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Immune Responses in Macaques to a Prototype Recombinant Adenovirus Live Oral Human Papillomavirus 16 Vaccine

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Immunization with human papillomavirus (HPV) L1 virus-like particles (VLPs) prevents infection with HPV. However, the expense and logistical demands of current VLP vaccines will limit their widespread use in resource-limited settings, where most HPV-induced cervical cancer occurs. Live oral adenovirus vaccines have properties that are well-suited for use in such settings. We have described a live recombinant adenovirus vaccine prototype that produces abundant HPV16 L1 protein from the adenovirus major late transcriptional unit and directs the assembly of HPV16 VLPs in tissue culture. Recombinant-derived VLPs potently elicit neutralizing antibodies in mice. Here, we characterize the immune response to the recombinant after dual oral and intranasal immunization of pigtail macaques, in which the virus replicates as it would in immunized humans. The immunization of macaques induced vigorous humoral responses to adenovirus capsid and nonstructural proteins, although, surprisingly, not against HPV L1. In contrast, immunization elicited strong T-cell responses to HPV VLPs as well as adenovirus virions. T-cell responses arose immediately after the primary immunization and were boosted by a second immunization with recombinant virus. T-cell immunity contributes to protection against a wide variety of pathogens, including many viruses. The induction of a strong cellular response by the recombinant indicates that live adenovirus recombinants have potential as vaccines for those agents. These studies encourage and will inform the continued development of viable recombinant adenovirus vaccines.

Live adenovirus vaccines have been used by the United States military for decades to prevent adenovirus type 4 (Ad4)- and Ad7-induced severe upper respiratory disease in recruits (1). Given orally as enteric-coated tablets, the vaccines contain lyophilized Ad4 and Ad7 that induce both humoral and cell-mediated immunity as they replicate asymptomatically in the gut of the vaccinee. With one dose, these vaccines confer long-lasting protection from Ad4 and Ad7 infection with great efficacy and exemplary safety (1). The Ad vaccines possess properties that are well-suited to the developing world, including low dosage and consequent economy of production, ease of administration, freedom from needles, and a single-dose regimen. Live recombinant adenoviruses (rAds) used in a similar manner might prove to be powerful tools for immunization against other pathogens, especially in low-resource settings.

Human papillomavirus (HPV) causes cervical cancer that kills about 275,000 women annually, predominantly in developing nations (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx?cancer=cervix). Two HPV vaccines have been licensed: Gardasil and Cervarix, which both contain HPV16 and HPV 18 virus-like particles (VLPs) composed of recombinant L1, the HPV major capsid protein. Both vaccines prevent persistent HPV infection and cervical disease induced by the HPV types included in the vaccine (2). However, about 80% of cervical cancer worldwide occurs in women in low-resource and/or remote settings who may never receive these vaccines, as the vaccines are costly and require multiple injections (3). The development of an improved HPV vaccine therefore remains a high priority and an attractive opportunity for assessing the utility of a replicating adenovirus vaccine.

To explore the applicability of the live rAd platform to other pathogens, we constructed replication-competent adenovirus recombinants that make novel use of the high-level gene expression characteristic of the adenovirus major late transcriptional unit (MLTU) to produce papillomavirus L1 proteins (4). Purified VLPs harvested from cells infected with a prototype expressing HPV16 L1 induced strong neutralizing-antibody responses in mice (4, 5). L1 expression by these recombinants requires virus replication (4), and responses to purified recombinant-derived VLPs in mice are therefore not likely to accurately predict responses to L1 produced by the recombinants as they replicate in a human vaccinee. Here, we characterize the immune responses of pigtail macaques to a prototype live recombinant HPV vaccine prepared using an Ad5 host range mutant that replicates in nonhuman primates. HPV exhibits strict host tropism and does not induce disease in monkeys. Therefore, we examined the immunologic surrogates used in humans and animal models in order to evaluate HPV L1 VLP vaccines (6–8).

MATERIALS AND METHODS

Virus. Ad5*hr*-FFIL[16] (*hr*, host range; FFIL, fiber-fiber-internal ribosome entry site-L1) Fig. 1A) has been described elsewhere (5). The recombinant is fully replication competent, expresses the HPV16 L1 gene from the MLTU, and directs the assemblies of highly immunogenic HPV16

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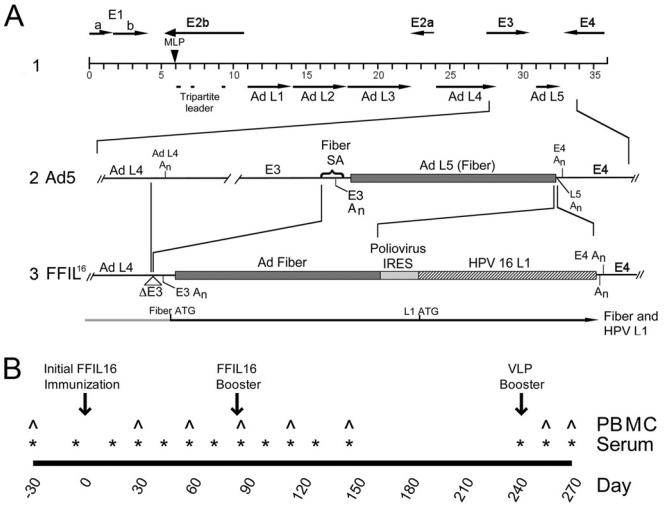

FIG 1 (A) FFIL^{16} Genomic structure and expression cassette. Line 1, the major late transcriptional unit (MLTU) of the adenovirus genome lies between the major late promoter (MLP) and early region 4 (E4). The primary ~27-kb transcript of the MLTU is alternatively polyadenylated and spliced to generate about 15 late mRNAs that comprise five late regions (Ad L1 to Ad L5) defined by the positions of their polyadenylation sites (arrowheads). Line 2, enlargement of the E3-fiber region of wild-type Ad5. In FFIL^{16} (line 3), late region 5 (Ad L5) has been expanded to include a poliovirus IRES and the HPV16 L1 gene downstream of the native fiber gene. FFIL^{16} also includes an E3 deletion of 2.8 kb (ΔE3) and a mutation in E2a (*hr*404; not shown) that confers the ability to grow in monkey cells. Both the fiber and L1 proteins are translated from a single L5 mRNA. (B) Immunization schedule. Blood samples were taken from animals to obtain PBMCs (^) and serum (*) at the times indicated. Monkeys were inoculated with 10^9 PFU of recombinant Ad5*hr*-FFIL^{16} virus at day 0 and day 85. At day 237, the monkeys were given a protein booster consisting of 20 μg of HPV16 L1 OptiPrep-purified VLPs made from recombinant baculovirus infections.

VLPs in tissue culture. The recombinant also carries the host range mutation *hr*404, which permits growth in monkey cells in culture and in macaques (9, 10). Ad5*hr*-FFIL^{16} titers were determined by plaque assay on 293 cells or by A_{260} (1.5×10^9 PFU/ml/A_{260} [11]). The purification of recombinants by CsCl density gradient centrifugation has been described (5).

Immunizations. Four female pigtail macaques (*Macaca nemestrina*) were immunized with CsCl density gradient purified recombinants by a dual intranasal-intragastric protocol. At weeks 0 and 12, 2.5×10^8 PFU of Ad5*hr*-FFIL^{16} was delivered to each nostril in 250 μl of phosphate-buffered saline (PBS), followed immediately by 5×10^8 PFU administered to the stomach via a feeding tube (1×10^9 PFU total) (9). The immunized monkeys were housed in isolation until viral DNA was no longer detected in the nasal and stool specimens. Following completion of the analysis of recombinant-induced immune responses at week 35, each animal also received 10 μg of OptiPrep-purified baculovirus-derived HPV16 VLPs suspended in PBS plus 0.5 M NaCl injected into each deltoid muscle (20 μg total).

Prior to and every 2 weeks after immunization, 5 ml of blood was drawn from each animal, clotted at room temperature, and incubated overnight at 4°C. Serum samples were collected, clarified, and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were purified as described previously (12) from 20 ml of blood drawn monthly and cryopreserved in 90% fetal bovine serum–10% dimethyl sulfoxide (DMSO). Serum samples and PBMCs were taken 7 and 21 days after the protein boost (Fig. 1B).

PCR to detect viral DNA. Nasal secretions were obtained by swabbing both nostrils with sterile cotton-tipped sticks. The swabs were eluted in PBS containing 0.1% bovine serum albumin (BSA), 0.01% thimerosal, and 10 μg/ml aprotinin and frozen at −80°C. PCR was performed on samples clarified by centrifugation (3 min at 10,000 rpm). Fresh stool samples were resuspended in a 10-fold volume of RPMI buffer supplemented with amphotericin B (Fungizone; LTI, Grand Island, NY), incubated at 100°C for 3 min, and clarified by centrifugation. Nested PCR of adenovirus fiber gene DNA and Southern blot confirmation were performed as described previously (9).

Ad5 and HPV16 neutralization. Adenovirus-neutralizing antibody was measured by plaque reduction (5). A 50% reduction compared to the no-serum controls was considered neutralizing. HPV16-neutralizing titers were determined by the secreted alkaline phosphatase method (13, 14). For secreted embryonic alkaline phosphatase (SEAP) assays, a serum dilution of 1:50 was the lowest tested, as higher serum concentrations can nonspecifically interfere with the SEAP assay. Prior to immunization, the animals had no detectable Ad5- or HPV16-neutralizing antibodies.

Baculovirus-derived HPV16 L1 VLP production. VLPs were purified as described from SF9 cells infected with a recombinant baculovirus expressing HPV16 L1 (John Schiller, National Cancer Institute [NCI]) 72 h postinfection (13). Control lysates from an irrelevant baculovirus recombinant were purified in parallel. VLP purity was estimated by Western blotting with rabbit anti-HPV16 polyclonal antibody.

Western blots. 293 cells (15) were transfected with an Ad5 L4-100K expression plasmid (Antony Rosen, Johns Hopkins University [JHU]) (16) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cell lysates prepared 24 h posttransfection were fractionated by SDS-PAGE and transferred electrophoretically to a nylon filter. Filter strips were incubated with monkey serum diluted 1:50. Goat anti-monkey IgG, IgM, and IgA horseradish peroxidase (HRP)-conjugated secondary antibodies (Open Biosystems, Huntsville, AL) were used at a 1:5,000 dilution.

T-cell proliferation. Cultures of cryopreserved PBMCs were stimulated with baculovirus-generated HPV16 VLPs (10 or 1 μg), a similarly prepared irrelevant baculovirus lysate (10 or 1 μg), purified Ad5 virions (5 μg), or phytohemagglutinin (PHA) (1 μg), and proliferation was measured by the incorporation of [^{3}H]thymidine (17). Stimulation index (SI) values were calculated by dividing the incorporated radioactivity of individual stimulated samples by the mean radioactivity of 6 unstimulated samples from the same day. The results of triplicate samples are listed as the mean SI values and their corresponding standard deviations. *P* values were calculated by a paired Student *t* test comparing postimmunization to preimmunization VLP10 SI values ($P < 0.05$ is considered significant). Cell viability is listed as a percentage and was determined by 7-aminoactinomycin (AAD) staining.

Alternatively, the PBMCs were suspended in 1 ml of PBS (pH 7.4), and 10^6 viable cells were incubated for 6 min at room temperature with carboxyfluorescein succinimidyl ester (CFSE) (3.3 μg/ml). The cells were quenched, diluted with AIM-V medium containing 10% fetal bovine serum (FBS), plated, and stimulated as described above. After 5 days in culture, the cells were harvested and labeled with 7-AAD and allophycocyanin (APC) fluorescent antibodies (clone L200; BD Pharmingen). Proliferation data were acquired on a FACSCalibur (BD) machine and analyzed with CellQuest Pro software.

Institutional approval. All animal experiments were approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

RESULTS

Ad5*hr*-FFIL^{16}. Ad5*hr*-FFIL^{16} (Fig. 1A) is a member of a novel class of rAds that abundantly express exogenous genes from the adenovirus MLTU (5). Ad5*hr*-FFIL^{16} carries a partially codon-optimized HPV 16 L1 gene inserted immediately downstream of the native fiber gene in late region 5 (Ad L5) of the MLTU, followed by an artificial polyadenylation signal. An intervening po-

TABLE 1 Viral shedding by immunized macaques

Macaque no.	Last day DNA was detected by PCR:			
	After dose 1		After dose 2	
	Nasal secretions	Stool	Nasal secretions	Stool
746	3	ND[a]	2	ND
803	10 (3)[b]	3	ND	2
811	10 (3)	ND	ND	ND
831	7	3	ND	2

[a] ND, no viral DNA detected in the first sample postinoculation. Sensitivity was <1 PFU per 10-μl sample.

[b] Numbers in parentheses indicate the last day on which infectious virus was recovered from macaques 803 and 811.

liovirus internal ribosome entry site (IRES) permits the expression of both fiber and L1 from a single bicistronic mRNA (5). A 2.8-kb sequence was deleted from E3 to accommodate the L1 gene, but unlike many rAd vaccine candidates, Ad5*hr*-FFIL[16] contains E1 and is viable in normal human cells. Finally, Ad5*hr*-FFIL[16] carries *hr*404, a point mutation that confers the ability to grow in monkey cells in culture and in macaques (9, 10). Critically, such host range mutants are available only in Ad5 and Ad2 backgrounds, restricting this study approach to those serotypes.

Immunization of macaques. Four female pigtail macaques were immunized using a combination intranasal-intragastric protocol with a total of 1.0×10^9 PFU of gradient-purified Ad5*hr*-FFIL[16] per monkey per immunization. Immunization and a specimen collection (Fig. 1B) were performed essentially as described with viable recombinant Ad-simian immunodeficiency virus (SIV) vaccines in rhesus macaques (9). The monkeys were weighed periodically, and complete blood counts were taken monthly. The vaccine was well tolerated, as judged by these parameters.

Ad5*hr*-FFIL[16] replication in macaques. Nested PCR (9) detected adenovirus DNA in the nasal swabs of all macaques, with two of four animals shedding for up to 10 days (Table 1). Two animals had detectable virus in their stools after the first inoculation. The duration of shedding was similar to that of replicating Ad5*hr*-SIV vectors (9, 18). Following the second immunization, virus was detected in the nasal secretions of one macaque and in the stools of two animals for 2 days. Southern blots of PCR products confirmed that the DNA amplified was viral. Nasal swab suspensions were also used to infect BSC-1 monkey kidney cells. Viral cytopathic effects (CPE) were observed in the cultures incubated with day-3 samples from macaques 811 and 831 (Table 1). Recovered DNA was confirmed as Ad5*hr*-FFIL[16] by HindIII digestion of radiolabeled nucleic acid and sequencing (not shown). No other sample yielded virus.

Antibody response to the viral late nonstructural protein 100K. Viral DNA detected by PCR in nasal secretions or stool samples may originate from the inoculum. To confirm that Ad5*hr*-FFIL[16] replicated and expressed late genes, we sought an antibody response to the nonstructural viral late protein 100K. Like other viral late proteins, 100K expression is dependent upon virus replication. However, unlike the other viral late proteins, 100K is absent from mature virions. Therefore, antibodies to 100K can arise only in animals in which viral replication has occurred. Macaque serum samples were assessed for reactivity to 100K by immunoblotting the lysates of 293 cells transfected with a 100K expression plasmid (16). Individual strips were incubated with preimmune serum and day 18 (d18) and d98 sera at a 1:50 dilution (Fig. 2A). Macaques 803 and 811 produced antibodies to 100K, which strongly supports that replication occurred in those animals. The response in 803 was evident on d18 and was maintained, while anti-100K antibody in 811 was first detectable at d98. Preimmune sera from macaques 746 and 831 reacted with a ~100K band in the lysates of untransfected cells, making it difficult to unequivocally detect 100K responses. The presence of the remaining viral late proteins in the virion and therefore in the inoculum precludes their use to assess replication in 746 and 831, and we can draw no firm conclusions concerning viral replication in those animals.

Neutralizing antibodies against Ad5. All four macaques developed anti-Ad5 neutralizing antibodies. Three macaques mounted a neutralizing response after the initial immunization (Fig. 2B). The titers peaked at 3 to 4 weeks postimmunization and waned over the next 2 months. The boost with recombinant virus at d85 induced a response in the remaining seronegative macaque and elevated titers in the other animals 10- to 1,000-fold. Interestingly, macaque 831, which did not respond to the initial immunization, achieved the highest titer after the boost. Again, the titers fell after immunization but remained higher than those measured prior to the boost.

Adenoviral antibodies also were assessed by immunoblot and immunoprecipitation (IP) using serum samples obtained at days 18 and 112 (not shown). All macaques, including 831, mounted a

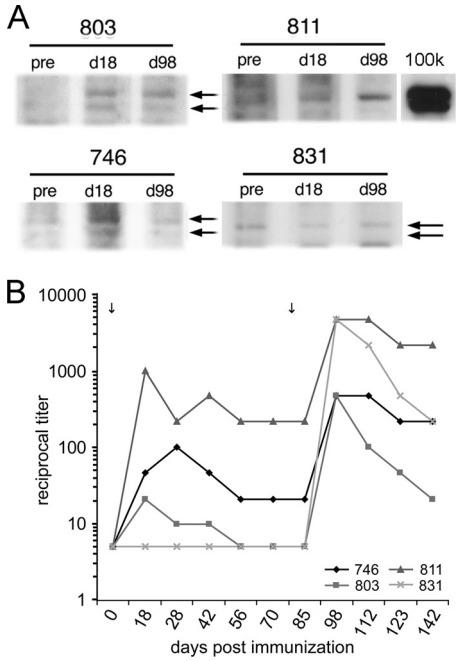

FIG 2 Immunized macaques (numbers in key) produce anti-adenovirus antibodies. (A) Immunized macaque sera were tested for reactivity with the nonvirion late protein 100K by Western blotting. Individual filter strips containing lysates of 293 cells transfected with a 100K expression plasmid were blotted with sera taken at the indicated times. A strip probed with anti-100K rabbit serum is shown at the right of the panel for macaque 811; the positions of the two 100K species present in the transfected cells are indicated by arrows for the others. For each animal, the density and contrast of all strips, as a group, were adjusted in Photoshop. (B) Ad5-neutralizing antibodies in sera drawn at the indicated time points were measured by plaque reduction. The lowest dilution tested was 1:10; values <10 were arbitrarily assigned a value of 5.

TABLE 2 HPV16 pseudovirion-neutralizing titers

Macaque no.	Data by day after first immunization							
	18	28	83	98[a]	110	121	254[b]	268
746	<50[c]	<50	<50	<50	<50	<50	<50	<50
803	<50	<50	<50	<50	<50	<50	<50	**50**[d]
811	<50	<50	<50	<50	<50	<50	<50	**100**
831	<50	<50	<50	<50	<50	<50	<50	<50
L1 Ab	**25,600**[e]							

[a] First time point following the virus boost.
[b] First time point following the VLP boost.
[c] Titer expressed as the reciprocal of the highest dilution that reduced SEAP activity to half or less of that of the corresponding preimmunization serum.
[d] Samples considered positive are in bold type.
[e] A typical value for positive-control serum.

response to the major capsid protein, hexon, by d18. Macaques 803 and 811 additionally responded to the external capsid protein penton or proteins penton and fiber, respectively, at d18. Following the boost, all monkeys maintained hexon reactivity with new or enhanced reactivity to the fiber, V (minor core), and inner capsid proteins VI and IIIa. Epitopes recognized in immunoblots or IPs may not be those targeted by neutralizing antibodies. However, increased anti-fiber antibodies correlated with the jump in neutralization titers following the boost, consistent with the role of the fiber protein in receptor attachment (19).

Ad5*hr*-FFIL^{16} immunization and HPV16-neutralizing antibodies. Neutralizing antibodies induced by L1 protect against HPV infection (20). HPV-neutralizing titers were determined at the peaks of the anti-adenovirus antibody responses after initial immunization (days 18 and 28) and immediately before and at the peak of the adenoviral response after the Ad5*hr*-FFIL^{16}boost (days 85, 98, and 112) (Table 2). Remarkably, despite the immunogenicity of recombinant-produced VLPs in mice and of the adenoviral vector, no macaque developed HPV-neutralizing antibodies after Ad5*hr*-FFIL^{16} immunization or boost. The absence of neutralizing antibody was confirmed by the lack of a detectable antibody response against HPV16 L1 in any of the macaques by enzyme-linked immunosorbent assay (ELISA), IP, or Western blots (not shown).

Our recombinants were designed with oral administration in mind. However, regimens involving a live rAd priming dose and subsequent purified protein boost have proved effective in inducing immune responses to SIV antigens in macaques and to influenza virus antigens in humans (21–23). To determine whether the Ad5*hr*-FFIL^{16} immunizations had primed a humoral response to HPV16 L1, each macaque was injected at week 35 with 10 μg of purified HPV16 VLPs. Macaques 803 and 831 developed HPV-neutralizing antibodies after VLP injection, suggesting that in those animals, the recombinant may have primed an L1 response (Table 2). Macaques 803 and 831 both also mounted an unequivocal Ad5 100K antibody response (Fig. 2A), which may indicate that recombinant replication was most vigorous in those macaques.

Macaques generate a strong T-cell response to L1 VLPs. Both replicating and replication-defective rAds induce strong T-cell responses. We investigated T-cell responses after Ad5*hr*-FFIL^{16} immunization using a PBMC proliferation assay (17). All animals mounted a robust T-cell response to L1 VLPs (Fig. 3 and Table 3). Following the first viral immunization, three of four animals (746, 803, and 811) had stimulation indices (SI) nearly 10-fold higher than their preimmunization levels when pulsed with 10 μg/ml VLPs. Proliferation declined with time but remained above background in all three monkeys. After the Ad5*hr*-FFIL^{16} boost at 3 months, the SI values rose again in macaques 746 and 811 while remaining largely unchanged for macaque 803. Monkey 831 displayed a delayed response to HPV VLPs: weak proliferation was seen at 28 and 56 days postimmunization followed by a strong response (SI, 12.6) at day 85 (prior to viral boost). Anti-adenovirus cell-mediated immunity was also induced by immunization and sustained throughout the study, with that of macaque 831 again being noticeably lower. Thus, replicating MLTU recombinants elicit robust cell-mediated responses to both viral and transgene products.

Following immunization with purified VLPs at day 247, macaques 803 and 831 showed an increase in T-cell responses to L1, with SIs 20- to 60-fold greater than those of the unstimulated controls. Macaque 746 remained unchanged from previously high

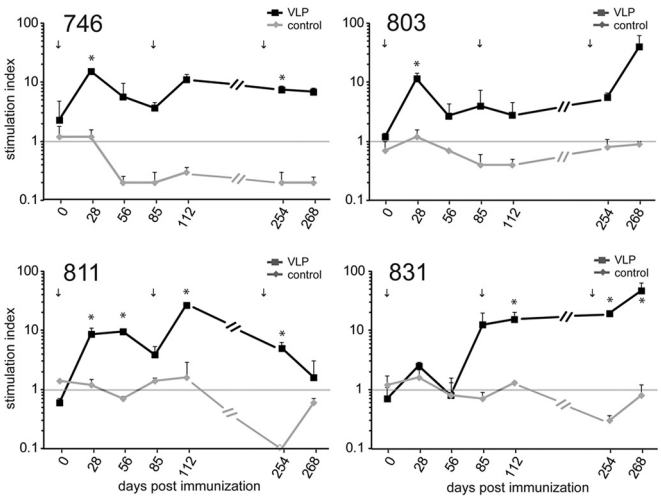

FIG 3 Cellular responses to immunization with Ad5*hr*-FFIL^{16}. Stimulation indices (SI) in response to 10 μg of HPV16 L1 VLPs (■) or an irrelevant baculovirus lysate (◆) are plotted for PBMCs drawn from each macaque at the indicated times. The arrows indicate immunizations; the third immunization was with purified HPV16 VLPs. The error bars represent the standard errors for the triplicate wells at each time point. The asterisks indicate the time points at which postimmunization and preimmunization responses differ statistically ($P < 0.05$). Interruptions in the plots indicate an extended period for which SI values were not determined. A horizontal line is drawn at an SI value of 1 for reference.

TABLE 3 Stimulation indices and viability for monkey PBMC

Macaque no. and day of testing[a]	Viability (%)[b]	SI value (mean ±SD) for: VLP10[c]	BAC10[d]	VLP1[c]	BAC1[d]	Ad5[e]	PHA[f]	*P* value[g]
M 746								
0	55.8	2.3 ± 2.6	1.2 ± 0.6	4.1 ± 3.5	1.4 ± 0.7	1.8 ± 1.1	415 ± 87	
28	58.1	15.4 ± 0.9	1.2 ± 0.4	2.6 ± 0.8	2.1 ± 0.3	8.1 ± 3.4	242.5 ± 35.8	**0.0203**
56	46	5.7 ± 4.1	0.2 ± 0.06	1.4 ± 0.9	1.1 ± 0.9	3.2 ± 1.1	499.7 ± 101	0.2118
85	57.3	3.7 ± 0.9	0.2 ± 0.1	2.2 ± 0.8	1 ± 0.7	3.6 ± 1	243.8 ± 4.8	0.2645
112	53.8	11.2 ± 2.4	0.3 ± 0.06	1.9 ± 0.8	2 ± 1.1	7 ± 0.6	81 ± 15	0.2926
254	65	7.5 ± 1.2	0.2 ± 0.1	2.7 ± 0.5	0.8 ± 0.6	2.8 ± 0.5	137.4 ± 8.4	**0.0244**
268	57.9	6.9 ± 1.1	0.2 ± 0.05	1.4 ± 0.6	1.3 ± 1.1	4.3 ± 1.2	139 ± 32	0.0962
M 803								
0	62	1.2 ± 0.2	0.7 ± 0.3	0.6 ± 0.3	0.6 ± 0.2	1.2 ± 0.3	47.5 ± 7	
28	54.4	11.6 ± 2.7	1.2 ± 0.4	0.7 ± 0.3	0.8 ± 0.1	3.4 ± 1.6	137.1 ± 4	**0.0196**
56	63.6	2.7 ± 1.7	0.7 ± 0.03	0.4 ± 0.05	0.3 ± 0.04	2.9 ± 1.5	123.1 ± 10.4	0.0596
85	63	4 ± 3.4	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	3.4 ± 0.8	172.5 ± 54.1	0.3468
112	57.5	2.8 ± 1.8	0.4 ± 0.1	0.8 ± 0.4	0.7 ± 0.03	1.4 ± 0.1	125.2 ± 6.2	0.0907
254	56.7	5.6 ± 1.1	0.8 ± 0.3	1.7 ± 0.7	1 ± 0.1	1.9 ± 0.4	276.8 ± 28.1	0.1439
268	62.8	40.5 ± 22.9	0.9 ± 0.09	3.4 ± 1.6	2.5 ± 2	7.2 ± 4	258.9 ± 29.6	0.148
M 811								
0	47.5	0.6 ± 0.1	1.4 ± 0.07	0.4 ± 0.2	0.8 ± 0.3	0.8 ± 0.3	9.3 ± 2.8	
28	56.4	8.7 ± 2.4	1.2 ± 0.3	0.6 ± 0.5	0.8 ± 0.2	5.1 ± 0.6	277 ± 4.8	**0.0293**
56	61	9.6 ± 0.5	0.7 ± 0.06	1.2 ± 0.6	0.7 ± 0.3	5.7 ± 1.6	320 ± 35.7	**0.0013**
85	58.9	3.9 ± 1.5	1.4 ± 0.2	0.4 ± 0.2	0.6 ± 0.3	6.9 ± 1.7	235 ± 17.6	0.0624
112	53.4	26.9 ± 2.1	1.6 ± 1.3	1.9 ± 1.3	1.6 ± 0.7	20.6 ± 1.6	282 ± 45	**0.0021**
254	62.2	5 ± 1.4	0.1 ± 0.02	1.3 ± 0.4	0.6 ± 0.1	4.2 ± 0.4	161 ± 3.1	**0.0361**
268	65.6	1.6 ± 1.5	0.6 ± 0.1	0.4 ± 0.2	0.7 ± 0.2	7.3 ± 2	234 ± 8.2	0.3902
M 831								
0	54.8	0.7 ± 0.4	1.2 ± 0.5	0.5 ± 0.2	0.7 ± 0.4	1 ± 0.4	33.4 ± 9.8	
28	60.6	2.5 ± 0.4	1.6 ± 0.6	0.7 ± 0.4	0.8 ± 0.2	1.1 ± 0.4	134.9 ± 3.6	0.0677
56	61.2	0.8 ± 0.8	0.8 ± 0.5	1 ± 0.7	0.6 ± 0.2	0.5 ± 0.03	24.8 ± 4.6	0.6047
85	68.5	12.6 ± 7.3	0.7 ± 0.2	0.9 ± 0.3	0.6 ± 0.3	2.2 ± 1	159.4 ± 7.8	0.1043
112	58.4	15.5 ± 4.8	1.3 ± 0.1	0.8 ± 0.4	0.5 ± 0.2	2 ± 0.6	281 ± 92.8	**0.0276**
254	66.5	19.5 ± 1	0.3 ± 0.06	2.7 ± 1.4	2.2 ± 1.5	1 ± 0.3	198 ± 13	**0.0002**
268	70	47.5 ± 17	0.8 ± 0.4	0.7 ± 0.3	0.5 ± 0.07	3.2 ± 1.9	241.9 ± 16.9	**0.0411**

[a] M, macaque.
[b] Determined by 7-AAD exclusion.
[c] Cultures stimulated by 10 or 1 μg HPV16 VLP.
[d] Cultures stimulated by 10 or 1 μg irrelevant baculovirus lysate.
[e] Cultures stimulated with purified adenovirus 5 particles.
[f] Cultures stimulated with phytohemagglutinin.
[g] Bold type indicates a *P* value of <0.05 for the VLP10 SI.

levels, while 811 declined substantially from its peak (SI, 26.9) at day 112. None of the PBMCs of the animals were stimulated by the control baculovirus-infected cell lysate.

T-cell subset responses against L1 VLPs. To identify the cells that proliferate in response to L1 VLPs, CSFE-stained PBMCs from monkeys 803 and 811 obtained prior to immunization, after each virus administration (days 28 and 123), and after the VLP booster (d254) were stimulated with either VLPs or medium, stained with antibody against human CD4 and with 7-AAD, and examined by flow cytometry. Lymphocytes were selected based on forward and side scatter, and dead cells (7-AAD$^+$) were excluded from the analysis. Figure 4 shows the lymphocytes obtained following the Ad5*hr*-FFIL[16] boost (day 112) in monkeys 803 and 811 after stimulation with HPV16 VLPs, which are representative of the other postimmunization PBMCs tested. CD4$^+$ lymphocytes do not proliferate in response to stimulation, but there was substantial CFSE dilution among CD4$^-$ lymphocytes. Our anti-human CD8 antibody did not recognize the monkey CD8 antigen, and we could not directly measure proliferation of CD8$^+$ T cells. However, the position of these cells is consistent with the preferential proliferation of CD8$^+$ T cells in response to HPV16 L1 VLPs.

DISCUSSION

The replication-competent adenovirus recombinant Ad5*hr*-FFIL[16] expresses the HPV16 L1 protein from the adenoviral major late transcriptional unit (MLTU) and produces HPV L1 VLPs abundantly in tissue culture. Purified recombinant-produced VLPs are highly immunogenic when injected into mice (5), but mice do not support human adenoviral replication and do not permit an assessment of the recombinant in its intended application as a replicating vaccine. In this study, we have exploited a

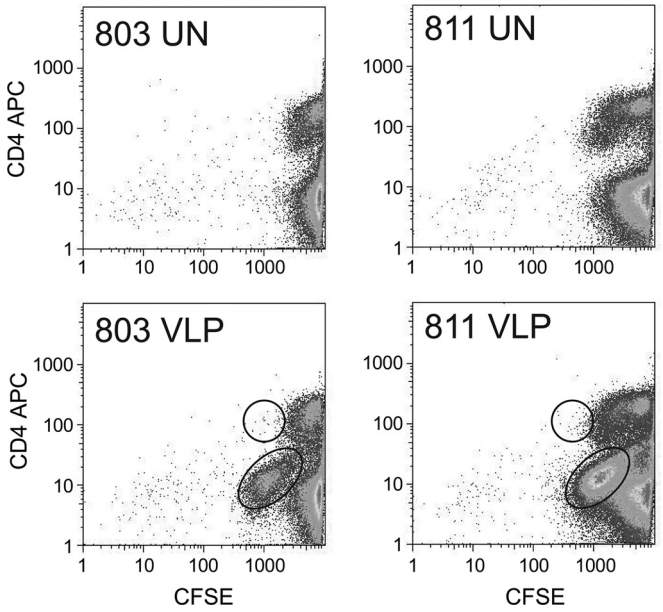

FIG 4 FFIL^{16} does not induce CD4$^+$ T-cell proliferation. PBMCs collected on day 112 from macaques 803 and 811 were labeled with CFSE and stimulated with medium (UN) or 10 μg/ml of HPV16 VLPs (VLP). Live cells were examined by flow cytometry after staining with APC-labeled anti-CD4 antibody and CFSE. The circles indicate where proliferating CD4$^+$ cells are expected. The CD4$^-$ proliferating populations are indicated by an ellipses.

permissive nonhuman primate model to test the hypothesis that under conditions that permit viral replication, Ad5*hr*-FFIL^{16} is capable of inducing immune responses that might protect against HPV infection.

Adenoviruses are species specific, and human adenoviruses do not grow in most animals. Among the few exceptions are macaques, which support growth of host range (*hr*) mutants of the group C adenovirus serotypes Ad5 and Ad2 (9, 10). The *hr* mutant/macaque model thus provides one of the few animal systems in which replicating adenovirus vaccine candidates can be evaluated. Importantly, no mutations similar to the host range mutants exist outside group C, and in particular, none is available in the Ad4 and Ad7 military vaccine types or in types under consideration as vaccine vectors because of low seroprevalence in humans (for example, Ad26 and Ad35 [24]). Further, host range mutations affect a region of the viral 72-k DNA binding protein (DBP) that bears no recognizable sequence homology to that of viruses outside group C, preventing their reconstruction in other vaccine candidate serotypes. Therefore, our experimental approach is restricted to Ad5/Ad2. Seropositivity to Ad5 is widespread and can blunt the efficacy of replication-defective adenovirus vaccine candidates (25, 26). Our replication-competent recombinants may be affected similarly. Therefore, it is important to stress that our Ad5 MLTU recombinants exploit viral gene expression strategies and genome architecture that are universal among adenoviruses, and that the construction of analogous MLTU recombinants in less seroprevalent adenoviral types should prove to be no major hurdle if necessary for the evasion of preexisting immunity.

Four female pigtail macaques were inoculated with Ad5*hr*-FFIL^{16} by intranasal and intragastric routes to present the virus to mucosal surfaces (9, 27). The prolonged detection of viral DNA in nasal swabs and stool samples, a vigorous neutralizing antibody response to Ad5, and development in two animals of antibodies to 100K, a late viral protein not present in the virion, confirmed replication in the monkeys.

We were surprised that despite its replication, the demonstrated potent immunogenicity of recombinant-derived HPV16 L1 VLPs and its induction of Ad5 antibody Ad5*hr*-FFIL^{16} did not elicit HPV16 antibodies. The lack of a strong CD4$^+$ T-cell response after Ad5*hr*-FFIL^{16} immunization is consistent with that result. It is generally accepted that antibodies but not cell-mediated responses, are protective against HPV infection (28), and for that reason, it is not likely that the recombinant in its present form will be useful in the prevention of HPV infection.

The reasons for the puzzling failure of Ad5*hr*-FFIL^{16} to elicit the anticipated antibody response are not clear. In mice immunized by intraperitoneal injection, a 5-fold mass excess of adenoviral particles completely inhibits the induction of antibodies by a dose of HPV16 VLPs that elicits high-titer antibodies if administered alone (M. G. Berg and G. Ketner, unpublished data). A similar phenomenon was observed in rabbits immunized with recombinant vesicular stomatitis viruses expressing HPV16 L1 (29). Such immunological interference might explain the poor induction of HPV antibodies by Ad5*hr*-FFIL^{16} *in vivo*. If so, increases in the stoichiometry of HPV VLPs to Ad5 particles might increase antibody induction. L1 production by MLTU recombinants varies widely depending on the specific regulatory elements used to drive expression (4). L1 production *in vivo* and immunogenicity therefore might be substantially improved by the use of different regulatory sequences in recombinant construction. Alternatively, the production of HPV L1 VLPs *in vivo* may be less than that expected from experiments in tissue culture. VLPs may not assemble efficiently in macaques, or the vigorous T-cell responses against infected cells might reduce VLP production by clearing VLP-producing cells prior to the release of the VLPs. Intracellular proteasomal degradation of L1 prior to VLP assembly and release might also preclude a strong B-cell response. In any of these cases, VLPs might fail to accumulate to levels in the local lymph nodes sufficient to activate antigen-specific B cells. The reported abilities of other live rAds to induce humoral immunity have been mixed. An SIV *env* recombinant elicited SIV-neutralizing antibody transiently after its second administration to macaques (9), but an rAd expressing influenza A virus H5N1 hemagglutinin protein (30) failed to induce antibodies in humans after three immunizations (23). Both of those recombinants primed an antibody response to subsequent immunization with purified protein. Notably, in both cases, the cell-mediated response to immunization with the recombinant included a CD4$^+$ T-cell response, which was not observed with Ad5*hr*-FFIL^{16}.

In contrast to the lack of a humoral response to L1, the cellular response induced by Ad5*hr*-FFIL^{16} was robust. While a direct comparison to the published results for HPV VLP trials in humans of our *in vivo* results may be only suggestive, the cell-mediated response by Ad5*hr*-FFIL^{16} substantially exceeds that generated in human volunteers to an HPV16 L1 VLP vaccine. Among 11 women given 3 doses of 50 μg of VLPs, the mean SI of PBMCs by HPV16 L1 VLPs increased only 2-fold over their prevaccination mean values (7, 17), while SI values rose >10-fold in each of our monkeys after one virus dose. T-cell responses to viable Ad7-HIV recombinants were of a magnitude similar to those seen here (9). In women immunized with HPV16 L1 VLPs, both CD4$^+$ and CD8$^+$ cells expand in response to VLP stimulation (17). We see no

$CD4^+$ response, and for technical reasons, we could not confirm that $CD8^+$ T cells proliferated in our experiments. However, adenoviral vectors frequently generate strong $CD8^+$ T-cell responses (31), and we believe that the VLP-responsive T cells in immunized macaques were $CD8^+$ cells. It must be emphasized that there is no compelling evidence linking cell-mediated immunity to protection from HPV infection. However, in at least one study, L1 RNA was present in 100% of cervical cancer biopsy specimens, and $CD8^+$ T cells were as effective in killing tumor cells *in vitro* as were E7-specific cytotoxic T lymphocytes (CTLs) (32), and in mice, immunization with L1 VLPs induced $CD8^+$ T-cell-mediated protection from challenge with HPV16-transformed cells that expressed a low level of L1 RNA and no detectable L1 protein (33). Therefore, a T-cell response to L1 might contribute to protection in individuals immunized with an Ad5*hr*-FFIL^{16}-like recombinant also optimized to increase antibody production.

The live oral military adenovirus vaccines safely induce vigorous humoral and cell-mediated responses and effective immunity against adenovirus-induced respiratory disease (1). The low dose of live virus required, the ease and safety of needle-free delivery, and the single-dose regimen that would be offered by recombinant vaccines patterned on the Ad4/Ad7 vaccine model hold considerable promise for immunization, especially in the developing world. The lack of a humoral response to HPV 16 L1 in Ad5*hr*-FFIL^{16}-immunized animals was therefore a disappointment in the context of HPV immunization. However, the cell-mediated response to HPV16 L1 in Ad5*hr*-FFIL^{16}-immuized animals suggests that MLTU recombinants will be of value in immunization at least against pathogens for which cell-mediated responses are protective. These include many pathogens with an obligate intracellular stage, such as most viruses, intracellular bacteria (*Mycobacteria* and *Francisella* spp. [34–36]), and parasitic protozoa (*Plasmodium* and *Leishmania* spp. [37, 38]). Studies of live MLTU recombinants expressing other antigens may therefore extend the advantages of the safe, effective, and practical live military adenovirus vaccines to a variety of important pathogens if not, at the moment, to HPV.

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REFERENCES

1. **Gaydos CA, Gray GC.** 2008. Adenovirus vaccine, p 1103–1122. *In* Plotkin SA, Orenstein MD, Offit P (ed), Vaccines, Saunders, Philadelphia, PA.
2. **Lu B, Kumar A, Castellsagué X, Giuliano AR.** 2011. Efficacy and safety of prophylactic vaccines against cervical HPV infection and diseases among women: a systematic review & meta-analysis. BMC Infect. Dis. **11:**13. http://dx.doi.org/10.1186/1471-2334-11-13.
3. **Lowy DR, Schiller JT.** 2012. Reducing HPV-associated cancer globally. Cancer Prev. Res. (Phila.) **5:**18–23. http://dx.doi.org/10.1158/1940-6207.CAPR-11-0542.
4. **Berg M, Difatta J, Hoiczyk E, Schlegel R, Ketner G.** 2005. Viable adenovirus vaccine prototypes: high-level production of a papillomavirus capsid antigen from the major late transcriptional unit. Proc. Natl. Acad. Sci. U. S. A. **102:**4590–4595. http://dx.doi.org/10.1073/pnas.0500933102.
5. **Berg M, Gambhira R, Siracusa M, Hoiczyk E, Roden R, Ketner G.** 2007. HPV16 L1 capsid protein expressed from viable adenovirus recombinants elicits neutralizing antibody in mice. Vaccine **25:**3501–3510. http://dx.doi.org/10.1016/j.vaccine.2006.06.080.
6. **Harro CD, Pang YY, Roden RB, Hildesheim A, Wang Z, Reynolds MJ, Mast TC, Robinson R, Murphy BR, Karron RA, Dillner J, Schiller JT, Lowy DR.** 2001. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. J. Natl. Cancer Inst. **93:**284–292. http://dx.doi.org/10.1093/jnci/93.4.284.
7. **Pinto LA, Viscidi R, Harro CD, Kemp TJ, García-Piñeres AJ, Trivett M, Demuth F, Lowy DR, Schiller JT, Berzofsky JA, Hildesheim A.** 2006. Cellular immune responses to HPV-18, -31, and -53 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. Virology **353:**451–462. http://dx.doi.org/10.1016/j.virol.2006.06.021.
8. **Tobery TW, Smith JF, Kuklin N, Skulsky D, Ackerson C, Huang L, Chen L, Cook JC, McClements WL, Jansen KU.** 2003. Effect of vaccine delivery system on the induction of HPV16L1-specific humoral and cell-mediated immune responses in immunized rhesus macaques. Vaccine **21:**1539–1547. http://dx.doi.org/10.1016/S0264-410X(02)00679-5.
9. **Buge SL, Richardson E, Alipanah S, Markham P, Cheng S, Kalyan N, Miller CJ, Lubeck M, Udem S, Eldridge J, Robert-Guroff M.** 1997. An adenovirus-simian immunodeficiency virus env vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. J. Virol. **71:**8531–8541.
10. **Klessig DF, Grodzicker T.** 1979. Mutations that allow human Ad2 and Ad5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA binding protein. Cell **17:**957–966.
11. **Challberg SS, Ketner G.** 1981. Deletion mutants of adenovirus 2: isolation and initial characterization of virus carrying mutations near the right end of the viral genome. Virology **114:**196–209.
12. **Sharma DP, Anderson M, Zink MC, Adams RJ, Donnenberg AD, Clements JE, Narayan O.** 1992. Pathogenesis of acute infection in rhesus macaques with a lymphocyte-tropic strain of simian immunodeficiency virus. J. Infect. Dis. **166:**738–746. http://dx.doi.org/10.1093/infdis/166.4.738.
13. **Buck CB, Pastrana DV, Lowy DR, Schiller JT.** 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. **78:**751–757. http://dx.doi.org/10.1128/JVI.78.2.751-757.2004.
14. **Pastrana DV, Buck CB, Pang YY, Thompson CD, Castle PE, FitzGerald PC, Krüger Kjaer S, Lowy DR, Schiller JT.** 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology **321:**205–216. http://dx.doi.org/10.1016/j.virol.2003.12.027.
15. **Graham FL, Smiley J, Russell WC, Nairn R.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74. http://dx.doi.org/10.1099/0022-1317-36-1-59.
16. **Andrade F, Bull HG, Thornberry NA, Ketner GW, Casciola-Rosen LA, Rosen A.** 2001. Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. Immunity. **14:**751–761. http://dx.doi.org/10.1016/S1074-7613(01)00149-2.
17. **Pinto LA, Edwards J, Castle PE, Harro CD, Lowy DR, Schiller JT, Wallace D, Kopp W, Adelsberger JW, Baseler MW, Berzofsky JA, Hildesheim A.** 2003. Cellular immune responses to human papillomavirus (HPV)-16 L1 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. J. Infect. Dis. **188:**327–338. http://dx.doi.org/10.1086/376505.
18. **Gómez-Román VR, Grimes GJ, Jr, Potti GK, Peng B, Demberg T, Gravlin L, Treece J, Pal R, Lee EM, Alvord WG, Markham PD, Robert-Guroff M.** 2006. Oral delivery of replication-competent adenovirus vectors is well tolerated by SIV- and SHIV-infected rhesus macaques. Vaccine **24:**5064–5072. http://dx.doi.org/10.1016/j.vaccine.2006.03.048.
19. **Philipson L, Lonberg-Holm K, Pettersson U.** 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2:**1064–1075.
20. **Frazer IH.** 2004. Prevention of cervical cancer through papillomavirus vaccination. Nat. Rev. Immunol. **4:**46–54. http://dx.doi.org/10.1038/nri1260.
21. **Patterson LJ, Malkevitch N, Pinczewski J, Venzon D, Lou Y, Peng B, Munch C, Leonard M, Richardson E, Aldrich K, Kalvanaraman VS, Pavlakis GN, Robert-Guroff M.** 2003. Potent, persistent induction and

modulation of cellular immune responses in rhesus macaques primed with Ad5hr-simian immunodeficiency virus (SIV) *env*/*rev*, *gag*, and/or *nef* vaccines and boosted with SIV gp120. J. Virol. **77:**8607–8620. http://dx.doi.org/10.1128/JVI.77.16.8607-8620.2003.

22. **Malkevitch N, Patterson LJ, Aldrich K, Richardson E, Alvord WG, Robert-Guroff M.** 2003. A replication competent adenovirus 5 host range mutant-simian immunodeficiency virus (SIV) recombinant priming/subunit protein boosting vaccine regimen induces broad, persistent SIV-specific cellular immunity to dominant and subdominant epitopes in Mamu-A*01 rhesus macaques. J. Immunol. **170:**4281–4289. http://dx.doi.org/10.4049/jimmunol.170.8.4281.
23. **Gurwith M, Lock M, Taylor EM, Ishioka G, Alexander J, Mayall T, Ervin JE, Greenberg RN, Strout C, Treanor JJ, Webby R, Wright PF.** 2013. Safety and immunogenicity of an oral, replicating adenovirus serotype 4 vector vaccine for H5N1 influenza: a randomised, double-blind, placebo-controlled, phase 1 study. Lancet Infect. Dis. **13:**238–250. http://dx.doi.org/10.1016/S1473-3099(12)70345-6.
24. **Abbink P, Lemckert AA, Ewald BA, Lynch DM, Denholtz M, Smits S, Holterman L, Damen I, Vogels R, Thorner AR, O'Brien KL, Carville A, Mansfield KG, Goudsmit J, Havenga MJ, Barouch DH.** 2007. Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. J. Virol. **81:**4654–4663. http://dx.doi.org/10.1128/JVI.02696-06.
25. **McCoy K, Tatsis N, Korioth-Schmitz B, Lasaro MO, Hensley SE, Lin SW, Li Y, Giles-Davis W, Cun A, Zhou D, Xiang Z, Letvin NL, Ertl HC.** 2007. Effect of preexisting immunity to adenovirus human serotype 5 antigens on the immune responses of nonhuman primates to vaccine regimens based on human- or chimpanzee-derived adenovirus vectors. J. Virol. **81:**6594–6604. http://dx.doi.org/10.1128/JVI.02497-06.
26. **Sumida SM, Truitt DM, Lemckert AA, Vogels R, Custers JH, Addo MM, Lockman S, Peter T, Peyerl FW, Kishko MG, Jackson SS, Gorgone DA, Lifton MA, Essex M, Walker BD, Goudsmit J, Havenga MJ, Barouch DH.** 2005. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J. Immunol. **174:**7179–7185. http://dx.doi.org/10.4049/jimmunol.174.11.7179.
27. **Patterson LJ, Malkevitch N, Zhao J, Peng B, Robert-Guroff M.** 2002. Potent, persistent cellular immune responses elicited by sequential immunization of rhesus macaques with Ad5 host range mutant recombinants encoding SIV Rev. and SIV Nef. DNA Cell Biol. **21:**627–635. http://dx.doi.org/10.1089/104454902760330165.
28. **Schiller JT, Lowy DR.** 2012. Understanding and learning from the success of prophylactic human papillomavirus vaccines. Nat. Rev. Microbiol. **10:**681–692. http://dx.doi.org/10.1038/nrmicro2872.
29. **Roberts A, Reuter JD, Wilson JH, Baldwin S, Rose JK.** 2004. Complete protection from papillomavirus challenge after a single vaccination with a vesicular stomatitis virus vector expressing high levels of L1 protein. J. Virol. **78:**3196–3199. http://dx.doi.org/10.1128/JVI.78.6.3196-3199.2004.
30. **Alexander J, Ward S, Mendy J, Manayani DJ, Farness P, Avanzini JB, Guenther B, Garduno F, Jow L, Snarsky V, Ishioka G, Dong X, Vang L, Newman MJ, Mayall T.** 2012. Pre-clinical evaluation of a replication-competent recombinant adenovirus serotype 4 vaccine expressing influenza H5 hemagglutinin. PLoS One **7:**e31177. http://dx.doi.org/10.1371/journal.pone.0031177.
31. **Bassett JD, Swift SL, Bramson JL.** Optimizing vaccine-induced CD8(+) T-cell immunity: focus on recombinant adenovirus vectors. Expert Rev. Vaccines **10:**1307–1319. http://dx.doi.org/10.1586/erv.11.88.
32. **Bellone S, El-Sahwi K, Cocco E, Casagrande F, Cargnelutti M, Palmieri M, Bignotti E, Romani C, Silasi DA, Azodi M, Schwartz PE, Rutherford TJ, Pecorelli S, Santin AD.** 2009. Human papillomavirus type 16 (HPV-16) virus-like particle L1-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are equally effective as E7-specific $CD8^+$ CTLs in killing autologous HPV-16-positive tumor cells in cervical cancer patients: implications for L1 dendritic cell-based therapeutic vaccines. J. Virol. **83:**6779–6789. http://dx.doi.org/10.1128/JVI.02443-08.
33. **De Bruijn ML, Greenstone HL, Vermeulen H, Melief CJ, Lowy DR, Schiller JT, Kast WM.** 1998. L1-specific protection from tumor challenge elicited by HPV16 virus-like particles. Virology **250:**371–376.
34. **Dannenberg AM, Jr.** 2010. Perspectives on clinical and preclinical testing of new tuberculosis vaccines. Clin. Microbiol. Rev. **23:**781–794. http://dx.doi.org/10.1128/CMR.00005-10.
35. **Kirimanjeswara GS, Olmos S, Bakshi CS, Metzger DW.** 2008. Humoral and cell-mediated immunity to the intracellular pathogen *Francisella tularensis*. Immunol. Rev. **225:**244–255. http://dx.doi.org/10.1111/j.1600-065X.2008.00689.x.
36. **Tarnvik A.** 1989. Nature of protective immunity to *Francisella tularensis*. Rev. Infect. Dis. **11:**440–451. http://dx.doi.org/10.1093/clinids/11.3.440.
37. **Chuang I, Sedegah M, Cicatelli S, Spring M, Polhemus M, Tamminga C, Patterson N, Guerrero M, Bennett JW, McGrath S, Ganeshan H, Belmonte M, Farooq F, Abot E, Banania JG, Huang J, Newcomer R, Rein L, Litilit D, Richie NO, Wood C, Murphy J, Sauerwein R, Hermsen CC, McCoy AJ, Kamau E, Cummings J, Komisar J, Sutamihardja A, Shi M, Epstein JE, Maiolatesi S, Tosh D, Limbach K, Evelina A, Bergmann-Leitner E, Bruder JT, Doolan DL, King CR, Carucci D, Dutta S, Soisson L, Diggs C, Hollingdale MR, Ockenhouse CF, Richie TL.** 2013. DNA prime/adenovirus boost malaria vaccine encoding *P. falciparum* CSP and AMA1 induces sterile protection associated with cell-mediated immunity. PLoS One **8:**e55571. http://dx.doi.org/10.1371/journal.pone.0055571.
38. **Singh OP, Gidwani K, Kumar R, Nylén S, Jones SL, Boelaert M, Sacks D, Sundar S.** 2012. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. Clin. Vaccine Immunol. **19:**961–966. http://dx.doi.org/10.1128/CVI.00143-12.