Foot-and-mouth disease virus (FMDV) leader protease (L\textsuperscript{pro}) 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 L\textsuperscript{pro} is also associated with degradation of nuclear factor \( \kappa \)B (NF-\( \kappa \)B), a process that requires L\textsuperscript{pro} nuclear localization. Additionally, we reported that disruption of a conserved protein domain within the L\textsuperscript{pro} coding sequence, SAP mutation, prevented L\textsuperscript{pro} nuclear retention and degradation of NF-\( \kappa \)B, resulting in \textit{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 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 days postinoculation and for at least 21 days postinoculation. Early protection correlated with a distinct pattern in the serum levels of proinflammatory cytokines in comparison to the levels detected in 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 L\textsuperscript{pro} plays a pivotal role in modulating several pathways of the immune response. Furthermore, manipulation of the L\textsuperscript{pro} coding region may serve as a viable strategy to derive live attenuated strains with potential for development as effective vaccines against foot-and-mouth disease.

Foot-and-mouth disease (FMD) is one of the most contagious diseases of livestock animals. The etiologic agent, FMD virus (FMDV), infects cloven-hoofed animals, including cattle and swine, causing a devastating disease that can significantly impact the economy of affected countries (33). The virus is the prototype member of the\textit{ Aphthovirus} genus of the\textit{ Picornaviridae} family and consists of a positive-sense single-stranded RNA genome of about 8,000 nucleotides surrounded by an icosahedral capsid containing 60 copies each of four structural proteins. Upon infection, the viral RNA is translated as a single polyprotein which is consequently processed by three virus-encoded proteins, leader (L\textsuperscript{pro}), 2A, and 3C\textsuperscript{pro}, into precursors and mature structural (VP1, VP2, VP3, and VP4) and nonstructural (NS) (L\textsuperscript{pro}, 2A, 2B, 2C, 3A, 3B\textsubscript{1,2,3}, 3C\textsuperscript{pro}, and 3D\textsuperscript{pol}) proteins (67).

Control of FMD is achieved by vaccination, inhibition of movement of susceptible animals, slaughter of infected and FMD-susceptible contact animals, and decontamination. The current commercial FMD vaccine, a chemically inactivated whole-virus preparation emulsified with adjuvant, is most commonly used in enzootic areas, and it has been very successful in reducing the number of outbreaks worldwide (33). However, this vaccine platform has some deficiencies: (i) the vaccine manufacturing requires a biosafety level 3 (BSL3) containment facility, (ii) unless highly purified, the vaccine does not allow differentiation between infected and vaccinated animals (DIVAs), (iii) there is a potential risk of developing asymptomatic disease carriers upon exposure of vaccinated animals to infectious virus, and (iv) affected countries need more time to regain FMD-free status and resume trading if vaccination rather than slaughter is used. To address some of the disadvantages of the inactivated vaccine, we have developed a new approach using a replication-defective adenovirus subunit vaccine expressing empty viral capsids that has been very successful in swine and cattle (36, 51, 63). Nevertheless, both the inactivated and the subunit vaccines require approximately 7 days to induce protection. It has been reported that rapid and long-lasting protection against viral infection is usually best achieved by vaccination with attenuated viral vaccines. Indeed, some viral diseases, including smallpox and rinderpest, have been eradicated using such vaccines (30, 56). So far, no attenuated vaccine has been successfully used against FMDV. Among others, a candidate attenuated vaccine was previously developed by deletion of the NS viral L\textsuperscript{pro} coding region (leaderless virus) (64). Despite the reduced pathogenicity of this virus in swine and cattle, vaccinated animals were not completely protected against homologous wild-type (WT) virus challenge, probably due to the slow and limited viral replication of the mutant strain.

FMDV has evolved several mechanisms to evade the host immune response, and L\textsuperscript{pro} plays a central role in pathogenesis (35). L\textsuperscript{pro} is a papain-like proteinase that autocatalytically removes itself from the growing polypeptide chain (74) and cleaves the host translation initiation factor eIF4G, resulting in the shutoff of host mRNA translation (22), a characteristic of most picornavirus infections (29). As mentioned above, it has been demonstrated that a virus lacking the L\textsuperscript{pro} coding region, leaderless virus, is highly attenuated in cattle and swine (12, 48, 64). Apparently, the reason for this attenuation is the inability of the virus to block type I interferon (alpha/beta interferon [IFN-\( \alpha/\beta \)])
transcription (14) and transcription of IFN-β (19), similar to other picornaviruses (5, 18, 46). Inhibition of IFN-β transcription is associated with L\(^{\text{Pmo}}\) translation to the nucleus of the infected cell and subsequent degradation of p65/RelA, a subunit of transcription factor nuclear factor kappa B (NF-κB) (20). We have recently identified a conserved protein domain within the L\(^{\text{Pmo}}\) coding region known as the \(\text{SAF-A/B, gcinus, and PIAS (SAP)}\) domain (21). SAP domains which are present in some DNA binding proteins usually involved in transcriptional control mediate protein-protein interactions between activators and repressors (1, 41). Mutations of two conserved amino acid residues in the SAP domain of FMDV L\(^{\text{Pmo}}\) altered the protein subcellular localization during the course of infection, making the virus (SAP-mutant virus [A12-SAP]) unable to induce degradation of NF-κB and thus resulting in upregulation of expression of several cytokines, chemokines, and interferon-stimulated genes (ISGs) (21).

Upon viral infection, NF-κB translocates to the nucleus of the cell, where it binds to its cognate promoter sites to activate transcription of an array of genes, including proinflammatory cytokines, chemokines, and adhesion molecules, most of them involved in the natural response against pathogens (50). Apart from a role in the adaptive immune response, NF-κB also has a very important function in innate immunity by the activation of the Janus kinase (JAK)/signal transducer and activators of transcription (STAT) antiviral pathway (39). Furthermore, proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), directly induced by NF-κB, are also involved in the establishment of an antiviral state against RNA viruses (7).

Inflammatory cytokines are divided into two major groups, depending on their action in the organism: proinflammatory cytokines (IL-1, IL-6, TNF-α, and IFNs) and anti-inflammatory cytokines (IL-10). A dynamic balance exists between pro- and anti-inflammatory components (57) that could be disrupted upon viral infection. Previous studies have shown that during acute FMDV infection in swine, the virus induces an immunosuppressive stage, characterized by T cell unresponsiveness and transient lymphopenia affecting all T cell subsets and correlating with the appearance of viremia (3, 26). One possible mechanism by which the virus induces immunosuppression might be related to the production of IL-10 (25), an immunosuppressive cytokine that plays an important stimulatory role in the function of B lymphocytes and the production of antibodies by B1 lymphocytes (2). In addition, FMDV interferes with expression of type I IFNs, which are important for activation of the proinflammatory response in combination with virus-induced lymphopenia may play an important role in allowing successful FMDV infection and spread within the host.

**MATERIALS AND METHODS**

**Cells and viruses.** Porcine kidney (IBRS-2) cell lines were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center. 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 nonessential amino acids. BHK-21 cells (baby hamster kidney cells, strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC; Manassas, VA), 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 nonessential amino acids. Cell cultures were incubated at 37°C in 5% CO\(_2\).

FMDV A12-WT was generated from the full-length serotype A12 infectious clone pRMC55 (66). A12-SAP-mutant virus, a derivative of A12-WT containing mutations I55A and L58A in the L\(^{\text{Pmo}}\) region, was constructed by site-directed mutagenesis (21). All viruses were propagated in BHK-21 cells, concentrated by polyethylene glycol precipitation, and stored at −70°C. 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 sequence detection system (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, 15 Yorkshire gilts (age, 5 weeks; weight, approximately 40 lb each) were divided into 5 groups of 3 animals each. Animals were inoculated i.d. in the heel bulb of the right hind foot with different doses of FMDV A12-WT (1 × 10\(^5\) or 1 × 10\(^6\) PFU/animal) or A12-SAP (1 × 10\(^5\), 1 × 10\(^6\), or 1 × 10\(^7\) 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 14 and 21 days 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 1 × 10\(^5\) PFU/pig of FMDV A12-WT i.d. 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). In a second experiment, 4 groups of 3 Yorkshire gilts each (age, 5 weeks; weight, approximately 40 lb) were subcutaneously (s.c.) vaccinated with attenuated FMDV A12-SAP (1 × 10\(^6\) PFU/animal) followed by challenge at different times postvaccination (2, 4, 7, and 14 days postvaccination [dpi]) with 5 × 10\(^4\) PFU/animal of virulent FMDV A12-WT i.d. in the heel bulb. One extra group of 3 pigs was inoculated with phosphate-buffered saline (PBS) and challenged 14 days later (control group). Serum samples were collected at 2, 4, 7, and 14 dpi. After the challenge, clinical signs were 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 to 70). Serial 10-fold dilutions of the samples were protected when challenged as early as 2 days after vaccination, and animals inoculated with A12-SAP were completely protected with 100% protection when challenged with A12-WT virus 21 days after inoculation. Interestingly, inoculated animals were also completely protected when challenged as early as 2 days after vaccination, a time when the adaptive immune response could not be detected.
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 37°C 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/ml of serum or nasal swab. The detection level of this assay is 5 PFU/ml.

Detection of FMDV RNA by rRT-PCR.  At 1 to 7 dpc, frozen serum samples from animals that had no detectable clinical disease were thawed and processed for RNA extraction and measurement of specific FMDV RNA by real-time reverse transcription-PCR (rRT-PCR) as previously described (58). Samples were considered positive when threshold cycle (Cₚ) values were <40.

Determination of neutralizing antibody titer.  Serum 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.

RIP of [³⁵S]methionine/[³⁸S]cysteine-labeled FMDV A12-infected cell lysates with swine serum samples.  Radiolabeled lysates of FMDV A12-infected BHK-21 cells were incubated with individual swine serum samples from 0 and 21 dpc and examined for the presence of antibodies specific to FMDV structural and NS polypeptides by radioimmunoprecipitation (RIP) (34). Convalvescent-phase serum from an 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 isotopes by enzyme-linked immunosorbent assay (ELISA).  The presence of FMDV-specific immunoglobulin M (IgM), IgG1, and IgG2 antibodies was detected by an indirect double antibody sandwich assay as described previously (17), with some modifications. Briefly, Costar enzyme immunoassay/radioimmunoassay 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 (17) 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 2 or 7 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 preimmune sera from the same animals.

Analysis of cytokines in serum.  IFN-α, IL-1β, IL-6, IL-10, and TNF-α protein concentrations in sera from infected animals were determined using an ELISA. IFN-α was detected using monoclonal antibodies (MAbs) K9 and F17 (PBL Interferon Source, Piscataway, NJ) as previously described (52). IL-10, CytoSet ELISA (Biosource-Invitrogen, Carlsbad, CA) and IL-1, primers 138FW (5’ TGGCCCTTGGAGCATCA) and 405RW (5’ CGGGTTATCTGAGGTTGAGA AAA) and probe FAM-CAACAGGCTGCCCAATG; and for TNF-α, primers 338FW (5’ TGGCCCCTTGGAGCATCA) and 405RW (5’ CGGGTTATCTGAGGTTGAGA AAA) and probe FAM-CAACAGGCTGCCCAATG; and for IFN-γ, primers 338FW (5’ TGGCCCCTTGGAGCATCA) and 405RW (5’ CGGGTTATCTGAGGTTGAGA AAA) and probe FAM-CAACAGGCTGCCCAATG. IFN-γ and TNF-α/Cytokine Duo Set ELISAs (R&D Systems, Minneapolis, MN) were performed following the manufacturers’ directions. All ELISAs were developed with 3,3’,5,5’-tetramethylethylenebenzide (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 on the basis of the optical densities obtained with the standards and are expressed in relative levels for each individual at different times postinfection with respect to its own level at day 0.

Detection of cytokines in PBMCs by real-time PCR.  Expression of several cytokines in PBMCs was analyzed. RNA was extracted from purified PBMCs, approximately 10⁷ 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: for IFN-α, primers 236FW (5’ TGGTGCAATGACGTCTCACA) and 290RW (5’ GCCGAGGCCTCT GTGCT) and probe FAM-CAGACCTGGAGCAGT (where FAM is 6-carboxyfluorescein); for IL-1β, primers 737FW (5’ TTGAATTGAGCT

RESULTS

**FMDV SAP mutant is attenuated in swine.** To compare the virulence of the FMDV WT, A12-WT, with that of an FMDV mutant containing mutations in the SAP domain of Lpro (A12-SAP) (21), groups of three pigs were inoculated i.d. in the rear heel bulb with different doses of either FMDV. We inoculated animals with 10⁵ or 10⁶ PFU/animal of A12-WT, doses that we had previously shown caused clinical disease in swine (12), and with 10⁷, 10⁸, and 10⁹ PFU/animal of A12-SAP. In animals inoculated with WT virus, disease was detectable as early as 2 dpc, but only the group inoculated with 10⁶ PFU/animal had temperatures of 40°C or higher. By 7 dpc, all animals inoculated with WT virus showed clinical signs of disease, with no statistically significant differences between the two groups (Fig. 1A). However, all animals inoculated with A12-SAP, even those inoculated with a 10-fold higher dose than WT (10⁷ PFU/animal), never showed clinical signs or elevated temperatures throughout the experiment (Fig. 1A).

Animals inoculated with A12-WT developed viremia on the day prior to (group inoculated with 10⁶ PFU) or concomitantly with (group inoculated with 10⁸ PFU) the appearance of clinical signs (Fig. 1B; *Cₚ* values = 33 to 37) and lymphopenia (data not shown). Interestingly, none of the animals inoculated with A12-
SAP had detectable viremia either by virus isolation (Fig. 1B) or by rRT-PCR ($C_T$ values $\geq 40$ [58]), nor did they develop lymphopenia. In parallel to viremia, animals inoculated with WT virus had detectable virus in nasal swabs starting at 2 to 3 dpi, and only one out of three animals inoculated with $10^7$ PFU of A12-SAP showed virus with a very low titer ($\leq 10$ PFU/ml) in nasal swabs at 5 dpi (Fig. 1B). These data indicate that A12-SAP FMDV displays significantly reduced virulence in swine compared to A12-WT.

FMDV A12-SAP and A12-WT elicit equivalent adaptive immune responses. It has previously been demonstrated that animals inoculated with an attenuated strain of FMDV lacking Lpro (leaderless FMDV) developed significant antibody titers against viral proteins in serum after 14 dpi (12, 48). In the current experiment, we observed that despite the absence of viremia, all the animals inoculated with A12-SAP developed significant levels of FMDV-specific neutralizing antibodies starting at 7 dpi, with a peak occurring at 14 dpi (Fig. 1B). These data indicate that A12-SAP FMDV displays significantly reduced virulence in swine compared to A12-WT.

In order to characterize the FMDV antibody response, the specific Ig isotype present in swine sera after inoculation was determined. The presence of IgM was detected by 7 dpi in all inoculated animals, and the level of IgM 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 that of WT virus, but does not cause vesicular lesions, viremia, or fever.

Animals inoculated with FMDV A12-SAP are completely protected when challenged with FMDV WT. In our previous studies, animals inoculated with the attenuated leaderless virus never showed clinical signs but were only partially protected when challenged with WT FMDV (12, 48). To test whether or not the animals inoculated with the FMDV SAP mutant were protected against FMD, we challenged the three groups of A12-SAP-inoculated swine with A12-WT ($10^5$ PFU/animal i.d. in the heel of the ear).
At 21 days. All challenged animals were protected, and none of the animals showed clinical signs (fever or vesicles) or the presence of virus in blood or nasal secretions (Table 1). As expected, the challenge acted as a boost, and the animals had increased neutralizing antibody titers by 7 dpc (Table 1).

FMDV Lpro is involved in reducing the expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α in swine. Previous in vitro studies demonstrated that FMDV Lpro antagonizes the innate immune response by limiting the expression of IFN and ISGs (11, 14, 19, 20, 74). Furthermore, WT infection induces production of the anti-inflammatory cytokine IL-10, impairing T-cell proliferation (25). These data suggest that Lpro might play a role in the induction of an anti-inflammatory state, thereby impairing rapid virus clearance. In order to test this hypothesis, 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 4 days after infection. Figures 3 and 4 show the levels of IFN-α, IL-10, IL-1β, IL-6, and TNF-α in the serum of A12-WT- or A12-SAP-inoculated swine. Relative values were plotted with respect to the basal levels of each cytokine at 0 dpi. In the case of IFN-α, animals infected with the highest dose of A12-WT showed a slight increase peaking at 2 dpi, although high variation within the group was observed. Similarly, there were not statistically significant differences in the groups inoculated with A12-SAP-mutant virus (Fig. 3A). In contrast, pigs inoculated with A12-WT or A12-SAP showed an increase of the anti-inflammatory cytokine IL-10, with a peak at 2 dpi (Fig. 3B). Although the variation of the levels of expression between individuals was high, all the animals had increased expression of IL-10 by 2 dpi compared with the levels observed at 0 dpi, and this difference was statistically signifi-
significantly ($P < 0.05$). Interestingly, the serum levels of the cytokines IL-1β, IL-6, and TNF-α dropped by 2 dpi only in the animals inoculated with A12-WT, independently of the inoculation dose and coinciding with the peak of viremia (Fig. 4). The difference at 2 dpi was statistically significant compared to the levels observed at day 0 ($P < 0.01$). In contrast, two of the three groups inoculated with A12-SAP ($1 \times 10^5$ and $1 \times 10^6$ PFU/animal) showed an increase in the levels of IL-1β and IL-6 compared to the levels observed at 0 dpi, and the differences with respect to the groups inoculated with A12-WT were also statistically significant by 2 dpi ($P < 0.01$) (Fig. 4). Surprisingly, this effect was not seen in the group of swine inoculated with the highest dose of A12-SAP ($1 \times 10^7$ PFU/animal). In the case of TNF-α, all A12-SAP-inoculated animals showed an increase by 2 to 3 dpi that was statistically significant ($P < 0.01$) for the low- and medium-dose groups ($1 \times 10^5$ and $1 \times 10^6$ PFU/animal, respectively). At 2 dpi, the relative levels of TNF-α were statistically significantly higher ($P < 0.01$) for the three A12-SAP groups than the A12-WT-inoculated groups (Fig. 4C).

**Vaccination with attenuated FMDV A12-SAP confers protection against challenge with FMDV A12-WT as early as 2 dpv.**

Inoculation of animals with live attenuated viral vaccines can induce early and long-lasting protection. As mentioned above, the FMDV SAP mutant not only was attenuated in vivo but also induced a robust adaptive immune response that conferred protection at 21 dpi. In order to determine if inoculation with this mutant virus could induce rapid protection, groups of three swine were s.c. vaccinated with $1 \times 10^6$ PFU/animal A12-SAP, followed by challenge with A12-WT virus at different times postvaccination (2, 4, 7, and 14 dpv). Another group inoculated with PBS was used as a control. We decided to use s.c. vaccination to determine if this route of inoculation would induce the same level of protection as that obtained by i.d. inoculation. s.c. 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 dpv, and by 7 dpv they had a maximum lesion score of 14 (Fig. 5), with fever starting at 3 dpv. In parallel to clinical signs, control animals showed the presence of virus in serum and nasal swabs with a peak at 3 dpv (Fig. 5). However, vaccination with A12-SAP-mutant virus conferred full protection, even in the group of animals vaccinated just 2 days prior to WT virus challenge. Only one animal in the group vaccinated 14 days before challenge showed one lesion, which was first apparent at 5 dpv, and virus was detected in nasal swabs but not in blood (Fig. 5).

**FIG 3 Cytokine protein profiles in serum after FMDV infection.** Levels of IFN-α (A) and IL-10 (B) in the serum of animals inoculated with FMDV A12-WT ($10^5$ or $10^6$ PFU/animal) or A12-SAP ($10^5$, $10^6$, or $10^7$ PFU/animal) during the first 4 days after infection were detected by sandwich ELISA. Amount of protein is expressed in relative levels for each individual animal at different times postinfection with respect to its own level at day 0. The gray areas represent time points at which a statistically significant difference from the amount at 0 dpi was observed ($P < 0.05$).

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

<table>
<thead>
<tr>
<th>A12-SAP dose (no. of PFU/animal)</th>
<th>Challenge result</th>
<th>Viremia</th>
<th>Nasal swabs</th>
<th>Neutralizing antibody PRN&lt;sub&gt;70&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal no.</td>
<td>0 dpc</td>
<td>7 dpc</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>90</td>
<td>Neg.</td>
<td>2.4</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>Neg.</td>
<td>3.3</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>Neg.</td>
<td>2.7</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>93</td>
<td>Neg.</td>
<td>3</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>Neg.</td>
<td>2.7</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>Neg.</td>
<td>1.8</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>96</td>
<td>Neg.</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>Neg.</td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>Neg.</td>
<td>2.1</td>
<td>&gt;3.1</td>
</tr>
</tbody>
</table>

*The dose of the A12-WT challenge virus was $1 \times 10^5$ PFU per animal.
*The animals were tested for 7 days after the challenge. Neg., negative (less than 5 PFU/ml).
*The neutralizing antibody titer is reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN<sub>70</sub>).
Detectable levels of humoral or cellular immunity do not directly correlate with early protection. It is widely established that in the primary adaptive immune response, several days are required for the clonal expansion and differentiation of lymphocytes into effector T cells and antibody-secreting B cells. However, it has been reported that different levels of antibodies can develop as early as 3 days after FMDV infection (27). We 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. 6). All but one animal

FIG 4 Cytokine protein profiles in serum after FMDV infection. Levels of IL-1β (A), IL-6 (B), and TNF-α (C) in the serum of animals inoculated with FMDV A12-WT (10^5 or 10^6 PFU/animal) or A12-SAP (10^5, 10^6, or 10^7 PFU/animal) during the first 4 days after infection were detected by sandwich ELISA. Amount of protein is expressed in relative levels for each individual animal at different times postinfection with respect to its own level at day 0. The gray areas represent time points at which a statistically significant difference from the amount at 0 dpi was observed (P < 0.01).
FIG 5 Clinical outcome of animals vaccinated with attenuated A12-SAP after FMDV A12-WT challenge. Groups of three pigs were s.c. vaccinated with A12-SAP (10⁶ PFU/animal) and i.d. challenged at different times post-vaccination (2, 4, 7, and 14 dpv) with 5 × 10⁶ PFU/animal of A12-WT, and clinical signs (bars) and 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 the Fig. 1 legend. The error bars represent the variation within the three animals from each group.

FIG 6 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 A12-SAP (10⁶ PFU/animal) were measured at the day of challenge (arrow) with A12-WT (5 × 10⁶ PFU/animal) and at 4, 7, and 14 dpc. Titers are expressed as described in the Fig. 2 legend.

FIG 7 Cell-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 (dpc) challenge with A12-WT and 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. A 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.

A12-SAP vaccination induced the expression of TNF-α in serum and PBMCs. As expected, animals that were challenged early after vaccination (2 dpv) did not show detectable levels of antibodies or cell-mediated immunity by the time of challenge. On the basis of these observations and results from the first animal experiment in which animals inoculated with WT FMDV showed a decrease in the level of some cytokines in serum 2 days after infection, while those inoculated with the attenuated SAP-mutant virus did not (Fig. 3 and 4), we decided to analyze the systemic levels of some pro- and anti-inflammatory cytokines by ELISA in serum and by real-time RT-PCR in PBMCs (Fig. 8). Control animals challenged with A12-WT showed a statistically significant increase in the levels of IL-10 by 2 to 3 days postinfection and a significant decrease in the levels of IL-1β, IL-6, and TNF-α starting at 1 dpi by ELISA (Fig. 8A). Similar results were observed when we analyzed the relative levels of cytokine mRNAs in PBMCs, in which we could detect an increase only of the relative levels of IL-10 in control A12-WT-infected animals (Fig. 8B). In the case of IFN-α, animals inoculated with A12-WT showed a decrease in the relative levels of protein by 3 dpi compared with the levels at 0 dpi (Fig. 8A), while no variation in the relative levels of mRNA was detected (data not shown). On the other hand, animals vaccinated with the SAP mutant showed a statistically significant increase in the levels of IL-10 by ELISA, as was observed in the first experiment (Fig. 3). For the other cytokines, there was an overall tendency toward increased protein levels at 2 to 3 dpi, but the variation was statistically significant only for TNF-α, which showed a peak at 3 dpi (Fig. 8A). IL-1β, IL-6, and TNF-α were upregulated, as detected by rRT-PCR starting at 2 dpi (Fig. 8B).

DISCUSSION

The FMDV NS protein L^pro plays a key role in antagonizing the innate immune response (35). We have previously reported that FMDV lacking L^pro is attenuated in swine and cattle; however, this virus is unable to completely protect animals against challenge with virulent FMDV (9, 12, 48). Recently, we constructed an FMDV mutant containing amino acid substitutions in a conserved domain of the L^pro coding region, A12-SAP, which is attenuated in vitro (21). 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 the FMDV A12-SAP mutant induces humoral and cellular immunity at levels equivalent to the levels found during infection with WT FMDV. More importantly, our study reveals that while animals inoculated with the SAP mutant significantly increase proinflammatory cytokines at early times postinoculation (2 to 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 (31). Although FMDV can infect DC precursors in vitro, interfering with proper maturation, infection in vivo is abortive in swine (25, 55) and does not affect the capability of at least skin DCs and monocyte-derived DCs (moDCs) to present antigen (25, 55). Similar to infection with WT FMDV, the high levels of neutralizing antibodies and the induction of cell-mediated immunity in the animals inoculated i.d. with A12-SAP, even in the absence of detectable viremia or virus shedding, suggest that local skin DCs might have taken up 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 (16, 69). However, in our vaccine experiment one animal inoculated with the FMDV A12-SAP mutant developed mild disease after challenge with WT virus, despite the presence of significant levels of neutralizing antibodies. We believe that this animal was a nonresponder. Previous studies have shown that protection against FMD does not always rely on the levels of neutralizing antibodies, since resistance to challenge has been observed in animals showing low levels of antibodies and disease has been detected even in the presence of significant antibody titers (49). Recently, a cytotoxic T cell lymphocyte (CTL) response during natural FMDV infection has been reported (37). The idea that a good vaccine against FMDV should combine stimulation of both humoral and cellular responses has been considered for a long time (4), and several attempts to include T cell stimulation in FMDV vaccine strategies have been pursued (6, 32, 38, 62). How-

FIG 8 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 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 of ≥2 are considered upregulated. The error bars represent the variation within the three animals from each group. *, P < 0.05; **, P < 0.01; ****, P < 0.001.
ever, none of the vaccine platforms evaluated 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 offer protection that better resembles 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 (10, 28). 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 markers for DIVAs 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 (20, 60, 61). Activated NF-κB promotes the expression of over 150 target genes, most of which participate in the host immune response (59), and among them there are several cytokines, such as TNF-α, IL-1, and IL-6 (40, 45). In the case of FMDV, NF-κB activation and translocation to the nucleus occur at a relatively early stage of infection, but at later times the p65/RelA subunit of NF-κB disappears from infected cells (20). Previously, we demonstrated that FMDV Lprov is necessary and sufficient for degradation of p65/RelA and that mutations in the Lprov SAP domain abolished this function (20, 21). Little is known about the influence of natural FMDV infection on the profile of proinflammatory cytokines in vivo. Increased mRNA levels of TNF-α and IL-1α have been reported in nasal tissue-associated lymphoid tissue of infected cattle at 7 days after infection and later (73). In our study, a consistent decrease in the levels of TNF-α, IL-1, and IL-6 in blood was detected concurrently with the peak of viremia in animals inoculated with FMDV WT. We observed similar effects in swine infected with FMDV serotypes Asia 1 and O1 Manisa (data not shown). Some viruses, such as cytomegalovirus (42), or, more specifically, some viral proteins, such as paramyxovirus V (47) and poliovirus 3A (15), 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 animals inoculated with FMDV WT. These cytokines play an important role in the acute inflammatory response to infection and in tissue repair (43). 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 to be molecular adjuvants involved in the maturation of DCs (71). It is possible that increased expression of TNF-α results from the inability of A12-SAP to cause degradation of NF-κB, which 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) (68). Although IL-6 does not have any known antiviral activity (47), its involvement in viral pathogenesis of vaccinia virus (44) or herpes simplex virus type 1 (54) has been demonstrated. Similarly, 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 (11, 14, 53). We have previously demonstrated that, in vitro, WT FMDV interferes with full induction of transcription of IFN-β (19). However, in vivo, IFN-α mRNA or protein has been detected in WT FMDV-infected bovine and swine (8, 65, 72). 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 (70), it is possible that differences in other IFN-α subtypes might exist but were not detected. Alternatively, differences in the IFN levels might be detectable only in specific tissues, correlating with the number of virus particles present at the specific site of infection (8). Nevertheless, we have previously observed that a relatively large amount of IFN (≥1,000 pg/ml serum) induces protection against FMDV, but in some cases, protection has been observed even when no systemic IFN was detected (23). Therefore, at this point there is no evidence that the levels of systemic IFN induced by infection with different strains of FMDV play a clear role in pathogenesis.

The other cytokine analyzed in our study, IL-10, showed an increase in both animals inoculated with WT virus and animals inoculated with SAP-mutant virus. 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 (25). Infection of animals with the SAP mutant triggers the expression of proinflammatory cytokines (IL-1, TNF-α, IL-6), presumably through stronger activation of the NF-κB pathway. Therefore, IL-10 expression may blunt the proinflammatory 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.

In summary, our results suggest that FMDV Lprov plays a pivotal role in modulating the innate and adaptive immune response to viral infection, affecting multiple overlapping pathways. Manipulation of the Lprov coding region has allowed us to derive a viable attenuated mutant virus that, when 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 Lprov mutant strains should help to provide a better understanding of virus-host interactions and, it is hoped, facilitate the development of improved FMD countermeasures.

ACKNOWLEDGMENTS

This research was supported in part by the Plum Island Animal Disease Research Participation Program, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Department of Agriculture (appointment of Fayna Diaz-San Segundo, Marcelo Weiss, and Camila C. Dias), by CRIS project number 1940-32000-052-00D, ARS, USDA (Telesa de los Santos and Marvin J. Grubman), and by National Pork Board grant number 11-005 (Telesa de los Santos, Fayna Diaz-San Segundo, and Marvin J. Grubman).

We are thankful to Juan M. Pacheco for helping in assaying the viremia by real-time RT-PCR and Beatriz G. Matias for everyday support in the lab. We also thank the animal care staff at the Plum Island Animal Disease Center for their professional support and assistance. Finally, we thank Noemi Sevilla for helpful discussions, suggestions, and critical reading of the manuscript.
REFERENCES

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