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Early Adaptive Immune Responses in the Respiratory Tract of Foot-and-Mouth Disease Virus-Infected Cattle

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Foot-and-mouth disease (FMD) is a highly contagious viral disease which affects both domestic and wild biungulate species. This acute disease, caused by the FMD virus (FMDV), usually includes an active replication phase in the respiratory tract for up to 72 h postinfection, followed by hematogenous dissemination and vesicular lesions at oral and foot epithelia. The role of the early local adaptive immunity of the host in the outcome of the infection is not well understood. Here we report the kinetics of appearance of FMDV-specific antibody-secreting cells (ASC) in lymphoid organs along the respiratory tract and the spleen in cattle infected by aerosol exposure. While no responses were observed for up to 3 days postinfection (dpi), all animals developed FMDV-ASC in all the lymphoid organs studied at 4 dpi. Tracheobronchial lymph nodes were the most reactive organs at this time, and IgM was the predominant isotype, followed by IgG1. Numbers of FMDV-ASC were further augmented at 5 and 6 dpi, with an increasing prevalence in upper respiratory organs. Systemic antibody responses were slightly delayed compared with the local reaction. Also, IgM was the dominant isotype in serum at 5 dpi, coinciding with a sharp decrease of viral RNA detection in peripheral blood. These results indicate that following aerogenous administration, cattle develop a rapid and vigorous genuine local antibody response throughout the respiratory tract. Time course and isotype profiles indicate the presence of an efficient T cell-independent antibody response which drives the IgM-mediated virus clearance in cattle infected by FMDV aerosol exposure.

Foot-and-mouth disease (FMD) is a highly contagious viral disease which affects a wide range of domestic and wild biungulate species. Although fatal cases usually occur in young animals, the high transmissibility and morbidity observed in adult animals infected by the FMD virus (FMDV) result in major economic losses to the livestock industry during disease outbreaks (1). The potentially devastating economic, social, and environmental consequences of the disease have been demonstrated dramatically during the last 2 decades by a number of different outbreaks of the disease reported around the world (2–4).

FMDV pathogenesis presents particular features depending on the host. Routes of entry, primary replication sites, and, consequently, the associated symptoms and immune responses elicited differ among species (reviewed in reference 5). Cattle are highly susceptible to FMDV, and the virus usually gains entry through the respiratory tract of these animals (6). Moreover, the ability of FMDV to replicate in tissues of the upper respiratory system, demonstrated in early reports (7) and widely confirmed later using more sensitive approaches, identified the soft palate and pharynx as sites of FMDV replication and persistence in ruminants (8, 9). Suttmoller and McVicar further expanded their initial findings to include the lung as an additional portal of virus entry (10), although many researchers considered the evidence for both sites of entry to be controversial. Recently, a detailed description of the previremic stages after experimental aerosol infection of cattle confirmed these early results, identifying both the pharynx and lung as primary sites for viral entry (11). Cattle infected by controlled aerosol exposure of infectious FMDV (12) exhibit a primary replication event in epithelial cells of the pharynx followed by extensive replication in pneumocytes in the lungs, which in turn allows the establishment of a sustained viremia (11).

The detection of adaptive immune responses at the local level after natural or experimental infection of cattle was performed in the past through the detection of secretory immunoglobulins in the oronasal cavities and esophageal-pharyngeal fluids (OPF), and most of the time these efforts were focused on the discovery of markers of infection or a carrier state (13–19). In an early report, Figueroa et al. (13) reported neutralizing activity in samples of saliva and nasal fluids taken from cattle infected by FMDV administered by intranasal spray. Later, McVicar and Suttmoller (18), also using an intranasal route for infection, detected neutralizing antibodies in saliva and OPF, suggesting a putative independence between the local response and the antibody response detected in serum. Other reports also showed the detection of FMDV-specific IgM and IgA in pharyngeal fluids 1 week after infection, but they proposed a systemic origin for these antibodies (14, 20). In these reports, only IgA antibodies detected at late infection times were attributed to genuine mucosal responses. None of these studies were able to identify a local immune response earlier than the 7th day postinfection.

Despite these studies, the role of the early host local immune response in the outcome of the infection is not well characterized.

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In this report, we approached the study of the local immunity against FMDV in cattle experimentally infected through the oronasal route by identifying organs and tissues involved in the local production of antibodies. Lymphoid tissues from the respiratory system were analyzed at early times postinfection by use of an FMDV-specific antibody-secreting cell enzyme-linked immunosorbent spot (FMDV-ASC ELISPOT) assay developed for this purpose. We provide evidence of the existence of antibody mucosal responses soon after infection, and we determined their origin, time course, and immunoglobulin isotype profiles. The results show a strong stimulation of FMDV-specific B lymphocytes to locally produce antibodies all along the respiratory tract, including in the tracheobronchial lymph nodes (TBL). This local response precedes the appearance of the systemic humoral immune response and demonstrates a close correlation with the disappearance of viremia, mediated mainly by FMDV-specific IgM antibodies.

MATERIALS AND METHODS

Experimental animals. Fifteen 15- to 18-month-old Hereford steers weighing 300 to 350 kg each were used for the experiments. Animals were obtained from an FMDV-free region and checked for the absence of FMDV-specific antibodies by a liquid-phase blocking enzyme-linked immunosorbent assay (IpELISA) before infection. During the experimental period, steers were kept in biosafety level 3A (BSL-3A) animal facilities according to internal and federal regulations on biosecurity and animal welfare (Institute of Virology, CICVyA-INTA, Argentina).

Virus, experimental infections, and clinical assessment of cattle. Virulent FMDV strain O1 Campos was provided by the OIE FMD Reference Laboratory at SENASA, Argentina. Experimental infection through the oronasal route was performed with a jet nebulizer attached to an aerosol delivery system (10^7 50% tissue culture infective doses [TCID₅₀] in a 2-ml volume per animal) according to the protocols described by Pacheco et al. (12). Cattle were monitored daily for clinical signs of FMD after challenge. These included vesiculation, lameness, increased salivation, loss of appetite, and fever (rectal temperature of $>39.5^\circ\text{C}$). Clinical scores were determined by assigning a score of 1 for fever or behavioral modifications, 1 for lesions in the oral and nasal cavities, and 1 for each foot that developed vesicles, with a maximum clinical score of 6.

Inactivated FMDV antigens. Inactivated suspensions of FMDV O1 Campos were kindly provided by Biogénesis-Bagó S.A. (Argentina), and whole viral particles were purified following a standard sucrose density gradient (SDG) centrifugation method (21) further optimized in our laboratory. Briefly, inactivated FMDV suspensions were mixed 4:1 (vol/vol) with 6% *N*-lauroylsarcosine (Sigma-Aldrich) in NET buffer (0.1 M NaCl-0.004 M EDTA-0.05 M Tris, pH 8.0). This preparation was placed on a discontinuous 15 to 45% SDG and ultracentrifuged at $45,000 \times g$ for 16 h at 4°C in an SW-28 rotor in a Beckman Coulter ultracentrifuge. Fractions were collected by selecting the peak absorbance at 260 nm and were pooled. This preparation was further centrifuged at $100,000 \times g$ for 16 h at 4°C , and pelleted virus was resuspended in NET buffer, quantified by spectrophotometry at 260 nm, and stored at -80°C . The integrity of 140S particles was assessed by SDG and a monoclonal antibody (MAb)-based indirect ELISA applied to each gradient fraction, as described previously (22).

Serum and tissue sample collection. Serum samples were collected on a daily basis from the arrival of the animals until the time of necropsy. One steer (C135) was euthanized and necropsied for tissue collection prior to the experimental infections to provide negative-control samples for immunological assays. The rest of the animals were aerosol inoculated and then euthanized and necropsied at different time points from 2 to 6 days postinfection (dpi), except for two steers that were kept until 14 dpi and were sampled daily only for serum. Tissue collection was based on previous reports showing the main sites of FMDV replication along the respi-

ratory tract (11, 23, 24). Samples obtained postmortem included 6 anatomically distinct organs and tissues: mandibular lymph nodes (ML), medial and lateral retropharyngeal lymph nodes (MRL and LRL, respectively), pharyngeal tonsils (PhT), tracheobronchial lymph nodes (TBL), and the spleen. 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.

Isolation of mononuclear cells from lymphoid tissues. All tissues were processed to obtain mononuclear cell suspensions according to previously published protocols (25), with minor modifications. Briefly, tissues were first ground in cell collectors with 80-mesh screens (Sigma-Aldrich, St. Louis, MO) to obtain single-cell suspensions. Cells were then pelleted, resuspended in 90% Percoll solution (GE Healthcare, Uppsala, Sweden), and centrifuged at $5,000 \times g$ (30 min at 4°C). Cell pellets were resuspended in 43% Percoll solution, transferred to a 70% Percoll solution, and centrifuged at $4,000 \times g$ (30 min at 4°C). Mononuclear cells were collected at the 43 to 70% interface and resuspended in complete medium (wash buffer with 10% fetal bovine serum), and their viability was determined by trypan blue exclusion.

ELISPOT assay for FMDV-specific ASC. An FMDV-ASC ELISPOT assay was developed for this study. Ninety-six-well nitrocellulose plates (Millipore, MA) were coated overnight with inactivated purified FMDV O1 Campos and blocked with 4% skim milk for 1 h at room temperature (RT). Mononuclear cells were seeded in FMDV-coated plates in 2-fold dilutions (5×10^6 and 2.5×10^6 cells per ml) in triplicate wells and incubated overnight at 37°C with 5% CO₂. After 5 washes with phosphate-buffered saline (PBS), mouse anti-bovine IgG1 or IgG2 monoclonal antibodies (BD-Serotec, Oxford, United Kingdom) were added (1:500 dilution) and incubated for 1 h at RT. Reactions were revealed with anti-mouse IgG horseradish peroxidase (HRP)-labeled conjugate (KPL, United Kingdom) for 1 h at RT, followed by addition of True Blue peroxidase substrate (KPL, United Kingdom). IgM and IgA ASC were detected with HRP-labeled sheep anti-bovine IgM and IgA sera (Bethyl, TX), diluted 1:5,000 and revealed as described above. Spots corresponding to ASC were visualized and counted manually under a stereomicroscope. Spots from control wells were subtracted from experimental wells, and results were expressed, unless otherwise indicated, as the mean number of ASC per 5×10^5 cells for triplicate wells.

FMDV RNA detection in serum samples. Specific sense and antisense primers were utilized to amplify a 259-nucleotide fragment of the viral polymerase gene (positions 7079 to 7338). Viral RNA was extracted from serum samples (140 μl) by use of a QIAamp viral RNA minikit (Qiagen), and reverse transcription was carried out using the antisense primer and reverse transcriptase (from Moloney murine leukemia virus [M-MLV]; Promega) under standard conditions. The resulting template cDNAs or 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) and ROX reference dye (Invitrogen). The reaction was 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 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. The standard curve presented a slope of -3.332 (primer efficiency, $>99\%$) and an R^2 value of 0.993. Samples and standards were run in triplicate in an ABI 7500 thermocycler (Applied Biosystems) and analyzed using model 7500 SDS software v 1.3.1. Both primers and the standard plasmid were kindly provided by Guido König.

IgM purification from serum samples. Total IgM was purified from pooled serum samples by affinity chromatography in an Äkta Explorer system (GE Healthcare). Serum samples were diluted 1:3 in binding buffer [20 mM sodium phosphate, 0.8 M (NH₄)₂SO₄, pH 7.5] and applied to HiTrap IgM HP purification columns (GE Healthcare). Unbound input

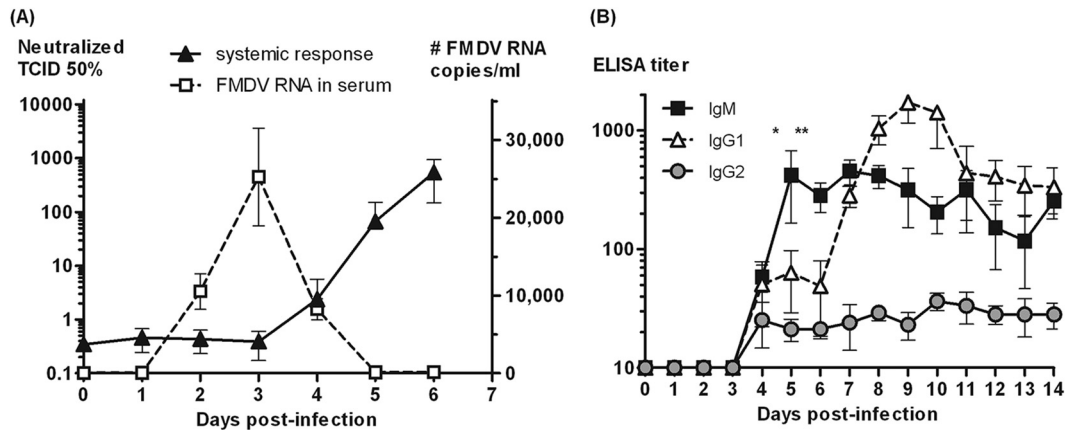


FIG 1 Time course of viral RNA detection and systemic adaptive immune responses in cattle after FMDV aerosol exposure. (A) Viral RNA detection in sera from infected cattle at each time point is indicated as the mean number of FMDV genome copies/ml \pm standard deviation (SD) (right axis). Systemic antibody responses are represented by the number of serum neutralizing doses detected for each animal, and results are expressed as the mean TCID₅₀ neutralized by the diluted sera from all surviving animals at each time \pm SD (left axis). (B) Isotypes of FMDV-specific serum antibodies were analyzed by an FMDV-specific isotype indirect ELISA. Each line corresponds to a particular isotype, and each point represents the average ELISA titer obtained for all animals assayed. Titers are expressed as the highest dilution of serum reaching an optical density of 0.2 (mean value for 25 negative sera plus 2 SD). *, significant differences between IgM titers at 4 and 5 dpi ($P < 0.05$); **, significant differences between IgM and IgG1 titers at 5 dpi ($P < 0.05$).

sample was collected and preserved as the IgM-depleted fraction, and IgM-purified fractions were eluted in 20 mM sodium phosphate buffer, pH 7.5. A purified commercial bovine IgM standard (Sigma) was also included as a control for the whole purification procedure. The IgM content in all fractions was confirmed by 10% SDS-PAGE and Western blot assay, using an HRP-labeled sheep anti-bovine IgM (Bethyl) as a probe (data not shown). All fractions assayed for virus-neutralizing activity were further adjusted to match a 1:8 dilution of the original whole-serum pools.

Serology determinations. FMDV-specific total antibody titers were determined by an IpELISA originally developed by Hamblin et al. (26) and further modified by Periolo et al. (27).

Neutralizing antibodies were detected by a microplate virus neutralization assay modified in our laboratory (28). Briefly, serum samples (50 μ l; 1:16 dilution in RPMI) were added to 96-well cell culture plates and incubated at 37°C for 1 h with four 10-fold dilutions of infective FMDV O1 Campos (50 μ l; 1 to 1,000 TCID₅₀). BHK-21 cell suspensions (100 μ l; 3×10^5 cells/ml) were then added and incubated for 72 h at 37°C with 5% CO₂. Virus dilutions were prepared from a 10⁸-TCID₅₀ FMDV O1 Campos stock suspension, and the concentration was assessed for each test, allowing a variation of $\pm 0.5 \log_{10}$ from the expected value. Neutralization assays performed for purified IgM and pooled sera were carried out essentially in the same way, except that samples were diluted 1:8 (50 μ l), mixed with the different virus dilutions (50 μ l), and applied to BHK-21 cell monolayers already seeded in 96-well cell culture plates. For both assays, neutralizing antibody titers were expressed as the TCID₅₀ neutralized by the diluted samples, according to the method of Reed and Muench (29).

FMDV-specific isotype ELISA was performed as reported by Capozzo et al. (30), except that sheep anti-bovine IgG1 and IgG2 HRP-conjugated antibodies were used at a dilution of 1:750, and sheep anti-bovine IgM HRP-conjugated antibody was used at 1:5,000 (BD-Serotec, Oxford, United Kingdom).

Statistical analysis. Differences in the number of ASC produced per isotype per animal and between FMDV-specific serum IgM titers at different times were analyzed by one-way analysis of variance (ANOVA), and pairwise comparisons were carried out using the Tukey test. Differences in mean FMDV-specific serum isotype titers at different times were analyzed by two-way ANOVA following the Bonferroni method. Analyses were performed using Graph Pad Prism 5.0 software (Graph Pad Software Inc.).

RESULTS

Progression of viremia and clinical symptoms following experimental oronasal FMDV infection in cattle. A total of 12 animals were experimentally infected by the oronasal route following the procedure described by Pacheco et al. (12). Clinical symptoms were similar across the infected cattle. Animals started exhibiting fever and general lameness between 1 and 2 days after exposure, and the first vesicles were observed from day 2 postinfection. As expected, clinical scores progressed in a time-dependent manner, up to the maximum score of 6, corresponding to a generalized infection with lesions in all feet and in oral and nasal cavities at 6 dpi (data not shown). FMDV RNA was detected in serum after 24 h postinfection; the number of viral genome copies sharply increased to a peak at 3 dpi and disappeared from the bloodstream of infected animals by 5 dpi, coinciding with the onset of systemic antibody responses (Fig. 1A).

Systemic adaptive antibody responses in FMDV-infected cattle. Circulating FMDV-neutralizing antibodies were initially detected at 4 dpi in 2 of 8 assayed animals, and they were observed in all animals ($n = 5$) at 5 dpi (Fig. 1A). Similar results were observed when total FMDV-reacting antibodies were studied by IpELISA (data not shown). Isotype profiles at 4 dpi showed that mean FMDV-specific IgM titers were at similarly low levels as IgG1 antibodies, while IgG2 was barely evident. Mean FMDV-specific IgM titers, however, increased significantly at 5 dpi ($P < 0.05$) and were significantly higher than IgG1 or IgG2 titers at 5 dpi ($P < 0.05$). FMDV-specific IgG1 and IgG2 levels remained unchanged between 4 and 6 dpi. Following this initial phase, IgG1 levels dramatically increased, peaking at 9 dpi and remaining stable between 11 and 14 dpi, the last sampling time point. IgM titers remained mostly unchanged from 5 dpi, showing little fluctuation between 12 and 14 dpi, and IgG2 titers remained low throughout the study period (Fig. 1B).

Mucosal adaptive antibody responses in FMDV-infected cattle. Animals were sacrificed at 2 ($n = 1$), 3 ($n = 3$), 4 ($n = 3$), 5 ($n = 2$), or 6 ($n = 3$) dpi to study FMDV-specific mucosal im-

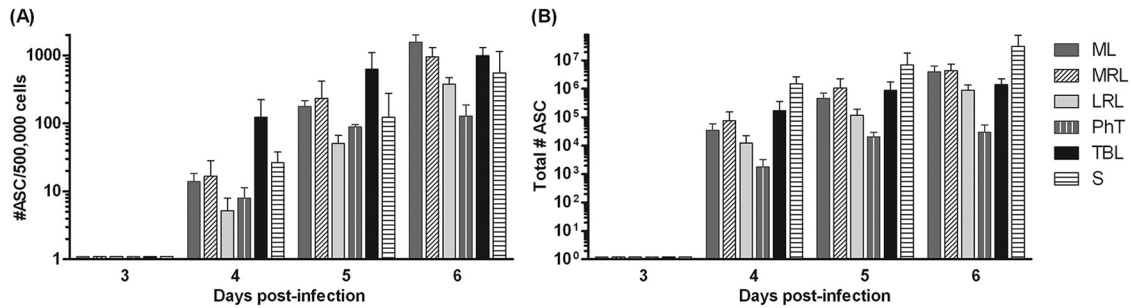


FIG 2 Detection of FMDV-specific antibody-secreting cells in lymphoid organs of the respiratory tract of infected cattle. After oronasal infections, FMDV-specific ASC were detected by an FMDV-ASC ELISPOT assay. Mandibular lymph nodes (ML), pharyngeal tonsils (PhT), lateral (LRL) and medial (MRL) retropharyngeal lymph nodes, tracheobronchial lymph nodes (TBL), and the spleen (S) were processed and assayed by the FMDV-ASC ELISPOT assay. Results are expressed as either the number of FMDV-specific ASC per 5×10^5 extracted mononuclear cells (A) or the total number of FMDV-specific ASC per organ (B). Each bar corresponds to the average value for 3 individuals (except for 5 dpi, where $n = 2$) plus SD for each particular organ.

immune responses along the respiratory tract by FMDV-ASC ELISPOT assay. An additional naïve animal (C135) was also sacrificed and processed for use as a negative control. Onset of local responses was established between 3 and 4 days postinfection. While FMDV-specific ASC were not detected in any of the individuals studied at 3 ($n = 3$) or 2 ($n = 1$) dpi, all animals analyzed at 4 dpi ($n = 3$) developed adaptive responses in most of the lymphoid tissues sampled. The mean number of FMDV-specific ASC (per 5×10^5 total mononuclear cells) detected in the organs of the respiratory tract, considering all animals and organs analyzed at each time, rose ~ 7 -fold between 4 (mean \pm standard deviation [SD], 166.4 ± 104.0) and 5 (mean \pm SD, $1,172.5 \pm 726.2$) dpi and ~ 3.4 -fold between 5 and 6 dpi (mean \pm SD, $4,011.0 \pm 1,003.9$) (see Table S1 in the supplemental material). A strong FMDV-specific antibody response was detected in all infected cattle at 6 dpi: FMDV-ASC represented up to 0.39% of the total mononuclear cells extracted from mandibular lymph nodes and 0.23% of those from the tracheobronchial lymph nodes (Fig. 2; see Table S1 in the supplemental material). When ELISPOT assay results were considered in terms of the total number of FMDV-specific ASC per organ, no major changes in the general profile were observed, with the exception of values corresponding to the largest organ, the spleen, and the smallest one, the pharyngeal tonsil.

Lymphoid organs involved in local production of FMDV antibodies in infected cattle. Five different lymphoid tissues from the lower and upper respiratory systems were sampled: mandibular and retropharyngeal (lateral and medial) lymph nodes, pharyngeal tonsil, and TBL. The spleen was also analyzed as a representative measurement of systemic responses elicited in each animal. TBL were the most reactive organs in all the individuals studied 4 days after FMDV infection (Fig. 2). The spleen (S), medial retropharyngeal (MRL), and mandibular (ML) lymph nodes followed the TBL in the mean number of anti-FMDV ASC, although no differences were registered among these organs compared with the lateral retropharyngeal lymph nodes (LRL) or the pharyngeal tonsils (PhT), which presented only a marginal reactivity.

A similar profile was found at 5 dpi, with TBL as the predominant organ for FMDV-specific ASC, followed by MRL (steer C118) or ML (steer C146). Differences in FMDV-specific ASC numbers in MRL and ML were small with respect to those in TBL and LRL; PhT had increased anti-FMDV ASC numbers relative to those in TBL. ASC numbers in the spleen were augmented in one

of the animals (steer C118), without exhibiting significant differences within the other organs. The remaining steer did not display a further increase in spleen stimulation relative to the value from 4 dpi (Fig. 2; see Table S1 in the supplemental material).

The increasing relevance of the upper respiratory organs in the mucosal response initiated at 5 dpi was confirmed at 6 dpi. ML were significantly the most reactive organs in all animals studied at this time ($n = 3$), followed by the TBL and MRL, with similar values for all animals analyzed. The number of FMDV-ASC in ML from the 3 steers increased ~ 9 -fold, on average, relative to the number from the previous day, while the LRL, which displayed relatively low levels at 4 and 5 dpi, also increased their average number >7 -fold. Rises registered in the PhT were moderate, reaching an average of 128 FMDV-specific ASC/ 5×10^5 mononuclear cells. Responses in the spleen were modestly higher than those of the previous day for two of the animals (steers C115 and C126), although the third animal analyzed (steer B995) presented a marked increase in the anti-FMDV reactivity in this organ as measured by ELISPOT assay, reaching a total of 1,220 ASC/ 5×10^5 mononuclear cells (see Table S1 in the supplemental material).

Immunoglobulin isotype profiles induced by local adaptive immunity in FMDV-infected cattle. As mentioned above, TBL were the most stimulated organs in all three animals 4 days after infection, with IgM as the dominant isotype among the FMDV-ASC developed in these organs. IgG1 was consistently second with regard to detection levels, with levels 3- to 4-fold lower than those for IgM. IgA and IgG2 ASC were detected at very low levels or were undetectable. This pattern was repeated overall, with lower magnitudes, in all the local organs assayed. FMDV-specific ASC in the spleen also exhibited increased levels of IgM-producing cells compared to cells expressing the rest of the isotypes. Splenic IgG2 ASC were not detectable at this time, and IgA and IgG1 values, which were 8 to 10 times lower than the IgM value, showed no significant differences between them (Fig. 3; see Table S1 in the supplemental material).

Five days after infection, TBL were still the most stimulated organs in both animals studied. The isotype pattern observed was similar to that of the previous day, although the IgM/IgG1 and IgM/IgA ratios increased 3- to 4-fold. The total number of FMDV-specific ASC in TBL was also augmented 5-fold relative to that on the previous day, and thus an important fraction of this increase was due to the IgM ASC. The same figure was registered in the

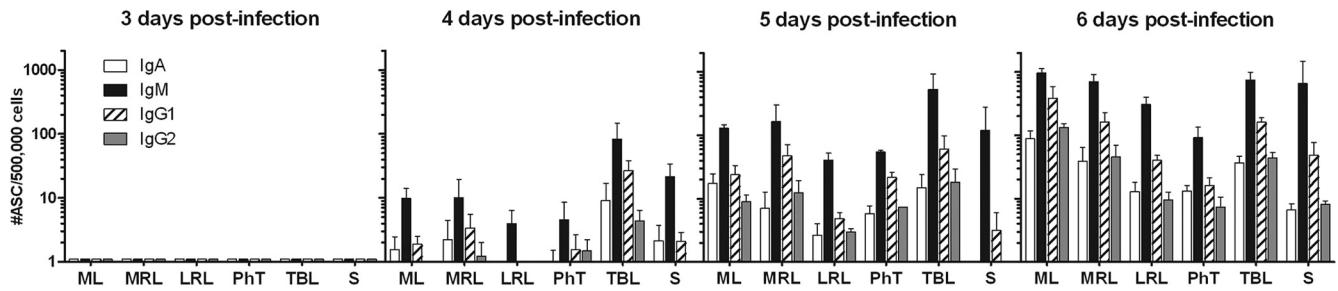


FIG 3 Isotype profiles of the FMDV-ASC developed in cattle after FMDV aerosol exposure. Mononuclear cells were purified from mandibular lymph nodes (ML), pharyngeal tonsils (PhT), lateral (LRL) and medial (MRL) retropharyngeal lymph nodes, tracheobronchial lymph nodes (TBL), and the spleen (S) and assayed by the FMDV-ASC ELISPOT assay, using monoclonal (IgG1 and IgG2) or polyclonal (IgM and IgA) antibodies against bovine immunoglobulin isotypes as probes. Results are expressed as the number of FMDV-specific ASC per 5×10^5 extracted cells, and each bar corresponds to the average value for 3 individuals (except for 5 dpi, where $n = 2$) plus SD for each particular isotype and organ.

spleen, since IgG1 and IgA isotypes showed little differences from the previous day, while mean IgM ASC numbers went from 21 to 118 ASC per 5×10^5 extracted cells (Fig. 3). IgM was also the dominant isotype in the FMDV-ASC detected in the rest of the organs tested, and differences with the other isotypes remained mostly constant.

The general isotype pattern at 6 dpi was still dominated by IgM ASC for all animals and organs assayed, although a relative rise in the numbers of FMDV-specific IgG1 and IgA ASC was observed, mainly in the ML, TBL, and spleen (Fig. 3). The number of FMDV-specific IgG2 ASC was also augmented, particularly in ML, reaching IgA levels. One of the animals (steer B995) developed a large number of FMDV-specific IgG1 ASC (789 ASC/ 5×10^5 cells), close to the IgM level in the same animal and organ (1,003 ASC/ 5×10^5 cells). Interestingly, this was the same individual that also presented a significantly larger number of anti-FMDV splenic ASC than the other 2 animals studied at that time (see Table S1 in the supplemental material).

Neutralizing activity of FMDV-specific serum IgM antibodies isolated from infected cattle. In order to evaluate the contributions of the different isotypes to the phenomenon of virus neutralization during infection in cattle, the neutralizing ability of purified serum IgM was tested using a pool of sera obtained daily from 4 to 6 days after infection. For each time point, serum IgM was affinity purified and its neutralizing activity compared to those of whole pooled sera, IgM-depleted serum fractions, and pooled sera from uninfected animals. As shown in Fig. 4, neutralizing activity of the tested samples was clearly detected after 4 dpi, and the serum IgM fractions from 5 and 6 dpi neutralized infective FMDV to levels similar to those with whole serum. In contrast, the neutralizing capacity of the IgM-depleted serum fractions resembled that of the normal serum for all time points. This result is in line with a central role of this immunoglobulin subclass in the early clearance of viremia in infected animals.

DISCUSSION

The detection of specific neutralizing antibodies in oronasal and esophageal-pharyngeal fluids has previously been described for cattle infected by oronasal routes with different FMDV O1 strains (13, 15, 18). All of these reports were based on results obtained starting at least 1 week after the experimental infections. Consequently, they did not clearly determine the onset of the local anti-FMDV antibody response in naturally infected cattle or indubitably identify the exact organ/tissue originating that response.

Neutralizing activity in cattle infected by nasal spray exposure was reported for nasal fluids and saliva 10 and 21 days after nasal spray infection, respectively (13), and around 2 weeks after instillation for OPF samples (18). An even earlier report by Kaaden and Matthaeus (16) revealed a poor correlation between serum and saliva neutralization titers, with IgA as the predominant isotype in salivary secretions. McVicar and Suttmoller (18) also compared the kinetics of the serum and mucosal antibody responses up to 30 weeks after primary infection and concluded that the lack of temporal correlation between them might reflect the independence of the FMDV-neutralizing activity at systemic and local levels. This perspective, however, was later set aside by other authors. Francis et al. (14) detected FMDV-specific IgM and IgA antibodies in pharyngeal fluid in naïve cattle 7 days after virus exposure but proposed that these were actually due to serum and tissue fluid escaping from the damaged mucosa during the acute inflammatory phase of infection. According to these authors, active local antibody production was evident only at later stages (20 to 60 days after virus exposure) and corresponded to the rise in specific IgA antibodies. Archetti et al. also followed the kinetics of serum and OPF antibodies in naïve cattle by use of ELISA (20). Even when the earliest positive reaction was detected at day 14 postinfection, they proposed that only the IgA responses peaking after healing of the vesicular lesions (between 4 and 8 weeks postinfection) were produced by mucosa-resident antibody-secreting B lymphocytes.

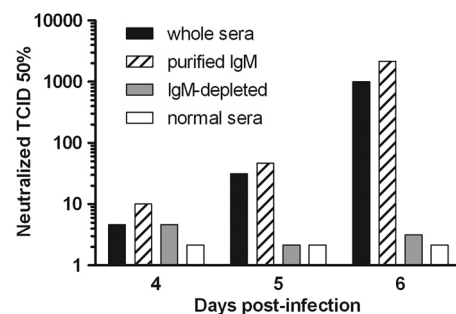


FIG 4 Virus-neutralizing activity in whole sera and antibody fractions. IgM antibodies were purified from pooled serum samples taken between 4 and 6 dpi. Neutralizing antibodies were determined for sera from nonimmune cattle, as well as pooled sera, IgM, and IgM-depleted fractions from infected animals, as described in Materials and Methods. Results are expressed as TCID₅₀ neutralized by the different diluted samples.

Therefore, the role of the early host local immune response in the outcome of the infection is still not well understood.

We approached the study of the mucosal humoral responses induced by FMDV infection by the aerosol route in cattle by analyzing the induction of ASC specific for the virus in lymphoid tissues located along the respiratory tract to obtain information about the source of the antibodies detected at local lymphoid organs in relation to tissues involved in the initial viral replication stages (11, 23, 24).

Interestingly, patterns of immune reactivity found in lymphoid organs after infection were associated with the early stages of replication of aerosolized FMDV. Arzt et al. (11) used the same aerosol exposure method to show that after primary replication (during 6 h postexposure) in the follicle-associated epithelium overlying the pharyngeal mucosa-associated lymphoid tissue, the virus followed an extensive replication in pneumocytes between 24 and 48 h postexposure. They proposed that this replication in the lungs allows the virus to establish and sustain viremia. Accordingly, our results demonstrated that the onset of local adaptive responses at 4 dpi was dominated by the TBL, with poor stimulation of the lymph nodes from the upper respiratory system, and that only at 6 dpi were lymph nodes from the upper tract able to exceed TBL ASC counts.

It is possible to hypothesize that this first extensive replication of FMDV in large organs such as the bovine lungs is directly related to the predominant stimulation of the tracheobronchial lymph nodes draining these tissues observed on days 4 and 5 after infection and thus independent from the humoral systemic response. It is probable that primary replication in nasopharynx and larynx tissues produced only limited amounts of FMDV, and therefore only a modest number of FMDV-specific B cells were activated in lymph nodes from the upper tract at the onset of the response. Only when the virus was extended in a generalized infection through the peripheral circulation could a larger number of FMDV-specific B lymphocytes be activated in these lymph nodes. Progression of the response from 6 dpi led to a more extended immunoglobulin class switching and to an increasing importance of the systemic immunity, as reflected by the spleen ASC responses and IgG1 serum levels detected between 7 and 14 dpi. Interestingly, IgM mediated most of the local ASC responses coexisting with the end of the viremia phase at 5 dpi.

The FMDV capsid possesses key structural features compatible with type 2 T cell-independent antigens, which are able to stimulate antibody production in the absence of major histocompatibility complex (MHC) class II-restricted T cell help (31). In addition, as demonstrated for poliovirus (32) and vesicular stomatitis virus (33), the high local antigen concentrations reached during infection, which allow replicating virus to be present at high levels for a prolonged time, represent a key factor in the efficient induction of T cell-independent responses (34). An early work from our laboratory demonstrated that FMDV-specific primary and short-term memory protective antibody responses were not dependent on the CD4⁺ T-helper cells in a mouse model (35). Only recently, Juleff et al. extended these results to cattle, showing that a rapid and class-switched antibody response could be elicited by FMDV infection in bovines transiently depleted of CD4⁺ T cells (36). Our results at local and systemic levels are consistent with these previous findings, even when differences can be pointed out regarding this later work. Juleff et al. first detected FMDV-specific serum IgG1 antibodies at 5 dpi, with FMDV-specific IgM antibodies

identified 1 day later. In our work, FMDV-specific IgM responses were rapidly induced at the local level 4 days after infection, and IgG1 ASC numbers were comparable to those of the IgM-secreting cells only at 6 dpi. Serum responses followed a similar kinetics; while specific IgM and IgG1 antibodies were detected between 4 and 5 dpi, IgM titers peaked at 5 dpi, exhibiting significantly higher titers than the other isotypes at that time.

A previous report by Abu Elzein and Crowther indicated that FMDV-neutralizing activity in infected cattle started between 4 and 5 dpi, showing a biphasic development with a slow initial rise until 10 dpi, when a sharp increase in titer was observed (37). These authors found that both FMDV-specific IgM and IgA were present from 4 dpi, but since IgG1 and IgG2 were also detected in smaller amounts, they could not attribute the neutralizing activity to a determined isotype. We showed here that anti-FMDV IgM predominated in the adaptive response at both the local and systemic levels, and interestingly, the peak of anti-FMDV IgM in serum closely correlated with a sharp decrease in viral RNA detection in blood at 5 dpi. Supporting the role of IgM as a critical element in controlling/clearing FMDV infection in cattle, we demonstrated here that a considerably large proportion of the neutralizing activity of sera sampled 5 and 6 days after infection was due to the FMDV-specific IgM antibodies.

Altogether, our observations suggest that the development of local adaptive responses coincides with tissues where the virus undergoes significant productive replication and that IgM antibodies are the main effectors mediating FMDV clearance from peripheral circulation and thus represent a key factor in the development of early protective responses.

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REFERENCES

1. Carpenter TE, O'Brien JM, Hagerman AD, McCarl BA. 2011. Epidemic and economic impacts of delayed detection of foot-and-mouth disease: a case study of a simulated outbreak in California. *J. Vet. Diagn. Invest.* 23:26–33.
2. Muroga N, Hayama Y, Yamamoto T, Kurogi A, Tsuda T, Tsutsui T. 2012. The 2010 foot-and-mouth disease epidemic in Japan. *J. Vet. Med. Sci.* 74:399–404.
3. Thompson D, Muriel P, Russell D, Osborne P, Bromley A, Rowland M, Creigh-Tyte S, Brown C. 2002. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Rev. Sci. Technol.* 21: 675–687.
4. Yang PC, Chu RM, Chung WB, Sung HT. 1999. Epidemiological characteristics and financial costs of the 1997 foot-and-mouth disease epidemic in Taiwan. *Vet. Rec.* 145:731–734.
5. Alexandersen S, Zhang Z, Donaldson AI, Garland AJ. 2003. The

- pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Pathol.* 129:1–36.
6. Alexandersen S, Mowat N. 2005. Foot-and-mouth disease: host range and pathogenesis. *Curr. Top. Microbiol. Immunol.* 288:9–42.
 7. McVicar JW, Suttmoller P. 1976. Growth of foot-and-mouth disease virus in the upper respiratory tract of non-immunized, vaccinated, and recovered cattle after intranasal inoculation. *J. Hyg. (Lond.)* 76:467–481.
 8. Zhang Z, Alexandersen S. 2004. Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. *J. Gen. Virol.* 85:2567–2575.
 9. Zhang ZD, Kitching RP. 2001. The localization of persistent foot and mouth disease virus in the epithelial cells of the soft palate and pharynx. *J. Comp. Pathol.* 124:89–94.
 10. Suttmoller P, McVicar JW. 1976. Pathogenesis of foot-and-mouth disease: the lung as an additional portal of entry of the virus. *J. Hyg. (Lond.)* 77:235–243.
 11. Arzt J, Pacheco JM, Rodriguez LL. 2010. The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation: identification of the nasopharynx as the primary site of infection. *Vet. Pathol.* 47:1048–1063.
 12. Pacheco JM, Arzt J, Rodriguez LL. 2010. Early events in the pathogenesis of foot-and-mouth disease in cattle after controlled aerosol exposure. *Vet. J.* 183:46–53.
 13. Figueroa F, Ohlbaum A, Contreras G. 1973. Neutralizing antibody response in bovine serum and nasal and salivary secretions after immunization with live or inactivated foot-and-mouth disease virus. *Infect. Immun.* 8:296–298.
 14. Francis MJ, Ouldrige EJ, Black L. 1983. Antibody response in bovine pharyngeal fluid following foot-and-mouth disease vaccination and, or, exposure to live virus. *Res. Vet. Sci.* 35:206–210.
 15. Hyslop NS. 1965. Secretion of foot-and-mouth disease virus and antibody in the saliva of infected and immunized cattle. *J. Comp. Pathol.* 75:111–117.
 16. Kaaden O, Matthaeus W. 1970. Detection and some characteristics of foot-and-mouth disease (FMD) antibodies in bovine saliva. Brief report. *Arch. Gesamte Virusforsch.* 30:263–266.
 17. Maddur MS, Gajendragad MR, Kishore S, Chockalingam AK, Suryanarayana VV, Gopalakrishna S, Singh N. 2008. Enhanced mucosal immune response in cattle persistently infected with foot-and-mouth disease virus. *Vet. Immunol. Immunopathol.* 125:337–343.
 18. McVicar JW, Suttmoller P. 1974. Neutralizing activity in the serum and oesophageal-pharyngeal fluid of cattle after exposure to foot-and-mouth disease virus and subsequent re-exposure. *Arch. Gesamte Virusforsch.* 44:173–176.
 19. Parida S, Anderson J, Cox SJ, Barnett PV, Paton DJ. 2006. Secretory IgA as an indicator of oro-pharyngeal foot-and-mouth disease virus replication and as a tool for post vaccination surveillance. *Vaccine* 24:1107–1116.
 20. Archetti IL, Amadori M, Donn A, Salt J, Lodetti E. 1995. Detection of foot-and-mouth disease virus-infected cattle by assessment of antibody response in oropharyngeal fluids. *J. Clin. Microbiol.* 33:79–84.
 21. Barteling SJ, Meloen RH. 1974. A simple method for the quantification of 140S particles of foot-and-mouth disease virus (FMDV). *Arch. Gesamte Virusforsch.* 45:362–364.
 22. Seki C, Robiolo B, Periolo O, Iglesias M, D'Antuono A, Maradei E, Barros V, La Torre J, Mattion N. 2009. Rapid methodology for antigenic profiling of FMDV field strains and for the control of identity, purity and viral integrity in commercial virus vaccines using monoclonal antibodies. *Vet. Microbiol.* 133:239–251.
 23. Brown CC, Meyer RF, Olander HJ, House C, Mebus CA. 1992. A pathogenesis study of foot-and-mouth disease in cattle, using in situ hybridization. *Can. J. Vet. Res.* 56:189–193.
 24. Burrows R, Mann JA, Garland AJ, Greig A, Goodridge D. 1981. The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle. *J. Comp. Pathol.* 91:599–609.
 25. Schaller JP, Saif LJ, Cordle CT, Candler E, Jr, Winship TR, Smith KL. 1992. Prevention of human rotavirus-induced diarrhea in gnotobiotic piglets using bovine antibody. *J. Infect. Dis.* 165:623–630.
 26. Hamblin C, Barnett IT, Hedger RS. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *J. Immunol. Methods* 93:115–121.
 27. Periolo OH, Seki C, Grigera PR, Robiolo B, Fernandez G, Maradei E, D'Aloia R, La Torre JL. 1993. Large-scale use of liquid-phase blocking sandwich ELISA for the evaluation of protective immunity against aphthovirus in cattle vaccinated with oil-adjuvanted vaccines in Argentina. *Vaccine* 11:754–760.
 28. Booth JC, Rweyemamu MM, Pay TW. 1978. Dose-response relationships in a microneutralization test for foot-and-mouth disease viruses. *J. Hyg. (Lond.)* 80:31–42.
 29. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493–497.
 30. Capozzo AV, Periolo OH, Robiolo B, Seki C, La Torre JL, Grigera PR. 1997. Total and isotype humoral responses in cattle vaccinated with foot and mouth disease virus (FMDV) immunogen produced either in bovine tongue tissue or in BHK-21 cell suspension cultures. *Vaccine* 15:624–630.
 31. Bachmann MF, Hengartner H, Zinkernagel RM. 1995. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur. J. Immunol.* 25:3445–3451.
 32. Szomolanyi-Tsuda E, Le QP, Garcea RL, Welsh RM. 1998. T-cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particles. *J. Virol.* 72:6665–6670.
 33. Ochsenbein AF, Pinschewer DD, Odermatt B, Ciurea A, Hengartner H, Zinkernagel RM. 2000. Correlation of T cell independence of antibody responses with antigen dose reaching secondary lymphoid organs: implications for splenectomized patients and vaccine design. *J. Immunol.* 164:6296–6302.
 34. Zinkernagel RM. 2000. Localization dose and time of antigens determine immune reactivity. *Semin. Immunol.* 12:163–171.
 35. Borca MV, Fernandez FM, Sadir AM, Braun M, Schudel AA. 1986. Immune response to foot-and-mouth disease virus in a murine experimental model: effective thymus-independent primary and secondary reaction. *Immunology* 59:261–267.
 36. Juleff N, Windsor M, Lefevre EA, Gubbins S, Hamblin P, Reid E, McLaughlin K, Beverley PC, Morrison IW, Charleston B. 2009. Foot-and-mouth disease virus can induce a specific and rapid CD4⁺ T-cell-independent neutralizing and isotype class-switched antibody response in naive cattle. *J. Virol.* 83:3626–3636.
 37. Abu Elzein EM, Crowther JR. 1981. Detection and quantification of IgM, IgA, IgG1 and IgG2 antibodies against foot-and-mouth disease virus from bovine sera using an enzyme-linked immunosorbent assay. *J. Hyg. (Lond.)* 86:79–85.

Table S1. Number of FMDV-specific ASC in all animals and organs assayed from 4 to 6 days post-infection.

Isotype	Organ	C181 / 4 dpi *		C165 / 4 dpi		C182 / 4 dpi		C118 / 5 dpi	
		mean±SD #	Significant Differences §	mean±SD	Significant Differences	mean±SD	Significant Differences	mean±SD	Significant Differences
IgM	ML	14.0±1.9	TBL	8.0±6.2	TBL	5.6±1.8	TBL	43.8± 8.1	TBL
	MRL	12.0±11.4	TBL	2.3±0.6	TBL	7.0±1.7	TBL	130.7±28.9	TBL
	LRL	5.0±3.8	TBL	3.3±2.0	TBL	2.0±1.0	TBL	24.3±5.1	TBL
	PhT	7.5±3.9	TBL	1.3±0.6	TBL	3.0±1.4	TBL	21.7±14.0	TBL
	TBL	53.5±21.4	all	41.7±11.4	all	180.8±79.2	all	328.3±84.3	all
	S	7.0± 2.0	TBL	4.0±1.7	TBL	21.6±18.0	TBL	74.5±52.2	TBL
IgG1	ML	2.5±0.8	TBL	1.8±1.0	TBL	0.5±0.8	TBL	33.3±13.0	MRL,LRL,TBL,S
	MRL	1.0±1.0	TBL	1.3±0.6	TBL	7.7±4.5	TBL	71.3±16.2	all
	LRL	1.0±1.0	TBL	0.0±0.0	TBL	1.0±1.0	TBL	0.0±0.0	ML,MRL,PhT,TBL
	PhT	0.0±0.0	TBL	1.0±1.0	TBL	3.7±1.5	TBL	26.0±5.3	MRL,LRL,TBL,S
	TBL	23.8±10.7	all	10.2±2.2	all	42.3±9.6	all	98.7±6.1	all
	S	2.3±1.5	TBL	0.7±1.1	TBL	3.3±3.0	TBL	0.3±0.6	ML,MRL,PhT,TBL
IgG2	ML	0.0±0.0	TBL	0.3±0.8	none	0.7±1.1	TBL	6.7±3.0	MRL,TBL
	MRL	2.7±1.1	TBL	0.0±0.0	none	1.0±1.0	TBL	19.3±4.2	ML,MRL,S
	LRL	0.7±0.8	TBL	0.0±0.0	none	0.3±0.6	TBL	3.3±2.3	MRL,TBL
	PhT	0.0±0.0	TBL	1.3±1.5	none	2.3±0.6	none	7.3±4.2	TBL
	TBL	6.3±2.6	all	1.3±2.8	none	5.3±3.2	all	29.3±8.3	ML,LRL,PhT,S
	S	1.0±1.7	TBL	2.7±3.5	none	1.3±1.1	none	0.0±0.0	MRL,TBL
IgA	ML	0.7±1.1	none	3.3±2.5	MRL,LRL,TBL,S	1.0±1.7	TBL	20.5±7.5	MRL,LRL,PhT,S
	MRL	0.0±0.0	none	0.0±0.0	ML	5.3±2.5	TBL	11.0±5.3	all
	LRL	0.0±0.0	none	0.0±0.0	ML	0.7±0.6	TBL	2.7±2.0	ML,MRL,TBL
	PhT	1.3±1.5	none	1.7±0.6	none	0.0±0.0	TBL	2.8±2.0	ML,MRL,TBL
	TBL	2.7±3.8	none	0.0±0.0	ML	23.7±7.0	all	22.5±4.0	MRL,LRL,PhT,S
	S	5.3±3.0	none	0.0±0.0	ML	1.0±1.7	TBL	0.0±0.0	ML,MRL,TBL

* steer number/days post-infection, # mean ASC number/5x10⁵ mononuclear cells ± standard deviation, § significant differences with other organs for this isotype and steer (p<0.05)

Table S1 (cont.). Number of FMDV-specific ASC in all animals and organs assayed from 4 to 6 days post-infection.

Isotype	Organ	C146 / 5 dpi		C115 / 6 dpi		C126 / 6 dpi		B995 / 6 dpi	
		mean±SD #	Significant Differences §	mean±SD	Significant Differences	mean±SD	Significant Differences	mean±SD	Significant Differences
IgM	ML	58.0±5.6	all	1,099.0±23.0	all	774.7±6.4	all	1,003.0±8.0	all
	MRL	34.3±4.9	ML,LRL,TBL,S	26.3±11.0	all	485.3±5.0	all	918.7±16.2	ML,LRL,PhT,S
	LRL	16.0±5.5	ML,MRL,TBL	698.7±26.2	all	313.3±3.0	all	218.0±30.8	all
	PhT	26.0±2.0	ML,TBL,S	397.3±10.1	all	139.3±5.1	all	56.7±3.0	all
	TBL	124.0±12.1	all	881.0±21.4	all	468.0±5.6	all	897.3±17.5	ML,LRL,PhT,S
	S	5.6±2.2	ML,MRL,PhT,TBL	198.0±21.1	all	165.0±7.5	all	1,101.0±20.6	all
IgG1	ML	14.7±5.3	MRL,S	200.0±7.2	LRL,PhT,S	162.3±3.2	all	789.3±10.3	all
	MRL	21.8±5.3	LRL,S	167.3±29.7	LRL,PhT,S	46.7±7.2	all	273.3±7.0	all
	LRL	2.1±1.8	ML,MRL,PhT,TBL	54.7±8.3	ML,MRL,TBL,S	26.7±2.0	ML,MRL,TBL	56.0±15.9	ML,LRL,TBL
	PhT	17.3±3.0	LRL,S	19.3±2.9	ML,MRL,TBL	23.3±2.0	ML,MRL,TBL	6.5±4.5	ML,MRL,TBL,S
	TBL	19.0±6.7	LRL,S	180.7±21.9	LRL,PhT,S	106.3±9.7	all	194.5±49.6	all
	S	4.5±2.6	ML,MRL,PhT,TBL	16.7±6.0	ML,MRL,LRL,TBL	24.7±5.0	ML,MRL,TBL	104.7±21.6	ML,MRL,PhT,TBL
IgG2	ML	11.3±3.0	MRL,LRL,S	168.7±21.9	all	101.0±5.6	all	64.3±71.0	LRL,PhT,S
	MRL	4.7±2.9	ML	94.0±30.8	ML,LRL,PhT,S	16.7±4.2	ML,TBL	19.3±8.7	none
	LRL	2.7±0.6	ML	17.0±15.6	ML,MRL,TBL	15.0±2.6	ML,TBL	5.2±2.4	ML
	PhT	7.3±1.1	none	9.0±1.0	ML,MRL,TBL	12.0±3.0	ML,TBL	1.0±0.9	ML
	TBL	7.0±4.0	none	62.0±9.9	ML,LRL,PhT,S	34.3±3.0	all	40.7±15.5	none
	S	2.0±1.0	ML	9.5±3.8	ML,MRL,TBL	10.3±1.5	ML,TBL	4.2±3.6	ML
IgA	ML	8.5±3.2	MRL,LRL,S	146.0±16.4	all	70.3±15.9	all	40.7±7.8	MRL,LRL,PhT,S
	MRL	1.3±1.5	ML	88.7±15.1	all	12.0±6.5	ML	23.7±8.3	ML,LRL,PhT
	LRL	1.3±1.1	ML	8.7±1.5	ML,MRL,TBL	23.3±2.0	ML	3.5±4.6	ML,MRL,TBL
	PhT	5.2±3.1	none	6.5±2.3	ML,MRL,TBL	18.7±0.6	ML	7.5±9.0	ML,MRL,TBL
	TBL	5.7±1.5	none	52.7±3.0	all	18.7±0.6	ML	33.4±11.7	LRL,PhT,S
	S	1.3±2.3	ML	7.7±2.0	ML,MRL,TBL	3.3±4.2	ML	11.5±6.0	ML,TBL

* steer number/days post-infection, # mean ASC number/5x10⁵ mononuclear cells ± standard deviation, § significant differences with other organs for this isotype and steer (p<0.05)