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Development of an adenovirus vector vaccine platform for targeting dendritic cells

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Abstract

Adenoviral (Ad) vector vaccines represent one of the most promising modern vaccine platforms, and Ad vector vaccines are currently being investigated in human clinical trials for infectious disease and cancer. Our studies have shown that specific targeting of adenovirus to dendritic cells dramatically enhanced vaccine efficacy. However, this was achieve using a molecular adapter, thereby necessitating a two component vector approach. To address the mandates of clinical translation of our strategy, we here sought to accomplish the goal of DC targeting with a single component adenovirus vector approach. To redirect the specificity of Ad vector vaccines, we replaced the Ad fiber knob with fiber-fibritin chimeras fused to DC1.8, a single domain antibody (sdAb) specific for murine immature DC. We engineered a fiber-fibritin-sdAb chimeric molecule using the coding sequence for DC1.8, and then replaced the native Ad5 fiber knob sequence by homologous recombination. The resulting Ad5 virus, Ad5FF1.8, expresses the chimeric fiber-fibritin sdAb chimera. Infection with Ad5FF1.8 dramatically enhances transgene expression in DC 2.4 dendritic cells compared to infection with native Ad5. Ad5FF1.8 infection of bone marrow derived DC demonstrates that Ad5FF1.8 selectively infects immature DC consistent with the known specificity of DC1.8. Thus, sdAb can be used to selectively redirect the tropism of Ad5 vector vaccines, providing the opportunity to engineer Ad vector vaccines that are specifically targeted to DC, or specific DC subsets.

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CONFLICTS OF INTEREST

No conflicts to disclose.

Keywords

Adenoviral vaccine; Targeted Ad5; dendritic cell; single domain antibody

INTRODUCTION

Ad vector vaccines have emerged as one of the most promising modern vaccine platforms,[1] and there are currently a number of human clinical trials ongoing in the infectious disease and cancer vaccine fields.[2–9] Ad vector vaccines have several key biologic properties that make them attractive[10, 11]: (1) Ad vector vaccines have been deleted in E1 and are replication-defective. They have an excellent safety profile in clinical translation; (2) Ad vector vaccines can be grown to high titer in cell culture, facilitating manufacture of clinical grade material, regulatory approval and clinical translation; (3) Multiple transgenes can be inserted into Ad vector vaccines, including not only the antigen of interest but also other genes to enhance the response to vaccination, such as cytokines or danger signals; and (4) Ad vector vaccines induce potent inflammatory responses following vaccination. These inflammatory responses are associated with the induction of pro-inflammatory cytokines and stimulation of both the innate and adaptive immune systems.[12–14]

A relatively unique property of Ad vector vaccines is the fact that the cellular attachment and entry processes are molecularly distinct. The Ad fiber knob domain initiates viral attachment via the coxsackie and adenovirus receptor (CAR), a receptor expressed ubiquitously on epithelial cells. Internalization of the viral particle is then mediated by distinct molecular interactions between Arg-Gly-Asp (RGD) motifs in the Ad penton base capsomer and cellular α_v integrins on the cell surface.[15, 16] This separation of the cellular attachment and entry processes provides an opportunity to re-direct the specificity of Ad cellular attachment without compromising internalization.

A major focus in the infectious disease and cancer vaccine fields is to improve primary and memory CD8 T cell responses. Efficient delivery of antigen to specialized antigen presenting cells (APC) appears to be critical for the generation of productive CD8 T cell responses. Dendritic cells (DC) are a highly specialized subset of APC that play a critical role in regulating immune responses with the ability to steer immune responses towards immunity or tolerance depending on the activation state, location, and specific subset of DC.[17] Previous studies have demonstrated that targeting antigen to DC can dramatically enhance the efficacy of vaccines, and this has proven to be true targeting multiple different DC receptors, in the context of multiple different vaccine platforms (reviewed in [18]).

In our earlier studies we sought to modify adenovirus tropism to enhance gene delivery to DCs as a strategy to improve vaccine efficacy.[19] These studies employed the general approach of a two component “adapter”, cross-linking the adenovirus fiber knob to a DC cell surface marker.[20, 21] In various studies, we were able to show that this approach dramatically enhanced the efficiency, and specificity, of Ad-mediated gene delivery to DCs. Most importantly, we could show that these vector gains translated directly into improved vaccine outcomes in murine models of cancer immunotherapy.[22, 23] Despite these efficacy gains, the two component design of this targeting strategy presented logistical challenges vis-à-vis

clinical development.[24–26] On this basis, we sought to develop a single component adenovirus vector capable of DC-selective gene delivery.

Single-domain antibodies (sdAb) are antigen-binding antibody fragments engineered from heavy-chain antibodies found in camelids. sdAb offer the high binding affinity and specificity of conventional antibodies, combined with the small size, stability, tissue penetration, and favorable pharmacokinetics of small molecules.[27, 28] Of note, the monomeric structure and small size of sdAb offer clear advantages at the level of protein engineering in the context of redirecting the specificity of Ad vector vaccines.[29] Based on the hypothesis that sdAb would allow the most flexible, precise and readily translated DC targeting strategy, we adapted our "fiber replacement" methodology [24, 30, 31] to allow incorporation of sdAb into the Ad5 capsid. We subsequently tested the ability of engineered Ad vector vaccines incorporating a sdAb specific for immature DC to specifically infect DC.

MATERIALS AND METHODS

Generation of DC-targeted Ad5

We employed a genetic fiber modification approach to ablate the native tropism of Ad5, and redirect cellular attachment.[32] The knob domain of the Ad capsid fiber protein, was replaced with the C-terminal 95 amino acid long foldon domain from the T4 phage fibritin protein to maintain fiber trimerization while allowing incorporation of targeting moieties.[31, 33] To selectively target DC, we employed DC 1.8, a camelid sdAb that is specific for murine immature bone marrow-derived DC (BMDC) *in vitro* and *in vivo*.[34] We constructed an Ad5-based genome carrying both firefly luciferase and eGFP expression cassettes in place of the deleted early E1 and E3 regions, respectively, and encoding chimeric fiber-fibritin-DC1.8 protein in place of the endogenous fiber knob gene. This replication incompetent genome was rescued using 211B cells[35] expressing both E1 and wildtype Ad5 fiber genes, allowing packaging of the Ad genome into capsid that incorporates modified fibers along with wild type fibers resulting in fiber-mosaic virus progeny as described previously.[32] Of note, fiber-mosaic virus is only an intermediate step in the production of the virus. The viral progeny were subsequently upscaled in 211B cells, and then used to infect 293 cells in order to amplify viral particles containing only chimeric fiber-fibritin-DC1.8 proteins. The resultant CsCl-purified Ad5FF1.8 vector preparation was then analyzed by western blot to confirm incorporation of only chimeric FF1.8 fiber in the context of assembled Ad5FF1.8 viral particles. We also constructed Ad5FF, a non-targeted Ad5 vector with a chimeric fiber-fibritin construct to serve as an isogenic control.

Mice and cell lines

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions in the animal facility at Washington University School of Medicine (WUSM). All experiments were carried out in 6–12 week old mice using an institutionally-approved protocol and in accordance with the guidelines established by the WUSM Animal Studies Committee. The DC2.4 cell line [36] was received from Dr. K.L. Rock (University of Massachusetts Medical School, Worcester, MA) while

PEA-10 (catalogue number CRL-2215) and SVEC4-10 (catalogue number CRL-2181) cell lines were obtained from the American Type Culture Collection.

Bone marrow-derived DC cultures

Femurs were harvested from mice and soaked in 70% ethanol on ice for 5 minutes. Epiphyses were cut, and the BM was then flushed out using a sterile syringe (26–28 gauge needle) filled with 200 μL of culture media. BM cells were treated with Tris-ammonium chloride at room temperature for 5 minutes to lyse RBC. Cells were then resuspended in culture medium consisting of Iscove's Modified Dulbecco's Medium supplemented with 2 mM L-Glutamine, 100 I.E./mL sodium penicillin, 100 μg/mL streptomycin, 2.5 μg/mL Amphotericin B, essential and non-essential amino acids, sodium pyruvate, HEPES buffer and 10% fetal bovine serum. BM cells were plated in 6 well plates at a density of 1×10^6 cells/mL in culture media supplemented with mouse Flt3L (200 ng/mL) and/or GMCSF (300 pg/mL) at 37°C in 5% CO_2. On day 6 floating cells were removed and the GM-CSF concentration was increased to 1 ng/mL. The cells growing in monolayers were incubated for an additional 48 hours before being infected with the indicated Ad5 vectors. For generation of GM-CSF and GM-CSF/IL-4 DC cultures we used media supplemented with either GM-CSF alone (20 ng/mL), or GM-CSF (20 ng/mL) and IL-4 (20 ng/mL) respectively. For activation of DC, TNFα (100 ng/mL) was added to the culture media on day 7. All the cytokines used were obtained from PeproTech (Rocky Hill, NJ). Analysis for IL-12 was performed by ELISA following the manufacturer's instructions (IL-12p70, BioLegend, San Diego, CA).

Gene transfer assay

Monolayers of DC2.4 and BMDC were washed one time with PBS, and then infected with 3000 viral particles/cell in triplicate. After one hour, the media was removed, the tissue culture wells were washed with PBS and fresh media was added. Then the infected cells were incubated for 24 hours at 37°C and 5% CO_2. GFP expression was evaluated using an inverted immunofluorescence microscope (EVOS FL Cell Imaging System, ThermoFisher Scientific).

Flow Cytometry

BMDC were stained with mAb specific for CD45RA (14.8-BV650, Cat. # 564360, BD Biosciences), B220 (RA3-6B2-PE/Cy7, Cat. # 103221, BioLegend), CD11c (N418-APC, Cat. # 117309, BioLegend), CD11b (M1/70-BV711, Cat. # 101241, BioLegend), class II MHC (M5/114.15.2-PerCP/Cy5.5, Cat. # 107625, BioLegend), CD8α (53-6.7-PE/Cy5, Cat. # 100709, BioLegend), CD24 (M1/69-Pacific Blue, Cat. # 10819, BioLegend), and SIRPα (P84-PE, Cat. # 14011, BioLegend). Cell sorting and analysis was performed on a LSRFortessa (BD Biosciences) instrument. Post acquisition analysis was performed using FlowJo software v10.1 (Tree Star Inc., Ashland, OR).

Luciferase Assay

The luciferase assay system (Promega) and ORION microplate luminometer (Berthold Detection systems, Oak Ridge, TN) were used for the evaluation of luciferase activity in

transduced cells. The DC Protein assay (Bio-Rad, Hercules, CA) was used to normalize the protein concentration of the cell lysate. Activity is reported in relative light units (RLU) per 1×10^6 cells.

In vivo characterization

In vivo experiments involving mice were carried out under protocol nos. 20140289 and 20110035 approved by the Washington University Animal Studies Committee. C57BL/6 mice at 10 weeks of age, obtained from Jackson Laboratory (Bar Harbor, ME, USA), were used in the present work. For *in vivo* distribution, mice were injected intradermally with 1×10^{11} particles of virus in 200 μL of saline. Seventy-two hours post virus administration, mice were anesthetized with 2.5% 2, 2, 2-tribromoethanol (Avertin, Sigma-Aldrich, St Louis, MO, USA), left -ventricle perfused with phosphate-buffered saline (PBS) followed by 10% neutral-buffered formalin. Mouse tissues were harvested, post-fixed in formalin for 2–4 hours at room temperature, cryopreserved in 30% sucrose for 16 hours at 4°C, and cryo-embedded in NEG50 (Thermo Fisher Scientific, Waltham, MA) over 2-methylbutane chilled in liquid nitrogen. For immunization experiments, mice were injected intradermally with 1×10^9 vp per mouse on days 0 and 7. Control mice received three immunizations with ovalbumin (OVA) cDNA at days 0, 3, and 6, as described.[37] All mice were analyzed for OVA-specific reactivity in vitro by interferon gamma (IFNγ) ELISPOT on day 11 using splenocytes. Splenocytes were tested for recognition of the OVA peptide SIINFEKL, p257–264, as described.[37]

Immunofluorescence Staining and Imaging

Sixteen-micrometer frozen sections were collected, air-dried briefly, rehydrated in PBS, blocked with protein block (5% donkey serum in PBS containing 0.1% Triton X-100), and incubated over night at 4 °C with primary antibodies including: chicken anti-GFP 1:400 (A10262, Life Technologies, Carlsbad, CA, USA) and anti-CD11c. After PBS washes for three times, the slides were incubated with corresponding Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibodies, 1:400, (Jackson ImmunoResearch Laboratories, West Grove, PA) and counterstained for nuclei with SlowFade Gold Antifade mounting reagent with 49,6-diamidino-2-phenylindole (DAPI) (Life Technologies). Immunofluorescence microscope images were collected using an FVII digital camera with Extended Focal Imaging (EFI) function (Olympus America, Center Valley, PA). The camera acquisition time for EGFP fluorescence was optimized and set a priori for each tissue.

Statistics

Data are presented as mean ± standard error of mean or standard deviation, as indicated. Graphs were drawn using Prism v6.0 (GraphPad Software, La Jolla, CA). Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The Ad5FF1.8 capsid contains chimeric fiber-fibritin-DC1.8 molecules

To redirect the specificity of Ad vector vaccines we created chimeric fiber-fibritin molecules incorporating the sdAb DC1.8 using methods we have previously described.[38] We validated

the successful incorporation of the chimeric molecules into the Ad5FF1.8 capsid by detecting similar sized protein bands using mAb against the N-terminus of the Ad fiber tail and against the polyhistidine-tag introduced into the C-terminus of DC1.8 (Fig. 1). The efficiency of chimeric fiber incorporation into the Ad5FF1.8 capsid was similar to that of the control Ad5 vector, which was constructed previously to encode wildtype fiber carrying the C-terminal polyhistidine-tag.[39]

Ad5FF1.8 efficiently transduces the murine dendritic cell line DC2.4

DC do not express CAR and the transduction efficiency using wildtype Ad5 vectors is very low. To assess whether Ad5FF1.8 could successfully transduce DC, we infected DC2.4, a murine DC cell line with Ad5FF1.8 and control Ad5. 30 ± 11% of the DC infected with Ad5FF1.8 expressed GFP compared to 3.5 ± 0.9 % infected with control Ad5 (Fig. 2A, B). The intensity of GFP expression was also higher in the DC infected with Ad5FF1.8 (MFI of GFP^+ cells 15,986 ± 411 vs 7,702 ± 106). Ad5FF1.8 was associated with higher transduction efficiency across multiple different time points (12, 24 and 48 hours) and multiple different multiplicity of infection (600, 1000, 3000 and 5000 MOI, data not shown). Similar differences were seen when we tested for expression of luciferase (69,258 vs 11,372 relative light units, data not shown). To demonstrate that replacement of the fiber knob domain of Ad5FF1.8 with chimeric fiber-fibritin molecules eradicates the broad tropism to CAR-expressing cells, we used the CAR-expressing cell lines PEA10 (mouse fibroblast) and SVEC4-10 (mouse vascular epithelium). Infection with Ad5FF1.8 was associated with a 12 to 15-fold reduction in gene transfer compared to control Ad5 in PEA10 and SVEC4-10 cells, respectively (Fig. 2C).

Ad5FF1.8 targeting specificity is mediated by the sdAb DC1.8

We infected DC in the presence or absence of soluble DC1.8 to determine if soluble sdAb could specifically interfere with DC infection. We used the BCII10 sdAb as a negative control (BCII10 is specific for the subunit 10 of the β-lactamase BC-II enzyme of *Bacillus cereus).* [40] Increasing concentrations of sdAb DC1.8 resulted in a dose-dependent inhibition of the ability of Ad5FF1.8 to infect DC, while BCII10 had no effect (Fig. 3). Control Ad5 showed poor infectivity of DC, and this was not affected by either DC1.8 or the control sdAb. Overall, these results provide additional evidence that the chimeric fiber-fibritin-sdAb mediates the targeting specificity of Ad5FF1.8.

Ad5FF1.8 specifically transduces immature DC in murine BMDC cultures

To determine if Ad5FF1.8 retains the specificity of DC1.8, we infected BMDC cultured in GM-CSF/IL-4 with Ad5FF1.8 and control Ad5 and harvested the cells after overnight incubation. We sorted live $CD11c^+$ cells into mature (MHC-II^{high}) and immature (MHC-II^{low}) subpopulations and compared GFP expression between the two (Fig. 4A). Infection with Ad5FF1.8 was associated with higher GFP expression in immature DC (MFI 852 ± 281 vs 459 ± 77), but similar levels of GFP expression in mature DC (MFI GFP 355 ± 29 vs 415 ± 67) (Fig 4C). To determine the specific subset(s) of DC infected by Ad5FF1.8, we added TNF-α to BMDC cultures to trigger maturation and infected the monolayers with Ad5FF1.8 or control Ad5. Infection with Ad5FF1.8 was associated with higher GFP expression in myeloid DC ($CD11b^{high}/CD11c^+$, MFI 1583 ± 279 vs 570 ± 9). There were similar levels of

GFP expression in lymphoid DC ($CD11b^{low}/CD11c^{+}$, MFI 291 ± 51 vs 384 ± 14) (Fig 4B, D). Next, we cultured murine bone marrow cells with Flt3L and GM-CSF according to established protocols and exposed the monolayers to Ad5FF1.8 and control Ad5 on day 8 of culture. At 24 hours post-infection we found that Ad5FF1.8 infection was associated with increased GFP expression in plasmacytoid DC (MFI 379 ± 81 vs 145 ± 21.5) and $CD8\alpha^{-}$ DC (MFI 455 ± 55 vs 130 ± 13.5), but there was no change in $CD8\alpha^{+}$ DC (MFI 202 ± 29 vs 117 ± 9) (Fig 4E, F, G). To assess if infectivity altered the maturation status of DC, Flt3 + GM-CSF-derived DC and GM-CSF + IL-4-derived DC were infected with either Ad5 or Ad5FF1.8, and both IL-12 production and cell surface expression of CD80 and CD86 was assessed. Unlike LPS-matured DC, Ad5 and Ad5FF1.8-infected DC did not produce detectable levels of IL-12 (data not shown). Comparison of DC maturation phenotypes by flow cytometry showed similar levels of expression of CD80 and slightly increased levels of CD86 after infection with Ad5FF1.8 (data not shown).

In vivo administration of Ad5FF1.8

We performed *in vivo* biodistribution studies demonstrating that incorporation of the DC1.8 sdAb into the Ad5 capsid (Ad5FF1.8 expressing GFP) significantly alters the *in vivo* biodistribution compared to wildtype Ad5 (control Ad5 expressing GFP). Specifically, there is a dramatic decrease in GFP expression at the injection site following injection with Ad5FF1.8 compared to Ad5. These studies validate that replacement of the fiber knob with a chimeric construct integrating a sdAb (with consequent ablation of CAR recognition), alters the biodistribution of Ad5FF1.8 (Fig. 5). While we attempted detection of CD11c dendritic cells, the CD11c immunofluorescence was suboptimal to undetectable in the skin and suboptimal in retroperitoneal, axillary, and cervical lymph nodes and spleen (data not shown). We also generated Ad5 vectors expressing the model antigen ovalbumin (Ad5-OVA and Ad5FF1.8-OVA), and used these vectors to test the immune response to OVA following vaccination. Vaccination with either Ad5-OVA or Ad5FF1.8-OVA resulted in robust immune responses (Fig. 6).

DC are a highly specialized subset of APC that play a critical role in orchestrating adaptive immune responses following vaccination. Seminal studies performed by Steinman et al. documented the potential of targeting antigen to DC via the C-type lectin receptor DEC-205.[41, 42] In these studies antigen was selectively delivered to DC using DEC-205-specific antibody-antigen fusion proteins. Subsequent studies confirmed the potential of targeting antigen to DC via multiple different receptors and vaccine platforms. Of note, these studies also highlighted that targeting antigen to DC is not sufficient to induce immunity; antigen must be delivered in the context of additional stimuli to induce potent and long-lived immune responses. Although initial clinical trials based on these studies are ongoing, we have sought to leverage these insights to enhance the efficacy of the Ad vector vaccine platform.

Natural ligands, antibodies and antibody mimetics have been investigated to redirect the specificity of Ad vectors, but most of these molecules are functionally incompatible with cytosolic Ad capsid synthesis and assembly. Typically, the redox state of the cytosol results in improper folding of targeting molecules, altering the structural configuration required for

binding specificity and antigen recognition. Previous attempts to retarget Ad vectors with chimeras integrating natural high affinity ligands have been challenged by the limited tolerance of the Ad fiber for genetic modifications,[43] and biosynthetic incompatibilities between viral capsid assembly in the nucleus and posttranslational processing of ligand molecules.[44] The paucity of naturally existing molecules that can be successfully incorporated into the Ad fiber prompted the use of affibody antibody mimetics, a novel type of artificial protein ligand derived from the three-helix bundle domain Z (dZ) of *Staphylococcus* protein A.[45] However, subsequent advances using this affibody technology have been mainly limited to targeting the human epidermal growth factor receptor type 2 (HER2).[46–50]

We have recently demonstrated the feasibility of Ad vector retargeting to selected cellular receptors using sdAb. sdAb are compatible with Ad vector biosynthesis and assembly and maintain high binding affinity after incorporation into fiber-fibritin chimeras.[38] Recently, Poulin et al. and Kaliberov et al. demonstrated that sdAb can be successfully incorporated into the Ad capsid and redirect Ad specificity.[38, 44] The compatibility of sdAb with phage panning/selection to allow exquisite target cell specificity adds to the appeal of sdAb as a candidate targeting moiety for targeting Ad vectors to DC. Indeed, Poulin et al. recently showed that expression of anti-EGFRvIII sdAb on the Ad capsid through fusion to pIX protein can be used to redirect Ad infection.[44] This strategy represents a significant advance over previous attempts to redirect the specificity of Ad vectors. In addition, effective particle-to-infectivity ratios and production yields suggest the compatibility of this vector design with human clinical translation applications.

In terms of strategies to retarget Ad vector vaccines to DC, we have previously demonstrated the utility of genetic modification of Ad fiber to display the functional TNF-like domain of human CD40 ligand (hCD40L). These engineered Ad were able to achieve selective DC transduction, activation and migration in a clinically relevant human skin explant model.[24] This was done using “mosaic” Ad vectors incorporating fiber proteins with a mutation in the CAR binding domain, and replacement of the fiber knob with a heterologous trimerization domain, derived from bacteriophage T4 fibritin protein and hCD40L.[31] Despite the promise of this strategy in preclinical models, clinical translation has been hampered by cGMP production issues related to the mosaic nature of the viral capsids.[26] Nevertheless, these data, in combination with our previous success using adapter-mediated CD40 targeting, [21, 22, 25, 51–56] provide strong rationale to pursue alternative strategies of Ad vector retargeting to DC.

In the current study we used the sdAb DC1.8 for proof-of-concept studies. It should be noted, however, that we do not think that the DC subset targeted by the sdAb DC1.8 is the ideal subset to target to induce CD8 T cell responses. In fact, previous studies have used the sdAb DC1.8 to target antigen to DC.[34] In this study, induction of CD8 T cell responses was not improved. The authors concluded that the inability of DC1.8 targeting to improve vaccine efficacy was likely related to the fact that DC1.8 does not target a dendritic cell subset involved in CD8 T cell priming. Second, in this manuscript we confirm the hypothesis that DC1.8 does not target the ideal DC subset for CD8 T cell priming, demonstrating that Ad5FF1.8 demonstrates superior transduction of immature DC, but not

CD8α$^+$/CD141$^+$ DC. Given these results, we are not surprised that targeting DC with sdAb DC1.8 did not enhance the ability of Ad5FF1.8 to prime CD8 T cell responses. We were impressed, however, that despite ablation of CAR-mediated transduction, Ad5FF1.8 was still able to mediate a very robust immune response.

DC have been subcategorized into various subsets with each possessing a distinct functional, transcriptional, and surface marker profile.[57, 58] Most DC can present antigen to CD4$^+$ T cells in the context of MHC-II molecules but the capability of processing exogenous antigens and presenting them on MHC-I molecules to CD8$^+$ T cells is restricted to only a few specialized DC subsets.[59] This capability, known as cross-presentation, is key for mounting an effective cytotoxic T cell response against tumor cells and provides strong rationale for targeting specific DC subsets to enhance T cell response following vaccination.[60] CD8α$^+$ DC in mice and CD141$^+$ DC in human are known to be proficient in antigen cross-presentation. We have recently made an important contribution to the understanding of CD8 T cell priming, demonstrating that CD8α$^+$ DC are required to prime CD8 T cell responses to DNA and cell-based vaccines.[37] XCR1 is selectively expressed on CD8α$^+$/CD 141$^+$ DC.[61, 62] Current evidence suggests that CD8α$^+$ DC arise from a non-myeloid thymic progenitor while CD8α $^-$ DC have a myeloid lineage.[63, 64] There are functional differences as well with CD8α$^+$ DC preferentially inducing a T_h1 response whereas CD8α $^-$ DC mainly activating CD4$^+$ cells.[65, 66]

The key role of DCs in regulating immunity has been exploited for therapeutic purposes. In addition to *ex vivo* preparation of antigen-expressing DCs followed by adoptive transfer, *in vivo* delivery of antigen to DCs through antigen-antibody conjugates is an attractive strategy. By targeting endocytic receptors, antigen is internalized, processed, and presented by MHC class I and/or II molecules. Endocytic receptors such as C type lectin, DEC-205 [67] have been targeted and proof-of-concept studies have demonstrated protective immunity can be induced (reviewed in Lehmann et al.,).[68] As our understanding of the physiological role of diverse DC subsets improves, the need for specific targeting becomes greater. For example, HIV antigen gag p24 linked with antibodies to molecules expressed by CD8α$^+$ DC such as Langerin/CD207, DEC205/CD205 and CLEC9A receptors, along with anti-CD40 antibody induced a more robust gag-specific Th1 and CD8$^+$T cell response than that obtained by targeting gag to CD8α $^-$ DC via DCIR.[69] Thus by targeting specific DC subsets one could potentially modulate the immune response against the vaccine antigen both qualitatively and quantitatively.

The recent isolation and validation of sdAb raised against murine bone marrow-derived DC with yet unidentified target specificity,[34] and integration of these sdAb into lentivirus using an envelope display technology, allowed Goyvaerts *et al.* to target of human and murine APC subsets, including DCs and macrophages.[70, 71] Of particular interest, sdAb DC1.8, which showed specific binding to immature BMDCs *in vitro,* [34] mediated selective lentiviral transduction of murine cDC subsets *in vivo.*[72] These findings contrast with our data showing sdAb DC1.8 mediated adenovirus transduction primarily of pDC and CD8α $^-$ cDc (Fig. 4). It should be noted that DC subsets were defined differently in both studies and our data were generated through transduction of bone marrow-derived DCs *in vitro* rather than *in vivo*.

We also studied the efficacy of transduction via our sdAb-targeted Ad in IL-12 release and DC expression of CD80 and CD86. In both instances there was no detectable DC activation and not a significant difference between targeted and un-targeted Ad. These findings contrast with our earlier studies whereby targeting via CD40 led to enhanced DC secretion of IL-12 and upregulation of CD86.[22] In this earlier study, vector targeting also allowed an enhanced induction of anti-tumor immunity. [22] As both CD40 and Clec9a are activating receptors on DC, we anticipate that Ads targeted to Clec9a will activate DC and trigger antitumor immunity.

In summary, we have developed an innovative strategy to specifically target Ad vectors to DC. We have replaced the Ad fiber knob with fiber-fibritin chimeras fused to single domain antibodies (sdAb) specific for DC. This flexible and robust strategy ablates the native tropism of Ad, and permits selective and efficient DC targeting based on the specificity of the sdAb. We successfully generated Ad5FF1.8, a novel Ad5 vector that incorporates DC1.8, a sdAb specific for immature DC. Ad5FF1.8 selectively transduces immature myeloid BMDC *in vitro*, but does not transduce CAR-expressing cells. These data, establishing a versatile and robust Ad vector vaccine platform for targeting DC, pave the way for testing DC-targeted Ad vector vaccines *in vivo*. Of note, the use of non-human primate Ads has recently provided a technology to circumvent immunity to human Ad-based vectors. On this basis, we are currently pursuing strategies to retarget gorilla Ads. Such a vector could thus allow DC targeting, even in the context of pre-formed anti-Ad5 immunity. We are convinced that Ad vectors incorporating fiber-fibritin-sdAb chimeras have tremendous potential, leveraging the ability to induce innate and adaptive immune responses with the ability to target specific subsets of DC. We hypothesize that specific targeting of Ad vaccines to DC will ensure that the DC presenting antigen will also be activated by viral infection, minimizing the risk of nonproductive immune responses.

Substantial differences exist between humans and mice with respect to the cellular populations within the dermis. These differences have impacted our ability in this report to document vector-mediated transduction of DC targets in context of intradermal delivery. On this basis, we have employed a human skin plug system to enable study of vector properties as would predicate efficacy in clinical applications. In this regard, we have been able to show that DC-targeted Ad species can accomplish DC selective gene delivery.[24] Importantly, this enhanced and selective transduction of DCs in this surrogate *in vivo* model achieves an enhanced cellular immune response.[56] Whereas the limitations of the mouse model have not allowed a similar analysis, murine vaccine studies have also shown a direct correlation between DC targeting and improved vaccine efficacy.[22, 23] On this basis, the studies in this report provide the technical basis to study these correlates in the context of the more precise DC targeting we achieve via these current methods.

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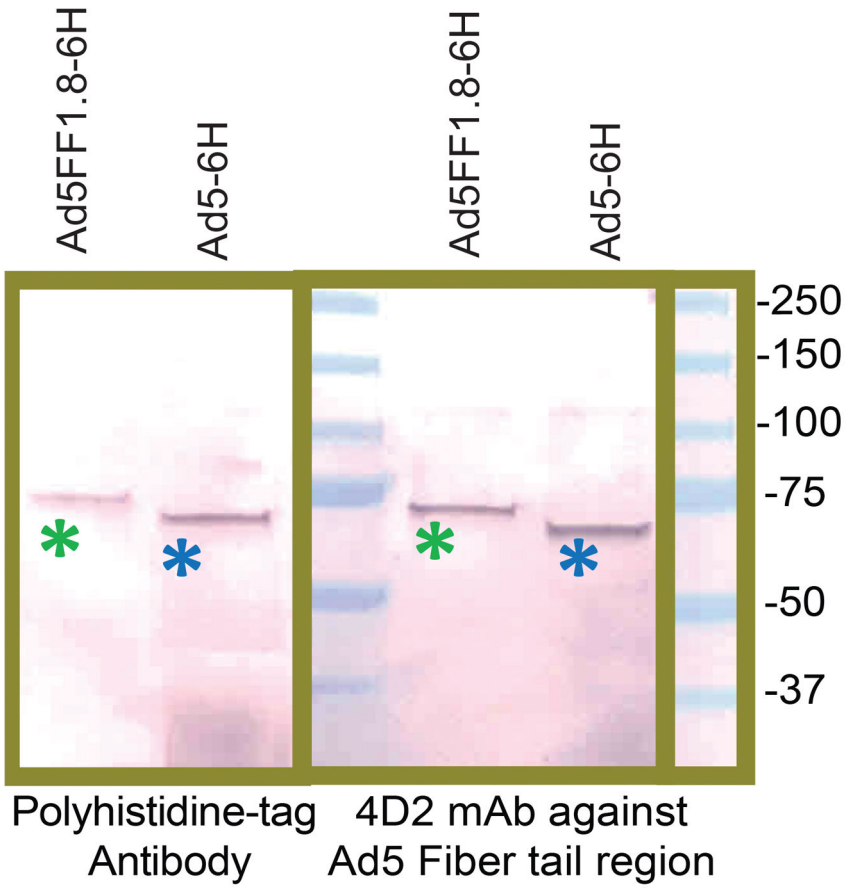

Figure 1. Validation of Nb-DC1.8 incorporation into the Ad5FF1.8 capsid

Purified samples of Ad5FF1.8 (lanes 1 and 4) and control Ad5-6H vector (lanes 2 and 5) were boiled in Laemmli sample buffer and run on 4-20% gradient SDS-PAGE. Viral proteins were transferred to PVDF membrane and incubated with either 4D2 mAb against Ad5 fiber tail region (left panel) or Penta-His mAb against poly-histidine tag (right panel). Molecular masses of Precision Plus marker proteins (MW) are indicated in kilodaltons (kDa) on the right. Protein bands corresponding to Ad5FF1.8 fiber with expected molecular mass of 66 kDa is marked with green asterisk, protein band corresponding to the control Ad5F-6H fiber of 63 kDa is marked with blue asterisk.

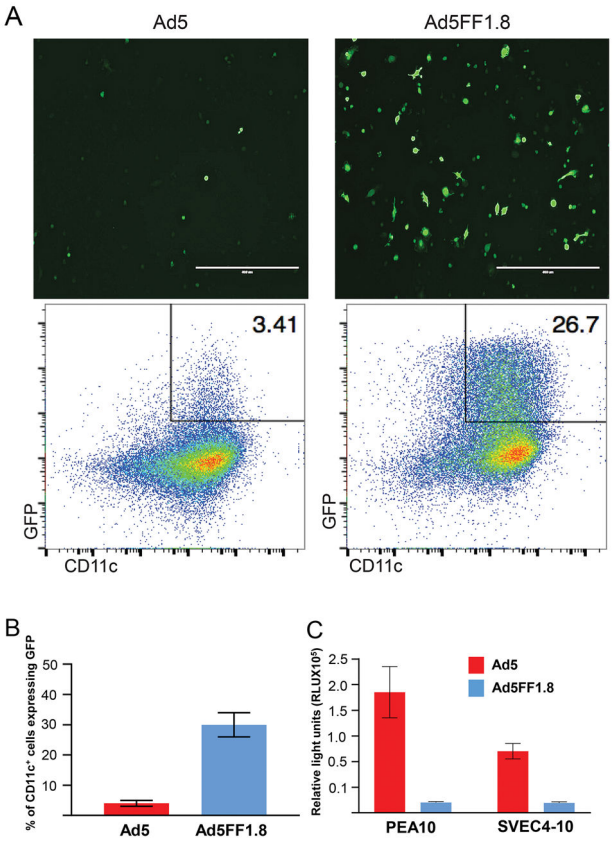

Figure 2. Ad5FF1.8 efficiently transduces dendritic cells in a CAR independent manner

(A) Comparison of GFP expression in DC2.4 cells by Ad5 and Ad5FF1.8 using immunofluorescence microscopy (20X objective) and flow cytometry. (B) Histogram representing increased transduction efficiency of Ad5FF1.8 in BMDC. (C) Deletion of fiber knob domain abrogates CAR-dependent gene transfer by Ad5FF1.8. Monolayers of SVEC4-10 (murine axillary lymph node/vascular epithelium) and PEA-10 (murine fibroblasts) cell lines were incubated with Ad5FF1.8 and Ad5 for 1 hour at increasing MOIs. Luciferase activity was measured after a further 24 hour incubation in virus free medium. Both SVEC4 and PEA-10 cells infected with Ad5FF1.8 showed a lower Luciferase expression as compared to cells infected with Ad5.

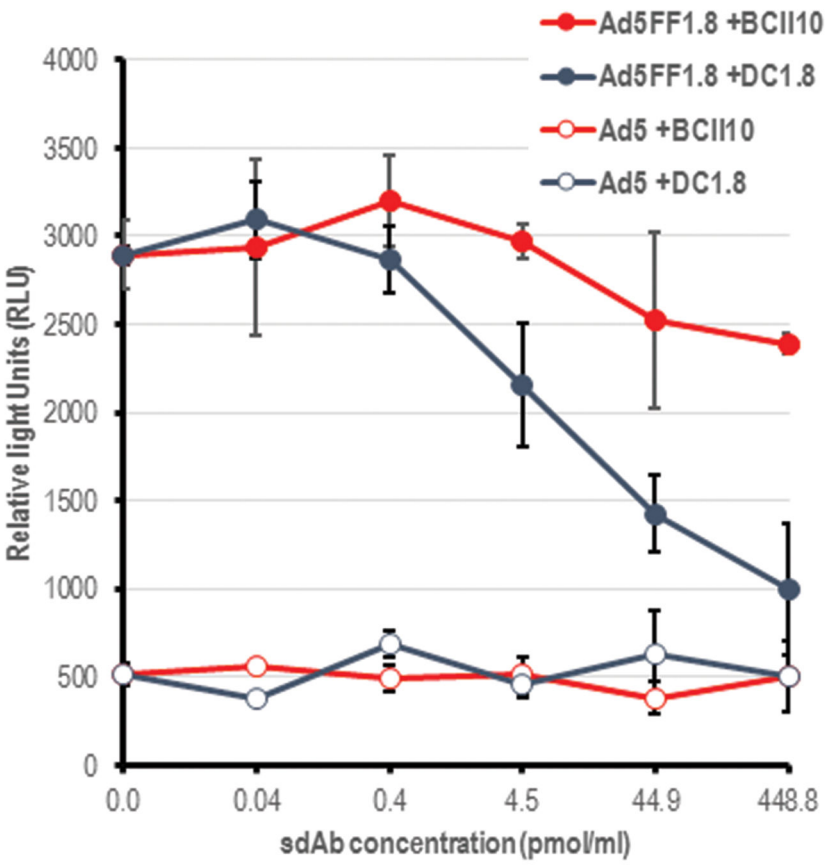

Figure 3. sdAb DC1.8 and Ad5FF1.8 compete for binding to DC

As the amount of DC1.8 added to the BMDC culture is increased, the expression of reporter gene Luciferase decreases in a dose dependent manner.

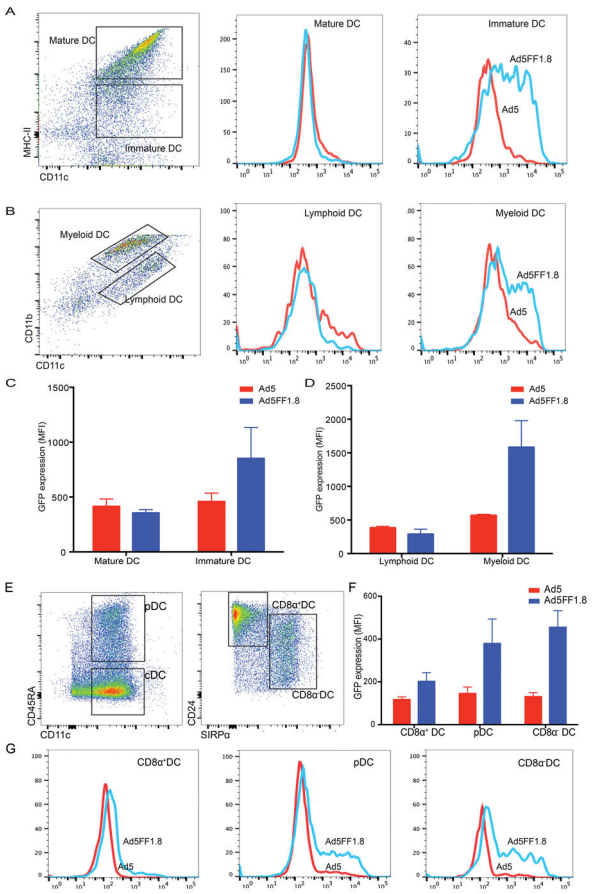

Figure 4. Subset specific targeting by Ad5FF1.8

(A) Gating strategy used to segregate MHC-II high and MHC-II low CD11c^{+} cells in GMCSF/IL4-supplemented BMDC culture. BMDC infected with Ad5FF1.8 (in blue) have an increased eGFP expression in immature DC but not in mature DC when compared to cells infected with Ad5 (in red). (B) Gating strategy used to segregate CD11c^{+} cells in GMCSF/IL-4 supplemented BMDC culture activated with TNFα as myeloid DCs (CD11b^{+}) and lymphoid (CD11b^{-} DCs). BMDC infected with Ad5FF1.8 (in blue) have an increased eGFP expression in the myeloid DC but not in lymphoid DC when compared to cells infected with Ad5 (in red). Histograms representing differential transduction specificity of Ad5FF1.8 and Ad5 in (C) GMCSF/IL4 supplemented BMDC culture and (D) GMCSF/IL-4 supplemented BMDC activated with TNFα. (E) Gating strategy used to segregate CD11c^{+} cells into pDC (CD45RA^{high} plasmacytoid dendritic cells), cDC (CD45RA^{low} conventional dendritic cells), CD8α^{+}DC (CD24^{high} Sirpa^{low}) and CD8α $^{-}$DC(CD24^{low} Sirpa^{high}). (F, G) Ad5FF1.8 causes an increased transduction of GFP in pDC and CD8α $^{-}$ DC but not in CD8α^{+} DC.

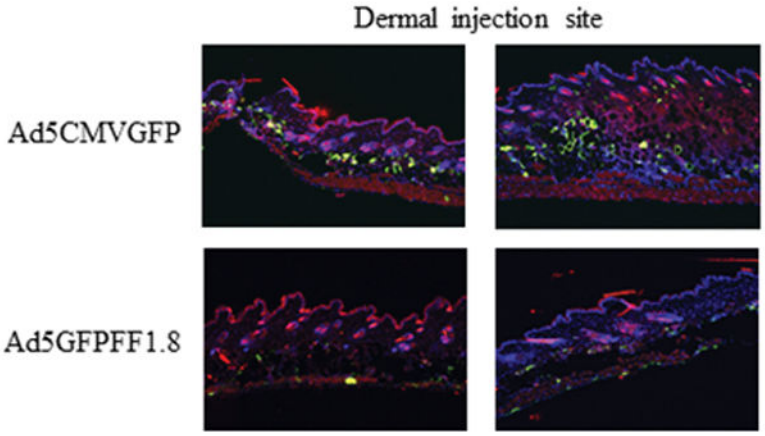

Figure 5. Biodistribution of Ad5 and Ad5FF1.8

Ad5CMVGFP and Ad5GFPFF1.8 were injected intradermally with 10^{11} vp in PBS per mouse. After 72-hours, mice were sacrificed and the injection site skin tissue was processed for analysis of GFP (green), CD11c (red) and DAPI (blue) by immunofluorescence. Results from two representative mice for each virus construct are shown.

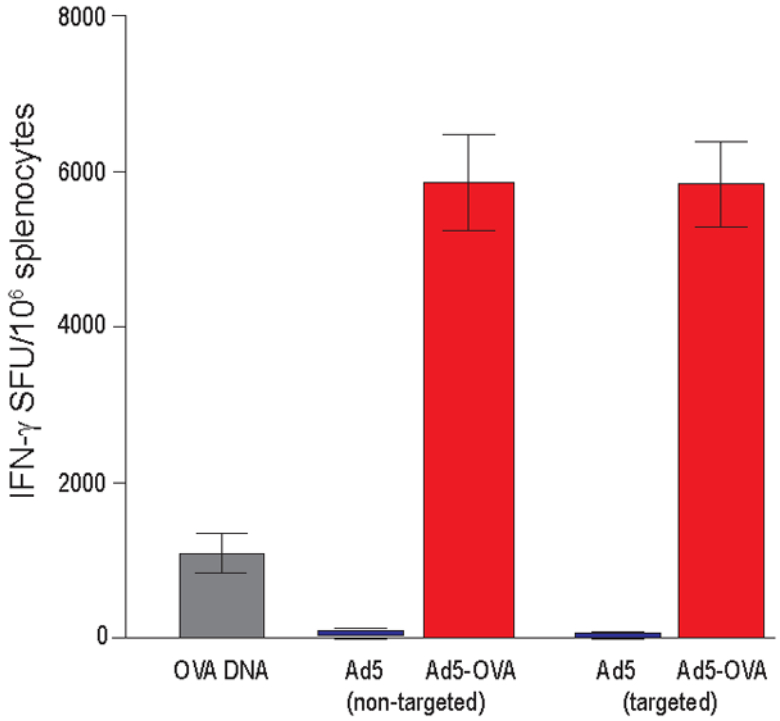

Figure 6. In vivo immunogenicity of Ad5 vs Ad5FF1.8

Mice were injected intradermally on days 0 and 7 with 10^9 vp in PBS or with OVA cDNA through gene gun on days 0, 3, and 6. Splenocytes were analyzed for recognition of the OVA peptide p257–264 on day 11 by INF**γ** ELISPOT assay. Data are presented as the number of Spot Forming Units (SFU) per 10^6 splenocytes.