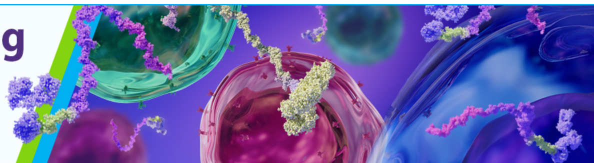

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J Immunol 2004; 172:6290-6297; ;
doi: 10.4049/jimmunol.172.10.6290
http://www.jimmunol.org/content/172/10/6290

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Immunogenicity of Recombinant Adenovirus Serotype 35 Vaccine in the Presence of Pre-Existing Anti-Ad5 Immunity[1]

Dan H. Barouch,[2]* Maria G. Pau,† Jerome H. H. V. Custers,† Wouter Koudstaal,† Stefan Kostense,† Menzo J. E. Havenga,† Diana M. Truitt,* Shawn M. Sumida,* Michael G. Kishko,* Janelle C. Arthur,* Birgit Korioth-Schmitz,* Michael H. Newberg,* Darci A. Gorgone,* Michelle A. Lifton,* Dennis L. Panicali,‡ Gary J. Nabel,§ Norman L. Letvin,* and Jaap Goudsmit†

The high prevalence of pre-existing immunity to adenovirus serotype 5 (Ad5) in human populations may substantially limit the immunogenicity and clinical utility of recombinant Ad5 vector-based vaccines for HIV-1 and other pathogens. A potential solution to this problem is to use vaccine vectors derived from adenovirus (Ad) serotypes that are rare in humans, such as Ad35. However, cross-reactive immune responses between heterologous Ad serotypes have been described and could prove a major limitation of this strategy. In particular, the extent of immunologic cross-reactivity between Ad5 and Ad35 has not previously been determined. In this study we investigate the impact of pre-existing anti-Ad5 immunity on the immunogenicity of candidate rAd5 and rAd35 vaccines expressing SIV Gag in mice. Anti-Ad5 immunity at levels typically found in humans dramatically blunted the immunogenicity of rAd5-Gag. In contrast, even high levels of anti-Ad5 immunity did not substantially suppress Gag-specific cellular immune responses elicited by rAd35-Gag. Low levels of cross-reactive Ad5/Ad35-specific CD4$^+$ T lymphocyte responses were observed, but were insufficient to suppress vaccine immunogenicity. These data demonstrate the potential utility of Ad35 as a candidate vaccine vector that is minimally suppressed by anti-Ad5 immunity. Moreover, these studies suggest that using Ad vectors derived from immunologically distinct serotypes may be an effective and general strategy to overcome the suppressive effects of pre-existing anti-Ad immunity. *The Journal of Immunology,* **2004, 172: 6290–6297.**

Recombinant adenovirus serotype 5 (rAd5)[3] vector-based vaccines have been shown to elicit high frequency cellular immune responses in animal models (1, 2). Candidate rAd5 vaccines for HIV-1 and other pathogens are therefore being advanced into large-scale clinical trials (3). A major limitation of this approach, however, is that the majority of the human population has pre-existing immunity to the Ad5 vector as a result of natural exposure. Such pre-existing antivector immunity may substantially reduce the immunogenicity and clinical utility of rAd5 vaccines. In fact, anti-Ad5 immunity has already been demonstrated to suppress the immunogenicity of rAd5 vaccines in studies in mice (4, 5), rhesus monkeys (6), and humans in early phase 1 clinical trials (7).

The development of adenovirus (Ad) vaccine vectors that elicit potent Ag-specific immune responses, but are not inhibited by pre-existing anti-Ad5 immunity, is therefore an important research priority. One strategy involves the development of Ad vectors from species other than humans (8, 9). For example, chimpanzee Ads are currently being developed as candidate vaccine vectors that are only marginally affected by anti-Ad5 immunity (10, 11). However, nonhuman Ads may pose significant regulatory challenges as a result of their unknown clinical disease associations in humans.

Another strategy involves the development of vaccine vectors from rare human Ad serotypes (12–15), but cross-reactive antivector immune responses between heterologous serotypes have been reported and could prove a major limitation of this approach (16). Ad35 is one of the rarest of the 51 known human serotypes, with a seroprevalence of $<7\%$, and thus may offer a significant advantage over Ad5 as a candidate vaccine vector (15). However, the immunogenicity of rAd35 vaccines and the extent of immunologic cross-reactivity between Ad5 and Ad35 have not previously been determined. We therefore investigated the impact of anti-Ad5 immunity on the immunogenicity of rAd5 and rAd35 vaccines expressing SIV Gag in mice.

Materials and Methods

Ad5 and Ad35 vector construction, production, and purification

E1/E3-deleted, replication-incompetent Ad5 or Ad35 vectors were generated in PER.C6/55K cells using pBR322-based adaptor plasmid pAdApt or pAdApt535 together with cosmid pWE.Ad.AflII-rITRΔE3 or pWE.Ad35.pIX-rITRΔE3 essentially as previously described (17, 18). The adaptor plasmids contained the left portion of the Ad genomes (nt 1–454 in Ad5 or nt 1–464 in Ad35), followed by transcriptional control elements and the adaptor Ad DNA region (nt 3511–6095 in Ad5 or nt 3401–4669 in Ad35). The SIVmac239 *gag* gene optimized for high levels of expression in mammalian cells (GeneART, Regensburg, Germany) was cloned into the expression cassette in the adaptor plasmids. The resulting pAdApt-Gag and pAdApt535-Gag plasmids expressed the SIVmac239 *gag* gene

*Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; †Crucell Holland, Leiden, The Netherlands; ‡Therion Biologics, Cambridge, MA 02142; and §Vaccine Research Center, National Institutes of Health, Bethesda, MD 20892

Received for publication December 19, 2003. Accepted for publication March 4, 2004.

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[1] This work was supported by National Institutes of Health Grants AI51223 (to D.H.B.), AI58727 (to D.H.B.), AI60368 (to D.H.B.), and P30 AI28691. D.H.B. is the recipient of a Doris Duke Clinical Scientist Development Award.

[2] Address correspondence and reprint requests to Dr. Dan H. Barouch, Research East Room 113, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail address: dbarouch@bidmc.harvard.edu

[3] Abbreviations used in this paper: rAd5, adenovirus serotype 5; Ad, adenovirus; NAb, neutralizing Ab; SFC, spot-forming cell; vp, virus particle.

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under transcriptional control of the human, full-length, immediate-early CMV promoter and the SV40 polyadenylation signal. These plasmids were linearized and transfected into PER.C6/55K cells together with the cosmid pWE.Ad.AflII-rITRΔE3 or pWE.Ad35.pIX-rITRΔE3 containing the right portion of the Ad genomes using Lipofectamine (Invitrogen, Breda, The Netherlands). Homologous recombination led to the generation of rAd5-Gag or rAd35-Gag virus. Ad vectors in crude lysates were plaque-purified using limiting dilutions and agar overlays, and Ad vector clones were analyzed for presence and expression of the transgene. Positive clones were amplified for large-scale production using PER.C6/55K cells in 24–48 triple-layer $3 \times 175\text{-cm}^2$ flasks. Stock viruses were purified by standard two-step CsCl gradient ultracentrifugation and dialyzed three times into PBS containing 5% sucrose. Purified Ad vectors were aliquoted and stored at −80°C. Virus particle (vp) titers were determined by HPLC. Infectivity was assessed by plaque assays using PER.C6/55K cells. SIV Gag expression was assessed by infection of A549 cells, followed by analysis of culture lysates using a commercial Gag ELISA kit (Murex Biotech, Dartford, U.K.). Purified rAd5-Gag and rAd35-Gag vectors were negative in replication-competent Ad assays. Replication-incompetent rAd5-luciferase, rAd35-luciferase, rAd5-empty, and rAd35-empty vectors were produced using similar methods.

Mice and immunizations

Six- to 8-wk-old C57/BL6 or BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). For rAd5 or rAd35 immunizations, mice were injected i.m. with 10^6–10^{10} vp replication-incompetent E1/E3-deleted rAd5 or rAd35 expressing SIVmac239 Gag in 100 μl of sterile PBS in the quadriceps muscles. For DNA immunizations, mice were injected i.m. with 50 μg of plasmid VRC-4307 expressing SIVmac239 Gag-Pol-Nef (Vaccine Research Center, National Institutes of Health, Bethesda, MD) in 100 μl of sterile saline. For rMVA immunizations, mice were injected i.p. with 10^8 PFU of rMVA-T338 expressing SIVmac239 Gag in 100 μl of sterile PBS (Therion Biologics, Cambridge, MA). To induce active anti-Ad5 immunity, mice were preimmunized once or twice, separated by a 4-wk interval, i.m. with 10^{10} vp of rAd5-empty containing no insert in 100 μl of sterile PBS.

Gag-specific ELISPOT

Gag-specific cellular immune responses were assessed by IFN-γ ELISPOT assays using murine splenocytes in response to individual Gag epitope peptides or a pool of overlapping 15-aa peptides covering the entire SIVmac239 Gag protein. Ninety-six-well multiscreen plates (Millipore, Bedford, MA) coated overnight with 100 μl/well of 10 μg/ml rat anti-mouse IFN-γ (PharMingen, San Diego, CA) in PBS were washed three times with endotoxin-free Dulbecco's PBS (Life Technologies, Gaithersburg, MD) containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37°C. The plates were washed three times with Dulbecco's PBS containing 0.25% Tween 20, rinsed with RPMI 1640 containing 10% FBS, and incubated in triplicate with 2×10^5 or 5×10^5 splenocytes/well in a 100-μl reaction volume containing 1 μg/ml peptide. For studies using the Gag peptide pool, each peptide in the pool was present at 1 μg/ml. After an 18-h incubation, the plates were washed nine times with Dulbecco's PBS containing 0.25% Tween 20 and once with distilled water. The plates were then incubated for 2 h with 75 μl/well of 5 μg/ml biotinylated rat anti-mouse IFN-γ (BD PharMingen), washed six times with Coulter Wash (Coulter, Miami, FL), and incubated for 2 h with a 1/500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After five washes with Coulter Wash and one wash with PBS, the plates were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, Rockford, IL), development was stopped by washing with tap water, and plates were air-dried and read using an ELISPOT reader (Hitech Instruments, Edgement, PA). For depletion studies, splenocytes were incubated with magnetic microbeads coated with anti-CD4 (L3T4) or anti-CD8 (Ly-2) mAbs (Miltenyi Biotec, Auburn, CA) and separated using MiniMACS columns before performing the ELISPOT assay. Cell depletions were >95% efficient.

Gag-specific ELISA

Serum anti-Gag Ab titers from immunized mice were measured by a direct ELISA. Ninety-six-well plates coated overnight with 100 μl/well of 1 μg/ml recombinant SIV Gag protein (Intracel, Cambridge, MA) in PBS were blocked for 2 h with PBS containing 2% BSA 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 1/2000 dilution of a peroxidase-conjugated, affinity-purified, rabbit anti-mouse secondary Ab (The Jackson Laboratory, Bar Harbor, ME). The plates were then washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD), stopped with 1% HCl, and analyzed at 450 nm with an MR5000 ELISA plate reader (Dynatech Laboratories, Chantilly, VA).

AL11-specific tetramer binding assays

Tetrameric H-2D^b complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) were prepared and used to stain peptide-specific CD8$^+$ T lymphocytes essentially as previously described (4, 19). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. After lysis of the RBCs, 0.1 μg of PE-labeled D^b/AL11 tetramer in conjunction with allophycocyanin-labeled anti-CD8α mAb (Ly-2; Caltag Laboratories, San Francisco, CA) was used to stain AL11-specific CD8$^+$ T lymphocytes. The cells were washed in PBS containing 2% FBS and fixed in 0.5 ml of PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry on a FACSCalibur (BD Biosciences, Mountain View, CA). Gated CD8$^+$ T lymphocytes were examined for staining with the D^b/AL11 tetramer. CD8$^+$ T lymphocytes from naive mice were used as negative controls and exhibited <0.1% tetramer staining.

Ad-specific ELISPOT

Ad5- or Ad35-specific cellular immune responses were assessed by IFN-γ ELISPOT assays using murine splenocytes from C57/BL6 mice in response to Ad5- or Ad35-infected syngeneic BLK CL4 stimulator cells (ATCC TIB-81; American Type Culture Collection, Manassas, VA) essentially as previously described (15). BLK CL4 cells were plated at a density of 1×10^6 cells/well in a six-well plate and infected with E1/E3-deleted rAd5-empty or rAd35-empty at a multiplicity of infection of 2×10^4 for 3 days. ELISPOT assays using splenocytes from immunized C57/BL6 mice were then performed as described above, using 5×10^5 splenocytes and 1×10^5 Ad-infected BLK CL4 stimulator cells/well in place of peptide Ags. For negative controls, splenocytes were incubated with uninfected BLK CL4 cells or medium alone.

Ad-specific neutralizing Ab assay

Ad5- or Ad35-specific neutralizing Ab (NAb) responses were assessed by luciferase-based virus neutralization assays essentially as previously described (20). A549 human lung carcinoma cells were plated at a density of 1×10^4 cells/well in 96-well plates. E1/E3-deleted rAd5-luciferase or rAd35-luciferase reporter constructs were then added at a multiplicity of infection of 500 with 2-fold serial dilutions of serum in 200-μl reaction volumes. After a 24-h incubation, luciferase activity in the cells was measured using the Steady-Glo Luciferase Reagent System (Promega, Madison, WI). Ninety percent neutralization titers were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

Ad-specific cellular proliferation assay

Ad5- or Ad35-specific CD4$^+$ cellular proliferative responses were assessed by [^{3}H]thymidine incorporation assays. Splenocytes from immunized mice were depleted of CD8$^+$ T cells using anti-CD8 (Ly-2)-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA). Cell depletions were >95% efficient. CD8-depleted splenocytes were then resuspended at 4×10^6 cells/ml in RPMI 1640 containing 5% FBS. One hundred microliters was added to each well in 96-well plates with 10^8 vp of rAd5-empty, 10^8 vp of rAd35-empty, or medium alone. After 4 days of culture, 1 μCi of [^{3}H]thymidine (ICN Biochemicals, Costa Mesa, CA) was added to each well. After a 16-h incubation, cells were harvested on glass filter paper, and radioactivity was measured in a 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD). The stimulation index was calculated as: (cpm with Ag stimulation)/(background cpm without Ag).

Statistical analyses

ELISPOT and tetramer data are presented as the mean and SE. Statistical analyses were performed with PRISM version 2.01 (GraphPad, San Diego, CA). Comparisons of mean ELISPOT responses among groups of mice were performed by two-tailed t tests for two groups of animals or by ANOVA for more than two groups. Bonferroni adjustments were included when appropriate to account for multiple comparisons. In all cases, $p < 0.05$ was considered significant.

Results

Immunogenicity of rAd5-Gag and rAd35-Gag in naive mice

We initiated studies to determine the immunogenicity of E1/E3-deleted, replication-incompetent rAd5-Gag and rAd35-Gag vaccines in naive mice. Groups of BALB/c and C57/BL6 mice (n =

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4/group) were immunized once i.m. with 10^{10} or 10^{8} vp of each vector. Vaccine-elicited cellular immune responses were assessed by ELISPOT assays using a pool of 15-aa peptides overlapping by 11 aa covering the entire SIV Gag protein. Vaccine-elicited humoral immune responses were assessed by Gag-specific ELISAs.

As shown in Fig. 1, both rAd5-Gag and rAd35-Gag elicited only marginal SIV Gag-specific cellular immune responses in BALB/c mice. In contrast, both vectors at a dose of 10^{10} vp elicited rapid and potent ELISPOT responses by 2 wk after vaccination in C57/BL6 mice. At the dose of 10^{8} vp, SIV Gag-specific cellular immune responses elicited by rAd35-Gag were ~2-fold lower than those elicited by rAd5-Gag. High titer anti-Gag Ab responses were elicited by rAd5-Gag in both BALB/c and C57/BL6 mice. However, no anti-Gag Ab responses were detected after immunization with rAd35-Gag. These data demonstrate that rAd35-Gag elicited slightly lower cellular immune responses, but markedly lower humoral immune responses, than rAd5-Gag.

Mapping D^b-restricted T lymphocyte epitopes within SIV Gag

The rapid emergence of high frequency, Gag-specific, cellular immune responses in C57/BL6 mice (Fig. 1*C*) suggested the presence of immunodominant D^b- or K^b-restricted $CD8^+$ T lymphocyte epitopes. We therefore used a matrix-based ELISPOT approach to identify candidate epitopes within SIV Gag to facilitate the development of quantitative peptide-specific cellular immune assays for rAd5-Gag and rAd35-Gag vectors. As depicted in Fig. 2, *A–C*, C57/BL6 mice immunized with 10^{10} vp of rAd5, 10^{10} vp of rAd35, or 50 μg of plasmid DNA expressing SIV Gag developed an immunodominant cellular immune response to the 15-aa P78 peptide (QTDAAVKNWMTQTLL) and a subdominant response to the P19 peptide (ENLKSLYNTVCVIWC). ELISPOT assays using splenocytes depleted of $CD4^+$ or $CD8^+$ T lymphocytes demonstrated that both P78 and P19 were, in fact, $CD8^+$ T lymphocyte epitopes.

We next fine-mapped these epitopes based on the peptide binding motifs of D^b (Asn at position 5 and hydrophobic Leu/Ile/Val C terminus) and K^b (Tyr at position 5 and hydrophobic Leu/Ile/Val C terminus). As shown in Fig. 2, *D–F*, candidate optimal peptides were assessed at log dilutions from 1 μg/ml to 100 fg/ml in peptide-specific ELISPOT assays. A D^b-restricted immunodominant AL11 (AAVKNWMTQTL) epitope within P78 and a D^b-restricted subdominant KV9 (KSLYNTVCV) epitope within P19 were identified. These $CD8^+$ T lymphocyte epitopes elicited ELISPOT responses when used at a concentration of 1 pg/ml and were confirmed by functional chromium release cytotoxicity assays using peptide-pulsed EL4 cells as well as Ltk cells transfected with D^b, but not Ltk cells transfected with K^b, as targets (data not shown). The LV10 peptide was similarly investigated as a potential K^b-restricted epitope, but this peptide was not confirmed in cytotoxicity assays using Ltk cells transfected with K^b as targets, suggesting that its reactivity in ELISPOT assays probably reflected KV9 contaminant peptide within the LV10 preparation.

Impact of low levels of anti-Ad5 immunity on the immunogenicity of rAd5-Gag and rAd35-Gag

We next determined the impact of low levels of anti-Ad5 immunity on cellular immune responses elicited by rAd5-Gag and rAd35-Gag. To model pre-existing anti-Ad5 immunity, C57/BL6 mice were preimmunized once with 10^{10} vp rAd5-empty 4 wk before immunization. As shown in Fig. 3*C*, mice preimmunized with rAd5-empty developed mean Ad5-specific NAb titers of 128, but no detectable Ad35-specific NAb titers (<16). These Ad5-specific NAb responses represent average titers found in individuals in the United States and Western Europe (15, 21). Ad-specific T lymphocyte responses in these mice were assessed by virus-specific ELISPOT assays using splenocytes stimulated with Ad5- or Ad35-infected syngeneic BLK CL4 cells. As shown in Fig. 3*D*, mice preimmunized with rAd5-empty developed mean Ad5-specific ELISPOT responses of 250 spot-forming cells (SFC)/10^{6} splenocytes, but no detectable Ad35-specific ELISPOT responses (<25 SFC/10^{6} splenocytes).

Groups of naive mice or mice with anti-Ad5 immunity (n = 4/group) were then immunized with 10^{10} or 10^{8} vp of rAd5-Gag or rAd35-Gag. Four weeks after immunization, vaccine-elicited cellular immune responses were assessed by Gag-pooled peptide and epitope-specific ELISPOT assays. As shown in Fig. 3, *A* and *B*, Gag- and epitope-specific cellular immune responses elicited by 10^{10} vp of rAd5-Gag were blunted by 75% in mice with anti-Ad5 immunity compared with those in naive mice. Cellular immune responses elicited by 10^{8} vp of rAd5-Gag were completely abrogated in mice preimmunized with rAd5-empty. These data demonstrate the marked suppressive effects of anti-Ad5 immunity on the immunogenicity of rAd5 vaccines. In contrast, Gag- and AL11-specific responses elicited by rAd35-Gag were not detectably blunted in mice with low levels of anti-Ad5 immunity. In fact, Gag-specific cellular immune responses elicited by rAd35-Gag were significantly higher than those elicited by rAd5-Gag in these mice ($p < 0.05$ comparing pooled peptide or peptide-specific

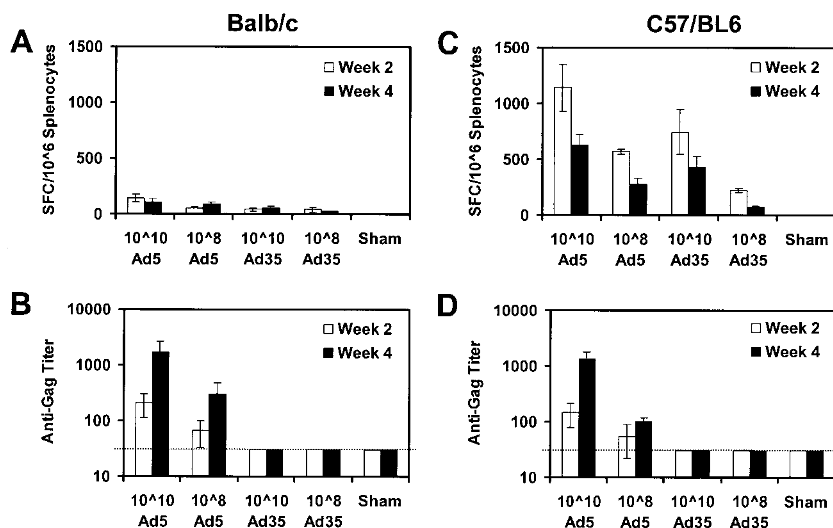

FIGURE 1. Immunogenicity of rAd5-Gag and rAd35-Gag in naive mice. Groups of naive BALB/c (*A* and *B*) or C57/BL6 mice (*C* and *D*) (n = 4/group) were immunized once with 10^{10} or 10^{8} vp of rAd5-Gag or rAd35-Gag. SIV Gag-specific cellular and humoral immune responses were assessed by pooled peptide ELISPOT assays (*A* and *C*) and ELISAs (*B* and *D*). The mean and SE response for each group are shown.

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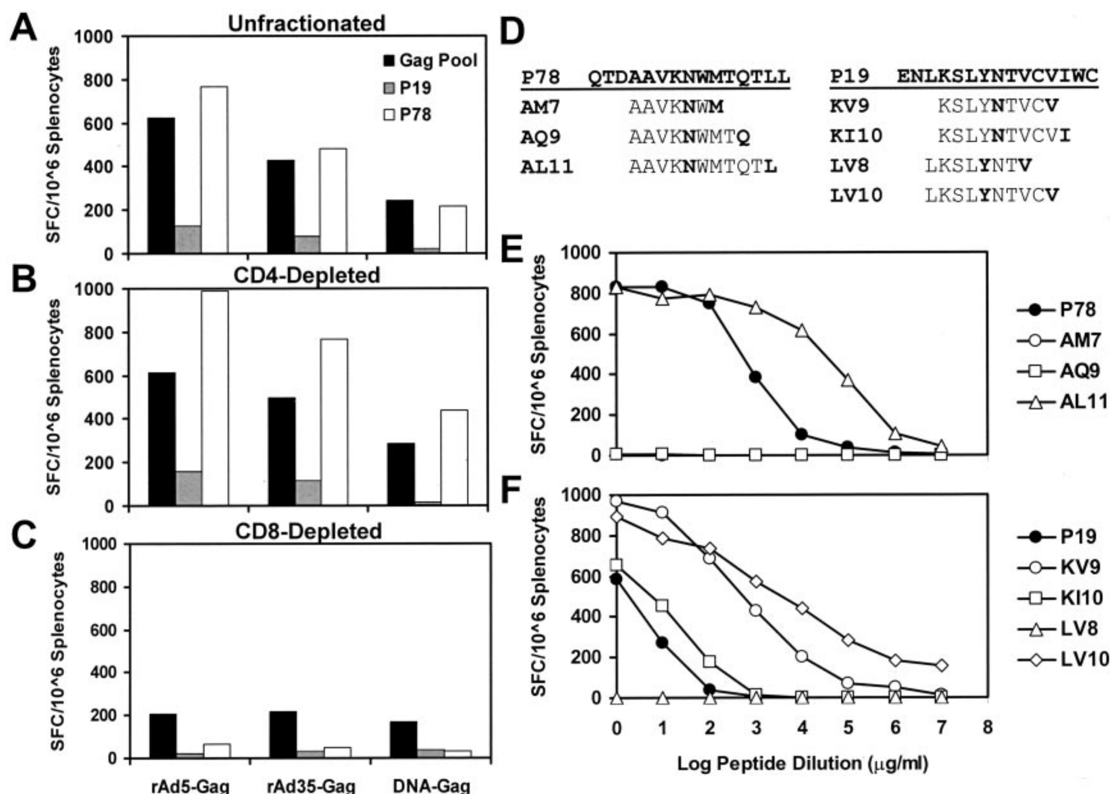

FIGURE 2. Epitope mapping studies. C57/BL6 mice were immunized with 10^{10} vp of rAd5, 10^{10} vp of rAd35, or 50 μg of plasmid DNA expressing SIV Gag. P78 and P19 peptide-specific ELISPOT assays were performed using unfractionated splenocytes (*A*) or splenocytes depleted of $CD4^+$ T lymphocytes (*B*) or $CD8^+$ T lymphocytes (*C*). *D*, Fine-mapping strategy for P19 and P78 based on the peptide binding motifs of D^b and K^b. Position 5 and C-terminal residues are shown in bold. Candidate optimal peptide epitopes for P78 (*E*) and P19 (*F*) were assessed in ELISPOT assays using log dilutions of each peptide from 1 μg/ml to 100 fg/ml.

ELISPOT responses using two-tailed *t* tests). Subdominant KV9-specific responses elicited by rAd35-Gag were low, but were similarly not blunted by anti-Ad5 immunity.

Vector-specific humoral and cellular immune responses were also assessed in these groups of mice. As shown in Fig. 3, *C* and *D*, naive mice immunized with rAd5-Gag or rAd35-Gag developed Ad serotype-specific immune responses. No detectable cross-reactive Ad5/Ad35-specific NAb or ELISPOT responses were observed in these mice, suggesting that these two vectors were largely immunologically distinct. As expected, mice preimmunized with rAd5-empty generated potent, anamnestic Ad5-specific NAb and ELISPOT responses after rAd5-Gag immunization. Interestingly, mice preimmunized with rAd5-empty generated unexpectedly potent Ad35-specific NAb responses after rAd35-Gag immunization (Fig. 3*C*). In fact, these responses were 10-fold higher than the Ad35-specific NAb responses generated in naive mice after the same rAd35-Gag immunization. These data suggest that preimmunization with rAd5-empty may have primed low levels of cross-reactive antivector immune responses that became apparent only after administration of the heterologous rAd35-Gag vector. However, these cross-reactive responses did not appear to suppress the immunogenicity of rAd35-Gag.

Impact of high levels of anti-Ad5 immunity on the immunogenicity of rAd5-Gag and rAd35-Gag

As the majority of individuals in the developing world have high titers of Ad5-specific NAbs, we assessed whether high levels of anti-Ad5 immunity would suppress cellular immune responses elicited by rAd35-Gag. To investigate this, mice were preimmunized twice with 10^{10} vp of rAd5-empty 8 and 4 wk before immunization. As shown in Fig. 4*C*, these mice developed mean Ad5-specific NAb titers of 16,384, but no detectable Ad35-specific NAb titers (<16). These NAb titers represent the upper limit of those typically found in sub-Saharan Africa (21). As shown in Fig. 4*D*, these mice also developed high frequency, Ad5-specific ELISPOT responses of 560 SFC/10^6 splenocytes, but no detectable Ad35-specific ELISPOT responses (<25 SFC/10^6 splenocytes). Thus, preimmunization of mice with two doses of rAd5-empty generated potent anti-Ad5 immunity.

Groups of naive mice or mice with these high levels of anti-Ad5 immunity ($n = 4$/group) were then immunized with 10^{10} vp of rAd5-Gag or rAd35-Gag. Four weeks after immunization, Gag-specific cellular immune responses were assessed. As shown in Fig. 4, *A* and *B*, high levels of anti-Ad5 immunity abrogated Gag- and epitope-specific ELISPOT responses elicited by 10^{10} vp of rAd5-Gag by 90%. Importantly, Gag- and epitope-specific cellular immune responses elicited by 10^{10} vp of rAd35-Gag were not suppressed by these high levels of anti-Ad5 immunity. These data demonstrate that even high levels of anti-Ad5 immune responses failed to suppress the immunogenicity of rAd35-Gag. Moreover, rAd35-Gag was markedly more immunogenic than rAd5-Gag in these mice with potent anti-Ad5 immunity ($p < 0.001$ comparing pooled peptide and peptide-specific ELISPOT responses using two-tailed *t* tests). As shown in Fig. 4, *C* and *D*, Ad5-specific NAb and ELISPOT responses in mice with high levels of anti-Ad5 immunity were not further increased after rAd5-Gag immunization. These data suggest that the rAd5-Gag vaccine vector was rapidly neutralized before eliciting substantial Ag- or vector-specific immune responses in these animals.

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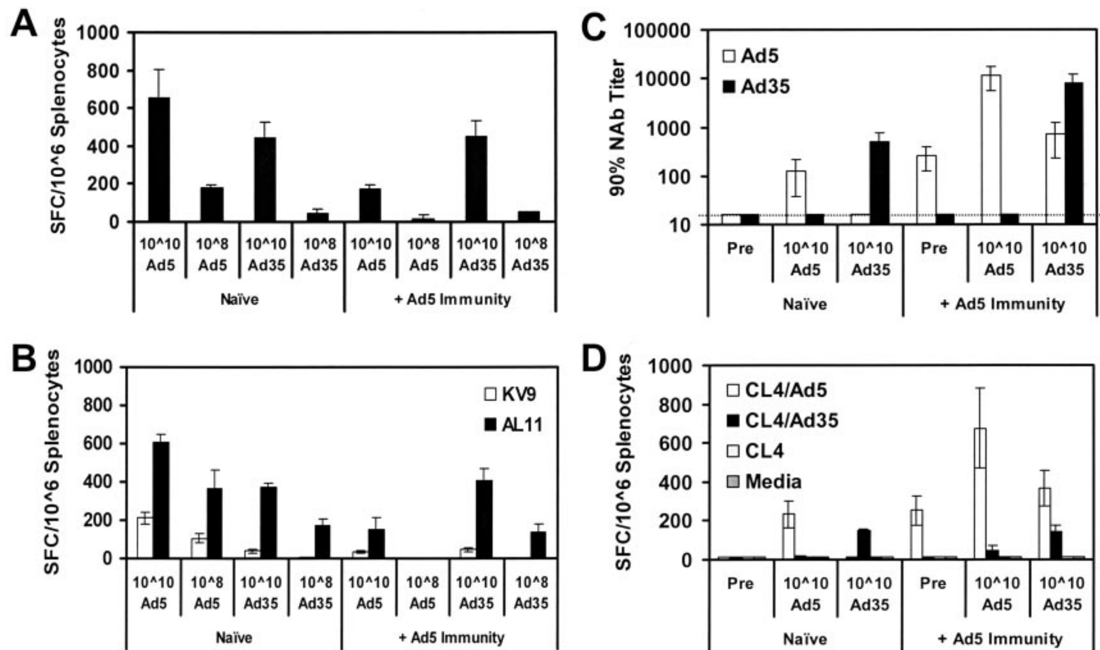

FIGURE 3. Immunogenicity of rAd5-Gag and rAd35-Gag in mice with low levels of anti-Ad5 immunity. C57/BL6 mice were preimmunized once with 10^{10} vp of rAd5-empty to induce anti-Ad5 immunity 4 wk before immunization. Groups of naive mice or mice with anti-Ad5 immunity ($n = 4$/group) were immunized with 10^{10} or 10^8 vp of rAd5-Gag or rAd35-Gag. Gag-specific cellular immune responses were assessed by Gag pooled peptide (*A*) and KV9 and AL11 epitope-specific (*B*) ELISPOT assays. Ad5- and Ad35-specific immune responses were also assessed both before and after immunization by Ad NAb (*C*) and Ad ELISPOT assays (*D*) using Ad5- or Ad35-infected syngeneic BLK CL4 stimulator cells. Note that preimmunization with one dose of 10^{10} vp of rAd5-empty induced mean Ad5-specific NAb titers of 128 and Ad5-specific ELISPOT responses of 250 SFC/10^6 splenocytes.

Tetramer binding assays

We next used tetramer binding assays to assess both the kinetics and the magnitudes of $CD8^+$ T lymphocyte responses specific for the immunodominant AL11 epitope. Tetrameric D^b/AL11 complexes were constructed essentially as previously described (4, 19) and were used to quantitate the emergence of AL11-specific $CD8^+$ T lymphocyte responses elicited by rAd5-Gag and rAd35-Gag in mice with or without anti-Ad5 immunity. C57/BL6 mice were preimmunized once with 10^{10} vp rAd5-empty 4 wk before immunization and developed Ad5-specific NAb titers of 128–256. Groups of naive mice or mice with anti-Ad5 immunity ($n = 4$/group) were then immunized with 10^{10} or 10^8 vp of rAd5-Gag or rAd35-Gag. D^b/AL11 tetramer binding to gated $CD8^+$ T lymphocytes was assessed at multiple time points after immunization. As shown in Fig. 5, *A* and *B*, anti-Ad5 immunity markedly blunted the emergence of $tetramer^+CD8^+$ T lymphocyte responses elicited by rAd5-Gag, but did not substantially affect the responses elicited by rAd35-Gag. These data confirm the absence of functionally suppressive, cross-reactive, antivector immune responses between Ad5 and Ad35. We also assessed the immunologic potencies of rAd5-Gag and rAd35-Gag administered at doses of 10^{10}, 10^9, 10^8, 10^7, and 10^6 vp in naive mice. As shown in Fig. 5*C*, the threshold dose required to elicit detectable $tetramer^+$ $CD8^+$ T lymphocyte responses in naive mice was 10^8 vp for rAd35-Gag compared with 10^7 vp for rAd5-Gag.

Immunogenicity of homologous and heterologous prime-boost regimens in naive mice

We next investigated the immunogenicity of homologous and heterologous prime-boost vaccine regimens in naive C57/BL6 mice. Groups of mice ($n = 4$/group) were primed at wk 0 with 10^{10} vp rAd5-Gag or rAd35-Gag and boosted at wk 4 with homologous or heterologous vectors. As shown in Fig. 6*A*, heterologous rAd5 prime-rAd35 boost and rAd35 prime-rAd5 boost regimens were more immunogenic than homologous prime-boost regimens. Readministration of homologous vectors did not substantially boost Gag-specific cellular immune responses under these conditions, presumably as a result of the generation of potent antivector immunity. Readministration of homologous vectors similarly failed to boost Gag-specific responses in mice with pre-existing anti-Ad5 immunity (data not shown).

The higher Ad35-specific NAb responses observed after rAd35-Gag immunization of mice with anti-Ad5 immunity compared with naive mice (Figs. 3*C* and 4*C*) suggested the presence of low levels of cross-reactive Ad5/Ad35-specific NAb or $CD4^+$ T lymphocyte responses. To confirm these findings, we assessed the generation of antivector immune responses in the mice in the previous experiment. As shown in Fig. 6*B*, potent NAb responses were observed after boost immunizations with both heterologous vectors. However, cross-reactive Ad5/Ad35-specific NAb responses were not detected even after boost immunizations with homologous vectors. Interestingly, as shown in Fig. 6*C*, low, but detectable, cross-reactive Ad5/Ad35-specific $CD4^+$ cellular proliferative responses were observed after administration of rAd5 or rAd35. These data suggest that low levels of cross-reactive Ad5/Ad35-specific Th lymphocytes led to the generation of potent serotype-specific NAb responses after heterologous vector administration.

Immunogenicity of heterologous prime-boost regimens in mice with anti-Ad5 immunity

Heterologous DNA prime-rAd5 boost vaccine regimens have been shown to elicit particularly potent immune responses in animal models and are therefore being investigated as candidate vaccine strategies in clinical trials (1–3). As the majority of humans have pre-existing anti-Ad5 immunity, we sought to assess the immunogenicity of various prime-boost regimens in animals with anti-Ad5

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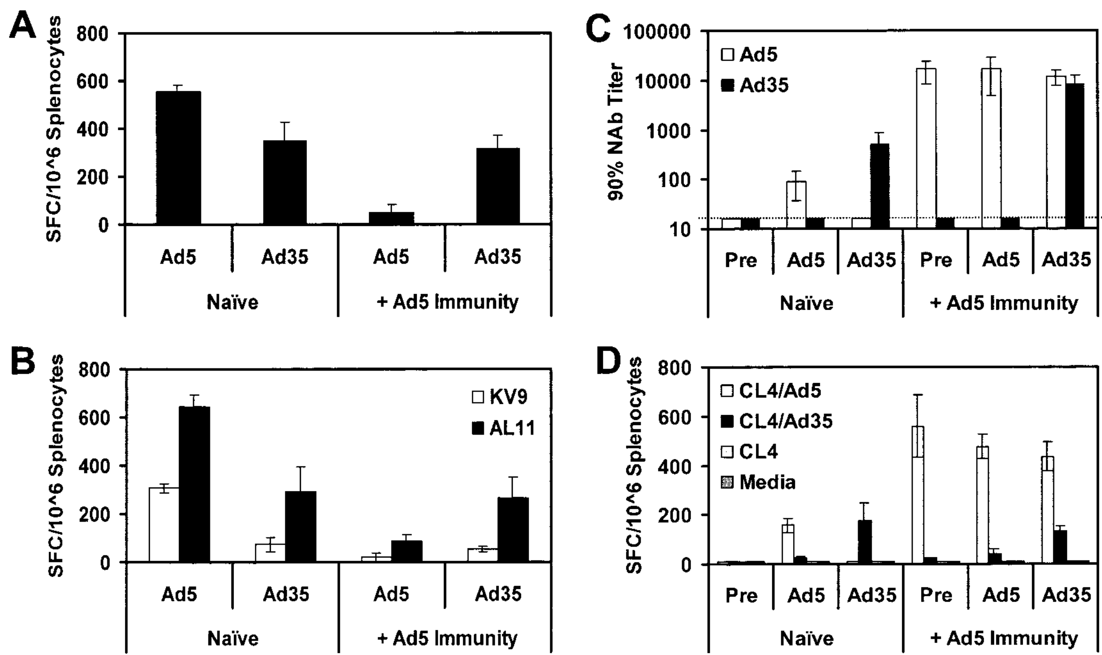

FIGURE 4. Immunogenicity of rAd5-Gag and rAd35-Gag in mice with high levels of anti-Ad5 immunity. C57/BL6 mice were preimmunized twice with 10^{10} vp of rAd5-empty to induce anti-Ad5 immunity at 8 and 4 wk before immunization. Groups of naive mice or mice with high levels of anti-Ad5 immunity (n = 4/group) were immunized with 10^{10} vp of rAd5-Gag or rAd35-Gag. Gag-specific cellular immune responses were assessed by Gag pooled peptide (*A*) and KV9 and AL11 epitope-specific (*B*) ELISPOT assays. Ad5- and Ad35-specific immune responses were also assessed both before and after immunization by Ad NAb (*C*) and Ad ELISPOT assays (*D*). Note that preimmunization with two doses of 10^{10} vp rAd5-empty induced mean Ad5-specific NAb titers of 16,384 and Ad5-specific ELISPOT responses of 560 SFC/10^6 splenocytes.

immunity. We therefore compared the immunogenicities of four heterologous prime-boost regimens in C57/BL6 mice that were preimmunized once with 10^{10} vp of rAd5-empty 4 wk before primary immunization. These mice had low Ad5-specific NAb titers of 128–256 (Fig. 7*C*). Groups of mice (n = 4/group) were primed at wk 0 with 50 μg of DNA or 10^{10} vp of rAd5 expressing SIV Gag and then boosted at wk 4 with 10^{10} vp of rAd5, 10^{10} vp of rAd35, or 10^8 PFU of rMVA expressing SIV Gag. Mice were sacrificed at wk 8 for immunologic assays.

As shown in Fig. 7, *A* and *B*, DNA vaccine priming elicited low to moderate Gag-specific ELISPOT responses, as expected. These responses were boosted only marginally by rAd5-Gag as a result of the anti-Ad5 immunity, but were boosted efficiently by rAd35-Gag. In fact, the DNA prime-rAd35 boost regimen elicited significantly higher pooled peptide- and epitope-specific ELISPOT responses compared with the DNA prime-rAd5 boost regimen in these mice ($p < 0.001$ comparing responses among groups of mice using ANOVA with Bonferroni adjustments to account for multiple comparisons). Similarly, rAd5-Gag priming elicited only low to moderate ELISPOT responses as a result of the anti-Ad5 immunity. Nevertheless, these responses were boosted by the heterologous viral vectors rAd35-Gag and rMVA-Gag. These data demonstrate that the DNA prime-rAd35 boost regimen and, to a lesser extent, the rAd5 prime-rAd35 boost and rAd5 prime-rMVA boost regimens were highly immunogenic in mice with pre-existing anti-Ad5 immunity.

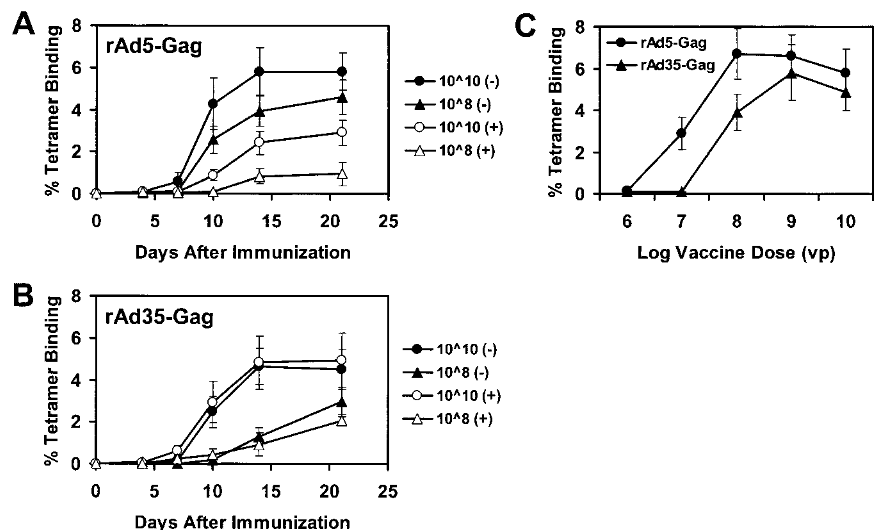

FIGURE 5. Tetramer binding assays. C57/BL6 mice were preimmunized once with 10^{10} vp of rAd5-empty to induce anti-Ad5 immunity 4 wk before immunization. Groups of naive mice (● and ▲) or mice with anti-Ad5 immunity (○ and △) were immunized with 10^{10} or 10^8 vp of rAd5-Gag (*A*) or rAd35-Gag (*B*). D^b/AL11 tetramer binding to $CD8^+$ T lymphocytes is shown at various time points after immunization. *C*, Groups of naive mice were immunized with 10^{10}, 10^9, 10^8, 10^7, or 10^6 vp of rAd5-Gag or rAd35-Gag, and D^b/AL11 tetramer binding to $CD8^+$ T lymphocytes was determined on day 14 after immunization.

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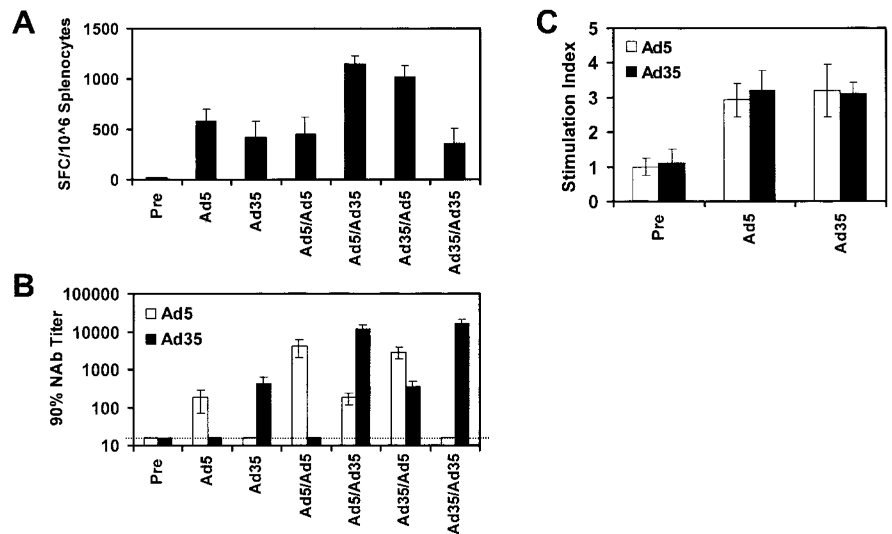

FIGURE 6. Immunogenicity of homologous and heterologous prime-boost regimens in naive mice. Groups of naive C57/BL6 mice (n = 4/group) were primed with 10^{10} vp of rAd5-Gag or rAd35-Gag at wk 0 and boosted with 10^{10} vp of homologous or heterologous vectors at wk 4. The various immunization regimens are depicted on the *x*-axis. Gag pooled peptide ELISPOT assays (*A*) and Ad5- and Ad35-specific NAb assays (*B*) were performed at wk 8. *C*, Ad5- and Ad35-specific cellular proliferative assays were performed using splenocytes depleted of $CD8^+$ T lymphocytes.

Discussion

The high prevalence of pre-existing anti-Ad5 immunity in human populations may substantially reduce the immunogenicity and clinical utility of rAd5 vector-based vaccines for HIV-1 and other pathogens. To circumvent this problem, adenoviral vectors can be developed from rare adenoviral serotypes, such as serotype 35. However, extensive cross-reactive cellular immune responses between heterologous Ad serotypes have been reported (10, 16, 22) and may prove a significant limitation of this strategy. In this study we assessed the degree of functionally significant, cross-reactive, antivector immune responses between Ad5 and Ad35. We demonstrate that a rAd35-Gag vaccine elicited potent cellular immune responses that were not detectably suppressed by pre-existing anti-Ad5 immunity in mice.

We did detect low levels of cross-reactive Ad5/Ad35-specific $CD4^+$ T lymphocyte responses. Cross-reactive, Ad-specific, Th lymphocytes have also been observed in humans (16, 22), suggesting that Ads from heterologous serotypes are not completely immunologically distinct. Moreover, these cross-reactive $CD4^+$ T lymphocyte responses probably contributed to the potent Ad-specific NAbs that developed after heterologous vector administration in mice (Fig. 6*B*). However, despite these cross-reactive immune responses, Gag-specific cellular immune responses elicited by rAd35-Gag were not detectably suppressed by pre-existing anti-Ad5 immunity. These data suggest that antivector NAb responses that are present at the time of immunization are more important than those that develop after immunization in determining their suppressive effects on vaccine immunogenicity.

In mice with anti-Ad5 immunity at levels typically found in humans, rAd35-Gag elicited significantly higher cellular immune responses than rAd5-Gag. These data demonstrate the potential utility of Ad35 as a vaccine vector in the presence of pre-existing anti-Ad5 immunity. In naive mice, however, rAd35-Gag elicited slightly lower cellular immune responses and substantially lower humoral immune responses compared with rAd5-Gag. These differences in immunogenicity are consistent with our previous finding that rAd35-mediated transgene expression was several-fold lower than rAd5-mediated transgene expression in mouse muscle (15). Ad5 interacts with the coxsackievirus and Ad receptor on the surface of cells with its long and flexible fiber protein (23–26). In contrast, the Ad35 fiber protein is shorter and more rigid than the Ad5 fiber (26, 27), and CD46 has recently been identified as an Ad35 receptor (28). As a result, Ad5 and Ad35 have different cellular tropisms (29–32) and intracellular trafficking pathways

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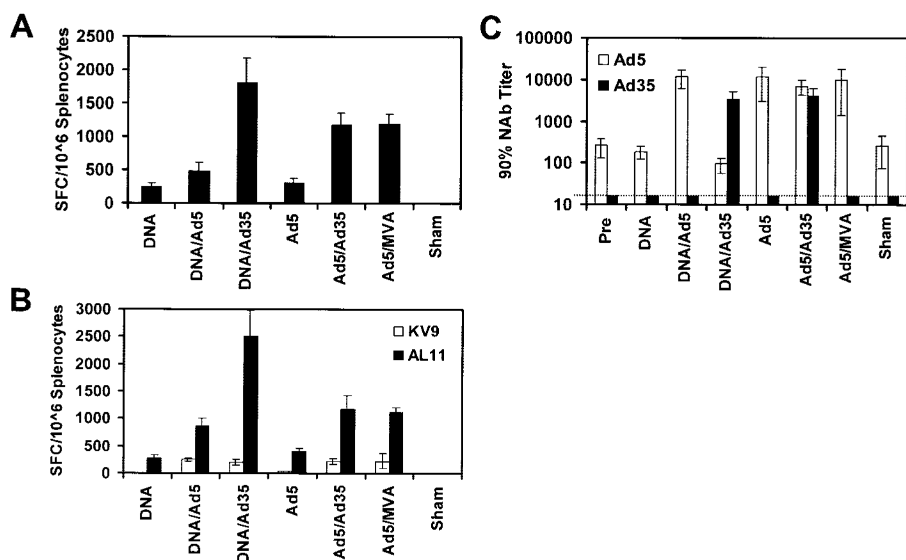

FIGURE 7. Immunogenicity of heterologous prime-boost regimens in mice with anti-Ad5 immunity. C57/BL6 mice were preimmunized once with 10^{10} vp of rAd5-empty to induce anti-Ad5 immunity 4 wk before primary immunization. These mice had Ad5-specific NAb titers of 128–256. Groups of mice with anti-Ad5 immunity (n = 4/group) were then primed with 50 μg of plasmid DNA or 10^{10} vp of rAd5 expressing SIV Gag at wk 0. Certain groups were boosted with 10^{10} vp of rAd5, 10^{10} vp of rAd35, or 10^8 PFU of rMVA expressing SIV Gag at wk 4. The various immunization regimens are depicted on the *x*-axis. Gag pooled peptide ELISPOT assays (*A*), KV9 and AL11 epitope-specific ELISPOT assays (*B*), and Ad5- and Ad35-specific NAb assays (*C*) were performed at wk 8.

(33). These differences may explain in part the differences in immunogenicity between rAd5-Gag and rAd35-Gag in this animal model. At present, it is not clear why we observed large differences between rAd5-Gag and rAd35-Gag in their ability to elicit Gag-specific antibodies. It is possible that a higher threshold of Ag is needed to generate Ab responses compared with T lymphocyte responses in this system.

Heterologous prime-boost vaccine strategies are commonly used to elicit high frequency, Ag-specific immune responses. We confirmed that boosting with heterologous vectors elicited higher immune responses than boosting with homologous vectors (Fig. 6*A*). However, regimens that include rAd5 will probably have substantially reduced immunogenicity and clinical utility in the presence of anti-Ad5 immunity. In mice with anti-Ad5 immunity, a DNA prime-rAd35 boost regimen was significantly more immunogenic than a DNA prime-rAd5 boost regimen (Fig. 7). Thus, DNA prime-rAd35 boost as well as rAd5 prime-rAd35 boost regimens warrant further investigation as candidate HIV-1 prime-boost vaccine strategies.

These data demonstrate the potential utility of Ad vaccine vectors derived from rare human serotypes to elicit immune responses in the presence of pre-existing anti-Ad5 immunity. It is possible that cross-reactive cellular immune responses between serologically distinct Ads may limit the utility of certain vector combinations, although this problem appears to be minimal for Ad5 and Ad35. Therefore, this strategy may prove an effective and general approach for developing vectors that avoid the suppressive effects of pre-existing anti-Ad5 immunity. However, the ultimate utility of this approach in humans will require data from clinical trials.

Acknowledgments

We thank N. Helmus, G. Penders, C. Ophorst, D. Lange, S. Verhaagh, A. Lemckert, E. Wherry, R. Ahmed, M. Seaman, S. Santra, J. Schmitz, and M. Kuroda for generous advice, assistance, and reagents. The SIV Gag peptides were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

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