

Vaccine-Induced Immunity in Baboons by Using DNA and Replication-Incompetent Adenovirus Type 5 Vectors Expressing a Human Immunodeficiency Virus Type 1 *gag* Gene

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The cellular immunogenicity of formulated plasmid DNA and replication-defective human adenovirus serotype 5 (Ad5) vaccine vectors expressing a codon-optimized human immunodeficiency virus type 1 *gag* gene was examined in baboons. The Ad5 vaccine was capable of inducing consistently strong, long-lived CD8⁺-biased T-cell responses and in vitro cytotoxic activities. The DNA vaccine-elicited immune responses were weaker than those elicited by the Ad5 vaccine and highly variable; formulation with chemical adjuvants led to moderate increases in the levels of Gag-specific T cells. Increasing the DNA-primed responses with booster doses of either Ad5 or modified vaccinia virus Ankara vaccines suggests a difference in the relative levels of cytotoxic and helper responses. The implications of these results are discussed.

Virus-specific cellular immunity, particularly that associated with CD3⁺ CD8⁺ cytotoxic T lymphocytes (CTLs) (6, 15, 23–25, 27), has been increasingly recognized to have an important role in controlling persistent human immunodeficiency virus type 1 (HIV-1) infection. Recently, we reported on the efficacy of using CRL1005 adjuvant-formulated DNA and recombinant adenovirus type 5 (Ad5) vectors that express simian immunodeficiency virus SIV_{mac239} *gag* to protect rhesus macaques (*Macaca mulatta*) against disease progression from a pathogenic infection with simian-human immunodeficiency virus SHIV_{89,6P} (25). In developing a vaccine against HIV-1, it is important to evaluate the cellular immunogenicity of equivalent vectors expressing HIV-1 *gag* in nonhuman primates. The most commonly used rhesus macaques weigh considerably less (3 to 12 kg) than human adults. In contrast, adult baboons (genus *Papio*) are nearly half the size of adult humans. They can be infected with several simian-human immunodeficiency virus strains (1, 14) and have been shown to develop AIDS-like disease symptoms when infected with HIV-2 (5). They thus provide a suitable alternative immunological model that may closely approximate vaccine-induced immune responses in humans. Prior to this work, vaccine testing in baboons (4, 9, 13, 17, 26) had largely involved candidates aimed at generating virus-specific neutralizing antibodies. Hence, techniques for quantitative analyses of antigen-specific T-cell responses in this primate system were limited.

In this study, we evaluated the cellular immune responses induced in adult baboons by using several vector approaches for treatment of HIV-1. A DNA vaccine was constructed by inserting a synthetic gene for HIV-1 CAM1 *gag*, which used human-biased codons (16), into the V1Jns plasmid under the control of the human cytomegalovirus-human intron A promoter and bovine growth hormone terminator (8, 25). The Ad5 vaccine construct is a prototypic vector containing an E1 deletion replaced with the expression cassette for the same codon-optimized *gag* gene. Methods for constructing and characterizing such vectors have been described earlier (25). The modified vaccinia virus Ankara (MVA) vector was constructed by inserting the same *gag* gene into the *PmeI* site of the pLW22 shuttle vector (20). *gag* was placed under the control of a synthetic early-late vaccinia virus promoter and was inserted into the site of deletion II in the MVA genome by homologous recombination. The generation of the vaccine in chicken embryo fibroblasts and the characterization of the recombinant MVA virus have been described elsewhere (20).

Groups of three baboons (*Papio cynocephalus*; 25 to 34 kg each) were given priming immunizations with (i) 5 mg of V1Jns–HIV-1 *gag* (the DNA vaccine) in phosphate-buffered saline, (ii) 5 mg of the DNA vaccine formulated with 700 μg of aluminum phosphate (HCI Biosector, Frederikssund, Denmark), (iii) 5 mg of the DNA vaccine formulated with 7.5 mg of the adjuvant CRL1005 (CytRx Corp., Atlanta, Ga.) and 0.75 mM benzalkonium chloride (Spectrum, Gardena, Calif.), or (iv) 10¹¹ viral particles (VP) of replication-incompetent Ad5–HIV-1 *gag* (the Ad5 vaccine). Each vaccine was prepared in a 1-ml volume and injected at a single site in the deltoid muscle of an anesthetized animal by using a tuberculin syringe. In

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TABLE 1. Immunization schedule and vaccines used^a

Animals	Priming immunization	Booster at wk 24 ^b
11017, 11361, 14232	10 ¹¹ VP of Ad5 at wk 0 and 4 (i.m.)	10 ¹¹ VP of Ad5 (i.m.)
10041, 11039, 11096	10 ¹¹ VP of Ad5 at wk 0 and 4 (s.c.)	10 ¹¹ VP of Ad5 (s.c.)
10432, 8698, 10875	None	10 ⁸ VP of Ad5 (i.m.)
9895, 11251, 14811	DNA at wk 0, 4, and 8 (i.m.)	10 ⁸ VP of Ad5 (i.m.)
8988, 9783, 10843	DNA-alum at wk 0, 4, and 8 (i.m.)	10 ⁸ VP of Ad5 (i.m.), 10 ⁹ PFU of MVA (i.m.), — ^c
10544, 11247, 11876	DNA-CRL1005 at wk 0, 4, and 8 (i.m.)	10 ⁹ PFU of MVA (i.m.), 10 ⁹ PFU of MVA (i.m.), 10 ⁸ VP of Ad5 (i.m.)
11029 (naive)	None	None

^a s.c., subcutaneously; Ad5, Ad5-HIV-1 *gag*; MVA, MVA-HIV-1 *gag*; DNA, 5 mg of V1Jns-HIV-1 *gag*; alum, 700 μg of aluminum phosphate; CRL1005, 7.5 mg of CRL1005 with 0.75 mM benzyalkonium chloride.

^b For the group in which a different booster vaccine is given to each animal, the order of booster vaccines listed corresponds to that of animals listed in the first column.

^c This animal died of an unrelated cause after week 12.

addition to intramuscular (i.m.) immunization, Ad5-HIV-1 *gag* was also injected subcutaneously at four proximal sites (250 μl per site) above the deltoid muscle area. Table 1 details the priming and booster immunizations that each baboon received. All animal care and treatment were performed in accordance with standards approved by the Institutional Animal Care and Use Committee.

The levels of Gag-specific T cells (Fig. 1) from peripheral blood mononuclear cell (PBMC) preparations were quantified by the enzyme-linked immunospot (ELISPOT) assay by using

the same reagent sets described elsewhere (2). All antigen-specific stimulation reactions were achieved by using a pool of 20-amino-acid (aa) peptides that overlapped by 10 aa and that encompassed the entire Gag product sequence (SynPep, Dublin, Calif.). The length of each peptide is inclusive to allow detection of both CD4⁺- and CD8⁺-T-cell determinants. Priming immunizations with 10¹¹ VP of the Ad5-HIV-1 *gag* vector induced in all six vaccinees high levels of Gag-specific T cells which peaked at levels ranging from 530 to 2,050 spot-forming T cells (SFC)/10⁶ PBMCs at weeks 8 to 12 (Fig. 1).

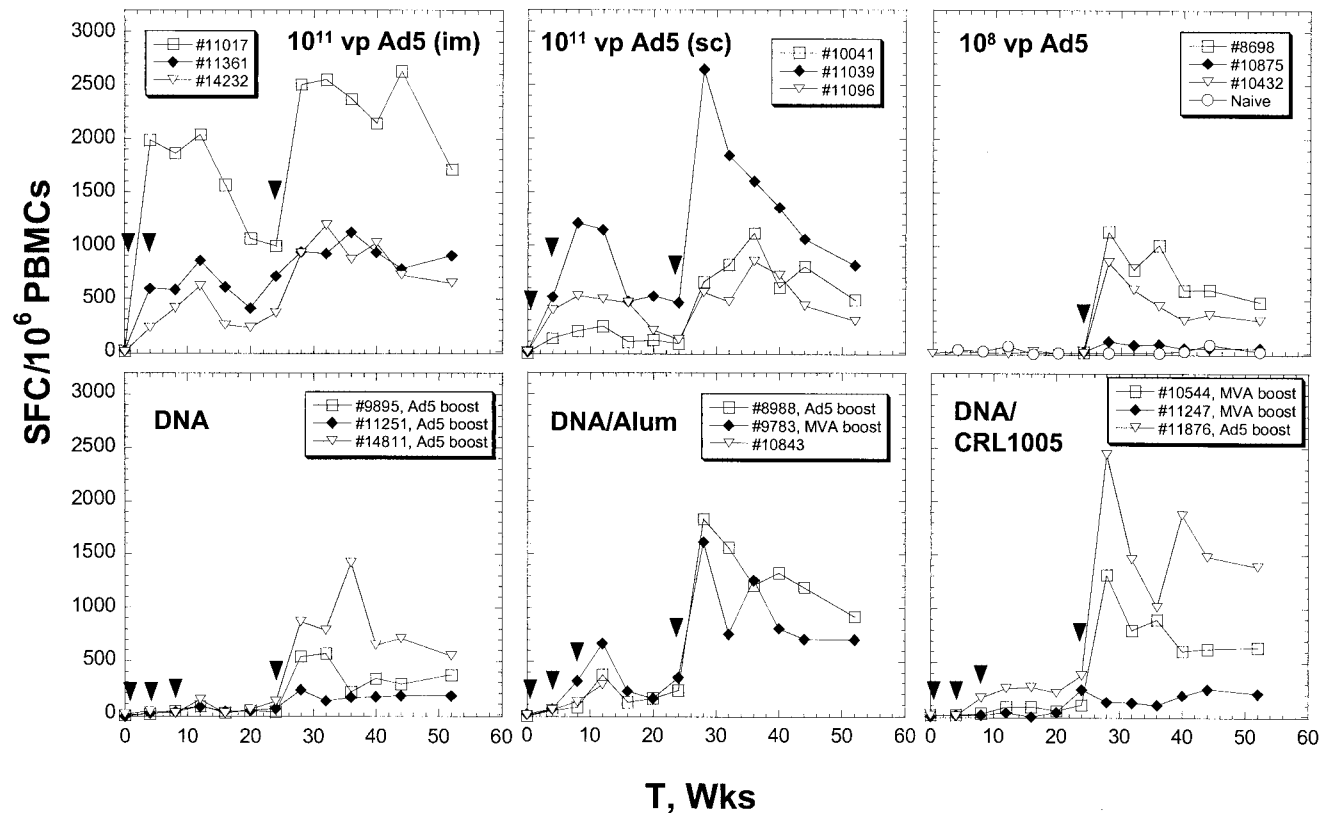


FIG. 1. Levels of Gag-specific T cells in baboons immunized with DNA and viral vaccine vectors as a function of time (T). For the ELISPOT assay, 2×10^5 to 4×10^5 PBMCs were plated on each well and the wells were incubated in the absence or presence of peptides at a 4-μg/ml concentration per peptide. The plotted values are differences in the numbers of IFN-γ SFC per 10⁶ PBMCs for a given sample mixed with the Gag peptide pool and the blank control. The average blank control count was about 18 SFC/10⁶ PBMCs with a standard deviation of 28 SFC/10⁶ PBMCs. Arrowheads indicate the times of immunization with the vectors described in each panel. The DNA-, DNA-aluminum phosphate (Alum)-, and DNA-CRL1005-vaccinated animals received booster doses of either 10⁸ VP of Ad5 or 10⁹ PFU of MVA-HIV-1 *gag* vector at week 24.

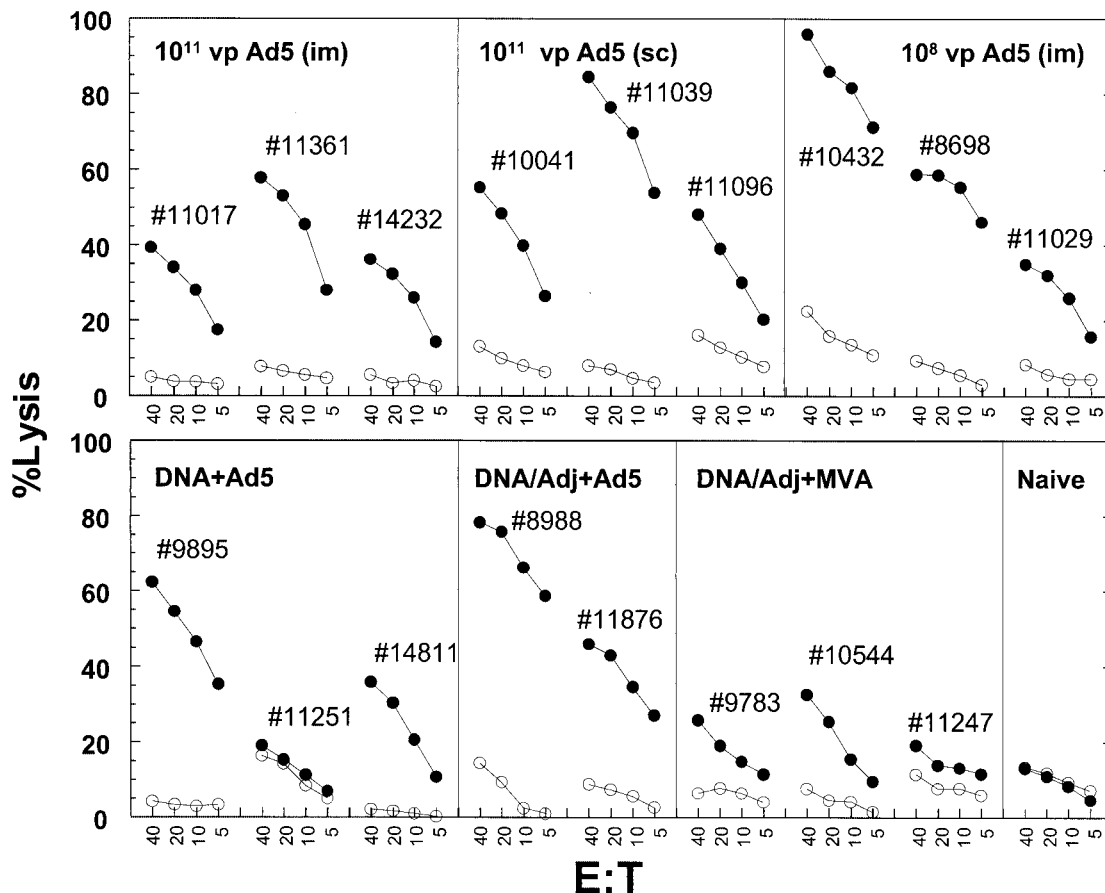


FIG. 2. ⁵¹Cr release assay of CTL killing by baboon effector cells of autologous target BLCLs that either were unpulsed (open circles) or were pulsed with a pool of 20-mer peptides derived from HIV-1 Gag (closed circles). Effector cells were prepared from PBMCs that were collected at week 36 (12 weeks after the booster dose) and were restimulated with a vaccinia virus expressing HIV-1 gag for 2 weeks. The percentages of specific lysis were recorded at effector-to-target cell ratios (E:T) ranging from 40 to 5. In all cases, the spontaneous lysis did not exceed 15% of the maximum ⁵¹Cr release values. Adj, adjuvant; sc, subcutaneous.

The magnitude of these responses was independent of the route of administration. Immunization of the Ad5 vaccinees with a third dose of the virus resulted in improved levels of circulating Gag-specific T cells (1.3- to 4-fold and 2- to 10-fold higher than the prebooster peak and set point levels, respectively). The anti-Ad5 neutralizing antibody titers were determined by testing serially diluted serum samples for the ability to block the expression of human secreted alkaline phosphatase from cells infected with an Ad5-human secreted alkaline phosphatase vector; the titers for the Ad5 vaccinees ranged from 139 to 4,000 at week 24. Animals 11017 and 11039 showed the lowest neutralizing antibody titers (139 and 201, respectively) and the largest absolute increases in T-cell responses. The fact that the immune responses were increased despite high levels of vector-specific neutralizing immunity suggests that only a small amount of the Ad5 vaccine was necessary to effect a significant expansion of the existing T-cell pool.

Baboons immunized with the DNA vaccine alone developed low levels of antigen-specific T cells following the priming immunizations (<138 SFC/10⁶ PBMCs) (Fig. 1). The responses (293 to 705 SFC per 10⁶ PBMCs at week 12) were improved by combining the DNA vaccine with aluminum phos-

phate. One animal in the DNA-aluminum phosphate-vaccinated group (animal 10843) died due to an unrelated cause during the course of the priming regimen. In the cohort vaccinated with DNA formulated with CRL1005 adjuvant, the levels of circulating Gag-specific T cells were not significantly higher than those in the DNA-alone control group in this animal model system. The animals that received DNA-alone vaccinees were given a low (10⁸ VP) booster dose of Ad5-HIV-1 gag at week 24. The low Ad5-HIV-1 gag booster dose is designed to simulate a case in which the subject has a pre-existing Ad5-specific immunity capable of 99.9% neutralization of a 10¹¹-VP dose. Each of three baboons in a control group was inoculated i.m. with a single dose of 10⁸ VP of the Ad5 vaccine (booster control group) at the same time that the other baboons received the booster immunization. The levels of Gag-specific T cells in the DNA-alone vaccinees following the booster dose increased to 200 to 1,500 SFC/10⁶ cells. These levels were comparable to those in the booster control group, suggesting that the DNA immunizations did not significantly prime for HIV-1 T-cell responses.

Several studies have shown that optimal HIV-1-specific T-cell responses are obtained by administering boosters of recombinant poxvirus vaccines such as the MVA vaccine to

animals receiving DNA priming immunizations (3, 11). We decided that the remaining five DNA-adjuvant vaccinees were best utilized by comparing the booster effects of the Ad5 vaccine with those of the MVA vaccine (Table 1). Gag-specific responses in all DNA-adjuvant vaccinees were augmented by immunization with either viral booster at week 24 (Fig. 1). The two animals receiving DNA-adjuvant priming and Ad5 booster immunizations (animals 8988 and 11876) developed peak T-cell responses in the range of 1,800 to 2,500 SFC/10⁶ cells, which were markedly higher than those of the booster control group animals. Animals that received the MVA booster likewise showed high postbooster T-cell levels (200 to 1,500 SFC/10⁶ cells), but these levels were slightly lower than those observed in animals from the same DNA-adjuvant groups that were given the Ad5 booster. A control cohort for the MVA booster was not included in this study. However, immunization of rhesus macaques with the same MVA-HIV-1 *gag* vector resulted in consistently weak Gag-specific T-cell responses (<150 SFC/10⁶ PBMCs) (7).

Cytotoxic functions of the vaccine-induced T-cell responses (Fig. 2) were examined by *in vitro* killing of autologous B-lymphoid cell lines (BLCLs) pulsed with the 20-aa peptide pool by using established protocols (10). At week 36, all animals (with the exception of animal 11251) that received at least a single injection of the Ad5 vaccine exhibited consistently strong CTL activity (>40% at an effector-to-target cell ratio of 40:1). Prior to the booster doses, none of the DNA vaccinees showed any Gag-specific cytotoxic activity above that of the unpulsed BLCL controls (data not shown). All three animals that received MVA boosters at week 24 exhibited much weaker CTL killing efficiencies than those of the DNA vaccinees that received Ad5 boosters (Fig. 2).

The intracellular cytokine staining method (ICS) has been widely utilized to distinguish the relative contributions of CD4⁺ and CD8⁺ cells to the overall T-cell responses (18). We have identified suitable commercial antibodies for staining markers (CD3, CD16, CD4, CD8, and gamma interferon [IFN- γ]) on baboon PBMCs. Anti-CD3 or anti-CD16 monoclonal antibodies can be used interchangeably in order to exclude cytokine-producing CD8⁺ NK cells (CD3⁻ CD16⁺) (19). At week 28, all six Ad5-HIV-1 *gag* vaccinees showed detectable levels of effector CD8⁺ T cells mixed with lower amounts of CD4⁺ T cells (range of anti-Gag CD8⁺:CD4⁺ cell ratios, 1.4:1 to 10:1) (Table 2; Fig. 3) following overnight restimulation with the 20-aa peptide pool. Prior to any viral boost, all DNA vaccinees exhibited largely CD4⁺ antigen-specific IFN- γ -producing cells (<0.14% of CD16⁻ T cells) (data not shown).

The types of responses in the adjuvant-formulated-DNA vaccinees after booster vaccinations with either the Ad5 or the MVA vector were also compared. Two of the three animals that received MVA-HIV-1 *gag* boosters exhibited responses with a stronger CD4⁺ component than a CD8⁺ component (CD4⁺:CD8⁺ cell ratios of 6:1 and 10:1 for animals 9783 and 10544, respectively) (Fig. 3); in the third animal, the overall response was much weaker, which was consistent with the ELISPOT assay results. It should be noted that the CD4⁺ and CD8⁺ distributions were similar when either a 15-mer peptide pool (with 11-aa overlaps) or a 20-mer peptide pool was used for stimulation (data not shown). In contrast, the two animals that received the Ad5 booster dose developed CD8⁺-biased

TABLE 2. Percentages of CD16⁻ T lymphocytes that are Gag-specific CD8⁺ or CD4⁺ cells in vaccinated animals^a

Vaccine (route)		Animal	% CD16 ⁻ T cells that were ^b :	
Priming	Booster		CD4 ⁺	CD8 ⁺
Ad5	Ad5	11017	0.17	1.84
		11361	0.10	0.25
		14232	0.06	0.52
Ad5 (s.c.)	Ad5 (s.c.)	10041	0.10	0.14
		11039	0.09	0.73
		11096	0.06	0.18
None	Ad5	8698	0.01	0.51
		10875	0.02	0.06
		10432	0.04	0.20
DNA	Ad5	9895	0.07	0.03
		11251	0.05	0.02
		14811	0.05	0.38
DNA-alum	Ad5	8988	0.07	0.56
DNA-CRL1005	Ad5	11876	0.26	0.67
DNA-alum	MVA	9783	0.35	0.02
DNA-CRL1005	MVA	10544	0.23	0.04
		11247	0.02	0.01

^a Data are based on IFN- γ ICS analyses of PBMCs collected at week 28 (4 weeks postbooster). s.c., subcutaneously; alum, aluminum phosphatase.

^b Mock-subtracted values.

T-cell responses (antigen-specific CD8⁺:CD4⁺ ratios of 2:1 and 8:1, respectively). This trend was likewise observed in rhesus macaques immunized with a heterologous DNA-CRL1005-Ad5 priming-booster regimen (7).

HIV-1-specific cellular responses in all vaccinees were assessed at week 52, or about 7 months after the final immunization. All animals maintained levels of Gag-specific effector T cells to within 30 to 80% of the postbooster peak levels. The antigen-specific T-cell counts were consistently higher than those at the set point (week 24) after the priming series, suggesting an immunological benefit of the booster treatment.

The successful development of an HIV-1 vaccine for either prophylactic or therapeutic application will require gene delivery systems that are potent and consistent in their ability to elicit effective cellular immune responses. Herein, we provide evidence that a nonreplicating human adenovirus vector is capable of inducing consistently strong and stable HIV-1-specific cytotoxic T-cell responses in a large nonhuman primate system. First, 10 out of the 11 baboons that received immunizations of the Ad5-HIV-1 *gag* vaccine one or more times during the study produced high levels of Gag-specific T cells (0.01 to 0.25% of lymphocytes) that were sustained for at least several months. Second, the majority of the Gag-specific cellular immune responses consistently originated from CD8⁺ T cells, a finding which is in agreement with the observed high efficiencies of *in vitro* killing by vaccinee PBMCs of peptide-pulsed autologous cell lines. Third, the virus is effective with even a single low-dose immunization of 10⁸ VP.

One limitation of viral vaccine vectors such as Ad5 or MVA involves the negative effect of preexisting vector-specific im-

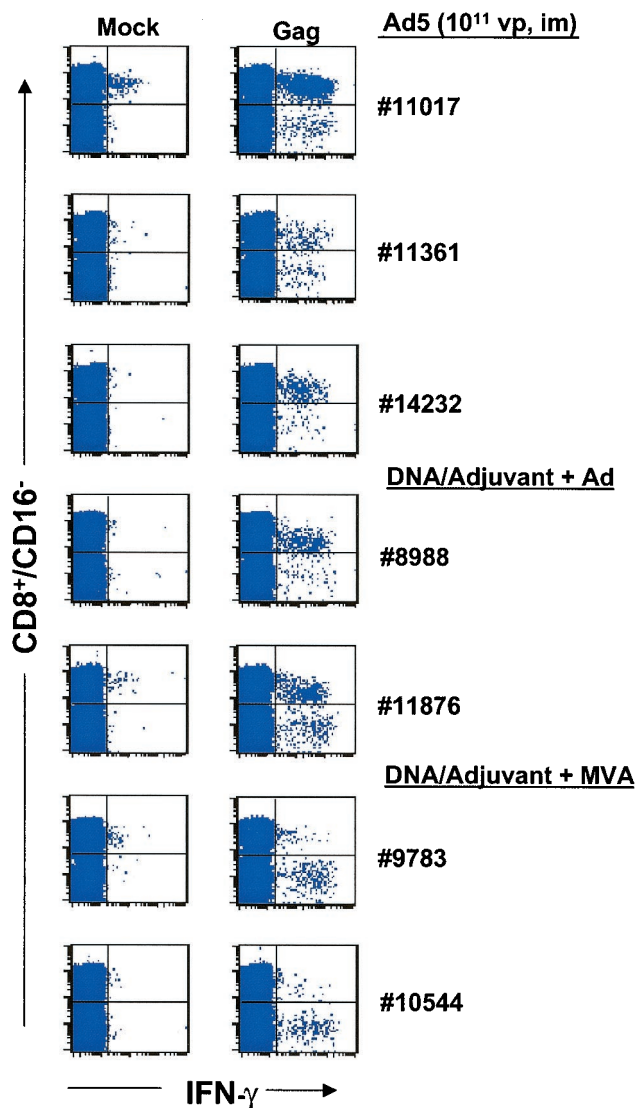


FIG. 3. Intracellular staining for IFN- γ production by PBMCs collected from selected baboons at week 28. The animals received multiple high doses of Ad5-HIV-1 *gag* or DNA-adjuvant priming immunizations followed by either an MVA or an Ad5 (Ad) booster. PBMCs were treated overnight with brefeldin, anti-human CD28 (anti-hCD28, clone L293; Becton Dickinson, Franklin Lakes, N.J.), and anti-hCD49d (clone L25; Becton Dickinson) with or without antigen stimulation. They were then stained with anti-hCD16-Cy-Cychrome (clone 3G8; BD-PharMingen), anti-hCD4-allophycocyanin (anti-hCD4-APC, clone SK3; Becton Dickinson), anti-hCD8-APC (clone SK1; Becton Dickinson), and fluorescein isothiocyanate-anti-hIFN- γ (clone 1D1K; MABTECH, Nacka, Sweden). The plots illustrate IFN- γ staining of CD16⁻ CD8⁺ and CD16⁻ CD8⁻ cells incubated in the absence (left panels) or presence (right panels) of the 20-aa Gag peptides. In every case, the numbers of CD16⁻ CD8⁻ IFN- γ ⁺ T cells normalized to 10⁶ lymphocytes matched those of CD16⁻ CD4⁺ IFN- γ ⁺ cells, suggesting that in the plots shown, the events in the CD8⁻ population represented helper responses.

munity on the induced immunity against the vaccine antigen. This limitation can be circumvented by priming with a nonimmunogenic vector system such as DNA and then following that with a heterologous viral booster (7, 28). Here we provide evidence suggesting that an effective priming vaccine using

adjuvant-formulated DNA combined with an Ad5 booster can result in a T-cell immune response that approaches those observed with multiple high doses of an Ad5-based vaccine.

Comparison of the immune responses of the adjuvant-formulated-DNA vaccinees given an Ad5 or an MVA booster is suggestive of differences in polarization in the phenotype of the Gag-specific T cells. The Ad5 booster was particularly potent in eliciting a CD8⁺-biased T-cell response. In contrast, none of the three animals that received the MVA vector produced a CD8⁺-biased Gag-specific immunity. It should be noted that the numbers of animals tested were limited and that these findings should be reinforced with studies of other non-human primate systems as well as of other poxviruses (12, 21). It has been suggested that potent CD8⁺- and CD4⁺-T-cell responses are both critical in controlling an HIV infection (22, 29), yet the relative magnitude and the intrinsic quality of both types of responses remain unclear. It would appear that such immunological parameters can be controlled by the nature of the vaccine modality. Ultimately, the potential of these vaccine approaches can be assessed only with clinical trials involving healthy and HIV-1-infected individuals.

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