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Comparative Analysis of Simian Immunodeficiency Virus Gag-Specific Effector and Memory CD8\(^+\) T Cells Induced by Different Adenovirus Vectors

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Adenovirus (Ad) vectors are widely used as experimental vaccines against several infectious diseases, but the magnitude, phenotype, and functionality of CD8\(^+\) T cell responses induced by different adenovirus serotypes have not been compared. To address this question, we have analyzed simian immunodeficiency virus Gag-specific CD8\(^+\) T cell responses in mice following vaccination with Ad5, Ad26, and Ad35. Our results show that although Ad5 is more immunogenic than Ad26 and Ad35, the phenotype, function, and recall potential of memory CD8\(^+\) T cells elicited by these vectors are substantially different. Ad26 and Ad35 vectors generated CD8\(^+\) T cells that display the phenotype and function of long-lived memory T cells, whereas Ad5 vector-elicited CD8\(^+\) T cells are of a more terminally differentiated phenotype. In addition, hepatic memory CD8\(^+\) T cells elicited by Ad26 and Ad35 mounted more robust recall proliferation following secondary challenge than those induced by Ad5. Furthermore, the boosting potential was higher following priming with alternative-serotype Ad vectors than with Ad5 vectors in heterologous prime-boost regimens. Anamnestic CD8\(^+\) T cell responses were further enhanced when the duration between priming and boosting was extended from 30 to 60 days. Our results demonstrate that heterologous prime-boost vaccine regimens with alternative-serotype Ad vectors elicited more functional memory CD8\(^+\) T cells than any of the regimens containing Ad5. In summary, these results suggest that alternative-serotype Ad vectors will prove useful as candidates for vaccine development against human immunodeficiency virus type 1 and other pathogens and also emphasize the importance of a longer rest period between prime and boost for generating optimal CD8\(^+\) T cell immunity.
gen challenge into effectors that are able to produce copious amounts of gamma interferon (IFN-γ) (14–16). On the other hand, TEM cells are present in nonlymphoid tissues and the periphery and can provide immediate effector responses at mucosal sites (15, 17). Here, we compare the phenotypic and functional properties of CD8+ T cell responses generated by Ad5, Ad26, and Ad35 vectors expressing simian immunodeficiency virus (SIV) Gag. Our data show that immunization with an Ad5 vector preferentially elicits more effector memory T cell-like responses characterized by low CD127, CD62L, and Bcl-2 expression and lower proliferative potential. On the other hand, immunization with alternative-serotype Ad vectors generates CD8+ T cells that are biased toward long-lived central memory T cell responses with higher frequencies of functional memory CD8+ T cells resulting in enhanced CD127, Bcl-2, and CD62L expression. Importantly, heterologous prime-boost vaccine regimens with alternative-serotype Ad vectors may be attractive vaccine vectors.

MATERIALS AND METHODS

Vector construction and production. E1/E3-deleted replication-competent recombinant Ad5, Ad26, and Ad35 vectors containing the full-length SIV gag gene insert were constructed as previously described (1). Vectors were produced by homologous recombination, propagated in adherent PER.C6 cells, and purified by double cesium chloride gradient ultracentrifugation as previously described (1). All viral vectors have comparable viral particle-to-PUF (vp/PUF) ratios.

Transgene expression and quantification. Transgene expression was evaluated as described previously (18). Various dosages of Ad-SIV-gag vectors were incubated with one million 293 cells in 24-well plates. Following 24 h of incubation at 37°C, SIV-gag in cell lysates was quantitated by enzyme-linked immunosorbent assay (ELISA).

Mice and immunization: primary response. Eight- to 12-week-old C57BL/6 mice were purchased from Jackson Laboratory. Mice were immunized with either 1 × 10^7, 1 × 10^8, or 1 × 10^9 VP of Ad5-, Ad26-, or Ad35-SIV-gag by intramuscular (i.m.) injections in both hind leg muscles. The vaccine vector was administered in 100 µl phosphate-buffered saline (PBS) injections (50 µl per quadriceps). Mice were anesthetized with 2.5% working stock of avertin (100% stock containing 10 g tribromoethanol [T4, 840-2; Aldrich] and 10 ml tertiary amyl alcohol [24, 048-6; Aldrich]) at 25 µl/g of body weight i.p. prior to vaccine immunization. Animals were housed at the Emory University School of Medicine animal facility, and experiments were performed according to approved IACUC protocol.

Heterologous prime-boost regimen. Eight- to 12-week-old C57BL/6 mice were purchased from Jackson Laboratory. Mice were primed with 1 × 10^7 VP of Ad5-, Ad26-, or Ad35-SIV-gag i.m. and rested for 30 or 60 days before heterologous boost with 1 × 10^6 VP of Ad26-, Ad35-, or Ad5-SIV-gag vectors. Immune responses were then tracked longitudinally in the blood and tissues at various time points.

Cell isolation, adoptive transfer, and virus challenge. Lymphocytes were isolated from the spleen, liver, lungs, lymph nodes, intestines, bone marrow, and blood as previously described (19). Total memory lymphocytes for adoptive transfers were isolated from the spleen and liver of memory B6 CD45.2 or CD45.1 mice by homogenizing the tissues and centrifugation through a Percoll gradient. The total numbers of CD8+ T cells in the spleen and liver were enumerated by using the frequency of major histocompatibility complex class I (MHC-I) DP^+AL11 tetramer staining with anti-CD8α antibody. Equal numbers of total antigen-specific CD8+ T cells were adoptively transferred from the spleen (20,000 cells) or liver (7,000 cells) to naive B6 mice bearing the allelic marker CD45.1 or CD45.2 to differentiate donor versus host CD8+ T cells. Memory CD8+ T cells were transferred by intravenous (i.v.) injections through the tail vein. Recipient mice were challenged with 2 × 10^6 PFU of MVA-SIV-gag (gift from Bernard Moss, NIAID, National Institutes of Health) delivered intramuscularly. Expansion of donor cells was then evaluated on day 14 postchallenge.

Antibody staining and flow cytometry. MHC-I (H-2D^β) tetramers were produced as previously described (15, 20, 21). The antibodies used were CD8α (clone 53-6.7), CD44 (clone IM7), CD62L (clone MEL-14), and CD127, Bcl-2, KLRGB-1, IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), CD45.1, CD45.2, CXCR3, Ly6C, CD122, T-bet, Eomesodermin, 2B4 (CD244), Lag3, Tim3, granzyme B, Bcl-2, and Live/Dead Near-IR antibody. All antibodies were purchased from BD except for CD127, eomesodermin (eBioScience), T-bet (Biolegend), KLRGB-1 (Southern Biotech), granzyme B (Caltech), and Live/Dead Fixable Near-IR antibody (Invitrogen). Optimal antibody dilutions and staining conditions were used according to titration or the manufacturer's recommendations. After staining, cells were fixed in 2% (w/v) paraformaldehyde. Events were acquired using a Canto II multiparameter flow cytometer (BD). Dead cells were excluded on the basis of forward and side light scatter and staining with Live/Dead Near-IR antibody. Data were analyzed by using Flowjo (Treestar) and GraphPad Prism.

Statistical analysis. Flow cytometry data were statistically analyzed by using a two-tailed unpaired Student's t test. Data are presented as standard errors of the means (SEM). P < 0.05 is considered significant.

RESULTS

Magnitude and kinetics of CD8+ T cell responses induced by recombinant adenovirus serotypes 5, 26, and 35. Studies were initiated to determine the relative transgene expression from Ad5, Ad26, and Ad35 vectors expressing SIV Gag in 293 cells. We infected 293 cells with escalating doses of the recombinant adenoviruses and observed comparable Gag expression from all Ad serotypes by ELISA (Fig. 1A). In order to determine the optimal dose of these recombinant Ad vectors to induce a uniform magnitude of immune responses, we immunized B6 mice with Ad5, Ad26, and Ad35 vectors expressing SIV gag utilizing 10^7, 10^8, and 10^9 viral particles (vp) of each vector, and we assessed longitudinal CD8+ T cell kinetics in the blood. Gag-specific CD8+ T cells were detected as early as day 8 postimmunization for all doses, and Ad5 generated between a 4- and 20-fold higher magnitude of Gag-specific CD8+ T cell responses compared to those of Ad26 and Ad35 (Fig. 1B). The peak of the Gag-specific CD8+ T cell response following Ad5 immunization was around day 15 with all three vaccine doses tested. However, the peak of the Gag-specific CD8+ T cell response after vaccination with the alternative-serotype Ad vectors was influenced by the dose (around day 37 with the low 10^7 dose and day 15 with the high 10^9 dose). At a 10^9-vp dose, we observed equivalent magnitude and uniform kinetics of peak Gag-specific CD8+ T cell responses among all serotypes (Fig. 1B). Therefore, in our subsequent experiments, we used the 10^9-vp dose for all vectors. We studied Gag-specific CD8+ T cell responses longitudinally by MHC-I H-2D^β AL11 tetramer binding assays in the blood and tissues as previously described (20–22). Consistent with our earlier observations, levels of Gag-specific CD8+ T cells elicited by Ad5 immunization were higher in magnitude than those of Ad26 and Ad35 at day 8 p.i. in the blood (P < 0.0001) (Fig. 1B and C). However, by day 15 p.i., Ad26 and Ad35 elicited greater Gag-specific CD8+ T cell responses than Ad5 (P = 0.009 for Ad5 versus Ad26 and P = 0.07 for Ad5 versus Ad35) (Fig. 1D). Subsequently, antigen-specific CD8+ T cells declined to a level of 8,000 to 13,000 cells per 10^6 peripheral blood mononuclear cells (PBMCs) at the memory phase by day 45 p.i. for all serotypes (Fig. 1D). We found that all serotypes exhibited similarly high numbers of Gag-specific CD8+ T cells in the
Recombinant adenovirus serotypes 5, 26, and 35 elicit high magnitudes of CD8\(^+\) T cell responses. (A) Equivalent expression of transgene product by recombinant adenoviruses encoding the full-length SIV gag gene inoculated with 10\(^7\) VP, 10\(^8\) VP and 10\(^9\) VP of Ad5, Ad26, and Ad35-SIV Gag in 10\(^6\) 293 cells. After 24 h of incubation at 37°C, SIV Gag production was assayed in cell lysates by ELISA. Groups of B6 mice were immunized with various doses of Ad5, Ad26, and Ad35 SIV Gag vaccine via intramuscular injections. (B) Kinetics of H\(^2\)Db-AL11 tetramer-positive CD8\(^+\) T cells in the blood of B6 mice immunized with escalating doses of 1 \times 10\(^7\), 1 \times 10\(^8\), and 1 \times 10\(^9\) vp of Ad5, Ad26, or Ad35-SIV Gag. The dotted line indicates the limit of detection. (C) Representative flow plots of AL11 tetramer-positive CD8\(^+\) T cell responses in the blood of mice immunized with 10\(^9\) vp of Ad5, Ad26, or Ad35 SIV Gag (n = 6 to 12 per group). Numbers on plots are percentages and are gated on live CD8\(^+\) T cells. (D) Summary of the numbers of AL11 tetramer-positive CD8\(^+\) T cells in the blood of immunized mice (n = 6 to 12). For days 8 and 15, \(P = 0.003\) and 0.009 (**), and \(P < 0.0001\) (***) for day 45, \(P = 0.0004\) (**) for Ad5 versus Ad26 and \(P = 0.0015\) (**) for Ad5 versus Ad35. (E) Numbers of AL11 tetramer-positive CD8\(^+\) T cells accumulated in both the lymphoid and nonlymphoid tissues at days 15 and 45 p.i. PBMCs (indicated after the dotted line) were enumerated based on per million cells (n = 3 per time point). *, \(P < 0.04\); **, \(P = 0.0052\). Graphs are presented in log scale to better represent the broad range of data. Data are representative of 4 independent experiments.
spleen, liver, and lung at day 15 p.i. (Fig. 1E). However, at day 45 p.i., Ad5 maintained 2- to 5-fold greater numbers of Gag-specific CD8+ T cells than Ad26 and Ad35 in the liver and the lymph nodes, but the numbers of the Gag-specific CD8+ T cells were not significantly different in the spleen, lung, and blood of all vaccinated groups (Fig. 1E). Thus, our data demonstrate that at the 10^9-vp dose, alternative Ad serotypes are immunogenic and elicit high magnitudes of Gag-specific CD8+ T cell responses that are essentially comparable to those of Ad5.

**Phenotype and functional characteristics of CD8+ T cells following immunization with Ad5, Ad26, and Ad35.** We sought to compare several phenotypic markers that are commonly used to differentiate memory CD8+ T cells, and we assessed their functional abilities to produce IFN-γ, TNF-α, and IL-2 following Ad5, Ad26, and Ad35 immunizations. In order to examine the quality of the memory CD8+ T cell response that was generated after vaccination with each Ad vector, we analyzed the expression of CD127 (homeostatic survival marker), CD62L (lymphatic trafficking receptor), Bcl-2 (antiapoptotic molecule), and KLRG-1 (marker for effector and terminally differentiated cells). At day 8 following immunization, all Gag-specific CD8+ T cells downregulated CD127, CD62L, and Bcl-2 and upregulated KLRG-1 and granzyme B in the blood (Fig. 2A to D). Similar expression profiles of these markers were also observed in the spleen, lung, and liver at day 15 p.i. (Fig. 2B and data not shown). However, by day 45 p.i., Gag-specific CD8+ T cells generated by Ad26 and Ad35 vectors expressed significantly higher levels of CD127 (P < 0.0001) and Bcl-2 (P < 0.004) in the blood and tissues than did Gag-specific CD8+ T cells generated by Ad5 vectors (Fig. 2A and B). Similar expression kinetics of CD127 were also observed with lower doses of Ad5, Ad26, and Ad35 (Fig. 2C), although the differences were less striking. Moreover, Gag-specific CD8+ T cells following Ad5 immunization appeared to be more effector-like than Gag-specific CD8+ T cells following alternative-serotype Ad immunization, as evidenced by increased expression of KLRG-1 in the blood lymphocytes (P = 0.0221) (Fig. 2A). No significant differences were observed among Ad serotypes in the expression kinetics of T-bet, Eomes, CD122, CD69, and CXCR3 (data not shown). At day 8 p.i., we observed higher expression of the inhibitory receptor PD-1 on Gag-specific CD8+ T cells of Ad5-immunized mice than of Ad26- and Ad35-immunized mice (the mean fluorescent intensity [MFI] of PD-1 for Ad5 was 872, for Ad26 was 605, and for Ad35 was 655) (data not shown). In addition, Tim3 expression was also elevated in Ad5 Gag-specific CD8+ T cells (Ad5 MFI, 993; Ad26 MFI, 450; and Ad35 MFI, 839; P < 0.05) in the blood of immunized mice. No differences were seen in the expression of 2B4 and Lag3 (data not shown). However, significant differences in the expression of Ly6C were observed. Interestingly, the frequency of Ly6C expression on Ad5-induced Gag-specific CD8+ T cells decreased after day 8 to 21 p.i. (P < 0.02) and only gradually increased to expression levels similar to those of Ad26 and Ad35 by day 50 p.i. (Fig. 2D). In contrast, Gag-specific CD8+ T cells elicited by Ad26 and Ad35 highly expressed Ly6C throughout the course of vaccination (Fig. 2D). Taken together, these results demonstrate that vaccination with Ad26 and Ad35 results in CD8+ T cell responses that differ substantially in phenotype compared to Ad5. In particular, Ad26 and Ad35 vaccination generated CD8+ T cells that displayed the phenotype and function of long-lived memory populations, whereas Ad5 induced CD8+ T cells appear to be more short-lived and terminally differentiated.

The ability to produce cytokines, such as IFN-γ, TNF-α, and IL-2, is one of the many functional abilities of effector and memory CD8+ T cells. Therefore, we next examined the capabilities of effector and memory CD8+ T cells elicited by these adenovirus serotypes to produce cytokines upon antigen restimulation. Splenocytes from immunized mice were isolated and restimulated with Gag AL11 peptide to measure cytokine expression. We found that on day 15 p.i., AL11-specific cells from all three Ad serotype vaccinations were equally functional in coproducing IFN-γ and TNF-α (Fig. 2E, left). However, at day 45 p.i. (memory time point), a higher frequency of polyfunctional Gag-specific CD8+ T cells was present in Ad26- and Ad35-immunized mice (P = 0.032 (for Ad5 versus Ad26, IFN-γ), 0.0432 (for Ad5 versus Ad35, IFN-γ), 0.046 (for Ad5 versus Ad26, IFN-γ and TNF-α coproducers), 0.0348 (for Ad5 versus Ad35, IFN-γ and TNF-α coproducers), and 0.01 (for Ad5 versus Ad35 IFN-γ, TNF-α and IL-2 coproducers) (Fig. 2E, right). These results demonstrate that immunizations with alternative Ad26 and Ad35 adenovirus-based vaccines result in CD8+ T cell responses that exhibit greater polyfunctional properties than Ad5-based vaccines.

**Nonlymphoid memory CD8+ T cells from mice vaccinated with alternative adenovirus serotypes are more robust in recall proliferation than Ad5-induced memory cells.** A hallmark feature of protective CD8+ T cell responses following vaccination is rapid proliferation of memory T cells upon reexposure to antigen. In order to analyze anamnestic responses of Gag-specific memory CD8+ T cells, we adoptively transferred equal numbers of splenic or hepatic CD45.2+ Gag-specific memory CD8+ T cells into naive CD45.1+ recipient mice. We then challenged the recipient mice with 2 × 10^6 PFU MVA-SIV Gag i.m. and analyzed donor cell recall proliferative potential at day 14 postchallenge (Fig. 3A). We did not observe any significant differences in the splenic memory recall proliferative potential in the tissues of recipient mice, except the livers, among the serotypes (data not shown). However, we found significant differences in the proliferative potential of hepatic-derived donor memory CD8+ T cells in the tissues and blood of recipient mice. In particular, more robust proliferative potential was observed in mice that received Gag-specific memory CD8+ T cells from Ad26- and Ad35-immunized mice than in mice that received Gag-specific memory CD8+ T cells from Ad5-immunized mice (spleen, 4- to 8-fold, P = 0.0024 (for Ad5 versus Ad26), P < 0.0001 (for Ad5 versus Ad35), liver, 2- to 5-fold, P = 0.0189 (for Ad5 versus Ad26), P = 0.0327 (for Ad5 versus Ad35), lung, 2- to 5-fold, P = 0.0126 (for Ad5 versus Ad26); and blood, 4- to 6-fold, P = 0.0062 (for Ad5 versus Ad26) and P = 0.0004 (for Ad5 versus Ad35) (Fig. 3B). These data imply that hepatic Gag-specific CD8+ T cells elicited by alternative-serotype Ad vectors exhibited superior recall potential compared to Ad5-induced memory cells.

**Length of antigen rest between priming and boosting impacts the magnitude and phenotype of CD8+ T cell responses during Ad26 and Ad35 heterologous prime-boost regimens.** Most vaccinations to induce robust adaptive immunity employ a prime-boost regimen. We sought to investigate the boosting potential of Gag-specific CD8+ T cells following an Ad26 or Ad35 prime-boost at different times. We first primed mice with 10^9 vp of Ad26 or Ad35. At day 30 or 60 postprime, we boosted mice heterologously with 10^9 vp of Ad26 or Ad35 (termed Ad26/Ad35 or Ad35/Ad26) and examined the kinetics, magnitude, and phenotype of Gag-specific CD8+ T cell responses (Fig. 4A). We ob-
FIG 2  Phenotype and functional characteristics of CD8⁺ T cells after Ad5, Ad26, and Ad35 vaccination. B6 mice were immunized with 10⁷ vp of Ad5, Ad26, or Ad35-SIV Gag intramuscularly. (A) Expression kinetics of CD127, CD62L, Bcl-2, and KLRG-1 in H2-D^b-AL11 tetramer-positive CD8⁺ T cells in PBMCs (n = 12 per group). *, P = 0.0221 (day 45 p.i. for Ad5 versus Ad26 KLRG-1); **, P < 0.004 (day 45 p.i. for Ad5 versus Ad35, Bcl-2); ***, P < 0.0001 (day 45 p.i. for Ad5 versus Ad26, CD127). (B) Expression of CD62L, CD127, KLRG-1, Bcl-2, and granzyme B in the spleen and liver of immunized mice on day 15 for AL11⁺ CD8⁺ effectors and day 45 for memory cells (n = 3 per group per time point). Numbers on histograms are representative percentages and mean fluorescence intensity (MFI) of expression. (C) Kinetics of CD127 expression in the PBMCs of mice immunized with 10⁷ and 10⁶ vp of Ad5, Ad26, and Ad35-SIV Gag. (D) Expression of granzyme B and Ly6C in AL11⁺ CD8⁻ T cells in the PBMCs of immunized mice (n = 12 per group). *, P < 0.02. (E) Splenocytes were isolated from vaccinated B6 mice at days 15 and 45 p.i., stimulated with AL11 peptide, and cultured for 5 hours at 37°C. Pie charts represent the proportion of functional CD8⁺ T cells coproducing IFN-γ, TNF-α, and IL-2 upon stimulation with peptide (n = 3). Samples were gated on live CD8⁻ T cells. Data are representative of 3 experiments (n = 3 per group). Asterisks indicate significance. IFN-γ⁺ Ad5 versus Ad26, P = 0.032; Ad5 versus Ad35, P = 0.0437; IFN-γ⁺ TNF-α⁺ Ad5 versus Ad26, P = 0.046; Ad5 versus Ad35, P = 0.0348; IFN-γ⁺ TNF-α⁺ IL-2⁺ Ad5 versus Ad26, nonsignificant; Ad5 versus Ad35, P = 0.01.
served only a modest secondary response that is of similar magni-
tude to the primary response after the 30-day prime-boost regimens (Fig. 4B). The peak response was a week earlier (day 8 postboost) than the primary response, which is a salient feature of secondary immune response (Fig. 4B). In striking contrast, in the 60-day heterologous prime-boost regimens, we found significant increases in the secondary responses for both groups that were about 2-fold higher (Ad26/Ad35, \( P < 0.04 \); Ad35/Ad26, \( P < 0.001 \)) than the primary responses (Fig. 4C). Similar to the groups boosted at day 30, the secondary response peaked at day 8 postboost for both combinations and declined thereafter (Fig. 4C). In comparison, significantly higher numbers of secondary effectors were present in the blood of the groups boosted at day 60 than in those boosted at day 30 at day 8 postboost (\( P = 0.0025 \) and \( P < 0.0001 \)) (Fig. 4D). Higher numbers of Gag-specific CD8\(^+\) T cells were also observed in both the lymphoid and nonlymphoid tissues of the groups boosted at day 60 than at day 30 (data not shown). Since we observed different response magnitudes between the day 30 and day 60 groups, we sought to analyze the CD8\(^+\) T cell phenotypes induced during these prime-boost vaccinations to ascertain if they are different in quality. We tracked the phenotypic expression of the memory markers CD127, CD62L, and Bcl-2 in the blood of the groups boosted at day 60 than in those boosted at day 30 at day 8 postboost (\( P = 0.0025 \) and \( P < 0.0001 \)) (Fig. 4D). Bcl-2 expression on Gag-specific CD8\(^+\) T cells was also higher in the groups boosted on day 60 (MFI, 850) than for the groups boosted on day 30 (MFI, 700) (Fig. 4E and 4F, right). However, CD62L expression was not significantly different among the groups (Fig. 4E and 4F, middle). Notably, there were no significant differences in the phenotypes and magnitudes of responses between Ad26/Ad35 and Ad35/Ad26 prime-boost combinations. Thus, a longer time interval between priming and boosting positively affected the expression of the prosurvival markers CD127 and Bcl-2. These data suggest that the time interval between priming and boosting is important to determine the magnitude and the longevity of anamnestic CD8\(^+\) T cell responses.

**FIG 3** Nonlymphoid memory CD8\(^+\) T cells from mice vaccinated with alternative adenovirus serotypes are more robust in recall proliferation. (A) Graphical representation of adoptive transfer. Hepatic lymphocytes were isolated from Ad5, Ad26, and Ad35-SIV Gag immunized memory CD45.2 mice after day 45 p.i. Equal numbers (7,000) of AL11 tetramer-positive CD8\(^+\) T cells were adoptively transferred to groups of naïve CD45.1 mice. Recipient mice were challenged with 2 × 10^6 PFU MVA-SIV Gag i.m. each day after adoptive transfer and were sacrificed on day 14 postchallenge. Blood and tissues were obtained for proliferation analysis of donor cells. (B) Numbers of donor hepatic AL11 CD8\(^+\) T cells expanded in both the lymphoid and nonlymphoid organs of recipient mice. Numbers of PBMCs indicated after the dotted line were enumerated based on per million PBMCs. Data are represented in log scale (\( n = 5 \) per group). *, \( P < 0.04 \); **, \( P < 0.008 \); ***, \( P = 0.0004 \). Data are representative of 2 experiments.
FIG 4 Importance of longer intervals between prime and boost for generating optimal memory CD8⁺ T cell responses. (A) B6 mice were primed with $10^9$ vp of Ad26 or Ad35-SIV Gag and rested for 30 or 60 days. Antigen-primed mice were heterologously boosted with Ad35 or Ad26-SIV Gag on day 30 or 60 postprime ($n = 6$ to 10 mice per group). (B) Kinetics of AL11-tetramer positive CD8⁺ T cell responses in the blood during Ad26 or Ad35-SIV Gag prime and heterologous boost at day 30 postprime. (C) AL11 tetramer-positive CD8⁺ T cell kinetics of response in the blood after heterologous boost with Ad26 or Ad35-SIV Gag at 60 days postprime. Comparison of peak primary response versus peak secondary response for Ad26/Ad35 (*, $P = 0.04$) and Ad35/Ad26 (**, $P = 0.001$). (D) Number of AL11 tetramer-positive CD8⁺ T cells on day 8 postboost in the blood of mice boosted heterologously on days 30 and 60. **, $P = 0.0025$; ***, $P < 0.0001$. Samples were gated on live CD8⁺ AL11⁺ T cells. (E) Kinetics of CD127, CD62L, and Bcl-2 (MFI, 700) expression in AL11⁺ CD8⁺ T cells in the blood after Ad26 or Ad35 priming and heterologously boosted at day 30 postprime. (F) Longitudinal analysis of tetramer-positive PBMC CD8⁺ T cells for expression of CD127, CD62L, and Bcl-2 (MFI, 850) after priming with Ad26 or Ad35-SIVgag and heterologously boosted at day 60 postprime ($n = 6$ per group).
A.

B.

C.

D.

E.
2-fold increase in the secondary response at day 8 postboost in the Ad5-primed groups (Ad5/Ad26, P = 0.0017; Ad5/Ad35, P = 0.0034) (Fig. 5A). However, the magnitude of secondary responses between the Ad26- or Ad35-primed and Ad5-primed groups were significantly different. The frequency of the peak secondary responses in the blood after Ad5 priming was about 2.5-fold less (P < 0.0001) than that of the Ad26/Ad5 and Ad35/Ad5 prime-boost regimens (Fig. 5A). In correlation with the higher frequency observed, the numbers of Gag-specific CD8⁺ T cells in the blood at day 8 postboost were also significantly higher in the Ad26/Ad5 and Ad35/Ad5 prime-boost groups (3- to 4-fold; P = 0.0025 and P < 0.0001) (Fig. 5B and C). However, the numbers of Gag-specific CD8⁺ T cells in the tissues were largely similar for all combinations at day 45 postboost, with the exception that the Ad35/Ad5 combination had significantly more Gag-specific CD8⁺ T cells in the spleen (P = 0.0075) and liver (P < 0.05) and Ad26/Ad5 in the blood (P < 0.05) (data not shown). Phenotypic expression of Bcl-2 on Gag-specific CD8⁺ T cells in the blood was significantly increased in the Ad26- or Ad35-boosted groups compared to the Ad5-boosted groups at day 40 postboost (P = 0.0002 and P = 0.0385) (Fig. 5D, right), CD127 and CD62L expression was similar in all Ad5 prime-boost combinations (Fig. 5D). The numbers of CD62Lhi CD127hi KLRG-1low Gag-specific CD8⁺ memory T cells in the spleen, liver, lung, inguinal lymph nodes, and bone marrow of mice vaccinated with the day 60 heterologous prime-boost regimens were next assessed. Significantly greater numbers of Gag-specific memory CD8⁺ T cells were observed in the spleen, liver, and bone marrow of mice vaccinated with Ad26/ Ad35 and Ad35/Ad26 heterologous prime-boost combinations than of mice vaccinated with the Ad5 prime-boost combinations (P = 0.0003, P = 0.0037, and P < 0.04, respectively) (Fig. 5E). These data demonstrate that heterologous prime-boost regimens with Ad26 and Ad35 elicited more memory cells that highly expressed the memory markers CD127 and Bcl-2 than those produced with any heterologous prime-boost regimens containing Ad5. These results further extend the previous phenotyping and recall data (Fig. 2 to 3) and show that Ad5 primes poorly. Together, these results indicate the immunologic superiority of alternative-serotype Ad vectors to prime immune responses compared with Ad5 vectors.

Heterologous prime-boost regimens with Ad26 and Ad35 produce more polyfunctional CD8⁺ T cells than any heterologous prime-boost regimen containing Ad5. To examine the functionality of vaccine-induced CD8⁺ T cells during heterologous vaccination with Ad5/Ad26 or Ad5/Ad35 and Ad26/Ad35 combinations, we isolated splenocytes from immunized mice at day 50 postboost and assessed functionality as described previously (Fig. 2D). We analyzed the ability of the memory CD8⁺ T cells to produce IFN-γ, TNF-α, and IL-2 simultaneously after restimulation with Gag AL11 peptide. We show that the frequency of CD8⁺ IFN-γ⁺ T cells coproducing IFN-γ⁺ and TNF-α was 74 and 76% (mean) in the Ad26/Ad35 combinations, whereas the Ad5/Ad26, Ad26/Ad5, Ad5/Ad35, and Ad35/Ad5 combinations were between 50 and 62% (means) (Fig. 6A, left). The frequency of IFN-γ and IL-2 coproducers was 12 and 15% (means) in the Ad26/Ad35 combinations, and the combinations that included Ad5 were between 8 and 10% (means) (Fig. 6A, right). To assess polyfunctionality of memory CD8⁺ T cells, we compared the functional quality of memory cells between Ad26 and Ad35 prime-boost regimens in conjunction with Ad5 and Ad26 or Ad35 prime-boost regimens at day 50 postboost. We found that the Ad26 and Ad35 prime-boost combinations were more polyfunctional than the Ad5 prime-boost with Ad26 or Ad35 combinations (P < 0.004 for Ad5/Ad26 combinations versus Ad26/Ad35 combination), IFN-γ, P < 0.003 for Ad5/Ad35 combinations versus Ad26/Ad35 combinations, IFN-γ, P < 0.008 for Ad5/Ad26 combinations versus Ad26/Ad35 combinations, IFN-γ and TNF-α coproducers, P = 0.02, P < 0.005, and P = 0.001 (for Ad5/Ad35 and Ad5/Ad26 combinations versus Ad26/Ad35 combinations, double cytokine coproducers, respectively) (Fig. 6B). Although the day 60 Ad5 prime-boost combinations had about 8 to 10% triple cytokine producers, there was also a much higher frequency of CD8⁺ T cells producing only IFN-γ (means, 36 to 49%) compared to Ad26 and Ad35 (means, 19 to 21%) day 60 heterologous prime-boost combinations (Fig. 6B). Therefore, the Ad26/Ad35 and Ad35/Ad26 regimens elicited more polyfunctional Gag-specific CD8⁺ T cells than any regimen that included Ad5, and they elicited dramatically more than Ad5 alone (compare Fig. 2E and 6B).

Secondary memory CD8⁺ T cells elicited by Ad26 and Ad35 heterologous prime-boost regimens proliferate more robustly than prime-boost regimens containing Ad5. To compare the recall proliferative potential of secondary memory CD8⁺ T cells induced by Ad26 and Ad35 day 30 and day 60 heterologous prime-boost combinations with Ad5 and Ad26 or Ad35 heterologous prime-boost combinations, we adoptively transferred secondary memory CD8⁺ T cells from the spleens and livers of immunized mice to different groups of naïve mice. The recipient mice were challenged with 2 × 10⁸ PFU of MVA-SIV Gag 1 day after adoptive cell transfers (Fig. 7A). We then analyzed the spleens, livers, and lungs of the recipient mice for the expansion of the donor cells on day 14 postchallenge. We compared the recall expansion abilities of memory CD8⁺ T cells of the Ad26/Ad35 prime-boost combinations and Ad5 with Ad26 or Ad35 prime-boost combinations with Ad5 primary memory recall. Our results showed that memory CD8⁺ T cells from the Ad26/Ad35 heterologous prime-boost combinations in general proliferated significantly better than the Ad5 prime-boost combinations and Ad5 alone in the spleen and
lung but not the liver of the recipient mice during splenic memory challenge and recall (Fig. 7B). We observed the most significant differences in the hepatic memory adoptive transfer and challenge experiment. In the Ad26/Ad35 combinations, secondary memory CD8$^+$ T cells from the day 30 and day 60 prime-boost groups proliferated significantly better than the secondary memory from the day 60 prime-boost regimens that included Ad5 in the tissues of the recipient mice (spleen, $P < 0.02$ (for Ad35/Ad26 D30 boost versus Ad35/Ad5 D60 boost; Ad26/Ad5 D60 boost versus Ad26/Ad35 D60 boost), $P < 0.003$ (for Ad5 versus Ad35/Ad26 D30 boost and Ad35/Ad5 D60 boost versus Ad35/Ad26 D60 boost), and $P < 0.0001$ (for Ad5 versus Ad26/Ad26 D30 boost and Ad26/Ad35 D30 boost versus Ad26/Ad5 D60 boost); liver, $P < 0.04$ (for Ad5 versus Ad26/Ad35 D30 boost; and Ad26/Ad5 D60 boost versus Ad26/Ad35 D60 boost), $P < 0.0098$ (for Ad5 versus Ad35/Ad36 D30 boost; Ad5 versus Ad26/Ad36 D60 boost; and Ad35/Ad26 D60 boost versus Ad35/Ad5 D60 boost), and $P < 0.0001$ (for Ad5 versus Ad35/Ad26 D60 boost; and Ad35/Ad26 D60 boost versus Ad35/Ad5 D60 boost)). In conclusion, these data show that secondary memory CD8$^+$ T cells elicited by the Ad26/Ad35 heterologous prime-boost vaccination regimen induced better quality CD8$^+$ T cells that proliferated more robustly than any Ad5-based regimen. The optimal regimen included both Ad26 and Ad35 vectors with a 60-day rest interval and completely excluded Ad5 vectors.

**FIG 6** Heterologous prime-boost regimens with Ad26 and Ad35 produce more polyfunctional CD8$^+$ T cells than heterologous prime-boost regimens including Ad5. Freshly explanted splenocytes were stimulated with AL11 peptide with 1.5 million cells per well and cultured in 10% FBS plus RPMI medium at 37°C for 5 h. (A) Representative flow plots of intracellular staining of IFN-γ, TNF-α, and IL-2 in stimulated and unstimulated memory CD8$^+$ T cells from the spleen at day 50 postboost. The left panel shows the frequency of IFN-γ$^+$ TNF-α$^+$ coproducers and the right panel shows IFN-γ$^+$ IL-2$^+$ coproducers among CD8$^+$ T cells. Numbers in the plots are representative percentages of cytokine producers, and percentages in parentheses are coproducers of the indicated cytokines from IFN-γ$^+$-producing CD8$^+$ T cells (n = 3 per group). (B) Comparison of polyfunctionality of stimulated CD8$^+$ T cells coproducing IFN-γ, TNF-α, and IL-2 at day 50 postboost. For IFN-γ$^+$ Ad5/Ad26 combinations versus Ad26/Ad35 combinations, $P < 0.004$ (**); Ad5/Ad35 combinations versus Ad26/Ad35 combinations, $P < 0.003$ (**); IFN-γ$^+$ TNF-α$^+$ Ad5/Ad26 combinations versus Ad26/Ad35 combinations, $P < 0.008$ (**); Ad5/Ad35 combinations versus Ad26/Ad35 combinations $P = 0.02$ (*), $P < 0.005$ (**), and $P = 0.001$ (**); all combinations of IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ producers were not statistically significant.
DISCUSSION

Although Ad5 vector-based vaccines are highly immunogenic, the high prevalence of anti-Ad5 immunity and a potential association with enhancement to HIV-1 infection (4) will likely preclude the use of Ad5 in future HIV-1 vaccine trials in the developing world. A recent study has shown that Ad26 and Ad35 vectors can protect rhesus monkeys against stringent SIVmac251 challenges (12), whereas Ad5 and DNA/Ad5 regimens have failed in this system.
(23, 24), suggesting important differences in T cell responses elicited by these adenoviral vectors. In this comparative report, we have identified key differences in the properties of primary and secondary SIV Gag-specific CD8\(^+\) T cell responses following Ad5, Ad26, and Ad35 vaccinations. Our analysis of SIV Gag-specific CD8\(^+\) T cells showed that alternative-serotype Ad vectors elicit magnitudes of antigen-specific CD8\(^+\) T cell responses comparable to those of Ad5 vector at a dose of 10\(^9\) vp in mice. Interestingly, Gag-specific CD8\(^+\) T cells generated by Ad26 and Ad35 vectors expressed significantly more of the homeostatic memory survival marker CD127 and the antiapoptotic molecule Bcl-2, suggesting enhanced memory CD8\(^+\) T cell differentiation compared to Ad5. Immunization with alternative-serotype Ad vectors also resulted in more multifunctional primary and secondary Gag-specific CD8\(^+\) T cell responses capable of producing IFN-γ, TNF-α, and IL-2 compared to Ad5 prime-boost or Ad5 alone. Importantly, antigen-specific hepatic memory CD8\(^+\) T cells induced by Ad26 and Ad35 prime-boost regimens proliferated more robustly during secondary challenge.

The quality of memory T cell responses is correlated with the expression of CD127, Bcl-2, and IL-2 (17, 25–27). Our data demonstrated that not only were all of these molecules more significantly upregulated but also that Ly6C, another TCM cellular marker implicated for preferential homing to the lymph nodes (19, 28), was also highly expressed in Ad26 and Ad35 elicted Gagspecific CD8\(^+\) T cells throughout the course of vaccination in mice compared to Ad5. Similar results were obtained by Yang et al. in a study utilizing an Ad5-Ova system, with low expression of CD62L, CD127, and Ly6C in Ova-specific CD8\(^+\) T cells 30 days postimmunization (29). In comparison, our data suggest that memory CD8\(^+\) T cells generated with alternative-serotype Ad vaccination are more long-lived and polyfunctional than Ad5. Indeed, in our adoptive transfer experiments, we observed significantly better recall responses of the liver memory CD8\(^+\) T cells from donor mice vaccinated with Ad26 and Ad35 compared to those of mice vaccinated with Ad5. This increased recall response was observed in both the lymphoid and nonlymphoid tissues after challenge of recipient mice with MVA-Gag. It is well established that Ad5, Ad26, and Ad35 differ in their tropism (30) and primary cellular receptors (5, 7–9, 27). A study by Kaufman et al. (10) examined the transgene expression, distribution, and magnitude of Ad5 and Ad26 vectors expressing SIV Gag and observed that all vectors induced comparable magnitudes and kinetics of T cell responses in various systemic and mucosal tissues following i.m. immunization with a 10\(^9\)-vp dosage. In addition, it has been shown that the transduction efficiencies of Ad5 and Ad35 in the muscle (which lacks CAR expression) are comparable during i.m. immunization (27). We have also shown that Ad5, Ad26, and Ad35 induce comparable expression profiles of CD127, CD62L, and PD-1 in CD46 transgenic mice compared to wild-type mice (30a).

In the heterologous prime-boost combinations with Ad26/Ad35, Ad5/Ad26, or Ad5/Ad35, we observed substantially different magnitudes of secondary responses between day 30 and day 60 boost in the Ad26/Ad35 and Ad35/Ad26 prime-boost regimens. Significantly higher magnitudes of antigen-specific response were observed after boosting at day 60 postprime than at day 30 postprime. This suggests that the duration of the antigenic rest before boosting is critical in enhancing secondary response. This phenomenon is not unusual and has been documented in other vaccination regimens, which showed that boosting after a longer interval enhanced the magnitude of the secondary effector response (16, 31). Intriguingly, there was only a very modest increase in the boosting response of CD8\(^+\) T cells in mice that received prime-boost regimens involving Ad5 primes (Fig. 5A). However, when Ad5 was used as a boosting immunogen, it was effective in boosting Ad26 and Ad35 primes, although the phenotype and functional quality after Ad5 boosting was not improved (Fig. 5A to D and 6A to B). This phenomenon cannot be attributed to cross-reactive vector-specific NAbs, as previous studies have shown that NAbs between these subgroups do not cross-react (1). Therefore, the low boosting capacity of Ad5-primed CD8\(^+\) T cells most likely is due to inherent defects in the differentiation of memory CD8\(^+\) T cells (Fig. 2A and B, 4E and F, and 5D) and perhaps also due to antigen persistence during Ad5 vaccination, as previously reported (11). Our results also demonstrate important differences in the order of Ad5 vector usage (as a prime or boost) and that Ad26 and Ad35 are efficient primers that result in high magnitude of secondary effector response when boosted by alternative-serotype Ads or Ad5.

Several studies have shown that both priming and boosting immunogens play a role in shaping the quality and phenotype of secondary memory cells (25, 32, 33). Our current study concurs with these studies, in that we observed differential phenotypic expressions of T cell memory markers when Ad5 or alternative Ad vectors were used in boosting. When Ad5 was used as a boosting immunogen, the expression of the antiapoptotic marker Bcl-2 deteriorated. Conversely, when Ad26 or Ad35 was used as boosting immunogen, Bcl-2 expression was significantly elevated (Fig. 5D). As shown in Fig. 6B, secondary CD8\(^+\) T cell responses from heterologous Ad26/Ad35 or Ad35/Ad26 prime-boost regimens produced more multifunctional T cell responses than when primed or boosted with Ad5 during a longer resting interval of 60 days before boost. These results suggest that both primary and secondary immunogens contribute to the differentiation programming of the functional quality of secondary T cell responses, which is in agreement with a previous study (25).

More contrasting results were found during our challenge and recall studies comparing the primary and secondary memory CD8\(^+\) T cells of the various Ad vectors used. Although we observed some improvement in the recall proliferation potential between the primary and secondary memory T cells during lymphoid memory challenge with cognate antigen, significantly more robust proliferation was observed in the challenge of nonlymphoid liver memory transfer. This finding is especially important as memory cells present in the periphery play an important role to immediately control pathogenic infection during a secondary attack (26, 34–36). Our challenge and recall results after Ad26 and Ad35 heterologous prime-boost regimens in mice are consistent with a recent study in monkeys in which partial protection against Ebola virus challenge was observed after one primary immunization with Ad26 vectors and complete protection after boosting with Ad35 vectors (37). In addition, prime-boost regimens involving Ad26/MVA and Ad35/Ad26 regimens expressing SIV Gag, Pol, and Env has been shown to confer partial protection against challenges with SIVmac251 (12). These observations in nonhuman primates suggest the physiological relevance of our murine observations.

Taken together, the data presented in this study show that Ad26 and Ad35 vectors elicit CD8\(^+\) T cells responses that are
qualitatively superior to those of Ad5 vectors. Our study also sheds light on the impaired memory differentiation program of CD8+ T cells following Ad5 immunization. Thus, these data merit consideration of the alternative-serotype Ad26 and Ad35 vectors as potentially improved platforms for vaccine development. Our study also highlights the importance of a longer interval between prime and boost for generating optimal memory CD8+ T cell responses.

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