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Potent immune responses and in vitro pro-inflammatory cytokine suppression by a novel adenovirus vaccine vector based on rare human serotype 28

Christoph A. Kahl, Jessica Bonnell, Suja Hiriyanna, Megan Fultz, Cassandra Nyberg-Hoffman, Ping Chen, C. Richter King^{*}, and Jason G. D. Gall^{*}
GenVec, Inc., 65 West Watkins Mill Road, Gaithersburg, MD 20878

Abstract

Adenovirus vaccine vectors derived from rare human serotypes have been shown to be less potent than serotype 5 (Ad5) at inducing immune responses to encoded antigens. To identify highly immunogenic adenovirus vectors, we assessed pro-inflammatory cytokine expression, binding to the CD46 receptor, and immunogenicity. Species D adenoviruses uniquely suppressed pro-inflammatory cytokines and induced high levels of type I interferon. Thus, it was unexpected that a vector derived from a representative serotype, Ad28, induced significantly higher transgene-specific T-cell responses than an Ad35 vector. Prime-boost regimens with Ad28, Ad35, Ad14, or Ad5 significantly boosted T cell and antibody responses. The seroprevalence of Ad28 was confirmed to be <10% in the United States. Together, this shows that a rare human serotype-based vector can elicit strong immune responses, which was not predicted by *in vitro* results.

Keywords

Adenovirus; vector; human vaccine; seroprevalence

1. Introduction

Replication-deficient adenovirus (Ad) vectors have been demonstrated to be effective carriers for genetic vaccines. Vectors derived from serotype 5 (Ad5) have been most commonly used in vaccine applications and immune responses to transgene products encoded by Ad5 vectors are potent and broad, encompassing both cellular and humoral responses [1,2]. The magnitude of Ad5-induced immune responses has been shown to be superior to naked plasmid DNA and poxvirus vectors [3-6]. Protective vaccination with Ad5 vectors has been demonstrated in multiple disease models, such as influenza [7,8], HIV [3,9,10], malaria [11,12], and herpes simplex virus 2 (HSV-2) [13]. However, administration of Ad5 vectors induces Ad5 neutralizing antibodies (NAb) which reduces the efficacy of homologous prime-boost regimens [3,14]. In addition, high levels of pre-existing immunity to Ad5 may limit the clinical application of Ad5-derived vectors.

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^{*}Address correspondences to: Jason G. D. Gall, Ph.D., GenVec, Inc., 65 West Watkins Mill Road, Gaithersburg, MD 20878, Phone: (240) 632-5547, Fax: (240) 632-0735, jgall@genvec.com.

Current address: International Aids Vaccine Initiative, 140 58th Street, Bldg A, Suite 8J Brooklyn, NY 11220

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The 52 human adenovirus serotypes are biologically diverse and are a rich resource for potential vaccine vectors. The seroprevalence of species C serotypes, such as Ad5, were shown to be high in many human populations, whereas species B and D serotypes were generally rare [15,16]. Several Ad vector systems based on rare human serotypes from species B and D have been engineered, but all have been markedly less immunogenic compared to Ad5-based vectors [15-18]. Non-human adenovirus vectors, such as those derived from great apes and monkeys, have also been evaluated in vaccine models, generally exhibiting inferior immunogenicity compared to Ad5 [19-21]. Some very recent studies with chimpanzee-based vectors have shown promise in challenge models of malaria [22,23]. However, there is still little clinical information available on the use of non-human adenoviruses in human subjects, thus the derivation of vaccine vectors from human serotypes with low seroprevalence and high immunogenicity is an important priority.

Induction of potent adaptive immune responses by vaccine vectors is dependent on the proper stimulation of the innate immune system. Adenoviruses are known to interact with primary immune cells, leading to changes in innate signaling pathways and cytokine cascades [24-29], and different serotypes can effect changes in innate signaling [26,27]. In turn, differential activation of these early pathways likely modulates type and magnitude of subsequent adaptive immune responses. It is currently unclear, however, which activation patterns lead to immune responses *in vivo* that are beneficial for vaccination with recombinant Ad vectors. In this study, we combined assessment of innate immune pathways *in vitro* and immunogenicity in a vaccine model to develop novel Ad vectors that are both low in seroprevalence and potent vaccine platforms *in vivo*. We report that species D serotypes induce cytokine secretion patterns *in vitro* that markedly differ from those induced by other adenovirus species. We describe the generation of a novel adenovirus vector derived from the D serotype Ad28 with a low seroprevalence in United States population samples. In comparisons of adaptive immune responses induced by Ad28 and species B and C vaccine vectors, the Ad28 vector was shown to induce significantly higher transgene-specific T cell responses than the species B-derived vectors, which was not predicted by *in vitro* cytokine expression results. Although responses induced by Ad28- were generally lower than those induced by an Ad5 vector, the differences in CD8⁺ T cells became indistinguishable at higher doses. Also, Ad28 did not require Coxsackie and Adenovirus Receptor (CAR) or CD46 cellular proteins for infection, and thus has a different receptor tropism than Ad5, Ad35, and Ad14 (species B). Finally, we showed that adenovirus vectors with low seroprevalence used with or without Ad5 in prime-boost regimens induced significant responses. This study demonstrates that an Ad28-derived vaccine vector with low seroprevalence and potent immunogenicity is an attractive candidate for vaccine development, and also points to species D for discovery of effective vaccine vectors based on rare human serotypes.

2. Materials and Methods

2.1. Cell lines and viruses

The cell lines 293- ORF6 [30], A549 (American Type Culture Collection (ATCC)), and BHK-CD46 (obtained from Silvio Hemmi, University of Zürich, Switzerland) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; HyClone, Logan, UT) supplemented with 2mM L-glutamine, and cells were kept at 37°C with 5% CO₂. Wild-type adenoviral serotypes 4 (VR-1572), 2 (VR-846), 5 (VR-5), 6 (VR-6), 7 (VR-848), 8 (VR-1368), 13 (VR-14), 14 (VR-15), 15 (VR-16), 18 (VR-19), 20 (VR-255), 24 (VR-259), 25 (VR-223), 26 (VR-224), 27 (VR-1105), 28 (VR-226), 29 (VR-272), 30 (VR-273), 32 (VR-625), 34 (VR-716), 35 (VR-718), 41 (VR-930), 42 (VR-1304), 43 (VR-1305), 44 (VR-1306), 45 (VR-1307), 46 (VR-1308), and 47 (VR-1309) were obtained from ATCC. The replication-deficient Ad vectors used in this

study contained either a deletion of the E1 region (Ad14, Ad28), combined deletions in E1/E3 (Ad35), or E1/E3/E4 (Ad5). Replication-deficient rAd5 and rAd35 vectors expressing influenza antigens were generated in 293-ORF6 cells essentially as described previously [31-33]. Derivation of Ad14-based vectors will be described elsewhere (C. Kahl, manuscript in preparation). Recombinant adenoviral vectors and wild-type adenoviruses were passaged on 293-ORF6 cells, purified by triple cesium chloride (CsCl) gradient centrifugation as previously described [32], and resuspended in final formulation buffer (FFB). Ad35, 14, and 28 viral preparations were analyzed for infectious adenoviral particles by fluorescence-focus unit (FFU) assay with a polyclonal antibody against the Ad2 hexon protein. Infectious titers of Ad5 viruses were determined with an FFU assay using monoclonal antibody 38-2 against the Ad5 DNA binding protein [34]. Total particle unit (pu) titer was determined by absorbance in the presence of SDS [35].

2.2. Engineering of E1-deleted Ad28 and Ad14 vector

Wild-type Ad28 virus lysate (strain BP-5, ATCC VR-226) was amplified on 293-ORF6 cells and purified on three successive cesium chloride density gradients. Viral DNA was isolated using a standard proteinase K, phenol-chloroform extraction method. All DNA manipulations were performed using previously published methodologies [32,36]. Briefly, the Ad28 genome was rescued into a small plasmid containing 442 bp and 502 bp of the left and right hand Ad28 genomic ends, respectively, using homologous recombination in *E. coli* strain BJDE3. The complete Ad28 genome in the plasmid was sequenced and submitted to GenBank (accession FJ824826). An E1-deletion shuttle plasmid was constructed with Ad28 sequences 1-459, a CMV-driven transgene expression cassette in reverse orientation to E1, and Ad28 sequences 3112-3998. The E1 region deletion was designed to delete the coding regions of E1 and maintain protein IX expression. The Ad28 genome plasmid was linearized by digestion with Eco R I in the E1 region and recombined with the shuttle plasmid. Ad28 E1-deleted vectors with transgenes for enhanced green fluorescent protein (eGFP), luciferase, hemagglutinin (HA), or nucleoprotein (NP) from influenza were generated by transfection of 293-ORF6 cells as described [32]. Production yields for Ad28 vectors as measured by total number of physical particle units (pu) were generally comparable to yields from Ad5 or Ad35 productions. Infectious viral titers for Ad28 as measured by the FFU assay or eGFP transgene expression on 293-ORF6 cells were generally 10-100 fold lower (data not shown). In contrast, titrations of Ad28 vectors done on other cell lines (A549, CHO, BHK) showed comparable infectious titers to Ad5 and Ad35 vectors, suggesting that infectious assays performed on the 293-ORF6 cell line may underestimate Ad28 infectious vector titers (data not shown). Therefore, for all experiments vector input doses were calculated using physical pu titers. The derivation of recombinant E1-deleted Ad14 vectors from Ad14 de Witt will be described elsewhere.

2.3. Cytokine assays

Cryopreserved human peripheral blood mononuclear cells (PBMC; Cellular Technology Ltd., Cleveland, OH) were rapidly thawed in a water bath, followed by drop wise addition of Roswell Park Memorial Institute (RPMI) 1640 medium containing 30% FCS, 2mM L-glutamine, and 50 units/ml benzonase (EMD Biosciences, Gibbstown, NJ) and incubation for 1 hour at 37°C. Cells were then washed with phosphate-buffered saline (PBS) and resuspended in RPMI containing 10% FCS and 2mM L-glutamine. For evaluation of pro-inflammatory cytokine secretion, PBMC (10^6 cells per well) were allowed to adhere in 96-well plates for two hours and non-adherent cells were washed off. Adherent cells (monocytes) were exposed to 10 ng/ml of IFN- γ (Pierce Biotechnology, Rockford, IL) and CsCl-purified wild-type adenoviruses. The next day, cells were stimulated with 1 μ g/ml of *E. coli* 0111:B4 lipopolysaccharide (LPS; Sigma, Saint Louis, MS). Supernatants were harvested 3 days post-infection and assayed by either IL-12 ELISA (R&D Systems,

Minneapolis, MN) or Searchlight[®] human inflammatory cytokine array 1 (Thermo Scientific Pierce #84619B). Arrays were scanned with the AlphaMager FluorChem SP and cytokine concentrations were determined with spot densitometry using AlphaEase FC software (both Alpha Innotech, San Leandro, CA). For determination of IFN- α induction, PBMC were plated at 2×10^5 cells per well in 96-well plates and exposed to increasing concentrations of CsCl-purified wild-type adenoviruses. Supernatants were harvested after 48 hours, and IFN- α concentration was measured by ELISA using a human IFN- α ELISA kit (R&D Systems, Minneapolis, MN).

2.4. CD46 receptor binding assay

White 96-well Nunc Maxisorp plates (Thermo Fisher Scientific, Rochester, NY) were coated with 0.1 μ g per well of murine monoclonal anti-FLAG M2 antibody (F1804, Sigma), following by blocking with a 3% bovine serum albumin (BSA) solution in TBST (150 mM NaCl, 10 mM Tris pH 8, 0.05% Tween 20). Then, wells were washed with 0.3% BSA in TBST and coated with conditioned media from Tn5 insect cells infected with baculovirus expressing the extracellular domain of CD46 with an N-terminal FLAG tag (sCD46). Each coating and blocking step was performed overnight at 4°C. After four washes with TBST, 10^9 or 10^{10} pu purified adenovirus were added per well and incubated at 37°C for 1 hour. Wells were washed four times with TBST and bound adenovirus capsids were lysed by adding TE buffer containing 0.1% SDS and incubation at 37°C for 30 minutes. Adenoviral DNA was detected using the Quant-iT PicoGreen[®] dsDNA Assay Kit (Molecular Probes, Eugene, OR), and quantitated with a fluorescence microplate reader (FLUOstar Optima, BMG Labtech Inc., Durham, NC).

2.5. Competition assays

Full length fiber protein with the shaft and knob regions from Ad35 (F-35) was expressed from baculovirus with an N-terminal 6x-His tag for detection and purification over a cobalt column. BHK-CD46 cells were seeded at 10^4 cells/well in flat-bottom 96-well plates (Fisher Scientific, Pittsburgh, PA) overnight. F-35 was serially diluted in plain DMEM from 20 μ g/L to 0.009 μ g/L. Diluted F-35 was added to the pre-seeded cells and incubated at 37°C/5%CO₂ for 1 hour. Adenovirus vectors diluted to 2×10^9 pu/ml were then added to the wells, and plates were incubated for two hours. Media from Tn5 insect cells infected with the FLAG-sCD46-expressing baculovirus was serially diluted in baculovirus-conditioned insect cell medium and incubated with adenovirus vectors at 2×10^9 pu/ml for 1 hour at 37°C/5%CO₂. Competitor dilutions ranged from undiluted sCD46-media to 1:2187 using three-fold dilutions. The sCD46/vector mixture was added to the cells and incubated for two hours. Transductions were stopped by overlaying with a FCS/DMEM solution yielding a final concentration of 5% FCS per well and incubated overnight. Plates were developed using a luciferase assay reagent system (Promega, Madison, WI) according to manufacturer's instructions and analyzed for luminescence via LMAX³⁸⁴ Microplate Luminometer (Molecular Devices, Sunnyvale, CA) with SoftmaxPro software (Molecular Devices).

2.6. Virus neutralization assays

A total of 240 human serum samples (New York Biologics, Inc, New York, NY) were tested in this study. The samples were collected from healthy male and female blood donors (age range 17-71, mean age 44, median age 47 years) from five geographical regions across the US with 48 samples per region. Sera were heat-inactivated at 56°C for 1 hour. To measure NAb activity, a transduction assay was used based on inhibition of luciferase expression [37]. To preserve the integrity of cell-surface receptors, adherent A549 cells were gently detached by adding EDTA (10 mM) to culture media and incubating for 15 minutes at 37°C. Meanwhile, diluted sera were incubated with the appropriate adenovirus vector for 30

minutes at room temperature in 96-well tissue culture plates. Then, 5×10^4 A549 cells were added per well, resulting in a final MOI of 500 pu/cell in a total volume of 200 μ l. After 24 hour incubation, luciferase activity was assayed with a luciferase assay reagent system (Promega) on a LMAX³⁸⁴ Microplate Luminometer (Molecular Devices). Initially, human serum samples were screened for anti-adenoviral NAb at a 1:16 dilution in a plus/minus format in triplicate. Sera scored positive for NAb when the mean luciferase activity was inhibited at least 90%. The IC₉₀ was calculated by determining the maximum luciferase activity in control wells containing cells and virus but no serum. Sera that scored positive for NAb were then retested in the same fashion, except using serial doubling dilutions, ranging from 1:16 to 1:2,048 in duplicate. Neutralization titers were defined as the maximum serum dilution that inhibited luciferase activity by 90%. To ensure assay consistency, positive mouse control sera for each vector with known neutralization titers were assayed in parallel with the human samples in each experiment.

2.7. Animals and immunizations

Female, six to eight week old Balb/c mice weighing 19-22g were purchased from Harlan, USA. These mice were fed with a standard diet, received water *ad libitum*, and were maintained in the GenVec animal facility under conventional conditions. The GenVec animal facility operates in accordance with the “NIH Guide for the Care and Use of Laboratory Animals” and complies with the PHS (Public Health Service) Policy on Humane Care and Use of Laboratory Animals. All of the experimental procedures conducted were in compliance with the NIH guidelines and PHS Policy. The studies were approved by the Institutional Animal Care and Use Committee (IACUC) of GenVec, Inc. Mice were immunized i.m. with the appropriate dose of adenovirus vaccine vector in 100 μ l FFB in both quadriceps muscles. For prime-boost studies, mice were boosted by i.m. injection in both tibialis anterior muscles 4 weeks post-prime.

2.8. Intracellular cytokine staining (ICS) assays

The magnitude and phenotype of NP-specific cellular immune responses in vaccinated BALB/c mice were assessed via multiparameter ICS assays. Splenocytes were isolated from mice and 1×10^6 cells were incubated for 13 h at 37°C with RPMI 1640 containing 10% FCS, 0.01% Penicillin/Streptomycin and 0.01% L-Glutamine, as well as 10 μ g/mL NP₁₄₇₋₁₅₅ peptide (Anaspec, San Jose, CA) or 2.5 μ g/mL of NP peptide pool (Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA). As controls, cells were incubated with 10 μ g/mL HA₃₂₂₋₃₄₀ peptide (Anaspec), or with 50 ng/mL phorbol myristate acetate (PMA) plus 500 ng/mL ionomycin (Sigma). Cultures contained monensin (GolgiStop; BD Biosciences, San Diego, CA). Cells were stained with pre-titrated amounts of anti-CD4-FITC (GK1.5; fluorescein isothiocyanate), anti-CD8a-PerCP-Cy5.5 (53-6.7; Peridinin-chlorophyll-protein Complex Cy5.5), and/or anti-CD8a-FITC (53-6.7; fluorescein isothiocyanate) antibodies and fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences). Cells were then stained intracellularly with anti-IFN- γ -PE (XMG1.2; phycoerythrin), anti-TNF- α -PE-Cy7 (MP6-XT22; phycoerythrin Cy7) anti-TNF- α -APC (MP6-XT22; allophycocyanin), and/or anti-IL-2-APC (JES6-5H4; allophycocyanin) antibodies and fixed with 0.25% Cytofix (BD Biosciences). Experiments were composed of two different staining strategies. The first employed co-staining with anti-CD4-FITC and anti-CD8a-PerCP-Cy5.5 in conjunction with anti-IFN- γ -PE and anti-TNF- α -APC. Later experiments were stimulated and stained in parallel implementing either anti-CD4-FITC or anti-CD8a-FITC with anti-IFN- γ -PE, anti-TNF- α -PE-Cy7, and anti-IL-2-APC. Samples were analyzed with a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). Approximately 150,000 to 300,000 events were collected per sample. Media backgrounds were typically <0.9% of gated CD8⁺ or CD4⁺ T lymphocytes; however, background was removed from individual animals by subtracting the

media control from NP peptide-stimulated samples. Responses in mock treated (FFB only) animals were typically <0.1% and were used to determine response thresholds for each experiment.

2.9. HA antibody ELISA

Serum samples obtained from ocular bleeds were collected on day 0 and then on days 14, 28, and 35 post-vaccination. 384-well MaxiSorp surface plates (VWR International, Bridgeport, NJ) were pre-coated with 0.01 µg/well purified recombinant HA protein (Protein Sciences Corporation, Meriden, CT) and blocked with a 3% BSA (EMD, Gibbstown, NJ) solution. Serum samples were diluted in 0.2% Tween 80 (Sigma P8074) in TBST at an initial dilution of 1:64. Serial doubling dilutions of the serum were performed for day 14 and 28 samples yielding a final dilution of 1:8192. Day 35 samples were titrated from 1:64 to 1:2×10⁶ to measure increased antibody titers post-boost. Samples were detected with a donkey anti-mouse IgG antibody conjugated to HRP (Millipore Corporation, Billerica, MA). Plates were developed with the SuperSignal ELISA Pico Chemiluminescent Substrate kit (Pierce Biotechnology Inc, Rockford, IL) and read on a fluorescence microplate reader (FLUOstar Optima). Resulting relative luminescent units (RLU) were fitted to a nonlinear curve using OriginPro version 8 software (OriginLab Corporation, Northampton, MA). The following formula, solved for x, was used to calculate the titers from the fitted data: $y = A1 + ((A2-A1)/((1+10^{(\text{Log}x^{0-x})p})))$. Background was calculated using the average RLU values of the 1:64 dilutions of the day 0 samples, plus 1 standard deviation. The above formula accounts for background luminescence when determining the antibody titers.

2.10. Statistical analyses

Statistical analyses were performed in OriginPro version 8. Immune responses between vaccine groups were compared using non-parametric methods (Mann-Whitney test) assuming non-normal sample distributions. Significance levels were adjusted by the number of group comparisons made using Bonferroni corrections, and are indicated for each experiment. Thresholds for positive cytokine responses were determined using mock (FFB)-immunized animals for each experiment.

3. Results

3.1. Species D serotypes suppress pro-inflammatory cytokine secretion and induce type I IFN secretion

Previously, it was shown that several rare human adenoviruses increase type I IFN secretion and decrease pro-inflammatory cytokine secretion *in vitro*, whereas Ad5 does not [26,27]. Vaccine vectors based on these serotypes have a significantly lower immunogenicity *in vivo* compared to Ad5 [38-40]. Therefore, we hypothesized that we could identify immunogenic Ad serotypes based on their cytokine secretion patterns. Twenty-eight wild-type human adenoviruses were analyzed for modulation of pro-inflammatory cytokine and type I IFN secretion from primary human blood cells. IFN-γ-primed monocytes were exposed to adenovirus and stimulated with LPS. Priming and stimulation with IFN-γ and LPS alone resulted in high levels of IL-12 (Fig. 1A). IL-12 secretion was not affected by exposure to species C serotypes and most species A, B, E, and F serotypes. In contrast, all tested species D serotypes reduced IL-12 secretion to background levels (>100-fold reduction). The inhibition of IL-12 secretion was dose-dependent, suggesting a virus-specific effect (Fig. 1B). In addition, secretion of other pro-inflammatory cytokines (IL-1α/β, IL-6, IL-10, and TNF-α) was significantly inhibited by species D serotypes (Fig. 1C). In this experiment, the concentration of IL-12 in supernatants from Ad28-treated cells was indistinguishable from the unstimulated cells, in good agreement with the prior experiments. Levels of IL-2 and IL-8 were unaffected by neither stimulation nor adenovirus. The converse effect was seen

for IFN- α induction. All species D adenoviruses induced high levels of IFN- α (Fig. 2). In contrast, species A, B, C, E, and F serotypes did not induce IFN- α secretion above background levels with the exception of Ad35, which induced a low level of the cytokine (Fig. 2). Together, these results show that serotypes from species D can markedly affect the secretion of both type I IFN and pro-inflammatory cytokines, a characteristic that appears to distinguish species D from other adenoviral species.

3.2. A number of species D serotypes do not bind to recombinant soluble CD46 *in vitro*

Suppression of secretion of pro-inflammatory cytokines such as IL-12 from monocytes has been previously associated with the use of CD46 as a cellular receptor by species B adenoviruses [27]. To assess whether species D serotypes utilize CD46, different concentrations of the viruses were applied to ELISA plates coated with sCD46 and viral binding was determined by quantitation of viral DNA labeled with a fluorescent dye (Materials & Methods 2.4). As an indication of the specificity of the assay, the fluorescent signal was high for the species B serotypes (7, 14, 34, and 35), indicating viral binding to the immobilized sCD46. There was no fluorescence detected in wells with Ad5 (Fig. 3A), consistent with the known receptor tropisms [41,42]. Furthermore, there was no fluorescence detected in wells with an Ad35 vector with a mutation in the GH loop of fiber that ablated binding to CD46 (J. Gall, manuscript in preparation). In general, wells incubated with species D serotypes did not show strong fluorescence over background, with the exception of serotypes 13, 32 and 45. These serotypes exhibited very low to moderate levels of fluorescence at the highest viral particle concentration. Although Ad41 demonstrated measurable binding to sCD46, Ad41 was previously convincingly shown to not utilize CD46 for cell entry[43]. The results from this biochemical binding assay showed that many species D serotypes do not bind to sCD46 *in vitro*, and thus may not utilize CD46 for cell entry.

3.3. Ad28 does not utilize CD46 or CAR receptors for infection

The profound effect on cytokine expression *in vitro* and absence of binding to sCD46 by many members of species D implied that gene transfer vectors based on these serotypes may have unique characteristics. The species D serotype 28 was chosen as the representative D serotype for further study as an adenovirus vector because seroprevalence was estimated to be less than 10% [15] and Ad28 has not been associated with human disease [44]. The absence of sCD46 binding suggested that the modulation of cytokine secretion by species D adenoviruses may not be related to CD46 receptor usage. To determine if Ad28 utilized CD46 for cell entry, transduction competition experiments based on luciferase activity were first performed on BHK-CD46 cells. The cells were incubated with full-length recombinant fiber derived from Ad35 (F-35), a fiber protein known to bind to CD46, prior to adding the viral vectors to the cells. The F-35 protein inhibited Ad35-mediated transduction in a dose-dependent fashion, with less than 10% luciferase activity remaining at the highest competitor concentration (Fig. 3B). In contrast, the level of Ad28- and Ad5-mediated luciferase activity at the maximal F-35 concentration was not different from the level of luciferase activity without competitor. Of note, transduction with the Ad14 luciferase vector was most strongly inhibited, with a reduction below 0.3% of luciferase activity at the highest competitor dose. We also evaluated the effect of preincubation of the vectors with sCD46 on transduction. Luciferase activity in Ad35 transduced cells was reduced up to about 73% in a dose-dependent fashion but transduction with Ad28 or Ad5 vectors was not affected at the maximum concentration of sCD46 (data not shown). Interestingly, preincubation of Ad14 with sCD46 did not affect luciferase activity, in contrast to the strong inhibition seen by the F-35 protein. In addition, titration of transduction by the Ad28, Ad5, Ad35, and Ad14 vectors was compared on the BHK-CD46. Ad28 and Ad5 transduction was comparable, whereas transduction with Ad14 and Ad35 vectors was markedly higher (data not shown). Lastly, there was no increase in transduction by Ad28 vectors in CHO cells over-expressing

CAR compared to wild-type CHO cells, indicating that Ad28 does not use the common adenovirus receptor CAR as their cellular receptor (Fig. 3C). Together, these results show that Ad28 does not utilize CD46 or CAR as a primary receptor for infection *in vitro*, and suggest that Ad28 uses CD46-independent pathways to modulate cytokine secretion.

3.4. Ad28 has a low seroprevalence in the United States

To determine the Ad28 seroprevalence levels in the United States (U.S.), 240 serum samples from various geographical regions across the U.S were tested in a neutralizing antibody assay based on luciferase vector transduction. All serum samples were first screened at a single dilution of 1:16 to determine whether each sample was positive or negative for neutralizing activity. On average across the U.S., the fraction of sera with neutralizing activity to Ad28 was 7.5% (Fig. 4A). As expected, a majority of samples (57.5%) were seropositive for Ad5. When assessing individual geographic regions, seroprevalence varied with a range of 35-71% and 2-17% for Ad5 and Ad28, respectively. End-point titrations of positive samples were then performed to determine individual NAb titers. The majority of individuals positive for Ad28 had low (<200) NAb titers (68.42%), some had moderate titers (26.32%), and only one individual of the 18 seropositives (5.26%) had a titer greater than 1,000 (Fig. 4B). In contrast, the majority of Ad5 seropositive individuals tested had moderate or high titers, 38% and 20%, respectively. Together, these data suggest that the seroprevalence of Ad28 in the U.S. is low.

3.5. Single immunization with Ad28 vaccine vector induces potent T cell responses

Low seroprevalence, weak neutralizing titers in seropositives, and unique *in vitro* properties of Ad28 encouraged us to test this serotype in vaccine applications. T-cell and antibody responses induced to Ad28 vectored antigens were determined in an influenza mouse model. T cell responses to NP in the BALB/c mouse have been extensively characterized, including identification of the immunodominant epitope, NP₁₄₇₋₁₅₅, responsible for the majority of CD8⁺ T cell responses [45]. To determine the magnitude of T-cell responses induced to NP after a single intramuscular immunization, mice were immunized with replication-deficient Ad28, Ad5, Ad14, and Ad35 vectors expressing NP and cellular responses were assessed in splenocytes by ICS 14 days after injection. The magnitude of IFN- γ ⁺ CD8⁺ T cells induced by Ad28 was significantly higher than that induced by Ad35 at 10⁸ pu and 10⁹ pu doses (>3-fold, $p < 0.001$) and approached that induced by Ad5 at the highest dose, 10⁹ pu (Fig. 5A). Additionally, Ad28, Ad35, and Ad14 induced fewer NP-specific CD8⁺ T cells expressing IFN- γ than Ad5 at 10⁷ pu and 10⁸ pu. A similar pattern for TNF- α ⁺ CD8⁺ T cell induction was observed; Ad28 induced more TNF- α ⁺ CD8⁺ T cells than Ad35 at 10⁸ pu, Ad28 approached Ad5 levels at 10⁸ and 10⁹ pu, whereas Ad35 and Ad14 induced significantly lower levels than Ad5 at all doses (Fig. 5B). Moreover, at the intermediate dose, 10⁸ pu, more animals immunized with Ad28 responded with IFN- γ ⁺ or TNF- α ⁺ CD8⁺ T cells than when immunized with Ad35 ($p < 0.01$) or Ad14 (Fig. 5A & B). In the CD4⁺ T cell compartment, IFN- γ ⁺ responses were comparable between Ad28, Ad35 and Ad14 and responses induced by the Ad5 vector were in most cases significantly higher than those seen with the other three vector types (Fig. 5C). No significant responses in the IL-2⁺ and TNF- α ⁺ CD4⁺ and the IL-2⁺ CD8⁺ T cell compartments were observed in any of the immunized vector groups (data not shown). Together, these results demonstrate that an Ad28-based vaccine vector can induce higher magnitudes of T cell responses than other rare serotype vectors, such as Ad14 and Ad35, which was not predicted by their *in vitro* cytokine modulation patterns.

3.6. Priming with Ad28 followed by Ad5 immunization potentially boosts T cell responses compared to single Ad5 vaccination

Since the choice of prime and boost vectors can influence the magnitude of the immune responses we first evaluated the performance of Ad28 as a prime immunization, with a standard Ad5 boost. Mice were immunized with a single administration or with a prime-boost regimen of the adenovirus vectors expressing NP. Similar to our previous experiments, a single administration of Ad5NP at a dose of 10^9 pu i.m. resulted in an average of about 6% IFN- γ^+ CD8 $^+$ T cells specific for the immunodominant NP₁₄₇₋₁₅₅ epitope (Fig. 6A). Roughly 12% IFN- γ secreting CD8 $^+$ T cells were detected after homologous Ad5NP prime-boost administration ($p < 0.005$ compared to single-dose Ad5NP immunization). Strikingly, over 25% IFN- γ positive CD8 $^+$ T cells were detected when priming with Ad28NP vector followed by heterologous boosting with Ad5NP ($p < 0.0005$ compared to homologous Ad5NP prime-boost regimens). The Ad28-Ad5 regimen also induced significantly higher responses among TNF- α^+ CD8 $^+$ T cells compared to the homologous Ad5-Ad5 regimen (Fig. 6B; $p < 0.005$). A comparable trend was also observed when priming with Ad14NP or Ad35NP vectors followed by Ad5NP boost, although the increase in TNF- α^+ CD8 $^+$ T cells responses did not reach statistical significance (Fig. 6A & B). Also, no significant boost in IL-2 $^+$ CD8 $^+$ T-cells or any CD4 $^+$ T cell compartments was observed (Fig. 6C and data not shown). Analysis of multicytokine-secreting cell populations revealed that Ad5 boosting increased IFN- γ single-positive and IFN- γ /TNF- α double-positive CD8 $^+$ effector T cells, whereas there was no significant increase in other CD8 $^+$ T cell subsets (TNF- α , IL-2, TNF- α /IL-2, IFN- γ /IL-2, and IFN- γ /TNF- α /IL-2 populations; data not shown). Together, these results show that antigen-specific CD8 $^+$ T cell responses can be potentially enhanced by heterologous prime-boost regimens when priming with several different rare Ad vaccine vectors in conjunction with an Ad5 vector boost.

3.7. Prime-boost regimens with Ad28 and a second rare serotype vector significantly boost T cell responses

A prime-boost regimen that utilized two rare serotype vectors would have the advantage of avoiding potential complications due to pre-existing viral immunity. Therefore, prime-boost experiments were performed using combinations of Ad28NP and Ad14NP vectors and compared to single-dose administration. In addition, these vectors were evaluated as potential boost immunizations for a potent priming vector, Ad5. As shown in Figure 7, both Ad14-Ad28 and Ad28-Ad14 prime-boost regimens induced significantly higher levels of NP-specific CD8 $^+$ T cell responses compared to single immunization with either vector. Mean increases in cells expressing IFN- γ^+ or TNF- α^+ cytokines were over 3-fold for the Ad28-Ad14 regimen and over 7-fold for the Ad14-Ad28 regimen. Significant boost effects were also noted in the IL-2 CD8 $^+$ T cell compartment, although responses were generally of low magnitude (Fig. 7C). Priming with Ad5NP followed by boosting with either Ad28NP or Ad14NP vectors resulted in an average of less than 2-fold increase in NP-specific IFN- γ^+ and TNF- α^+ CD8 $^+$ T cell populations compared to single administration of Ad5NP, and in most cases did not reach statistical significance. These results show that Ad14 and Ad28-based vectors in prime-boost regimens can effectively boost T cell responses compared to single vector administration.

3.8. Ad28 prime, Ad-boost regimens potentially boost antibody responses

To determine the induction of transgene-specific antibody by Ad28-based vaccine vectors, mice were immunized with vectors expressing the influenza HA antigen. BALB/c mice were primed with 10^9 pu of either Ad28 or Ad35 HA vectors and boosted with homologous or heterologous Ad vectors 28 days later. There were comparable antibody responses to HA on days 14 and 28 following priming with Ad28 (Fig. 8A) and Ad35 (Fig. 8B). Priming with Ad28 followed by Ad28 or Ad35 boost resulted in significantly increased HA antibody titers

($p < 0.05$ for day 28 vs. day 35; Fig. 8A and B). Antibody titers were also boosted in the homologous Ad35 prime-boost group, but not in the Ad35 prime – Ad28 boost group (Fig. 8B). In addition, Ad5 immunization on day 28 resulted in greater than 100-fold increases in HA antibody titers on day 35 in animals primed with either Ad28 or Ad35 ($p < 0.0001$). Together, these results suggest that vectors based on rare serotypes, such as Ad28 and Ad35, can be combined in prime-boost regimens to elicit significantly increased antibody responses.

4. Discussion

The immunogenicity profile of a species D-derived vaccine vector based on the rare (less than 10% seroprevalence in the United States) serotype Ad28 was tested in an influenza model in comparative dose-response and prime-boost studies. Single immunization with the Ad28 vector expressing influenza NP induced higher NP-specific T cell responses compared to a species B-derived Ad35 vector. In particular, the Ad28 vector elicited significantly higher levels of antigen-specific IFN- γ^+ and TNF- α^+ CD8 $^+$ T cells compared to the Ad35 vector at low to moderate dose levels (10^8 and 10^9 pu). In contrast, dose escalation with a second species B vector, Ad14, showed a similar magnitude of CD8 $^+$ T cell response as the Ad35 vector. CD8 $^+$ T cell responses induced by the Ad28 vector were lower than those induced by the Ad5 vector at the low to moderate doses. Interestingly, responses induced by Ad28 vector approached those elicited by Ad5 vector at the highest dose presented here, 10^9 pu. Consistent with that observation, responses induced by Ad28 and Ad5 with 10^{10} pu were very similar (data not shown). Although the reason for this is unclear, it is possible that the adjuvant effect of the adenovirus vector reached a plateau in this mouse model. The results of this study indicate that Ad28-based vectors induce potent T cell responses.

Vaccination strategies based on prime-boost regimens typically generate higher magnitude immune responses compared to single immunization. Adenoviral prime and boost vectors should ideally be of immunologically distinct serotypes to allow efficient boosting without interference by immune responses generated to the priming vector. Here we demonstrate effective prime-boost regimens for cellular and humoral immune responses that do not include a high seroprevalent adenovirus serotype. In particular, when Ad28 was used as the prime, Ad35 boosted antibody responses. Marked boosting of T cell responses (up to ~7-fold) was also observed in the prime-boost regimens of rare serotypes. Overall, magnitudes of CD8 $^+$ T cell responses induced by the combinations of alternative serotype vectors were somewhat lower than those observed with heterologous Ad5 boosting. Priming with adenovirus vectors based on rare serotypes such as Ad28 provided for potent boosting of T cell and antibody responses by Ad5, in good agreement with previous findings based on other rare serotype vectors [3,18,46-48]. Levels of IFN- γ^+ /CD8 $^+$ NP-specific T cells reached over 25% in Ad5-boosted animals. The magnitude of IFN- γ^+ and TNF- α^+ CD8 $^+$ T-cell responses was comparable to or higher than those in an influenza protection study using DNA prime – Ad5 boost [49]. The DNA prime – Ad5 boost regimen induced IFN- γ^+ and TNF- α^+ CD8 $^+$ T-cells up to ~10% and effectively protected in the mouse model against homotypic and heterotypic influenza challenge. Thus, the adenovirus prime – Ad5 boost regimens tested here induced potent T-cell responses. There was no difference in the T-cell boost between Ad28, Ad14, or Ad35 prime and Ad5 boost regimens. In contrast, Ad28 prime followed by Ad28 or Ad35 boost, as well as homologous prime-boost with Ad35 vector, elicited significant increases in HA antibodies, whereas Ad35 prime and Ad28 boost did not. It is possible that priming with Ad35 vector induces immune responses that interfere with the subsequent Ad28 boost, or that Ad28 is less efficient at boosting anti-HA antibodies than Ad35. Together, the results suggest that prime-boost vaccine regimens using combinations of rare Ad vectors without Ad5 may be feasible. Recently, it was reported that regimens combining Ad26 prime with an Ad5 boost in rhesus macaques resulted in higher T

cell responses and improved immune function and survival after SIV challenge compared to either Ad5-Ad5 or Ad35-Ad5 regimens [50]. Combined with the current study, this indicates certain species D-derived vectors can induce relevant T cell responses, and suggests that additional species D serotypes should be explored for the development of novel candidates for vaccine vector testing.

In this study, we evaluated a large number of human adenovirus serotypes for interactions with innate signaling pathways. Through a comparison of the effects of human adenoviruses on pro-inflammatory cytokine expression *in vitro* we found that species D serotypes, such as Ad28, had a unique pattern relative to the other species. Cytokine secretion patterns elicited by species D adenoviruses differed from the prototype Ad5 virus, as they strongly suppressed pro-inflammatory cytokines such as IL-12 and potently induced the secretion of IFN- α . Thus, it might be expected that a species D adenovirus vector would be less potent of a vaccine vector than even the species B adenoviruses Ad14 and Ad35. Ad28 was less potent than Ad5, however, Ad28 induced significantly higher T cell responses compared to Ad14 and Ad35 *in vivo*. Together, this showed that while adenovirus interactions with the innate immune system were serotype-dependent, particular patterns of innate immune modulation *in vitro* did not correlate with *in vivo* immunogenicity.

A previous report described suppression of IL-12 secretion and induction of type I interferons by a component of the Ad35 capsid [26,27]. The Ad35 fiber protein was presented on the Ad5 particle, in contrast to the current study in which we studied complete wild type Ad35 and Ad14 viral capsids. Our results were consistent with Ad35 inducing low levels of alpha-interferon, but we did not detect significant suppression of IL-12 or other pro-inflammatory cytokines. Despite the different results with wild type Ad35 in our study and the Ad5-Ad35 fiber vector there was good agreement with the species D viruses analyzed. In that study, the species D serotype Ad37 suppressed IL-12 production, and was also shown separately to induce type I interferon and suppress other pro-inflammatory cytokines [26]. This is consistent with the effects of the eighteen species D serotypes analyzed for IL-12 suppression, the induction of alpha-interferon by Ad28, and the suppression of pro-inflammatory cytokines by Ad28 in our study.

The receptors for species B and C (CAR) have been the subject of much investigation [41,42]. In contrast, relatively little is known about receptor usage of the members of the human adenovirus species with the most serotypes, D. The D serotypes 9, 15, and 19 have been shown to use CAR [51], 8, 19a, and 37 can utilize sialic acid as a cellular receptor [52,53] yet more likely primarily utilizes CD46 [54]. Our results using a CD46 receptor binding assay indicate that many species D serotypes do not interact with CD46. Alternatively, it is possible that adenoviruses could bind differently to a truncated soluble form of CD46 compared to binding in the context of a cell membrane-expressed viral receptor. Transduction studies using competitors of CD46-binding were performed in CD46-expressing BHK cells, indicating that the D serotype Ad28 does not use CD46 as a cellular receptor for infection. We also did not detect an effect of CAR overexpression on transduction with Ad28. Together, this implies that Ad28 does not utilize the major adenovirus receptors CAR or CD46, and the receptor for Ad28 remains to be identified. In contrast, the species B serotypes Ad14 and Ad35 efficiently bound to sCD46 *in vitro* and transduction with these viruses was sensitive to competition with Ad35 fiber knob. However, Ad14-mediated transduction was not abrogated by virus preincubation with sCD46, consistent with the report that the Ad14 fiber knob binds to CD46 with lower affinity and higher off-rate constants compared to Ad35 [55]. This is also consistent with our observation that Ad14 transduction was efficiently inhibited by the full length Ad35 fiber protein. Therefore, Ad14 likely interacts with CD46 on the cell surface and may utilize it and possibly a second, alternative receptor for entry.

Our results suggest that the mechanisms that modulated pro-inflammatory and type I IFN pathways and determined magnitude of adaptive immune responses were not related to CD46 receptor usage. Firstly, Ad28 does not bind to or use the CD46 receptor, demonstrating that suppression of pro-inflammatory cytokine secretion and induction of type I IFN by Ad28 are mediated by CD46-independent pathways. This may also be the case for other species D adenoviruses that were found not to bind to sCD46 protein. Secondly, recent studies comparing T-cell responses induced by several rare adenoviruses identified the species D virus Ad26 as a serotype with increased immunogenicity that appears to utilize CD46 for infection [16,47]. Together, this suggests that neither modulation of the innate signaling pathways studied here *in vitro* nor CD46 receptor utilization *in vivo* can be used to predict immunogenicity of a serotype.

Mouse models for viral vectored vaccine evaluation can be limited by the absence of viral receptors. Most mouse tissues do not express CD46 [56] and it is not known whether relevant mouse cell types express a receptor for Ad28. The relative immunogenicity of Ad5 and Ad35 was unchanged between mouse and Rhesus macaque models and the performance of Ad28 in a non-human primate model remains an open question. Our prime-boost studies in mice showed that two rare serotype vectors, whether utilizing CD46 or not, can contribute to enhanced immune responses; suggesting that further testing of Ad28 in non-human primate models of vaccination for a specific disease target would be justified.

It is currently unknown which innate signaling pathways are necessary to elicit strong adaptive immune responses to adenovirus vaccine vectors. Previous studies with other vaccine systems and adjuvants in knockout mouse models have shown varying effects. The pro-inflammatory cytokine IL-12 is a downstream effector of the Toll-like receptor (TLR) pathway and is considered a key inducer of cell-mediated immune responses via the Th1 pathway [57]. However, here the Ad28 vector induced superior CD8⁺ T cell responses compared to other rare serotype vectors, despite its ability to potentially downregulate IL-12 secretion *in vitro*. Therefore, IL-12 suppression *in vitro* is not a useful predictor of adenovirus immunogenicity. The immunogenicity of DNA plasmid vaccines depends on signaling through an intact type I IFN pathway, but is independent of TLR signaling [58]. In contrast, protein vaccination with CpG-A adjuvant in mice elicits antigen-specific CD8⁺ T cell responses that are critically dependent on both type I IFN and TLR pathways [59]. Similar *in vivo* studies will be necessary to elucidate the pathways necessary for mediating Ad vaccine vector immunogenicity.

Vaccine vectors derived from Ad5 have been explored for multiple vaccine applications and can induce potent transgene-specific T cell and antibody responses. However, the utility of Ad5-based vectors may be limited by the high seroprevalence of this serotype in human populations [15,16,60,61], prompting the development of vaccine vectors that avoid pre-existing immunity. For this reason, despite the high HA antibody responses induced by Ad5-containing prime-boost regimens, we would prefer to avoid neutralizing antibodies by using heterologous prime-boost regimens containing only adenovirus vectors with a low seroprevalence. In addition, results from the recent HIV STEP trial have raised concerns about use of Ad5-based vaccine vectors and underscore the need to develop alternative adenovirus vaccine vector platforms for which there is much lower prevalence of prior exposure [62,63]. Recombinant Ad vectors have previously been derived from rare species B and D serotypes, such as Ad35, but induced significantly lower cell-mediated immune responses in HIV and HCV animal vaccine models [16,18,19]. The data presented here demonstrates that vectors based on the low seroprevalent serotype Ad28 are attractive candidates for vaccine development, and also points to species D for discovery of potent vaccine vectors based on rare human serotypes.

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Figure 1. Species D serotypes suppress secretion of pro-inflammatory cytokines from stimulated human monocytes

(A) IL-12 secretion after adenoviral exposure at 10⁴ pu/cell and (B) IL-12 secretion as a function of viral dose. IL-12 p70 subunit concentrations were determined by ELISA (R&D Systems). (C) Secretion of IL-1 α/β , IL-2, IL-6, IL-8, IL-10, IL-12, and TNF- α after adenoviral exposure at 10⁴ pu/cell measured by chemoluminescent protein array (Thermo Scientific, Searchlight # 84619B). Panels B and C show representative serotypes of species B (Ad14, Ad35), species C (Ad5), and species D (Ad28). Results are representative of three independent experiments. Stim. = Cells stimulated with IFN- γ and LPS only; Non-stim. = non-stimulated cells.

Figure 2. Species D serotypes induce IFN-α secretion from human PBMC

Human PBMC were exposed to CsCl-purified wild-type adenoviruses at 10^4 pu/cell. Supernatants were harvested after 48 hours and IFN-α concentration was measured by ELISA. Results are representative of three independent experiments. N.S. = Non-stimulated.

Figure 3. Ad28 does not bind to CD46 and does not utilize the CD46 receptor or CAR for infection

(A) CD46 Binding Assay. 96-well ELISA plates were coated with soluble CD46 receptor (sCD46) and exposed to CsCl-purified wild-type adenoviral serotypes at 10^9 (white bars) or 10^{10} (black bars) pu/well. Adenoviral binding to sCD46 was quantitated by measuring PicoGreen dye binding to viral DNA with a fluorescence microplate reader. Ad35GH is an Ad35-derived vector with a mutation in the GH loop of fiber that ablates CD46 binding. (B) BHK cells expressing the CD46 receptor (BHK-CD46) were incubated with recombinant Ad35 fiber (F-35) at varying concentrations, followed by infection with 2×10^9 pu/mL of Ad5, Ad14, Ad28 or Ad35 vectors expressing luciferase. Vector transduction was quantitated 24 hours post infection by luminescence assay. Results show the percentage of luciferase activity remaining compared to uncompetited transductions and are representative of three independent experiments. (C) Comparative transduction of CHO cells that do not express CAR (CHO) and cells engineered to express CAR (CHO-CAR). Cells were incubated with Ad5 or Ad28 vectors expressing luciferase and cell extracts were analyzed for luciferase activity at 24 hours post-infection.

Figure 4. Ad28 has a low seroprevalence in the United States

(A) NAb titers to Ad5 and Ad28 were determined in a total of 240 serum samples drawn from five geographic regions across the United States (48 samples per region). Sera were screened in triplicate at a 1:16 dilution in a transduction inhibition assay. Samples scored positive for NAb when the mean luciferase activity was inhibited by 90% compared to uninhibited control transductions. Shown are the percentages of NAb-positive sera in each region and the combined average. (B) Distribution of Ad5 and Ad28 neutralization titers. Samples that scored positive in the initial screen were retested by performing serial doubling dilutions, ranging from 1:16 to 1:2,048 in duplicate. All Ad28-seropositive samples (n=19) and a randomly selected subset of Ad5-seropositive samples (n=50) were retested. Neutralization titers were defined as the maximum serum dilution that inhibited luciferase activity by 90%.

Figure 5. Single immunization with Ad28 vaccine vector induces potent T cell responses Mice were injected i.m. with 10⁷, 10⁸, or 10⁹ pu Ad5, Ad14, Ad28, or Ad35 vectors expressing influenza NP. Two weeks post-immunization NP-specific responses were assessed by intracellular cytokine staining (ICS) in splenocytes stimulated with a single peptide for the immunodominant epitope (NP₁₄₇₋₁₅₅) or a peptide pool spanning NP. Shown are results for NP pool-stimulated cells (A, C; open symbols) and cells stimulated with NP₁₄₇₋₁₅₅ (B; closed symbols). Two independent experiments were performed with a total of n = 10/group. Thresholds above which responses were considered positive are indicated by dotted lines and were determined using mock-treated (FFB) animals. Bars represent mean values. Individual groups were compared with Bonferroni-adjusted Mann-Whitney tests, resulting in p < 0.01 as the significance threshold. * = p < 0.01; ** = p < 0.001.

Figure 6. Priming with Ad28 followed by Ad5 immunization potently boosts T cell responses compared to single Ad5 vaccination

Mice were primed i.m. with 10^9 pu Ad5, Ad14, Ad28, or Ad35 vectors expressing influenza NP or vehicle (FFB) and then boosted four weeks later with 10^9 pu Ad5NP. Two weeks post-boost NP-specific responses were assessed by ICS in splenocytes stimulated with a single peptide for the immunodominant epitope (NP₁₄₇₋₁₅₅) or a peptide pool spanning NP. Immune responses were comparable for both stimulation conditions, and results are shown for NP₁₄₇₋₁₅₅-stimulated cells (closed symbols). Two independent experiments were performed with a total of n = 12/group. Thresholds above which responses were considered positive are indicated by dotted lines and were determined using mock-treated (FFB) animals. Bars represent mean values. Individual groups were compared with Bonferroni-adjusted Mann-Whitney tests, resulting in p < 0.005 as the significance threshold. * = p < 0.005; ** = p < 0.0005.

Figure 7. Prime-boost regimens using Ad28 together with another rare serotype vector significantly boost T cell responses

Mice were primed i.m. with 10^9 pu Ad5, Ad14, or Ad28 vectors expressing influenza NP or vehicle (FFB). Four weeks later a heterologous boost was performed at the same dose. Two weeks post-boost NP-specific responses were assessed by ICS in splenocytes stimulated with a single peptide for the immunodominant epitope (NP₁₄₇₋₁₅₅) or a peptide pool spanning NP. Immune responses were comparable for both stimulation conditions, and results are shown for NP₁₄₇₋₁₅₅-stimulated cells (closed symbols). Two independent experiments were performed with a total of $n = 12$ /group. Thresholds above which responses were considered positive are indicated by dotted lines and were determined using mock-treated (FFB) animals. Bars represent mean values. Individual groups were compared with Bonferroni-adjusted Mann-Whitney tests, resulting in $p < 0.0125$ as the significance threshold. * = $p < 0.0125$; ** = $p < 0.005$.

Figure 8. Prime-boost regimens using Ad28 or Ad35 together with Ad5 potentially boost antibody responses

Mice were primed i.m. with 10^9 pu of (A) Ad28 or (B) Ad35 vectors expressing influenza HA. Twenty-eight days later an Ad vector boost was performed at the same dose. Sera were assayed for antibody titers to HA by ELISA at 14 and 28 days post-prime and 7 days post-boost (Day 35) using serial doubling dilutions and a background threshold of 1:128. Two independent experiments were performed with a total of $n = 12$ /group. HA antibody responses on days 28 and 35 were compared using Mann-Whitney tests. * = $p < 0.05$, **** = $p < 0.0001$.