Increased Mucosal CD4+ T Cell Activation in Rhesus Macaques following Vaccination with an Adenoviral Vector

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ABSTRACT

The possibility that vaccination with adenovirus (AdV) vectors increased mucosal T cell activation remains a central hypothesis to explain the potential enhancement of HIV acquisition within the Step trial. Modeling this within rhesus macaques is complicated because human adenoviruses, including human adenovirus type 5 (HAdV-5), are not endogenous to macaques. Here, we tested whether vaccination with a rhesus macaque-derived adenoviral vector (simian adenovirus 7 [SAdV-7]) enhances mucosal T cell activation within rhesus macaques. Following intramuscular SAdV-7 vaccination, we observed a pronounced increase in SAdV-7-specific CD4+ T cell responses in peripheral blood and, more dramatically, in rectal mucosa tissue. Vaccination also induced a significant increase in the frequency of activated memory CD4+ T cells in SAdV-7- and HAdV-5-vaccinated animals in the rectal mucosa but not in peripheral blood. These fluctuations within the rectal mucosa were also associated with a pronounced decrease in the relative frequency of naive resting CD4+ T cells. Together, these results indicate that peripheral vaccination with an AdV vector can increase the activation of mucosal CD4+ T cells, potentially providing an experimental model to further evaluate the role of host-vector interactions in increased HIV acquisition after AdV vector vaccination.

IMPORTANCE

The possibility that vaccination with a human adenovirus 5 vector increased mucosal T cell activation remains a central hypothesis to explain the potential enhancement of human immunodeficiency virus (HIV) acquisition within the Step trial. In this study, we tested whether vaccination with a rhesus macaque-derived adenoviral vector in rhesus macaques enhances mucosal CD4+ T cell activation, the main cell target of simian immunodeficiency virus (SIV)/HIV. The results showed that vaccination with an adenoviral vector indeed increases activation of mucosal CD4+ T cells and potentially increases susceptibility to SIV infection.

The pursuit of an efficacious human immunodeficiency virus type 1 (HIV-1) vaccine is an ongoing effort to minimize or halt the burden of this disease worldwide. Numerous T cell-directed HIV vaccines have aimed to target immune responses that would either decrease the viral load if an individual is infected or restrict HIV replication entirely (1, 2). Notably, Merck’s Step study was a large, phase Ib, placebo-controlled clinical trial testing a replication-defective recombinant human adenovirus serotype 5 (HAdV-5) vector to deliver the HIV-1 antigens gag, pol, and nef, with the intention of preventing HIV-1 infection by generating T cell immunity. This candidate HIV vaccine trial was halted in September 2007 due to statistical futility and the finding of a trend toward increased rates of HIV acquisition among vaccinated men with anti-HAdV-5 seropositivity (3). Another phase Ib clinical trial using the same adenovirus vector is referred to as the Phambili trial, which yielded the same results of increased acquisition of HIV infection in some vaccinees (4). The underlying mechanism of these apparent increases in acquisition remains to be defined.

During the Step trial, only peripheral blood mononuclear cells (PBMCs) were collected, and many follow-up studies seeking to elucidate the Step outcome have investigated this compartment exclusively without the use of mucosal biopsy specimens (3, 5–7). Importantly, the gut mucosa is both the potential site of HIV transmission in infected Step participants (6, 7) as well as a site of AdV persistence (8). Expansion of the enteric virome, including mucosal adenovirus infection, has even been associated with pathogenic simian immunodeficiency virus (SIV) infection (9). Compartmental differences in the immunological milieu between the blood and mucosa are well documented, and alterations in phenotype, functionality, activation state, or distribution of CD4+ T cells within these compartments may offer clues to the Step outcome. In the peripheral blood of HAdV-5 vaccine recipients, we found no difference in the expression of Ki67, a proliferation marker, or α4β7, a gut homing marker (6), in agreement with others who saw no increase in cellular activation (5, 10). Similarly, there was no increase in α4β7 or CCR5 expression levels on peripheral blood CD4+ T cells in vaccinees during the Step study (5, 6); however, in vitro studies suggest that HAdV-5 can induce α4β7 and CCR5 expression on expanded AdV-specific memory CD4+ T cells (7, 11), raising the possibility that HAdV-5 could have a specific effect in the mucosa. In those studies, im-
Mucosal CD4+ T Cell Activation after AdV Vaccination

Mucosal immune responses to adenovirus were evaluated by using HAdV-5. It is important to note that there are currently 65 serologically distinct human adenoviruses, and responses to adenovirus may be influenced by the type of adenovirus used in the study. Moreover, similar studies in monkeys used human adenovirus to reproduce the Step trial in monkeys (12–14). It is important to note that adenovirus can also be found in macaques and that these adenoviruses are different from those isolated from humans (15), which suggest that the use of a vector based on an endogenous rhesus macaque AdV may more accurately model host-vector interactions than the use of an endogenous human adenoviral vector, such as HAdV-5. Vaccination with a human AdV vector in the macaque model may produce an incomplete understanding of potential outcomes, as was seen in studies leading up to the Step trial (12–14).

To address this, we examined whether vaccination of rhesus macaques with a simian adenovirus serotype 7 (SAdV-7) (16)–based vector vaccine differentially influences peripheral and mucosal CD4+ T cell responses and cellular activation compared to an HAdV-5–based vector. We find that both SAdV-7 and HAdV-5 vector vaccination can increase AdV–specific CD4+ T cell cytokine responses in blood and, more dramatically, rectal mucosa. Importantly, however, both vectors heightened the cellular activation profile of total and AdV–specific CD4+ T cells specifically within the rectal mucosa. This increase in rectal mucosa CD4+ T cell activation was most pronounced after the vaccine prime and in SAdV-7–vaccinated animals was accompanied by a prolonged decrease in resident naive CD4+ T cells within the rectal mucosa. These data highlight the importance of assessing tissue-specific effects induced by vaccine platforms and provide a potential mechanism by which adenovirus vaccine vectors might influence susceptibility to HIV, as was observed in the Step study (3).

MATERIALS AND METHODS

Adenovirus vectors. Wild-type SAdV-7 was purchased from the ATCC (ATCC VR-201, originally isolated from rhesus monkey kidney cells). HAdV-5 was generated from a molecular clone (Clontech) by Vector Core (University of Pennsylvania). SAdV-7–based vectors were constructed as previously described (17). Briefly, the viral genome was molecularly cloned, and expression cassettes (severe acute respiratory syndrome [SARS] coronavirus spike protein driven by a chicken beta-actin promoter or beta-galactosidase [beta-gal] driven by a cytomegalovirus [CMV] promoter) were inserted in place of an E1 deletion to generate the replication-incompetent vectors SAdV-7–SARS spike and SAdV-7–LacZ, respectively. These vectors as well as the HAdV-5 vectors used in this study were propagated on HEK 293 cells and purified on CsCl gradients. Moreover, both vectors heightened the cellular activation profile of total and AdV–specific CD4+ T cells specifically within the rectal mucosa. This increase in rectal mucosa CD4+ T cell activation was most pronounced after the vaccine prime and in SAdV-7–vaccinated animals was accompanied by a prolonged decrease in resident naive CD4+ T cells within the rectal mucosa. These data highlight the importance of assessing tissue-specific effects induced by vaccine platforms and provide a potential mechanism by which adenovirus vaccine vectors might influence susceptibility to HIV, as was observed in the Step study (3).

Animals. Seventeen captive-bred rhesus macaques of Indian origin were purchased from Covance Research Products (Alice, TX) and enrolled in this study. All animals were treated and cared for at the Nonhuman Primate Research Program (NPRP) facility of the Gene Therapy Program of the University of Pennsylvania (Philadelphia, PA) during the study. The study was performed according to a protocol approved by the Environmental Health and Radiation Safety Office, the Institutional Biosafety Committee, and the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. All macaques were intramuscularly (i.m.) immunized 3 times with SAdV-7 or HAdV-5 at a dose of 1 × 1011 particles/injection (see Fig. 2 for the immunization schedule).

Endoscopic sampling of macaque rectum and isolation of lamina propria lymphocytes. All nonhuman primates (NHPs) were fasted the evening before the procedure, with free access to water at all times. NHPs were sedated, and biopsy specimens were obtained by utilizing an alligator jaw-style endoscopic biopsy pinch held freehand. Twenty biopsy specimens were taken, spaced far enough apart so as not to weaken the rectal wall. Biopsy specimens were placed into RPMI medium, and lamina propria lymphocytes (LPLs) were isolated by using collagenase type II as previously described (18).

Blood collection and isolation of peripheral blood mononuclear cells. PBMCs were isolated from whole blood collected into heparin-containing Vacutainer tubes after Ficoll (Amersham Bioscience) density gradient centrifugation at 1,000 × g for 25 min. Cells were collected from the interphase and washed with phosphate-buffered saline (PBS). PBMCs were incubated with ammonium chloride potassium lysing buffer to lyse red blood cells and washed and resuspended in complete RPMI medium (Mediatech) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES, 50 μg/ml gentamicin sulfate, and penicillin-streptomycin.

AdV neutralizing antibody assay. Anti-SAdV-7 or anti-HAdV-5 neutralizing antibody (NAb) titers in serum samples were measured by assessing the ability of serum to inhibit transduction of the corresponding reporter vector, SAdV-7–LacZ or HAdV-5–LacZ, respectively, into HEK 293 cells. The reporter vector was incubated with 2-fold serial dilutions (initial dilution, 1/5) of heat-inactivated sera for 1 h at 37°C. Serum samples were diluted with naive mouse serum (Sigma-Aldrich) so that the final serum concentration at all dilutions was 5%. Subsequently, the serum-vector mixture was added to HEK 293 cells in 96-well flat-bottomed plates (at a multiplicity of infection [MOI] of 10 virus particles [VP] per cell) and incubated for 18 to 22 h. Cells were then washed twice in PBS and lysed, and the lysate was developed with the mammalian β-galactosidase assay kit for bioluminescence, in accordance with the manufacturer’s protocols (Applied Biosystems), and measured in a microplate luminometer (Clarity; BioTek). The NAb titer was reported as the highest serum dilution that inhibited AdV-CMV-LacZ transduction (β-gal expression) by 50% compared with the mouse serum control (protocol number S3509; Sigma).

Antibody reagents. Antibodies used for surface staining included anti-CD14 Qdot 655, anti-CD20 Qdot 655, anti-CD8 phycoerythrin (PE) (Texas Red; Invitrogen, Carlsbad, CA), anti-CD28 PE-Cy7 (eBioscience, San Diego, CA), anti-CD45R Pac Blue (Biolegend, San Diego, CA), anti-CD25 allophycocyanin (APC)-Cy7, anti-CD95 PE-Cy5, and anti-HLA-DR peridinin chlorophyll protein (PerCP)-Cy5.5 (BD Pharmingen, San Diego, CA). Antibodies used for intracellular staining included anti-interleukin-2 (IL-2) Alexa 700 (700; Biolegend), anti-gamma interferon (IFN-γ) Alexa 700 (Invitrogen), anti-tumor necrosis factor alpha (TNF-α) Alexa 700, anti-CD69 APC, and anti-Ki67 fluorescein isothiocyanate (FITC) (BD Pharmingen).

Cell processing and stimulation. Rectal biopsy specimens were processed within 6 h of being collected. Rhesus macaque PBMCs were cryopreserved in FBS (ICS HyClone, Logan, UT) containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA) and stored in liquid nitrogen until use. After washing of fresh LPL cells or PBMCs thawed once in RPMI medium (Mediatech Inc., Manassas, VA), both PBMCs and LPL cells were resuspended in complete medium (RPMI medium supplemented with 10% FBS, 1% l-glutamine [Mediatech Inc.], and 1% penicillin-streptomycin [Lonza, Walkersville, MD], sterile filtered) at a concentration of 1 × 106 to 2 × 106 cells/ml medium in fluorescence-activated cell sorter (FACS) tubes. Cells were split into three stimulation conditions, at a volume of 1 ml each, with either no stimulation, 1 μl Staphylococcus enterotoxin B (SEB) at a concentration of 1 mg/ml (Sigma-Aldrich, St. Louis, MO) as a positive control, or 1 × 109 particles/ml of the SAdV-7 vector. Cells were stimulated overnight at 37°C in 5% CO2.

FACS staining assay. Stimulation tubes were removed from the incubator in the early morning to add monein (0.7-μg/ml final concentration; BD Biosciences) and brefeldin A (1-μg/ml final concentration; Sigma-Aldrich, St. Louis, MO) and incubated for an additional 6 h. Cells were then washed once with PBS and stained for viability with Aqua amine-reactive dye (Invitrogen) for 10 min in the dark at room temperature. A mixture of antibodies used for staining of surface markers was...
added to the cells and kept at room temperature for 20 min. Cells were washed with PBS containing 1% bovine serum albumin (BSA; Fisher Scientific) and 0.1% sodium azide (Fisher Scientific) and permeabilized for an additional 20 min at room temperature by using the Cytofix/Cytperm kit (BD Pharmingen). Next, cells were washed in Perm/Wash buffer (BD Pharmingen). A mixture of antibodies used for staining of intracellular markers was added to the cells and incubated in the dark for 1 h at room temperature. Cells were again washed with Perm/Wash buffer and fixed with PBS containing 1% paraformaldehyde (Sigma-Aldrich). Fixed cells were stored in the dark at 4°C until being collected for flow cytometric analysis. Biopsy samples from four animals at the baseline time point were stained with a different fluorophore panel and were not included in the analysis. These samples are identified in Results as “lost.”

Flow cytometric analysis. For each sample, between 3 × 10^6 and 1 × 10^7 total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems, San Jose, CA) equipped to detect up to 18 fluorescent parameters. Antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each antibody used in the experiment. Data analysis was performed by using FlowJo version 9.0.1 (TreeStar, Ashland, OR). Percent expression is shown after background subtraction, where values were calculated as the difference between cells that were stimulated with the SAdV-7 vector overnight and those that were left unstimulated. AdV-specific percentages are reported as the population of CD4^+ memory T cells which expressed IL-2, IFN-γ, and/or TNF-α within each compartment. While we did not include a placebo-only group, AdV-specific T cell responses prior to immunization at baseline should reflect this appropriately. To separate naive cells from memory, effector memory, and effector T cells, we stained cells with fluorochrome-conjugated antibodies for CD28 and CD95, where CD28^+ CD95^- CD4^+ T cells indicated the naive subset, whereas all other cells were grouped as memory cells. Naïve CD4^+ T cells were gated by using FlowJo for each macaque at all time points in both compartments. Although a CD3 antibody was not included in the panel, a CD14/CD20 exclusion gate as well as careful gating, including CD4^+ bright cells while excluding CD8^+ cells, provided assurance in the gating strategy.

Figures. Prism software version 5.0 (Graphpad, La Jolla, CA) was used to create the figures.

Statistical analysis. Activation levels were summarized overall and at each time point. Graphical methods were employed to assess distributional assumptions of the data. SAdV-7 and HAdV-5 group levels were modeled over time by using GEEs (generalized estimating equations), which are a standard statistical approach for testing hypotheses involving repeated-measures data (19). They have the advantage over traditional repeated-measures analysis of variance (ANOVA) of being able to handle randomly missing observations of animals. GEEs adjust for the fact that one has data from an animal over time by linking those data. They are similar to repeated-measures ANOVA models in that they model the inherent correlation from measurements made for the same animal at different times; however, they can handle data that are missing completely at random. GEE models are flexible, allowing overall group comparisons, comparisons at each time point, as well as within-group comparisons to their respective baseline levels. The GEE models used were of the form \( f(\text{outcome}) = \text{constant} + \text{group} + \text{time} + \text{group} \times \text{time} \). Time was included as a categorical (rather than continuous) variable to allow for potentially nonlinear changes from one time point to another. The transformation \( f \) used for the outcome was based on whether the outcome of interest was normally distributed, Poisson distributed, or binomial (standard transformations for each type were used). Once the model was fit (and assessed for goodness of fit), the average differences between groups at each time point, and also the differences within a group from the baseline, were calculated. The \( P \) values for these pairwise comparisons were calculated by using the Holm-Bonferroni method in order to preserve the overall type I error rate of 0.05. This was done to make sure that we did not incorrectly find a comparison significant by chance just because of the number of comparisons being performed. To investigate naïve CD4^+ T cells over the time course, four analyses were completed. Namely, the LPL outcome was evaluated separately for the two groups of macaques determined by the type of vaccine administered (SAdV-7 and HAdV-5), and a similar series of analyses was conducted for PBMCs for the two vaccine groups. For each analysis, GEE models were fit to accommodate the repeated measures of macaques over time. Specifically, linear GEE models were fit for each continuous outcome (LPLs and PBMCs), adjusting for a categorical “time” effect and assuming an exchangeable correlation structure. Model-based differences of predicted outcomes based on each respective fitted model are presented by vaccine type. Finally, raw \( P \) values from the pairwise differences comparing outcomes to each subsequent follow-up time point as well as comparing week 16 and week 33 were adjusted by using the Holm-Bonferroni method to control for multiple comparisons.

RESULTS

Simian adenovirus type 7 immunity and vaccination. Adenovirus vectors are known to elicit potent T cell responses to the encoded transgenes and have the potential to be an important component of a defense strategy against infectious agents such as HIV, where T cell-based immunity can have an important part to play in either preventing infection or containing virus replication. However, prior exposure to adenoviruses in vaccinees can result in a memory response to the payload adenoviral capsid antigens (20). This response is likely to be strongly influenced by the epitopes that are shared between capsid antigens present in the adenoviral vaccine and the epitopes in adenoviruses that have caused prior infections in the host. Furthermore, it is likely that both humans and macaques are chronically infected by adenoviruses (21, 22), resulting in ongoing restimulation of memory responses. The use of an adenovirus homologous to the species used in the study may be important to restimulate this response and properly model the immune parameters corresponding to an adenoviral vaccination, including the magnitude and the homing of the response. A phylogenetic analysis of monkey adenovirus hexon protein sequences (Fig. 1) indicates that they are divergent from all human adenoviral species. Other macaque adenovirus proteins also form clusters distinct from human adenoviruses (data not shown). Thus, it is plausible that vaccination of monkeys by a macaque-derived adenovirus such as SAdV-7 would result in anamnestic responses that may similarly replicate what might be expected in human subjects vaccinated with an HAdV-5 vector.

We vaccinated 12 rhesus macaques three times intramuscularly with 1 × 10^11 virus particles (VP) of an E1-deleted, replication-defective, SAdV-7-derived vector expressing SARS spike (see Materials and Methods for a description of the construct). As a control, we vaccinated five additional rhesus macaques with an E1-deleted, replication-defective, HAdV-5-derived vector containing the same transgene and promoter. Ten sample collection time points were interspersed around the vaccinations (Fig. 2). Prior to vaccination, baseline levels of SAdV-7 neutralizing antibody (NAb) titers ranged from undetectable (<5) to 640, with 11/17 macaques displaying an SAdV-7 titer of ≥10 (Table 1). All macaques had undetectable levels of HAdV-5 NAbs at baseline. Vaccination with either the SAdV-7 or HAdV-5 vector occurred at weeks 0, 17, and 31, while rectal biopsy specimens and peripheral blood were collected at −2 (baseline), 2, 5, 9, 16, 19, 21, 25, 29, and 33 weeks after the first immunization. Baseline rectal biopsy specimens from four rhesus macaques (macaques 04C010, 05C069, 05D007, and 05D079) were lost during analysis. Additionally, an LPL sample from week 21 (macaque 04C058) and a peripheral
blood sample from week −2 (baseline) (macaque 04C059) were unable to be processed. Cells were stained for multiple markers, with a particular focus on memory markers (CD28 and CD95), activation markers (HLA-DR, CD25, CD69, and Ki67), and cytokine functionality (IL-2, IFN-γ, and TNF-α), for flow cytometric analysis (representative gating is shown in Fig. 3).

**Adenovirus-specific CD4+ T cells expand postvaccination.**

We first examined AdV-specific CD4+ T cells at baseline and whether AdV vector vaccination influenced these frequencies. We assessed cytokine expression on memory CD4+ T cells after stimulation with either SEB or SAdV-7 (or no stimulation) in rectal lamina propria lymphocytes (rLPLs) and peripheral blood (PBMCs) from vaccinated macaques (Fig. 4). Cells from HAdV-5-vaccinated animals were stimulated with SAdV-7 to determine whether vaccination boosted cross-reactive responses against the heterologous SAdV-7 vector. SAdV-7-specific CD4+ T cell responses were determined by the production of IFN-γ, TNF-α, and/or IL-2 at each collection time, as shown in Fig. 4. Several animals had detectable AdV-specific CD4+ T cells (a response of >0.05% was considered positive) in both rectal lamina propria and peripheral blood at baseline, prior to AdV vector vaccination (4/9 SAdV-7 rLPLs [3 lost to testing], 4/12 SAdV-7 PBMCs, 3/4 HAdV-5 rLPLs, and 4/4 PBMCs [1 lost to testing]). Only one SAdV-7-immunized animal (macaque 04C068) had detectable AdV-specific CD4+ T cells within both tissues, and one HAdV-5-immunized animal (macaque 02C061) had AdV-specific CD4+ T cells within both tissues. We found no statistically significant correlation between baseline AdV-specific CD4+ T cell responses and preexisting baseline SAdV-7-specific NAbs in PBMCs or rLPLs (data not shown). While preexisting AdV-specific CD4+ T cells were present in both compartments, the magnitude of the response was consistently higher in the rectal mucosa, with 4 macaques exhibiting >1% expression in rLPLs (ranging from 0 to 1.26% for SAdV-7 and 0 to 1.5% for HAdV-5), while 0.139% was the highest cytokine response in PBMCs (ranging from 0 to 0.095% for SAdV-7 and 0 to 0.139% for HAdV-5).

**FIG 1** Phylogenetic relationships between hexon proteins from human and monkey adenoviruses. (The amino acid sequences were aligned by using ClustalW version 2.0.3 and refined by using Gblocks version 0.91b. The alignment was used to construct the phylogenetic tree by using PhyML version 3.0 aLRT and rendered by using TreeDyn 198.3. Branch support values are indicated as percentages. The scale bar indicates the number of substitutions per site.) Representatives of human adenoviruses of species A through G (serotypes in parentheses and including HAdV-5, in boldface type) and several monkey adenoviruses are shown. Monkey adenoviruses (including SAdV-7, which was used to construct vectors used in this study [highlighted in red]) are seen to belong to a phylogenetically distinct group (gray box).

**FIG 2** Vaccination and sample collection timeline. Shown is the macaque trial design with 10 tissue (PBMCs and rLPLs) collection time points and either SAdV-7 or HAdV-5 vector vaccinations interspersed at weeks 0, 17, and 31.
After both the first and second immunizations, SAdV-7-specific CD4+ T cell cytokine responses increased substantially in many animals and reached statistical significance as a group relative to baseline values within the rectal mucosa at week 5 postprime \((P < 0.01)\) (Fig. 4). We found a general association between the peak of the AdV-specific CD4+ T cell response and preexisting AdV NAb titers in the rLPLs of SAdV-7-vaccinated macaques, with the three highest responses being found in macaques with a NAb titer of \(\geq 10\). No association between AdV-specific CD4+ T cell responses and preexisting AdV NAbs was found in blood or in HAdV-5-vaccinated macaques (data not shown). The highest SAdV-7-specific cytokine responses were seen in the rLPLs of SAdV-7-vaccinated macaques, peaking at 14.42% (macaque 04C058) and 16.84% (macaque 04C066) at week 5 postprime and at 15.62% (macaque 05D007) at week 9 postprime (Fig. 4). An increase in the frequency of SAdV-7-cross-reactive CD4+ T cells in the rLPLs of HAdV-5-vaccinated macaques was also noted for 3 animals with a >3% AdV-specific CD4+ T cell response at week 5 postprime. While increases in AdV-specific responses were also observed in PBMCs, they were largely <1.5%, with singular exceptions of SAdV-7-vaccinated macaques at isolated time points. In both SAdV-7- and HAdV-5-vaccinated macaques, rLPLs that were stimulated with the SAdV-7 vector were predominantly central memory CD4+ T cells (CD28+ CD95-), while PBMCs were a mix of naive (CD28+ CD95-) and central memory phenotypes (data not shown). These results indicate that there are preexisting AdV-specific T cell responses in both the peripheral blood and rectal mucosa and that AdV vector vaccination can strongly increase AdV-specific T cell frequencies in the rectal mucosa, with peak mucosal responses being at least 3-fold higher on average than those seen in peripheral blood.

**Adenovirus vector vaccination increases the frequency of activated memory CD4+ T cells in the rectal mucosa.** To determine whether SAdV-7 vaccination affected the activation state of total CD4+ T cells in the rectal lamina propria or in blood, we assessed the expression of four different activation markers simultaneously (CD25, CD69, HLA-DR, and Ki67) on unstimulated CD4+ T cells from these sites (Fig. 1, bottom). At baseline, rectal lamina propria lymphocytes from SAdV-7-vaccinated macaques had a slightly higher percentage of activated memory CD4+ T cells (range, 16.8 to 29.5% and 11.7 to 19.6% in SAdV-7- and HAdV-5-vaccinated macaques, respectively) than did PBMCs (range, 7.1 to 20.4% and 9.8 to 14% in SAdV-7- and HAdV-5-vaccinated macaques, respectively) (Fig. 5). We found no association between baseline SAdV-7 NAb titers and the frequency of activated memory CD4+ T cells over the immunization period (data not shown). Similarly to the rLPLs of unstimulated cells of SAdV-7-vaccinated macaques at baseline, the rLPLs of SAdV-7 vector-stimulated cells (Fig. 6) from SAdV-7-vaccinated macaques had the highest ex-
expression levels of activation markers on memory CD4\(^+\) T cells compared to the blood or HAdV-5-vaccinated macaques. Stimulation ex vivo with the whole SAdV-7 vector overnight noticeably increased the percentage of activated cells at baseline relative to unstimulated cells.

Following vaccination, rectal LPL CD4\(^+\) T cell activation in unstimulated cells increased in several macaques at week 2 (Fig. 5). In two SAdV-7-vaccinated macaques (macaques 04C010 and 04C066), these levels reached nearly 50%, and SAdV-7-vaccinated macaques averaged a 1.42-fold increase from the baseline at week 2 (\(P < 0.01\)). These macaques maintained heightened activation for several weeks, peaking at up to a 1.5-fold group average increase from baseline at week 16. Interestingly, we also observed an increase in mucosal activation levels following priming and boosting in some HAdV-5-vaccinated animals, with one macaque (macaque 05D079) reaching nearly 40% total activated CD4\(^+\) memory T cells at week 2. At week 9, HAdV-5-vaccinated macaques had a 1.7-fold average increase from baseline values, with average values ranging between 1.35- and 1.7-fold throughout the duration of this study. We did not find a significant increase of PBMC activation levels in unstimulated cells of SAdV-7- or HAdV-5-vaccinated macaques at any study time point (Fig. 5).

For the SAdV-7-stimulated conditions (Fig. 6) at week 2, >40% of the rLPLs consisted of activated CD4\(^+\) memory T cells in 7/12 SAdV-7-vaccinated macaques and 2/5 HAdV-5-vaccinated macaques. Changes in mucosal SAdV-7-specific activation averaged consistently above 1.49-fold (SAdV-7 vaccinated) and 1.18-fold (HAdV-5 vaccinated) during weeks 2 to 16. Thus, after the SAdV-7 prime, a marked increase in activation levels of both resting and SAdV-7-specific memory CD4\(^+\) T cells was noted for the rLPLs, which was maintained at a heightened level relative to baseline levels for several weeks. In contrast, a significant reduction in the number of activated CD4\(^+\) memory T cells was observed for PBMCs (Fig. 6). These data indicate that immunization with an AdV vector, whether of macaque or human origin, can cause increased activation marker expression in rectal lamina propria CD4\(^+\) T cells which is not observed for peripheral blood.

**Increased frequency of rectal mucosa naive CD4\(^+\) T cells after adenovirus vector vaccination.** Given the increased frequencies both activated and cytokine-expressing memory CD4\(^+\) T cells in the rectal mucosa after vaccination, we examined whether there were overt changes in the naive CD4\(^+\) T cell population (Fig. 7), defined as CD28\(^+\)CD95\(^-\) cells (Fig. 7B), in the peripheral blood or rectal lamina propria following vaccination. We found pronounced decreases in the relative frequency of naive CD4\(^+\) T cells within the lamina propria of SAdV-7-vaccinated macaques up to week 16, after which these frequencies slowly returned to around the baseline level by week 33 (Fig. 7B). In contrast, the frequency of naive CD4\(^+\) T cells within the PBMCs of SAdV-7-vaccinated macaques increased steadily above baseline levels as a group average postvaccination. At baseline, PBMCs had a higher frequency of naive CD4\(^+\) T cells, ranging between 18

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**FIG 4** AdV vector vaccination increases memory AdV-specific CD4\(^+\) T cell percentages. (A) Representative memory CD4\(^+\) T cell IL-2/IFN-\(\gamma\)/TNF-\(\alpha\) response after stimulation with 1 \(\times\) 10\(^{10}\) particles/ml of whole SAdV-7 vector. The number at the bottom right of the plot represents the proportion of cells that express IL-2/IFN-\(\gamma\)/TNF-\(\alpha\). (B) Percent CD4\(^+\) memory T cell IL-2/IFN-\(\gamma\)/TNF-\(\alpha\) expression in response to whole SAdV-7 vector stimulation (SAdV-7 stimulated) overnight after background (no stimulation) subtraction. Levels of IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) production were measured on the same fluorochrome. Peripheral blood and rectal lamina propria T cells from 12 SAdV-7-vaccinated and 5 HAdV-5-vaccinated rhesus macaques (symbols for each macaque are listed in the key) were collected at 10 time points, including baseline (week -2), interspersed with three AdV vector immunizations. Significance at any time point was calculated as the percent increase relative to the baseline value and was determined as described in Materials and Methods. Statistically significant increases relative to the baseline value are indicated above the red bars (week 19 approached statistical significance). +, animals with no rectal biopsy data at the baseline time point.
and 44.4% and between 17.4 and 39.7% for SAdV-7- and HAdV-5-vaccinated macaques, respectively, while the frequencies of rLPLs varied from 1.5 to 24% and 1.6 to 9.6%, respectively, for these groups. There was no evidence for an association between baseline SAdV-7 NAb titers and percentages of naive CD4⁺ T cells over time (data not shown). No such overall decrease was noted for naive rLPL CD4⁺ T cells after HAdV-5 vaccination in the control animals.

FIG 5 Increased activation marker expression on total unstimulated memory CD4⁺ T cells in rectal mucosa after AdV vector vaccination. Shown are percentages of total unstimulated memory CD4⁺ T cells expressing at least one of four activation markers (HLA-DR, CD25, Ki67, and CD69). Peripheral blood and rectal lamina propria T cells from 12 SAdV-7-vaccinated and 5 HAdV-5-vaccinated rhesus macaques (symbols for each macaque are listed in the key) were collected at 10 time points, including baseline (week −2), interspersed with three AdV vector immunizations. Significance at any time point was calculated as the percent change relative to the baseline value, shown by red lines above each plot, and was determined as described in Materials and Methods. +, animals with no rectal biopsy data at the baseline time point.

FIG 6 Increased activation marker expression on total SAdV-7-stimulated memory CD4⁺ T cells in rectal mucosa after AdV vector vaccination. Shown are percentages of total memory CD4⁺ T cells stimulated with whole SAdV-7 vector overnight and expressing at least one of four activation markers (HLA-DR, CD25, Ki67, and CD69). Cells are from the same rhesus macaques and time points as those in Fig. 5, with the same statistical significance. +, animals with no rectal biopsy data at the baseline time point.
The importance of T cell responses in the control of HIV infection is well documented (13, 23, 24), and HAdV-5 vector-based vaccines have been shown to be highly immunogenic for inducing cell-mediated immunity (25, 26). The Step and Phambili studies were able to generate HIV-specific T cell immunogenicity in many participants but did not accomplish their goal of either preventing HIV-1 infection or decreasing the viral load set point (3), a finding mirrored in the recently discontinued HVTN 505 trial. Perhaps more troubling is the clear evidence of a vaccine-mediated enhancement effect on HIV acquisition reported by the Step and Phambili studies. The underlying mechanism for this AdV vaccine-mediated enhancement of infection has remained controversial. It has been speculated that the presence of both cross-reactive AdV-specific T cells and preexisting antibodies to AdV, due to previous natural exposure to any adenoviral serotype, had a direct causal effect on increased HIV infection, although it is as yet unclear if these may be surrogates for a different mechanism. Other proposed theories are that the HAdV-5 vaccine is unable to elicit a sufficiently broad immune response to counteract HIV-1 strain diversity or that the AdV vector induced a response that was of an altered, undesirable quality (5, 27).

In this study, we used a rhesus macaque model to investigate the hypothesis that the rectal mucosa is a site for increased T cell activation, either global or antigen specific, after AdV vector vaccination. Furthermore, while we did not test it here, persistent adenovirus presence (18, 28) is a driver for T cell migration to the gut, possibly increasing the population of activated T cells for SIV to infect. Our data show that vaccination with an AdV vector, whether of human or macaque origin, increased the percentage of total and AdV-specific activated CD4⁺ T cells in the rectal mucosa, an initial site of HIV/SIV transmission. This effect was not observed in the blood. Additionally, our analyses reveal that AdV vector vaccination selectively increases AdV-specific memory CD4⁺ T cell responses in the rectal mucosa above already existing levels. In contrast, little change in CD4⁺ T cell activation was found in the peripheral blood. We do not expect that SAdV-7-specific CD4⁺ T cells are any more or less susceptible to SIV infection than any other activated CD4⁺ T cell, but it is clear from our results that there is an increase in activated mucosal CD4⁺ T cells in vaccinated animals relative to baseline levels. Peripheral blood adenovirus-specific CD4⁺ T cells within these animals (n = 3) did not appear to express different levels of CD4, CCR5, or macrophage inflammatory protein b (MIP-1b) upon stimulation compared to non-adenovirus-specific CD4⁺ T cells (activated or resting) (data not shown). Future studies will be necessary to determine whether mucosal adenovirus-specific CD4⁺ T cells express differential levels of these markers after vaccination. Furthermore, our results showed that priming with the SAdV-7 vector decreased the percentage of naive CD4⁺ T cells in the rectal lamina propria, but not in blood, prior to boosting. Together, these findings emphasize the value of sampling from mucosal sites...
in the context of vaccination with putative HIV vaccine platforms. Indeed, mounting evidence for unique phenotypes between the peripheral blood and gut mucosa (28, 29) indicates that analyzing one compartment alone is insufficient for guiding further vaccine development.

Previous studies prior to the Step trial had examined the impact of HAdV-5 vector vaccination followed by SIV infection in macaques, but these models consistently failed to predict adverse effects of vaccination (12–14, 16). When we compared the immunological effects of SAdV-7 to those of HAdV-5 vector vaccination, we anticipated increased frequencies of activated and AdV-specific CD4+ T cells specifically in SAdV-7 vector-vaccinated macaques. Instead, we were surprised to find that HAdV-5 vector-vaccinated macaques showed significant responses as well, suggesting cross-reactivity of memory AdV-specific T cell epitopes between species-specific AdV serotypes. With 65 known strains of human adenovirus and at least 25 nonhuman primate strains (21, 22, 30, 31), it is likely that AdV-specific T cell memory and cross-reactivity may affect more rare serotypes, in addition to common AdV serotypes (20). We have demonstrated AdV T cell cross-reactivity in monkeys and humans in this study as well as previously (18) and recognize that changes in activation levels or trafficking patterns depend on the specific adenovirus vector used as well as the degree of cross-reactivity of the humoral and cellular immune responses. Furthermore, we have also shown that human intestinal tissue, particularly intestinal lymphocytes, can frequently be found to harbor adenoviral DNA (22) and that captive macaques likely accumulate from exposure to multiple AdVs (6, 10) and not solely SAdV-7. Since we have shown previously that HAdV-5 vector vaccination in humans expands HAdV-5-specific CD8+ T cells (34), we believe that intercurrent adenoviral infections in macaques and humans are likely modeling each other, as they both exhibit increases in AdV-specific T cells. Furthermore, one unresolved observation was the downward trend of naive CD4+ T cells in the rectal lamina propria of SAdV-7-vaccinated macaques following the vaccine prime, which appeared to return to baseline levels following the vaccine boosts. The initial decrease in the frequency of naive T cells may indicate trafficking of activated memory AdV-specific CD4+ T cells into the gut mucosa. Alternatively, our observations may reflect the loss of naive cells, expansion of memory cells, transition of naive to memory cells, or altered trafficking of naive cells out of the gut mucosa. Regardless of the underlying mechanism, the net result of this effect is a temporary increase in the frequency of activated memory CD4+ T cells in the mucosa, creating a potentially favorable dynamic for increased susceptibility to HIV infection.

We have shown that vaccination with both endogenous macaque as well as human adenoviral vectors can lead to significant increases in the frequencies of AdV-specific and activated CD4+ T cells in the rectal lamina propria of macaques but not in the peripheral blood. While it is unclear at this time whether this induction of mucosal CD4+ T cell activation is sufficient to increase susceptibility to SIV infection, these results clearly suggest that adenovirus vaccination might indeed create such an environment. Previous adenovirus vaccine platform studies have not indicated an increase in SIV susceptibility, but importantly, SIV challenge in these models did not take place within the vaccine-induced mucosal CD4+ T cell activation window that we found here. Ultimately, a low-dose SIV challenge study following AdV-based vector vaccination within the activation period that we have found will offer a proof of concept for the effects of such activation. In the meantime, our findings raise a note of caution regarding the development of HIV vaccine vectors based upon mucosal pathogens and suggest that greater consideration should be placed upon the induction of vector-specific immunity and more generalized activation effects within appropriate tissue sites. More broadly, the effects on mucosal CD4+ T cell activation that we have observed may not be restricted to adenovirus and may be difficult to circumvent for the induction of effective mucosal immune responses by vaccination.

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