Immunogenicity of Recombinant Fiber-Chimeric Adenovirus Serotype 35 Vector-Based Vaccines in Mice and Rhesus Monkeys

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Preexisting immunity to adenovirus serotype 5 (Ad5) has been shown to suppress the immunogenicity of recombinant Ad5 (rAd5) vector-based vaccines for human immunodeficiency virus type 1 (HIV-1) in both preclinical studies and clinical trials. A potential solution to this problem is to utilize rAd vectors derived from rare Ad serotypes, such as Ad35. However, rAd35 vectors have appeared less immunogenic than rAd5 vectors in preclinical studies to date. In this study, we explore the hypothesis that the differences in immunogenicity between rAd5 and rAd35 vectors may be due in part to differences between the fiber proteins of these viruses. We constructed capsid chimeric rAd35 vectors containing the Ad5 fiber knob (rAd35k5) and compared the immunogenicities of rAd5, rAd35k5, and rAd35 vectors expressing simian immunodeficiency virus Gag and HIV-1 Env in mice and rhesus monkeys. In vitro studies demonstrated that rAd35k5 vectors utilized the Ad5 receptor CAR rather than the Ad35 receptor CD46. In vivo studies showed that rAd35k5 vectors were more immunogenic than rAd35 vectors in both mice and rhesus monkeys. These data suggest that the Ad5 fiber knob contributes substantially to the immunogenicity of rAd vectors. Moreover, these studies demonstrate that capsid chimeric rAd vectors can be constructed to combine beneficial immunologic and serologic properties of different Ad serotypes.

Recombinant adenovirus serotype 5 (rAd5) vector-based vaccines have been shown to elicit high-frequency, protective immune responses in a variety of animal models (22, 23, 26, 27). As a result, rAd5 vaccines for human immunodeficiency virus type 1 (HIV-1) and other pathogens are currently being advanced into large-scale clinical trials (5, 14, 24). However, the high prevalence of preexisting anti-Ad5 immunity in human populations may limit the immunogenicity and clinical utility of rAd5 vector-based vaccines, particularly in the developing world (12, 29, 30). Importantly, anti-Ad5 immunity has already been shown to suppress the immunogenicity of rAd5 vaccines for HIV-1 in both preclinical studies (2, 3, 7, 8, 28, 29, 33) and clinical trials (24).

A potential solution to this problem is to develop rAd vectors from rare human Ad serotypes, such as Ad35 and Ad11 (11, 23, 30). We have recently reported that rAd35 and rAd11 vectors expressing simian immunodeficiency virus (SIV) Gag were immunogenic and were not suppressed by anti-Ad5 immunity in mice, demonstrating the potential feasibility of this approach (3, 13). However, preclinical studies from our laboratory and others have suggested that rare serotype rAd vectors are intrinsically less immunogenic than rAd5 vectors in both mice and rhesus monkeys (3, 13, 23, 24).

In this study, we explore the hypothesis that the differences

in immunogenicity between rAd5 and rAd35 vaccines may be due in part to the differences between the Ad5 and Ad35 fiber proteins. The Ad5 fiber knob interacts with the Ad5 receptor CAR (coxsackievirus and adenovirus receptor) on the surfaces of cells and mediates efficient viral attachment (6, 18, 19) prior to viral entry that is facilitated by the penton base and cellular integrins (31). In contrast, the Ad35 fiber knob does not interact with CAR but instead utilizes CD46 as a high-affinity receptor (9). Differences in receptor binding also likely contribute to the different tropisms of these viruses. For example, rAd5 but not rAd35 vectors exhibit significant liver tropism (30). In addition, the different fiber knobs direct these viruses into different intracellular trafficking pathways. The Ad5 fiber knob facilitates rapid viral escape from early endosomes into the cytosol, leading to efficient translocation of viral genomes into the nucleus, whereas the Ad35 fiber knob results in retention of virus particles in late endosomes and recycling of a large fraction back to the cell surface (21). The Ad5 fiber knob thus may contribute to the potent immunogenicity of rAd5 vectors by facilitating efficient viral attachment to cells, by determining viral tropism, and by leading to efficient intracellular trafficking of viral particles to the nucleus.

To assess the role of the Ad5 fiber knob in contributing to the immunogenicity of rAd vectors, we constructed capsid chimeric rAd35 vectors containing the Ad5 fiber knob (rAd35k5) and compared the immunogenicities of rAd5, rAd35k5, and rAd35 vectors. We observed that rAd35k5 vectors elicited higher immune responses than rAd35 vectors in both mice and rhesus monkeys. These data show that the Ad5 fiber knob

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contributes substantially to the immunogenicity of rAd vectors. These results further demonstrate that capsid chimeric rAd vectors can be constructed to combine beneficial immunologic and serologic properties of different Ad serotypes.

MATERIALS AND METHODS

Vector construction, production, and purification. E1/E3-deleted, replication-incompetent rAd35k5 vectors were generated in PER.C6/55K cells essentially as described previously (30). The region encoding the Ad5 fiber knob (amino acids 133 to 314) was synthesized by GeneART (Regensburg, Germany) and was cloned as a BsiWI-NheI fragment into the pBR.Ad35.PacI-rITR.dE3 plasmid to replace the corresponding region encoding the Ad35 fiber knob. The mutant pBR.Ad35k5.PacI-rITR.dE3 plasmid together with the pWE.Ad35.pIX-EcoRV cosmid and pAdApt35 adaptor plasmids encoding SIVmac239 Gag or HIV-1 89.6P Env was cotransfected into PER.C6/55K cells. Double homologous recombination yielded the rAd35k5-Gag or rAd35k5-Env vector. These vectors were plaque purified, analyzed for transgene expression, amplified in 24 to 48 triple-layer T175 flasks, purified by double CsCl gradient ultracentrifugation, and dialyzed into phosphate-buffered saline (PBS) containing 5% sucrose. Purified rAd vectors were stored at -80°C . Virus particle titers were determined by high-performance liquid chromatography. Infectivity was assessed by PFU assays. SIV Gag expression was confirmed by infection of A549 cells, followed by intracellular monoclonal antibody (MAb) staining for Gag p27 and analysis by flow cytometry. HIV-1 Env expression was confirmed by Western blotting and enzyme-linked immunosorbent assays (ELISAs). Replication-incompetent rAd5 and rAd35 vectors were produced using similar methods.

Animals and immunizations. Six- to eight-week-old C57BL/6 or BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were injected intramuscularly (i.m.) with various doses of replication-incompetent rAd5, rAd35k5, or rAd35 vectors expressing SIV Gag or HIV-1 Env in 50 μl sterile PBS in both quadriceps muscles. To induce active antivector immunity, mice were preimmunized once or twice separated by a 4-week interval i.m. with 10^{10} virus particles (vp) rAd5-Empty in 100 μl sterile PBS.

Adult rhesus monkeys (*Macaca mulatta*) that did not express the major histocompatibility complex class I allele *Mamu-A*01* were housed at New England Primate Research Center (Southborough, MA). At week 0, monkeys were primed i.m. with 10^{11} vp replication-incompetent rAd5, rAd35k5, or rAd35 vector expressing SIV Gag and HIV-1 Env in 1 ml sterile PBS in the quadriceps muscles. At week 12, monkeys received a homologous boost immunization. All animal studies were approved by our Institutional Animal Care and Use Committees.

Tetramer binding assays. Tetrameric H-2D^b complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) (3) were prepared and utilized to stain peptide-specific CD8⁺ T lymphocytes from C57BL/6 mice as described previously (1, 3). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. Following lysis of red blood cells, 0.1 μg of phycoerythrin-labeled D^b/AL11 tetramer in conjunction with allophycocyanin-labeled anti-CD8 α MAb (Ly-2; Caltag, San Francisco, CA) was utilized to stain AL11-specific CD8⁺ T lymphocytes. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry with FACS Calibur (BD Pharmingen, San Diego, CA). Gated CD8⁺ T lymphocytes were examined for staining with the D^b/AL11 tetramer. CD8⁺ T lymphocytes from naive mice were utilized as negative controls and exhibited <0.1% tetramer staining.

ELISPOT assays. Gag- and Env-specific cellular immune responses in vaccinated mice and rhesus monkeys were assessed by gamma interferon (IFN- γ) enzyme-linked immunospot (ELISPOT) assays as described previously (3, 4). Overlapping 15-amino-acid peptides spanning the SIVmac239 Gag and HIV-1 89.6P Env proteins were obtained from the NIH AIDS Research and Reference Reagent Program. Ninety-six-well multiscreen plates (Millipore, Bedford, MA) were coated overnight with 100 μl /well of 10 $\mu\text{g}/\text{ml}$ anti-mouse or anti-human IFN- γ (BD Pharmingen, San Diego, CA) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20 (D-PBS-Tween), blocked for 2 h with D-PBS containing 5% FBS at 37°C , washed three times with D-PBS-Tween, rinsed with RPMI 1640 containing 10% FBS to remove the Tween 20, and incubated with 2 $\mu\text{g}/\text{ml}$ each peptide and 5×10^5 murine splenocytes or 2×10^5 monkey peripheral blood mononuclear cells (PBMC) in triplicate in 100- μl reaction volumes. Following an 18-h incubation at 37°C , the plates were washed nine times with D-PBS-Tween and once with distilled water. The plates were then incubated with 2 $\mu\text{g}/\text{ml}$ biotinylated anti-mouse or anti-human IFN- γ (BD Pharmingen, San Diego, CA) for 2 h at

room temperature, washed six times with Coulter Wash (Coulter Corporation, Miami, FL), and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Following five washes with Coulter Wash and one with PBS, the plates were developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, Rockford, IL), reactions were stopped by washing of plates with tap water, plates were air-dried, and results were read using an ELISPOT reader (HiTech Instruments, Edgement, PA). Spot-forming cells (SFC) per 10^6 cells were calculated. Medium backgrounds were consistently <15 spot-forming cells per 10^6 cells.

ELISA. Serum antibody titers specific for HIV-1 Env or SIV Gag from immunized mice were measured by a direct ELISA as described previously (2, 29). Ninety-six-well plates coated overnight with 100 μl /well of 1 $\mu\text{g}/\text{ml}$ recombinant HIV-1 MN Env gp120 or SIVmac239 Gag p27 (ImmunoDiagnostics, Woburn, MA) in PBS were blocked for 2 h with PBS containing 2% bovine serum albumin and 0.05% Tween 20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with a 1:2,000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary antibody (Jackson Laboratories, Bar Harbor, ME). The plates were washed three times and developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD), reactions were stopped with 1% HCl, and results were analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader.

Virus neutralization assay. Ad5- and Ad35-specific neutralizing antibody (NAb) responses were assessed by luciferase-based virus neutralization assays as described previously (25). A549 human lung carcinoma cells were plated at a density of 1×10^4 cells per well in 96-well plates and infected with E1/E3-deleted, replication-incompetent rAd5-Luciferase or rAd35-Luciferase reporter constructs at a multiplicity of infection of 500 with twofold serial dilutions of serum in 200- μl reaction volumes. Following a 24-h incubation, luciferase activities in the cells were measured using a Steady-Glo luciferase reagent system (Promega, Madison, WI). Neutralization titers were defined as the maximum serum dilutions that neutralized 90% of luciferase activity.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 4.01 (GraphPad Software, Inc., 2004). Immune responses among groups of mice are presented as means with standard errors. Comparisons of mean immune responses were performed by two-tailed *t* tests for two groups of animals or by analyses of variance for more than two groups. Bonferroni's adjustments were included when appropriate to account for multiple comparisons. In all cases, *P* values of less than 0.05 were considered significant.

RESULTS

Construction of capsid chimeric rAd35k5 vectors. We initiated studies by constructing E1/E3-deleted, replication-defective capsid chimeric rAd35 vectors containing the Ad5 fiber knob (rAd35k5). As shown in Fig. 1A, rAd35k5 vectors were designed by replacing the Ad35 fiber gene with a chimeric fiber gene consisting of the Ad35 fiber tail and shaft (amino acids 1 to 132) and the Ad5 fiber knob (amino acids 133 to 314). Vectors expressing SIVmac239 Gag and HIV-1 89.6P Env gp120 were produced in PER.C6/55K cells as described in Materials and Methods and as previously reported (30). Growth rates and yields of rAd35k5 vectors were comparable with those of parental rAd35 vectors (data not shown). Ratios of virus particles to PFU, however, were approximately 10-fold higher for rAd35k5-Gag vectors (range, 61 to 595; median, 189) than for rAd35-Gag vectors (range, 1 to 54; median, 13).

We assessed receptor usage by rAd35k5 vectors with cell lines stably expressing either the Ad5 receptor CAR or the Ad35 receptor CD46. Cells were infected with rAd5-Gag, rAd35k5-Gag, or rAd35-Gag at a multiplicity of infection of 1,000 for 2 h and were then washed and incubated for 2 days. Virus infectivity was assessed by intracellular MAb staining for SIV Gag, followed by analysis by flow cytometry. As shown in Fig. 1B, rAd5-Gag and rAd35k5-Gag but not rAd35-Gag efficiently transduced CHO-CAR cells. Conversely, as depicted in

FIG. 1. Construction of rAd35k5 vectors. (A) rAd35k5 vectors were constructed by replacing the Ad35 fiber gene with a chimeric fiber gene consisting of the Ad35 tail, Ad35 shaft, and Ad5 knob. Genetic organization of the Ad35 genome is shown (ITR, E2, E3, E4, L1-L3, L4, L5). Receptor usage was determined by assessing the abilities of rAd5, rAd35k5, and rAd35 vectors expressing SIV Gag to infect cells transfected with (B) the Ad5 receptor CAR or (C) the Ad35 receptor CD46. Transgene expression was assessed by intracellular expression of SIV Gag.

Fig. 1C, rAd35-Gag but not rAd5-Gag or rAd35k5-Gag readily transduced B16F10-CD46 cells. Thus, rAd5 utilized the CAR receptor, whereas rAd35 utilized the CD46 receptor, as has been previously reported (6, 9, 18, 19). Importantly, engineering the rAd35 vector by replacing the Ad35 fiber knob with the Ad5 fiber knob effectively switched receptor usage from CD46 to CAR.

Immunogenicities of rAd5, rAd35k5, and rAd35 vectors in mice. We next assessed the immunogenicities of rAd5, rAd35k5, and rAd35 vectors expressing SIV Gag in mice. Groups of naïve C57BL/6 mice ($n = 8/\text{group}$) were immunized once i.m. with 10^9 vp rAd5-Gag, rAd35k5-Gag, or rAd35-Gag. Gag-specific CD8⁺ T lymphocyte responses to the immunodominant AL11 epitope (AAVKNWMTQTL) (3) were measured by D^b/AL11 tetramer binding assays. As shown in Fig. 2A, all three vaccine vectors elicited comparable mean tetramer⁺ CD8⁺ T lymphocyte responses following vaccination at this dose.

We next repeated this experiment with a lower dose (10^8 vp)

FIG. 2. Immunogenicities of rAd5, rAd35k5, and rAd35 vectors expressing SIV Gag in mice. Naïve C57BL/6 mice were immunized with (A) 10^9 vp or (B) 10^8 vp rAd5-Gag, rAd35k5-Gag, or rAd35-Gag. To assess the impact of preexisting anti-Ad5 immunity, C57BL/6 mice were preimmunized with (C) one or (D) two injections of 10^{10} vp rAd5-Empty prior to immunization with 10^8 vp rAd5-Gag, rAd35k5-Gag, or rAd35-Gag. In all cases, Gag-specific CD8⁺ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization.

of each vector. As shown in Fig. 2B, mean tetramer⁺ CD8⁺ T lymphocyte responses elicited by rAd35-Gag were lower than those elicited by rAd5-Gag at this dose, consistent with our prior observations (3, 13). Responses elicited by rAd35k5-Gag, however, were only slightly lower than those induced by rAd5-Gag (*P* value was not significant) and were significantly higher than those elicited by rAd35-Gag at this dose (*P* of <0.01, comparing mean tetramer responses among groups of mice on day 14, using analyses of variance with Bonferroni's adjustments to account for multiple comparisons). These tetramer data were confirmed by pooled peptide IFN- γ ELISPOT assays (data not shown). Thus, inclusion of the Ad5 fiber knob enhanced the immunogenicity of rAd35 vectors in C57BL/6 mice.

We next sought to evaluate the impact of preexisting anti-Ad5 immunity on the immunogenicity of these vectors. Groups of C57BL/6 mice (*n* = 4/group) were preimmunized once with 10¹⁰ vp rAd5-Empty 4 weeks prior to vaccination to generate low/moderate levels of anti-Ad5 immunity. Ad5-specific NAb titers in these mice were 64 to 128 (3, 13, 25). As shown in Fig. 2C, tetramer⁺ CD8⁺ T lymphocyte responses elicited by 10⁸ vp rAd5-Gag were essentially ablated in these mice. In contrast, 10⁸ vp rAd35-Gag and rAd35k5-Gag responses were not substantially affected by this level of anti-Ad5 immunity. Importantly, rAd35k5-Gag proved more immunogenic than both rAd5-Gag (*P* < 0.001) and rAd35-Gag (*P* < 0.05) in these mice on day 14 following immunization.

The ability of rAd35k5-Gag to evade low/moderate levels of anti-Ad5 immunity is consistent with our previous findings that Ad5-specific NABs are primarily directed against the Ad5 hexon protein (29). However, we also detected low levels of NABs directed against the Ad5 fiber protein in this prior study (29). We therefore repeated this experiment for mice with high levels of anti-Ad5 immunity. Mice were preimmunized twice with 10¹⁰ vp rAd5-Empty 8 weeks and 4 weeks prior to vaccination. Ad5-specific NAb titers in these mice were 8,192 to 16,384 (3, 13, 25), comparable with the highest titers found in individuals in sub-Saharan Africa (12, 29). As shown in Fig. 2D, tetramer⁺ CD8⁺ T lymphocyte responses elicited by rAd35k5-Gag were reduced by approximately 50% in these mice and were comparable with those induced by rAd35-Gag (*P* value was not significant). Thus, high levels of anti-Ad5 immunity partially suppressed the immunogenicity of rAd35k5 vectors.

To evaluate in further detail vector-specific immunity elicited by the chimeric rAd35k5-Gag vectors, we performed heterologous prime-boost studies as well as virus neutralization assays. Groups of naïve C57BL/6 mice (*n* = 4/group) were primed at week 0 with 10⁹ vp rAd35k5-Gag and were boosted at week 4 with 10⁹ vp rAd5-Gag or rAd35-Gag. As shown in Fig. 3A, tetramer⁺ CD8⁺ T lymphocyte responses elicited by rAd35k5-Gag were efficiently boosted by rAd5-Gag but not by rAd35-Gag. These data suggest that rAd35k5 and rAd35 induced substantial cross-reactive antivector immunity, whereas rAd35k5 and rAd5 were largely immunologically distinct. Consistent with these observations, mice immunized with rAd35k5-Gag generated Ad35-specific NABs comparable with those induced by rAd35-Gag, but Ad5-specific NABs were substantially fewer than Ad35-specific NABs in these mice (Fig. 3B). Thus,

FIG. 3. Antivector immunity elicited by rAd35k5 vectors in mice. (A) Naïve C57BL/6 mice were primed at week 0 with 10⁹ vp rAd35k5-Gag and were boosted at week 4 with 10⁹ vp rAd5-Gag or rAd35-Gag. Arrows indicate immunizations. Gag-specific CD8⁺ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays. (B) Serum samples from mice injected with 10⁹ vp rAd5-Gag, rAd35k5-Gag, or rAd35-Gag were assessed in Ad5 and Ad35 luciferase-based virus neutralization assays.

rAd35k5 vectors exhibited primarily the serologic profile of rAd35 vectors rather than rAd5 vectors.

To assess the generalizability of these results, we evaluated the immunogenicity of rAd35k5 vectors expressing a second antigen in a different strain of mice. Groups of naïve BALB/c mice (*n* = 4/group) were immunized once i.m. with 10⁹ vp rAd5-Env, rAd35k5-Env, or rAd35-Env. Cellular and humoral immune responses elicited by rAd35k5-Env vectors were intermediate between those induced by rAd5-Env vectors and rAd35-Env vectors, as measured by IFN- γ ELISPOT assays (Fig. 4A) and Env-specific ELISAs (Fig. 4B).

Immunogenicities of rAd5, rAd35k5, and rAd35 vectors in rhesus monkeys. To confirm the immunogenicity studies in mice, we performed a pilot study to evaluate the immunogenicities of rAd5, rAd35k5, and rAd35 vectors in rhesus monkeys. Nine *Mamu-A*01*-negative rhesus monkeys (*n* = 3/group) were immunized i.m. with 10¹¹ vp rAd5, rAd35k5, or rAd35 vectors expressing SIV Gag and HIV-1 Env (total dose, 2 × 10¹¹ vp). We utilized this relatively high vaccine dose since a previous study reported that rAd35 vectors elicited relatively weak immune responses in rhesus monkeys (23). Monkeys were primed at week 0 and received a homologous boost immunization at week 12. Figure 5 depicts antigen- and vector-specific immune responses in these animals. Gag- and Env-specific cellular immune responses were quantitated by pooled peptide IFN- γ ELISPOT assays at multiple time points follow-

FIG. 4. Immunogenicities of rAd5, rAd35k5, and rAd35 vectors expressing HIV-1 Env in mice. Naïve BALB/c mice were immunized with 10^9 vp rAd5-Env, rAd35k5-Env, or rAd35-Env. (A) Env-specific cellular immune responses were assessed by pooled peptide and MI10 epitope peptide-specific IFN- γ ELISPOT assays. (B) Env-specific humoral immune responses were assessed by ELISA.

ing immunization. Vector-specific NAb titers were determined by luciferase-based virus neutralization assays.

The rAd5 vectors elicited high-frequency ELISPOT responses following the primary immunization (mean Gag-specific plus mean Env-specific responses of 538 SFC/10⁶ PBMC at week 12), but these responses were not substantially increased following the homologous boost immunization (mean, 608 SFC/10⁶ PBMC at week 16) (Fig. 5A). The failure to boost these responses presumably reflected the rapid generation of high titers of Ad5-specific NAb in these animals (Fig. 5B). The rAd35 vectors elicited antigen-specific ELISPOT responses that were approximately twofold lower than those induced by rAd5 vectors following the initial immunization (mean, 248 SFC/10⁶ PBMC at week 12). Interestingly, these responses increased substantially following the homologous boost immunization (mean, 876 SFC/10⁶ PBMC at week 16) (Fig. 5C), consistent with the lower titers of vector-specific NAb initially generated in these animals (Fig. 5D). Thus, although rAd35 vectors elicited lower antigen-specific immune responses than did rAd5 vectors, rAd35 vectors also induced lower vector-specific NAb that allowed for an effective homologous boost immunization in rhesus monkeys. These differences between vector-specific NAb generated by rAd5 and rAd35 vectors were not apparent with mice (Fig. 3B).

The rAd35k5 vectors elicited antigen-specific ELISPOT responses comparable to those induced by rAd5 vectors following the primary immunization (mean, 578 SFC/10⁶ PBMC at

week 12) (Fig. 5E). Importantly, these responses were also substantially enhanced following the homologous boost immunization (mean, 1,736 SFC/10⁶ PBMC at week 16), presumably reflecting the relatively low vector-specific NAb initially generated in these monkeys (Fig. 5F). In fact, following the boost immunization, rAd35k5 vectors elicited twofold to threefold higher Gag- and Env-specific ELISPOT responses than either rAd5 or rAd35 vectors, although the small numbers of animals per group precluded a formal statistical analysis.

All three vectors also elicited both CD4⁺ and CD8⁺ T lymphocyte responses, as determined by ELISPOT assays using CD8-depleted and CD4-depleted PBMCs (Fig. 6). Consistent with the unfractionated ELISPOT data (Fig. 5), both CD4⁺ and CD8⁺ T lymphocyte responses elicited by rAd35k5 vectors were twofold to fourfold higher than those induced by either rAd5 or rAd35 vectors at week 16 following immunization.

DISCUSSION

The high prevalence of preexisting anti-Ad5 immunity, particularly in the developing world, has led to the development of rAd vectors derived from rare Ad serotypes such as Ad35 (13, 29, 30). Importantly, studies to date with mice (3, 13) and rhesus monkeys (23) have shown that rAd35 vectors effectively evade anti-Ad5 immunity but are intrinsically less immunogenic than rAd5 vectors. In this study, we explored the importance of the Ad5 fiber knob in contributing to rAd vector immunogenicity by constructing capsid chimeric rAd35 vectors containing the Ad5 fiber knob (rAd35k5). The rAd35k5 vectors utilized the Ad5 receptor CAR rather than the Ad35 receptor CD46 and exhibited enhanced immunogenicity compared with that of rAd35 vectors in both mice and rhesus monkeys. These studies demonstrate that capsid chimeric rAd vectors can be constructed to combine beneficial immunologic and serologic properties of different Ad serotypes.

The potent immunogenicity of rAd35k5 vaccine vectors suggests a functionally relevant role of the Ad5 fiber protein. The Ad5 fiber knob determines binding to the high-affinity receptor CAR (6, 9, 18, 19) and also plays a key role in the efficient intracellular trafficking of viral particles to the nucleus (21). Differences in receptor usage also likely contribute to the different viral tropisms, for example, the liver tropism of rAd5 vectors but not rAd35 vectors (30). In addition, the Ad5 fiber knob has been shown to contribute to dendritic cell maturation via an NF- κ B-dependent pathway (15, 16). It is likely that the enhanced immunogenicity of rAd35k5 vectors reflects the biologic functions of the Ad5 fiber knob, although the precise mechanism remains to be determined.

The rAd35k5 vectors exhibited primarily the neutralization profile of rAd35 vectors and thus effectively evaded low/moderate levels of anti-Ad5 immunity (Fig. 2C). These observations are consistent with previous studies demonstrating that Ad5-specific NAb are directed primarily against the Ad5 hexon protein (10, 29, 32, 34). Consistent with these findings, rAd5 vectors containing the Ad35 fiber (rAd5f35 vectors) failed to circumvent anti-Ad5 immunity and elicited lower humoral and cellular immune responses than rAd5 vectors in both mice and nonhuman primates (17). High levels of anti-Ad5 immunity, however, partially reduced the immunogenicity

FIG. 5. Immunogenicities of rAd5, rAd35k5, and rAd35 vectors in rhesus monkeys. Adult rhesus monkeys were primed at week 0 with 10^{11} vp (A and B) rAd5, (C and D) rAd35, or (E and F) rAd35k5 vectors expressing HIV-1 Env and SIV Gag. At week 12, all monkeys received a homologous boost immunization. (A, C, and E) Env- and Gag-specific cellular immune responses were assessed by pooled peptide IFN- γ ELISPOT assays at multiple time points following immunization. (B, D, and F) Vector-specific NAb titers were assessed by Ad5 and Ad35 virus neutralization assays. Arrows indicate immunizations.

of rAd35k5 vectors (Fig. 2D), likely reflecting lower titer but clearly detectable NAb directed against the Ad5 fiber protein (Fig. 3B, 5F) (29). Importantly, the partial reduction of rAd35k5 immunogenicity by anti-Ad5 immunity was observed only at high Ad5-specific NAb titers. Future studies could evaluate whether chimeric rAd35 vectors containing fiber knobs of other CAR-binding Ad serotypes may circumvent this problem.

Another limitation of rAd35k5 vectors may be their relatively high virus particle/PFU ratios, which were approximately 10-fold higher than those typically observed with rAd35 vectors. This observation suggests that viral integrity and stability may be partly compromised by the chimeric fiber protein, which could potentially complicate large-scale vector production and may limit the ultimate utility of these vectors in their current form. However, we speculate that these vectors could be further improved and optimized by studies exploring the effects of exchanging selected fiber domains.

The rhesus monkey studies compared the immunogenicities of rAd5, rAd35k5, and rAd35 vectors (Fig. 5 and 6) and confirmed the results obtained with mice (Fig. 2 and 4). This is

FIG. 6. CD4⁺ and CD8⁺ T lymphocyte responses. To assess fractionated CD4⁺ and CD8⁺ T lymphocyte responses from the study in rhesus monkeys described in the legend for Fig. 5, pooled peptide IFN- γ ELISPOT assays were performed using CD8-depleted (Depl) and CD4-depleted PBMCs from monkeys at week 16. Monkeys received 10¹¹ vp (A) rAd5, (B) rAd35, or (C) rAd35k5 vectors expressing HIV-1 Env and SIV Gag at weeks 0 and 12.

important since mice lack the optimal Ad35 receptor, CD46, and thus could potentially underestimate the immunogenicity of rAd35 vectors (9). After the primary immunization in rhesus monkeys, the rAd35 vectors were approximately twofold less immunogenic than the rAd5 vectors. Thus, rAd35 vectors were clearly less immunogenic than rAd5 vectors in nonhuman primates, but they appeared more potent than previously reported (23), suggesting that minor differences in vector backbones or manufacturing processes may impact vector immunogenicity.

Importantly, the rAd5 vectors elicited rapid and high titers of vector-specific NAb that prevented a homologous boost immunization, consistent with prior studies with rhesus monkeys (20, 22). In contrast, both rAd35 and rAd35k5 vectors elicited lower vector-specific NAb titers than did rAd5 vectors, which facilitated efficient boosting of these responses following readministration of homologous vectors. The particularly robust immune responses observed with the monkeys that received rAd35k5 vectors likely reflected the fact that these vectors both primed robust initial responses and were efficiently boosted.

Our studies demonstrate that rAd35 vectors containing the

Ad5 fiber knob were more immunogenic than rAd35 vectors in both mice and monkeys. These data suggest that the Ad5 fiber protein contributes substantially to the immunogenicity of rAd vectors. Constructing capsid chimeric rAd vectors thus represents a novel strategy for the generation of improved, second-generation rAd vectors for both vaccination and gene therapy.

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