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Magnitude and Phenotype of Cellular Immune Responses Elicited by Recombinant Adenovirus Vectors and Heterologous Prime-Boost Regimens in Rhesus Monkeys[▽]

Jinyan Liu,[1] Bonnie A. Ewald,[1] Diana M. Lynch,[1] Matthew Denholtz,[1] Peter Abbink,[1] Angelique A. C. Lemckert,[2] Angela Carville,[3] Keith G. Mansfield,[3] Menzo J. Havenga,[2] Jaap Goudsmit,[2] and Dan H. Barouch[1]*

Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215[1]; Crucell Holland BV, 2301 CA, Leiden, The Netherlands[2]; and New England Primate Research Center, Southborough, Massachusetts 01772[3]

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Recombinant adenovirus serotype 5 (rAd5) vaccine vectors for human immunodeficiency virus type 1 (HIV-1) and other pathogens have been shown to elicit antigen-specific cellular immune responses. Rare serotype rAd vectors have also been constructed to circumvent preexisting anti-Ad5 immunity and to facilitate the development of novel heterologous rAd prime-boost regimens. Here we show that rAd5, rAd26, and rAd48 vectors elicit qualitatively distinct phenotypes of cellular immune responses in rhesus monkeys and can be combined as potent heterologous prime-boost vaccine regimens. While rAd5-Gag induced primarily gamma interferon-positive (IFN-γ^+) and IFN-γ^+/tumor necrosis factor alpha$^+$ (TNF-α^+) T-lymphocyte responses, rAd26-Gag and rAd48-Gag induced higher proportions of interleukin-2$^+$ (IL-2$^+$) and polyfunctional IFN-γ^+/TNF-α^+/IL-2$^+$ T-lymphocyte responses. Priming with the rare serotype rAd vectors proved remarkably effective for subsequent boosting with rAd5 vectors. These data demonstrate that the rare serotype rAd vectors elicited T-lymphocyte responses that were phenotypically distinct from those elicited by rAd5 vectors and suggest the functional relevance of polyfunctional CD8$^+$ and CD4$^+$ T-lymphocyte responses. Moreover, qualitative differences in cellular immune responses may prove critical in determining the overall potency of heterologous rAd prime-boost regimens.

Replication-incompetent, recombinant adenovirus serotype 5 (rAd5) vectors have been explored as candidate vaccines both for human immunodeficiency virus type 1 (HIV-1) and other pathogens (5, 25, 30) but may be substantially limited by preexisting anti-Ad5 immunity. A variety of novel serotype rAd vectors have therefore been generated to circumvent this problem and to facilitate the development of heterologous rAd prime-boost regimens (1, 10, 32). We recently described the construction and immunogenicity of several rare serotype rAd vectors, including rAd11, rAd35, and rAd50 from Ad subgroup B, as well as rAd26, rAd48, and rAd49 from Ad subgroup D (1). These rare serotype rAd vectors utilized CD46 rather than the Ad5 receptor CAR for cellular entry, suggesting fundamental biologic differences among these viruses. These rAd vectors also exhibited low seroprevalence in human populations in sub-Saharan Africa and proved immunogenic in both mice and rhesus monkeys. However, the phenotypes of the cellular immune responses elicited by these novel rAd vectors and the immunogenicity of heterologous rAd prime-boost regimens in rhesus monkeys have not previously been investigated.

In this study, we evaluated the magnitude and phenotypes of the cellular immune responses elicited by rAd5, rAd26, and rAd48 vectors and heterologous rAd prime-boost regimens in rhesus monkeys. The rAd26 and rAd48 vectors induced CD8$^+$ and CD4$^+$ T-lymphocyte responses that were more balanced and cytokine secretion responses that were more polyfunctional than those elicited by rAd5 vectors. Moreover, the cellular immune responses primed by rAd26-Gag and rAd48-Gag were boosted remarkably effectively by rAd5-Gag in both mice and rhesus monkeys, suggesting that the qualitative aspects of T-lymphocyte responses may prove critical in determining the overall potency of heterologous rAd prime-boost regimens.

MATERIALS AND METHODS

Vector production. Replication-incompetent, E1/E3-deleted rAd5, rAd35, rAd26, rAd48, and rAd49 vectors expressing the simian immunodeficiency virus SIVmac239 Gag were prepared as previously described (1, 10, 32). Briefly, adaptor plasmids containing the transgene expression cassette and cosmids containing the majority of the Ad genomes were linearized prior to transfection of PER.C6 or PER.55K cells with Lipofectamine in T25 flasks. Cells were passaged into T75 flasks after 48 h and maintained until virus cytopathic effect was observed. The vectors were plaque purified, analyzed for transgene expression, amplified in 24 triple-layer T175 flasks, purified by double CsCl gradient ultracentrifugation, and dialyzed into phosphate-buffered saline (PBS) containing 5% sucrose. Purified rAd vectors were stored at −80°C. Virus particle (vp) titers were determined by spectrophotometry (13). Specific infectivity was assessed by PFU assays.

Animals and immunizations. Six- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or Taconic (Hudson, NY). Mice were injected intramuscularly (i.m.) with 10^9 vp replication-incompetent rAd vectors expressing SIVmac239 Gag in 100 μl sterile PBS in both quadriceps muscles. To induce anti-Ad5 immunity, mice were preimmunized twice i.m., separated by a 4-week interval, with 10^{10} vp rAd5-Empty in 100 μl

* Corresponding author. Mailing address: Research East Room 213, Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone: (617) 667-4434. Fax: (617) 667-8210. E-mail: dbarouch@bidmc.harvard.edu.

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sterile PBS. The Ad5 neutralizing antibody titers were determined by luciferase-based virus neutralization assays (27).

Adult rhesus monkeys (*Macaca mulatta*) that did not express the major histocompatibility complex class I allele *Mamu-A*01* were housed at New England Primate Research Center, Southborough, MA. Monkeys were immunized i.m. with 10^{11} vp replication-incompetent rAd vectors expressing SIVmac239 Gag in 1 ml sterile PBS containing 5% sucrose in both quadriceps muscles. All animal studies were approved by our Institutional Animal Care and Use Committees (IACUC).

Tetramer binding assays. Tetrameric H-2D^b complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) (3) were prepared and utilized to stain peptide-specific CD8$^+$ T lymphocytes from C57BL/6 mice as described previously (2, 3). Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. Following lysis of red blood cells, 0.1 μg of phycoerythrin (PE)-labeled D^b/AL11 tetramer in conjunction with anti-CD8α-allophycocyanin (APC [Ly-2]; Caltag, San Francisco, CA) was utilized to stain AL11-specific CD8$^+$ T lymphocytes. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analyzed by two-color flow cytometry on a fluorescence-activated cell sorter array (BD Biosciences, San Diego, CA). Gated CD8$^+$ T lymphocytes were examined for staining with the D^b/AL11 tetramer. CD8$^+$ T lymphocytes from naïve mice were utilized as negative controls and exhibited <0.1% background tetramer staining.

ELISPOT assays. Gag-specific cellular immune responses in vaccinated mice or rhesus monkeys were assessed with gamma interferon (IFN-γ) ELISPOT assays as described previously (3, 16). Overlapping 15-amino-acid peptides spanning the SIVmac239 Gag protein were obtained from the NIH AIDS Research and Reference Reagent Program. Ninety-six-well multiscreen plates (Millipore, Bedford, MA) were coated overnight with 100 μl/well of 10 μg/ml anti-mouse or anti-human IFN-γ (BD Biosciences, San Diego, CA) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20 (D-PBS–Tween), blocked for 2 h with D-PBS containing 5% FBS at 37°C, washed three times with D-PBS–Tween, rinsed with RPMI 1640 containing 10% FBS to remove the Tween 20, and incubated with 2 μg/ml of each peptide and 5×10^5 murine splenocytes or 2×10^5 rhesus monkey peripheral blood mononuclear cells (PBMC) in triplicate in 100-μl reaction mixture volumes. Following an 18-h incubation at 37°C, the plates were washed nine times with PBS-Tween and once with distilled water. The plates were then incubated with 2 μg/ml biotinylated anti-mouse or anti-human IFN-γ (BD Biosciences, San Diego, CA) for 2 h at room temperature, washed six times with PBS-Tween, and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Following five washes with PBS-Tween and one with PBS, the plates were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, Rockford, IL), stopped by washing with tap water, air dried, and read using an ELISPOT reader (Cellular Technology Ltd., Cleveland, OH). The numbers of spot-forming cells (SFC) per 10^6 cells were calculated. The medium background levels were typically <15 SFC per 10^6 cells.

Intracellular cytokine staining (ICS) assays. The magnitude and phenotype of Gag-specific cellular immune responses in vaccinated rhesus monkeys were assessed by multiparameter ICS assays. PBMC (3×10^6) were incubated for 6 h at 37°C with RPMI 1640 containing 10% FBS alone as the negative control, the SIVmac239 Gag peptide pool consisting of 2 μg/ml of each peptide, or 10 pg/ml phorbol myristate acetate and 1 μg/ml ionomycin (Sigma-Aldrich, St. Louis, MO) as the positive control. The cultures contained monensin (GolgiStop; BD Biosciences, San Diego, CA) and 1 μg/ml anti-CD49d monoclonal antibody (MAb) (BD Biosciences). Cells were then stained with pretitered amounts of anti-CD3-Alexa Fluor 700 (Alexa700) (SP34), anti-CD4-AmCyan (L200), anti-CD8-APC-Cy7 (SK1), anti-CD28-peridinium chlorophyll protein (PerCP)-Cy5.5 (L293), and anti-CD95-PE (DX2) MAbs, fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences). Cells were then stained intracellularly with anti-IFN-γ–PE–Cy7 (B27), anti-tumor necrosis factor alpha (TNF-α)–fluorescein isothiocyanate (FITC) (MAb11), and anti-interleukin-2 (IL-2)–APC (MQ1-17H12) MAbs and fixed with 1% paraformaldehyde. Samples were analyzed with an LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). Approximately 500,000 to 1,000,000 events were collected per sample. Central memory (CM) and effector memory (EM) CD8$^+$ and CD4$^+$ T-lymphocyte subpopulations were defined by CD28 and CD95 expression as previously described (17, 19). The medium control background levels were typically <0.02% of the levels of gated CD8$^+$ or CD4$^+$ T lymphocytes.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 4.01 (GraphPad Software, Inc., 2004). The results for immune responses among groups are presented as means with standard errors. Comparisons of mean immune responses were performed by using two-tailed *t* tests with Bonferroni adjustments to account for multiple comparisons. *P* values of less than 0.05 were considered significant.

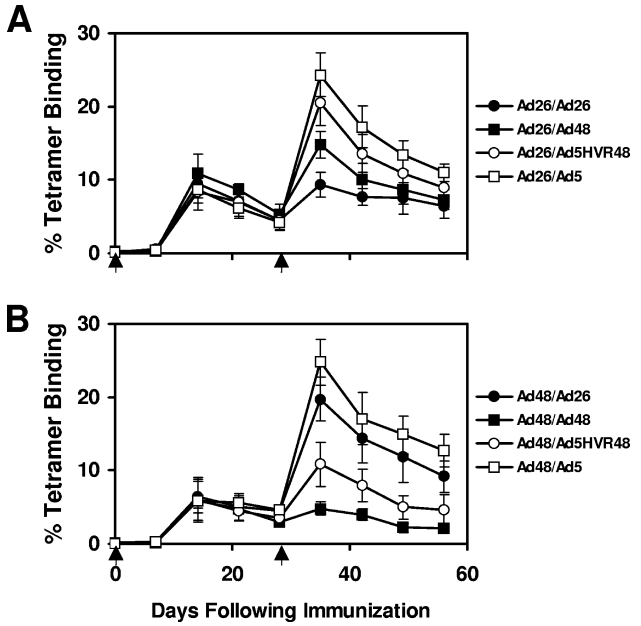


FIG. 1. Immunogenicity of heterologous rAd prime-boost regimens in mice. Naïve mice (n = 4/group) were primed on day 0 with 10^9 vp rAd26-Gag (A) or 10^9 vp rAd48-Gag (B) and were boosted on day 28 with 10^9 vp rAd26-Gag, rAd48-Gag, rAd5HVR48-Gag, or rAd5-Gag as shown. Arrows indicate immunizations. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization. Mean responses with standard errors are shown.

RESULTS

Immunogenicity of heterologous rAd prime-boost regimens in mice. We initiated studies by exploring the immunogenicity of heterologous rAd prime-boost regimens in mice. Groups of C57BL/6 mice (n = 4/group) were primed on day 0 with 10^9 vp rAd26-Gag or rAd48-Gag and were boosted on day 28 with 10^9 vp rAd26-Gag, rAd48-Gag, rAd5HVR48-Gag, or rAd5-Gag. The rAd5HVR48 vector is a hexon-chimeric rAd5 vector in which the seven hexon hypervariable regions (HVRs) have been exchanged with the corresponding regions from Ad48 as previously described (22). Gag-specific CD8$^+$ T-lymphocyte responses specific for the dominant AL11 epitope (AAVKN WMTQTL) (3) were assessed with D^b/AL11 tetramer binding assays at multiple time points following immunization.

As shown in Fig. 1A, the CD8$^+$ T-lymphocyte responses primed by rAd26-Gag were boosted efficiently and similarly by rAd5-Gag and rAd5HVR48-Gag but less well by rAd48-Gag (1). Responses primed by rAd26-Gag were not boosted effectively by a second injection of rAd26-Gag, presumably as a result of anti-Ad26 immunity generated by the priming immunization. Similarly, as depicted in Fig. 1B, the responses primed by rAd48-Gag were boosted efficiently by the heterologous vectors rAd5-Gag and rAd26-Gag. The responses primed by rAd48-Gag were boosted less well by rAd5HVR48-Gag, however, as a result of the generation of anti-Ad48 im-

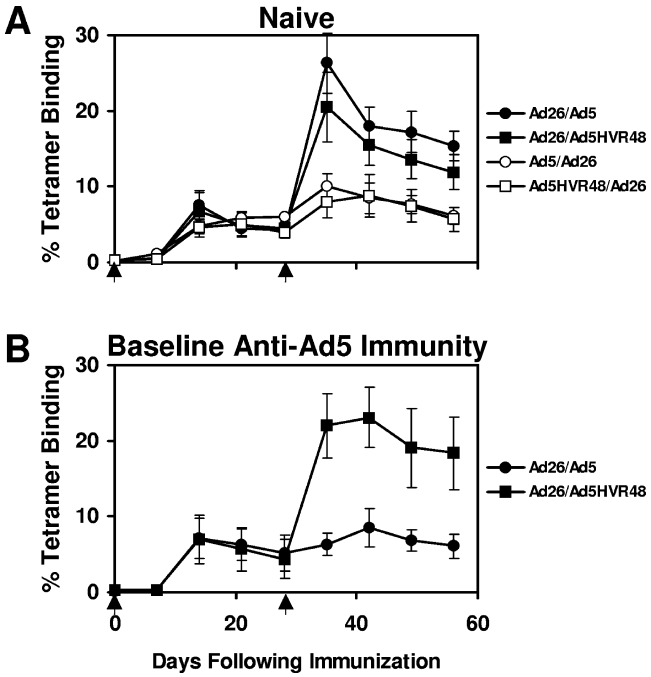


FIG. 2. Order of vector administration in heterologous rAd prime-boost regimens in mice. Naïve mice (A) or mice preimmunized with two injections of 10^{10} vp rAd5-Empty with Ad5 neutralizing antibody titers of 8,192 to 16,384 (B) ($n = 4$/group) were primed on day 0 and were boosted on day 28 with 10^9 vp rAd26-Gag, rAd5-Gag, or rAd5HVR48-Gag as shown. Arrows indicate immunizations. Gag-specific cellular immune responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization. Mean responses with standard errors are shown.

munity by the priming immunization that suppresses the chimeric rAd5HVR48 vector (22). These data are consistent with our previous observations that dominant vector-specific neutralizing antibodies are directed primarily against the hexon HVRs (22, 28). As expected, the responses primed by rAd48-Gag were not boosted effectively by a second injection of rAd48-Gag. These data demonstrate that optimal heterologous rAd prime-boost regimens require two vectors that are serologically distinct (11, 31) and that lack hexon HVR cross-reactivity.

Order of vector administration in heterologous rAd prime-boost regimens in mice. We next assessed the importance of the order of vector administration in heterologous rAd prime-boost regimens in mice. C57BL/6 mice ($n = 4$/group) were primed on day 0 and were boosted on day 28 with rAd26-Gag, rAd5-Gag, or rAd5HVR48-Gag as shown in Fig. 2A. The $CD8^+$ T-lymphocyte responses primed by rAd26-Gag were boosted efficiently and comparably by rAd5-Gag and rAd5HVR48-Gag. Interestingly, the responses primed by rAd5-Gag or rAd5HVR48-Gag were boosted less effectively by rAd26-Gag. These data suggest that the order of administering rAd vectors may be important in determining the overall potency of heterologous rAd prime-boost regimens. We similarly observed that rAd35/rAd5 regimens were more potent than rAd5/rAd35 regimens in mice (data not shown), suggesting the generalizability of these findings.

We also evaluated the impact of anti-Ad5 immunity on the optimal rAd26/rAd5 and rAd26/rAd5HVR48 regimens in this model. In mice with preexisting anti-Ad5 immunity, the rAd5-Gag vector was not effective as a boosting vector, and the rAd26/rAd5HVR48 regimen proved optimal in this setting (Fig. 2B). Functional IFN-γ ELISPOT assays revealed similar results (data not shown). These data confirm our prior findings that rAd5HVR48-Gag effectively circumvented anti-Ad5 immunity (22) and suggest that optimal heterologous rAd prime-boost regimens should involve two vectors that are serologically distinct and that evade preexisting antivector immunity.

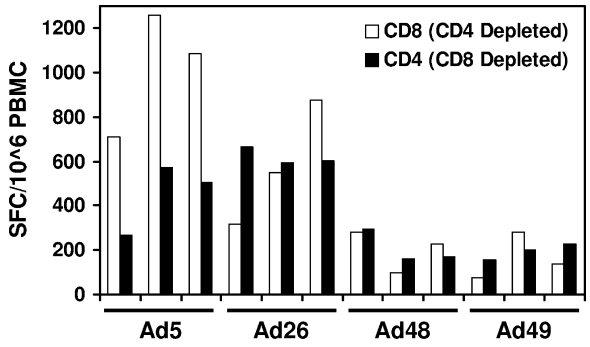


FIG. 3. Immunogenicity of rAd vectors in rhesus monkeys. Adult rhesus monkeys ($n = 3$/group) were immunized with a single injection of 10^{11} vp rAd5-Gag, rAd26-Gag, rAd48-Gag, or rAd49-Gag. CD4- and CD8-depleted IFN-γ ELISPOT assays with pooled Gag peptides were performed at week 4 following immunization. Responses of individual animals are shown.

Immunogenicity of rAd vectors in rhesus monkeys. The importance of the order of vector administration in heterologous rAd prime-boost regimens in mice suggested that there could be important qualitative differences in the phenotypes of cellular immune responses elicited by various serotype rAd vectors. We explored this possibility in rhesus monkeys by assessing the magnitude and phenotype of $CD8^+$ and $CD4^+$ T-lymphocyte responses elicited by various novel rAd vectors. We have recently reported unfractionated IFN-γ ELISPOT responses in rhesus monkeys ($n = 3$/group) following immunization with 10^{11} vp rAd5-Gag, rAd26-Gag, rAd48-Gag, or rAd49-Gag (1). Here we expand these studies by evaluating fractionated $CD8^+$ and $CD4^+$ T-lymphocyte responses in these animals at week 4 following primary immunization. As shown in Fig. 3, CD4-depleted and CD8-depleted IFN-γ ELISPOT assays demonstrated that rAd5-Gag elicited $CD8^+$ T-lymphocyte responses of higher magnitude than rAd26-Gag but that both vectors elicited $CD4^+$ T-lymphocyte responses of comparable magnitude. Thus, rAd5-Gag elicited skewed $CD8^+ > CD4^+$ T-lymphocyte responses, whereas rAd26-Gag elicited more balanced $CD8^+$ and $CD4^+$ T-lymphocyte responses. The rAd48-Gag and rAd49-Gag vectors also induced balanced $CD8^+$ and $CD4^+$ T-lymphocyte responses but at lower magnitude.

Phenotypes of T-lymphocyte responses elicited by rAd vectors in rhesus monkeys. We next utilized multiparameter flow cytometry to evaluate the phenotypes of T-lymphocyte responses elicited by these novel rAd vectors. We selected rAd26 and rAd48 for detailed study, since rAd26 proved the most immunogenic and rAd48 had the lowest seroprevalence of the rare serotype rAd vectors in our previous study (1). We per-

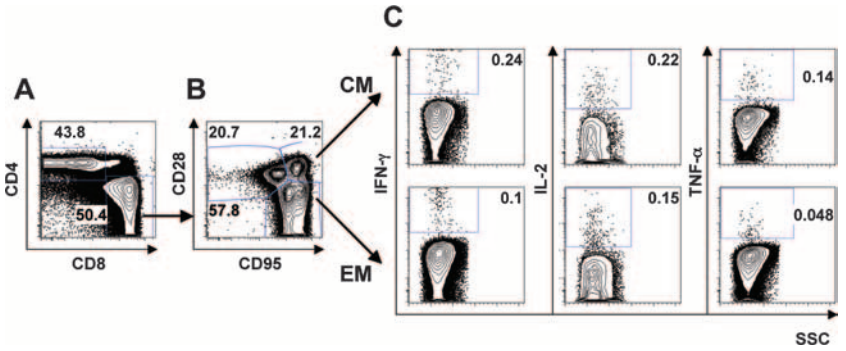


FIG. 4. Gating strategy for multiparameter ICS assays. Eight-color ICS assays were performed using CD3-Alexa700, CD4-AmCyan, CD8-APC-Cy7, CD28-PerCP-Cy5.5, CD95-PE, IFN-γ–PE–Cy7, TNF-α–FITC, and IL-2–APC MAbs. Gated $CD3^+$ lymphocytes were assessed for $CD4^+$ and $CD8^+$ expression (A) and CD28 and CD95 expression (B) to determine CM and EM subpopulations and for IFN-γ, IL-2, and TNF-α expression (C) to determine functional cytokine profiles following stimulation with pooled SIV Gag peptides. The results for a representative monkey vaccinated with rAd26-Gag as described for Fig. 3 are shown. Medium control background levels were typically <0.02% of gated $CD8^+$ or $CD4^+$ T lymphocytes. Numbers indicate percentages of gated cells. SSC, side scatter.

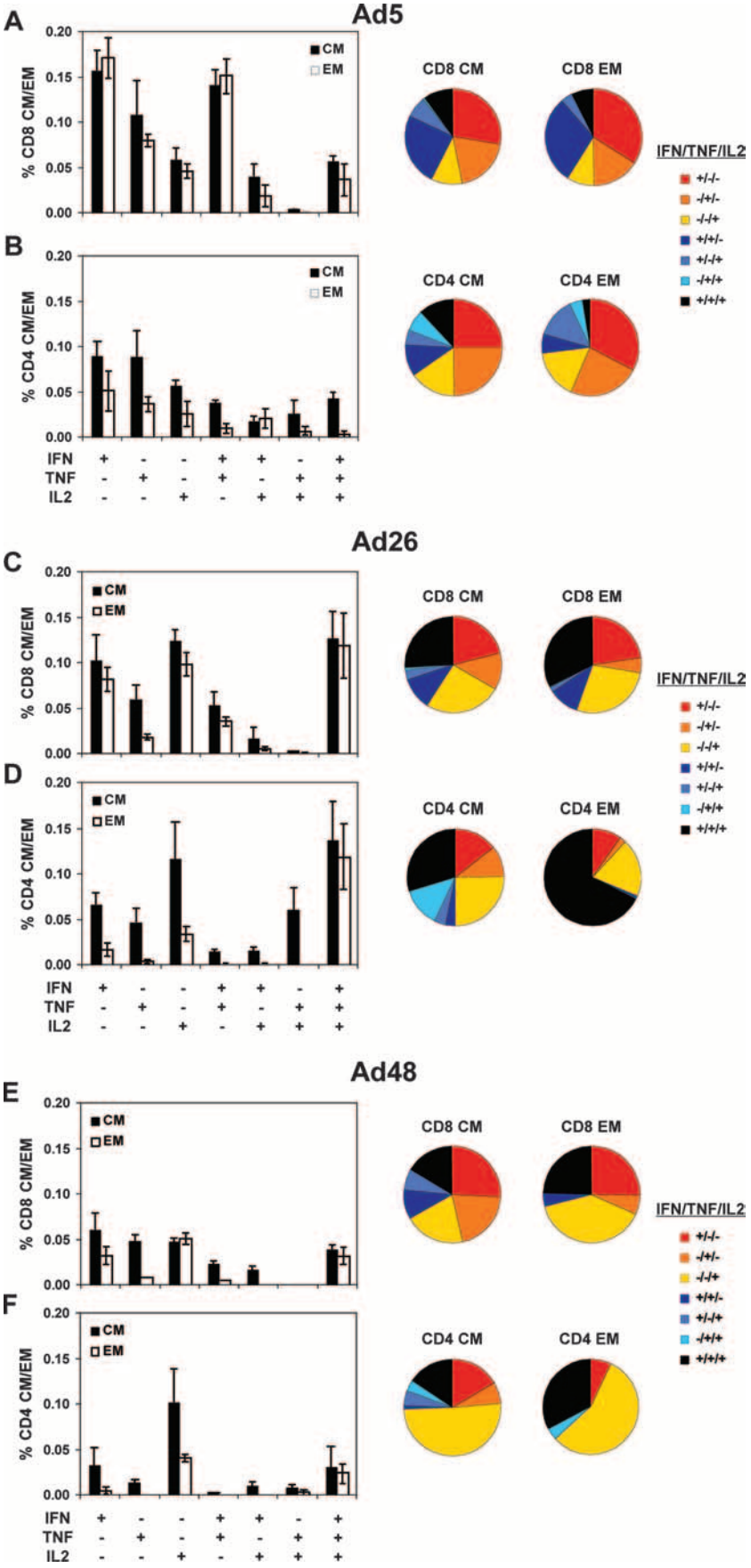
formed eight-color ICS assays utilizing the following MAbs: CD3-Alexa700, CD4-AmCyan, CD8-APC-Cy7, CD28-PerCP-Cy5.5, CD95-PE, IFN-γ–PE–Cy7, TNF-α–FITC, and IL-2–APC. Figure 4 depicts the gating strategy. CM and EM T lymphocytes were defined, respectively, as $CD28^+$ $CD95^+$ and $CD28^-$ $CD95^+$ T lymphocytes (17, 19).

As shown in Fig. 5A and B, rAd5-Gag elicited primarily IFN-γ-positive (IFN-γ^+), TNF-α^+, and IFN-γ^+/TNF-α^+ $CD8^+$ T-lymphocyte responses but only low levels of IL-2^+ and polyfunctional IFN-γ^+/TNF-α^+/IL-2^+ cells. In contrast, as depicted in Fig. 5C and D, rAd26-Gag elicited lower levels of IFN-γ^+, TNF-α^+, and IFN-γ^+/TNF-α^+ $CD8^+$ T-lymphocyte responses but substantially higher levels of IL-2^+ and polyfunctional IFN-γ^+/TNF-α^+/IL-2^+ cells. Means of 27% of $CD8^+$ CM T lymphocytes and 33% of $CD8^+$ EM T lymphocytes elicited by rAd26-Gag secreted all three cytokines, whereas only 10% of $CD8^+$ CM T lymphocytes and 7% of $CD8^+$ EM T lymphocytes elicited by rAd5-Gag were polyfunctional. Similarly, the proportion of $CD4^+$ CM and $CD4^+$ EM T lymphocytes induced by rAd26-Gag that secreted all three cytokines was substantially greater than that induced by rAd5-Gag. As shown in Fig. 5E and F, rAd48-Gag induced higher proportions of IL-2^+ and polyfunctional IFN-γ^+/TNF-α^+/IL-2^+ cells than rAd5-Gag, although the magnitude of the responses elicited by rAd48-Gag was severalfold lower than those induced by rAd5-Gag. These data demonstrate that various serotype rAd vectors elicit different phenotypes of cellular immune responses in rhesus monkeys. Specifically, rAd26-Gag induced larger proportions, as well as greater absolute numbers, of IL-2^+ and polyfunctional $CD8^+$ and $CD4^+$ T lymphocytes, whereas rAd5-Gag induced greater numbers of IFN-γ^+ and TNF-α^+ $CD8^+$ T lymphocytes. Similar phenotypes of Gag-specific T-lymphocyte responses were observed at week 16 compared to those at week 4 (data not shown).

To assess the reproducibility of these findings, we evaluated the magnitude and phenotype of $CD8^+$ and $CD4^+$ T-lymphocyte responses in 10 additional rhesus monkeys that were similarly immunized with rAd5-Gag ($n = 4$) or rAd26-Gag ($n = 6$). These data proved comparable with those shown in Fig. 5A to D (data not shown). A combined analysis of the results of both experiments demonstrated that rAd5-Gag elicited significantly higher IFN-γ^+ ($P = 0.023$) and IFN-γ^+/TNF-α^+ ($P = 0.0019$) $CD8^+$ CM T-lymphocyte responses as well as greater IFN-γ^+ ($P = 0.0053$) and IFN-γ^+/TNF-α^+ ($P < 0.0001$) $CD8^+$ EM T-lymphocyte responses than rAd26-Gag. In contrast, rAd26-Gag induced significantly higher IL-2^+ ($P = 0.0014$) and polyfunctional ($P = 0.012$) $CD8^+$ CM T-lymphocyte responses as well as greater IL-2^+ ($P = 0.023$) and polyfunctional ($P = 0.0014$) $CD8^+$ EM T-lymphocyte responses than rAd5-Gag. Similarly, rAd26-Gag induced significantly higher polyfunctional $CD4^+$ CM T-lymphocyte responses ($P = 0.012$) and $CD4^+$ EM T-lymphocyte responses ($P < 0.0001$) than rAd5-Gag.

Immunogenicity of heterologous rAd prime-boost regimens in rhesus monkeys. Given the results of our preliminary studies in mice and the polyfunctionality of T-lymphocyte responses elicited by the rare serotype rAd vectors in rhesus monkeys, we hypothesized that priming with rare serotype rAd-Gag vectors may prove highly effective for subsequent boosting with rAd5-Gag in rhesus monkeys. To explore this possibility, groups of rhesus monkeys ($n = 3$/group) were immunized with various candidate vaccine regimens as follows: rAd5/rAd5, rAd26/rAd5, rAd48/rAd5, rAd49/rAd5, rAd5/rAd26, rAd35/rAd5, and rAd5/rAd35. All vectors expressed the same SIV Gag insert, and the boost immunization was performed 24 weeks after the priming immunization. The mean IFN-γ ELISPOT responses following the boost immunization are shown in Fig. 6A and B.

As depicted in Fig. 6A, monkeys primed with rAd5-Gag were not efficiently boosted by a second injection of rAd5-Gag, presumably due to anti-Ad5 immunity generated by the priming immunization (16, 23, 26). In contrast, monkeys primed with rAd26-Gag were boosted remarkably well by rAd5-Gag to mean ELISPOT responses of 2,553 SFC/10^6 PBMC against the single SIV Gag antigen at week 2 following the boost immunization. These responses were >eightfold higher than those generated by the homologous rAd5/rAd5 regimen, and the differences between groups of monkeys persisted for at least 16 weeks. Monkeys primed with rAd48-Gag or rAd49-Gag were also boosted by rAd5-Gag, although to a lesser extent. Although the limited numbers of animals in this study precluded a formal statistical analysis, it is clear that the heterologous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rAd prime-boost regimens were substantially more immunogenic than the homologous rAd5 regimen in rhesus monkeys. Moreover, as shown in Fig. 6B, we also observed that the order of vector administration impacted the overall potency of heterologous rAd prime-boost regimens in rhesus monkeys. Consistent with the results of our mouse studies, the rAd26/rAd5 regimen proved more potent than the rAd5/rAd26 regimen, and the rAd35/rAd5 regimen was more potent than the rAd5/rAd35 regimen. The ability of rAd26-Gag to prime responses for a subsequent efficient boost by rAd5-Gag suggests the functional relevance of the polyfunctional responses elicited by rAd26 vectors.

We next characterized the phenotype and breadth of cellular immune responses in the rhesus monkeys that received the optimal rAd26/rAd5 boost regimen. As shown in Fig. 6C and D, the phenotype of the $CD8^+$ and $CD4^+$ T-lymphocyte responses following the boost immunization appeared to be intermediate between those for the rAd5 (Fig. 5A and B) and rAd26 (Fig. 5C and D) vectors. We evaluated breadth by assessing IFN-γ ELISPOT responses against all 125 individual SIV Gag peptides. As shown in Fig. 7A, at least two epitope-specific responses were observed in each animal at week 4 following the rAd26-Gag priming immunization. As depicted in Fig. 7B, we observed a marked, 10-fold increase in the magnitude of these epitope-specific responses at week 4 following the rAd5-Gag boost immunization. We also observed the emergence of numerous additional epitope-specific responses following the boost immunization. These data indicate that heterologous rAd boosting augmented not only the magnitude but also the breadth of Gag-specific cellular immune responses in rhesus monkeys.

DISCUSSION

Novel serotype rAd vectors have recently been developed to overcome the problem of preexisting anti-Ad5 immunity and to facilitate the development of potent heterologous rAd prime-boost regimens. Previous studies have described the magnitude of cellular immune responses elicited by rare serotype rAd vectors (1, 3, 8, 16, 22), but the qualitative phenotypes of these responses have not been previously reported. In this study, we show that rAd5 vectors and rare serotype rAd vectors elicit qualitatively distinct profiles of T-lymphocyte responses in rhesus monkeys. In particular, rAd5 vectors elicited skewed, $CD8^+ > CD4^+$ T-lymphocyte responses that were characterized primarily by IFN-γ^+, TNF-α^+, and IFN-γ^+/TNF-α^+ cells. In contrast, rAd26 vectors elicited lower $CD8^+$ T-lymphocyte responses but more balanced $CD8^+$ and $CD4^+$ responses that included a higher proportion of IL-2^+ and polyfunctional IFN-γ^+/TNF-α^+/IL-2^+ cells.

It is not surprising that rAd5 and rAd26 vectors elicited qualitatively distinct cellular immune responses, since these viruses utilize different cellular receptors and thus could have substantially different biologic properties (1). Whereas rAd5 and rAd5HVR48 utilize the CAR receptor, rAd26 and rAd48 utilize CD46 for cell entry (1). Our results suggest that not only the magnitude but also the phenotype of cellular immune responses generated by vaccine vectors may be important in determining their practical utility. In particular, qualitative differences among various rAd serotypes may underlie the overall potency of heterologous rAd prime-boost regimens. We speculate that the efficiency of rAd26-Gag priming reflects the ability of this vector to elicit polyfunctional $CD8^+$ and $CD4^+$ T-lymphocyte responses (Fig. 5C and D). Similarly, the potency of rAd5-Gag boosting likely reflects the capacity of this vector to drive potent IFN-γ^+ $CD8^+$ T-lymphocyte responses (Fig. 5A and B). Consistent with this model, the cellular immune responses elicited by the optimal heterologous rAd26/rAd5 prime-boost regimen were remarkably high in magnitude (Fig. 6A and B), with an intermediate phenotype (Fig. 6C and D) and substantial breadth (Fig. 7). It remains to be determined, however, whether the higher magnitude and improved phenotype of the cellular immune responses elicited by rAd26-based vaccine regimens will afford greater protective efficacy against SIV challenges in rhesus monkeys than the homologous rAd5 regimen. In addition to these qualitative benefits, rare serotype rAd vectors may also prove particularly important given the apparent increased risk of acquisition of HIV-1 infection following rAd5-Gag/Pol/Nef vaccination in individuals with preexisting anti-Ad5 immunity in a recent phase 2b efficacy study (7).

The results of prior studies from our laboratory and others have shown that heterologous rAd prime-boost regimens elicited potent cellular immune responses in mice (1, 11, 18, 31). The combination of a chimpanzee rAd vector with rAd5 has also been reported to induce potent immune responses in rhesus monkeys (15, 21). Our studies confirm and extend these prior observations by investigating for the first time the detailed phenotypes of $CD8^+$ and $CD4^+$ T-lymphocyte responses elicited by various rAd vectors and by performing a comprehensive comparison of the immunogenicity of various heterologous rAd prime-boost regimens in rhesus monkeys. The rAd26 prime, rAd5 boost regimen proved optimal in the present study, suggesting that this regimen should be explored further as a potential vaccine regimen. In populations with preexisting anti-Ad5 immunity, the rAd26 prime, rAd5HVR48 boost regimen may prove optimal (Fig. 2B).

Our data are consistent with the results of prior studies that have suggested the potential importance of polyfunctional T-lymphocyte responses. Polyfunctional HIV-1-specific $CD8^+$ T-lymphocyte responses were shown to be correlated with control of viral replication in HIV-1-infected patients and with clinical nonprogression (4). Moreover, highly effective vaccines, such as vaccinia virus, have been demonstrated to induce

FIG. 5. Phenotype of rAd5, rAd26, and rAd48 vectors in rhesus monkeys. Cytokine secretion phenotypes of $CD8^+$ (A, C, E) and $CD4^+$ (B, D, F) T lymphocytes elicited by rAd5-Gag (A, B), rAd26-Gag (C, D), and rAd48-Gag (E, F) vectors in the study described for Fig. 3 were determined by multiparameter flow cytometry at week 4 following immunization. Gag-specific absolute (bar graphs) and proportional (pie charts) IFN-γ, TNF-α, and IL-2 responses in all combinations are depicted in $CD8^+$ and $CD4^+$ CM and EM T-lymphocyte subpopulations. Mean responses with standard errors are shown. +, present; −, absent.

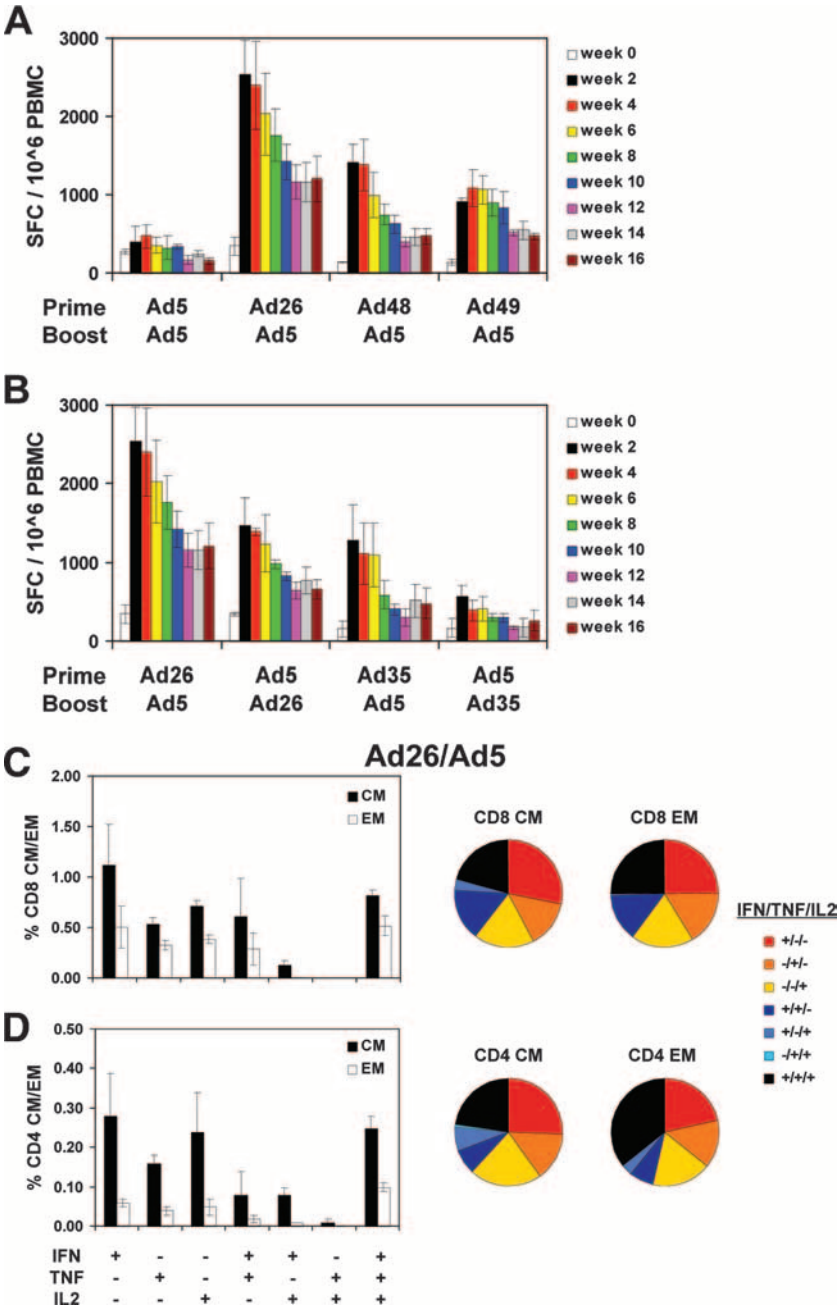

FIG. 6. Immunogenicity of heterologous rAd prime-boost regimens in rhesus monkeys. Groups of rhesus monkeys (n = 3/group) were primed at week 0 and were boosted at week 24 with the following regimens expressing SIV Gag: rAd5/rAd5, rAd26/rAd5, rAd48/rAd5, rAd49/rAd5, rAd5/rAd26, rAd35/rAd5, and rAd5/rAd35. (A, B) Gag-specific IFN-γ ELISPOT responses are shown at multiple time points following the boost immunization. Results for the same rAd26/rAd5 group are presented in both panels for clarity of comparison. (C, D) Gag-specific absolute (bar graphs) and proportional (pie charts) IFN-γ, TNF-α, and IL-2 $CD8^+$ and $CD4^+$ T-lymphocyte responses as described for Fig. 5 are shown for the monkeys that received the rAd26-Gag prime, rAd5-Gag boost vaccine regimen at week 4 following the boost immunization. Mean responses with standard errors are shown. +, present; −, absent.

polyfunctional $CD8^+$ T-lymphocyte responses in humans (20). In addition, polyfunctional $CD4^+$ T-lymphocyte responses have been shown to be required for optimal protection against *Leishmania major* challenges in mice, and different doses of rAd5 vectors have also been shown to influence the quality of T-lymphocyte responses (6). Taken together, these studies suggest that the generation of polyfunctional $CD8^+$ and $CD4^+$ T-lymphocyte responses may be highly desirable for vaccine strategies. Our findings are also consistent with those of prior reports showing that priming with other vaccine modalities, such as DNA vaccines or proteins with Toll-like receptor agonists, can influence the magnitude and quality of T-lymphocyte responses following rAd boost immunizations (12, 14, 33, 34).

Several groups have also reported the critical importance of

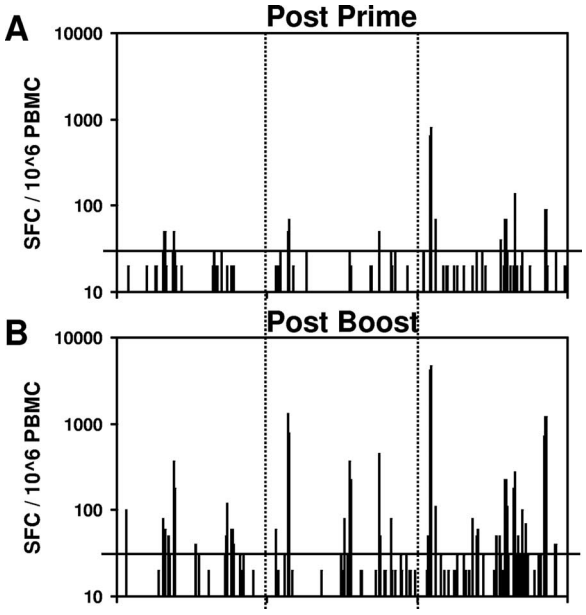

FIG. 7. Breadth of responses elicited by the heterologous rAd26/rAd5 regimen in rhesus monkeys. Breadth of Gag-specific cellular immune responses is shown for the rhesus monkeys ($n = 3$) that received the rAd26-Gag prime, rAd5-Gag boost regimen described for Fig. 6. IFN-γ ELISPOT responses to each of the 125 individual SIV Gag peptides were assessed 4 weeks following the rAd26-Gag priming immunization (A) and 4 weeks following the rAd5-Gag boost immunization (B). Data presentation uses a logarithmic *y* axis to emphasize the breadth of epitope-specific cellular immune responses. The horizontal bars indicate an arbitrary definition of 30 SFC/10^6 PBMC positive responses.

$CD4^+$ T-cell help in determining the overall functionality of $CD8^+$ T-lymphocyte responses. In particular, "helped" $CD8^+$ T lymphocytes primed in the presence of adequate $CD4^+$ T-cell help were able to expand much more efficiently following subsequent boost immunizations than "helpless" $CD8^+$ T lymphocytes primed without $CD4^+$ T-cell help (9, 24, 29). Consistent with this model, we speculate that the efficient priming by rAd26 vectors may be related to the balanced $CD8^+$ and $CD4^+$ T-lymphocyte responses elicited by this vector, although the precise degree of T-cell help required for optimal $CD8^+$ T-lymphocyte function has not yet been determined.

The present studies demonstrate that priming with one rAd vector and boosting with a serologically distinct rAd vector can elicit remarkably potent and broad cellular immune responses in nonhuman primates. Our data further suggest that not only the magnitude but also the phenotype of cellular immune responses may be critical in determining the overall potency of heterologous rAd prime-boost regimens. Heterologous rAd prime-boost regimens that exhibit improved magnitude, breadth, and phenotype of cellular immune responses compared with those of homologous rAd5 regimens thus warrant further investigation as candidate vaccines for HIV-1 and other pathogens.

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