Vaccines Based on Novel Adeno-Associated Virus Vectors Elicit Aberrant CD8+ T-Cell Responses in Mice

Jianping Lin, Yan Zhi, Lauren Mays, and James M. Wilson*

Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania,
125 S. 31st Street, Philadelphia, Pennsylvania 19104

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We recently discovered an expanded family of adeno-associated viruses (AAVs) that show promise as improved gene therapy vectors. In this study we evaluated the potential of vectors based on several of these novel AAVs as vaccine carriers for human immunodeficiency virus type 1 Gag. Studies with mice indicated that vectors based on AAV type 7 (AAV7), AAV8, and AAV9 demonstrate improved immunogenicity in terms of Gag CD8+ T-cell and Gag antibody responses. The quality of these antigen-specific responses was evaluated in detail for AAV2/8 vectors and compared to results with an adenovirus vector expressing Gag (AdC7). AAV2/8 produced a vibrant CD8+ T-cell effector response characterized by coexpression of gamma interferon and tumor necrosis factor alpha as well as in vivo cytolytic activity. No CD8+ T-cell response generated by any of the AAVs was effectively boosted with AdC7, a result consistent with the finding of a relative lack of cells expressing interleukin-2 (IL-2) or a central memory phenotype at 3 months after the prime. The primary response to an AdC7 vaccine differed from that generated by AAVs in that the peak effector response evolved into populations of Gag-specific T cells expressing high levels of cytokines, including IL-2, and with effector memory and central memory phenotypes. A number of mechanisms could be considered to explain the aberrant activation of CD8+ T cells by AAV, including insufficient inflammatory responses, CD4 help, and/or chronic antigen expression and T-cell exhaustion. Interestingly, the B-cell response to AAV-encoded Gag was quite vibrant and easily boosted with AdC7.

A number of vaccine strategies have been developed for preventing and treating human immunodeficiency virus type 1 (HIV-1)-related diseases. These have ranged from protein adjuvant formulations to inactivated HIV-1 to a variety of genetic vectors (7, 31, 32, 35). Merck has developed vaccines based on HIV-1 antigens by methods such as recombinant adenovirus vectors (7, 31, 32, 35). Merck has developed vaccines based on human adenovirus serotype 5 that have progressed to phase II clinical trials. These studies have been encouraging in that individuals without significant preexisting immunity to the vector have demonstrated a high frequency of responses in terms of antigen-specific T cells (http://www.laviereport.org/Issues/Issue10-1/immunity.asp). One problem, however, is that vaccine efficacy is diminished in some individuals with preexisting immunity to adenovirus serotype 5, which in the United States occurs at a frequency of 30 to 50% (11), and in many developing countries at >90% (M. Cleveland et al., presented at AIDS Vaccine 2005, Montreal, Quebec, Canada, 2005).

An alternative platform for genetic vaccines is based on adeno-associated viruses (AAVs). This group of paroviruses was identified over 30 years ago as contaminants in laboratory preparations of adenoviruses (1, 3, 4, 16, 17, 25, 28). Six serotypes of AAV were identified. The initial application of vectors based on these viruses was for gene therapy. In these studies, impressive results were achieved following in vivo administration of vector in terms of the efficiency and stability of transgene expression without significant toxicity.

A number of investigators have pursued the use of AAV type 2 (AAV2) as a vaccine carrier. Manning et al. demonstrated T- and B-cell responses to herpes simplex virus type 2 glycoproteins B and D following intramuscular (i.m.) injection in mice (22). AAV2 expressing human papillomavirus E7 eliminated human papillomavirus-expressing tumors in a syngeneic mouse model (21). Intranasal administration of AAV2 expressing HA from influenza virus resulted in protection against a challenge with influenza virus (41). A number of investigators have demonstrated encouraging results with AAV2-based vaccines administered orally. AAV2 expressing a receptor to a neurotransmitter found in the brain was shown to induce autoantibodies that prevented clinical sequelae in experimentally induced stroke and epilepsy (10).

AAV2 vectors have been evaluated as vaccine carriers for HIV-1 antigens as well. Xin et al., injected AAV2 vectors expressing HIV-1 Env, Tat, and Rev into muscles of BALB/c mice and showed persistent HIV antibodies based on an enzyme-linked immunosorbent assay (ELISA) and T cells based on cytotoxic T-lymphocyte (CTL) assays (41). Orally administered AAV2 expressing HIV-1 Env resulted in systemic and regional immunity and significantly reduced the viral load of a vaccinia virus expressing HIV-1 Env following rectal administration (40). Johnson and colleagues have shown detectable Gag-specific T cells by an enzyme-linked immunospot assay in monkeys who had been immunized i.m. with AAV2 vectors (20). Based on these encouraging results, this group progressed to a phase I clinical trial that recently completed ac-
crucial of research subjects; the vector appeared to be safe, though not very immunogenic (J. van Lunzen et al., presented at the 14th Conference on Retroviruses and Opportunistic Infections, 2007).

We recently discovered an expanded family of AAVs from humans and nonhuman primates based on recovery of latent forms of the genome using PCR techniques (12–14). More than 110 isolates were categorized into six different clades representing a broad distribution of potential AAV biology. Application of these novel viruses as vectors for gene therapy has yielded impressive results, with transduction efficiencies superior to those achieved with vectors based on serotypes 1 to 6.

In this study we evaluate the potential of novel AAVs as vaccine carriers for HIV-1 antigens.

MATERIALS AND METHODS

Mice and immunization. All animal procedures were performed in accordance with protocols approved by the institutional animal care and use committees of the University of Pennsylvania. (BALB/c × C57Bl/6)F1, hybrid mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were immunized with recombinant AAV vectors diluted in 50 μl of phosphate-buffered saline (PBS) and administered i.m.

Vaccine vectors. All AAV vectors used in this study were manufactured as described previously (36) by PenaVector at the University of Pennsylvania (Philadelphia, PA). In brief, the cDNA carrying a truncated codon-optimized form of HIV-1 clade B gag was cloned into an AAV cis-plasmid vector containing the cytomegalovirus promoter, poly(A), and the AAV2 inverted terminal repeats. The recombinant vectors were packaged by triple transfection of HEK293 cells with an adenovirus helper plasmid, a chimeric packaging construct in which the AAV2 Rep gene was fused to the Cap gene derived from a different AAV serotype, and the vector plasmid to produce pseudotyped AAV2/1, AAV2/2, AAV2/7, and AAV2/9 carrying HIV Gag. AAV2 vector vaccines were purified by a single-step gravity-flow heparin column procedure. AAV1/1, -2/7, -2/8, and -2/9 vector vaccines were purified by three rounds of cesium chloride gradient centrifugation. The genome titers (genome copies [GC] per milliliter) of AAV vectors were determined by real-time PCR.

Peptides. The H2-Kd-restricted immunodominant CTL epitope contained in the p24 portion of the Gag protein consists of amino acids 197 to 205 (AMQLKETI). This peptide was synthesized from mimotopes (Chiron Mimotopes, Clayton, Victoria, Australia) and dissolved in dimethyl sulfoxide at 1 mg/ml. Dimethyl sulfoxide concentrations were kept below 0.1% (vol/vol) in all final assay mixtures.

MHC class I tetramer staining and phenotypic analysis. The phycoerythrin (PE)-conjugated major histocompatibility complex (MHC) class I H2-Kd-AMQLKETI tetramer complex was obtained from Beckman Coulter (Fullerton, CA). At various time points after vector injection, tetramer staining was performed on heparinized whole blood and on lymphocytes isolated from various tissues. Cells were stained for 30 min at room temperature with PE-conjugated tetramers, a fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (Ly-2, BD Biosciences Pharmingen, San Diego, CA), a PE-Cy5-conjugated anti-CD127 antibody, and a PE-Cy7-conjugated anti-CD62L antibody (ebioscience, CA). Red blood cells were then lysed, and cells were fixed with tAg MHC tetramer lysing solution with fix solution (Beckman Coulter) for 10 min at room temperature. The cells were then washed three times in PBS and eventually fixed again with BD CytoFix (BD Biosciences) for 20 min at 4°C. Data were gathered with an FC500 flow cytometer (Beckman Coulter) and were analyzed with FlowJo analysis software (Tree Star, San Carlos, CA). In the analysis, lymphocytes were selected on the basis of forward and side scatter characteristics, followed by selection of CD8 cells. Tetramer-positive CD8 T cells were further used for phenotypic analysis based on the staining of anti-mouse CD127 and CD62L antibodies.

Intracellular cytokine staining (ICS). Measurement of cytokine production and granzyme B (GzB) content in CD8+ T cells was performed by combined surface and intracellular staining with monoclonal antibodies and subjected the four-color flow cytometric analysis. Cytokine-secreting CD8 T cells were detected using the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus kit; PharMingen, CA). In brief, infiltrating lymphocytes were isolated from the mouse spleen, liver, or lung, and the lymphocytes obtained (1 × 10^6/sample) were incubated with 4 μg/ml of the HIV Gag peptide (AMQL KETI) for 5 h at 37°C in 96-well round-bottom microtiter plate wells in Dulbecco’s modified Eagle medium supplemented with 2% fetal bovine serum, 5 × 10^-5 M 2-mercaptoethanol, and 1 μg/ml of GolgiPlug (BD PharMingen, San Diego, CA). Cells were washed and stained with an FITC-conjugated anti-mouse CD8 antibody (Ly-2), permeabilized in 100 μl of Cytofix/Cytoperm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anti-cytokine antibodies including PE-conjugated anti-mouse gamma interferon (IFN-γ), PE-Cy7-conjugated tumor necrosis factor alpha (TNF-α), and allophycocyanin (APC)-conjugated interleukin-2 (IL-2) (BD Biosciences Pharmingen, CA) at 4°C for 30 min, followed by flow cytometric analysis. In order to measure the intracellular GzB content, the same protocol was followed except that FITC-conjugated anti-mouse CD8 antibody (Ly-2) and a PE-conjugated Gag tetramer complex were used for surface staining and anti-human Gb-Apc (Invitrogen) was used for intracellular staining. Previous studies showed that this antibody cross-reacts with mouse Gb (18).

ELISA. Polypropylene 96-well plates (USA Scientific Inc., FL) were coated with 100 μl of 0.5 μg/ml recombinant HIV-1 IIIB p24 (Immuno Diagnostics, Inc.) overnight at 4°C. Plates were blocked with PBS containing 1% fetal calf serum for 2 h at 25°C. Serum samples were twofold serially diluted with PBS containing 1% fetal calf serum and added to the plates for 2 h of incubation at 25°C. The plates were then washed three times with PBS containing 0.1% Tween 20 and incubated for 1 h with peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (diluted 1:1,000 in PBS) (Sigma). After three washes, the plates were incubated with 100 μl/well of tetramethylbenzidine (TMB) substrate (Sigma) for 15 min at room temperature. The reaction was stopped using 100 μl/well of 2N H2SO4. The optical density was read at 450 nm (OD450) with an enzyme-linked immunosorbent assay (ELISA) reader. The IgG titers are reported as the reciprocal of the highest serum dilution at which the OD450 is close to the cutoff value for naive mice.

In vivo CTL assay. The in vivo CTL assay was performed as previously described with modifications (8). Target cells were isolated from the spleens (10^7 cells) of naive BALB/c mice and were either pulsed with the Gag dominant peptide (2 μM) or with a medium without peptides at 37°C for 1 h. Severe acute respiratory syndrome coronavirus spike protein S366-374 was used to pulse a separate population of naive spleen cells as a nonspecific control. After a wash, the peptide-pulsed cells were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE™) cells and unpulsed cells were labeled with 0.2 μM CFSE (CFSE™) in PBS at 25°C for 5 min. After a wash, 10^7 CFSE™ cells and 10^7 CFSE™ cells were mixed together in ice-cold PBS and injected intravenously into naive or AAV-injected mice. Recipient mice were killed 18 h after cell transfer. The transferred spleenocytes were isolated from the blood, spleens, and livers of the recipient mice and analyzed by flow cytometry. The percentage of specific killing was calculated as the ratio of recovery of non-peptide-treated control spleen cells to that of peptide-sensitized spleen cells [(percentage of CFSE™ cells)/(percentage of CFSE™ cells)]. The percentage of specific lysis was calculated as 100 × [1 – (ratio of cells recovered from naive mice/ratio of cells recovered from infected mice)].

Isolation of infiltrating lymphocytes from livers and lungs. Liver or lung tissue was diced using sterile blades into 1-mm-thick pieces in RPMI 1640 containing 1 mg/ml collagenase IV (Sigma) and then incubated at 37°C for 1 h. After enzymatic digestion, tissue was passed through a nylon mesh filter (pore size, 100 μm) to remove cell clumps and nondissociated tissue. Cells were washed three times in PBS to remove collagenase IV, and the cell suspension was layered on a Percoll density gradient (64%/56%) and centrifuged for 30 min at 2,000 rpm. The lymphocyte band was then recovered from the interface between 56% and 64% Percoll and was further washed three times in PBS.

RESULTS

Immunogenic comparison between various serotypes of AAV-based vaccines. An initial objective was to compare the relative potencies of AAV vectors with different capsid structures in eliciting T- and B-cell responses to an HIV-1 target antigen. The target antigen used in these studies was HIV-1 Clade B Gag, for which a dominant, H2-Kd-restricted epitope has been mapped in BALB/c mice (23). A truncated gag open reading frame was cloned into an expression cassette driven by a cytomegalovirus promoter between AAV2 inverted terminal repeats. This vector plasmid was used to generate AAV vector
particles with capsids from AAV1, AAV2, AAV5, AAV7, AAV8, and AAV9. The corresponding vectors are named according to the vector genome, which in each case is derived from AAV2, and the vector capsid, such as serotype 8 (e.g., AAV2/8).

BALB/c mice were injected i.m. with $10^{11}$ GC of each vector, and animals were monitored for Gag-specific T cells by ICCS of IFN-γ (A) and p24 antigen production (B). IgG titers were reported by the reciprocal of the highest serum dilution with the OD$_{50}$ close to the cutoff value for naïve mouse sera. Data are means ± standard deviations for four mice per group.

FIG. 1. Activation of CD8$^+$ T cells specific for HIV-1 Gag in BALB/c mice after i.m. injection of AAV vectors. Mice were immunized with $10^{11}$ GC of AAV vaccines expressing HIV Gag in AAV serotypes 1, 2, 5, 7, 8, and 9. At the indicated time points, the mice were sacrificed and lymphocytes were isolated from spleen. Gag immunoreactivity was characterized by ICCS of IFN-γ (A) and p24$_{agg}$ antibody production (B). IgG titers were reported by the reciprocal of the highest serum dilution with the OD$_{50}$ close to the cutoff value for naïve mouse sera. Data are means ± standard deviations for four mice per group.

subset of vectors previously characterized, including AAV2/1, AAV2/7, and AAV2/8. Lymphocytes from different tissues, including the spleen, liver, and lung, were evaluated by ICCS for coexpression of the cytokines IFN-γ, TNF-α, and IL-2 (Fig. 3A). AAV2/8 again yielded the highest numbers of activated T cells, although the differences were less dramatic for tissue-derived cells than for PBMCs. In each case there was a substantial population of cells that coexpressed both IFN-γ and TNF-α as well as cells that expressed IFN-γ alone; very few cells expressed IL-2. The cytolytic activity of the Gag-specific T cells was evaluated in vivo using the following assay. Splenocytes from naïve animals either were stained with high concentrations of CFSE and loaded with the dominant Gag CDS$^+$ T-cell epitope (AMQMLKETI) or were stained with a lower concentration of CFSE and were not loaded with epitope. The two populations were mixed equally and injected into BALB/c mice previously vaccinated with AAV2/1, -2/7, or -2/8 as well as into nonvaccinated controls. At 18 h posttransfer, splenocytes were isolated from blood, spleens, and livers and were analyzed for the persistence of cells containing CFSE. The data showed that the Gag peptide-loaded cells were completely eliminated 18 h after cell infusion from animals vaccinated with AAV2/1, -2/7 or -2/8 Gag vaccines; in each case there was no diminution in the number of labeled cells that were not peptide loaded. No significant decrease was observed in the number of peptide-loaded cells after infusion into naïve animals (Fig. 3B) or in vaccinated animals infused with CFSE cells loaded with an irrelevant peptide (data not shown).

Comparison of Gag-specific CD8$^+$ T cells between AAV2/8 and AdC7 HIV Gag vaccines. A more detailed analysis of the Gag-specific T cells activated by AAV was performed. Studies were performed with (BALB/c × C57BL6)F₁ hybrid mice to overcome the potential bias in the immune response that could occur in BALB/c mice caused by the deficiency of the IL-12 receptor. These hybrid animals retain the H-2$^d$ haplotype, allowing the use of the dominant CDS$^+$ T-cell epitope and the associated tetramer complex. Pilot studies were performed with AAV2/1, AAV2/7, and AAV2/8 vectors in hybrid mice,
and the results were compared with those obtained for BALB/c mice. The following observations were made (data not shown): in hybrid mice, the absolute levels of Gag-specific CD8+ T cells were higher, with less contraction of the T-cell responses, although the relative activities between serotypes were similar (i.e., highest for AAV2/8, intermediate for AAV2/7, and lowest for AAV2/1). Detailed studies were performed with AAV2/8, and the results were directly compared to those obtained with the chimpanzee-derived adenovirus AdC7, which is known to effectively activate CTLs (7).

Hybrid mice injected i.m. with AAV2/8 or AdC7 were monitored for Gag-specific CD8+ T-cell responses in PBMCs by Gag tetramer staining and were sacrificed for more-detailed analyses at the peak (day 14) and after the initial phase of contraction (day 90). The phenotypes of the Gag-specific T cells and their functions in terms of activation of cytokine and GrB expression are summarized in Table 1 for AAV2/8 and in Table 2 for AdC7. The memory phenotype of tetramer-positive cells was characterized by measuring the expression of CD127 and CD62L as TE (effector T-cell) (CD62L+ CD127+), TEM (effector memory T-cell) (CD62L+ CD127+), and TCM (central memory T-cell) (CD62L+ CD127+) subsets, as described by Bachmann et al. (2).

For AAV2/8 injected animals, the frequencies of tetramer-positive cells at peak were extremely high, with tetramer-positive cells equal to 41, 35, 80, and 71% of all CD8+ T cells in PBMCs, spleens, livers, and lung, respectively (Table 1). There was some contraction over a 76-day period, which was greater in central compartments (PBMCs and spleen) than in periph-
eral organs (liver and lung). The phenotype of tetramer-positive cells at peak was almost exclusively TE (98 to 99%), with modest conversion to TEM (2.4 to 5.2%) and even smaller appearance of TCM (0.1 to 2.9%). ICCS demonstrated an equivalent distribution of cytokine-expressing cells between populations expressing IFN-γ alone and those expressing both IFN-γ and TNF-α, which shifted to an IFN-γ TNF-α double-positive predominant population over time (e.g., in the spleen, the proportions expressing IFN-γ alone versus both IFN-γ and TNF-α went from 7.7 and 4.1% at peak to 1.8 and 2.3% at day 90, respectively). There was very little expression of IL-2 at any time point in any cell population. Interestingly, tetramer staining detected higher numbers of antigen-specific cells than ICCS; the ratio of detection by ICCS to detection by tetramer staining was 0.31 to 0.34 (including variation) at peak and 0.25 to 0.63 at day 90. GrB expression, which is a measure of CTL activity, was highest in liver-derived cells and was stable over time.

Identical studies of animals injected with AdC7 yielded important differences (Table 2). As we showed previously, AdC7 is potent activator of CD8+ T cells; furthermore, there is little contraction over the 90-day period. The memory phenotype was qualitatively different, with an even distribution between TE and TEM cells at peak and a predominance of TEM with a substantial TCM population at day 90. The cytokine profile of Gag-specific T cells was predominately IFN-γ TNF-α at peak, which shifted by day 90 to an even greater proportion of double-cytokine-expressing cells as well as the emergence of triple-cytokine-expressing (IFN-γ TNF-α IL-2) cells. At peak the frequency of Gag-specific T cells detected by cytokine staining was actually in excess of that measured by tetramer staining, although this ratio diminished with time. GrB expression was again highest in liver-derived cells and was stable over time.

To more carefully compare the functions of Gag-specific T cells elicited by AAV2/8 and AdC7, the actual ICCS histograms are presented in Fig. 4, demonstrating some of the data noted in Tables 1 and 2. This depiction of the data illustrates differences in the levels of cytokines expressed following peptide activation (i.e., the absolute amount of cytokine expressed per cell). At peak there is no substantial difference in the quantities of IFN-γ or TNF-α between AAV2/8 and AdC7 (Fig. 4A). These profiles do not change over time following injection of the AAV2/8 vector (Fig. 4B). However, the total amount of IFN-γ expressed per cell does increase over time following AdC7 administration, as does the appearance of IL-2 coexpression (Fig. 4B).

### TABLE 1. Phenotypic and functional analysis of CD8 T cells following vaccination with AAV2/8 Gag

<table>
<thead>
<tr>
<th>Cell type/cell population</th>
<th>% Expression of phenotype or function in cell population</th>
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<tbody>
<tr>
<td></td>
<td>Peak</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
</tr>
<tr>
<td>Tetramer+/CD8</td>
<td>40.8 ± 7.2</td>
</tr>
<tr>
<td>TE+/tetramer</td>
<td>99.55</td>
</tr>
<tr>
<td>TEM+/tetramer</td>
<td>0.43</td>
</tr>
<tr>
<td>TCM+/tetramer</td>
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<tr>
<td>GrB+/Ter+/CD8</td>
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<tr>
<td>IFN-γ+/CD8</td>
<td>–</td>
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<tr>
<td>TNF-α+/CD8</td>
<td>–</td>
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<tr>
<td>IFN-γ+ TNF-α+/CD8</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ+ TNF-α+ IL-2+/CD8</td>
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* –, not measured; Ter, tetramer.

### TABLE 2. Phenotypic and functional analysis of CD8 T cells following vaccination with AdC7 gag

<table>
<thead>
<tr>
<th>Cell type/cell population</th>
<th>% Expression of phenotype or function in cell population</th>
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<tr>
<td></td>
<td>Peak</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
</tr>
<tr>
<td>Tetramer+/CD8</td>
<td>6.07 ± 1.1</td>
</tr>
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<td>TE+/tetramer</td>
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</tr>
<tr>
<td>TEM+/tetramer</td>
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<td>TCM+/tetramer</td>
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<tr>
<td>GrB+/Ter+/CD8</td>
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<tr>
<td>IFN-γ+ TNF-α+ IL-2+/CD8</td>
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* –, not measured; Ter, tetramer.
FIG. 4. Comparative study of CD8+ T cells specific for HIV-1 Gag following i.m. injection of AdC7 and AAV2/8 in hybrid mice. The mice were immunized with $1 \times 10^{10}$ viral particles of AdC7 or $1 \times 10^{11}$ GC of the AAV2/8 vector expressing HIV Gag. Populations of CD8 cells expressing single or multiple cytokines were measured as indicated in the key at day 14 (A) and day 90 (B). For each histogram (fluorescence analysis), cytokine staining is measured on the y axis and CD8 staining is measured on the x axis.
Data are means identical dose of AdC7 was injected i.m. into naïve mice (Ad peak) following the boost (peak boost), and the peak obtained when an immediate prior to the boost (preboost), the peak response following the prime (peak prime), the Gag tetramer responses (B) from sera and PBMCs were monitored. The production of anti-p24 gag following the boost relative to the response obtained with AdC7 in naïve animals; and a boost potency of 2-9 vector expressing HIV gag at doses equal to 10^9, 10^{10}, or 10^{11} GC, followed with 5 x 10^9 viral particles of AdC7 gag 60 days later. The adaptive immune responses were represented by the percentage of Gag tetramer-specific CD8^+ T cells and anti-p24 antibody production. The boost potency, calculated as (peak boost — preboost)/ (Ad peak), is presented on the y axis for the indicated doses of priming AAV (x axis). Individual data sets for each vector (AAV2/2, -2/7, -2/8, and -2/9) are presented for tetramer-positive CD8^+ T cells (“T cells”) and anti-Gag antibodies (“B cells”).

FIG. 5. CD8^+ T-cell and antibody responses to HIV-1 Gag following an AAV prime and an AdC7 boost. AAV2/1, -2/7, and -2/8 HIV Gag vectors (5 x 10^{10} GC) were injected i.m. into BALB/c mice, followed by 5 x 10^9 viral particles of AdC7 gag boost 90 days later. A similar prime-boost study was performed in C57 F1 hybrid mice using 10^{10} GC of AAV2/8 gag followed with a 10^{10} AdC7 gag viral particles boost. The production of anti-p24 gag antibodies (A) and CD8^+ T-cell Gag tetramer responses (B) from sera and PBMCs were monitored. Data are the peak response following the prime (peak prime), the measure immediately prior to the boost (preboost), the peak response following the boost (peak boost), and the peak obtained when an identical dose of AdC7 was injected i.m. into naïve mice (Ad peak). Data are means ± standard deviations for a group of five mice.

AAV prime actually diminishes the receptivity of the Gag-specific T cell populations to a boost.

An additional study was performed to confirm these findings with BALB/c mice in which the dose of the AAV prime was varied (10^9, 10^{10}, and 10^{11} GC) for AAV2/2, AAV2/7, AAV2/8, and AAV2/9, and the resulting Gag tetramer frequencies and anti-Gag antibody levels were measured. The data are presented as the boost potency as described above. A boost potency of 1 means there is no impact of the prime on the ability of AdC7 to elicit antigen-specific responses; a boost potency of <1 means that the prime has diminished the ability of the boost relative to the response obtained with AdC7 in naïve animals; and a boost potency of >1 means that the prime has enhanced the activity of AdC7 to boost over that with naïve animals. The data are presented in Fig. 6. In terms of antibody responses, each AAV effectively primes an AdC7 boost in a dose-dependent manner. For AAV2/7, -2/8, and -2/9, the boost potency after a high-dose prime is 20- to 50-fold, leading to a peak boost of anti-Gag antibodies almost 10-fold higher than the peak prime. The T-cell data confirm the results in Fig. 5, with increasing doses of all AAVs tested leading to a less effective boost than that observed with AdC7 alone; in a few cases, the T-cell frequencies actually dropped immediately after the boost. The generation of nonresponsive CD8^+ T cells by AAV was indeed dose dependent, as would be expected if the prime actively perturbed the function of the pool of naïve progenitors. There was no direct correlation, however, between the peak prime or preboost frequencies of tetramer-positive T cells after an AdC7 boost (data not shown). This suggests that the AAV prime actually diminishes the receptivity of the Gag-specific T cell populations to a boost.
assessed in order to more thoroughly evaluate the role of the AdC7 boost in the phenomenon. Animals were primed with AAVs of different serotypes (2/1, 2/2, 2/7, and 2/9), boosted 12 weeks later with a serologically distinct AAV (2/8), and evaluated for Gag-specific CD8+ T cells in splenocytes at various time points, including peak prime (either 2, 3, or 4 weeks after the prime), preboost (week 12), and peak boost (2.5 weeks after the boost). Controls included animals immunized with just AAV2/8 alone or primed and boosted with AAV2/8 (homologous prime-boost). The data are summarized in Fig. 7. As noted above (Fig. 1), all vectors except AAV2/2 yielded substantial levels of Gag-specific T cells following a single i.m. injection. Each of these vectors primed a T-cell response that was not effectively boosted with heterologous or homologous AAV vectors; in each case, the boost potency was less than 1. The only exception was the AAV2/2 prime–AAV2/8 boost, which yielded a boost potency of 1.5.

**DISCUSSION**

A number of investigators have used AAV2-based vaccines expressing HIV-1 antigens to generate anti-HIV T cells and antibodies (20, 40, 41). The present study evaluated novel AAV isolates as potentially better vaccine carriers for HIV-1.

Previous work with the novel AAVs for gene therapy applications did indeed demonstrate substantially improved performance over that of vectors based on AAV1 to -6 in terms of gene transfer efficiency (12–14). In the present vaccine studies, vectors based on AAV2/7, AAV2/8, and AAV2/9 do indeed outperform AAV2/1 and -2/2 vectors in terms of Gag-specific T cells and antibodies. This confirms the recent findings of Xin et al. (39). It is unclear what the precise mechanism(s) is for the capsid-dependent immunogenicity of the gag transgene. Levels of expression in muscles with resulting cross presentation may play a role (29). Analysis of injected muscles for expression of Gag demonstrated a general correlation of the levels of Gag expression with the resulting antigen-specific responses (much higher expression from AAV2/7 and -2/8 than from AAV2/2 [data not shown]). However, this cannot be the only explanation, since Gag-specific T-cell frequencies did not decline in proportion to the dose of AAV vector in hybrid mice (data not shown). Structural variation in the capsids could also affect interactions with receptors that could modify the activation and transduction of the antigen-presenting cell or cells that regulate the immune response (27).

We next sought to study the biology of the immune response to AAV-encoded Gag in terms of the level of antibody produced and the quality/phenotype of the CD8+ T-cell responses. Previous studies of AAV-directed transgene immune responses have focused on reporter genes and applications of gene therapy and have been confusing. The first demonstration of AAV gene therapy in vivo was i.m. injection of LacZ-expressing AAV2 vectors into mice (33). Expression was indeed stable without detectable cytolytic activity to β-galactosidase. Our initial studies comparing the immunology of AAV- versus Ad-based LacZ vectors suggested a lack of effective antigen presentation with AAV2 in muscles, leading to a state of immunologic ignorance and no activation of CD8+ T cells (29). Other studies indicated that the nature of the antigen and the inflammatory state of the target tissue can impact CD8+ T-cell formation (37, 43). The blunted CD8+ T-cell response to transgene products observed with i.m.-injected AAV2 does not necessarily translate to B-cell responses, especially when the antigen is secreted or is on the cell surface, where high levels of antibodies to the transgene can be formed (29). A paucity of both CD8+ T-cell and B-cell responses to transgene products is observed when AAV vectors are targeted to the liver (9, 26). Herzog and coworkers suggest that this may be due to the activity of regulatory T cells that suppress the function of both effectors in an antigen-specific manner (6).

Our studies indicate that i.m. injection of AAV2/8 leads to a very vibrant activation of Gag-specific T cells across multiple organs; these T cells demonstrate cytolytic activity in vivo, express GrB, have an effector phenotype, and express both IFN-γ and TNF-α. The peak CD8+ T-cell response obtained with AAV2/8 is similar to that achieved with AdC7 with respect to kinetics, cytokine expression, and in vivo cytolytic activity. However, several important differences were observed at the peak, including (i) disproportionately lower detection of Gag-specific T cells by ICCS than by tetramer staining following AAV2/8 compared to AdC7 and (ii) substantially more TEM cells from AdC7-immunized animals than from AAV2/8-immunized animals, which produced only TE cells. This suggested qualitative differences in the initial activation of CD8+ T cells between the two vector systems.

Differences in the CD8+ T-cell responses between the AAV2/8 and AdC7 vectors continued to evolve as the CD8+ T-cell response contracted and matured. In the AdC7-injected animals, the quantity of cytokines expressed from Gag-specific CD8+ T cells increased substantially, and triple-cytokine-expressing cells appeared concurrently with the appearance of cells with a TCM phenotype. These events did not occur following injection of AAV2/8. In light of these data (i.e., no IL-2 expression and no TCM phenotype following AAV2/8 injection), it was not surprising that the Gag-specific CD8+ T cells were not effectively boosted with AdC7 in animals previously exposed to the AAV vectors.

The mechanisms responsible for the dysfunctional CD8+ T-cell response that occurs following AAV2/8 compared to

![FIG. 7. Activation of Gag-specific T cells following prime-boost with different AAV serotypes. Another prime-boost study was performed with BALB/c mice by priming with AAV2/1, -2/2, -2/7, -2/8, or -2/9 vectors expressing HIV Gag at doses of 1011 GC followed with a boost of 1011 GC of AAV2/8 gag 90 days later. The specific CD8+ T-cell immune responses, expressed as the percentage of IFN-γ-producing CD8+ T cells upon stimulation with a Gag peptide in vitro, were measured by ICCS of splenocytes. The data are presented in the same way as in Fig. 5 in terms of the definition of peak prime, preboost, peak boost, and AAV peak. Data are means ± standard deviations for four mice per group.](http://jvi.asm.org/Downloaded from October 31, 2017 by guest)
AdC7 administration remain unexplained. The presence of substantial numbers of nonresponsive Gag-specific T cells at peak (i.e., higher numbers by tetramer staining than by ICCS) and the total absence of any memory phenotypes suggest problems in the initial activation of T cells by antigen-presenting cells. In fact, AAV is associated with little activation of innate immunity as opposed to Ad, which serves as a potent self-adjuvant through interactions of capsid and vector DNA with Toll-like receptors (42, 44). It is also possible that there is inadequate CD4+ T-cell help in the setting of AAV gene transfer, which is a step that is more important for the formation of memory than the initial CD8+ T-cell effector response (19, 30). Finally it is possible that persistent expression of Gag from AAV may lead to a condition of T-cell exhaustion that renders the Gag-specific T cells unresponsive to recall (38).

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This cannot explain, however, the abnormal characteristics of the T cells at peak and in the early contraction phase. One very encouraging aspect of the AAV platform for vaccine development is the vibrant B-cell response that ensues. The Gag-specific T cells are unresponsive to recall (38). Inadequate CD4+ adjuvant through interactions of capsid and vector DNA with cells. In fact, AAV is associated with little activation of innate immunity as opposed to Ad, which serves as a potent self-adjuvant through interactions of capsid and vector DNA with Toll-like receptors (42, 44). It is also possible that there is inadequate CD4+ T-cell help in the setting of AAV gene transfer, which is a step that is more important for the formation of memory than the initial CD8+ T-cell effector response (19, 30). Finally it is possible that persistent expression of Gag from AAV may lead to a condition of T-cell exhaustion that renders the Gag-specific T cells unresponsive to recall (38).
virus type 1 envelope from the primary isolate (Bx08) after synthetic DNA prime and recombinant adenovirus 5 boost. J. Gen. Virol. 84:203–213.


