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Citation for published version:
https://doi.org/10.1128/JVI.05127-11

Digital Object Identifier (DOI):
10.1128/JVI.05127-11

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Journal of Virology

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Differential Effects of Viral Vectors on Migratory Afferent Lymph Dendritic Cells In Vitro Predict Enhanced Immunogenicity In Vivo

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Received 15 May 2011/Accepted 27 June 2011

Targeting dendritic cells (DC) is key to driving effective immune responses. Lymphatic cannulation provides access to the heterogeneous populations of DC draining peripheral sites in rodents and ruminants. Afferent lymph DEC-205⁺ CD11c⁺ SIRPα⁺ DC were preferentially infected ex vivo with three vaccine viral vectors: recombinant human replication-defective human adenovirus 5 (rhuAdV5), recombinant modified vaccinia virus Ankara (rMVA), and recombinant fowlpox virus (rFPV), all expressing green fluorescent protein (GFP). The rhuAdV5-infected cells remained viable, and peak GFP expression was observed 16 to 24 h posttransduction. Increasing the incubation period of DC with rhuAdV5 enhanced GFP expression. In contrast, DC infected with rMVA-GFP or rFPV-GFP became rapidly apoptotic and GFP expression peaked at 6 h postinfection. Delivery of foot-and-mouth disease virus (FMDV) A₂₂ antigen to DC by rhuAdV5-FMDV-A₂₂ ex vivo resulted in significantly greater CD4⁺ T cell proliferation than did delivery by rFPV-FMDV-A₂₂. Delivery of rhuAdV5-GFP in oil adjuvant in vivo, to enhance DC-vector contact, resulted in increased GFP expression in migrating DC compared to that with vector alone. Similarly, CD4⁺ T cell responses were significantly enhanced when using rhuAdV5-FMDV-A₂₂ in adjuvant. Therefore, the interaction between viral vectors and afferent lymph DC ex vivo can predict the outcome of in vivo immunization and provide a means of rapidly assessing the effects of vector modification.

Dendritic cells (DC) are potent antigen-presenting cells capable of priming naïve T lymphocytes and are central to the induction of immune responses following infection or vaccination. To present antigens to naïve T cells, DC must migrate from peripheral tissues to the closest lymph node through the lymphatic vessels. Lymph, containing the migrating DC, can be collected by cannulation of the afferent lymphatic vessels (22, 24). The collection of migrating DC using this method has provided invaluable information on their phenotype and functions. It has been possible to cannulate the lymphatic vessels of a number of animal species; however, the procedure remains technically challenging and successful only for the thoracic duct in the rat and other lymph vessels in large animals (2, 16, 24, 44). These lymph DC are a valuable reagent to study the interaction with potential vaccine antigens because they can be used directly ex vivo without extensive in vitro manipulations. The development of techniques to purify and/or mature dendritic cells from bone marrow, blood monocytes (MoDC), or spleen has been an essential first step toward understanding the physiologic function of these cells. However, these models have limitations, especially in terms of relevance in vaccination protocols which rely on the delivery of antigen parentally. Another drawback for using MoDC is that these are poorly transduced by one of the most promising recombinant vaccine vectors, human replication-deficient recombinant adenovirus 5 (rhuAdV5) (6, 23, 39).

Afferent lymphatic DC (ALDC) represent a major population of migrating DC (11, 12, 19, 60) with functional and phenotypic heterogeneity. In cattle, various populations of skin-draining ALDC have been described by their differential expression of SIRPα (CD172a), CD11a, CD26, and CD13 (4, 19, 20, 30). These populations have been shown to interact with and stimulate T cells differently and have differential cytokine secretion profiles (25, 30, 43, 54). An example of the heterogeneity of these DC is the presence of a population of migratory DC characterized by the lack of expression of SIRPα (18, 37, 40, 60). This subpopulation of DC is present at a higher percentage in lymph draining from mucosal surfaces and has been proposed to be involved in the maintenance of tolerance (18, 31). A detailed understanding of in vivo populations of DC at relevant anatomical sites is important to vaccine design.

In vivo targeting of dendritic cells by vaccines is an attractive approach to improve vaccination strategies (7). However, the study of vaccine-DC interactions ex vivo is limited by the availability of the relevant dendritic cells, that is, DC draining the head mucosae in intranasal vaccination protocols and DC draining the skin in subcutaneous/intradermal/intramuscular (i.m.) vaccination approaches.

The viral vectors (rhuAdV5, recombinant modified vaccinia virus Ankara [rMVA], and recombinant fowlpox virus [rFPV]) are being put through trials for use as vaccine vectors; therefore, we have tried to elucidate mechanisms to improve antigen uptake by DC. Most vaccination strategies use the subcutaneous and intramuscular delivery of antigen (33, 46). Therefore,
we have used a number of recombinant vectors expressing green fluorescent protein (GFP) to identify the dendritic cell subsets which are naturally targeted by these vectors.

In this study, we use dendritic cells collected from calves which had their cervical lymph nodes surgically removed and the draining lymphatic vessels cannulated for the collection of cells draining the skin, providing a source of cells relevant in subcutaneous/intramuscular vaccination protocols. We analyzed the interaction of these ALDC with human adenovirus 5 (rAd5), recombinant modified vaccinia virus Ankara (rMVA), and recombinant fowlpox virus (rFPV) and performed ex vivo functional studies. We show that the results of our ex vivo studies, with the most promising vaccine candidate being rAd5, could be used to predict the outcome of in vivo vaccination studies.

**MATERIALS AND METHODS**

**Pseudoafflent lymphatic cannulation.** Outbred conventionally reared 6-month-old Friesian Holstein calves (Bos taurus) from the Institute for Animal Health (IAH) herd were used for these studies. Cannulations were performed essentially as previously described (24). Lymph was collected into sterile plastic bottles containing heparin (10 U/ml), penicillin, and streptomycin. Either the lymph collected was fresh (for uptake studies), or it was centrifuged (300 × g, 8 min) and resuspended in fetal calf serum (FCS)-10% dimethyl sulfoxide (DMSO) and the cells were stored in liquid nitrogen prior to use. For uptake studies, the mononuclear cells were isolated from the afferent lymph by density gradient centrifugation over Histopaque (1083; Sigma). Animal experiments were approved by the IAH ethics committee according to national United Kingdom guidelines.

**MAB and flow cytometry.** Fluorochrome-labeled mouse anti-bovine monoclonal antibodies (MAB) used in this study have been described in detail previously (24, 26–30, 50). These were CC28-allophycocyanin (APC) (anti-DEC205), CC21-phycoerythrin (PE) (anti-CD21), CC14-PE (anti-CD1b), CC19-peridinin chlorophyll protein (PerCP)/Cy5.5 (anti-SIRPA), ILA-16-Alexa Fluor 680 (anti-CD11c), ILA-21-PE (anti-major histocompatibility complex class II [anti-MHC II]), ILA-156-PE (anti-CD4), ILA-252/3-PE (anti-CD80), ILA-159-PE (anti-CD86), CC30-APC/Cy5.5 (anti-CD4), CC36-APC/CF70 (anti-CD8), ILA-111-Alexa Fluor 610/PE (anti-CD25), and CC302-PE (anti-gamma interferon [anti-IFN-γ]). Expression of mannose receptor (MR) was detected with a commercial MAB (clone CD206) from Beckman Coulter. Control MAB were isotype- and concentration-matched anti-avian MAB (24, 59). The cells were analyzed using an LSRFortessa cell analyzer (Becton Dickinson), and staining was assessed using FCS Express (DeNovo Software).

**Cell sorting.** Cells were sorted into the various dendritic cell populations using an FACSaria cell sorter (Becton Dickinson), and purities were confirmed by flow cytometry using FACSDiva v5 (Becton Dickinson). T cell subsets were magnetically isolated using MACS technology (Miltenyi Biotech, Germany) according to the manufacturer’s instructions. Briefly, GFP or FMDV A22, Iraq P1-2A-3B-3C was cloned into pENTR4 followed by LR clonase into the E1- and E3-deleted pAd/PL-DEST AdHu5 genome vector.

**Recombinant vectors.** The generation of recombinant MVA and FPV expressing GFP has been described previously (3, 13). Replication-deficient human adenovirus 5 (rHuAd5) recombinant modified vaccinia virus Ankara (rMVA), and recombinant fowlpox virus (rFPV) and performed ex vivo functional studies. We show that the results of our ex vivo studies, with the most promising vaccine candidate being rHAd5, could be used to predict the outcome of in vivo vaccination studies.

**Infection of afferent lymph cells.** Afferent lymph cells were cultured in tissue culture medium (TCM; Iscove’s modified Dulbecco’s medium [IMDM] containing 10% FCS [Autogen Bioclear, United Kingdom], 10−5 M 2-mercaptoethanol [Sigma-Aldrich, Poole, United Kingdom]) with the recombinant viruses at optimal multiplicities of infection (MOI); these were determined by titrating the recombinant viruses on ALDC (MOI of 1, 10, 100, and 1,000), and the optimal ratios were found to be as follows: rAd5, 100; rFPV, 1 PFU/cell. The frequency of cells expressing GFP was less than 1%, and approximately 8% were apoptotic, when infected with rHAd5 at MOI of 1 and 10; similar values were detected in mock-infected cultures. When cells were infected with rHAd5 at an MOI of 100 or above, approximately 34% of cells expressed GFP and approximately 9% of cells were apoptotic. In rMVA- and rFPV-infected cultures, no effect on cell viability was seen at an MOI of 0.1 but approximately 55% of cells were apoptotic at MOI of 1 and 10 after 3 h of infection. No GFP expression was detected in cells infected at an MOI of 0.1, but approximately 20% of cells were GFP positive at an MOI of 1. Subsequently, studies were performed by incubating cells at 37°C with rHAd5 at an MOI of 100 for 16 to 24 h and with rMVA and rFPV at an MOI of 1 for 6 h. Cells were extensively washed following infection and fixed with 1% formaldehyde for 30 min prior to flow cytometric analysis.

**Recombinant virus uptake by afferent lymph veiled cells in vivo.** For in vivo analysis of uptake, 5 × 10^6 cells of rHAd5-GFP (46) in 0.5 ml phosphate-buffered saline (PBS) were emulsified with 0.5 ml Montanide ISA-200 (Seppic, France) and injected either intramuscularly or subcutaneously in the area draining to the pseudoafferent lymphatic vessel (24). Lymph was collected at 4-hour intervals up to 12 h and then at 48 h and 72 h. The lymph cells were cultured for 12 h at 37°C in the presence of penicillin-streptomycin-gentamicin (1 μg/ml), to allow the expression of GFP. Then, cells were fixed for 30 min with 1% formaldehyde and subjected to multicolor flow cytometric analysis.

**Apoptosis assays.** Cells were resuspended to 1 × 10^6 cells per 0.1 ml in mixed prepared reagents of the TACS annexin apoptosis kit (R&D Systems, Minneapols, MN) according to the manufacturer’s instructions and analyzed by flow cytometry.

**Antigen presentation and T cell responses.** Populations of dendritic cells were purified by fluorescence-activated cell sorting (FACS), washed five times with phosphate-buffered saline (PBS), and incubated with recombinant virus or TCM for 60 min at 37°C. Cells were incubated with 5 × 10^6 cells of rHUAd5 at an MOI of 100 or above, approximately 34% of cells expressed GFP and approximately 9% of cells were apoptotic. In rMVA- and rFPV-infected cultures, no effect on cell viability was seen at an MOI of 0.1, but approximately 20% of cells were GFP positive at an MOI of 1. Subsequently, studies were performed by incubating cells at 37°C with rHAd5 at an MOI of 100 for 16 to 24 h and with rMVA and rFPV at an MOI of 1 for 6 h. Cells were extensively washed following infection and fixed with 1% formaldehyde for 30 min prior to flow cytometric analysis.

**Peptide antigen presentation and T cell responses.** Populations of dendritic cells were purified by fluorescence-activated cell sorting (FACS), washed five times with phosphate-buffered saline (PBS), and incubated with recombinant virus or TCM for 60 min at 37°C. Cells were incubated with 5 × 10^6 cells of rHUAd5 at an MOI of 100 or above, approximately 34% of cells expressed GFP and approximately 9% of cells were apoptotic. In rMVA- and rFPV-infected cultures, no effect on cell viability was seen at an MOI of 0.1, but approximately 20% of cells were GFP positive at an MOI of 1. Subsequently, studies were performed by incubating cells at 37°C with rHAd5 at an MOI of 100 for 16 to 24 h and with rMVA and rFPV at an MOI of 1 for 6 h. Cells were extensively washed following infection and fixed with 1% formaldehyde for 30 min prior to flow cytometric analysis.
Vaccination protocols. To assess whether expression of antigen by DC transduced by rhuAdV5 would improve vaccine immunogenicity in vivo, groups of outbred weight- and age-matched conventionally reared Friesian Holstein cattle (n = 3) were vaccinated intramuscularly with 1 ml of rhuAdV5-FMDV-A22 (5 × 10^8 IU/animal) in the presence or absence of water-in-oil-in-water adjuvant (Montanide ISA-206V; Seppic, France) or conventional inactivated FMDV vaccine (O, Manisa/A22, frag; Intervet, United Kingdom) or PBS.

**Statistical analyses.** Data are presented as means ± standard deviations, and groups were compared using one-way analysis of variance (ANOVA) and pairwise comparison using GraphPad Prism v5. Data for in vivo migration of DC following inoculation of rhuAdV5-GFP were analyzed using linear mixed models including time and treatment and an interaction between these factors as fixed effects and animal as a random effect. This interaction was significant (as judged by the Akaike information criterion) for all analyses. Results for “adjuvant only” effects and animal as a random effect. This interaction was significant (as judged by the Akaike information criterion) for all analyses. Results for “adjuvant only” were excluded from all analyses except percent DEC-205, because the observations for this factor were all zero.

**RESULTS**

Dendritic cells draining the skin are effectively targeted by recombinant vectors. The subsets of dendritic cells that drain the skin of cattle (27) and an improved method for collecting such cells have been described previously (24). Afferent lymph cells draining the skin were isolated and cultured in vitro with either rhuAdV5, rMVA, or rFPV. In initial experiments, viruses expressing GFP were utilized for ease of detection by flow cytometry. A significant increase of GFP expression was observed as early as 3 h postinfection with rMVA and rFPV (P = 0.0003) but no earlier than 12 h following transduction with rhuAdV5 (P = <0.0001) (Fig. 1A). Peak GFP expression was observed at 6 h with both rMVA and rFPV, in cells remaining viable, after which time GFP expression remained stable (rMVA) or decreased significantly (rFPV). Expression of GFP by ALDC transduced with rhuAdV5 continued to increase up to 24 h. Virus replication was not productive in ALDC infected with rMVA or rFPV as measured by plaque assay and immunofluorescence to detect virus capsids using nonspecific sera (data not shown).

Dendritic cells infected with all three recombinants expressed GFP, with rhuAdV5 transducing a higher percentage of FSC^high^ DEC-205^+^ cells and fluorescence intensity being higher than that with both rMVA and rFPV (Table 1). Interestingly, total numbers of FSC^high^ DEC-205^+^ cells in the cultures infected with rMVA and rFPV were reduced (Fig. 1B and Table 1). To identify whether this was due to virus-induced cytotoxicity and subsequent reduction in GFP expression, we measured cell death using propidium iodide and annexin V-fluorescein isothiocyanate (FITC) at various time points postinfection. Cells transduced with rhuAdV5 remained viable throughout the infection period. In contrast, DC infected with rMVA and rFPV became apoptotic (Fig. 1C).

In contrast to ALDC, bovine monocyte-derived DC (MoDC) and bone marrow-derived DC matured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were not infected by rhuAdV5, and the quantity and kinetics of GFP expression in rMVA- and rFPV-infected MoDC and bone marrow-derived DC were similar to those of ALDC (data not shown).

**Virus vectors infect various subsets of DC.** Dendritic cells draining the skin and muscle were defined as FSC^high^ DEC-205^+^ (Fig. 1D); all of these DC were CD11c^+^CD8^+^ (Fig. 1E). Within the DEC-205^+^ DC, there were subpopulations of DC which differentially expressed CD1b, CD21, and SIRPα. To identify virus vector tropism in subsets of DC, we added GFP-expressing viruses to freshly isolated pseudoafferent lymph cells and utilized multicolor flow cytometry to identify those DC which expressed GFP. All three viral vectors preferentially infected DEC-205 SIRPα^+^ dendritic cells (Fig. 1F), with significant GFP expression being observed in SIRPα^+^ cells (40.92, 31.57, and 26.02% of GFP expression for rhuAdV5, rMVA, and rFPV, respectively) but not SIRPα^-^ cells. Within this subpopulation, rhuAdV5 preferentially transduced CD1b^+^ DC; rMVA infected CD1b^+^ and rFPV infected CD1b^-/+^ dendritic cells (Fig. 1G). There was evidence for downregulation of CD1b by rFPV. CD1b^+^ DC were purified and then infected with rFPV; after 6 h in culture, viable CD1b^-^ GFP-positive cells were detected (data not shown). rhuAdV5 and rMVA infected both CD21^+^ and CD21^-^ cells; in contrast, GFP expression in rFPV-infected DC was mostly detected in CD21^-^ cells (Fig. 1H).

To further assess whether vector tropism was limited to the SIRPα^+^ DC, we purified FSC^high^ DEC-205^+^ SIRPα^+^ and SIRPα^-^ DC and cultured these with the recombinant viral vectors expressing GFP. Interestingly, SIRPα^-^ DC, which were not targeted by recombinant vectors in mixed culture (Fig. 1F), showed GFP expression similar to that observed in SIRPα^+^ DC (Fig. 2A) when purified cell cultures were utilized. Significant differences between the percentages of SIRPα^-^ and SIRPα^-^ DC expressing GFP were observed only for rMVA (P = 0.03). The enhanced expression of GFP (mean fluorescence intensity [MFI]) in total DC populations (Table 1) infected with rhuAdV5 compared to those infected with rMVA and rFPV was also observed with purified SIRPα^-^ and SIRPα^-^ DC populations (P = 0.001; Fig. 2B).

**rMVA and rFPV regulate costimulatory molecules.** Dendritic cells draining the skin and muscle express the costimulatory molecules MHC II, CD40, CD80, and CD86 (24). Culturing cells in tissue culture medium for 6 h without the addition of cytokines, or viral vectors, increased the expression of CD40 and CD86, but not of CD80 and MHC II, on SIRPα^+^ DC (Table 2); similar results were seen in gated SIRPα^-^ DC populations (data not shown). To identify whether the recombinant vectors had any effect on the expression of costimulatory molecules, freshly isolated DC were infected with recombinant viruses and analysis was performed on gated SIRPα^-^ DC. Transduction with rhuAdV5 did not have a significant effect on the expression of any of the costimulatory molecules measured 16 h posttransduction compared to cells cultured for the same time period in medium alone, although expression of each molecule was significantly increased compared to the control (P < 0.005). In contrast, at the time point where peak GFP expression was observed in DC infected with rMVA (6 h), there was significant downregulation of CD40, CD86, and MHC II (Table 2) but not of CD80 compared to DC cultured in medium alone. Recombinant fowlpox virus-infected DC showed downregulation of CD40 only; CD80, CD86, and MHC II were not affected at 6 h (Table 2). No significant changes in costimulatory molecule expression were observed in the gated SIRPα^-^ DC populations after incubation with the recombinant vectors compared to culture in medium alone.

SIRPα^-^ DC efficiently present antigen to T lymphocyte subsets. We investigated the ability of DC transduced with rhuAdV5 or infected with rFPV to present foot-and-mouth
FIG. 1. Dendritic cells infected with the recombinant viruses expressed GFP. (A) Afferent lymph cells were transduced with rhuAdV5, rMVA, or rFPV and analyzed by flow cytometry. Dendritic cells were gated as FSC<sup>high</sup> DEC-205<sup>+</sup> cells, and the percentage of GFP-expressing cells was calculated at the indicated time points (n = 4). NoV, no virus. (B) Dot plots of afferent lymph cells expressing DEC-205<sup>+</sup> following infection with recombinant viruses; green events represent GFP<sup>+</sup> cells. (C) Dendritic cells were gated as FSC<sup>high</sup> DEC-205<sup>+</sup> cells, and dot plots show the percentages of apoptotic/dead cells measured using propidium iodide (PI) and annexin V. (D) Dendritic cells draining the skin were gated as FSC<sup>high</sup> DEC-205<sup>+</sup>. (E) Expression of CD11c<sup>+</sup> and CD8α<sup>+</sup> was assessed. (F) Vector-encoded GFP expression was analyzed in subsets of FSC<sup>high</sup> DEC-205<sup>+</sup> DC expressing SIRPα<sup>+</sup>. (G) Expression of FSC<sup>high</sup> DEC-205<sup>+</sup> SIRPα<sup>+</sup> CD1b<sup>+</sup>. (H) Expression of FSC<sup>high</sup> DEC-205<sup>+</sup> SIRPα<sup>+</sup> CD21<sup>+</sup>. (F to H) Dendritic cells were infected with rhuAdV5-GFP for 16 h (n = 5) or infected with rMVA or rFPV for 6 h (n = 5). Error bars indicate standard deviations.
disease virus (FMDV) antigens to T cells in vitro. Recombinant MVA was not tested since the expression of the FMDV antigen cassette has not been possible in vaccinia virus. Initially, various ratios of DC to T cells were tested and the optimal ratio to induce T cell activation was found to be 1:100 (data not shown). FSC\textsuperscript{high} DEC-205\textsuperscript{+}, and SIRP\textalpha\textsuperscript{+} DC were transduced with rhuAdV5-FMDV-A\textsubscript{22} or infected with rFPV-FMDV and cultured with purified autologous CD4\textsuperscript{+} T cells isolated from the cattle at 3 weeks postvaccination. DC transduced with rhuAdV5-FMDV-A\textsubscript{22} induced antigen-specific T cell proliferation more efficiently than did DC infected with rFPV-FMDV (P = 0.0003) (Fig. 3A). Both viruses induced significantly lower proliferation than did purified protein antigen (P = 0.061 and P = 0.003 for rhuAdV5 and rFPV, respectively). To identify which DC subset was responsible for antigen presentation following delivery of antigen using recombinant viruses, FACS-purified populations of DC were tested for their ability to activate purified CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from FMDV-vaccinated cattle. Only SIRP\textalpha\textsuperscript{+} DC were able to induce significant FMDV-specific IFN-\gamma expression by both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (P > 0.001 compared to SIRP\textalpha\textsuperscript{−}) (Fig. 3B and 3C). rhuAdV5-FMDV induced a higher expression of IFN-\gamma than did purified protein antigen as a positive control for CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells, respectively (P = 0.0003 and P = 0.0471, respectively). rFPV-FMDV induced more IFN-\gamma than did the FMDV antigen on CD4\textsuperscript{+} T cells only (P = 0.002).

Interestingly, SIRP\textalpha\textsuperscript{−} DC subsets were infected in purified cultures, but they were unable to present antigen to CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (Fig. 3B and 3C, respectively).

Skin-draining DEC-205\textsuperscript{+} dendritic cells are efficiently transduced by recombinant adenovirus in vivo. Subsequent to demonstrating the transduction capacity of rhuAdV5 on antigen-presenting DC and their capacity to effectively present antigen in vitro, we investigated the ability of rhuAdV5 delivered either intramuscularly or subcutaneously to infect skin-draining DC in vivo. Cattle were inoculated subcutaneously above the site of cannulation with rhuAdV5-GFP (5 \times 10\textsuperscript{7} IU/animal) in the presence or absence of a water-in-oil-in-water adjuvant (Montanide ISA-206V). Draining DC were collected at 4-h intervals, and the phenotype of the draining DC and GFP expression was measured by flow cytometry. There was no significant change over time in the frequency of migrating DC (FSC\textsuperscript{high} DEC-205\textsuperscript{+}) in the groups of animals inoculated with rhuAdV5-GFP without adjuvant or those inoculated with adjuvant alone. However, there was a significant rise (P < 0.0003) during the first 15 h in the frequency of migrating DC in the groups of animals vaccinated with rhuAdV5-GFP in the presence of adjuvant (Fig. 4A). We analyzed the phenotypic composition of subsets of DC following inoculation. The frequencies of migration of GFP-positive FSC\textsuperscript{high} DEC-205\textsuperscript{+}, FSC\textsuperscript{high} DEC-205\textsuperscript{+} SIRP\textalpha\textsuperscript{−}, and FSC\textsuperscript{high} DEC-205\textsuperscript{+} SIRP\textalpha\textsuperscript{+} CD1b\textsuperscript{+} DC (Fig. 4B, C, and D) were significantly (P = 0.0348, P = 0.0092, and P = 0.0395, respectively) increased following subcutaneous inoculation of rhuAdV5 in the presence of adjuvant. GFP expression in DC was evident as early as 5 h postinoculation and detectable by flow cytometry for up to 15 h in the FSC\textsuperscript{high} DEC-205\textsuperscript{+} subset (Fig. 4B), the FSC\textsuperscript{high} DEC-205\textsuperscript{+} SIRP\textalpha\textsuperscript{−} subset (Fig. 4C), and the FSC\textsuperscript{high} DEC-205\textsuperscript{+} SIRP\textalpha\textsuperscript{+} CD1b\textsuperscript{+} subset (Fig. 4D). Similar GFP expression profiles were observed in animals inoculated intramuscularly with rhuAdV5-GFP in the presence of water-in-oil-in-water adjuvant (data not shown).

Induction of antigen-specific responses in vivo with rhuAdV5-FMDV-A\textsubscript{22}. Further to demonstrate that rhuAdV5 is capable of transducing DC both in vitro and in vivo and that this enhances antigen presentation in vitro, we investigated the possibility of enhancing the immune response to FMDV using

### TABLE 1. Infection of dendritic cells in vitro by recombinant vectors

<table>
<thead>
<tr>
<th>DC No.</th>
<th>No virus</th>
<th>rhuAdV5</th>
<th>rMVA</th>
<th>rFPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>% expressing GFP</td>
<td>0.34 ± 0.01</td>
<td>34.09 ± 8.1</td>
<td>29.98 ± 9.4</td>
<td>24 ± 8.7</td>
</tr>
<tr>
<td>MFI (GFP)</td>
<td>109 ± 11</td>
<td>570 ± 33</td>
<td>172 ± 22</td>
<td>257 ± 31</td>
</tr>
<tr>
<td>No. expressing GFP</td>
<td>41 ± 5</td>
<td>3677 ± 98</td>
<td>683 ± 35</td>
<td>550 ± 15</td>
</tr>
</tbody>
</table>

\( ^{a} \) Dendritic cells draining the skin were gated as FSC\textsuperscript{high} DEC-205\textsuperscript{+}. Dendritic cells were infected with rAdV5-GFP for 16 h (n = 5) or infected with rMVA or rFPV for 6 h (n = 5). Standard deviations are shown.

![FIG. 2. Purified subsets can be more readily infected than mixed populations. FACS-sorted DC subsets were transduced for 16 h with rhuAdV5 (n = 5) or infected for 6 h with rMVA and rFPV (n = 10). GFP expression was measured by flow cytometry. (A) Frequency of GFP-expressing DC subsets. (B) Mean fluorescence intensity (MFI) of GFP expression. Error bars indicate standard deviations.](image-url)
rhuAdV5 plus adjuvant. Groups of calves (n = 3) were inoculated intramuscularly (i.m.) with rhuAdV5-FMDV-A22 at 5 × 10^9 IU/animal, the same dose used in previous vaccination studies (46), with or without adjuvant, PBS prepared in the same adjuvant, or a commercially available inactivated FMDV vaccine. Both groups of cattle immunized with rhuAdV5-FMDV-A22 developed FMDV-specific IgG antibody responses comparable to but not statistically different from those of the animals vaccinated with conventional vaccine; the negative-control group did not seroconvert (data not shown).

CD4^+ and CD8^+ FMDV-specific proliferation was measured by CFDA-SE dilution; neither group immunized with rhuAdV5-FMDV-A22 showed a significantly increased T cell proliferative response (Fig. 5A and B).

FMDV-specific cellular responses were also measured by ELISpot assay and intracellular cytokine staining. MACS-sorted CD4^+ T cells from animals vaccinated with recombinant vector in adjuvant showed significantly higher IFN-γ release to FMDV antigen than did those animals vaccinated with recombinant vector in the absence of adjuvant or animals vaccinated with inactivated FMDV (P = 0.0002) (Fig. 5C).

We also analyzed the phenotype of T cell responses following vaccination by flow cytometry. The frequency of CD4^+ IFN-γ^+ and CD4^+ tumor necrosis factor alpha-positive (TNF-α^+) (Fig. 5D and E, respectively) T cells was increased in the group immunized with rhuAdV5-FMDV-A22 formulated in adjuvant compared to the group immunized with rhuAdV5-FMDV-A22 alone. There was no significant difference in the frequencies of CD8^+ IL-4^+ T cells in the two groups immunized with rhuAdV5-FMDV-A22 (Fig. 5F). There was no significant difference in the frequencies of CD8^+ T cells expressing IFN-γ and TNF-α among the vaccinated groups (data not shown).

**DISCUSSION**

The main role of dendritic cells is the presentation of antigen to T cells, in particular, priming of naive T cells within the lymph node (47, 55). Most systems used to investigate DC-T cell interactions rely on the isolation of monocytes or macrophages from blood or tissues (such as spleen or bone marrow) (49, 53), followed by maturation with IL-4 and GM-CSF, or the harvesting of tissues followed by isolation of resident DC. Published results suggest that peripheral dendritic cells in the epithelia take up antigen by pinocytosis, macropinocytosis, or receptor-mediated endocytosis (5, 30, 42). Peripheral dendritic cells then migrate via the afferent lymphatic ducts to the draining lymph nodes, losing their ability to uptake antigen but becoming very potent antigen-presenting cells. This model is challenged by the identification of dendritic cells from afferent lymph, the afferent lymph veiled cells (42), which migrate from peripheral tissues which are able to process antigen and are potent stimulators of resting and naive T cells.

We have previously shown that dendritic cells draining the

![FIG. 3](image-url)
skin of cattle which were collected by cannulation of pseudo-afferent lymphatic vessels (24) express high levels of DEC-205 (CD205). DEC-205 is an endocytic receptor which mediates uptake of antigens on dendritic cells. DEC-205 is currently the only receptor which has been visualized on most DC in the T cell area of secondary lymphoid tissues, which are the sites for the generation of immunity and tolerance in mouse models and humans (7, 21). Targeting of DEC-205-expressing DC by either chimeric antigen-antibody complexes or recombinant vectors has been proposed to be an effective approach to improve immune responses (7, 57).

DC draining the skin were found to be FSC high DEC-205+/H11001 CD11c+/H11001 CD8+/H11002 cells. Within this population, two major subpopulations can be identified, those cells expressing SIRP+/H9251 and those not expressing SIRP+/H9251 (30, 42). Although both populations express high levels of costimulatory molecules (CD40, CD80, and CD86) and MHC II, it has previously been shown that only the SIRP+/H9251+/H11001-expressing cells present antigen efficiently (30).

We now show that in mixed cultures, only the DC expressing SIRP+/H9251 are efficiently targeted by the recombinant replication-deficient viral vectors human adenovirus 5 (rhuAdV5), modified vaccinia virus Ankara (rMVA), and fowlpox virus (rFPV), which all have potential use as safe vaccine vectors (14, 35, 41, 56, 61). Surprisingly, engagement of Toll-like receptor (TLR) ligands did not increase vector uptake, the quantity of antigen expression, or upregulation of cell surface markers (data not shown). Interestingly, physical separation of SIRP+/H9251 cells allowed for efficient infection by the recombinant viruses; however, these cells did not efficiently present antigen to T cells, supporting previous data (30, 37). This observation is more interesting given that SIRP+/H9251 cells represent up to 30% of ALDC and express high levels of costimulatory molecules. The DC expressing SIRP+/H9251 are represented by a heterogeneous mix of DC all capable of presenting antigen. Recombinant vectors differentially target these various subpopulations so that rhuAdV5 targets CD1b+/H11001 CD21+/H11001 DC, rMVA targets CD1b+/H11001 and both CD21+/H11001 and CD21−/H11001 DC, and rFPV targets CD1b−/H11002 and both CD21+/H11001 and CD21−/H11001 DC. The biological significance of this differential targeting of DC is still unknown.

Interestingly, during our pilot experiments we found that bovine monocyte-derived DC grown in the presence of GM-CSF and IL-4 were poorly transduced by the rhuAdV5 vector, supporting data described for the human system (6). It was also interesting to observe the frequency of vector-induced cytolysis following infection of ALDC with rMVA and rFPV even at low frequencies (MOI = 1). Although there are reports of rMVA-induced cytolysis in monocyte-derived and bone marrow-derived dendritic murine DC (36), to our knowledge
this is the first report of rMVA- and rFPV-induced cytotoxicity of dendritic cells draining tissues which would normally be targeted in vaccine delivery protocols. It is important to point out that apoptosis was observed 12 to 24 h postinfection in human MoDC and macrophages infected with MVA (62), whereas in our system we observed cell death as soon as 3 h postinfection. Apoptosis induction by live vector vaccines may have important effects on the resulting immune response. While rapid apoptosis may limit antigen expression and thus adversely impact on specific immunity to vector-encoded antigens, apoptotic responses may also be desirable for antigen uptake and presentation through the alternative class I presentation pathway (antigen cross-priming) (17, 48, 51).

We demonstrated that rhuAdV5 was the most efficient of the three vectors studied at stimulating T cell responses in vitro. MVA- and FPV-infected dendritic cells did not secrete inhibitory cytokines as measured by ELISA (data not shown), and therefore the reason for differential capacity to present vector-encoded antigens is still unknown. We also confirmed previous studies that DEC-205+/H11001SIRP+/H9251/H11001DC were the most efficient stimulators of T cell responses in vitro. Subsequently, we performed in vivo experiments in which rhuAdV5-GFP was administered to cannulated calves. We observed that the majority of GFP-expressing cells were FSChigh DEC-205+, FSChigh DEC-205+ SIRPα+, and FSChigh DEC-205+ SIRPα+ CD1b+. GFP expression in FSChigh DEC-205+ SIRPα+ cells

FIG. 5. T cell responses to FMDV following vaccination. Groups of animals (n = 3) were vaccinated with PBS, inactivated FMDV, rhuAdV5-FMD, or rhuAdV5-FMDV-A22 with adjuvant. (A) CD4+ T cell CFDA-SE proliferation at 3 weeks postvaccination. (B) CD8+ T cell CFDA-SE proliferation at 3 weeks postvaccination. (C) FMDV-specific IFN-γ from MACS-sorted CD4+ T cells was measured by ELISpot assay 6 weeks postvaccination. Results are shown as median spot-forming units (SFU) per 10⁶ cells. (D to F) FMDV-specific intracellular cytokine expression ex vivo on CD4+ T cells gated at 6 weeks postvaccination. (D) Percent IFN-γ+ on CD4+ T cells. (E) Percent TNF-α+ on CD4+ T cells. (F) Percent IL-4+ on CD4+ T cells. N.S., no significant difference. Error bars indicate standard deviations.
Similarly, the frequency of CD4+ T cells decreased after introduction of an inflammatory stimulus (32). In light of the results of our in vitro and in vivo studies, we immunized animals with rAdV5-expressing FMDV antigens in the presence or absence of a water-in-oil-in-water adjuvant. Interestingly, the use of adjuvant had a significant effect on the frequency of CD4+ IFN-γ+ and CD4+ TNF-α+ T cells. Similarly, the frequency of CD4+ T cells secreting antigen-specific IFN-γ was increased in animals vaccinated with rAdV5-FMDV in the presence of adjuvant. Our studies demonstrate that the quantity of antigen delivered by viral vectors to DC can be enhanced by using oil adjuvant formulations, with a subsequent enhancement of specific T cell responses (1, 45, 52). Furthermore, ex vivo studies of the interaction between viral vector constructs with DC from afferent lymph could be used to predict the outcome of vaccination studies with novel vaccine formulations.

ACKNOWLEDGMENTS

We gratefully acknowledge the staff at IAH for care of cattle. We thank Simon Gubbins for his help in performing statistical analysis. This work was funded by the Biotechnology and Biological Sciences Research Council, United Kingdom, and industrial partners awards from Pfizer, J. C. Hope, S. C. Gilbert, and B. Charleston are Jenner Institute Investigators.

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