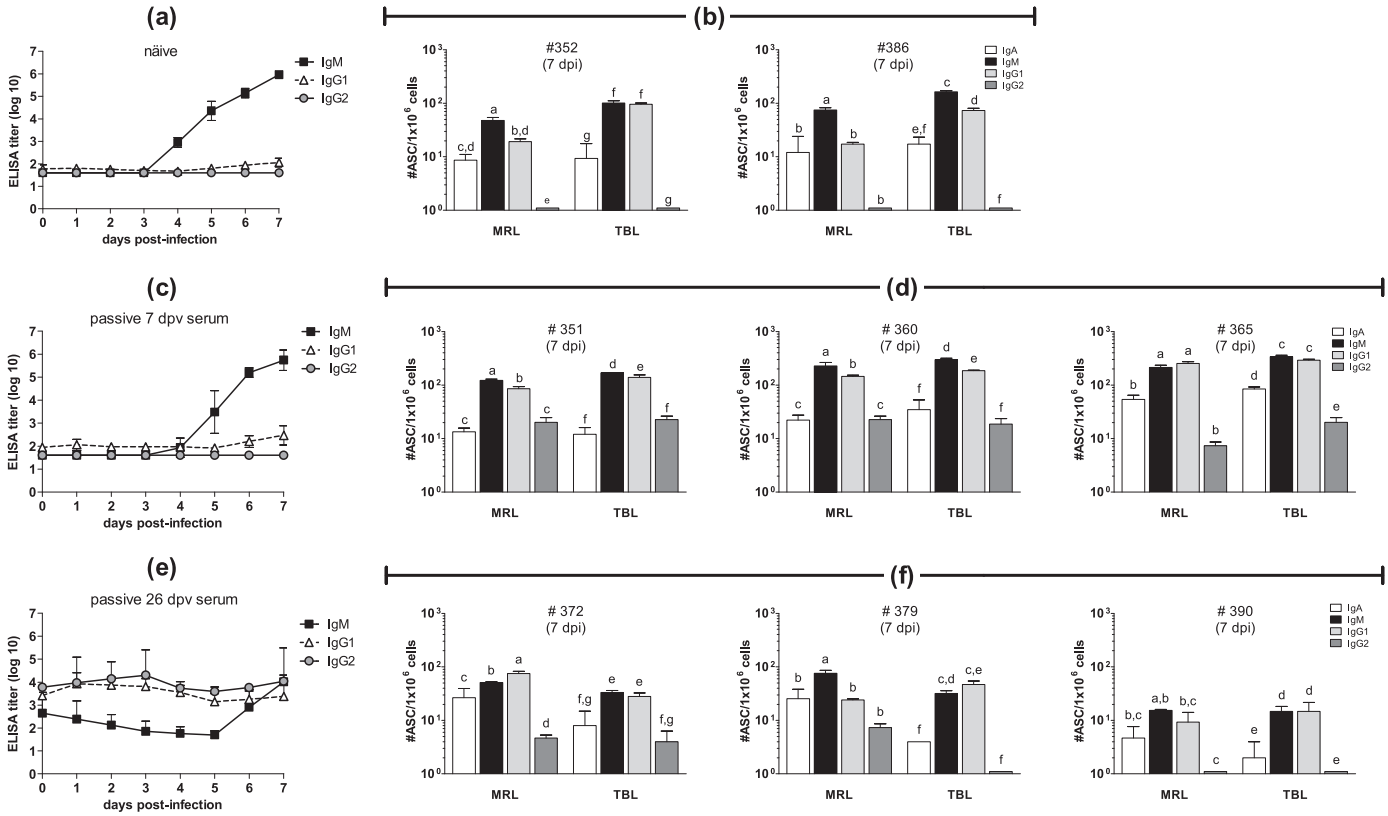

Fig. 3. Antibody responses at systemic and mucosal levels after FMDV oronasal challenge in naïve or passively immunized animals: (a, b) naïve animals; (c, d) passively immunized with 7-dpv serum; (e, f) passively immunized with 26-dpv serum. (a, c and e) Isotype responses detected after experimental challenge in circulating antibodies. Results are expressed the $\log_{10}$ of the ELISA titer for each isotype and correspond to the mean titer ± SD for each time (n = 2 for naïve animals; n = 3 for passively immunized cattle). (b, d, f) FMDV-specific ASC responses in the medial retropharyngeal (MRL) and tracheobronchial (TBL) lymph nodes of each animal at 7 dpi. Bars represent the mean #ASC/10^6 MNC ± SD of 3 replicates for each isotype, organ and animal. Lowercase letters above each bar indicate significant differences with other isotypes in that same tissue and animal.

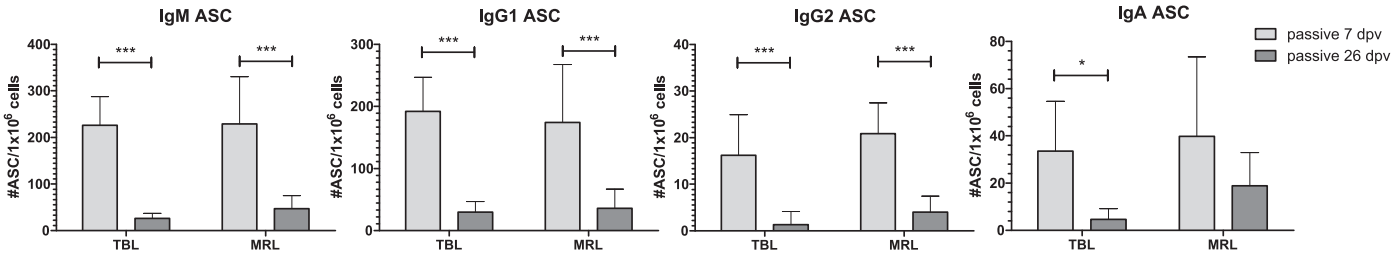

Fig. 4. Mean FMDV-specific ASC isotype responses in TBL and MRL lymph nodes from calves passively immunized with 7- or 26-dpv serum. Bars represent the mean #ASC/10^6 MNC of three animals measured in three replicates ± SD of each animal at 7 dpi. Significant differences between groups: $*P < 0.05$, $***P < 0.001$ (two-way ANOVA).

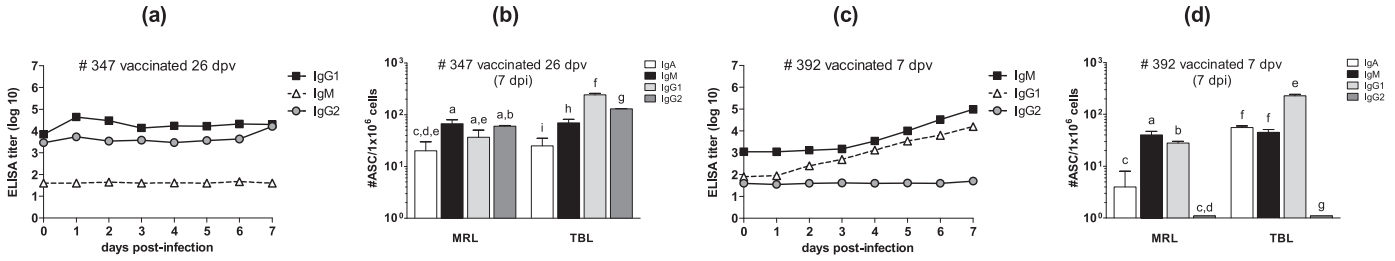

Fig. 5. Isotype antibody responses at systemic and mucosal levels in cattle vaccinated and challenged by the oronasal route at 7 or 26 dpv. (a and c) Isotype responses detected after experimental challenge in bovines at 26 (a) or 7 (c) dpv; results are expressed a $\log_{10}$ of the ELISA titer for each isotype. (b and d) FMDV-specific ASC responses in the TBL and MRL lymph nodes at 7 dpi for bovines challenged at 26 (b) or 7 (d) dpv. Bars represent the mean #ASC/10^6 MNC ± SD of 3 replicates for each isotype, organ, and animal. Lowercase letters above each bar indicate significant differences with other isotypes in that same tissue and animal.

different from all of the groups previously described. Aerosol challenge at 26 dpv did not modify significantly the isotype composition observed at the systemic level just before infection. IgG1 and IgG2 were the predominant isotypes and IgM remained at low levels among circulating antibodies during the whole week following infection (Fig. 5a). This switched isotype composition observed systemically, was also detected one week after infection at the local level. Mean numbers of IgG1 ASC present at MRL were similar to those of IgM and IgG2, while IgA ASC counts were slightly lower. On the other hand, IgG1 was the most abundant isotype among ASC from TBL (243.33 ± 25.17), followed in decreasing order by IgG2 (130.00 ± 2.00; $P < 0.001$), IgM (70.00 ± 20.00; $P < 0.01$) and IgA ASC (25.00 ± 10.00; $P < 0.05$). These observations coincide with those we previously reported, describing the development of a strong secondary response after oronasal challenge in FMD-vaccinated cattle infected at 30 dpv (Pega et al., 2015). Accordingly, this treatment presented the lowest viremia and clinical scores (Fig. 1a and b), being the only one where systemic IgM titers did not increase after challenge (Fig. 5a).

Interestingly, we found that steer #392, challenged 7 days after vaccination, also exhibited a predominant number of IgG1 FMDV-specific ASC (229,33 ± 25,71) over the other isotypes (IgM: 45,33 ± 10,06, and IgA: 56,00 ± 4,00; $P < 0.001$) at the TBL one week after infection. This isotype switch registered locally after infection was also observed at the systemic level, with progressively higher titers of FMDV-specific IgG1 already at 2dpi, in a similar trend as observed for IgM since 4 dpi. Such a post-infection isotype profile, resembling a secondary response pattern, was radically different from those observed in the naïve or 7-dpv passively immunized groups after infection. To analyze these observations further, we studied the post-challenge progression in the total anti-FMDV antibodies titers as well as in the avidity of the immune sera.

As it is shown in Fig. 6a, the steer challenged at 26 dpv (#347) presented high anti-FMDV antibody titers before infection. Total antibody titers remained constant until 5 dpi, when they showed a two-fold increase. A similar figure was obtained for the avidity indexes, with elevated values already before oronasal challenge, most probably related to the maturation of the antibody affinities since its vaccination 26 days before. Experimental infection also provoked a further increase in the avidity, only evident after the fifth day post-challenge and coincident with the total antibody titer increase (Fig. 6b).

Steer challenged at 7 dpv (#392) presented low anti-FMDV antibody titers before infection. However, soon after challenge (1 dpi), antibody titers exhibited a continuous increasing tendency to end with a 10-fold increase by the conclusion of the experiment, reaching similar LPBE titers as the other vaccinated steer (#347, Fig. 6a). Serum avidity followed an analogous increasing trend as that described for total anti-FMDV antibodies (Fig. 6b). Starting at basal levels by the time of infection, AI rapidly grew after 1 day to reach values of steer #347 by 6 dpi. This progression of the AI was also coincident with the time-course of the FMDV-specific IgG1 and IgM titers at the systemic level (Fig. 5c). In contrast, naïve animals and those passively immunized with the 7-dpv serum presented a typical primary response pattern with antibody titers rising after 4 dpi and avidity in basal levels until 6 days after infection, 1 or 2 days after the onset of IgM antibody responses at the systemic level (Fig. 3A). Steers passively immunized with the 26-dpv serum did not show any change in the titers nor in the avidity of anti-FMDV antibodies (Fig. 6). This observation is also in line with our previous results for this group, showing a reduced antibody response at the local level after the aerogenous infection. Collectively, these analyses indicate that enhanced responses in total Ab titers and serum avidity were restricted to those animals with a preexisting primary response, thus concurring with the ELISA isotype and ASC ELISpot results (Fig. 5) and suggesting the development of secondary responses at systemic and mucosal levels after oronasal challenge in both vaccinated steers.

4. Discussion

Understanding the immune mechanisms behind the protection against FMD in susceptible species represents a crucial step in the development of more effective vaccines and prophylactic strategies to control the disease under diverse epidemiologic scenarios. The generation of systemic humoral immunity has been regarded as an important parameter to evaluate the potential outcome of the infection in vaccinated cattle. However, systemic FMD vaccination in the bovine model promotes a number of immunological processes, even at local tissues and organs distal from the inoculation site (Pega et al., 2015), which hinders the dissection and clear interpretation of the role and

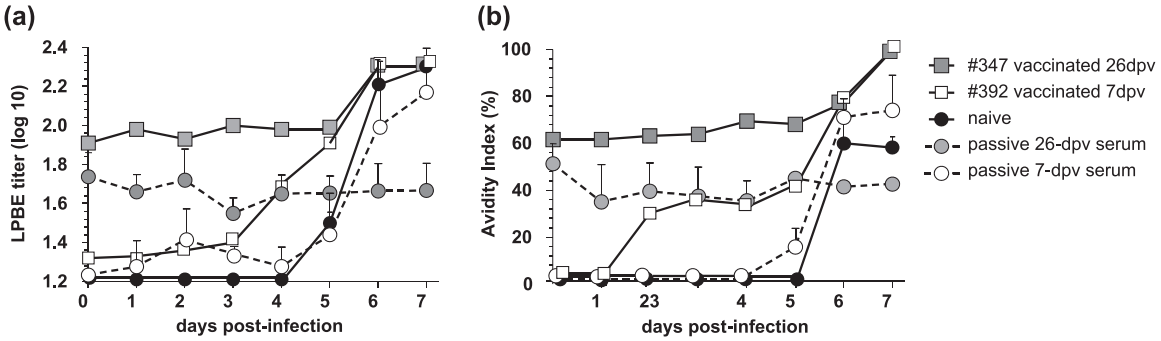

Fig. 6. Progression of the avidity and titers of total FMDV-specific antibodies after infection. (a) Total antibody responses measured by LPBE. Results are expressed as the $\log_{10}$ of the LPBE titer at each time-point after oronasal challenge (b) Avidity of serum samples measured by Avidity ELISA. Results are expressed as the avidity index (AI) at each time-point after oronasal challenge (described in Material and Methods). In both charts, values for the naïve (n = 2) and passively immunized (both n = 3) groups correspond to the mean titer ± SD.

importance of each one of them.

Early reports tried to understand the importance of systemic antibodies in the protection against FMDV infections entering through the respiratory system in the bovine model. Sutmoller et al. showed that steers (n = 12) passively immunized using immune sera (obtained from convalescent cattle, 3–5 months after infection) were not protected when challenged with 10^4 or 10^6 plaque-forming units (PFU) of infective FMDV of the homologous strain (A-4691) applied through the pharyngeal or intranasal routes (Sutmoller et al., 1968). A few years later these authors performed a new set of experiments using the same pool of immune sera to passively immunize steers which were further challenged by the intranasal route using 10^4 or 10^6 PFU of the same infective FMDV strain (Sutmoller and McVicar, 1972). Three out of four steers, all presenting high to medium titers of FMDV-specific antibodies prior to infection, showed symptoms of the disease (two of them challenged with 10^6 PFU and one challenged using 10^4 PFU). On the contrary, none of the steers carrying low FMDV-specific antibodies titers before infection (using 10^4 PFU per animal) showed any symptoms of FMD. As a whole, these results were not conclusive in establishing a correlation between the pre-exposure FMDV-specific antibody titers and the clinical outcome after challenge through the airways. Consequently, the role and efficacy of the circulating antibodies in preventing disease generalization for FMDV infections gaining access through the airways, as well as its impact on other immune mechanisms triggered after infection, could not be determined in these early works.

In order to elucidate the mechanisms involved in the induction of protective responses, we designed an *in vivo* aerosol challenge experiment using two groups of steers presenting only systemic immunity due to the passive transference of two different pools of bovine anti-FMDV sera obtained at 7 or 26 dpv. Animals were challenged within 24 h after passive immunization, following a well-established aerosol infection protocol (Pacheco et al., 2010) which proved to produce consistent FMD symptoms in the bovine model (Arzt et al., 2010; Pacheco et al., 2013; Pega et al., 2013, 2015).

Our experiment showed that animals receiving the 26-dpv serum before challenge did not present generalized FMD, in a similar way to steers vaccinated and challenged at 7 or 26 dpv. On the contrary, those immunized using the 7-dpv serum all showed generalization of the disease, with similar clinical progression as naïve-infected controls. We first analyzed these results by studying the existence of correlations between clinical scores, viremia and NAb titers measured just before oronasal challenge. As expected, we found a direct relationship between the viremia levels and severity of the FMD symptoms. Correlation studies also showed significant inverse associations between pre-challenge NAb titers *vs.* maximum clinical score or total viremia, demonstrating that the outcome of the experimental infection, at least in the passively immunized steers, was related to the level of circulating FMDV-neutralizing antibodies prior to aerosol challenge.

These results indicate that circulating antibodies will actually act as a first line of defense to prevent spreading of the virus within the organism. When these antibodies are in sufficient titers, the virus is restrained after primary replication preventing the progress towards FMD, as it was described for cattle vaccinated with adenovirus-vectored FMD vaccines (Stenfeldt et al., 2015). In the case that virus-specific antibodies are in low numbers, and in the absence of an active primary immunity, the infective viral particles would rapidly override the barrier created by the antibodies to reach tissues that will support its replication in high numbers, gaining access to blood circulation as it was observed in the 7-dpv serum passively immunized group.

Preexisting antibodies also affected the development of primary immune responses against the virus triggered after aerosol infection. Steers receiving the 26-dpv serum did not exhibit significant increases in total or isotype antibody titers, nor in the avidity of the circulating antibodies at least up to 7 days after challenge. In addition, numbers of FMDV-specific ASC at lymph nodes from the respiratory tract were significantly lower than in the 7-dpv passively immunized animals. Interestingly, Sutmoller et al. also observed in their early works that cattle with the highest pre-exposure antibody titers had the longest lag period in serum antibody production (Sutmoller and McVicar, 1972). Thus, based on the previous reports about the progression of the infection following this same aerosol challenge protocol (Arzt et al., 2010), it might be possible that antibodies would neutralize FMDV before reaching the lungs which act as amplifiers for the virus, preventing its access to the bloodstream and the subsequent high titer viremia. This would also severely decrease the availability of virus to act as stimulating antigen and to promote the generation of FMDV-specific antibodies and ASC, at both systemic and local levels.

Immune responses to the oronasal challenge in the 26-dpv vaccinated steer were concurrent with those observed in animals infected at 30 dpv and already described by our laboratory (Pega et al., 2015). Vaccination stimulated the development of a mature antibody response at 26 dpv, with high titers of IgG1 and IgG2, only basal levels of IgM and with an AI above 60%. Oronasal infection rapidly prompted secondary responses at systemic and mucosal levels, with predominant numbers of IgG1 ASC, followed by IgG2 and then IgM ASC at the TBL lymph node as observed at one week after infection, and a significant rise of the avidity of systemic antibodies to reach almost 100% in the AI after 6 dpi.

Interestingly, the animal challenged at 7 dpv was protected from FMD generalization, although carrying low pre-challenge anti-FMDV antibody levels, only slightly above those of the 7-dpv serum passively immunized group as measured by VNT and LPBE. Systemic vaccination, thus, offered additional immune mechanisms as observed in both animals immunized with conventional FMD vaccines.

Numerous reports indicate the early protection afforded by FMD vaccines at early times post-vaccination in the presence of low levels of FMDV-specific antibodies before infection (Doel et al., 1994; Elnekave et al., 2013; Golde et al., 2005). However, the immune mechanisms

behind those results are not fully elucidated. In the present study, oronasal challenge triggered a boosted response characterized by: (i) a rapid isotype switch already evident at 2 dpi; (ii) a significant rise in total anti-FMDV Ab titers; and (iii) an enhancement in the avidity of the FMDV-specific antibodies against the virus. Regarding this last observation, avidity increases at early times post-vaccination may reflect a rise in the number and variety of the antibodies present in serum (Newman et al., 1992) rather than changes in monovalent affinities of the antibodies produced as previously reported for booster responses at later times post-vaccination (Mulcahy et al., 1992). At the lower respiratory tract where the FMDV undergoes an extensive pre-viremic replication upon entering through the airways (Arzt et al., 2011), the presence at 7 dpi of predominant numbers of IgG1 ASC, followed by IgA and IgM ASC, coincided with the development of an amnesic response, being this local isotype induction pattern restricted only to both actively immunized steers. Thus, this second line of defense constituted by memory antibody responses, may turn active after challenge in vaccinated cattle irrespective of whether vaccination and infection routes coincide or not, and could be simultaneously detected at local and systemic levels.

Such enhanced post-challenge antibody responses may also be found in animals infected at later time points post-vaccination when, in the absence of antigenic re-stimulation, antibody titers drop below the protective-related levels due to the natural waning of the circulating anti-FMDV humoral response. In a recent work, Scott et al. demonstrated complete protection against homologous challenge at 162 days post-primary vaccination in different groups of cattle immunized with experimental formulations carrying stabilized SAT2 recombinant FMDV and in the presence of low titers of antigen-specific antibodies prior to infection. Interestingly, clinical protection in these animals occurred alongside significant increases in post-challenge antibody titers as registered by VNT and ELISA, in agreement with our observations for the vaccinated animals (Scott et al., 2017).

As it was previously described, type-2 T-independent antigenic attributes of the FMDV capsid (Borca et al., 1986; Juleff et al., 2009) impose significant limitations in terms of the generation of long-lasting antibody and B-cell memory responses after vaccination or infection (Grant et al., 2016). However, from these results it is evident that, at least within a relatively limited timeframe, a further encounter with the infectious virus may prompt the development of boosted antibody responses in FMD-vaccinated cattle. The existence of virus-specific memory B-cells is in line with the induction of antigen-specific CD4 + T-cell responses reported for FMD vaccines (Carr et al., 2013), including T-cell memory responses (Bucafusco et al., 2015). Moreover, the detection of low numbers of FMDV-specific circulating memory B-cells has been described for re-vaccinated steers soon after booster: our lab reported anti-FMDV IgG1 memory B-cells 4 days post-revaccination (Pega et al., 2015) and Grant and colleagues also found FMDV-specific memory B-cells between 7 and 14 days post-booster (Grant et al., 2016).

As a whole, our results show that systemic antibodies in sufficient amounts may be protective to the aerogenous FMDV challenge in the absence of other immune mechanisms. Additionally, we found that classical FMD vaccination promoted the generation of early and FMDV-specific antibody reactions soon after challenge, even at early times post-vaccination. Based on their particular time-course, isotype composition and increase of the serum avidity, such booster responses might be associated to the activation of memory B-cells generated by vaccination. These rapid, isotype switched and high titer antibody responses observed at both systemic and local levels and only in the vaccinated steers, resulted in complete protection to the FMD generalization even when challenged in the presence of non-protective antibodies titers at short times post-vaccination.

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