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J Immunol 2013; 190:2720-2735; Prepublished online 6 February 2013;
doi: 10.4049/jimmunol.1202861
http://www.jimmunol.org/content/190/6/2720

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/02/06/jimmunol.1202861.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Comparative Analysis of the Magnitude, Quality, Phenotype, and Protective Capacity of Simian Immunodeficiency Virus Gag-Specific CD8+ T Cells following Human-, Simian-, and Chimpanzee-Derived Recombinant Adenoviral Vector Immunization


Recombinant adenoviral vectors (rAds) are the most potent recombinant vaccines for eliciting CD8+ T cell–mediated immunity in humans; however, prior exposure from natural adenoviral infection can decrease such responses. In this study we show low seroreactivity in humans against simian- (sAd11, sAd16) or chimpanzee-derived (chAd3, chAd63) compared with human-derived (rAd5, rAd28, rAd35) vectors across multiple geographic regions. We then compared the magnitude, quality, phenotype, and protective capacity of CD8+ T cell responses in mice vaccinated with rAds encoding SIV Gag. Using a dose range (1–109 particle units), we defined a hierarchy among rAd vectors based on the magnitude and protective capacity of CD8+ T cell responses, from most to least, as: rAd5 and chAd3, rAd28 and sAd11, chAd63, sAd16, and rAd35. Selection of rAd vector or dose could modulate the proportion and/or frequency of IFN-γ+TNF-α+IL-2+ and KLRG1+CD127+CD8+ T cells, but strikingly ∼30–80% of memory CD8+ T cells coexpressed CD127 and KLRG1. To further optimize CD8+ T cell responses, we assessed rAds as part of prime-boost regimens. Mice primed with rAds and boosted with NYVAC generated Gag-specific responses that approached ∼60% of total CD8+ T cells at peak. Alternatively, priming with DNA or rAd28 and boosting with rAd5 or chAd3 induced robust and equivalent CD8+ T cell responses compared with prime or boost alone. Collectively, these data provide the immunologic basis for using specific rAd vectors alone or as part of prime-boost regimens to induce CD8+ T cells for rapid effector function or robust long-term memory, respectively. The Journal of Immunology, 2013, 190: 2720–2735.

Most approved vaccines against viral and bacterial infections mediate protection through Ab production. In contrast, there are no highly effective vaccines for infections in which Th1 CD4+ T cells, CD8+ T cells, or both play critical roles in pathogen control or elimination, such as Mycobacterium tuberculosis infection (Tb), malaria, or HIV (1–3). The development of vaccines capable of generating potent and durable T cell immunity has been limited by the availability of suitable vectors and adjuvants. Accordingly, replication-deficient recombinant adenoviral vectors (rAds) have held great promise based on their ability to generate strong T cell immunity in mice, nonhuman primates (NHPs), and humans (4–8). As a reflection of their potential importance, rAds have been and are being tested in a number of clinical vaccine studies against HIV, Tb, and malaria (6, 7, 9–13). The vaccine vector based on adenovirus serotype 5 (rAd5) has been the most comprehensively studied rAd in humans and was the first to be assessed in clinical efficacy trials against HIV (6, 7).

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Received for publication October 12, 2012. Accepted for publication January 8, 2013.

This work was supported in part by a grant from the Foundation for the National Institutes of Health with support from Collaboration for AIDS Vaccine Discovery Award OPP1039775 from the Bill and Melinda Gates Foundation.

Abbreviations used in this article: BFA, brefeldin A; Env, gp140 envelope protein; HAd, human adenovirus; HVTN, HIV Vaccine Trials Network; KLRG1, killer cell lectin-like receptor subfamily G member 1; LCMV, lymphocytic choriomeningitis virus; ListeriaGag, recombinant Listeria monocytogenes expressing SIV Gag; MPEC, memory precursor effector cell; MVA, modified vaccinia virus Ankara; NHP, nonhuman primate; PU, particle unit; rAd, recombinant adenoviral vector; rVACV:Gag, recombinant vaccinia virus expressing SIV Gag; SLEC, short-lived effector cell; Tb, Mycobacterium tuberculosis infection.

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The online version of this article contains supplemental material.
However, the clinical utility of rAd5 may be limited in populations that are key targets for HIV, malaria, and Tb vaccines, such as sub-Saharan Africa, owing to high prevalence of pre-existing immunity from prior natural infection (4, 14). Prior immunity to rAd5 has been shown to decrease Ag expression presumably by inhibiting infection of target cells, leading to suboptimal conditions for induction of immune responses (6, 13, 15–17), particularly within the CD8+ T cell compartment (17). Moreover, prior immunity to rAd5 may transiently increase the relative risk of infection with HIV through undefined mechanisms (18–20). To circumvent these potential limitations, a major research goal has been to develop rAd vectors from lower seroprevalence human-derived adenoviruses (4, 21, 22) or from nonhuman sources, such as monkeys and apes (23–26). These nonhuman vectors can minimize issues of seroprevalence but potentially retain mechanisms of adenoviral immune activation and potency. There are 65 serologically distinct adenoviruses that have been isolated from humans (HAd) and they can be organized into at least seven subgroups, denoted by the letters A–G (27, 28). Sequencing information of the common hexon gene can also be used to classify animal-derived adenoviruses into these same subgroups. The rAd5 vector was derived from an HAd in subgroup C (29), the rAd35 vector from a subgroup B virus (21), and the rAd26 and rAd28 vectors from subgroup D viruses (4, 22). HAdB-35 exhibits much lower seroprevalence than does HAdC-5 globally (4, 14, 21), whereas exposure rates to HAdD-26 and HAdD-28 are low in the United States but marginally higher in target populations for Tb, malaria, and HIV vaccines (14, 22). The rAd5 vector has been evaluated in numerous preclinical studies, as have rAd35, rAd26, and rAd28 to a lesser extent, and a hierarchy has emerged according to which rAd5 induces the most robust CD8+ T cell responses, followed by rAd26/rAd28 and then rAd35 (4, 5, 22). More recently, a number of simian- and chimpanzee-derived rAds have also been developed. The simian-derived vectors, sAd11 and sAd16, were developed from monkey adenovirus strains, but their phylogenetic classification based on the human subgrouping system has not yet been defined and their seroprevalence in human populations is unknown. Ertl, Wilson, and colleagues (23, 24, 30, 31) were the first to report on the potency of chimpanzee-derived rAds and, more recently, chAd3 and chAd63 have been developed and used in clinical studies (25, 26, 32). Hexon sequencing suggests that chAd3 and chAd63 classify into subgroups C and E, respectively (26). The chAd3 vector is of particular interest, as it clusters by phylogeny in the same subgroup as rAd5. It has also been evaluated in clinical trials and shown to prime robust T cell responses against hepatitis C virus to levels consistent with protective immunity (32). Preliminary assessment of both of these chimpanzee-derived rAds has demonstrated low seroprevalence in a European human population (26), although seroreactivity to chimpanzee-derived rAds can be higher in sub-Saharan Africa where chimpanzees are endemic (33). The in vitro activity or phylogenetic similarities of novel vectors compared with established rAd vectors has been used to make predictions about how these vectors will behave in vivo (21, 22, 26). However, there has not been a comprehensive comparative analysis in a single study using the same Ag insert to characterize the magnitude, quality, phenotype, and protective capacity of CD8+ T cells using human-, simian-, and chimpanzee-derived rAd vectors in a prime and/or boost setting.

In this study, we first evaluate rates of seroreactivity against the seven vectors, that is, rAd5, rAd28, rAd35, sAd11, sAd16, chAd3, and chAd63, across different vaccine target populations, demonstrating that simian- and chimpanzee-derived vectors have very low seroprevalence. Using a mouse model, we then directly compare rAds over a broad dose range with respect to the magnitude, quality, phenotype, and protective capacity of CD8+ T cells elicited using SIV Gag as the target Ag. This approach illustrates that titration of vectors is critical to correlate results obtained for immunogenicity and protection in mouse models with response hierarchies observed in NHP and human clinical studies. Importantly, we show that rAd5 and chAd3 vectors are similarly protective in a CD8+ T cell–dependent liseterial infection model, consistent with their phylogenetic similarities. We also demonstrate qualitative and phenotypic differences in CD8+ T cells induced across rAd vectors and doses, and we show that rAd vaccination induces a substantial population of cells at memory that coexpress CD127 and killer cell lectin-like receptor subfamily G member 1 (KLRG1). Finally, we assessed rAds both as prime vaccines with a heterologous pox-derived vector boost and as boost vaccines after priming with DNA or a heterologous rAd vector. The data demonstrate the versatility of rAds, which were effective as primes or boosts, in comparison with the pox vector, which was an ineffective prime but a robust boost for CD8+ T cell responses. These insights should inform a rational approach for using rAd vectors in prime-boost regimens to optimize robust and durable CD8+ T cell immunity, which is critical for the development of preventive and therapeutic vaccines against a variety of infections.

Materials and Methods

Adenovirus serum neutralization assay
Sera from volunteers were collected in accordance with local Institutional Review Board approvals and evaluated to determine the relative concentration of Ad-neutralizing Abs using the method described by Sprangers et al. (34). Briefly, sera were heat inactivated for 60 min at 56°C and serially diluted (covering a final sample dilution range from 1:12 to 1:8748) in a final sample volume of 50 μl D10 (DMEM supplemented with 10% heat-inactivated FBS, penicillin at 100 U/ml, and streptomycin at 100 μg/ml). An optimized dilution of rAd vector, each encoding a luciferase reporter gene, was added to each well in a volume of 50 μl. The rAd and sera were coincubated for 30 min at room temperature followed by addition of 1 × 105 A549 cells (human lung carcinoma), or 293T/17 cells for chAd63, per well in 100 μl D10. The samples were incubated at 37°C in 5% CO2 for 24 h. To evaluate luciferase activity, cells were pelleted and resuspended in 100 μl Glo lysis buffer (Promega) after removal of the culture medium. The cell suspension was transferred to a black-and-white Isolplate (PerkinElmer) and 100 μl Steady-Glo luciferase assay system reagent (Promega) was added per well. After incubation for 15 min at room temperature, luminescence was measured on a luminometer. The 90% inhibition serum titer was determined to be the serum dilution that could be interpolated to have 10% of the maximum luciferase activity, as determined by the assay run without the presence of a serum sample.

Mice
C57BL/6 mice, for use with vectors encoding SIV Gag, or BALB/c mice, for use with vectors encoding HIV gp140 envelope protein (Env), were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at the Vaccine Research Center Biomedical Research Unit (Bethesda, MD). Mice were 6–12 wk old at the time of vaccination. All experimental animal protocols were approved by the Vaccine Research Center Animal Care and Use Committee.

Vectors and vaccinations
The vector stocks were grown and purified with a two-step cesium chloride purification protocol and stored at −70°C or lower. Virus stocks were titrated to determine the particle units (Pu) per milliliter via HPLC. rAd5, rAd28, rAd35, sAd11, and sAd16 expressing SIV Gag were obtained from GenVec (Gaithersburg, MD). sAd11 and sAd16 vectors were derived from the wild-type viruses simian adenovirus 11 (ATCC VR-196) and simian adenovirus 16 (ATCC VR-944). Vectors were derived, built, and produced as described previously for rAd28 and rAd35 (22, 35). chAd3 and chAd63 backbones were obtained from OkaIors (Rome, Italy) and SIV Gag was cloned into these vectors before purification of viral particles as previously described (25, 26). All rAd vectors were rendered replication deficient through targeted deletion of the E1 adenoviral gene, although the E3 gene was additionally deleted in chAd3 and chAd63 and E3 and E4 genes in...
rAd5. The SIV Gag gene was inserted into the E1 locus for all constructs and is under the control of the CMV promoter. NYVAC expressing SIV Gag, DNA encoding SIV Gag (provided by Zhi-Yong Yang, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), rAd28 encoding SIV Gag, and modified vaccinia virus Ankara (MVA) encoding HIV Env were used for prime-boost experiments. All vectors, except DNA, were prepared in PBS and the indicated doses were administered s.c. in the rear footpads, given as a 100-μl dose split into 50 μl per footpad. DNA was prepared in PBS and given i.m. as a 100-μl dose split into 50 μl per gluteal muscle; two doses were administered 3 wk apart. All vectors contained a codon-optimized version of Gag/Pol from SIV strain mac239, except for the rAd vectors and MVA used in Fig. 7E–G and Supplemental Fig. 1C, 1D, which contain Env from the HIV hybrid strain IIIb/Bal.

**Tetramer staining**

Peripheral blood was harvested and RBCs were lysed using ACK lysis buffer (Lonza, Basel, Switzerland). Splenocytes were harvested, homogenized to single-cell suspensions, and RBCs were lysed using ACK lysis buffer (Lonza). Cells were washed in PBS and stained with Live/Dead Fixable Red viability dye (Life Technologies, Grand Island, NY). Subsequently, cells were stained with PE-labeled H-2D^d^ tetramer loaded with the immunodominant SIV Gag peptide AL11 (A1VKKNMWTQTL) (16) or PE-labeled H-2D^d^ tetramer loaded with the immunodominant HIV Bal Env peptide PA9 (IGPGRAFYA) (26). After blocking with anti-FcγRIII Ab (clone 2.4G2; 5 μg/ml; BD Pharmingen, San Diego, CA), cells were surface stained with anti–CD8-allophycocyanin (clone 53-6.7; BD Pharmingen) to confirm depletion of target T cell populations. Depleted cells were then fixed and permeabilized using the Fix/Perm and Perm/Wash buffer system (BD Pharmingen, San Diego, CA) and stained with anti–CD3-PerCP-Cy5.5 (clone 145-2C11; BD Pharmingen). Finally, 100 μl tetramethylbenzidine one-step substrate system (Dako, Carpinteria, CA) was added followed by 100 μl 2 N H_2SO_4. Absorbance was measured at 450 nm and the endpoint titer for each dilution series was calculated as 3 SDs above the mean of the PBS-vaccinated control group.

**Infections and Ab-mediated depletions**

For infection challenge, we used either attenuated *Listeria monocytogenes* (*ΔactA, ΔintB*) or vaccinia virus (thymidine kinase-deficient Western Reserve strain), each expressing Gag from SIV strain mac239. Recombinant *L. monocytogenes* expressing SIV Gag (*Listeria* Gag) was provided by ANZA Therapeutics (Concord, CA) and recombinant vaccinia virus expressing SIV Gag (rVACV-Gag) was provided by Glennys Reynoso (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). A 2 × 10^6^ CFU dose of *Listeria* Gag was i.v. administered in a 300-μl volume. Spleens were harvested 42 h later, mechanically homogenized in 1 ml PBS using a TissueRuptor (Qiagen, Valencia, CA), and plated in duplicate as a 10-fold dilution series on brain heart infusion agar (Difco, Detroit, MI). Plates were incubated at 37°C for 24–32 h before colonies were counted and back-calculated to yield values for total CFU per spleen. Alternatively, a 6.5 × 10^6^ PFU dose of rVACV-Gag was intranasally administered to anesthetized mice in a 50-μl volume. Mice were subsequently bled individually and weighed daily to assess infection-induced weight loss until 6 d postinfection.

For Ab-mediated depletion of T cells, 1 mg control (clone J11.2; rat IgG2b, anti-influenza NP), anti–CD4 (clone GK1.5; rat IgG2b), or anti–CD8 (clone 2.43; rat IgG2b) Ab was administered i.p. 3 d before infection with *Listeria* Gag. Mice were bled the day before infection and stained with noncompeiting Abs targeting CD4 (clone RM4-4) and CD8 (clone 53-6.7) to confirm depletion of target T cell populations. Depleting Abs were provided by Fred Finkelman (University of Cincinnati, Cincinnati, OH).

**Statistical analysis**

Statistical significance was calculated using a two-tailed Student *t* test for qualitative and phenotypic data using SPICE software or a two-tailed Mann–Whitney *U* test for all other data using Prism software.

**Results**

Low seroreactivity against simian- and chimpanzee-derived rAd vectors in vaccine target populations

Previous studies have established that there is high and modest pre-existing immunity to rAd5 and rAd26, respectively, in geographic areas where vaccines against HIV, malaria, and Tb are required, such as sub-Saharan Africa (4, 14). In contrast, although there is limited prior exposure to rAd35 globally, it is the least immunogenic human-derived rAd, particularly within the CD8^+^ T cell compartment (4, 14).

To assess the potential utility of novel simian- and newly described chimpanzee-derived vectors as vaccine candidates for the induction of CD8^+^ T cell immunity, we examined seroreactivity in cohorts of adults from several geographic regions where such vectors might be used: the United States, South America, the Caribbean, India, Southern Africa, Eastern Africa, and Central Africa. This was evaluated using a serum neutralization assay with a panel of representative rAd vectors expressing the luciferase reporter gene, including rAd5, rAd28, rAd35, sAd11, sAd16, chAd3, and chAd63 (Fig. 1). Neutralization of the rAd26 vector was also evaluated and was similar to rAd28 (data not shown). Seroreactivity directed against rAd5 was common, with titers >1000 (represented by red sections in the pie graphs) observed in ~50% of individuals; rAd28 exhibited somewhat lower titers, and rAd5 was the least seroreactive, consistent with prior studies. Titers were markedly lower across populations for all simian- and chimpanzee-derived vectors compared with rAd5, rAd28, and even rAd55, with sAd11 and chAd63 displaying the lowest titers. Collectively, these data suggest that multiple simian- or chimpanzee-derived rAds could be useful in human vaccine target populations, depending on their potency.
Development of SIV Gag-specific CD8+ T cell immunity after rAd vaccination

To determine the relative potency of human-, simian-, and chimpanzee-derived rAds for induction of CD8+ T cell responses, mice were vaccinated subcutaneously with $1 \times 10^9$, $1 \times 10^8$, or $1 \times 10^7$ PFU each vector. The vectors each expressed full-length Gag Ag from SIV strain mac239, which contains the immunodominant MHC class I epitope denoted AL11 (16). Gag-specific CD8+ T cell responses in peripheral blood were followed over time using an MHC class I tetramer loaded with the AL11 peptide. As shown in Fig. 2A, rAd5 induced robust and comparable CD8+ T cell responses at all doses. In contrast, responses fell below the limit of detection in some individual samples for mice vaccinated either with rAd35 at the two lower doses or with rAd28 at $1 \times 10^7$ PFU (Fig. 2A). The simian- and chimpanzee-derived vectors induced substantial CD8+ T cell responses at all doses (Fig. 2A), with some decrease in magnitude for sAd11, sAd16, and chAd63 at the $1 \times 10^7$ PFU dose. For these vectors, the effect of dose titration manifested primarily as a delay in response kinetics with a lower peak magnitude. A comparison of CD8+ T cell responses induced by all vectors at the $1 \times 10^7$ PFU dose (Fig. 2B) revealed a clear hierarchy between vectors as follows, from most to least potent: rAd5; chAd3, sAd11 and chAd63; sAd16; rAd28; and lastly rAd35.

SIV Gag-specific cytokine production by CD8+ T cells

To extend the immunologic analysis, intracellular cytokine staining and multiparameter flow cytometry were used to assess the magnitude of Gag-specific CD8+ T cell cytokine responses following rAd vaccination at peak and memory time points. In our analysis of peripheral blood, peak responses varied from day 14 to 28 for different rAds and different doses (Fig. 2A). Therefore, the time point used to approximate the “peak” for all vectors and doses was 23 d after vaccination, but this may antecede or precede maximal responses, particularly in mice that received the lowest dose of $1 \times 10^7$ PFU. Additionally, the “memory” time point used was 70 d after vaccination, but later time points (>100 days) have been performed with $1 \times 10^8$ PFU, and similar results to the following were observed (data not shown).

Analysis of total Gag-specific cytokine (IFN-γ, IL-2, or TNF-α) production by splenocytes after in vitro stimulation with the AL11 peptide revealed a pattern similar to the hierarchy observed in the peripheral blood with tetramer staining. At $1 \times 10^9$ PFU, all vectors...
induced high-frequency CD8+ T cell responses at both peak (Fig. 3A, 3B) and memory (Fig. 3C, 3D) that were generally comparable to rAd5. At 1 x 10^11 PU, all vectors again induced potent CD8+ T cell responses, with the exception of rAd35; in this case, responses could not be detected at peak and were significantly lower at memory. At 1 x 10^7 PU, rAd5 still induced robust CD8+ T cell responses at peak and memory. In comparison, rAd28 and sAd16 were significantly lower at peak, chAd63 was significantly lower at memory, and rAd35 did not exhibit detectable responses at either peak or memory. Although chimpanzee-derived vectors induced responses comparable to or even greater than rAd5 at peak at all doses, these responses contracted substantially by memory, which mirrors the contraction of Gag-specific CD8+ T cell responses observed in the peripheral blood with tetramer staining (Fig. 2). We also assessed tetramer staining and cytokine responses in the lung for all vectors at 1 x 10^8 PU at peak and memory time points and a similar hierarchy was observed (data not shown). Thus, a consistent hierarchy of immunogenicity across rAd vectors emerges from both Ag-specific cytokine production and tetramer staining across various tissues.

Multiple mechanisms could account for differences in the potency and durability of CD8+ T cell responses after rAd vaccination. CD4+ T cells can influence CD8+ T cell maintenance and expansion (38, 39) and have been shown to augment CD8+ T cell responses after rAd vaccination (40). CD4+ T cell responses primed by rAd vectors encoding SIV Gag in C57BL/6 mice were detectable but low at peak (Supplemental Fig. 1A, 1B) and undetectable by memory time points (data not shown). Generally, responses were low and variable for rAd5, rAd28, rAd35, sAd11, and sAd16, but chimpanzee-derived vectors induced modest responses that were significantly higher than rAd5 at 1 x 10^8 or 1 x 10^7 PU (Supplemental Fig. 1B). Similar results were obtained with rAdS encoding HIV Env as a target Ag in BALB/c mice (Supplemental Fig. 1C, 1D). We also assessed SIV Gag-specific

FIGURE 2. Tetramer+CD8+ T cell responses in peripheral blood after vaccination with dose titrations of rAd vectors. C57BL/6 mice (n = 4–5) were vaccinated with 1 x 10^6, 1 x 10^7, or 1 x 10^8 PU of the indicated rAd vector expressing SIV Gag. Peripheral blood samples were analyzed sequentially at days 14, 21, 28, 35, and 70 using tetramer staining to identify SIV Gag-derived AL11-specific cells. (A) Longitudinal comparison of frequencies of CD8+ T cells that were tetramer+ after vaccination with the indicated dose of each vector. (B) Longitudinal comparison for each vector at the lowest dose (1 x 10^7 PU), where rAd5 was compared with rAd28, rAd35, sAd11, and sAd16 (left panel) or compared with chAd3 and chAd63 (right panel) in independent experiments. Data points and error bars represent mean ± SEM. Each time course is representative of at least two independent experiments. The p values for each vector at 1 x 10^7 PU compared with rAd5 are displayed in the table: *p ≤ 0.05, **p ≤ 0.01.
MHC class I and II epitopes from SIV Gag in C57BL/6 mice. The frequencies of CD8+ T cells that produced cytokine in response to Ag (i.e., stained in independent experiments. At day 23 after vaccination, splenocytes were processed for in vitro stimulation with peptides encoding the immunodominant positive for IFN-γ experiments. Significant differences in frequency were assessed for each vector compared with rAd5 at the equivalent dose; *p ≤ 0.05.

Ab responses and observed a hierarchy between rAds similar to that observed for CD8+ T cells at both peak and memory (Supplementary Fig. 1E–H). Overall, we cannot attribute differences in the potency of CD8+ T cell responses after vaccination with rAds to differences in the corresponding CD4+ T cell responses because, in this model, rAd vaccination strongly biases toward induction of CD8+ as opposed to CD4+ T cell immunity.

**Qualitative profiles of SIV Gag-specific memory CD8+ T cells**

After evaluating CD8+ T cell responses induced by rAd vectors in terms of total magnitude, we then evaluated the quality of the response in terms of IFN-γ, TNF-α, and IL-2 coexpression. The term “quality” refers to functional markers (cytokine expression, phenotypic or cytolytic markers) possessed by individual T cells (41–43). T cells with multiple markers are designated “multifunctional,” can secrete higher amounts of cytokine per cell, and their presence correlates with protection in vaccine models of CD4+ T cell immunity and disease nonprogression in HIV infection (42, 43); thus, responses that induce a higher proportion of multifunctional cells may be predictive of cellular fate. A well-established model for

![Graph showing cytokine production by CD8+ T cells in the spleen 23 d (peak) and 70 d (memory) after rAd vaccination.](http://www.jimmunol.org/)

**FIGURE 3.** Ag-specific cytokine production by CD8+ T cells in the spleen 23 d (at peak) (**A**, **B**) or 70 d (memory) (**C**, **D**) after rAd vaccination. C57BL/6 mice (n = 4–5) were vaccinated with 1 × 10⁹, 1 × 10⁸, or 1 × 10⁷ PU rAd5, rAd28, rAd35, sAd11, sAd16 (A, C), or rAd5, chAd3 or chAd63 (B, D) in independent experiments. At day 23 after vaccination, splenocytes were processed for in vitro stimulation with peptides encoding the immunodominant MHC class I and II epitopes from SIV Gag in C57BL/6 mice. The frequencies of CD8+ T cells that produced cytokine in response to Ag (i.e., stained positive for IFN-γ, TNF-α, or IL-2) were determined. Bars and error bars represent mean ± SEM. Each group is representative of at least two independent experiments. Significant differences in frequency were assessed for each vector compared with rAd5 at the equivalent dose; *p ≤ 0.05.

Individual CD8+ T cells also possess distinct phenotypic profiles that may be predictive of cellular fate. A well-established model for...
classification of effector and memory CD8+ T cells in mice is based on expression of CD127, the IL-7 receptor α-chain, and KLRG1 (44, 45). In the lymphocytic choriomeningitis virus (LCMV) infection mouse model, CD8+ T cells that expressed KLRG1 without coexpression of CD127 were shown to be terminally differentiated effector cells and designated as short-lived effector cells (SLECs) (45). Conversely, CD8+ T cells that expressed CD127 without coexpression of KLRG1 were longer lived and more likely to contribute to the subsequent memory population, and so were termed memory precursor effector cells (MPECs) (44).

By applying these markers to SIV Gag-specific CD8+ T cells, we observed substantial populations of both CD127-KLRG1+ SLECs (yellow; 42%) and CD127+KLRG1+ MPECs (dark gray; 21.7%) 23 d after rAd5 vaccination (Fig. 5A). Strikingly, we also observed a substantial population of Gag-specific CD8+ T cells coexpressing CD127 and KLRG1 (intermediate gray; 29%) (Fig. 5A). Unlike the SLEC and MPEC populations, the potential contribution of the CD127+KLRG1+ population to either immediate effector function or memory development after primary vaccination is not well defined.
Phenotypic assessment of CD8+ T cells in the spleen 23 d (peak) and 70 d (memory) after rAd vaccination. C57BL/6 mice (n = 4–5) were vaccinated with 1 × 10^9, 1 × 10^8, or 1 × 10^7 PU of the indicated rAd vector expressing SIV Gag. At days 23 and 70, splenocytes were tetramer stained to identify SIV Gag-derived AL11-specific cells. Boolean gating was used to define subsets of Gag-specific CD8+ T cells expressing any of the four possible combinations of CD127 and KLRG1. (A) Representative plots after rAd5 vaccination to illustrate gating of CD8+ T cells for phenotypic analysis. CD3+ lymphocytes were gated on CD8+ events (left plot), then tetramer+ events (middle plot), and finally assessed for KLRG1 and CD127 expression (right plot). Gag-specific CD8+ T cells expressing KLRG1 but not CD127 are termed SLECs and cells expressing CD127 but not KLRG1 are termed MPECs. (B and C) The frequency of total CD8+ T cells (B) and proportion of Gag-specific CD8+ T cells (C) that express each combination of CD127/KLRG1 at day 23 after rAd5 vaccination at each dose. (D and E) The frequency of total CD8+ T cells (D) and proportion of Gag-specific CD8+ T cells (E) that express each combination of CD127/KLRG1 at day 70 after rAd5 vaccination at each dose. (F and G) The frequency of total CD8+ T cells (Figure legend continues)
The frequency and relative proportions of these populations at peak and memory after rAd5 vaccination were then determined. At peak, SLECs were the predominant population induced at all doses (Fig. 5B, 5C). By memory, the frequency of total Gag-specific CD8+ T cells had contracted markedly owing to a striking reduction in the frequency of SLECs and a modest reduction in MPECs (Fig. 5D). This is most clearly seen using the relative proportions of each cell population, where there is a marked decrease in SLECs from peak to memory (Fig. 5C, 5E). In contrast, the frequency of CD127+KRLG1+CD8+ T cells was well maintained (Fig. 5B, 5D), and thus the overall proportion of this population increased over time (Fig. 5C, 5E). These data show that rAd vaccination induces a large CD8+ T cell population that coexpresses CD127 and KLRG1, which is at least as stable as the MPEC population.

We also observed that the dose of vector could alter the phenotypic profile of the induced CD8+ T cell population. This effect was most evident for rAd5 at memory, where a substantial decrease in the proportion of SLECs was most evident for rAd5 at memory, which induced a substantial decrease in SLECs while increasing the proportion of total CD127+ cells (Fig. 5E). This effect was not shown). Overall, these data show that vector selection and dose reduction can be used to alter the phenotypic profile of CD8+ T cells to protection, mice that had been vaccinated previously with rAd5 were treated with anti-CD4 depleting Ab, anti-CD8 depleting Ab, or both prior to challenge with Listeria:Gag, rAd5-mediated protection was completely abrogated in mice treated with anti-CD8 or combined anti-CD4 and CD8 depleting Abs prior to infection (Fig. 6C), indicating that an Ag-specific CD8+ T cell response is essential for rapid vaccine-mediated control of listerial infection.

rVACV:Gag has also been used as a model to assess the protective efficacy of vaccines that elicit T cell immunity (46–48). Mice immunized with rAds were challenged intranasally with rVACV:Gag and the loss in body weight during the next 6 d was monitored to indicate disease severity (Supplemental Fig. 4). At 6 d postinfection, mice vaccinated with rAd5 had maintained their original body weight at all doses. The groups that received 1×10^9 or 1×10^8 PU sAd11, chAd3, or chAd63, or 1×10^6 PU sAd11, also maintained their weight. For rAd28, the group that received 1×10^6 PU maintained their original weight but those that received lower doses began to succumb. The rAd35 vector provided only partial protection at 1×10^9 PU, with all other doses succumbing. These data substantiate the potency of rAd5 and illustrate that this potency can extend to simian- and chimpanzee-derived rAds.

SIV Gag-specific immunity mediates protection following Listeria:Gag or rVACV:Gag challenge

There is no infectious challenge available in mice to directly model HIV infection in humans. Nonetheless, we wanted to evaluate whether Gag-specific adaptive responses at memory after rAd vaccination, and CD8+ T cells in particular, were functional in vivo. To test this, we used two infectious challenges, L. monocytogenes and vaccinia virus.

First, mice were administered an attenuated strain of L. monocytogenes that expressed SIV Gag (Listeria-Gag) 70 d after vaccination with rAd vectors. Spleen (Fig. 6) and liver (data not shown) tissue was harvested ∼42 h later to determine bacterial load. When compared with the control group, rAd5 vaccinated mice significantly reduced bacterial load (∼2.5 logs) at all doses (Fig. 6A, 6B). Mice vaccinated with rAd28 or sAd11 also significantly reduced bacterial loads at all doses compared with controls and did not differ significantly from vaccination with rAd5 at the equivalent dose, although some mice vaccinated with rAd28 or sAd11 at 1×10^8 PU exhibited higher loads, suggesting a slight loss of protection (Fig. 6A). Mice vaccinated with 1×10^7 PU rAd35 or sAd16 were protected, but protection was diminished at the 1×10^6 PU dose and bacterial loads were equivalent to controls at the 1×10^5 PU dose. Mice vaccinated with chAd3 significantly reduced bacterial load at all doses and did not differ from rAd5 at the equivalent dose (Fig. 6B). Groups vaccinated with chAd63 were protected compared with controls, but the group that received 1×10^7 PU trended toward higher bacterial loads, suggesting incomplete protection at the lowest dose (Fig. 6B). Thus, rAd5 and chAd3 consistently conferred the highest protective efficacy, followed by rAd28 and sAd11, chAd63, and finally sAd16 and rAd35.

To determine the relative contribution of CD4+ and CD8+ T cells to protection, mice that had been vaccinated previously with rAd5 were treated with anti-CD4 depleting Ab, anti-CD8 depleting Ab, or both prior to challenge with Listeria:Gag, rAd5-mediated protection was completely abrogated in mice treated with anti-CD8 or combined anti-CD4 and CD8 depleting Abs prior to infection (Fig. 6C), indicating that an Ag-specific CD8+ T cell response is essential for rapid vaccine-mediated control of listerial infection.

rAd priming followed by NYVAC boost induces potent CD8+ T cell responses

Heterologous prime-boost regimens can be used to generate high-magnitude vaccine-induced CD8+ T cell populations (49). Various regimens combining rAd vectors with other modalities, such as poxvirus-derived vectors, DNA vaccines, or heterologous rAd vectors, have been tested in NHPs and humans for the prevention of HIV and malaria (5, 11, 50–54). We therefore administered the rAd vectors as primes for a common boost or as boosts following a common prime to compare their potency in prime-boost vaccine regimens.

Priming with rAd vectors and boosting with pox vectors can induce high-magnitude CD8+ T cell responses in mice, NHPs, and humans (11, 50–54). We therefore used the pox vector, NYVAC...
expressing SIV Gag, as a common boost for the human- and chimpanzee-derived rAds. Mice were vaccinated with 1 × 10^7 PU each rAd to best model the hierarchy of rAd-primed responses in NHPs and humans and then boosted with 1 × 10^7 PFU NYVAC. Mice primed with rAd5 had a modestly higher frequency of CD8^+ T cells at the time of boost compared with the other rAd vectors (Fig. 7A). NYVAC did not induce detectable responses in unprimed mice, but it robustly boosted all rAd-primed mice that had detectable responses at the time of boost, with the frequency of Gag-specific CD8^+ T cells increasing 6- to 12-fold to ~60% of total CD8^+ T cells at peak for most rAds (Fig. 7B). The frequency of Gag-specific CD8^+ T cells after NYVAC boosting did not differ significantly between groups primed with rAd5, chAd3, and chAd63 at peak (Fig. 7B) or memory (Fig. 7C). In contrast, the rAd28-primed group was boosted by NYVAC but exhibited frequencies that were significantly lower than the rAd5-primed group at peak (Fig. 7B) and trended lower at memory (Fig. 7C). Lastly, priming with rAd35 at 1 × 10^7 PU did not prime detectable responses at the time of boost (Fig. 7A) and did not enable boosting in response to NYVAC (Fig. 7B, 7C). However, priming with higher doses of rAd35 induced responses that were potently boosted by NYVAC (Fig. 7D). Additionally, we performed experiments using rAd vectors encoding HIV Env as a target Ag and another pox vector, MVA, expressing Env as a common boost. Consistent with the results above, there was robust boosting of Env-specific CD8^+ T cell responses with all vectors (rAd5, chAd3, chAd63) that primed detectable responses at the time of boost (Fig. 7E–G). Taken together, these data show that pox vectors potently boost CD8^+ T cell responses after priming with rAds, even when priming is suboptimal.

rAd5 and chAd3 potently boost DNA or rAd28-primed responses

rAd vectors are also being evaluated in NHP and clinical trials as boosts for priming vaccines such as DNA (55–57) or heterologous rAds (5, 54). Accordingly, we compared rAd vectors as boosts after DNA or rAd28 priming. Mice were primed with either 100 μg DNA (two doses given 3 wk apart) or 1 × 10^8 PU rAd28 and then boosted 7 wk later with 1 × 10^7 PU rAd35, rAd5, or chAd3. After DNA priming, both rAd5 and chAd3 boosted to similar magnitudes (Fig. 8A). In contrast, rAd35-boosted responses were significantly lower than groups boosted with rAd5 or chAd3, although they were significantly higher than the DNA prime or rAd35 boost alone (Fig. 8A). As demonstrated above, the 1 × 10^8 PU dose of rAd35 primes CD8^+ T cell responses that are very low or undetectable by tetramer staining (Figs. 2A, 7D), so this dose may be suboptimal for boosting with rAd35. Nevertheless, in the setting of a sufficiently primed CD8^+ T cell response, rAd35 is an effective boost. By day 70 after vaccination, CD8^+ T cell responses contracted to frequencies that did not differ significantly from groups that received the vectors as boosts alone (Fig. 8B). In contrast, mice that were primed with DNA and boosted with rAd35 maintained their responses relative to peak at significantly higher levels compared with the rAd35 boost alone (Fig. 8B).

A similar pattern was evident for rAd28 priming, where rAd5 and chAd3 strongly boosted CD8^+ T cell responses at peak compared with rAd35 (Fig. 8A). Subsequently, rAd5 and chAd3 boosted responses had contracted significantly by day 70, whereas rAd35 responses were well maintained (Fig. 8B). Notably, rAd28-primed responses achieved a higher magnitude at peak and memory after boosting with any rAd vector compared with DNA-primed responses. This may reflect the higher frequencies of Gag-specific CD8^+ T cells present at the time of boosting after rAd28 compared with DNA priming. Overall, rAd5 and chAd3 similarly boosted robust CD8^+ T cell responses that then contracted, whereas rAd35 boosted to lower frequencies but effectively sustained these responses into memory.

**Discussion**

In this broad comparative analysis of seven different human-, simian-, and chimpanzee-derived rAds, a dose titration approach was used to delineate differences between vectors based on the magnitude, quality, phenotype, and protective capacity of SIV Gag-
specific CD8⁺ T cell responses. For each vector, the magnitude of CD8⁺ T cell responses at memory directly correlated with the protective capacity against *L. monocytogenes* infection. The rAd5 and chAd3 vectors induced the most robust and comparable CD8⁺ T cell–mediated protective immunity, followed by sAd11 and rAd28, chAd63, sAd16, and finally rAd35. Our conclusion that rAd5 is more potent than rAd28 and rAd35 is consistent with prior studies in mice and NHPs using rAd26 and rAd35 with SIV Gag (4, 5) and confirms the relative potency of chAd3 and chAd63 recently described in mice, NHPs, and humans (25, 26, 32). The dose titration approach also illustrated that, at the highest vector dose of 1 × 10⁹ PU, there was little ability to discriminate between the different rAd vectors in the mouse model. This is likely due to there being sufficient Ag expression at the highest dose to prime comparable responses. Indeed, the most common dose of rAd vectors used in humans is 1 × 10¹⁰ PU, which is only 10-fold higher than the highest and most protective dose for all rAds used in this study. Additionally, viral vectors target specific receptors that mediate uptake, but receptor distribution may differ from mice to humans and data generated using mouse models should be interpreted with this in mind. For example, rAd35 utilizes membrane cofactor protein (CD46) (58), which is broadly expressed across nucleated cells in humans but is limited in mice to the testes. This could apply to other vectors, because receptors and soluble mediators of rAd5 uptake are incompletely characterized (59) and the primary receptors for rAd28 (22, 60) and for the simian- and chimpanzee-derived adenoviruses have not been defined. Nevertheless, this study was able to replicate the hierarchy between rAd5, rAd28, and rAd35 seen in NHPs and humans and thus illustrates that preclinical mouse studies at lower doses provide predictive value for CD8⁺ T cell immunity in humans.

### FIGURE 7. Assessment of priming by rAd vectors for a common boost. C57BL/6 mice (*n* = 5) were primed with 1 × 10⁷ PU rAd vectors and boosted 8 wk later with 1 × 10⁷ PFU NYVAC. At the indicated time points, SIV Gag-derived AL11-specific CD8⁺ T cells were quantified in peripheral blood by tetramer staining. (A) Frequency of CD8⁺ T cells that were tetramer⁺ at the time of boost, 8 wk after priming. (B) Frequency of CD8⁺ T cells that were tetramer⁺ at the peak of the response to the boost, 2 wk after boosting. (C) Frequency of CD8⁺ T cells that were tetramer⁺ at memory, 10 wk after boosting. (D) Mice were primed with 1 × 10⁹, 1 × 10⁸, or 1 × 10⁷ PU rAd35 and boosted with 1 × 10⁷ PFU NYVAC. Gag-specific CD8⁺ T cells were quantified by tetramer staining over time after boosting. Alternatively, BALB/c mice (*n* = 4–5) were primed with 1 × 10⁷ PU of the indicated vectors and boosted 4 wk later with 1 × 10⁷ PFU MVA. A group vaccinated with chAd3 expressing SIV Gag was used as a negative control for Env priming. At the indicated time points, HIV Env-derived PA9-specific CD8⁺ T cells were quantified in peripheral blood by tetramer staining. (E) Frequency of CD8⁺ T cells that were tetramer⁺ at the time of boost. (F) Frequency of CD8⁺ T cells that were tetramer⁺ at the peak of the response to the boost, 2 wk after boosting. (G) Frequency of CD8⁺ T cells that were tetramer⁺ at memory, 10 wk after boosting. Bars or points on the line graph and error bars represent mean ± SEM. Each group is representative of at least two independent experiments. Significant differences in frequency were assessed compared with rAd5 primed animals; *p* ≤ 0.05.
cines or in prime-boost regimens. There is clear evidence that vaccine-mediated protection against malarial or SIV infection correlates with the induction of higher magnitude CD8^+ T cell responses (61–63). A stand-alone vaccine must achieve this magnitude in one vaccination, whereas prime-boost regimens can attain a similar or much higher magnitude in a stepwise manner over a prolonged period of time (49). rAd5 has been the most widely studied and remains the most potent rAd vector for inducing high magnitude CD8^+ T cell responses with a single immunization. Recent evidence suggests that T cell responses induced by rAd5 display a more differentiated profile based on surface markers (64) or qualitative assessments of cytokine production compared with other vaccine platforms (5, 43). Additionally, CD8^+ T cells induced by rAd5 vaccination are less proliferative on a per cell basis compared with CD8^+ T cells induced by L. monocytogenes, vaccinia virus, or LCMV infection and display an “exhausted” gene expression profile compared with LCMV (S. Sarkar, V. Kalia, and R. Ahmed, personal communication). Models for the differentiation state of T cells propose that cells sequentially lose functions, such as the production of IL-2 and TNF-α, after successive or prolonged Ag encounters, becoming terminally differentiated cells that produce IFN-γ only (43) or potentially exhausted cells (65, 66). Exhausted Ag-specific CD8^+ T cells are identified through high expression of markers, such as programmed death-1 (67) and KLRG1 (68, 69), or functional assessments, such as diminished proliferative capacity (65, 66). Our study shows that vaccination with rAd5 at 1 × 10^8 PU induced a higher proportion Gag-specific CD8^+ T cells that were IFN-γ- (1+) or IFN-γ-TNF-α (2+) rather than multifunctional IFN-γ/IL-2/TNF-α (3+) cells and a higher proportion of cells that expressed KLRG1 without CD127 coexpression. Taken together, these data suggest a more differentiated functional and memory phenotype, which is likely due to low-level Ag that can persist after rAd5 vaccination (17, 64, 70, 71). Nevertheless, the high-magnitude CD8^+ T cell response induced by rAd5 in this study was sufficient to rapidly control pathogen load after infection, regardless of qualitative or phenotypic profiles of the CD8^+ T cell population. Similarly, vaccination of NHPs with a 1 × 10^{10} PU dose of rAd5 but not rAd26 or rAd35 confers protection against Ebola, a rapidly progressing viral infection, by inducing a high magnitude CD8^+ T cell response (72, 73). Overall, when a stand-alone vector is required, high doses of rAd vectors such as rAd5 or chAd3 are capable of inducing high-magnitude CD8^+ T cell responses that may mediate rapid control of infections. Alternatively, lower doses of rAd vectors can improve the qualitative and phenotypic profiles of CD8^+ T cell responses, albeit at reduced magnitudes. Further work is needed to establish whether the favorable phenotypic and qualitative profiles induced with lower doses of rAds are beneficial for boosting of primed CD8^+ T cells. However, lowering the dose of rAds for priming purposes runs the risk of inducing CD8^+ T cell responses below a threshold necessary for efficient heterologous boosting, particularly in a diverse human population.

A striking finding in this study is that rAd vaccination induces a substantial population of CD8^+ T cells expressing both CD127 and KLRG1 at memory. The original study by Joshi et al. (45), which assessed expression of both CD127 and KLRG1 on CD8^+ T cells, defined CD127^+KLRG1^- and CD127^+KLRG1^- CD8^+ T cells as SLEC and MPEC populations, respectively, but there was limited analysis of CD127^-KLRG1^-CD8^+ T cells. This population has been observed at low levels in various vaccine and infection models (74, 75). In one study, a high frequency of CD127^-KLRG1^-CD8^+ T cells was detected after prime-boosting, and these cells were shown to have a modestly lower proliferative potential compared with MPECs (76). Consistent with prior reports, we observed that the frequencies of SLEC were highest at peak and decreased over time, whereas frequencies of MPECs and CD127^-KLRG1^- cells were relatively stable (Fig. 5B–E). These data substantiate the paradigm that SLEC are short-lived whereas MPECs are more stable, but they also suggest that CD127^-KLRG1^- cells represent a relatively stable CD8^+ T cell memory population that can be induced after primary vaccination. The degree to which different vaccines and infection models induce CD127^-KLRG1^-CD8^+ T cells is likely related to Ag persistence and/or repetitive antigenic stimulation. Thus, whereas acute LCMV infection is efficiently cleared (66, 77), prime-

![FIGURE 8.](http://www.jimmunol.org/) Assessment of boosting by rAd vectors after a common prime. C57BL/6 mice (n = 5) were primed with either 2 × 100 μg DNA, 3 wk apart, or 1 × 10^8 PU rAd28 and boosted 7 wk after initial priming with 1 × 10^9 PU of either rAd5, rAd35, or chAd3. At the indicated time points, SIV Gag-derived AL11-specific CD8^+ T cells were quantified in peripheral blood by tetramer staining. (A) Frequency of CD8^+ T cells that were tetramer^+ at the peak of the response, 2 wk after boosting. (B) Frequency of CD8^+ T cells that were tetramer^+ at memory, 10 wk after boosting. Bars and error bars represent mean ± SEM. Each group is representative of at least two independent experiments. Significant differences in frequency were assessed in primed/rAd-boosted animals compared with mice that received the equivalent rAd boost alone; *p < 0.05, **p ≤ 0.01.
induced higher magnitude CD8+ T cell responses than did two shots. Response magnitudes comparable to a single immunization with responses in humans more efficiently than DNA, as a DNA vaccine (Fig. 7D). rAd35 may be valuable as a boost (82), especially given its low rates of seroreactivity in human populations and relative amenability to large-scale production for clinical use.

As a practical concern, the development of a large array of well-characterized rAd vectors allows versatility in their clinical application. Clinical trials have been performed using rAd vectors for multiple infections, including malaria, Tb, and HIV (9, 12, 13, HVTN 077). Until recently, a limited number of rAd vectors (rAd5, rAd26, and rAd35) were available for clinical use, which complicated the application of a restricted pool of vectors to target multiple infections because prior immunity limits the potency of subsequent vaccination. The development of simian- and chimpanzee-derived rAds has provided a broader array of vectors to choose from, with low seroprevalence and considerable potency (23, 30). Accordingly, despite the constraint that prior immunity could place on repeated rAd administration, having a wide range of vectors will mitigate this issue.

In conclusion, these data can be used to both refine clinical selection of currently available rAd vectors as well as extend our understanding of mechanisms that control the potency of rAd vectors. Our data indicate that low-potency vectors such as rAd35 would not be sufficient as stand-alone vaccines for prophylactic vaccination against infections requiring CD8+ T cell immunity, but multiple rAd vectors such as rAd5 and chAd3 can efficiently induce such responses. In contrast, after priming with DNA or a heterologous rAd, all rAd vectors including rAd35 are capable of boosting CD8+ T cell immunity. Importantly, rAd vectors such as NYVAC and MVA provide truly heterologous and very robust boosting to any rAd prime and this may be the optimal combination for induction of high-magnitude CD8+ T cell responses. Finally, these data permit us to dissect requirements for induction of robust CD8+ T cell immunity. We characterized quantitative, qualitative, and phenotypic differences in CD8+ T cell immunity induced by rAd vectors, but early mechanisms after rAd vaccination responsible for subsequent induction of potent CD8+ T cell immunity remain unknown. A number of mechanisms have been suggested, including increased uptake or targeting of specialized APC subsets (83), expression of large amounts of Ag for a relatively prolonged time (17, 64, 70, 71), and robust activation of innate immune mechanisms (21, 22). In ongoing work, we are examining the mechanistic bases for differences observed in this study in potency of CD8+ T cell responses between rAd vectors by exploring differences in innate immunity, the amount and duration of Ag expression, and Ag presentation (K.M. Quinn and R.A. Seder, manuscript in preparation). Taken together, our direct comparison of rAd efficacy and the definition of mechanisms leading to potent CD8+ T cell responses will facilitate rational development of rAd-based vaccination strategies that address the unique requirements of different infections.

Acknowledgments

We acknowledge the U.S. Military HIV Research Program, the International AIDS Vaccine Initiative, and the HIV Vaccine Trials Network for provision of sera samples used to establish rAd neutralizing titers, in particular the following site investigators: Efthia Vardas (Perinatal HIV Research Unit, University of the Witwatersrand, Soweto, South Africa), Heather Jaspan (Desmond Tutu HIV Centre, University of Cape Town, Cape Town, South Africa), Gavin Churchyard (Aurum Institute for Health Research, Klerksdorp, South Africa), Ramesh Paranjape (National AIDS Research Institute, Pune, India), Jean Pape (Cornell University, New York, NY, and Centres Le Groupe Haitien d’Etude du Sarcome de Kaposi et des Infections Opportunistes, Port-au-Prince, Haiti), J. Peter Figueroa (Epidemiology Research and Training Unit, Ministry of Health, Kingston, Jamaica), Noreen Jack (Caribbean Care, Prevention, and Research Institute, Port of...
Disclosures

J.G.D.G. is an employee of GenVec, Inc. A.N., A.F., S.C., and R.C. are employees of Okairos AG. The other authors have no financial conflicts of interest.

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