Alternative Serotype Adenovirus Vaccine Vectors Elicit Memory T Cells with Enhanced Anamnestic Capacity Compared to Ad5 Vectors

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The failure of the adenovirus serotype 5 (Ad5) vector-based human immunodeficiency virus type 1 (HIV-1) vaccine in the STEP study has led to the development of adenovirus vectors derived from alternative serotypes, such as Ad26, Ad35, and Ad48. We have recently demonstrated that vaccines using alternative-serotype Ad vectors confer partial protection against stringent simian immunodeficiency virus (SIV) challenges in rhesus monkeys. However, phenotypic differences between the T cell responses elicited by Ad5 and those of alternative-serotype Ad vectors remain unexplored. Here, we report the magnitude, phenotype, functionality, and recall capacity of memory T cell responses elicited in mice by Ad5, Ad26, Ad35, and Ad48 vectors expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP). Our data demonstrate that memory T cells elicited by Ad5 vectors were high in magnitude but exhibited functional exhaustion and decreased anamnestic potential following secondary antigen challenge compared to Ad26, Ad35, and Ad48 vectors. These data suggest that vaccination with alternative-serotype Ad vectors offers substantial immunological advantages over Ad5 vectors, in addition to circumventing high baseline Ad5-specific neutralizing antibody titers.

Adenoviruses have emerged as potent vaccine vectors due to their high insert capacity and proven immunogenicity in multiple experimental systems (1–4). However, an initial evaluation of an Ad5-gag/pol/nef HIV-1 vaccine showed no protection against HIV-1 acquisition in humans (5). A substantial limitation of Ad5 vectors is the high baseline neutralizing antibody titers to the Ad5 vector in human populations, particularly in the developing world (1, 6). As a result, our laboratory and others have developed Ad vectors from alternative serotypes with lower baseline neutralizing antibody titers, including Ad26, Ad35, and Ad48 (1, 3, 6, 7). We have recently demonstrated the protective efficacy of alternative-serotype Ad vectors against both high-dose intravenous and repetitive low-dose intrarectal SIV challenges in rhesus monkeys (8, 9). However, a detailed comparison of the memory T cell phenotypes elicited by Ad5 vectors to those with alternative-serotype Ad vectors has not been previously reported.

Acute and chronic viral infections result in distinct T cell responses that differ in their phenotype and functionality. Following an acute viral infection, highly functional memory T cells are typically generated and often provide lifelong protection upon reinfection with the same pathogen. Importantly, expression of CD127 (the interleukin-7R α [IL-7R α] chain) defines the precursors that will enter the pool of long-lived memory T cells (10). In addition, expression of CD62L endows memory T cells with the ability to circulate throughout lymphoid tissues, and this marker is also used to identify central memory cells that persist in the host (11, 12). In contrast, during a chronic viral infection, T cells undergo a transcriptional program that renders them inefficient at controlling infection (13). Upregulation of inhibitory receptors, such as PD-1, is associated with T cell functional exhaustion, and therapeutic blockade of PD-1 receptors results in restoration of T cell proliferative capacity and function (14–17). Analysis of these phenotypic markers can be used to characterize T cell function following vaccination or establishment of a chronic infection.

The lymphocytic choriomeningitis virus (LCMV) system in

mice has been a standard model for analyzing T cell responses in the context of viral clearance or viral persistence. Infection with the LCMV Armstrong strain results in an acute infection that is cleared within 8 days and is characterized by the generation of highly functional memory T cells. Conversely, infection with the LCMV CI-13 strain results in a chronic infection and the generation of dysfunctional T cell responses. Moreover, findings from the acute and chronic LCMV systems have been generalized to various acute and chronic infections in humans (18–21).

In this study, we demonstrate that vaccination using the alternative-serotype Ad vectors Ad26, Ad35, and Ad48 results in substantially different T cell phenotypes than those from vaccination with Ad5 vectors, including enhanced memory conversion and improved functional and proliferative capacity. Although T cell responses elicited by Ad5 vectors were high in magnitude, they expressed high levels of PD-1 and exhibited functional exhaustion, decreased anamnestic potential, and reduced protective capacity compared to T cell responses elicited by alternative-serotype Ad vectors.

MATERIALS AND METHODS

Mice and infections. Six- to 8-week-old female C57BL/6 mice (from Jackson Laboratories) were used for all immunization experiments. Mice were immunized intramuscularly with 10^{10} viral particles of replication incompetent E1/E3 deleted adenoviruses (1) expressing LCMV glycoprotein (GP). For chronic viral challenge, LCMV CI-13 (22) was injected intravenously via the lateral tail vein (2×10^6 PFU). For acute viral challenge,

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FIG 1 Immunization with Ad26, Ad35, or Ad48 results in improved memory conversion of GP-specific CD8 T cells compared to immunization with Ad5. (A) Experimental outline. (B) Representative FACS plots showing the percentages of GP-specific CD8 T cells in blood. (C) Numbers of GP-specific CD8 T cells in blood. (D) Numbers of GP-specific CD8 T cells in spleen, lymph nodes, and liver. (E) Percent expression of memory markers CD127 (top) and CD62L (bottom) on GP-specific CD8 T cells in the liver. (F) Representative FACS plots showing expression of CD127 and CD62L on GP-specific CD8 T cells in liver. Tissue data are from day 60 postimmunization. All immunizations were at a dose of 10^{10} VP injected intramuscularly. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.02$. Error bars indicate standard errors of the means (SEM).

LCMV Armstrong (22) was injected intravenously (2×10^6 PFU). As a more stringent challenge model, a lethal dose of recombinant *Listeria monocytogenes* expressing the LCMV GP33-41 epitope (Lm-GP33-41) was injected intravenously (2×10^5 CFU). All experiments were performed with approval of the Institutional Animal Care and Use Committee (IACUC).

Viral titration. Titration of LCMV was performed on Vero cell monolayers by standard plaque assay or by reverse transcription-PCR (RT-PCR) (23). For plaque assays, serial 10-fold dilutions from serum samples or homogenized tissues were aliquoted on top of the Vero cell monolayers in 6-well plates. Plates were incubated for a total of 60 min (manually rocking every 15 min). A 1:1 solution of 1% agarose in $2 \times$ 199 medium

FIG 2 Decreased expression of PD-1 and increased functional capacity of memory CD8 T cells following immunization with Ad26, Ad35, or Ad48 compared to that with Ad5. (A) Representative FACS plots showing percentage of GP-specific CD8 T cells that express PD-1. (B) Mean fluorescence intensity (MFI) of PD-1 staining on virus-specific CD8 T cells. (C) Functionality of GP-specific CD8 T cells in spleen as measured by tetramer (top) and IFN- γ (bottom) expression. Each column represents tetramer and intracellular cytokine stains following peptide stimulation from the same mouse. (D) MFI of IFN- γ staining after peptide stimulation in spleen. Peptide stimulations were performed for 5 h at 37°C. Tissue data are from day 60 postimmunization. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.02$. Error bars indicate SEM.

then was overlaid on top of the monolayers, and 4 days later a 1:1 solution of 1% agarose in 2X199 medium with 1:50 neutral red was added to the top of the wells. Plaques were counted at day 5. For RT-PCR, total RNA was isolated from four pooled tissues (RNeasy; Qiagen), followed by reverse transcription and PCR amplification of cDNA coding for LCMV GP using the following primers: GP-reverse (GCAACTGCTGTGTCCCGA AAC) and GP-forward (CATTCACCTGGACTTTGTCAGACTC).

Degranulation assay. Spleen cells were stimulated for 5 h with 0.2 μ g/ml of LCMV peptides together with a 1:1,000 dilution of brefeldin A (GolgiPlug) and monensin (GolgiStop) and with anti-CD107a and anti-CD107b at 1:200. Cells were stained with anti-CD8 and anti-CD44 and then were fixed and permeabilized prior to intracellular staining with anti-gamma interferon (IFN- γ) and anti-tumor necrosis factor alpha (TNF- α) (24). All of these reagents were purchased from BD Biosciences.

FIG 3 Immunization of CD46 transgenic mice with Ad26, Ad35, or Ad48 also results in improved memory conversion and decreased PD-1 expression on GP-specific CD8 T cells. (A) Representative FACS plots showing the percentage of GP-specific CD8 T cells in blood. (B) Numbers of GP-specific CD8 T cells in blood. (C) Numbers of GP-specific CD8 T cells in spleen, lymph nodes, and liver. (D) Representative FACS plots showing expression of CD127 and CD62L on GP-specific CD8 T cells in liver. (E) Representative FACS plots showing percentages of GP-specific CD8 T cells that express PD-1. Tissue data are from day 60 postimmunization. All immunizations were at a dose of 10^{10} VP and were injected intramuscularly. Data are from 2 experiments, with $n = 6$ mice per group. *, $P \leq 0.05$. Error bars indicate SEM.

Antibodies and flow cytometry. Single-cell suspensions were stained with anti-CD8 α (53-6.7), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD127 (A7R34), anti-CD62L (MEL-14), anti-granzyme B (MHGB04), anti-CD107a (1D4B), anti-CD107b (ABL-93), and anti-PD-1 (RMP1-30). All antibodies were purchased from BD Pharmingen, except for CD44 (Biolegend), PD-1 (Biolegend), and granzyme B (Invitrogen). A combination of DbGP33-41/KbGP34-41 tetramers was used for analyzing the dominant GP-specific CD8 T cell responses responding to LCMV GP33-41. Biotinylated major histocompatibility complex class I monomers were from the NIH tetramer facility at Emory University. Intracellular cytokine staining was used for measuring the functionality of GP-specific T cell responses using 0.2 μ g/ml of LCMV GP33-41 (CD8 T cell peptide) or GP61-80 (CD4 T cell peptide). Intracellular cytokine staining for IFN- γ , TNF- α , and interleukin-2 (IL-2) was performed with the Cytofix/Cytoperm kit (BD Biosciences). Fixed cells were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo (Treestar).

Statistical analysis. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney tests (GraphPad Prism).

RESULTS

Improved memory conversion after immunization with Ad26, Ad35, and Ad48 vectors compared to Ad5 vectors. To compare the generation of T cell responses after immunization with Ad5 compared to that with alternative-serotype Ads, we assessed immune responses in C57BL/6 mice at several time points following intramuscular vaccination with 10^{10} viral particles of Ad5, Ad26, Ad35, or Ad48 vectors expressing lymphocytic choriomeningitis

virus glycoprotein (LCMV GP) (Fig. 1A). At day seven postimmunization, Ad5 vectors elicited 4-fold higher levels of GP-specific (GP33-41/GP34-41) CD8 T cells in blood compared to alternative-serotype Ad vectors (mean for Ad5, 7.8%; Ad26, 2.3%; Ad35, 2.2%; and Ad48, 1.7%) (Fig. 1B). However, the peak of the CD8 T cell responses for all vectors at day 15 was comparable in magnitude, and all vaccinated groups showed similar GP-specific CD8 T cell levels by day 60 (Fig. 1C).

To compare the magnitude of the memory CD8 T cell responses in tissues, we sacrificed mice at day 60 postimmunization. Interestingly, both Ad5 and alternative-serotype Ad vectors elicited similar numbers of GP-specific CD8 T cells in lymphoid tissues such as the spleen and the lymph nodes (the P value was not significant), but Ad5 induced more than 6-fold higher numbers of GP-specific CD8 T cells in the liver (mean of GP-specific CD8 T cells after vaccination with Ad5, 5.12×10^5 ; Ad26, 7.0×10^4 ; Ad35, 6.6×10^4 ; and Ad48, 9.8×10^4 ; $P \leq 0.002$) (Fig. 1D).

GP-specific CD8 T cells from Ad26-, Ad35-, and Ad48-immunized mice demonstrated increased expression of the memory homeostatic survival marker CD127 (Fig. 1E, top) and the lymphoid tropic marker CD62L (Fig. 1E, bottom) compared to Ad5. There was a 6-fold higher percentage of GP-specific CD8 T cells that converted to CD127⁺ following Ad26, Ad35, and Ad48 immunization compared to that following Ad5 immunization ($P \leq 0.02$). In addition, there was a 4-fold greater percentage of GP-specific

FIG 4 Vaccination with Ad26, Ad35, or Ad48 vectors expressing LCMV GP results in improved recall CD8 T cell responses in blood compared to Ad5 vectors following LCMV CI-13 challenge. (A) Experimental outline. (B) Number of PBMCs in blood. (C) Number of CD8 T cells in blood. Cell counting was performed in 100 μ L of blood after challenge with LCMV CI-13 at day 3. (D) Representative FACS plots showing the percentage of anamnestic GP-specific CD8 T cells in blood. (E) Numbers of anamnestic GP-specific CD8 T cells in blood. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.05$. Error bars indicate SEM. Unvax, unvaccinated.

FIG 5 Vaccination with Ad26, Ad35, or Ad48 vectors expressing LCMV GP results in improved anamnestic CD8 T cell responses in multiple tissues after an LCMV Cl-13 challenge. (A) Representative FACS plots showing the percentage of anamnestic GP-specific CD8 T cell responses in various tissues. (B) Percentage of GP-specific CD8 T cells that coexpress IFN- γ , TNF- α , and IL-2 cytokines in spleen (top) and liver (bottom). (C) Numbers of GP-specific CD8 T cells from spleen after GP33-41 peptide stimulation. (D) MFI of IFN- γ , TNF- α , and IL-2 in GP-specific CD8 T cells from liver (mean fluorescence intensity). GP33-41 peptide stimulations were performed for 5 h. Tissue data are from day 19 after LCMV Cl-13 challenge. Spleen, lymph node, and liver data are from 3 experiments, with $n = 12$ mice per group. Kidney, gut intraepithelial lymphocytes (IEL), and lung data are from 2 experiments, with $n = 8$ mice per group. *, $P \leq 0.05$. Error bars indicate SEM.

CD8 T cells that were CD62L⁺ following alternative-serotype Ad vector immunization compared to Ad5 immunization at day 60 postvaccination ($P \leq 0.02$). Representative fluorescence-activated cell sorter (FACS) plots of CD127 and CD62L expression on GP-specific CD8 T cells are shown (Fig. 1F). These results demonstrate that memory conversion was superior following immunization with alternative-serotype Ads than with Ad5.

Ad5 vectors, but not Ad26, Ad35, and Ad48 vectors, induce functional exhaustion of virus-specific CD8 T cells. PD-1 is a marker for functional exhaustion during chronic infections in mice and humans (14, 18). We therefore analyzed the expression of this immunoinhibitory receptor on GP-specific memory CD8 T cells at day 60 postimmunization. Interestingly, the vast majority of GP-specific CD8 T cells from mice vaccinated with Ad5 but not

FIG 6 Immunization with Ad26, Ad35, or Ad48 induces greater granzyme B (GzB) and CD107 expression on GP-specific CD8 T cells following LCMV CI-13 challenge compared to immunization with Ad5. (A) Representative histograms showing granzyme B expression on GP-specific CD8 T cells in spleen and liver. (B) Summary showing granzyme B expression on GP-specific CD8 T cells in spleen. (C) Representative histograms showing CD107 expression on GP-specific CD8 T cells in spleen and liver following GP33-41 peptide stimulation. (D) Summary showing CD107 expression on GP-specific CD8 T cells in spleen. Data are from day 19 after LCMV CI-13 challenge. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.03$. Error bars indicate SEM.

alternative Ad serotypes expressed PD-1, suggestive of functional exhaustion (Fig. 2A). Moreover, per cell expression of PD-1 was increased 2-fold on GP-specific CD8 T cells in the spleen ($P \leq 0.0002$), lymph nodes ($P \leq 0.005$), and peripheral blood mononuclear cells (PBMCs) ($P \leq 0.0002$) and 3-fold on GP-specific CD8 T cells in the liver ($P \leq 0.0002$) of Ad5-vaccinated mice compared to alternative-serotype Ad-vaccinated mice (Fig. 2B). Cytokine expression on GP-specific CD8 T cells was also different among vaccinated groups. Representative tetramer and cytokine FACS plots from individual mice are shown for spleen (Fig. 2C) and liver (Fig. 2D). Per cell expression of IFN- γ was increased on GP-specific CD8 T cells of Ad26-, Ad35-, and Ad48-vaccinated mice compared to Ad5-vaccinated mice ($P \leq 0.05$) (Fig. 1E). These data show that Ad5 immunization elicited GP-specific memory CD8 T cells with high expression of inhibitory PD-1, which was associated with impaired memory conversion and reduced cytokine production, indicating functional exhaustion.

The PD-1⁺ CD127⁺ CD62L⁺ phenotype and reduced cytokine production are associated with T cell exhaustion during chronic viral infections in both mice and humans (14, 18, 21, 25). Although Ad5 has been reported to persist *in vivo* (26), we did not detect Ad5 by RT-PCR in spleen or liver, suggesting at most low levels of antigen persistence (limit of detection, 10 mRNA copies; data not shown). We nevertheless observed similar T cell phenotypic and functional differences among Ad serotypes when administered at a lower dose (10^9 VP; data not shown) and in transgenic mice expressing the human CD46 receptor (Fig. 3).

CD8 T cells elicited by Ad5 exhibit reduced anamnestic expansion following secondary antigen challenge compared to alternative-serotype Ad vectors. We next compared anamnestic T cell responses in mice vaccinated with Ad5, Ad26, Ad35, and Ad48 vectors expressing LCMV GP. We challenged mice with LCMV CI-13 (see Materials and Methods) and analyzed anamnestic T cell responses (Fig. 4A). Interestingly, Ad5-vaccinated mice did not

FIG 7 Vaccination with Ad26, Ad35, or Ad48 results in improved anamnestic CD4 T cell responses compared to Ad5 vaccination after LCMV CI-13 challenge. (A) Representative FACS plots showing the percentage of GP-specific CD4 T cells that coexpress IFN- γ and TNF- α in spleen and liver following GP61-80 stimulation. (B) MFI of IFN- γ staining in spleen (left) and liver (right). GP61-80 peptide stimulations were performed for 5 h. Data are from day 19 after LCMV CI-13 challenge. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.05$. Error bars indicate SEM.

show the characteristic LCMV-induced day 3 lymphopenia (27), suggesting potential defects in T cell migration (Fig. 4B and C). LCMV-induced lymphopenia at day 3, however, was observed in alternative-serotype Ad-vaccinated and control mice (Fig. 4B and C), which also exhibited reduced frequencies of GP-specific CD8 T cells in blood at this time point (Fig. 4D).

Intriguingly, the absence of day 3 lymphopenia following LCMV CI-13 challenge in Ad5-immunized mice was also associated with reduced anamnestic expansion of GP-specific CD8 T cells. By day 19, there were 4-fold higher levels of GP-specific CD8 T cells in alternative-serotype Ad-immunized mice than in Ad5-immunized mice (mean percentage for Ad5, 12%; Ad26, 46%; Ad35, 61%; and Ad48, 54%) (Fig. 4D).

The absolute numbers of anamnestic GP-specific CD8 T cells in blood were also increased in mice vaccinated with alternative-serotype Ads compared to mice vaccinated with Ad5 (Fig. 4E). Similar recall kinetics were also observed after acute LCMV Armstrong challenge (data not shown), demonstrating that Ad26, Ad35, and Ad48 vectors elicit virus-specific CD8 T cells with greater recall capacity than Ad5 vectors.

Significantly enhanced recall T cell responses in various tissues after vaccination with alternative-serotype Ad vectors compared to those after Ad5 vector vaccination. We next analyzed the distribution of recall responses in various tissues at day 19 following LCMV CI-13 infection. Recall CD8 T cell responses were higher in every tissue tested in mice that were vaccinated with Ad26, Ad35, and Ad48 than with Ad5 (Fig. 5A). LCMV CI-13 viral challenge in alternative-serotype Ad-vaccinated mice also resulted in enhanced IFN- γ , TNF- α , and IL-2 expression in GP-specific

CD8 T cells compared to those of Ad5-vaccinated mice (Fig. 5B). GP-specific CD8 T cells elicited by Ad26, Ad35, and Ad48 showed significant coexpression of IFN- γ , TNF- α , and IL-2 after LCMV CI-13 challenge in both lymphoid and nonlymphoid tissues (Fig. 5B). There were also higher numbers of splenic GP-specific CD8 T cells in the mice that were immunized with alternative-serotype Ad vectors compared to Ad5 vectors following LCMV CI-13 challenge ($P \leq 0.02$) (Fig. 5C). Similar increases in the total numbers of GP-specific CD8 T cells were also observed in other tissues (data not shown). Expression levels of IFN- γ , TNF- α , and IL-2 were also enhanced in GP-specific CD8 T cells of mice that were immunized with alternative-serotype Ads rather than Ad5 (liver data are shown; $P < 0.05$) (Fig. 5D).

In addition, the expression of granzyme B on GP-specific CD8 T cells of Ad26-, Ad35-, and Ad48-vaccinated mice was greater than that of Ad5-vaccinated mice following LCMV CI-13 challenge (Fig. 6A and B) ($P \leq 0.02$). The degranulation potential of GP-specific CD8 T cells, as measured by CD107 staining, was also enhanced in alternative-serotype Ad-immunized mice compared to Ad5-immunized mice ($P \leq 0.03$) (Fig. 6C and D). These observations suggest that GP-specific CD8 T cells elicited by Ad26, Ad35, and Ad48 vectors exhibited superior cytotoxic and degranulation potential compared to Ad5 vectors.

We observed similar results for GP-specific memory CD4 T cell responses following LCMV CI-13 challenge. Similar to CD8 T cell responses, CD4 T cell responses specific for the immunodominant GP61-80 epitope were also enhanced in mice that were vaccinated with alternative-serotype Ad vectors rather than Ad5 (Fig. 7A and B) ($P \leq 0.05$). Taken together, these results indicate that

FIG 8 Increased PD-1 expression on anamnestic CD8 T cells in Ad5-vaccinated mice after LCMV Cl-13 challenge compared to alternative-serotype Ads. Expression of inhibitory PD-1 receptor (MFI) on GP-specific CD8 T cells in multiple tissues. Data are from day 10 after LCMV Cl-13 challenge. Data are from 3 experiments, with $n = 12$ mice per group. *, $P \leq 0.004$. Error bars indicate SEM.

Ad26, Ad35, and Ad48 vectors elicited greater recall CD8 and CD4 T cell responses than Ad5 vectors following secondary antigen challenge.

All vaccinated mice exhibited undetectable LCMV viral loads at day 10 following LCMV Cl-13 challenge (data not shown). These data suggest that the LCMV system does not have the resolution to evaluate differences in protective efficacy of the different Ad vectors, likely due to a low threshold of anamnestic T cell responses that are needed to control an LCMV Cl-13 infection following viral challenge. Nevertheless, we noted that the expression of PD-1 remained higher on anamnestic GP-specific CD8 T cells in Ad5-immunized mice than in alternative-serotype Ad-immunized mice following LCMV Cl-13 challenge (Fig. 8) ($P \leq 0.004$).

Significantly enhanced protective efficacy in mice primed with alternative-serotype Ad vectors compared with Ad5 vectors. We next developed a more stringent challenge model to assess potential differences in protective efficacy afforded by the various Ad-GP vectors utilizing a lethal dose of recombinant *Listeria monocytogenes* expressing the LCMV GP33-41 epitope. We immunized mice with the various Ad-GP vectors and boosted them with LCMV Armstrong prior to intravenous challenge

with 2×10^5 CFU Lm-GP33-41 (Fig. 9A). Ad26-, Ad35-, and Ad48-primed mice exhibited enhanced anamnestic responses compared to Ad5-primed mice (Fig. 9B), consistent with our prior results. Importantly, priming with Ad26, Ad35, and Ad48 vectors afforded complete control of *L. monocytogenes* bacterial loads to undetectable levels in liver and spleen, whereas priming with Ad5 only afforded partial efficacy ($P \leq 0.05$) (Fig. 9C). None of the Ad vectors were able to fully control the Lm-GP33-41 challenge without the common LCMV Armstrong boost, although similar trends were observed (data not shown). Thus, in this highly stringent challenge model, we observed that priming with Ad26, Ad35, and Ad48 vectors afforded significantly improved protective efficacy compared to that with Ad5 vectors, suggesting the clinical relevance of the less exhausted and more functional T lymphocyte responses elicited by the alternative-serotype Ad vectors.

DISCUSSION

In this study, we demonstrate marked differences in the phenotype and functionality of memory T cells elicited by different serotype Ad vectors. Importantly, Ad5 vectors expressing LCMV GP elicited memory CD8 T cells that appeared partially exhausted. In

FIG 9 Priming with alternative-serotype Ad vectors followed by boosting with LCMV Armstrong results in significantly enhanced immune protection following a lethal Lm-GP33-41 challenge compared with priming with Ad5 vectors. (A) Experimental outline. (B) Numbers of GP-specific CD8 T cells in blood. (C) Listeria titers in livers and spleens at day 2 following challenge. Data are from 2 experiments, with $n = 4$ mice per group. *, $P \leq 0.05$. Error bars indicate SEM. I.M., intramuscular; I.P., intraperitoneal; I.V., intravenous.

contrast, Ad26, Ad35, and Ad48 vectors expressing LCMV GP induced more functional CD8 T cell responses, increased upregulation of the lymphoid trafficking molecule CD62L and the homeostatic proliferation marker CD127, and enhanced anamnestic capacity and improved protective efficacy. CD127 is expressed on memory precursors, and its upregulation is tightly associated with long-term homeostatic maintenance (10, 11, 28). CD127 signaling on T cells has been shown to be important for the upregulation of prosurvival (antiapoptotic) molecules (29–31). As a result, higher CD127 expression on GP-specific CD8 T cells following Ad26, Ad35, or Ad48 immunization suggests enhanced memory homeostatic turnover compared to Ad5 immunization.

We also observed significant upregulation of inhibitory PD-1 on GP-specific memory CD8 T cells of Ad5-immunized mice. PD-1 has been demonstrated to be a marker for T cell exhaustion during chronic infections, such as chronic LCMV infection in mice, or HIV, hepatitis B virus, and hepatitis C virus in humans (14, 15, 18, 32). Constitutive PD-1 signaling is associated with decreased cytotoxic function during situations of antigen persistence, such as chronic infections (14, 15, 17, 18) and cancers (33, 34), although we did not detect mRNA transcripts expressed by Ad5 at memory time points by RT-PCR (data not shown).

To test the recall potential of Ad-elicited T cells, we challenged Ad-immunized mice with LCMV CI-13. Compared to Ad5-vaccinated mice, alternative-serotype Ad-vaccinated mice showed significantly enhanced recall expansion of GP-specific CD8 T cells in blood and tissues, especially in the lymph nodes and gut (Fig. 5). Since the lymph nodes and the gut are organs of active viral replication during an HIV-1 infection (35–37), this finding may be relevant for the development of an HIV-1 vaccine.

Virus-specific CD4 T cells from alternative-serotype Ad-vacci-

nated mice also showed more robust cytokine coexpression upon LCMV CI-13 challenge. CD4 T cell responses are important for sustaining virus-specific CD8 T cells during chronic infection (38–40) and for facilitating long-term humoral responses (41, 42). Moreover, HIV-1-specific CD4 T cells during the acute phase of HIV-1 infection have been associated with enhanced long-term control of HIV-1 (43, 44).

We were unable to detect differences in protective efficacy among the various serotype Ad-GP vectors following LCMV CI-13 challenge, likely due to the low stringency of this challenge model, since it has been shown that LCMV CI-13 can be completely cleared with only $\sim 10^5$ LCMV-specific T cells (45). We therefore developed a more stringent challenge model in which we challenged mice with a lethal dose of Lm-GP33-41. In this challenge model, we observed improved protective efficacy with Ad26, Ad35, and Ad48 vectors for priming compared to Ad5 vectors, suggesting the functional relevance of the different T lymphocyte phenotypes elicited by these Ad vectors.

Several factors may explain the potential superiority of alternative-serotype Ad vectors over Ad5 vectors as vaccine platforms. First, preexisting Ad5-specific neutralizing antibodies may result in virus neutralization before complete delivery of vaccine antigens to the immune system. This issue is partially circumvented with the use of more rare Ad serotypes with lower baseline vector-specific neutralizing antibody titers (1, 6). Second, since the hepatic tropism of Ad5 markedly differs from that of alternative-serotype Ads (7, 46, 47), these different viral vectors may trigger specific innate pathways that may affect the phenotype of adaptive immune responses (48–53). Moreover, Ad5 uses the CAR receptor, which is highly expressed on epithelial tight junctions, liver, and red blood cells, among other cell types (54), whereas Ad26,

Ad35, and Ad48 utilize the complement receptor CD46 (1, 7, 47, 55, 56), which is ubiquitously expressed on all nucleated cells (55, 57). These differences in tropism and receptor usage may contribute to the distinct immune responses elicited by Ad5 compared to Ad26, Ad35, and Ad48.

In summary, our data demonstrate that alternative-serotype Ad vectors elicit memory T cell responses with increased functionality and improved recall potential compared to Ad5 vectors. Alternative-serotype Ad vectors also offer the advantage of circumventing high baseline Ad5 neutralizing antibody titers. Whether the partial T cell exhaustion that is observed after vaccination with Ad5 vectors will also be seen with other Ad serotypes that utilize the CAR receptor remains to be determined. Ongoing and future clinical trials will assess whether alternative serotype Ad vectors will prove immunogenic and protective against HIV-1 and other pathogens in humans.

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