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Evaluation of a Fiber-Modified Adenovirus Vector Vaccine against Foot-and-Mouth Disease in Cattle

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Novel vaccination approaches against foot-and-mouth disease (FMD) include the use of replication-defective human adenovirus type 5 (Ad5) vectors that contain the capsid-encoding regions of FMD virus (FMDV). Ad5 containing serotype A24 capsid sequences (Ad5.A24) has proved to be effective as a vaccine against FMD in livestock species. However, Ad5-vectored FMDV serotype O1 Campos vaccine (Ad5.O1C.2B) provides only partial protection of cattle against homologous challenge. It has been reported that a fiber-modified Ad5 vector expressing Arg-Gly-Asp (RGD) enhances transduction of antigen-presenting cells (APC) in mice. In the current study, we assessed the efficacy of a fiber-modified Ad5 (Adt.O1C.2B.RGD) in cattle. Expression of FMDV capsid proteins was superior in cultured cells infected with the RGD-modified vector. Furthermore, transgene expression of Adt.O1C.2B.RGD was enhanced in cell lines that constitutively express integrin $\alpha_v\beta6$, a known receptor for FMDV. In contrast, capsid expression in cattle-derived enriched APC populations was not enhanced by infection with this vector. Our data showed that vaccination with the two vectors yielded similar levels of protection against FMD in cattle. Although none of the vaccinated animals had detectable viremia, FMDV RNA was detected in serum samples from animals with clinical signs. Interestingly, $CD4^+$ and $CD8^+$ gamma interferon $(IFN\text{-}\gamma)^+$ cell responses were detected at significantly higher levels in animals vaccinated with Adt.O1C.2B.RGD than in animals vaccinated with Ad5.O1C.2B. Our results suggest that inclusion of an RGD motif in the fiber of Ad5-vectored FMD vaccine improves transgene delivery and cell-mediated immunity but does not significantly enhance vaccine performance in cattle.

Foot-and-mouth disease (FMD) is a contagious disease that targets cloven-hoofed animals. The etiological agent, FMD virus (FMDV), is a single-stranded, positive-sense RNA virus of the *Picornaviridae* family (1–3). In infected animals, FMD is manifested by fever, lameness, and the appearance of vesicles in the extremities, snout, and teats, which can rapidly result in deterioration of the overall health of the animals (1, 4). Accidental or intentional introduction of FMDV into areas that are free of the disease can have serious economic consequences (5). Nonvaccination emergency methods for control of the disease include inhibition of movement of susceptible animals, stamping-out procedures, and decontamination (6). Current World Organisation for Animal Health (OIE) regulations allow for the use of emergency vaccines for the control of FMD; however, previously disease-free countries prefer either slaughter or vaccination followed by slaughter of infected or in-contact animals, in order to regain FMD-free status as early as possible.

Vaccines against FMD have classically utilized a chemically inactivated whole-virus preparation in combination with adjuvant (7–9). Although effective, these vaccine formulations have several limitations, including the difficulty of differentiating infected from vaccinated animals (DIVA), the need for high-containment manufacturing facilities, and the potential for release of live virus during the manufacturing process or when a final product that has not been properly inactivated is unintentionally used. Efforts to develop new vaccines have been under way for many years (10–16). Ideally, new vaccines need to be safe, pure, potent, efficacious, cost-effective, and DIVA compliant. Currently, the most successful approach to the development of new FMDV vaccines has been the production of a replication-defective human serotype 5 adenovirus (Ad5) that delivers the capsid coding region of FMDV serotype A (Ad5.A24 or Ad5.A12), including nonstructural (NS) proteins required for capsid precursor protein processing (i.e., 3C protease) (12, 13). This platform has been demonstrated to be highly efficient for delivering antigens to swine and cattle and eliciting protective immune responses against FMDV challenge in an experimental setting (12–15). In fact, Adt.A24 was the first recombinant vaccine granted conditional license for use in cattle by the U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service Center for Veterinary Biologics (17). Nonetheless, given the high antigenic variability of FMDV, which consists of 7 serotypes and multiple strains within each serotype, additional vaccine candidates of different serotypes are being produced and tested for protection in swine and cattle (17).

Ad5 FMDV vaccines are safe and highly pure; however, they are relatively expensive for veterinary use, demanding continuous

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efforts to improve their potency and efficacy. In fact, we reported previously that modifications of the Ad5 FMDV vector, such as (i) changes in the promoter to enhance expression (18) and (ii) addition of the full-length coding region of FMDV NS protein 2B (18, 19), improved vaccine performance in swine and cattle in an experimental setting. In addition, a combinatorial study evaluating an Ad5 FMD vaccine with an Ad5 delivering a type I interferon (IFN) gene demonstrated enhanced protection (15). Strategies to alter Ad5 vector tropism by making changes in the fiber may also improve Ad5 vaccine performance. Attachment of Ad5 to the cell occurs upon interaction between the fiber and the coxsackie and adenovirus receptor (CAR) (20, 21); this is followed by interactions between the RGD (Arg-Gly-Asp) motif of the penton base and cell surface integrins, which mediate internalization of the virus into the cell (20, 22, 23). Genetically engineered Ads that have an RGD motif inserted in the HI loop of the fiber can use $\alpha_v\beta3$ and $\alpha_v\beta5$ integrins to attach to host cells (24, 25), broadening the tropism (26, 27). This strategy is particularly important for targeting dendritic cells (DCs), which are professional antigen-presenting cells (APC) critical for the initiation of immune responses. Since DCs lack CAR, prolonged exposure to high levels of Ad normally has been required to achieve significant transgene expression (28). Despite inefficient transduction of DCs, vectored vaccines derived from Ad5 can elicit robust adaptive immune responses, predominantly T helper type 1 (Th1), as reported for experiments with mice (29–32). Because DCs express $\alpha_v\beta3$ and $\alpha_v\beta5$, incorporation of an RGD motif in the fiber of Ad5 has gained importance in recent years and has been used to enhance transduction of target cells for different experimental immunotherapeutic approaches (33, 34). Moreover, a recent study suggested that a limited subset of DCs expressing high levels of $\alpha_v\beta3$ integrins is preferentially transduced by Ad vectors and activates $CD8^+$ T cell responses against Ad-encoded antigens (35).

In this study, we sought to evaluate the efficacy of a modified vaccine platform based on Ad5 O1 Campos containing an RGD modification in the fiber (Adt5.O1C.2B.RGD), in comparison to an unmodified similar vector. Cattle were vaccinated with Adt.O1C.2B.RGD or Adt.O1C.2B, with homologous challenge 21 days later. Evaluation of clinical signs and a number of serological and cellular parameters suggested that, although inclusion of an RGD motif in the fiber of an Ad5-vectored FMD vaccine improves transgene delivery and cell-mediated immunity, it does not significantly improve vaccine efficacy in cattle.

MATERIALS AND METHODS

Cells and viruses. Porcine kidney (LF-PK) and LF-PK-$\alpha_V\beta6$ (36, 37) cell lines were obtained from the Foreign Animal Disease Diagnostic Laboratory at the Plum Island Animal Disease Center (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 nonessential amino acids. Chinese hamster ovary (CHO) and CHO-$\alpha_V\beta6$ cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and, with LF-PK cells, were used to measure FMDV transgene expression following infection with recombinant Ad (rAd), as described previously (13). The replication-defective recombinant human Ad5 vectors were provided by GenVec Inc. (Gaithersburg, MD) and contained deletions in the E1, E3, and E4 regions (Adt). Recombinant Ad5 encoding a cassette containing the P1-2A region of FMDV serotype O1 Campos and the 2B and 3C regions of FMDV serotype A12 (Adt.O1C.2B) (17) was modified to encode RGD in the HI-loop of the fiber (Adt.O1C.2B.RGD). A vector lacking the FMDV coding regions (Adt.Null) was used as a negative control. Ad5 titers were determined using a focus-forming unit (FFU) assay, as described previously (38). Briefly, 293-ORF6 cells (39) were infected with various dilutions of test article, and adenoviral DNA-binding protein was immunocytochemically detected 24 h after infection. FFU per milliliters were determined by counting positive cells through fluorescence microscopy and applying a dilution factor and a microscope constant. FMDV O1C, which was used as a challenge virus, was produced by experimental infection of cattle; a pool of vesicular fluid was harvested, and titers of the virus were determined. The 50% bovine infectious dose (BID_{50}) was determined by titration of the challenge virus via intradermolingual inoculation of cattle.

SDS-PAGE and immunoblot analysis. LF-PK and CHO cell monolayers were grown in 24-well plates and infected overnight with Adt vectors at various multiplicities of infection (MOIs). MOIs ranging from 100 to 5,000 were selected, depending on the sensitivity of the cells to detect differences and the toxicity induced by Adt infection. The cells were washed twice with phosphate-buffered saline (PBS), lysed with 100 μl of lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40), and centrifuged at 10,000 × *g*. Supernatants were run on 12% polyacrylamide gels (NuPAGE; Invitrogen), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), and examined by Western blotting using polyclonal rabbit antibodies against VP1 and VP3 (18). Following incubation with goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (HRP) (Pierce, Rockford, IL), protein bands were visualized using an Immun-Star HRP chemiluminescent kit (Bio-Rad, Hercules, CA), according to the manufacturer's directions, and reactions were detected with X-ray film (Kodak). Densitometric measurement of VP1 signal intensity, normalized to that of tubulin, was performed using ImageJ software (40).

Immunomicroscopy. Localization of Adt and transgene products (FMDV capsid proteins) in cell lines was determined by the multichannel immunofluorescence (mIF) technique as described previously (33–35), with minor modifications. Primary monoclonal antibody (MAb) 12FB, directed against FMDV type O1 140S and 12S capsid subunits (41), and a MAb against the Adt hexon protein (clone 2Q1850; United States Biological, Swampscott, MA) were used in combination for mIF assays. For mIF assays, detection was performed using isotype-specific secondary antibodies labeled with Alexa Fluor dyes (Invitrogen). Slides were evaluated with a wide-field epifluorescence microscope, and images were captured with a Nikon DS-Qi1 digital camera. Quantification was performed by enumerating fluorescent cells per visual field in at least three separate representative fields, and the percentage of positive cells was calculated based on the number of positive cells divided by the total number of cells per field for at least three different representative fields.

Vaccine trials in cattle. Two vaccine experiments were performed in the high-containment facilities of the PIADC under animal protocol no. 244, which was approved by the Institutional Animal Care and Use Committee of PIADC (USDA/APHIS/AC certificate no. 21-F-0001). Animals were purchased from Thomas D. Morris, Inc., and were acclimated at the PIADC for 5 to 7 days before the start of the experiments. In all experiments, Holstein steers (6 months of age and weighing approximately 400 to 500 lb each) were divided into groups of three or four, according to the vaccination regimen, and the groups were housed in separate rooms. All animals were vaccinated subcutaneously at two sites, on both sides of the neck, in a total volume of 2 ml of PBS. In the first experiment, each group of four animals was vaccinated with 5×10^9 FFU of Adt.O1C.2B.RGD, Adt.O1C.2B, or Adt.Null (control group). In the second experiment, groups of three animals each were vaccinated with 1×10^9 FFU of the same Adt vaccine vectors. All animals were challenged at 21 days postvaccination (dpv) by intradermolingual inoculation with 10^4 BID_{50} of FMDV O1C, followed by monitoring of clinical signs, body temperature, viremia, virus shedding, and humoral and cellular immune responses for 21 days, as described below. Animals were humanely euthanized according to approved Institutional Animal Care and Use Committee protocols.

Clinical monitoring and sampling. After challenge, animals were examined and clinical scores were recorded daily for 10 days; a final clinical examination was performed at 21 days postchallenge (dpc). Clinical scores were determined by the number of feet presenting FMD lesions and the presence of secondary lesions in the mouth/snout. The maximum score was 5, and lesions restricted to the site of challenge were not counted. Rectal temperature was monitored on a daily basis throughout the experimental period. Nasal swabs used to collect nasal fluid were obtained daily to assess virus shedding 7 days after challenge and to test for the presence of viral nucleic acids, while serum samples were obtained 0, 3, 7, and 14 dpv and 0, 2, 4, 7, 14, and 21 dpc and were assayed for viremia and viral nucleic acids. In both experiments, serum samples were heat inactivated and assayed for FMDV-specific neutralizing antibodies or were left untreated and assayed for viremia and viral nucleic acids. Nasal swab specimens were collected in 2.5 ml MEM containing 2% antibiotics, 0.2% bovine serum albumin (BSA), and 20 mM HEPES and were clarified for 10 min at 1,600 × *g*.

Evaluation of humoral immune responses. Heat-inactivated serum samples were tested for the presence of FMDV-specific neutralizing antibodies, as described previously (42). Neutralizing activity was assayed by determining the neutralization index, expressed as $\log_{10}$ of input FMDV O1C (50% tissue culture infective dose [$TCID_{50}$]), in LF-PK cells neutralized to the 50% endpoint with serum samples diluted 1:2.

Virus titration in blood and nasal secretions. Untreated serum samples and nasal swabs were assayed for the presence of virus by $TCID_{50}$ endpoint titration on LF-PK cells, as described previously (15). Virus titers were expressed as $TCID_{50}$ per milliliter of serum or nasal swab. A duplicate set of serum samples and nasal swabs was also assayed for the presence of viral nucleic acids (RNA) by real-time reverse transcription (rRT)-PCR, as described previously (19). Briefly, 1 to 7 dpc, frozen serum samples were thawed and processed for RNA extraction, and FMDV RNA was measured by rRT-PCR using specific primers. Samples were considered positive when threshold cycle (C_T) values were <40.

Flow cytometric analysis. Heparinized blood samples were diluted with an equal amount of PBS and layered onto Uni-Sep lymphocyte tubes (Novamed, Jerusalem, Israel). Cells were centrifuged for 20 min at 600 × *g* at 20°C. Bands containing peripheral blood mononuclear cells (PBMCs) were collected and washed twice with PBS. Contaminating red blood cells were eliminated by lysis in 9 volumes of sterile water, followed immediately by addition of 1 volume of 10× PBS. Cells were further centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FBS, HEPES, L-glutamine, and 1% antibiotics. PBMCs were stimulated overnight with FMDV O1C (MOI of 10) or for 4 h with a nonspecific stimulator (Leukocyte Activation Cocktail with GolgiPlug; BD Bioscience, Franklin Lakes, NJ), at 37°C in 5% CO_2. In addition, FMDV-stimulated cells were incubated with GolgiStop (BD Bioscience) for 4 h at 37°C in 5% CO_2, centrifuged, and resuspended in PBS containing 10% FBS. To analyze the expression of cell surface molecules, mouse anti-bovine CD4-FITC and mouse anti-bovine CD8-AF647 (AbD Serotec, Raleigh, NC) were used. Samples were incubated for 30 min at 4°C, washed, fixed with BD Cytoperm/Cytofix (BD Biosciences) for 10 min at 25°C, and subsequently permeabilized with Permeabilization Solution 2 (BD Biosciences) for 10 min at 25°C. Cells were then washed in staining buffer three times and stained intracellularly with mouse anti-bovine gamma interferon (IFN-γ)-R-phycoerythrin (RPE) (AbD Serotec) for 30 min at 4°C. Background levels of RPE fluorescence were determined by staining with the corresponding IgG1-RPE isotype controls (AbD Serotec). Cells were acquired using a FACSCalibur flow cytometer (BD Bioscience). Data were analyzed with CellQuest Pro software (BD Bioscience). For statistical analysis, Student's *t* test was performed using Microsoft Excel.

Cytokine profile analysis. RNA was extracted from approximately 1×10^7 PBMCs (2 dpv) by utilizing an RNeasy miniprep kit (Qiagen, Valencia, CA). A quantitative rRT-PCR assay was used to evaluate the mRNA levels of several cytokine/chemokine genes. *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization of the values for each sample. The sequences of primers and probes used in this analysis are listed in Table 1. All reactions were performed in an ABI Prism 7000 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by C_T analysis, utilizing the samples from the Adt.Null control groups as the reference. Two-fold or greater induction was considered upregulation of gene expression.

Evaluation of Adt transduction in cattle-derived APC populations. Cattle PBMCs were isolated from total blood as described previously (42). Approximately 1.5×10^9 PBMCs were used for depletion of T cells, B cells, NK cells, and monocytes following the method described by Sei et al. (43), with modifications. Briefly, PBMCs were resuspended in staining buffer (1× calcium- and magnesium-free PBS, 2 mM EDTA [Life Technologies, Grand Island, NY], and 0.5% BSA fraction V [Life Technologies]) and stained with unlabeled mouse anti-bovine CD3, CD11b, CD14, and IgM antibodies (all IgG1; from Washington State University [Pullman, WA] and AbD Serotec [Raleigh, NC]) for 30 min on ice. PBMCs were then incubated with anti-mouse IgG conjugated to microbeads (Miltenyi, San Diego, CA), following the manufacturer's instructions. Cells were passed through a negative-selection LD column (Miltenyi) at 5×10^8 cells per column. Unbound cells were collected and designated a "depleted or antigen-presenting cell (APC)-enriched cell population." This enriched sample was resuspended in infection medium (RPMI 1640 medium, 2% FBS, 1% nonessential amino acids, 1% antibiotics, 0.05 mM 2-mercaptoethanol, 5 mM L-glutamine, 1% sodium pyruvate, and 25 mM HEPES) and plated in six-well plates (1×10^6 cells/well). A fraction of this cell preparation was stained with a mouse anti-bovine MHC II antibody (AbSerotec) for further characterization. The enriched APC population was infected with Adt.O1C.2B or Adt.O1C.2B.RGD at an MOI of 1 and was incubated at 37°C in 5% CO_2. Mock-infected cells or cells infected with the empty vector (Adt.Null) were used as negative controls. After 18 h, the cells were treated with a protein transport inhibitor containing monensin (GolgiStop; BD Bioscience, San Jose, CA) and incubated for 6 h. Cells were then collected, resuspended in PBS, and stained using a Live/Dead fixable yellow dead cell stain kit (Molecular Probes, Grand Island, NY) for 30 min. Cells were washed, fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience), and stained with mouse monoclonal anti-VP1 FMDV structural protein, with detection with Alexa Fluor 488-conjugated goat anti-mouse IgG (44). Cell data were acquired using an LSR II fluorescence-activated cell sorting (FACS) system (BD Biosciences), and the data were analyzed with FACSDiva software (BD Bioscience). Validation of the staining/detection FACS method was performed on epithelial cells (CHO or CHO-$\alpha_v\beta6$ cells) that had been mock infected or infected with the Adt vectors at MOIs of 1 to 100, followed by incubation at 37°C in 5% CO_2. After 18 h, the cells were treated with monensin and incubated for 6 h. Cells were washed with PBS and scraped off the plates, followed by staining as described above.

Statistical analysis. The statistical significance of differences was evaluated by Student's *t* test using Microsoft Excel. Differences were considered significant at *P* values of <0.05.

RESULTS

RGD-fiber-modified Adt enhances gene transduction. In order to evaluate transgene expression of RGD-fiber-modified Adt, CHO cells were infected with Adt.O1C.2B.RGD or Adt.O1C.2B, and FMDV capsid protein expression was analyzed by Western blotting using polyclonal antibodies against VP1 and VP3. Our results showed that expression of VP1 and VP3 was strong in samples infected with Adt.O1C.2B.RGD (Fig. 1A, lanes 4 and 5), compared to samples infected with Adt.O1C.2B (Fig. 1A, lanes 2 and 3). Interestingly, infection with the fiber-modified Adt in CHO cells constitutively expressing the integrin $\alpha_V\beta6$ yielded a qualitatively stronger signal for FMDV capsid protein expression (Fig. 1A, lanes 9 and 10) than CHO cells lacking this integrin

TABLE 1 Primers used for cytokine profiling

Gene	Primer and probe set	Sequence	GenBank accession no.
CCL3	*Bos taurus* CCL3 F	AGCCAGGTCTTCTCGGCAC	EU276059
	Bos taurus CCL3 R	AGAAGCAGCAGGCCGTTG	
	Bos taurus CCL3 T	ATTTGGCGCTGACACC	
CCL5	*Bos taurus* CCL5 F	CGCTCCTGCTTCTGCCTC	BC102064
	Bos taurus CCL5 R	TATAGGCAAAGCAGCAGGGC	
	Bos taurus CCL5 T	CATATGCCTCGGACACC	
IL-1B	*Bos taurus* IL-1B F	AACTGGTACATCAGCACTTCTCAAA	EU276067.1
	Bos taurus IL-1B R	ATATCCTGGCCACCTCGAAA	
	Bos taurus IL-1B T	AGAAAGGCCCGTCTTCCTGGGACA	
IL-6	*Bos taurus* IL-6 F	GACACTGGCAGAAAATAAGCTGAA	NM_173923.2
	Bos taurus IL-6 R	TCGCCTGATTGAACCCAGA	
	Bos taurus IL-6 T	CAAAAATGGAGGAAAAGGACGGATGCTT	
CXCL10	*Bos taurus* RIG-I F	GTCATTCCTGCAAGTCAATCCTG	EU276062
	Bos taurus RIG-I R	CCCATTCTTTTTCATTGTGGC	
	Bos taurus RIG-I T	CCACGTGTCGAGATTA	
IRF-1	*Bos taurus* IRF-1 F	CCGGTGGAAATTGTGCCA	BC102766
	Bos taurus IRF-1 R	GGGCGACACCTGAAAGTTGT	
	Bos taurus IRF-1 T	CAGCACCAGCGACC	
IRF-7	*Bos taurus* IRF-7 F	GGACTGTGACACGCCCATCT	BC151518
	Bos taurus IRF-7 R	CCCGGAACTCCAGCAGTTC	
	Bos taurus IRF-7 T	ACTTCGGCACCTTCT	
TNF	*Bos taurus* TNF F	AGCCTCAAGTAACAAGCCGGT	EU276079
	Bos taurus TNF R	TGCCCCGGAGAGTTGATG	
	Bos taurus TNF T	CCCACGTTGTAGCCG	
IFN-γ	*Bos taurus* IFN-γ F	GGAGGACTTCAAAAAGCTGATTCA	FJ263670
	Bos taurus IFN-γ R	GCTTTGCGCTGGATCTGC	
	Bos taurus IFN-γ T	TTCCGGTGGATGATC	
IL-10	*Bos taurus* IL-10 F	GGTGATGCCACAGGCTGAG	EU276074
	Bos taurus IL-10 R	AGCTTCTCCCCCAGTGAGTTC	
	Bos taurus IL-10 T	CCACAGGCTGAGAACCACGGGC	
IFN-α	*Bos taurus* IFN-α F	AGCACACCTTCCAGCTTTTCA	XM_001250455
	Bos taurus IFN-α R	GGAGGCTCTTGTCCCACACA	
	Bos taurus IFN-α T	CAGAGGGCTCGGCC	
IFN-β	*Bos taurus* IFN-β F	CTACAGCTTGCTTCGATTCCAA	M15477
	Bos taurus IFN-β R	CTGCCCCAGGAGTTTCTGAC	
	Bos taurus IFN-β T	ACGTCAGAGCCTTAAA	
CD80	*Bos taurus* CD80 F	GGCTCTCTCAGCTCTTGGTG	NM_001206439
	Bos taurus CD80 R	TTTGCCAATAGATCCGAAGG	
	Bos taurus CD80 T	ACCCCAAAGAGTGTGACCAA	
CD86	*Bos taurus* CD86 F	CCAAACACCCAAAACCTCAG	NM_001038017
	Bos taurus CD86 R	CAAGGTCCAACTGTCCTGGT	
	Bos taurus CD86 T	TCCCAAGTATATAGGCCGCA	
CCR7	*Bos taurus* CCR7 F	CAAGAAGGATGTGCGGAACT	NM_001024930
	Bos taurus CCR7 R	GGGAGGGTCAGAAGGAAGAG	
	Bos taurus CCR7 T	CGTACCTGCTCAACCTAGCC	

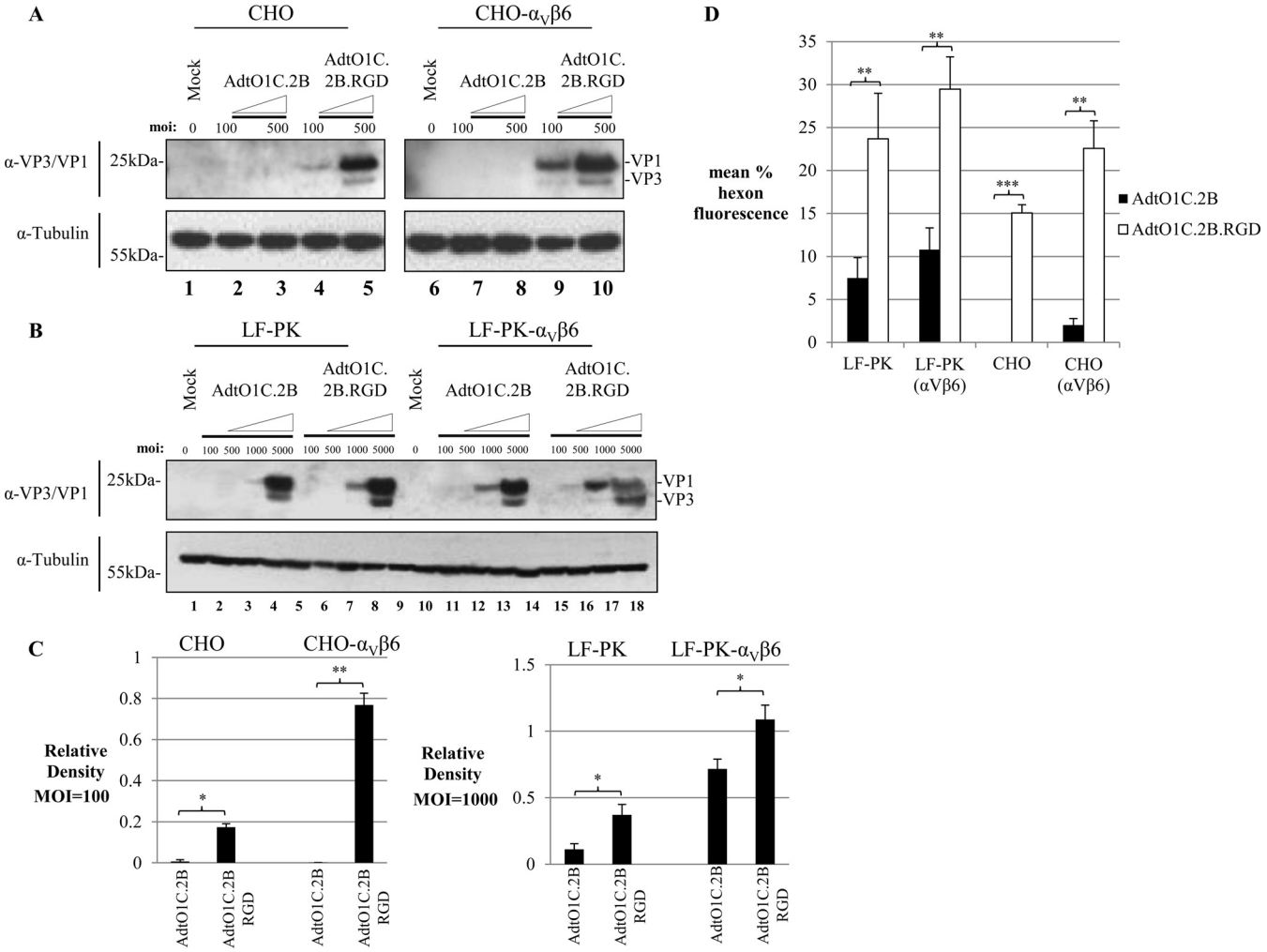

FIG 1 Fiber modification of the Ad5 vector vaccine enhances the expression of FMDV structural proteins in mammalian cells. (A and B) SDS-PAGE analysis of VP1 and VP3 expression. CHO and CHO-$\alpha_V\beta6$ cells (A) and LF-PK and LF-PK-$\alpha_V\beta6$ cells (B) were infected with either AdtO1C.2B or AdtO1C.2B.RGD at the indicated MOIs. Twenty-four hours postinfection, cell lysates were prepared and proteins were resolved by SDS-PAGE and visualized by Western blotting using polyclonal antibodies against FMDV capsid proteins VP1 and VP3. (C) Quantitation of FMDV VP1 protein in panels A and B by densitometry. (D) Relative quantitation of Ad5 hexon protein detected in infected (MOIs of 500) LF-PK, LF-PK-$\alpha_V\beta6$, CHO, and CHO-$\alpha_V\beta6$ cells. All experiments depicted were repeated at least three times, with similar results. Error bars represent standard deviation of the mean. Statistical significance was determined with Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 1A, lanes 4 and 5). Quantification of band intensity by densitometry (samples infected at an MOI of 100) indicated an approximately 4-fold increase in VP1 signal (Fig. 1C, left). The presence of cell surface $\alpha_V\beta6$ in CHO cells did not improve transgene expression of non-fiber-modified Adt.O1C.2B (Fig. 1A, lanes 7 and 8). These results are consistent with previous reports indicating that CAR is not expressed in CHO cells (21, 45), and they indicate that addition of the RGD motif circumvents the dependency of CAR expression on target cells.

We also evaluated transgene expression in porcine kidney cells (LF-PK cells), which are susceptible to FMDV infection (37). Expression of FMDV capsid proteins was not qualitatively different in cells infected with either Adt vector (Fig. 1B, lanes 2 to 9). However, quantitative analysis by densitometry showed a statistically significant increase in VP1 signal in samples infected with fiber-modified Adt (samples infected at an MOI of 1,000) (Fig. 1C, right). Examination of FMDV capsid protein expression in LF-PK cells expressing the *Bos taurus* integrin $\alpha_V\beta6$ (LF-PK-$\alpha_V\beta6$) and infected with either Adt vector (Fig. 1B, lanes 11 to 18) showed statistically significantly improved detection of VP1 signal, as measured by densitometry (Fig. 1C, right). These results showed that cells that are refractory to Ad infection (i.e., CHO cells) can become susceptible to infection by engaging the RGD-binding integrin receptor, thereby enhancing expression of FMDV capsids.

Using immunomicroscopy, we evaluated the efficiency of Adt infection (mean percent values ± SD) using an antibody against the Ad5 hexon protein. LF-PK, LF-PK-$\alpha_V\beta6$, CHO, and CHO-$\alpha_V\beta6$ cells were infected (MOI of 500) with Adt.O1C.2B.RGD or Adt.O1C.2B (Fig. 1D). Hexon expression levels in LF-PK or LF-PK-αVβ6 cells infected with the Adt.O1C.2B vector reached only 7.49% or 10.81%, respectively; in contrast, when cells were infected with Adt.O1C.2B.RGD, expression of the hexon protein showed significant increases of 3.1-fold and 2.7-fold, respectively.

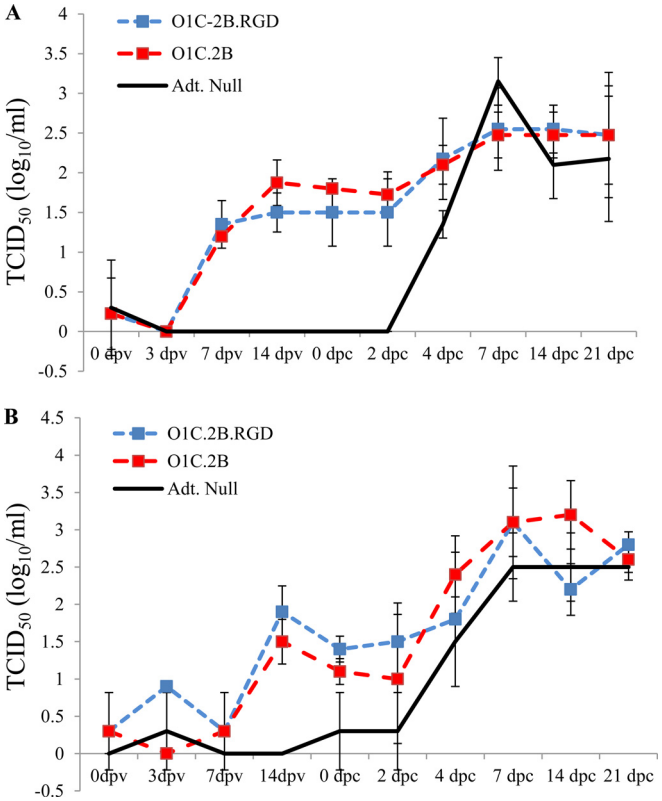

FIG 2 Kinetics of neutralizing antibodies against FMDV O1 Campos in trial 1 (A) and trial 2 (B). Serum samples were collected 0, 4, 7, and 14 dpv, on challenge day (0 dpc), and 2, 4, 7, 14, and 21 dpc. Titers are expressed as the inverse dilution of serum yielding a 50% reduction in $TCID_{50}$. Values represent means ± standard deviations.

In CHO cell lines, hexon expression followed the same pattern as observed in Fig. 1A. CHO and CHO-$\alpha_V\beta6$ cells were transduced more efficiently with Adt.O1C.2B.RGD, reaching hexon fluorescence values of 15.06% and 22.57%, respectively, compared to the minimal fluorescence values (1.02% and 2.01%, respectively) observed in cells infected with Adt.O1C.2B (Fig. 1D). These results confirm our Western blot data and indicate that cells with greater expression of surface integrins are targeted more effectively by RGD-fiber-modified adenoviruses, by overcoming the dependency on CAR expression.

Vaccine trials. (i) Virus neutralization titers after immunization. We performed two separate vaccine efficacy studies, utilizing unmodified and RGD-fiber-modified Adt vectors. All animals vaccinated with Adt vectors encoding FMDV antigens developed specific FMDV-neutralizing antibody responses, with an observable peak at 14 dpv. Unexpectedly, all animals that received Adt.O1C.2B.RGD did not show any significant difference in the levels of neutralizing antibodies, compared to unmodified vector vaccine (Adt.O1C.2B), at any time following vaccination (Fig. 2A). No anti-FMDV titers were detected in the Adt.Null control group until after challenge. In the second study, the vaccine dose was decreased to 1×10^9 FFU per animal. Similar to the first study, all animals vaccinated with Adt vectors encoding FMDV antigens had a peak of detectable antibodies at 14 dpv, followed by a second peak at 7 to 14 dpc (Fig. 2B). However, no significant differences were detected between the groups vaccinated with Adt vectors with versus without RGD.

(ii) Clinical evaluations. All animals inoculated with Adt.Null (control groups in both studies) developed lesions in the feet and snout as early as 2 dpc, and 5 of 7 animals reached a maximum score of 5 at 4 dpc (Tables 2 and 3). Viremia ($TCID_{50}$) and the presence of FMDV RNA in serum (determined by rRT-PCR) were detected in all animals in the control groups. The onset of viremia was observed as early as 1 dpc, and generally viremia was present for 3 days. Nucleic acids also were first detected at 1 dpc, and they were present for up to 4 to 6 days.

In the first study, 50% of the animals vaccinated with either Adt-FMDV vector did not show clinical disease, while the other animals presented mild clinical signs, as indicated by low clinical scores of 1 to 2, in contrast to the Adt.Null control group (Table 2). No infective FMDV particles ($TCID_{50}$) were detected in the serum of any of the Adt-FMDV vector-vaccinated animals. However, FMDV RNA was detected by qRT-PCR in the two Adt.O1C.2B-vaccinated animals that showed clinical disease and in one animal that did not develop clinical disease, although at much lower levels (C_T values ranging from ~34 to 40) than in the control animals (C_T values ranging from ~23 to 25). Viral RNA was detectable only in the two animals that developed clinical disease in the Adt.O1C.2B.RGD group. Virus shedding, as measured by $TCID_{50}$ per milliliter of nasal wash medium, occurred in all groups. Importantly, in the Adt-O1C.2B.RGD-vaccinated group, virus shedding was 32-fold lower than that in the control group and 16-fold lower than that in the Adt.O1C.2B-vaccinated group (Table 2) (fold decreases were based on the mean values for each group). The duration of shedding was, on average, 2 days and 1 day shorter in the Adt.O1C.2B.RGD- and Adt.O1C.2B-vaccinated groups, respectively, than in the control group.

In the second study (Table 3), the vaccination dose was reduced to 1×10^9 FFU per animal. At this dose, clinical disease was observed in one of the three Adt.O1C.2B.RGD-vaccinated animals (animal 47), with a clinical score of 4. Two of the three Adt.O1C.2B-vaccinated animals (animals 52 and 53) had visible lesions, with clinical scores of 5 and 1, respectively, detected at 8 dpc. None of the Adt-FMDV-vaccinated animals had detectable viremia, but FMDV RNA was detected in four of six animals in the two vaccinated groups (animals 45, 47, 52, and 53) (Table 3). The Adt.O1C.2B.RGD-vaccinated cattle had smaller amounts of viral RNA in serum (average C_T, 36.9) than did the Adt.O1C.2B-vaccinated group (average C_T, 33.9). Virus shedding occurred in all animals in the RGD group and in two of the three animals in the non-RGD group. Importantly, average virus shedding in the RGD group was 1,000-fold lower than that in the control group and 56-fold lower than that in the non-RGD group, with a duration about 2 days shorter than that in the control group (Table 3).

(iii) Cytokine profiles. The lack of enhancement in vaccine protection prompted us to examine whether any different innate responses triggered by the RGD-fiber modification could be detected in PBMCs isolated from animals vaccinated with the different Adts (trial 1). For this analysis, samples were taken 2 dpv, and comparison of the Adt-FMDV-vaccinated groups and the Adt.Null groups was performed. Figure 3 shows that, except for CXCL10 and IRF7, there was an overall upregulation in RNA levels for most of the analyzed cytokines, chemokines, and DC maturation markers in the cells isolated from the RGD-vaccinated group, compared to the non-RGD-vaccinated group (Fig. 3). However, the observed changes in mRNA levels were not statistically significant ($P > 0.05$). Interestingly, the mRNA levels for

TABLE 2 Summary of clinical data from trial 1

Vaccine	Cow no.	Dose (Adt FFU/2 ml)	Serum neutralization titer (log_{10} $TCID_{50}$)		No. of lesions at:			Viral RNA[a]			Viremia[b]			Virus shedding[c]		
			0 dpc	21 dpc	2 dpc	4 dpc	8 dpc	Time of viral RNA detection (dpc)	RT-PCR result	Duration of detectable viral RNA (days)	Time of viremia onset (dpc)	Maximal viremia ($TCID_{50}$/ml)	Duration of viremia (days)	Time of shed virus detection (dpc)	Maximal virus shedding ($TCID_{50}$/ml)	Duration of shedding (days)
Adt.O1C.2B.RGD	1	5×10^9	1.5	3.3	0	1	1	2	SP	1	0	0	0	2	1×10^3	2
	2		2.1	2.4	0	0	0	ND	Neg	Neg	0	0	0	2	3.2×10^3	2
	3		1.2	1.8	0	0	0	ND	Neg	Neg	0	0	0	2	5.6×10^3	1
	4		1.2	2.4	0	2	2	1	SP	4	0	0	0	2	1×10^5	2
Adt.O1C.2B	5	5×10^9	1.8	2.4	0	1	1	3	WP	1	0	0	0	2	5.6×10^3	3
	6		1.8	1.8	0	0	0	3	WP	1	0	0	0	2	1.8×10^3	3
	7		1.8	2.1	0	0	0	ND	Neg	Neg	0	0	0	2	5.6×10^2	2
	8		1.8	3.6	0	1	1	2	WP	1	0	0	0	2	1.8×10^6	3
Adt.Null	13	5×10^9	<0.3	3.3	4	5	5	1	SP	5	1	5.6×10^2	3	1	3.2×10^6	4
	14		<0.3	1.5	4	5	5	1	SP	4	1	1.8×10^3	3	2	1.8×10^5	3
	15		<0.3	1.2	3	4	4	1	SP	4	1	5.6×10^3	3	1	5.6×10^4	5
	16		<0.3	1.2	1	4	4	1	SP	5	1	1.8×10^3	3	2	3.2×10^4	3

[a] Detection of viral RNA in serum samples by RT-PCR. Results are expressed as follows: Neg, negative (C_T of ≥40); WP, weak positive (C_T of 35 to <40); SP, strong positive (C_T of <35); ND, not detected.

[b] Detection of virus in serum samples. Virus titers are expressed as $TCID_{50}$/ml.

[c] Detection of shed virus in nasal discharge fluid. Virus titers are expressed in $TCID_{50}$/ml.

type I IFN (IFN-α/β) and interleukin 10 (IL-10) were the most upregulated in the RGD group versus the non-RGD group, despite the high variability observed among animals.

(iv) T lymphocyte responses to vaccination. Previous studies by Moraes et al. (19) showed that Ad5.O1C.2B vectors could induce significant increases in IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, compared to an Ad5-O1C.2B vector lacking the complete 2B coding region. In order to examine differences in T lymphocyte responses triggered by the RGD-modified-vector vaccine, we measured IFN-γ production by $CD4^+$ and $CD8^+$ T cells, by using multiparametric flow cytometry, during the course of the first animal study. As expected, minimal levels of T cell responses were detected at 0 dpv (Fig. 4A). In contrast, at 7 dpv (Fig. 4B) we observed a tendency for the Adt.O1C.2B.RGD-vaccinated animals to generate higher levels of IFN-γ-producing $CD4^+$ and $CD8^+$ cells, although the differences were not statistically significant. Interestingly, at 14 dpv (Fig. 4C), more IFN-γ-producing $CD4^+$ and $CD8^+$ cells were detected in the RGD-vaccinated group than in the non-RGD-vaccinated group. Statistically significantly greater numbers of $CD4^+$ IFN-γ^+ cells ($P < 0.0003$) and $CD8^+$ IFN-γ^+ cells ($P < 0.0002$) were evident for the RGD-vaccinated group. However, these responses waned to basal levels by the time of challenge (0 dpc/21 dpv) (Fig. 4D). After challenge, substantial increases in the numbers of IFN-γ^+ $CD4^+$ cells and IFN-γ^+ $CD8^+$ cells were observed at 14 and 21 dpc in both groups vaccinated with Ad vectors, independent of the RGD insert (Fig. 4E and F). However, we found significantly greater numbers of $CD4^+$ IFN-γ^+ lymphocytes ($P < 0.04$) for the RGD-vaccinated group at 21 dpc (Fig. 4F). The numbers of $CD8^+$ IFN-γ^+ T cells were also found to be increased, but the differences in comparison with the non-RGD and control groups did not reach statistical significance.

RGD-fiber-modified Adt does not enhance FMDV capsid expression in APC-enriched populations. Ad5 fiber modification with an RGD motif has been shown to enhance the infectability of DCs, leading to enhanced transgene-specific humoral and cellular responses in mouse models (28, 29). Since our *in vivo* data showed no significant enhancement in protection after challenge, we hypothesized that RGD-fiber-modified Adt might not have targeted bovine APCs or their transduction might not have been effectively enhanced in bovine cells. In order to test these possibilities, we followed an APC enrichment protocol described by Sei et al. (43). Characterization of the bovine APC-enriched population indicated that the majority of the cells present after CD3, CD14, CD11b, and soluble IgM depletion were MHC II-positive cells (Fig. 5A to D). Enriched bovine APCs were mock infected or infected with Adt.Null, Adt.O1C.2B, or Adt.O1C.2B.RGD, and FMDV capsid protein expression was detected by flow cytometry (FACS) using a specific monoclonal antibody against VP1 (Fig. 5). Control experiments were performed with CHO cells to verify that the results obtained by FACS analysis reproduced the results obtained by Western blotting (see Fig. S1 in the supplemental material). As shown in the histograms (Fig. 5E), enriched APCs infected with either Adt vector showed comparable percentages of cells expressing VP1 at 24 h postinfection (36.6% for Adt.O1C.2B.RGD versus 37.5% for Adt.O1C.2B). In contrast, infection of CHO-$\alpha_V\beta6$ cells clearly yielded different responses (7.1 to 93.9% for Adt.O1C.2B.RGD versus 1.5 to 1.8% for Adt.O1C.2B) (see Fig. S1 in the supplemental material). With both enriched cattle APCs and CHO cells, little or no VP1 was detected in mock-infected or

TABLE 3 Summary of clinical data from trial 2

Vaccine	Cow no.	Dose (Adt FFU/2ml)	Serum neutralization titer (log_{10} $TCID_{50}$)			No. of lesions at:			Viral RNA[a]			Viremia[b]			Virus shedding[c]		
			0 dpc	21 dpc	2 dpc	4 dpc	8 dpc	Time of viral RNA detection (dpc)	RT-PCR result	Duration of viral RNA detection (days)	Time of viremia onset (dpc)	Maximal viremia ($TCID_{50}$/ml)	Duration of viremia (days)	Time of shed virus detection (dpc)	Maximal virus shedding ($TCID_{50}$/ml)	Duration of shedding (days)
Adt.O1C.2B. RGD	45	1×10^9	1.5	2.7	0	0	0	4	WP	2	0	0	0	1	3.2×10^3	3
	46		1.5	2.7	0	0	0	ND	Neg	Neg	0	0	0	1	1.8×10^3	3
	47		1.2	3	1	4	4	2	WP	2	0	0	0	1	3.2×10^5	4
Adt.O1C.2B	51	1×10^9	1.2	2.7	0	0	0	ND	Neg	Neg	0	0	0	2	5.6×10^4	2
	52		1.2	2.7	4	4	5	1	SP	3	0	0	0	2	5.6×10^6	3
	53		0.9	2.4	1	1	1	2	WP	2	0	0	0	0	None	None
Adt.Null	54	1×10^9	0.9	2.7	2	5	5	1	SP	6	1	5.6×10^3	6	1	3.2×10^5	5
	55		<0.3	3.6	3	5	5	1	SP	4	2	5.6×10^3	3	1	3.2×10^6	4
	56		<0.3	3.3	5	5	5	1	SP	4	1	5.6×10^4	3	1	1.8×10^6	5

[a] Detection of viral RNA in serum samples by RT-PCR. Results are expressed as follows: Neg, negative (C_T of ≥40); WP, weak positive (C_T of 35 to <40); SP, strong positive (C_T of <35); ND, not detected.
[b] Detection of virus in serum samples. Virus titers are expressed in $TCID_{50}$/ml.
[c] Detection of shed virus in nasal discharge fluid. Virus titers are expressed in $TCID_{50}$/ml.

2 dpv

	Adt.O1C.2B	Adt.O1C.2B.RGD
CCL3	1.15 ± 0.72	3.32 ± 2.47
CCL5	1.27 ± 1.00	2.06 ± 2.47
IL-1B	1.72 ± 1.35	1.74 ± 1.54
IL-6	1.71 ± 3.33	7.82 ± 12.42
CXCL10	21.10 ± 39.68	0.24 ± 0.29
IRF-1	1.17 ± 0.49	1.31 ± 0.75
IRF-7	70.36 ± 98.24	0.73 ± 1.44
TNF	1.25 ± 0.56	2.40 ± 1.79
IFN-γ	1.51 ± 1.91	2.88 ± 3.5
IL-10	1.16 ± 1.51	7.76 ± 9.65
IFN-α	1.89 ± 3.71	19.39 ± 31.78
IFN-β	1.12 ± 2.13	9.10 ± 13.74
CD80	1.13 ± 0.90	2.95 ± 4.71
CD86	1.24 ± 1.44	3.12 ± 3.38
CCR7	1.61 ± 2.91	1.61 ± 1.67

Fold Induction	
<2	
2-5	
5-10	
>10	

FIG 3 Analysis of mRNA expression in PBMCs. The expression of cytokine mRNAs was measured by rRT-PCR in PBMCs isolated from animals in trial 1 at 2 days following vaccination. Data are depicted as fold increase in gene expression for animals vaccinated with Adt.O1C.2B or Adt.O1C.2B.RGD relative to animals vaccinated with Adt.Null (control) in samples taken at 2 dpv.

Adt.Null-infected cells, indicating that the VP1 signal was specific. These results indicate that RGD modification of the Adt fiber does not enhance Adt transduction of enriched APC populations derived from cattle.

DISCUSSION

Recombinant adenoviral (rAd) vectors are considered a promising platform for the development of viral vector-based vaccines (17). However, new strategies to enhance their potency and efficacy are currently being explored. Modification of the fiber through incorporation of an RGD motif in rAd vectors has been shown to elicit strong immune responses to encoded antigens, by expanding the tropism and targeting antigen-presenting cells such as DCs (28, 33, 46). In this study, we hypothesized that an RGD motif in the fiber of the Ad5.O1C.2B vector would improve the antigenicity and potency of the vaccine. In addition, we evaluated the ability of the recombinant Adt.O1C.2B.RGD vector vaccine to induce protection in cattle.

Our *in vitro* data supported our hypothesis and revealed a substantial increase in FMDV capsid protein expression in cells lacking CAR (CHO cells) when they were transduced with Adt.O1C.2B.RGD (Fig. 1). Moreover, we observed that cell lines overexpressing $\alpha_V\beta6$ integrins but lacking CAR expression (CHO-$\alpha_V\beta6$ cells) were more permissive to Adt.O1C.2B.RGD than to the unmodified rAd vector. These data are in agreement with a number of studies demonstrating that RGD-modified rAd vectors improve the transduction efficiency of integrin-rich cell types such as endothelial cells, smooth muscle cells, and DCs (25, 28, 47). The initial *in vitro* experiments were extended to examine the efficacy of the RGD-modified fiber of the Adt.O1C.2B vector *in vivo*. In the first trial, we used a previously tested vector dose that protected 50 to 75% of cattle (18); in the second trial, we used a 5-fold lower dose. The two groups of vaccinated animals (RGD-fiber-modified and non-RGD-fiber-modified vectors) in the first trial had similar neutralizing antibody titers on the day of chal-

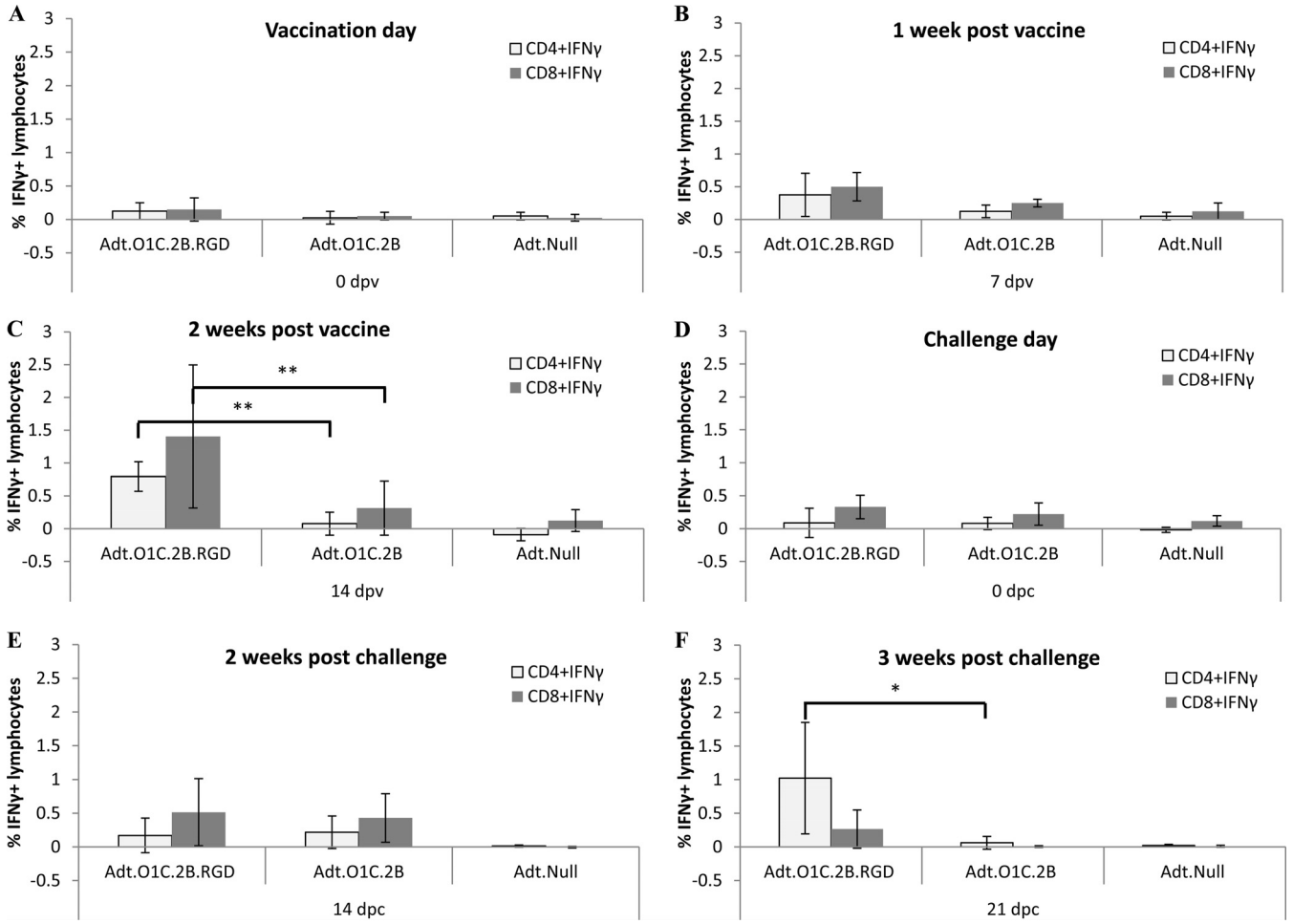

FIG 4 Cell-mediated immune responses of cattle vaccinated with Adt vectors and challenged with FMDV O1 Campos. Flow cytometric data corresponding to the numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ lymphocytes on vaccination day (A), 1 week postvaccination (B), 2 weeks postvaccination (C), on the day of challenge (D), 2 weeks postchallenge (E), and 3 weeks postchallenge (F). Error bars represent standard deviation of the mean. Statistical significance was determined with Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

lenge, and 50% of the animals in each group were protected (2 of 4 animals without vesicular lesions); however, one of the animals in the Adt.O1C.2B-vaccinated group without vesicles had detectable FMDV RNA in the serum (Table 2). Noticeably, none of the animals without clinical manifestations in the Adt.O1C.2B.RGD-vaccinated group was PCR positive for FMDV RNA in the serum. In the second study, we observed that 2 of the vaccinated animals in the RGD group were free of lesions, compared to 1 of 3 in the non-RGD group. Although none of the vaccinated animals in either group had detectable viremia at any time point, 2 of 3 animals in each group had detectable levels of viral RNA in the serum (Table 3). Although we did not observe a more pronounced protective response with the vector bearing the RGD modification in the fiber, compared to the vector lacking RGD, the detected differences between the two groups are worth noting. First, we observed that clinical manifestations after challenge in vaccinated animals in the second study (Table 3) showed slightly improved responses in the Adt.O1.2B.RGD-inoculated group (67% protection), which correlated with a moderate but nonsignificant increase in the humoral response (Fig. 2B). This suggests that, at doses of vaccine vector that induce limited protection, the presence of the RGD sequence in the fiber may provide sufficient additional expression of FMDV capsids in antigen-presenting cells to protect vaccinated animals. To support this hypothesis, additional experiments using lower doses of both vaccines and larger numbers of animals are required. Second, examination of the T cell responses (first animal study) revealed significant differences in animals vaccinated with the RGD-Ad vector (Fig. 4). In the present study, we were able to demonstrate statistically significant increases in the detection of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ prior to challenge in animals vaccinated with the RGD-Adt vector, compared to the non-RGD vector and control groups. We also observed a gradual increase in T cell responses after challenge, with significant differences in $CD4^+$ IFN-γ^+ T cell levels in the RGD-Adt-vaccinated groups at 21 dpc.

Analysis of the cytokine profile in PBMCs by qRT-PCR did not show significant differences between the groups vaccinated with RGD-Adt versus non-RGD-Adt. However, high variability in animals that are not inbred is usually expected and observed in this type of analysis (48). Nevertheless, greater induction of type I IFN and IL-10 expression was detected in the RGD-Adt-treated group. Both IFN and IL-10 have been shown to play roles in the adaptive

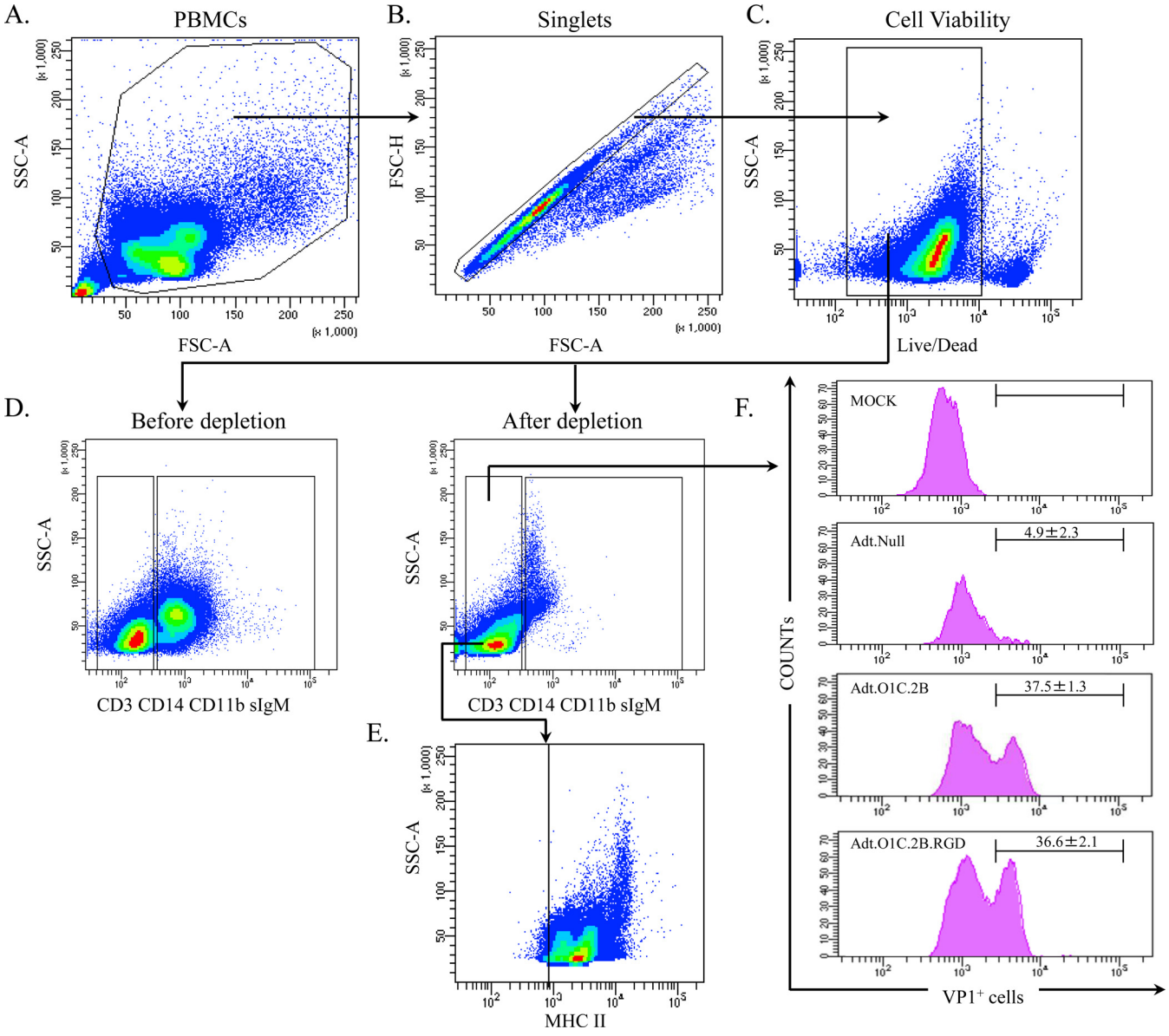

FIG 5 Infection of the enriched APC population with Adt.O1C.2B or Adt.O1C.2B.RGD. (A) PBMCs from cows were subjected to an APC enrichment process. (B and C) Doublets (B) and dead cells (C) were excluded from the total PBMC population. (D) Using lineage-specific antibodies (anti-CD3, anti-CD14, anti-IgM, and anti-CD11b), T cells, monocytes, B cells, and NK cells were excluded. (E) The APC-enriched population was characterized using anti-MHC II antibody. (F) Lineage-negative cells were then infected with Adt.O1C.2B, Adt.O1C.2B.RGD, or Adt.Null at an MOI of 1 or were mock infected. Histograms show CD3-, CD11b-, CD14-, and IgM-negative cell populations that were stained for the VP1 FMDV structural protein. Data are representative of three independent experiments. Numbers on histograms, average percentages of cells expressing VP1 staining. SSC-A, side-scattered area; FSC-A, forward-scattered area; FSC-H, forward-scattered height.

immune responses against FMDV. Type I IFN can act as an adjuvant for Ad5-FMDV in swine when animals are challenged 42 dpv (49); IL-10 is produced in response to FMDV infection to support a T cell-independent antibody response (50). Therefore, more experiments are required to evaluate whether the differences in the levels of IFN and IL-10 observed in RGD-Adt-vaccinated animals versus non-RGD-Adt-vaccinated animals may play a role in long-term protection. We speculate that, overall, the observed differences in our study could be enhanced by using more experimental subjects (>3 subjects) and other experimental designs. Alternatively, the species (cattle) used in these studies might have played an important role, and enhanced protection might have been masked by a relatively low vaccine dose/animal mass ratio. Moreover, it is known that FMDV serotype O vaccines require greater antigenic mass than do serotype A vaccines (18, 51), which suggests that the efficacy of the Ad serotype O1 Campos vaccine used in this study is dependent on the amount of transgene expressed in cells transduced with this vector. In this regard, and as mentioned above, no differences in transgene expression were detected when enriched APC populations isolated from cattle were infected with the RGD-Adt versus non-RGD-Adt vectors. These results could explain the lack of improvement in efficacy *in vivo* when animals were challenged 21 dpv.

Naive $CD4^+$ T cells differentiate into functional subsets, termed T helper type 1 (Th1) and T helper type 2 (Th2) cells, based on their production of the cytokines IFN-γ and IL-4, respectively (52). $CD8^+$ T cells mediate their effector functions through production of cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α) and/or by cytolytic mechanisms (53). Therefore, higher frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells obtained from blood from the RGD-vaccinated groups (Fig. 3 and 4) may suggest more effective induction of Th1 and/or Th2 immune responses despite the lack of improved transgene expression in enriched APC populations derived from cattle. It is possible that other cells localized at the site of vaccine inoculation, such as fibroblasts or mast cells, were better transduced by the subcutaneous route of immunization, potentially skewing immune responses toward cell-mediated immunity rather than humoral immunity.

In conclusion, our results show that insertion of an RGD motif in the fiber of the adenovirus vector that delivers FMDV serotype O1 Campos vaccine does not improve vaccine efficacy. Consistently, this modification does not improve transduction of bovine blood cell populations enriched in professional APCs. However, enhanced T cell responses and presumably enhanced memory T cell responses in animals vaccinated with the RGD construct may play a role in improving long-term protection from challenge. Further studies with larger statistical samples and challenges at later times after vaccination (e.g., 6 to 8 weeks), as well as development of more-sensitive techniques to identify specific subpopulations of immune cells, may contribute to a better understanding of the possible effects of RGD modification of the Ad5-FMDV fiber on humoral and cellular immunity in cattle.

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REFERENCES

1. **Grubman MJ, Baxt B.** 2004. Foot-and-mouth disease. Clin Microbiol Rev **17:**465–493. http://dx.doi.org/10.1128/CMR.17.2.465-493.2004.
2. **Arzt J, Juleff N, Zhang Z, Rodriguez LL.** 2011. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. Transbound Emerg Dis **58:**291–304. http://dx.doi.org/10.1111/j.1865-1682.2011.01204.x.
3. **Alexandersen S, Brotherhood I, Donaldson AI.** 2002. Natural aerosol transmission of foot-and-mouth disease virus to pigs: minimal infectious dose for strain O1 Lausanne. Epidemiol Infect **128:**301–312.
4. **Rodriguez LL, Grubman MJ.** 2009. Foot-and-mouth disease virus vaccines. Vaccine **27**(Suppl 4)**:**D90–D94. http://dx.doi.org/10.1016/j.vaccine.2009.08.039.
5. **World Organisation for Animal Health.** 2015. Manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris, France. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/.
6. **Rodriguez LL, Gay CG.** 2011. Development of vaccines toward the global control and eradication of foot-and-mouth disease. Expert Rev Vaccines **10:**377–387. http://dx.doi.org/10.1586/erv.11.4.
7. **Bahnemann HG.** 1975. Binary ethylenimine as an inactivant for foot-and-mouth disease virus and its application for vaccine production. Arch Virol **47:**47–56. http://dx.doi.org/10.1007/BF01315592.
8. **Barteling SJ, Vreeswijk J.** 1991. Developments in foot-and-mouth disease vaccines. Vaccine **9:**75–88. http://dx.doi.org/10.1016/0264-410X(91)90261-4.
9. **Doel TR.** 2003. FMD vaccines. Virus Res **91:**81–99. http://dx.doi.org/10.1016/S0168-1702(02)00261-7.
10. **Pacheco JM, Brum MC, Moraes MP, Golde WT, Grubman MJ.** 2005. Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. Virology **337:**205–209. http://dx.doi.org/10.1016/j.virol.2005.04.014.
11. **Montiel N, Smoliga G, Arzt J.** 2012. Early detection and visualization of human adenovirus serotype 5-viral vectors carrying foot-and-mouth disease virus or luciferase transgenes in cell lines and bovine tissues. Vaccine **30:**1690–1701. http://dx.doi.org/10.1016/j.vaccine.2011.12.082.
12. **Mayr GA, Chinsangaram J, Grubman MJ.** 1999. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. Virology **263:**496–506. http://dx.doi.org/10.1006/viro.1999.9940.
13. **Moraes MP, Mayr GA, Mason PW, Grubman MJ.** 2002. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. Vaccine **20:**1631–1639. http://dx.doi.org/10.1016/S0264-410X(01)00483-2.
14. **Grubman MJ, Lewis SA, Morgan DO.** 1993. Protection of swine against foot-and-mouth disease with viral capsid proteins expressed in heterologous systems. Vaccine **11:**825–829. http://dx.doi.org/10.1016/0264-410X(93)90357-4.
15. **Moraes MP, Chinsangaram J, Brum MCS, Grubman MJ.** 2003. Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and foot-and-mouth disease virus subunit vaccine. Vaccine **22:**268–279. http://dx.doi.org/10.1016/S0264-410X(03)00560-7.
16. **Porta C, Kotecha A, Burman A, Jackson T, Ren J, Loureiro S, Jones IM, Fry EE, Stuart DI, Charleston B.** 2013. Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. PLoS Pathog **9:**e1003255. http://dx.doi.org/10.1371/journal.ppat.1003255.
17. **Grubman MJ, Schutta C, Barrera J, Neilan J, Ettyreddy D, Butman B, Brough D, Brake D.** 2010. Adenovirus serotype 5-vectored foot-and-mouth disease subunit vaccines: the first decade. Future Virol **5:**51–64. http://dx.doi.org/10.2217/fvl.09.68.
18. **Pena L, Moraes MP, Koster M, Burrage T, Pacheco JM, Diaz-San Segundo F, Grubman MJ.** 2008. Delivery of a foot-and-mouth disease virus empty capsid subunit antigen with nonstructural protein 2B improves protection of swine. Vaccine **26:**5689–5699. http://dx.doi.org/10.1016/j.vaccine.2008.08.022.
19. **Moraes MP, Segundo FD, Dias CC, Pena L, Grubman MJ.** 2011. Increased efficacy of an adenovirus-vectored foot-and-mouth disease capsid subunit vaccine expressing nonstructural protein 2B is associated with a specific T cell response. Vaccine **29:**9431–9440. http://dx.doi.org/10.1016/j.vaccine.2011.10.037.
20. **Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW.** 1997. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science **275:**1320–1323. http://dx.doi.org/10.1126/science.275.5304.1320.
21. **Tomko RP, Xu R, Philipson L.** 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci U S A **94:**3352–3356. http://dx.doi.org/10.1073/pnas.94.7.3352.
22. **Greber UF, Willetts M, Webster P, Helenius A.** 1993. Stepwise dismantling of adenovirus 2 during entry into cells. Cell **75:**477–486. http://dx.doi.org/10.1016/0092-8674(93)90382-Z.
23. **Wickham TJ, Mathias P, Cheresh DA, Nemerow GR.** 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell **73:**309–319. http://dx.doi.org/10.1016/0092-8674(93)90231-E.
24. **Sengupta S, Ulasov IV, Thaci B, Ahmed AU, Lesniak MS.** 2011. En-

hanced transduction and replication of RGD-fiber modified adenovirus in primary T cells. PLoS One **6:**e18091. http://dx.doi.org/10.1371/journal.pone.0018091.

25. **Coughlan L, Vallath S, Saha A, Flak M, McNeish IA, Vassaux G, Marshall JF, Hart IR, Thomas GJ.** 2009. In vivo retargeting of adenovirus type 5 to αvβ6 integrin results in reduced hepatotoxicity and improved tumor uptake following systemic delivery. J Virol **83:**6416–6428. http://dx.doi.org/10.1128/JVI.00445-09.
26. **Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, Wickham TJ, Kovesdi I, Roelvink P, Crystal RG.** 1999. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest **103:**579–587. http://dx.doi.org/10.1172/JCI5309.
27. **Barnett BG, Crews CJ, Douglas JT.** 2002. Targeted adenoviral vectors. Biochim Biophys Acta **1575:**1–14. http://dx.doi.org/10.1016/S0167-4781(02)00249-X.
28. **Worgall S, Busch A, Rivara M, Bonnyay D, Leopold PL, Merritt R, Hackett NR, Rovelink PW, Bruder JT, Wickham TJ, Kovesdi I, Crystal RG.** 2004. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. J Virol **78:**2572–2580. http://dx.doi.org/10.1128/JVI.78.5.2572-2580.2004.
29. **Okada N, Tsukada Y, Nakagawa S, Mizuguchi H, Mori K, Saito T, Fujita T, Yamamoto A, Hayakawa T, Mayumi T.** 2001. Efficient gene delivery into dendritic cells by fiber-mutant adenovirus vectors. Biochem Biophys Res Commun **282:**173–179. http://dx.doi.org/10.1006/bbrc.2001.4527.
30. **Wickham TJ, Tzeng E, Shears LL, II, Roelvink PW, Li Y, Lee GM, Brough DE, Lizonova A, Kovesdi I.** 1997. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J Virol **71:**8221–8229.
31. **Lindsay RW, Darrah PA, Quinn KM, Wille-Reece U, Mattei LM, Iwasaki A, Kasturi SP, Pulendran B, Gall JG, Spies AG, Seder RA.** 2010. $CD8^+$ T cell responses following replication-defective adenovirus serotype 5 immunization are dependent on $CD11c^+$ dendritic cells but show redundancy in their requirement of TLR and nucleotide-binding oligomerization domain-like receptor signaling. J Immunol **185:**1513–1521. http://dx.doi.org/10.4049/jimmunol.1000338.
32. **Suleman M, Galea S, Gavard F, Merillon N, Klonjkowski B, Tartour E, Richardson J.** 2011. Antigen encoded by vaccine vectors derived from human adenovirus serotype 5 is preferentially presented to $CD8^+$ T lymphocytes by the $CD8\alpha^+$ dendritic cell subset. Vaccine **29:**5892–5903. http://dx.doi.org/10.1016/j.vaccine.2011.06.071.
33. **Okada N, Saito T, Masunaga Y, Tsukada Y, Nakagawa S, Mizuguchi H, Mori K, Okada Y, Fujita T, Hayakawa T, Mayumi T, Yamamoto A.** 2001. Efficient antigen gene transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. Cancer Res **61:**7913–7919.
34. **Harui A, Roth MD, Vira D, Sanghvi M, Mizuguchi H, Basak SK.** 2006. Adenoviral-encoded antigens are presented efficiently by a subset of dendritic cells expressing high levels of $\alpha_v\beta_3$ integrins. J Leukoc Biol **79:**1271–1278. http://dx.doi.org/10.1189/jlb.1105694.
35. **Yang TC, Dayball K, Wan YH, Bramson J.** 2003. Detailed analysis of the $CD8^+$ T-cell response following adenovirus vaccination. J Virol **77:**13407–13411. http://dx.doi.org/10.1128/JVI.77.24.13407-13411.2003.
36. **Swaney LM.** 1988. A continuous bovine kidney cell line for routine assays of foot-and-mouth disease virus. Vet Microbiol **18:**1–14. http://dx.doi.org/10.1016/0378-1135(88)90111-3.
37. **LaRocco M, Krug PW, Kramer E, Ahmed Z, Pacheco JM, Duque H, Baxt B, Rodriguez LL.** 2013. A continuous bovine kidney cell line constitutively expressing bovine αvβ6 integrin has increased susceptibility to foot-and-mouth disease virus. J Clin Microbiol **51:**1714–1720. http://dx.doi.org/10.1128/JCM.03370-12.
38. **Brake DA, McIlhaney M, Miller T, Christianson K, Keene A, Lohnas G, Purcell C, Neilan J, Schutta C, Barrera J, Burrage T, Brough DE, Butman BT.** 2012. Human adenovirus-vectored foot-and-mouth disease vaccines: establishment of a vaccine product profile through in vitro testing. Dev Biol (Basel) **134:**123–133.
39. **Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovesdi I.** 1996. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. J Virol **70:**6497–6501.
40. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods **9:**671–675. http://dx.doi.org/10.1038/nmeth.2089.
41. **Stave JW, Card JL, Morgan DO.** 1986. Analysis of foot-and-mouth disease virus type O1 Brugge neutralization epitopes using monoclonal antibodies. J Gen Virol **67:**2083–2092. http://dx.doi.org/10.1099/0022-1317-67-10-2083.
42. **Diaz-San Segundo F, Dias CC, Moraes MP, Weiss M, Perez-Martin E, Salazar AM, Grubman MJ, de los Santos T.** 2014. Poly ICLC increases the potency of a replication-defective human adenovirus vectored foot-and-mouth disease vaccine. Virology **468–470:**283–292.
43. **Sei JJ, Ochoa AS, Bishop E, Barlow JW, Golde WT.** 2014. Phenotypic, ultra-structural, and functional characterization of bovine peripheral blood dendritic cell subsets. PLoS One **9:**e109273. http://dx.doi.org/10.1371/journal.pone.0109273.
44. **Baxt B, Morgan DO, Robertson BH, Timpone CA.** 1984. Epitopes on foot-and-mouth disease virus outer capsid protein VP1 involved in neutralization and cell attachment. J Virol **51:**298–305.
45. **Bergelson JM, Krithivas A, Celi L, Droguett G, Horwitz MS, Wickham T, Crowell RL, Finberg RW.** 1998. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. J Virol **72:**415–419.
46. **Krause A, Whu WZ, Qiu J, Wafadari D, Hackett NR, Sharma A, Crystal RG, Worgall S.** 2013. RGD capsid modification enhances mucosal protective immunity of a non-human primate adenovirus vector expressing *Pseudomonas aeruginosa* OprF. Clin Exp Immunol **173:**230–241. http://dx.doi.org/10.1111/cei.12101.
47. **Staba MJ, Wickham TJ, Kovesdi I, Hallahan DE.** 2000. Modifications of the fiber in adenovirus vectors increase tropism for malignant glioma models. Cancer Gene Ther **7:**13–19. http://dx.doi.org/10.1038/sj.cgt.7700104.
48. **Diaz-San Segundo F, Moraes MP, de Los Santos T, Dias CC, Grubman MJ.** 2010. Interferon-induced protection against foot-and-mouth disease virus infection correlates with enhanced tissue-specific innate immune cell infiltration and interferon-stimulated gene expression. J Virol **84:**2063–2077. http://dx.doi.org/10.1128/JVI.01874-09.
49. **de Avila Botton S, Brum MC, Bautista E, Koster M, Weiblen R, Golde WT, Grubman MJ.** 2006. Immunopotentiation of a foot-and-mouth disease virus subunit vaccine by interferon alpha. Vaccine **24:**3446–3456. http://dx.doi.org/10.1016/j.vaccine.2006.02.011.
50. **Díaz-San Segundo F, Rodríguez-Calvo T, de Avila A, Sevilla N.** 2009. Immunosuppression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. PLoS One **4:**e5659. http://dx.doi.org/10.1371/journal.pone.0005659.
51. **Doel TR, Williams L, Barnett PV.** 1994. Emergency vaccination against foot-and-mouth disease: rate of development of immunity and its implications for the carrier state. Vaccine **12:**592–600. http://dx.doi.org/10.1016/0264-410X(94)90262-3.
52. **O'Garra A.** 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity **8:**275–283. http://dx.doi.org/10.1016/S1074-7613(00)80533-6.
53. **Seder RA, Ahmed R.** 2003. Similarities and differences in $CD4^+$ and $CD8^+$ effector and memory T cell generation. Nat Immunol **4:**835–842. http://dx.doi.org/10.1038/ni969.