RESEARCH REPORT
SWINE HEALTH

Title: Rational design of attenuated foot-and-mouth disease virus strains for development of improved disease countermeasures – **NPB #11-005**

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Industry Summary: FMD is the one of the most feared viral disease that can affect livestock, including swine. Although this disease appeared to be eliminated from most developed nations by the end of last century, recent outbreaks in Europe, Japan, Taiwan, South Korea, Eastern Russia, etc, have demonstrated that infection can spread as wild fire affecting any nation and causing devastating economic and social consequences. Furthermore, post 9/11 the US is threatened by the potential deliberate release of FMDV by terrorist groups. Therefore, it is essential to develop new control strategies that could confer very early protection and stop disease spread. It has been demonstrated for other viral diseases that live-attenuated vaccines are one of the best choices to obtain a strong early and long-lasting protection. The current FMD vaccine is a formulation of inactivated WT virus antigen prepared in high containment bio-security level 3 facilities. This vaccine requires 7 days to induce protection, a time during which vaccinated animals are still susceptible to one of the fastest replicating viruses in nature. In addition the vaccine is prepared from highly virulent circulating virus strains that despite extreme caution and care in the manufacturing process, could result in outbreaks by accidental virus release as it happened in the United Kingdom in 2007 (estimated cost $2B). Our goal is to develop alternative control strategies that could improve current FMD countermeasure programs. An attenuated vaccine is expected to elicit more rapid innate immunity and a long lived adaptive immunity to effectively control disease. Moreover, induction of innate immunity could result in early protection against multiple FMDV serotypes. From the production perspective, use of an attenuated FMDV strain will reduce the consequences of accidental outbreaks caused by accidental release of virus from vaccine manufacturing facilities. Importantly, attenuated strains are excellent new tools to study the interactions between FMDV and the host immune system and ultimately could lead to the development of novel strategies to counteract FMD. During the past year and with the support of NPB we have successfully derived a mutant strain of FMDV that did not cause disease in swine (FMDV-SAP mutant). Interestingly inoculation of swine with this mutant strain induced a strong immune response that protected animals against infection with the parental (wild type) virus, as early as two days post vaccination. Studies in animals and in cultured swine cells demonstrated that, in contrast to the parental wild type virus, the mutant variant was unable to block some inflammatory responses thus limiting dissemination of the virus beyond the original site of inoculation. Furthermore

we have started studies to add more mutations to this virus aiming to increase the stability of the original mutations therefore decreasing the probability of reversion to virulence. Our results indicated that manipulation of the viral genome in the region that encodes for the leader protein is a viable alternative to derive less pathogenic FMDV strains that could be used as the basis for live attenuated vaccines against FMD or as seeds to grow the virus for manufacturing safer inactivated vaccines. Although this strategy is far from being used in countries that are FMD free without vaccination, it could be an affordable alternative to control FMD in regions of the world where the disease is enzootic thereby decreasing the risks of dissemination to disease-free nations. Ultimately a combination of strategies tailored to each region of the world will eventually succeed to eradicate this feared disease.
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Keywords: Foot-and-Mouth Disease, FMDV, attenuated FMDV strains, leader protein, inflammatory response, SAP-mutant.

Scientific Abstract: Foot-and-mouth disease virus (FMDV) leader proteinase (Lpro) cleaves itself from the viral polyprotein and cleaves the translation initiation factor eIF4G. As a result, host cell translation is inhibited, affecting the host innate immune response. We have demonstrated that Lpro is also associated with degradation of nuclear factor κB (NF-κB), a process that requires Lpro nuclear localization. Additionally, we reported that disruption of a conserved protein domain within the Lpro coding sequence, SAP mutation, prevented Lpro nuclear retention and degradation of NF-κB, resulting in *in vitro* attenuation. Here we report that inoculation of swine with this SAP mutant virus does not cause clinical signs of disease, viremia or virus shedding even when inoculated at doses 100-fold higher than those required to cause disease with wild type (WT) virus. Remarkably, SAP mutant virus inoculated animals developed a strong neutralizing antibody response and were completely protected against challenge with WT FMDV as early as 2 and for at least 21 days post inoculation. Early protection correlated with a distinct pattern in the serum levels of pro-inflammatory cytokines in comparison to animals inoculated with WT FMDV that developed disease. In addition, animals inoculated with the FMDV SAP mutant displayed a memory T cell response that resembled infection with WT virus. Our results suggest that Lpro plays a pivotal role in modulating several pathways of the immune response. Furthermore, manipulation of the Lpro coding region may serve as a viable strategy to derive live attenuated strains with potential for development as effective vaccines against FMD.

Introduction: FMD is a highly contagious disease of domestic and wild cloven-hoofed animals including swine, cattle, sheep, goats, deer and buffalo that quickly replicates in the host spreading to susceptible animals by contact and aerosol. The disease is characterized by fever, lameness and vesicular lesions on the tongue, feet, snout and teats resulting in high morbidity but low mortality, except in young animals.

FMD is enzootic in all continents except for Australia and North America. Although the U.S. has been FMD free since 1929, recent natural outbreaks in previously disease-free countries have significantly increased the public awareness about this disease. In 2001, outbreaks in the United Kingdom (U.K.) that spread to several European countries resulted in losses to the agricultural industry surpassing $15B with more than 10M animals slaughtered. In August of 2007 a new outbreak affected the U.K. but in this instance the cause was attributed to accidental viral escape from the Pirbright campus which houses a commercial FMD vaccine production. More recently, in April 2010, an outbreak in Japan resulted in the slaughter of 300,000 animals and over a $2B loss. The U.S. is the world's No. 2 pork producer and exporter, and the No. 3 importer. An outbreak in our country would result in devastating economic losses considering the $100B/year livestock industry. Furthermore, world globalization and the events of

September 11, 2001 have demonstrated that the U.S. is vulnerable to the deliberate or accidental introduction of FMDV. Thus it is imperative to develop novel strategies to effectively control this disease, not only in case of an outbreak but also to reduce the number of cases in enzootic countries, resulting in less risk for FMDV-free countries.

The World Organization of Animal Health (OIE) includes FMD on the notifiable list of diseases and requires the immediate official report of confirmed FMD cases with cessation of trading of susceptible animals including their products. In case of an FMD outbreak, OIE demands restriction of animal/animal-product movement, slaughtering of infected/in contact animals, disinfection of premises and optional ring vaccination with an inactivated vaccine. To regain FMD-free status OIE regulations require that a country demonstrate the absence of disease for 3 months, when slaughtering and disinfection are used as control methods, or for 6 months when animals are vaccinated but not slaughtered (Grubman and Baxt, 2004).

An available control measure to FMD incursion includes the use of an inactivated vaccine, although FMD-free countries are reluctant to use this vaccine for several reasons: vaccine manufacturing requires a biosafety level 3 (BSL3) containment facility, the vaccine does not allow for differentiation between vaccinated and infected animals, there is a potential risk of deriving asymptomatic disease carriers upon exposure of vaccinated animals to infectious virus, and affected countries need more time to regain FMDV-free status when vaccine is used. In addition, the current vaccine does not confer protection before 7 days post vaccination (dpv). To overcome some of these limitations, Moraes et al., (2002) have constructed a recombinant vaccine delivered by a replication-defective human adenovirus type 5 (Ad5) vector. Although effective, protection induced by this new platform does not occur until 7 dpv either, and duration of immunity is shorter than the one conferred by natural FMDV infection.

Many viruses, including FMDV, are susceptible to interferons (IFNs). Using the same Ad5 vector, Chinsangaram et al., (2003) have expressed porcine type I IFN and demonstrated complete protection of swine when challenged one day after the Ad5-IFN treatment. However, this approach is limited by the relatively short-lived protection induced by IFN which lasts 3-5 days after administration. Since inoculation of an inactivated FMDV vaccine or a recombinant vaccine induces an adaptive immune response in approximately 7 days, even when IFN and vaccine are used in combination, there is a window of opportunity, between the 5th and 7th day, when the virus can successfully grow and spread.

It has been reported that rapid and long lasting protection against viral infection is usually best achieved by vaccination with attenuated viral vaccines. Indeed, using attenuated viral vaccines, both, smallpox and very recently rinderpest, have been eradicated. So far, no attenuated vaccine has been successfully used against FMDV. A candidate for such a vaccine was previously developed at PIADC, ARS, USDA, by deletion of the coding region for the nonstructural viral protein Lpro, (leaderless virus). Despite the reduced pathogenicity of this virus in swine and cattle, the induced protection against viral challenge was incomplete probably due to the very slow and limited replication of the mutant strain. More recently, we have identified conserved protein motifs within the Lpro coding region. Mutation of two conserved amino acid residues in the so called SAP (SAF-A/B, Acinus and PIAS domain)domain of Lpro resulted in a virus slightly attenuated in primary cultured cells, but still able to replicate better than FMDV leaderless virus. Here we report that inoculation of swine with this SAP mutant virus does not cause clinical signs of disease, viremia or virus shedding even when inoculated at doses 100-fold higher than those required to cause disease with wild type (WT) virus. Remarkably, SAP mutant virus inoculated animals developed a strong neutralizing antibody response and were completely protected against challenge with WT FMDV as early as 2 and for at least 21 days post inoculation. Early protection correlated with a distinct pattern in the serum levels of pro-inflammatory cytokines in comparison to animals inoculated with WT FMDV that developed disease.

Recently we have focused in trying to understand the interactions between FMDV WT or SAP mutant with the host immune cells. Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that have the capacity to indentify and recognize pathogens via Toll-like receptors (TLRs), resulting in

the subsequent up-regulation of expression of co-stimulatory molecules, proinflammatory cytokines, chemokines and enhancement of antigen presentation. After antigen up-take, DCs maturate and migrate to the draining lymph nodes where they present the antigen to effector T cells, ultimately mounting an effective cell-mediated response. Monocyte DC (moDC) differentiation is accompanied by an increased expression of several surface molecules that facilitate T cell stimulation, such as MHC class II (SLA II for porcine moDCs), a molecule that plays a central role in developmentally restricting antigen presentation. Previous studies have shown that WT FMDV infects swine DCs progenitors CD172+ cells leading to inhibition of maturation and antigen presentation. Additionally, in swine, an increased production of IL-10 by DCs exposed to FMDV, impairs activation of T cells during acute infection favoring a Th2 response that results in the production of FMDV specific neutralizing antibodies. We have comparatively analyzed the response of DCs and total PBMCs to infection with FMDV WT and SAP mutant *ex vivo*. Consistent with previous reports, WT virus affected moDC development while the SAP mutant did not.

Interestingly the levels of proinflammatory cytokines were significantly augmented in PBMCs exposed to FMDV SAP mutant virus consistently with the results obtained in vivo.

We have also identified another conserved region within the Lpro coding sequence (FHA domain) that may be involved in interactions with host factors. In preliminary studies we found that mutation of a conserved residue in this domain also yielded a viable virus with an attenuated phenotype in cell culture. These results suggest that Lpro plays a pivotal role in modulating several pathways of the immune response and manipulation of the Lpro coding region may serve as a viable strategy to derive live attenuated strains with potential for development as effective vaccines against FMD.

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Objectives:

1. Test the virulence of SAP mutant FMDV in swine.

2. Test the induction of protection against challenge with wild type (WT) FMDV at different times post-inoculation with SAP mutant FMDV.

3. Perform pathogenesis studies utilizing SAP mutant in comparison to WT FMDV.

4. Initiate experiments to construct and characterize in vitro, FMDV strains with mutations in the Lpro FHA domain alone or in combination with mutations in the SAP domain.

Materials & Methods:

Cells and viruses: Porcine kidney (IBRS-2) cell lines were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the PIADC. These cells were maintained in minimal essential medium (MEM, GIBCO BRL, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and non-essential amino acids. BHK-21 cells (baby hamster kidney cells strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC, Rockville, MD) were used to propagate virus stocks and to measure virus titers. BHK-21 cells were maintained in MEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with 1% antibiotics and non-essential amino acids. Cell cultures were incubated at 37ºC in 5% CO2.

FMDV A12-WT was generated from the full-length serotype A12 infectious clone, pRMC35 (Rieder et al., 1993). A12-SAP mutant virus, a derivative of A12-WT containing mutations I55A and L58A in the Lpro region, was constructed by site directed mutagenesis using a QuikChange® (Qiagen, Valencia, CA) kit and following the manufacturer's direction. All viruses were propagated in BHK-21 cells, concentrated by polyethylene glycol precipitation, titrated on the same cells and stored at -70oC. Viruses of passage #6 for A12-WT and passage #5 for A12-SAP were used for all experiments and the full length sequences were confirmed by DNA sequencing of derived viral cDNA using an ABI prism 7000 (Applied Biosystems, Foster City, CA).

Animal experiments: Animal experiments were performed in the high-containment facilities of the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. In a first experiment, fifteen Yorkshire guilts (five weeks old and weighing approximately 40 lbs each) were divided in five groups of three animals each. Animals were inoculated intradermally (ID) in the heel bulb of the right hind foot with different doses of FMDV A12-WT ($1x10^5$ or $1x10^6$ pfu/animal) or A12-SAP ($1x10^5$, $1x10^6$ or $1x10^7$ pfu/animal). Rectal temperatures and clinical signs, including lameness and vesicular lesions, were monitored daily during the first week postinoculation and samples of serum and nasal swabs were collected on a daily basis. Serum samples were also collected at days 14 and 21 postinoculation (dpi). Clinical scores were determined by the number of toes presenting FMD lesions plus the presence of lesions in the snout and/or mouth. The maximum score was 17, and lesions restricted to the site of inoculation were not counted. Those pigs inoculated with A12-SAP were challenged 21 days later with $1x10^5$ pfu/pig of FMDV A12-WT, ID in the heel bulb of the left hind limb. Clinical signs and samples were collected on a daily basis for 7 days and serum samples were also collected at 14 and 21 days postchallenge (dpc).

A second experiment was performed to test protective responses after inoculation with SAP mutant. Ten swine were divided in 3 groups. Two groups of 3 animals each, were either subcutaneously or intradermally (ID) inoculated with SAP mutant virus; a contact naïve animal was included in the room with ID SAP inoculated animals to test the possibility of passive animal to animal virus transfer. The third group was inoculated with PBS as a control. At 21 days post inoculation all animals were challenged with WT FMDV.

In a third experiment, 4 groups of 3 Yorkshire guilts each (five weeks old and weighing approximately 40 lbs) were subcutaneously (SC) vaccinated with attenuated FMDV A12-SAP ($1x10^6$ pfu/animal) followed by challenge at different times post-vaccination (dpv) (2, 4, 7 and 14 dpv) with $5x10^5$pfu/animal of virulent FMDV A12-WT, ID in the heel bulb. One extra group of 3 pigs was inoculated with PBS and challenged 14 days later (control group). For the last two experiments, serum samples were collected at 2, 4, 7 and 14 dpv. After the challenge, clinical disease was monitored daily during the first week and samples were collected as described for the first experiment.

Virus titration in serum and nasal swabs: Serum and nasal swabs were assayed for the presence of virus by plaque titration on BHK-21 cells (passage levels 60-70). Serial ten-fold dilutions of the samples were allowed to adsorb on monolayers of BHK-21 cells grown in 6-well plates. Following 1 h adsorption, the inoculum was removed and 2 ml of MEM containing antibiotics, essential amino acids and 0.6 % gum tragacanth was added to each well. The plates were incubated for 24 h at 37ºC in a humidified atmosphere containing 5% CO2 and then stained with a crystal violet-formalin solution to visualize the plaques. Virus titers were expressed as log10 pfu per ml of serum or nasal swab. The detection level of this assay is 5 pfu/ml.

Detection of FMDV RNA by real-time RT-PCR (rRT-PCR): One to 7 dpc frozen sera samples from animals that had no detectable clinical disease were thawed and processed for RNA extraction and measurement of specific FMDV RNA by rRT-PCR as previously described. Samples were considered positive when Ct values were <40.

Determination of neutralizing antibody titer: Sera samples were tested for the presence of FMDV-specific neutralizing antibodies by a plaque reduction neutralization assay as previously described (48). Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN70) induced by FMDV A12-WT in BHK-21 cells.

Radioimmunoprecipitation (RIP) of [35S]-methionine/[35S]-cysteine labeled FMDV A12 infected cell lysates with swine sera samples: Radiolabeled lysates of FMDV A12 infected BHK-21 cells were incubated with individual swine sera samples from 0 and 21 dpc and examined for the

presence of antibodies specific to FMDV structural and NS polypeptides by RIP. Convalescent serum from a FMDV infected bovine was used as a positive control. After 60 min incubation at room temperature, antibodies were precipitated with Staphylococcus aureus protein A. Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography.

Quantification of antibody isotypes by ELISA: The presence of FMDV-specific IgM, IgG1, IgG2 antibodies was detected by an indirect double antibody sandwich assay as described previously with some modifications. Briefly, Costar EIA/RIA high binding 96-well flat-bottom plates (Corning, NY) were coated with anti-FMDV antibody and incubated with an optimal dilution of either positive or negative FMDV antigen prior to addition of test sera. Positive control sera for IgM or for IgG1 and IgG2 were obtained from a swine inoculated with virulent FMDV A24 at 7 or 21 dpc, respectively. Positive control sera were chosen for their ability to generate a definitive signal in their respective isotype-specific assays. Negative control sera for each assay were pre-immune sera from the same animals.

Analysis of cytokines in serum and supernatants of PBMCs and monocyte derived dendritic cells (moDCs): IFN-α, IL-1β, IL-6, IL-10 and TNF-α protein concentrations were determined in sera from infected animals or supernatants of cultured cells using specific ELISAs. IFN-α was detected using mAbs K9 and F17 (PBL Interferon Source, Piscataway, NJ) . IL-10 Cytoset ELISA (Biosource-Invitrogen, Carlsbad, CA) and IL-1β, IL-6 and TNF-α Duo Set ELISAs (R&D Systems, Minneapolis, MN) were performed following the manufacturer's directions. All ELISAs were developed with 3, 3', 5, 5', tetramethylbenzidine (TMB) from KPL (Gaithersburg, MD). The absorbance at 450 nm was measured in an ELISA reader (VersaMax, Molecular Devices, Sunnyvale, CA). Cytokine concentrations were calculated based on the optical densities obtained with the standards and are expressed in relative levels for each individual at different times post infection with respect to its own level at day 0.

Detection of cytokines in PBMCs by Real Time PCR: Expression of several cytokines was analyzed in PBMCs. RNA was extracted from purified PBMCs, approximately 10^7 cells, by utilizing an RNeasy miniprep kit (Qiagen, Valencia, CA). A quantitative rRT-PCR method was used to evaluate the mRNA levels of several cytokines (IFN-α [236FW 5' TGGTGCATGAGATGCTCCA, 290RW 5' GCCGAGCCCTCTGTGCT and probe 6FAM-CAGACCTTCCAGCTCT], IL-1β [737FW 5' TTGAATTCGAGTCTGCCCTGT, 812RW 5' CCCAGGAAGACGGGCTTT and probe 6FAM-CAACTGGTACATCAGCACCTCTCAAGCAGAA], IL-6 [478FW 5' AATGTCGAGGCTGTGCAGATT, 559RW 5' TGGTGGCTTTGTCTGGATTCT and probe 6FAM-AGCACTGATCCAGACCCTGAGGCAAA], IL-10 [138FW 5' TGAGAACAGCTGCATCCACTTC, 241RW 5' TCTGGTCCTTCGTTTGAAAGAAA and probe 6FAM-CAACCAGCCTGCCCCACATGC] and TNF-α [338FW 5'TGGCCCCTTGAGCATCA , 405RW 5' CGGGCTTATCTGAGGTTTGAGA and probe 6FAM-CCCTCTGGCCCAAGGACTCAGATCA]). Porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the values for each sample [327FW5' CGTCCCTGAGACACGATGGT, 380RW5' CCCGATGCGGCCAAAT and 6FAM-AAGGTCGGAGTGAACG]. Reactions were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by comparative cycle threshold analysis (user bulletin 2; Applied Biosystems) utilizing as a reference the samples at 0 dpi.

Intracellular cytokine staining (ICCS): CD8+IFN-γ producing cells were analyzed by flow cytometry in total PBMCs after specific stimulation with FMDV A12-WT or with a non-specific stimulator (PMA plus Ca Ionophore) for 18 h at 37°C in 5% CO2. Non-specific stimulus was used as negative control. Cells were thereafter incubated with Golgi Stop (BD Bioscience, Franklin Lakes, NJ) according to the manufacturers' recommendations, followed by pelleting and resuspension in staining buffer (10% FBS in PBS). To analyze the expression of cell surface molecules, biotinylated mouse anti-porcine CD8 (Southern Biotech, Birmingham, AL) detected by Streptavidin-PerCP (BD-Pharmingen) were used. After

staining, cells were fixed and permeabilized with BD Cytoperm/Cytofix (BD Biosciences) for 30 min at 4°C, washed in BD perm/wash three times, and finally stained intracellularly with mouse anti- porcine IFN-γ-RPE (BD-Pharmingen). Cells were acquired using a FACS Calibur flow cytometer (BD Bioscience). Dead cells were excluded on the basis of forward and side light scatter. Data was analyzed with CellQuest software (BD Bioscience).

Extraction of PBMCs and generation of porcine moDCs: PBMCs from naïve pigs were obtained from heparanized blood by density gradient centrifugation at 800xg for 20 min, over polysucrose - sodium metrizoate medium (Uni-sep lymphocyte separation tubes, Novamed, Jerusalem, Israel). Cells expressing CD172 + were purified from total PBMCs (97% purity) using an anti- CD172 porcine pan-myeloid cell marker (monoclonal antibody BL1H7, AbD Serotec, Raleigh, NC, USA) by magnetic cell sorting using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and positive selection LS column. These cells were cultured for 7 days in RPMI medium supplemented with 10% (v/v) fetal calf serum and the recombinants porcine cytokines granulocyte-macrophage colony-stimulating factor (rpGM-CSF) at 150 ng/ml (Invitrogen, Carlsbad, CA, USA) and recombinant porcine interleukin-4 (rpIL-4) (100 ng/ml, Biosource, Nivelles, Belgium) to allow differentiation of DCs. Fresh cytokines were added every 2 days. To drive DC to complete maturation, non-adherent cells were collected at day 7 of incubation and resuspended in culture medium containing rpGM-CSF, rpIL-4 and Tumor Necrosis Factor-alpha (TNF-α) (5 ng/ml, Sigma, St Louis, MO, USA), and incubated for another 24 hours PBMCs or CD172+ cells after magnetic separation (D0) or after 4 days of culture in rpGM-CSF and rpIL-4 (D4) were either infected with A12-WT or A12-SAP mutant. In either case, cells were infected in six-well plates at an MOI of 10. Viral attachment was performed during one hour at 37°C, and subsequent washing with MES-buffer pH 5.9 as explained before.
After moDCs maturation process, infected or mock treated cells were pelleted by centrifugation and resuspended in staining buffer (PBS containing 2% [vol/vol] fetal calf serum and 0.2% [wt/vol] NaN3) for flow cytometry. To analyze the expression of cell surface molecules we used monospecific antibodies, fluorochrome dyes and flow cytometry. The primary antibodies used were mouse-anti SLA-IIDR-biotin (clone 1F12, BD Pharmingen, San Jose, CA, USA) and human CD152 (CTLA-4) murine immunoglobulin/FITC fusion protein (IG2a), that binds swine CD80/86, purchased from Ancell. In a second step, streptavidin conjugated to PE (BD Pharmingen) was used. After staining, cells were fixed in PBS/1% fetal bovine serum/4% PFA (wt/vol). Cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Dead cells were excluded on the basis of forward and side light scatter. Data were analysed with CellQuest software (Becton Dickinson). Supernatants of infected cells were collected and the concentration of different cytokines were determined by ELISA as described above.

Statistical analyses: Data handling, analysis and graphic representation were performed using Prism 5.0 (GraphPad Software, San Diego, CA) or Microsoft Excel. Statistical differences were determined using a Student's t test ($P < 0.05$[*], $P < 0.01$[**], $P < 0.005$ [***]).

Results

1. Virulence of SAP mutant FMDV in swine.

To compare the virulence of FMDV WT, A12-WT, with an FMD mutant virus containing mutations in the SAP domain of Lpro (A12-SAP), groups of three pigs were inoculated intradermally (ID) in the rear heel bulb with different doses of either FMDV. Animals were inoculated with 10^5 or 10^6 pfu/animal of A12-WT, doses previously shown to cause clinical disease in swine, and with 10^5, 10^6, and 10^7 pfu/animal of A12-SAP. In animals inoculated with WT virus, disease was detectable as early as 2 dpc, and only the group inoculated with 10^6 pfu/animal had temperatures of 40ºC or higher. By 7 dpc all animals inoculated with WT virus showed clinical signs of disease with no statistically significant differences between the groups (**Fig. 1A**). However, all animals inoculated with

A12-SAP, even those inoculated with a 10-fold higher dose than the WT virus inoculated group (10^7 pfu/animal), never showed clinical signs or elevated temperatures throughout the experiment. Animals inoculated with A12-WT developed viremia on the day prior to (group inoculated with 10^6pfu) or concomitantly (group inoculated with 10^5pfu) with the appearance of clinical signs. Interestingly, none of the animals inoculated with A12-SAP had detectable viremia either by virus isolation (**Fig. 1B**) or by rRT-PCR (data not shown). In parallel to viremia, animals inoculated with WT virus had detectable virus in nasal swabs starting at 2-3 dpi and only one out of three animals inoculated with 10^7 pfu of A12-SAP showed virus in nasal swabs at 5 dpi with a very low titer (<10 pfu/ml) (**Fig. 1B**).

These data indicated that A12-SAP FMDV displays significantly reduced virulence in swine as compared to A12-WT .

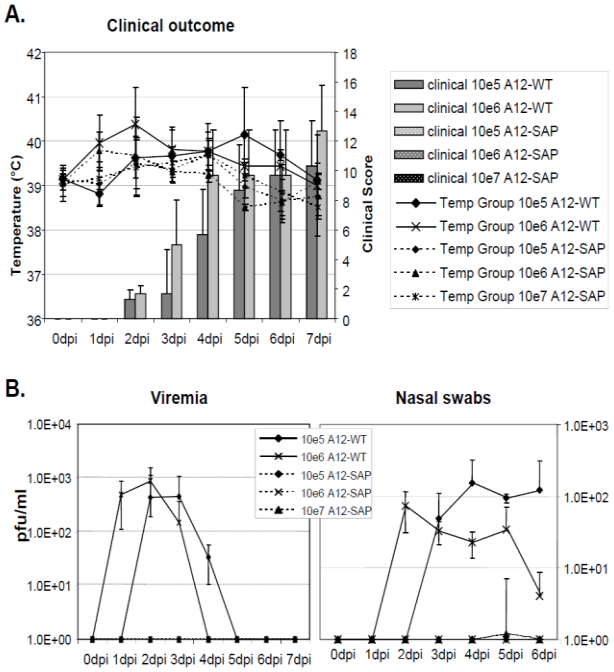


Figure. 1: Clinical outcome. Groups of three pigs were ID inoculated with different doses of A12-WT (10^5 or 10^6pfu/animal) or A12-SAP (10^5, or 10^7pfu/animal) and temperature, clinical signs and the presence of virus in serum and nasal swabs were monitored daily during 7 dpi. **A**. Clinical score is expressed as number of toes showing lesions plus one more point scored when lesions were present in either the mouth or snout both (maximum score is 17). Temperature above ^oC was considered as high fever. **B**. Virus levels expressed as the number of plaque forming units ml (pfu/ml) in plasma (viremia) or in media in which the swabs were collected. Each data point represents the mean (± S.D) of each group.

The levels of neutralizing antibodies were determined in all inoculated animals. As expected, all animals inoculated with A12-WT virus, developed high titers by day 7 post inoculation consistent with the signs of clinical disease. *Interestingly, all the animals inoculated with A12-SAP developed significant levels of FMDV specific neutralizing antibodies starting at 7 dpi with a peak at 14 dpi despite the absence of disease and detectable viremia* (**Fig. 2A**). No statistically significant differences were detected between animals inoculated with A12-WT and A12-SAP. Total antibodies, including those raised against structural and non-structural (NS) viral proteins, were determined by radio-immunoprecipation using the serum of inoculated animals at 21 dpi. All A12-WT inoculated animals developed antibodies against structural (VP4/2, VP1 and VP3) and NS (3Dpol) viral proteins, indicative of productive viral replication. Interestingly, all but one A12-SAP inoculated animals also had significant levels of antibodies against 3Dpol despite the absence of detectable viremia or virus shedding (**Fig. 2B**). All the sera from the animals were negative for the presence of antibodies at 0 dpi (data not shown). Immunoglobulin (Ig) isotype switching from IgM to IgG is expected when the antibody response is strong. The presence of IgM was detected by 7 dpi in all inoculated animals, peaked at 14 days and declined by 21 days while the levels of IgG1 and IgG2 increased in all inoculated animals (**Fig. 2C**).

Together, these data indicate that A12-SAP replicates in the animal, eliciting a strong adaptive immune response, comparable to WT virus, but does not cause vesicular lesions, viremia or fever.

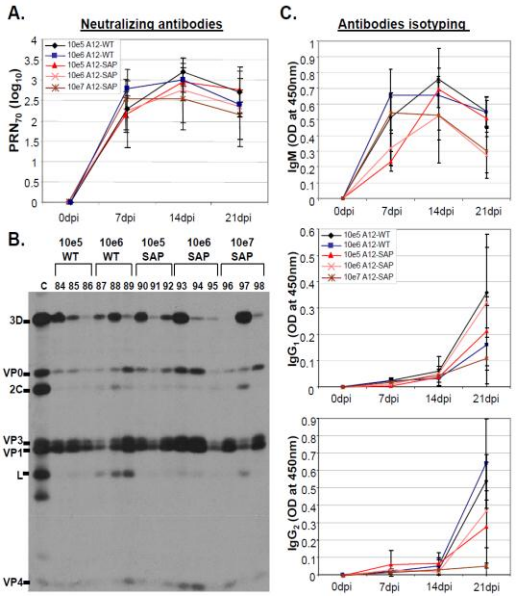


Figure 2: Presence of antibodies in serum of inoculated animals. **A**. The levels of neutralizing antibodies of swine inoculated with different doses of A12-WT or A12-SAP were determined at 0, 7, 14 and 21 dpi. Titers are expressed as the $\log_{10}$ of the inverse dilution of serum yielding a 70% reduction in the number of plaques (PRN_{70}). **B.** Immunoprecipitation of structural and NS viral proteins with serum of animals inoculated with different doses of FMDV A12-WT and A12-SAP. Cytoplasmic extract of FMDV A12-WT infected BHK-21 cells labeled with [^{35}S] Met /[^{35}S] Cys were immunoprecipitated with serum of infected animals after 21 dpi and with convalescent serum (C) positive control . Products were examined by SDS-PAGE on a 15% gel. **C.** Antibody isotype profiles in swine sera after infection. FMDV-specific IgM, IgG_1 and IgG_2 were detected by sandwich ELISA at 7, 14 and 21 dpi. Each data point represents the mean (± S.D) of each group.

2. Test the induction of protection against challenge with WT FMDV at different times post-inoculation with SAP mutant FMDV.

2a. To test whether the animals inoculated with A12-SAP were protected from WT FMDV challenge, the three groups of swine inoculated with different doses of A12-SAP in the previous experiment, were ID challenged with 10^5 pfu/animal of A12-WT at 21 days post-A12-SAP inoculation. **Table 1** shows that all animals were protected from challenge with WT virus. No clinical signs (fever or vesicles) or the presence of virus in plasma or nasal secretions was detected. Interestingly, the challenge acted as a boost and the animals showed an increase of neutralizing antibody titers by 7 dpc.

Table 1. Clinical outcome and presence of neutralizing antibodies in animals challenged with FMDV A12-IC WT 21 days after FMDV A12-SAP mutant inoculation.

Group	*Animal*	*Challenge virus*	*Dose*[a]	*Viremia (dpc, day of onset, duration)*[b]	*PFU in nasal swabs (dpc, day of onset, duration)*[c]	*Neutralizing antibodies PRN_{70}*[d] *0dpc*	*7dpc*
A12-SAP	90	A12-IC WT	$1x10^5$	Neg[e]	Neg.	2.4	>3.1
$1x10^5$pfu/animal	91			Neg.	Neg.	3.3	>3.1
	92			Neg.	Neg.	2.7	>3.1
A12-SAP	93	A12-IC WT	$1x10^5$	Neg.	Neg.	3	>3.1
$1x10^6$pfu/animal	94			Neg.	Neg.	2.7	>3.1
	95			Neg.	Neg.	1.8	>3.1
A12-SAP	96	A12-IC WT	$1x10^5$	Neg.	Neg.	1.5	3.0
$1x10^7$pfu/animal	97			Neg.	Neg.	2.7	3.1
	98			Neg.	Neg.	2.1	>3.1

[a] Dose of challenge virus expressed as number of plaque forming units (PFU) per animal.
[b] Number of PFU per ml of serum. The dpc value is the day after challenge that the maximum level of viremia was detected; the onset value is the first day postchallenge that viremia was detected; and the duration value is the number of days of viremia.
[c] Number of PFU per ml of nasal secretion. The dpc, onset and duration values are as defined in footnote b.
[d] The neutralizing antibodies is reported as the serum dilution yielding a 70% reduction in the numer of plaques (PRN_{70}).
[e] Neg., negative (less than 5 PFU/ml).

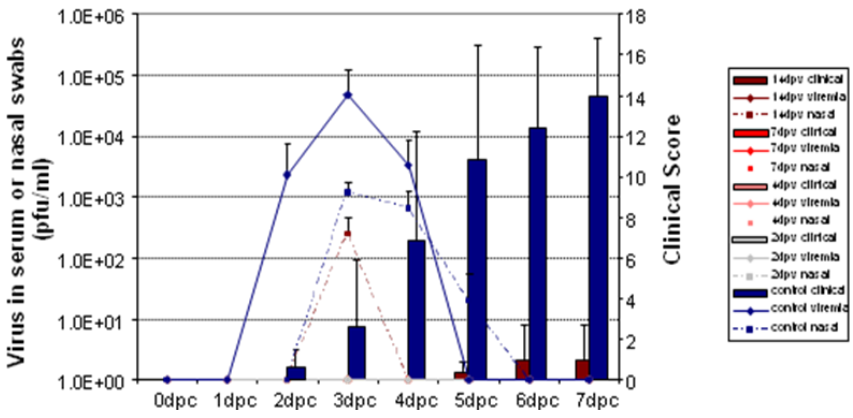
2b. A second experiment was performed to test protective responses after inoculation with SAP mutant. Ten swine were divided in 3 groups. Two groups of 3 animals each, were either subcutaneously or intradermally (ID) inoculated with SAP mutant virus; a contact naïve animal was included in the room with ID SAP

inoculated animals to test the possibility of passive animal to animal virus transfer. The third group was inoculated with PBS as a control. At 21 days post inoculation all animals were challenged with WT FMDV. **Table 2** shows the outcome of the experiment. Whereas, the 3 control animals developed FMD by 3 days post challenge, none of the 6 animals inoculated with SAP mutant FMDV developed disease during the course of the experiment (42 days). The contact animal co-housed with the A12-SAP ID inoculated animals also developed disease as the PBS inoculated control group.

These results confirmed that swine inoculated with SAP mutant virus develop a strong protective adaptive immune response. In addition, although preliminary, our results indicated that SAP mutant does not transmit to naïve in contact animals.

Table 2. Clinical outcome and presence of neutralizing antibodies in animals challenged with FMDV A12-IC WT 21 days after FMDV A12-SAP mutant inoculation.

2c.



WT

A third experiment was performed to determine if inoculation with the SAP mutant virus could induce rapid protection. Groups of three swine each were SC vaccinated with $1x10^6$ pfu/animal A12-SAP followed by challenge with A12-virus at different time post-vaccination (2, 4, 7, and 14 dpv). Another group inoculated with PBS was used as control. We decided to use SC vaccination to determine if this route of inoculation would induce the same level of protection as that obtained by ID inoculation. SC vaccination is a practical approach in the field and is commonly used for live vaccines. Control animals developed clinical signs of disease as early as 2 dpc and by 7 dpc they had a maximum lesion score of 14 (**Fig. 3**), with fever starting at 3 dpc. In parallel to clinical signs, control animals showed the presence of virus in serum and nasal swabs with a peak at 3 dpc (**Fig. 3**). However, vaccination with A12-SAP mutant virus conferred full protection, even in the group of animals vaccinated just two days prior to WT virus challenge. Only one animal in the group vaccinated 14 days before challenge, showed one lesion that was first apparent at 5 dpc, and virus in nasal swab but not viremia (**Fig. 3**).

Group	Animal #	Challenge virus[a]	Cinical score[b] (day of onset, score)	Viremia[c] (day of onset, maximum amount, duration)	Nasal shedding[d] (day of onset, maximum amount, duration)[d]	Neutralizing antibodies PRN_{70}[e] 0dpc	7dpc
A12-SAP	52	A12-IC WT	Neg[f]	Neg	Neg.	2.1	3.6
$1x10^6$pfu/animal	53		Neg	Neg.	Neg.	0.9	3.6
SC in neck	54		Neg	Neg.	Neg.	0.9	2.1
A12-SAP	55	A12-IC WT	Neg	Neg.	Neg.	2.1	3.9
$1x10^6$pfu/animal	56		Neg	Neg.	Neg.	1.5	3.6
ID in heel bulb.	57		Neg	Neg.	Neg.	2.1	3.0
Naïve contact animal	58		2,15	2, $4.0x10^7$,2	4, $2.5x10^2$,3	0	3.3
PBS	59	A12-IC WT	4,12	3, $1.4x10^4$,2	2, $2.5x10^2$,3	0	3.0
SC in neck	60		3,15	3, $8.7x10^4$,2	2, $5.0x10^2$,3	0	3.0
	61		2,10	Neg	2, $1.5x10^3$,3	0	2.4

[a] Challenge was performed at 21 days post vaccination with FMDV A12-IC wild type (WT), $1.0x10^5$ pfu/ animal.
[b] Clinical score is described as time after challenge when lesions were first detected and maximum score observed. Score refers to number of lesions in limbs and snout (Maximum possible score is 17 lesions/animal (4 lesions/limb and 1 lesion in snout).
[c] Viremia represents virus PFU per ml of serum. Viremia is reported as the day post challenge when it was first detected (onset), the maximum detected titer and the duration in days.
[d] Nasal shedding represents virus PFU per ml of nasal secretion. Values are as defined in footnote c.
[e] The neutralizing antibodies is reported as the serum dilution yielding a 70% reduction in the numer of plaques (PRN_{70}).
[f] Neg., negative (less than 5 PFU/ml).

Figure 3: Clinical outcome of animals vaccinated with attenuated A12-SAP, after FMDV A12-WT challenge. Groups of three pigs were SC vaccinated with A12-SAP (10^6pfu/animal) and ID challenged at different times post-vaccination (2, 4, 7 and 14 dpv) with $5x10^5$pfu/animal of A12-WT and clinical signs (bars), the presence of virus in serum (solid lines) and nasal swabs (dashed lines) were monitored daily during various dpc. Clinical score and virus levels are expressed as described in figure 1 legend. The error bars represent the variation within the three animals from each group.

We also evaluated the titers of neutralizing antibodies and stimulation of CD8+ T cells in all vaccinated groups before and after challenge. With the exception of the group challenged at 2 dpv, all the other vaccinated animals (4, 7 and 14 dpv) had detectable levels of neutralizing antibodies in serum prior to challenge (**Fig. 4**). All but one animal belonging to the 14 dpv group were completely protected against disease. Moreover, by 4 dpc animals challenged at 2 dpv showed equivalent levels of neutralizing antibodies as the control group, even when viremia or virus in nasal swabs was never detected (**Fig. 4**). As expected, FMDV challenge acted as a boost in all groups which showed higher neutralizing antibodies by 4 dpc.

A12-

day

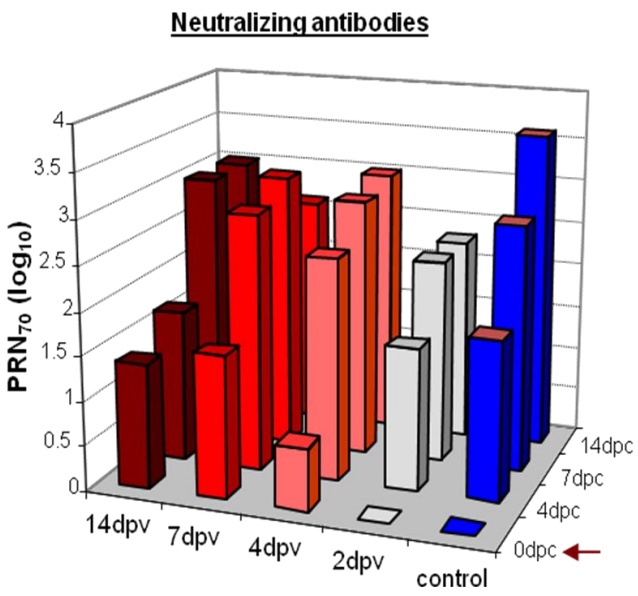


Figure 4: Serum neutralization titers of vaccinated and control animals at the day of challenge and up to 14 dpc. The neutralizing antibodies of swine vaccinated at different time points (2, 4, 7 and 14 dpv) with attenuated SAP (106pfu/animal) were measured at the of challenge (arrow) with A12-WT (5x105pfu/animal), and at 4, 7 and 14 dpc. Titers are expressed as described in figure 2 legend.

Analysis of a specific T cell response revealed a modest induction at 7 and 14 dpv. As shown in **Fig. 5**, before the challenge, there were statistically significant differences in the levels of CD8+ T IFN-γ producing cells at 14 dpv ($P < 0.05$). After the challenge, control animals developed specific cellular mediated immunity detected between 4 and 7 dpc. Interestingly, at 4 dpc vaccinated animals showed a clear increase in the number of IFN-γ producing CD8+ T cells as compared to the day of challenge and to control animals ($P < 0.01$), showing the rapid capacity of response in primed individuals as compared with control animals.

Taken together, these results suggested that inoculation with SAP mutant virus induced innate as well as adaptive protective immune responses highlighting its potential for being developed as a live attenuated vaccine.

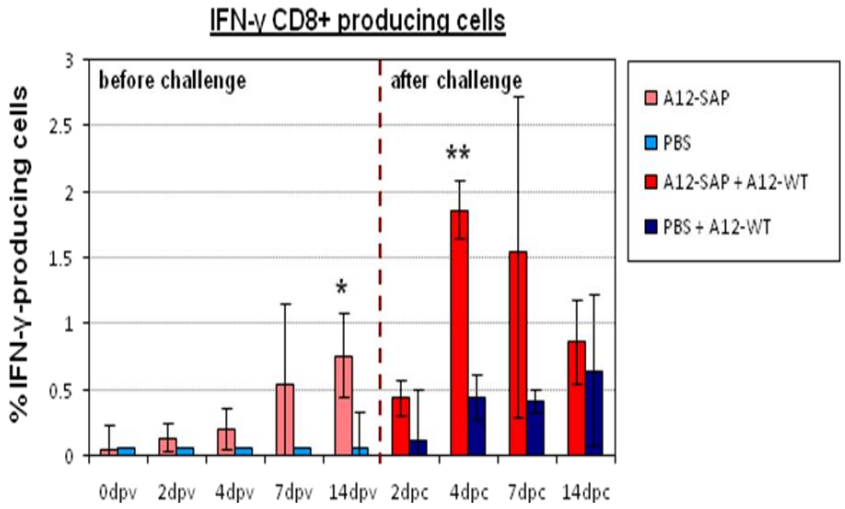


Figure 5: Cellular mediated immunity induced by A12-SAP vaccination. Specific cellular response was measured by intracellular cytokine staining (ICCS). PBMCs from A12-SAP-vaccinated and control animals were extracted at different times before (dpv) and after challenge (dpc) with A12-WT, stimulated with homologous FMDV A12-WT and the capacity of CD8+ T cells to produce IFN-γ was evaluated by ICCS. The percentage of CD8+ T cells that produce IFN-γ is shown. Vertical dashed line separates the data between vaccination and challenge. The error bars represent the variation within the three animals from each group ($P < 0.05$[*], $P < 0.01$[**]).

3. Pathogenesis studies.

3a. Previous in vitro studies demonstrated that FMDV Lpro antagonizes the innate immune response by limiting the expression of IFN and ISGs. Furthermore, WT infection induces production of the anti-inflammatory cytokine IL-10 impairing T-cell proliferation. These data suggest that Lpro might play a role in the induction of an anti-inflammatory state, thereby impairing rapid virus clearance. To better understand the interactions of FMDV with the host we analyzed the expression of pro- and anti-inflammatory cytokine protein levels in the sera of animals inoculated with A12-WT and A12-SAP for four days after infection. We analyzed the expression of IFN and anti-inflammatory (IL-10) or pro-inflammatory (TNF-α, IL-6 and IL-1β) cytokines in the sera of all animals of the experiment described in 2 c. Higher induction of IFN was detected in the SAP as compared to the WT inoculated animals, although no statistically significant increases could be determined (**Fig. 6**). With respect to IL-10

serum protein levels all animals showed an increase by 2 dpi and this difference was statistically significant ($P < 0.05$). Interestingly, the serum levels of the cytokines IL-1β, IL-6 and TNF-α dropped by 2 dpi only in the animals inoculated with FMDV A12-WT, independently of the inoculation dose and coinciding with the peak of viremia. The difference at 2 dpi was statistically significant when compared to the levels observed at day 0 ($P < 0.01$). In contrast, two of the three groups inoculated with A12-SAP ($1x10^5$ and 10^6pfu/animal) showed an increase in the levels of IL-1β and IL-6 as compared to 0 dpi and the differences with respect to the groups inoculated with A12 WT, were also statistically significant by 2 dpi ($P < 0.01$). The relative levels of TNF-α were statistically significantly higher ($P < 0.01$) for the three A12-SAP groups when compared at 2 dpi to the A12-WT inoculated groups.

These results suggested that FMDV Lpro is involved in reducing the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in swine.

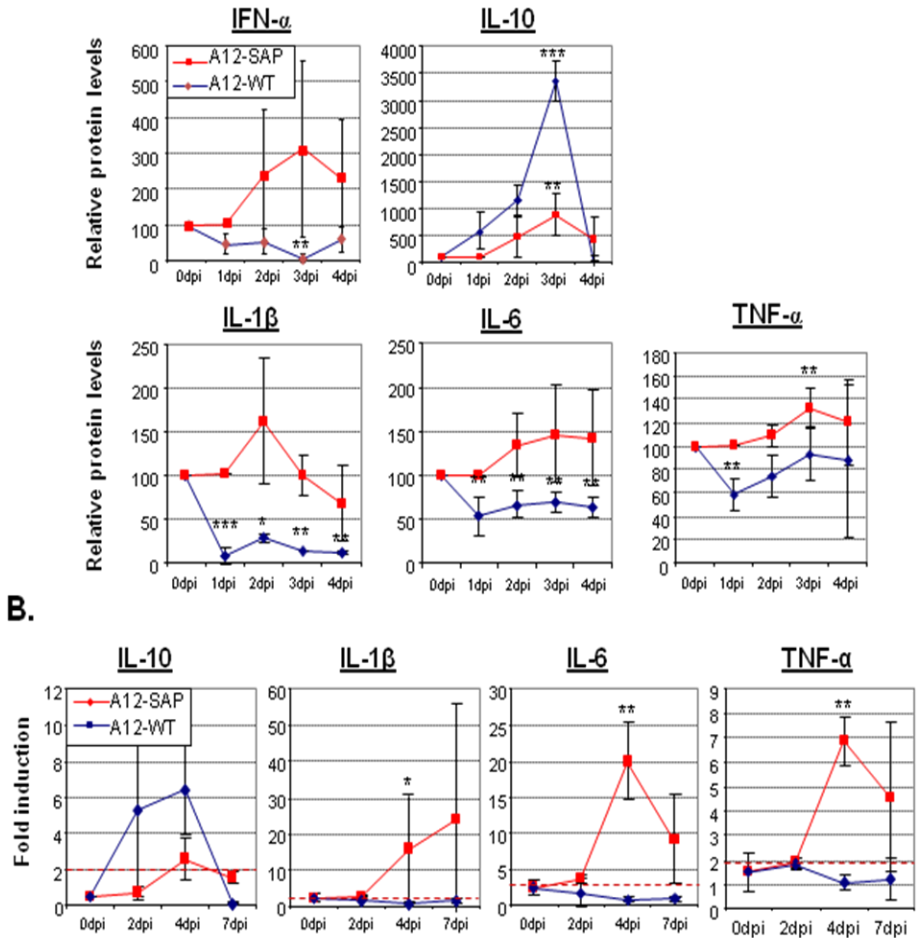


Figure 6: Cytokine profile in animals inoculated with FMDV A12-SAP or FMDV A12-WT.Pro and anti-inflammatory cytokines were detected in serum by ELISA (A) or in PBMCs by real Time rRT-PCR (B). A: levels of IFN-α, IL-10, IL-1β, IL-6 and TNF-α are expressed relative to the amount detected at day 0. B: Relative mRNA levels of IL-10, IL-1β, IL-6 and TNF-α were determined by comparative cycle threshold analysis utilizing as a reference the samples at 0 dpi. Only values ≥ 2 are considered up-regulated. The error bars represent the variation within the three animals from each group ($P < 0.05$[*], $P < 0.01$[**], $P < 0.001$[***]).

3c. As mentioned above analyses of various cytokines in the sera of infected animals showed a distinct pattern of expression depending on the virus used for inoculation. In general and with the exception of IL10, the levels of systemic cytokines were higher in animals inoculated with FMDV A12-SAP than in animals inoculated with A12-WT. Interestingly strong protective innate and adaptive immune responses were mounted in all the animal groups despite the fact that A12-SAP inoculated animals did not develop disease or viremia. In order to determine if the inhibition or induction of proinflammatory cytokines detected in serum could derive from circulating PBMCs in response to virus infection, isolated PBMCs were exposed to WT and SAP mutant virus in vitro. Consistent with the results observed in vivo, infection with WT virus inhibited the expression of TNF-α, IL1-β and IL-6 (**Fig. 7**). No significant effect was detected in the levels of IFN although a modest increase was detected in IL10 in the cells treated with the SAP mutant virus.

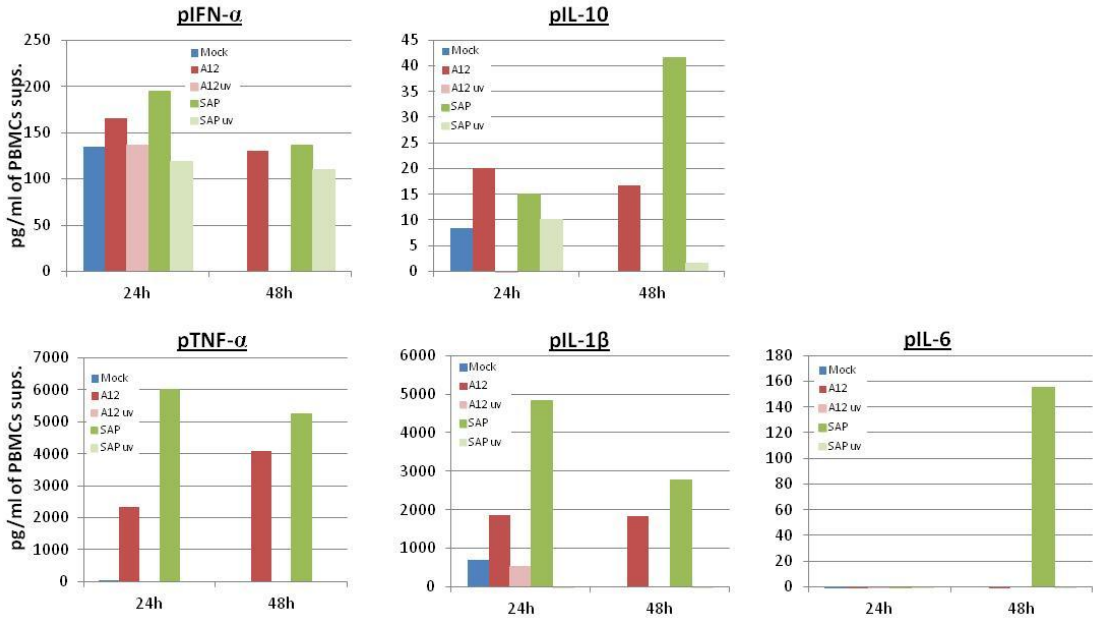


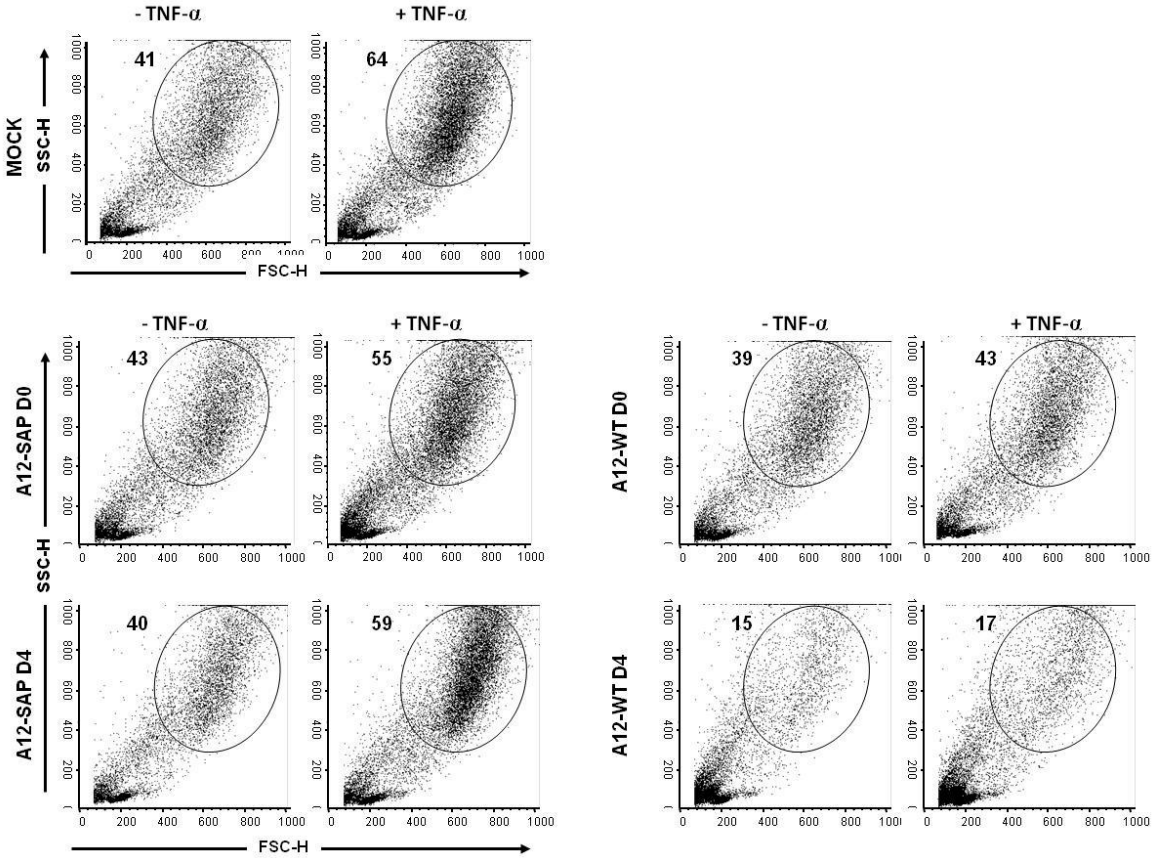
Figure 7. Live A12-SAP mutant virus induces production of pro-inflammatory cytokines in PBMCs. PBMCs from naïve pigs were extracted and infected with wt FMDV (A12-WT)or SAP mutant virus (A12-SAP) at MOI=10 for 24 to 48 hours. PBMCs were also exposed to UV inactivated WT or SAP mutant viruses and a non-infected cell control (Mock) was used. After incubation, supernatant of infected /treated cells were harvested to determine the levels of porcine cytokines by ELISA.

We also determine the effect of virus infection in moDC cell development. moDCs at different stages of maturation were exposed to WT or SAP mutant virus. We initially studied by flow cytometry the profile of forward scatter (FSC) and side scatter (SSC) in order to determine cell size and granularity evolution in the process of maturation. Uninfected (Mock) cultures underwent a dramatic increase in size and granularity after TNF-α treatment, according to mature MoDC phenotype (**Fig. 8A**).

Likewise, CD172+ cells either at D0 or D4 0f maturation infected with FMDV SAP mutant showed similar maturation pattern. In contrast, when cells were infected with FMDV WT (A12-WT), either at D0 or D4, the FACS analyses did not show an increase in size and granularity after adding TNF-α (**Fig. 8A**). Furthermore, expression of SLA II, a cellular marker for moDCs maturation,did increase on Mock and SAP mutant infected cells after adding TNF-α, although WT infected moDCs did not show this increase (**Fig. 8B**). However no differences in the expression of co-stimulatory molecule CD80/CD86 was detected between the different infected cells (data not shown).

These results indicated that infection with WT virus affected moDCs development and maturation.

A.



B.

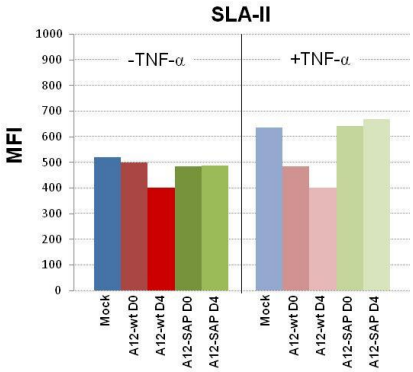


Figure 8. **A.** WT FMDV, but not FMDV-SAP mutant, interferes with ex vivo MoDC development. Dot plots show forward scatter (FSC-H) and side scatter (SSC-H) for uninfected (Mock), FMDV-SAP-infected (A12-SAP) at D0 or D4 and FMDV-WT-infected (A12-WT) at D0 and D4 before TNF-α stimulation (-TNF-α) and upon TNF-α treatment (+TNF-α). Note the lack of size increased in FMDV-infected MoDC after TNF-α at either D0 or D4 compared with uninfected control cells. It is shown the population with MoDCs phenotype in a circle and the percentage of this population is indicated. This is the results from a representative experiment (n = 4). **B.** The mean fluorescence intensity (MFI) of surface molecule SLA-II expressed on MoDCs. Each bar represents the MFI of SLA class II on uninfected (Mock), FMDV-SAP-infected (A12-SAP) at D0 or D4 and FMDV-WT-infected (A12-WT) at D0 and D4 before and after TNF-α addition.

4. Construction of new FMDV Lpro mutants. Based on the predictive structure of Lpro, a mutant in the FHA domain was constructed using the infectious FMDV clone A12-IC (A12-H*) and a commercially available site directed mutagenesis kit. Mutant viruses have been obtained and amplified in tissue culture to high titers for further characterization (**Fig. 9**).

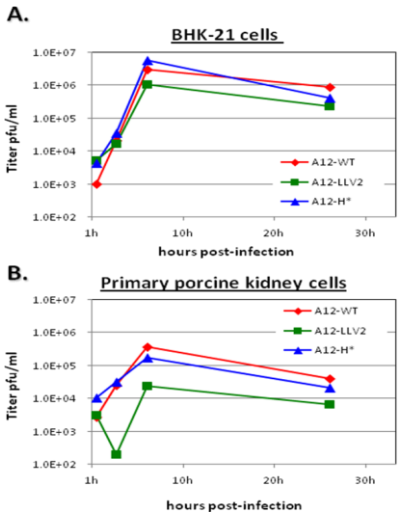


Figure 9. Kinetics of A12-H* mutant. Growth curves on BHK-21 (**A**) or primary porcine kidney (**B**) cells. Cells were infected with indicated viruses wild type [**A12-WT**], leaderless [**A12-LLV2**] or Lpro FHA mutant [**A12-H***] FMDV at MOI=10 and after 1h, unabsorbed virus was removed by washing with 150mM NaCl, 20mM MES (pH=6.0) followed by addition of complete media. Samples were taken at 1, 3, 6 and 24hpi and virus titers were determined by plaque assay on BHK-21 cells.

These viruses will be characterized in the future and combined mutations will be included in new FMDV strains for vaccine development.

VIII. Discussion

The FMDV NS protein Lpro plays a key role in antagonizing the innate immune response. We have previously reported that FMDV lacking Lpro is attenuated in swine and cattle; however this virus is unable to completely protect animals against challenge with virulent FMDV. Recently we have constructed an FMDV mutant containing amino-acid substitutions in a conserved domain of the Lpro coding region, A12-SAP, which is attenuated *in vitro*. Here we show that A12-SAP mutant FMDV is also attenuated *in vivo*. Remarkably, mutation of just two amino acid residues contained within the Lpro SAP domain prevented virus spread and disease, but was sufficient to induce complete protection against WT challenge. We show that inoculation with FMDV A12-SAP mutant induces humoral and cellular immunity equivalent to levels found during infection with WT FMDV. More importantly, our study reveals that while animals inoculated with SAP mutant significantly increase pro-inflammatory cytokines at early times post-inoculation (2-3 dpi), WT FMDV infection results in suppression of this response as reflected

by lower levels of these cytokines in serum. This, in turn, correlates with complete protection against WT FMDV challenge early after vaccination with SAP mutant virus (2 dpv). The most important question arising from these observations relates to the early activation of cytokine networks by the SAP mutant and the integration of these findings into the host capacity to mount an effective anti-FMDV immune response.

Dendritic cells (DCs) are the professional antigen-presenting cells responsible for mounting an effective adaptive immune response. Although FMDV can infect DC precursors *in vitro* interfering with proper maturation, infection *in vivo* is abortive in swine and does not affect the capability of at least skin DCs and monocyte derived DCs (moDCs) to present antigen. Similar to infection with WT FMDV, the high levels of neutralizing antibodies and the induction of cell-mediated immunity in the animals inoculated ID with A12-SAP, even in the absence of detectable viremia or virus shedding, suggests that local skin DCs might have up-taken antigen or become infected with FMDV, followed by lymphatic migration to the draining lymph nodes, thus eliciting a significant adaptive immune response. It is well characterized that during natural infection FMDV induces a strong neutralizing antibody response that ultimately clears the infection. Recently, a cytotoxic T-cell lymphocyte (CTL) response during natural FMDV infection has been reported. The idea that a good vaccine against FMDV should combine stimulation of both humoral and cellular responses has been considered for a long time and several attempts to include T cell stimulation in FMDV vaccine strategies have been pursued. However, none of the vaccine platforms evaluated up-to-date are able to induce the same immune response as the natural infection. It is expected that use of a live attenuated vaccine platform could better resemble the protection afforded by natural infection. One of the main concerns of attenuated vaccines is the possibility of reversion to wild type, especially for FMDV, given the high error rate of viral RNA replication and its quasispecies nature. Tissue culture passage of SAP mutant virus displayed remarkable stability of the SAP mutation for at least 12 passages (data not shown), suggesting that this mutant could potentially be developed as a live attenuated vaccine candidate. Inclusion of DIVA markers and additional mutations that stabilize the attenuated phenotype decreasing the probability of reversion to WT should be considered.

The transcription factor NF-κB can be activated by a variety of stimuli, including infection with picornaviruses. Activated NF-κB promotes the expression of over 150 target genes most of which participate in the host immune response and among them there are several cytokines such as TNF-α, IL-1 and IL-6. In the case of FMDV, NF-κB activation and translocation to the nucleus occurs at a relatively early stage of infection, but at later times the p65/RelA subunit of NF-κB disappears from infected cells. Previously, we demonstrated that FMDV Lpro is necessary and sufficient for degradation of p65/RelA and that mutations in the Lpro SAP domain abolished this function. Little is known about the influence of natural FMDV infection on the profile of pro-inflammatory cytokines in vivo. In our study, a consistent decrease in the levels of TNF-α, IL-1 and IL-6 was detected in animals inoculated with FMDV WT concurrently with the peak of viremia. We observed similar effects in swine infected with FMDV serotypes Asia 1 and O1 Manisa (data not shown). Some viruses, such as cytomegalovirus or more specifically, some viral proteins such as poliovirus 3A can inhibit the secretion of IL-1 or IL-6. However, animals inoculated with SAP mutant virus showed significant induction of TNF-α and maintained the levels of IL-1 and IL-6 in contrast to inoculation with FMDV WT. These cytokines play an important role in the acute inflammatory response to infection and in tissue repair. Several roles involved in the regulation of the adaptive immune response have also been described for these cytokines. For FMDV, TNF-α and IL-6 have been reported as molecular adjuvants involved in the maturation of DCs. It is possible that increased expression of TNF-α, results from the inability of A12-SAP to cause degradation of NF-κB that ultimately induces an innate immune response sufficient to neutralize the virus, preventing the appearance of disease while improving the development of the adaptive immune response. Furthermore, IL-1 has been demonstrated to have antiviral activity against RNA viruses including vesicular stomatitis virus (VSV). It is possible that IL-1 and IL-6 play a role in controlling FMDV replication and spread in vivo.

Another important molecule modulated by NF-κB that could be involved in early protection against challenge is IFN. It has been demonstrated that FMDV is sensitive to the action of IFN. We have previously demonstrated that, *in vitro*, WT FMDV interferes with full induction of transcription of IFN-β. However, *in vivo*, IFN-α mRNA or protein have been detected in WT FMDV infected bovine and swine. We did not detect significant differences in the amount of IFN-α protein in the serum of animals inoculated with A12-WT or A12-SAP. Since there are 17 different types of IFN-α in pigs, it is possible that differences in other IFN-α subtypes might exist but were not detected. Alternatively, differences in the IFN levels might only be detectable in specific tissues, correlating with the number of virus particles present at the specific site of infection. Nevertheless, we have previously observed that a relatively high amount of IFN (≥ 1000 pg/ml serum) induces protection against FMDV but, in some cases, protection has been observed even when no systemic IFN was detected. Therefore at this point there is no evidence that the levels of systemic IFN induced by infection with different strains of FMDV plays a clear role in pathogenesis.

The other cytokine analyzed in our study, IL-10, showed an increase in both, animals inoculated with WT or with SAP mutant viruses. It has been demonstrated that FMDV infection causes the induction of IL-10, a molecule that modulates DC function early after infection, possibly favoring a Th2 cell/cytokine-like environment thus inducing FMDV specific neutralizing antibodies. Infection of animals with SAP mutant triggers the expression of pro-inflammatory cytokines (IL-1, TNF-α, IL-6) presumably through stronger activation of the NF-κB pathway. Therefore, IL-10 expression may blunt the pro-inflammatory cytokine response to avoid an exaggerated cytokine production that could lead to inflammation-mediated disease. Our results expand the concept that IL-10 is a key regulatory cytokine.

Our preliminary pathogenesis studies analyzing the cytokine profile of isolated PBMCs exposed to WT and SAP mutant supported these hypotheses.

In summary, our results suggest that FMDV Lpro plays a pivotal role in modulating the innate and adaptive immune response to viral infection affecting multiple overlapping pathways. Manipulation of the Lpro coding region has allowed us to derive a viable attenuated mutant virus that used as a vaccine was able to induce complete protection from challenge as early as 2 days post vaccination. This observation highlights the potential of using live attenuated vaccine candidates to fight FMDV and deserves further consideration. Moreover, a comprehensive study of viral pathogenesis with WT and FMDV Lpro mutant strains should help to better understand virus-host interactions and hopefully facilitate the development of improved FMD countermeasures.