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Novel Vaccination Protocol with Two Live Mucosal Vectors Elicits Strong Cell-Mediated Immunity in the Vagina and Protects against Vaginal Virus Challenge

Zhongxia Li,* Manxin Zhang,* Chenghui Zhou,* Xinyan Zhao,* Norifumi Iijima,† and Fred R. Frankel²*

Most HIV infections result from heterosexual transmission to women. Because cellular immunity plays a key role in the control of the infection, we sought to strengthen cellular immune responses in vaginal tissue. We explored a novel prime-boost protocol that used two live mucosal agents that trigger different pathways of innate immunity and induce strong cellular immunity. Adenovirus serotype 5 (Ad5) has frequently been used as a boost for DNA vaccines. In this study we used attenuated, recombinant L. monocytogenes-gag (rLm-gag) to prime mice by various mucosal routes—oral, intrarectal, and intravaginally (ivag)—followed by a systemic or mucosal boost with replication-defective rAd5-gag. Mice primed with a single administration of rLm-gag by any route and then boosted with rAd5-gag intramuscularly exhibited abundant Gag-specific CD8 T cells in spleen and vaginal lamina propria. Conversely, when boosted with rAd5-gag ivag, the immune response was reoriented toward the vagina with strikingly higher CD8 T cell responses in that tissue, particularly after ivag immunization by both vectors (ivag/ivag). Five weeks to 5 mo later, ivag/ivag-immunized mice continued to show high levels of effector memory CD8 T cells in vagina, while the pool of memory T cells in spleen assumed a progressively more central memory T cell phenotype. The memory mice showed high in vivo CTL activity in vagina, a strong recall response, and robust protection after ivag vaccinia-gag challenge, suggesting that this prime-boost strategy can induce strong cellular immunity, especially in vaginal tissues, and might be able to block the heterosexual transmission of HIV-1 at the vaginal mucosa. The Journal of Immunology, 2008, 180: 2504–2513.

S
ome 40 million people are currently infected with HIV/AIDS as this pandemic becomes one of the most devastating in human history. HIV subverts the immune system by infecting and depleting CD4⁺ T cells of mucosal surfaces and becomes an integral part of the host cell genetic material. Over half of all infected individuals are women (1). Sexual transmission of the virus occurs at the vaginal epithelium where virus particles and virus-infected cells from semen are deposited on the mucosal surface. The virus is then transported through the epithelial barrier via tissue abrasions or by attaching to or infecting embedded CD4⁺ T cells or Langerhans cells and subsequently infects the abundant T cells and macrophages that reside in the submucosa (2–4). Although the process is inefficient (5–9), its success leads to the establishment of founder populations of infected cells that rapidly invade the entire host (10). The vigorous immune response that ensues fails to keep pace with the virus, leading to profound immunodeficiency and death if unchecked.

The development of an effective vaccine is very likely the only means to halt the pandemic. Several vaccine strategies depend on prime-boost protocols that produce a selective increase of memory T cells (TEM) specific for the shared Ag of the vectors. These cells show increased avidity (11, 12) and are predominantly effector memory cells that localize to peripheral nonlymphoid tissues, the major sites of pathogen encounter (13). Frequently, DNA vaccines have been used for the primary immunization. However, responses to DNA vaccines tend to be weak unless supplemented with molecules that induce additional inflammatory reactions that mimic responses to a pathogen, suggesting that more effective vaccines may be those capable of engaging multiple innate receptors that can act synergistically to trigger cell-mediated immunity and polyvalent responses (14–16).

Because HIV and other infectious agents enter the body at mucosal surfaces, mucosal immune responses function as a first line of defense against infection. Yet, the vast majority of vaccines presently in use are administered systemically, and immunity in the genital mucosa has largely been unexplored. Some studies indicate that the route of vaccination can result in a selective distribution of tissue lymphocytes (17–24) and that mucosal vaccination can provide effective local protection at the site of Ag encounter (25–31). In this report we examined a strategy designed to enhance protection against vaginal virus transmission by using a unique prime-boost protocol that employs two live mucosal vectors, Listeria monocytogenes (Lm)³ and adenovirus type 5 (Ad5), to elevate the frequency of memory CD8⁺ T cells in the vaginal lamina propria.

³Abbreviations used in this paper: Lm, Listeria monocytogenes; Ad5, adenovirus type 5; CD62L, CD62 ligand; ig, oral gavage; im, intramuscularly; ivag, intravaginally; ivag/ivag, intravaginal administration of both vectors, rLm-gag and rAd5-gag; TCM, central memory T cell; TEM, effector memory T cell; vps, virus particles.

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Materials and Methods

Mouse immunization

Six- to 8-week-old female BALB/c mice were obtained through the National Cancer Institute (Frederick, MD). Animal procedures met federal guidelines and institutional policies of the University of Pennsylvania Animal Care and Use Committee (Philadelphia, PA). For most infections by L. monocytogenes we used a recombinant attenuated strain, L. monocytogenes dal1 dal2 Bacillus subtilis dal1 HIV-1-gag (referred to here as attenuated Lm-gag; Z. Li, M. Zhang, C. Zhou, X. Zhao, and F. R. Frankel, manuscript in preparation), which contains a single chromosomal copy of the dal gene of B. subtilis that partially complements the Lm dal gene deletion (32, 33), and shows a systemic LD50 of 1 x 10^7 (vs wild-type Lm LD50 of ~1 x 10^7). For oral infections, the bacteria were administered by gavage (g) at a dose of 1.5 x 10^9 CFU. Intracutaneous infections were delivered by a 2-cm catheter at the same dose. For intravaginal (ivag) infections, 5 x 10^10 bacteria in a small volume (20 μl) were deposited into the vaginal canal. Intravaginal infections by all vectors (Lm-gag, rAd5-gag, and rVac-gag) were preceded on day −5 with a s.c. injection of 2 mg of medroxyprogesterone acetate (Depo-Provera) to thin the vaginal mucosa. At the time of infection the animals were anesthetized by an i.p. injection of ketamine (150 mg/kg body weight) plus xylazine (10 mg/kg) in 150 mM NaCl solution, vaginal mucus was removed by the use of a fine calcium alginate swab, and the vector was deposited and left in the vagina during a 60-min period of anesthesia. Replication-defective E1- and E3-deleted adenovirus type 5-HIV-gag (rAd5-gag) was administered intramuscularly (im) at 5 x 10^7 virus particles (vps) or at 1 x 10^10 vps intravaginally.

Isolation of lymphocytes

Splenocyte suspensions were obtained by pressing the tissue through a nylon mesh screen followed by lysis of RBC using ACK (ammonium chloride potassium) lysis buffer. Lymphocytes from Peyer’s patches were prepared by digestion with collagenase D and DNase I at 37°C for 30 min and then incubated in the presence of 5 mM EDTA for 5 min as described (34). The digested tissues were teased into suspension and filtered through nylon mesh to remove debris. Liver and lung lymphocytes were obtained from mice perfused with PBS plus heparin (35) and purified between 44 and 67.5% Percoll layers (Pharmacia) followed by ACK lysis of RBC. Vaginal tissue was minced, incubated with 0.5 mg/ml Dispase II (Roche Diagnostics) in PBS at 37°C for 15 min, and then further incubated in 5 ml of PBS containing 0.22 μg of collagenase D (Roche Diagnostics), 75 μg of Dnase (Roche Diagnostics), and 500 U of hyaluronidase (Sigma-Aldrich) at 37°C for 45 min with shaking and then pressed through 70-μm mesh. Cells were washed two times with PBS and one time in 2.5% BSA and 2 mM EDTA in PBS.

Flow cytometric analysis and other immune assays

Surface staining was performed using freshly prepared single cell suspensions. Cells were stained in PBS and 1% FCS for 60 min at 4°C with FITC-anti-CD11a (clone M17/4; eBioscience), PE-anti-CD8 (clone 53-6.7; BD Pharmingen), and allophycocyanin-conjugated H-2k-MHC class I-Gag197-205 tetramers (MHC Tetramer Core Facility, National Institute of Allergy and Infectious Diseases, Atlanta, GA). The cells were then washed, fixed in 2% (w/v) parafomaldehyde/PBS, and analyzed on a FACS Calibur flow cytometer. For vaginal lymphocytes, cells were blocked with anti-CD16/32 at 4°C for 10 min and then stained in 2.5% BSA with 2 mM EDTA in PBS at 1 h at 4°C, adding 7-AAD (BD Pharmingen) to identify dead cells during the last 15 min, washed with 2 mM EDTA in PBS, and analyzed. FlowJo software (Tree Star) was used to interpret the data. To analyze the phenotype of effector and memory Gag-specific CD8 T cells, lymphocytes from spleen and vagina were stained for 60 min at 4°C with FITC-anti-CD44, Alexa Fluor 750 anti-CD8 and PE-Cy7-anti-CD127 (BD Pharmingen), and allophycocyanin-conjugated H-2kd-MHC class I tetramers to analyze the phenotype of effector and memory Gag-specific CD8 T cells, lymphocytes from spleen and vagina were stained for 60 min at 4°C with FITC-anti-CD44, Alexa Fluor 750 anti-CD8, and PE-Cy7-anti-CD127 (eBioscience), PE-anti-CD62 ligand (CD62L; BD Pharmingen), 7-AAD, and allophycocyanin-labeled Gag tetramers. Following six-color staining, cells were analyzed using a FACS Canto flow cytometer.

To stain for intracellular IFN-γ and TNF-α, lymphocytes were cultured for 5 h in RPMI 1640 (Invitrogen Life Technologies) containing GolgiStop (BD Pharmingen) with 5 μg/ml HIV-1 Gag197-205 peptide AMQMLKETI118 (36) or the nonrelevant peptide lymphocytic choriomeningitis virus-nucleoprotein p130 ~ p126 (LCMV-NP130 ~ 126). Cells were then stained for the surface molecules CD8 and CD11a and fixed, and cell membranes were permeabilized in Cytofix/Cytoperm solution and stained with FITC-anti-IFN-γ (clone XMG 1.2; BD Pharmingen) and allophycocyanin-anti-TNF-α (clone MP6-XT22; eBioscience) in Perm/Wash solution. Cells were washed and fluorescence intensity was measured using a FACS Calibur flow cytometer. To determine whether Gag-specific activated CD8 T cells secreted IFN-γ, surface staining of lymphocytes included allophycocyanin-conjugated Gag tetramers.

For the measurement of in vivo cytolytic activity (37), target cells were pooled splenocytes from naive mice labeled at 2 x 10^5 cells/ml with 6 μM CFSE (CFSEphos or 0.3 μM CFSE (CFSEphos) (Molecular Probes) for 10 min in the dark and quenched with an equal volume of 100% FCS at room temperature for 1 min followed by two washes with RPMI 1640 medium.
and 10% FCS. The CFSE<sup>high</sup> cells were pulsed with 10<sup>−6</sup> M HIV-1 Gag<sub>197–205</sub> peptide. After washing, 1 × 10<sup>6</sup> cells from each population were mixed together and injected i.v. into groups of recipient mice. At 2, 3, or 16 h after injection, lymphocytes from various tissues were prepared and analyzed for CFSE<sup>high</sup> and CFSE<sup>low</sup> populations, assaying 10<sup>6</sup> cells per sample. Naive control groups were also sampled at 2, 3, and 16 h. In vivo cytosis was calculated as (1 − (ratio naive/ratio immune)) × 100, where the ratio = percentage CFSE<sup>high</sup>/CFSE<sup>low</sup>.

To determine the role of CD4 or CD8 T cells during in vivo cytosis, immune or control animals were individually depleted of these T cells by depleted rLm(ivag)/rAd5(ivag)-immunized mice, while normal rat Ig had no effect on the numbers of CD4 or CD8 T cells.

To assess protection against virus challenge, systemic challenges were administered i.p. with 1.2 × 10<sup>7</sup> PFU of the recombinant vaccinia-gag virus Vvkl (rVac-gag) or 1 × 10<sup>6</sup> recombinant vaccinia nef (rVTFNef; rVac-nef) 5 wk after a final immunization. Intravaginal challenge was revealed with rVac-gag at 1.6 × 10<sup>7</sup> PFU. Six days after challenge, ovaries and oviducts were removed, homogenized through nylon mesh, and plaque-assayed for virus by infecting BSC-1 cell monolayers.

**Results**

An *L. monocytogenes*/adenovirus type 5 prime-boost protocol

Our aim in this work was to characterize the cellular immune response elicited by a novel prime-boost immunization protocol. We first determined whether oral vaccination with an attenuated, recombinant strain of *L. monocytogenes* expressing HIV-gag (rLm-gag; Z. Li, M. Zhang, C. Zhou, X. Zhao, and F. R. Frankel, manuscript in preparation) could be effectively boosted with replication-defective recombinant adenovirus type 5-HIV-gag (rAd5-gag). Mice were immunized one or more times with rLm-gag, rested four weeks, and then boosted with a single i.m. dose of the rAd5-gag vector. The experiment shown in Fig. 1 indicates that the prime-boost protocol generated far higher levels of Gag-tetramer<sup>+</sup> CD8<sup>+</sup> T cells in spleen than were seen after vaccinations with the individual vectors. Thus, a single prime with rLm-gag followed by a boost with rAd5-gag elicited 21.8% Gag-specific CD8<sup>+</sup> T cells in spleen, increasing to 43.7% if three oral *Listeria* primes were followed by the rAd5-gag boost (Fig. 1A). Conversely, control mice at 4 wk after three oral infections with rLm-gag alone (3 × Lm (memory)) contained only 0.9% Gag-specific CD8<sup>+</sup> T cells relative to the total CD8<sup>+</sup> T cell population, and at
To determine whether this protocol elicited functionally active CD8\(^+\) T cells was elicited. Responses to the prime-boost protocol in Peyer’s patches were lower (Fig. 1B). However, the vaginal lamina propria of these animals showed strong responses, from 13.5\% (after one rLm-gag prime) to 35.3\% (after three rLm-gag primes) Gag-tetramer\(^+\)CD8\(^+\) T cells (Fig. 1C). Thus, while the responses to the individual vectors were modest, the experiment demonstrated a strong prime-boost effect in spleen as well as a robust response in vagina.

**CD8 T cell effector function after rLm-gag(ig)/rAd5-gag(im)**

To determine whether this protocol elicited functionally active CD8\(^+\) T cells, splenocytes were stimulated with the Gag epitope AMQLMKETI (36) and assayed for expression of IFN-γ and TNF-α. As shown in Fig. 2A, depending on the number of rLm-gag primes, 27.8 to 49.9\% of all CD8\(^+\) T cells were positive for both cytokines. That this effector function was expressed by Gag-specific CD8\(^+\) T cells is shown in Fig. 2B, which demonstrates a strong correlation between effector function and TCR specificity, with 89\% of the Gag-specific CD8\(^+\) T cells expressing IFN-γ.

The clearance of a pathogen depends in large part on in vivo killing. Thus, in an immune assay with strong biological relevance, the in vivo cytolytic activity against Gag-peptide-labeled target cells was assessed. As shown in Fig. 2C, after the infusion of targets into immunized mice high cytolytic activity was seen as early as 2 h on Gag-labeled target cells, while targets not expressing Gag were spared. Immunization of mice with the individual vectors resulted in lower cytolytic activities (data not shown in figure). As shown later (Fig. 6B), this in vivo cytolyis is dependent on the presence of Ag-specific CD8\(^+\) T cells.

**CD8 T cell memory after rLm-gag(ig)/rAd5-gag(im)**

The in vivo cytolytic activity seen in effector mice, as described above, continued to be expressed in memory mice, as shown in Fig. 2C, with 85\% cytolyis even in the stringent 2-h assay conducted 5 wk after completion of the prime-boost protocol. At this time, the Gag-specific CD8\(^+\) T cell pool in spleen represented 9.6\% of total CD8\(^+\) T cells in memory mice (Fig. 3A). These cells showed a strong recall response when a cohort of the animals was challenged i.p. with recombinant vaccinia-gag virus (rVac-gag). The result was an expansion of the Gag-specific CD8\(^+\) T cells to 40\% of total CD8\(^+\) T cells (Fig. 3A), while rVac-gag infection alone of naive mice resulted in the induction of only 1\% Gag-specific CD8\(^+\) T cells. In contrast, there was no change in the pool of Gag-specific CD8\(^+\) T cells of 5-wk memory mice after challenge with rVac-nef (although the percentage of Gag-specific CD8\(^+\) T cells relative to the total pool of CD8\(^+\) T cells decreased due to the expansion of non-Gag-specific CD8\(^+\) T cells; data not shown). Finally, based on rVac-gag titer in ovaries, these vaccinia-infected prime-boost memory mice were able to block most or all replication of the virus (Fig. 3B), whereas at this high-dose challenge (1.2 × 10\(^7\) pfu) the individual vectors had only a small effect on virus accumulation. No protection was seen against the challenge with rVac-nef (not shown).

**Mucosal vaccination by the ivag route**

Together, these data support the efficacy of the Lm/Ad5 prime-boost protocol. We then considered the possibility that other routes of vaccine administration might more effectively elicit T cell immunity in the vagina. Because *L. monocytogenes* has been reported to infect mice by mucosal routes other than ingestion (38–40), the possibility of vaginal infection by this organism was examined. When a small volume of wild-type *Listeria* was deposited onto the tissue surface of the mouse vagina, the bacteria were indeed found to translocate across the vaginal epithelial barrier and migrate to the iliac and inguinal draining lymph nodes of the vagina and then to the spleen and liver (Fig. 4A). When the attenuated strain of *Listeria* used in the studies described above, rLm-gag, was applied intravaginally, a strong Ag-specific immune response was elicited in spleen, lung, and liver (1.83, 7.04, 11.2\% of total CD8\(^+\) T cells, respectively), with a weaker response in vagina (1.53\%) (Fig. 4B).

Adenovirus has previously been shown to infect the mouse vagina (41). Advantage was therefore taken of this broad tissue tropism of both agents to compare various prime-boost protocols initiated by a single dose of attenuated rLm-gag deposited either on the vaginal mucosa, the rectal mucosa, or administered orally. At 5 wk this was followed by infection with rAd5-gag by either a systemic route (im) or mucosally into the vaginal canal (ivag).

All of the mucosal rLm-gag primes, when followed with a systemic boost by rAd5-gag, elicited high levels of Gag-specific T cells in spleen, ranging from 17.6 to 20.8\% of total CD8\(^+\) T cells in the experiment shown (Fig. 5A). In the vagina the percentage of Gag-specific CD8\(^+\) T cells was also high, ranging from 13.5 to 25.7\%.
25.8%. Strikingly, when the mucosal rLm-gag primers were followed with a vaginal (mucosal) boost by rAd5-gag (Fig. 5B), a significant redistribution of CD8 T cells to the lamina propria of the vagina was observed. As a result of this mucosal prime/mucosal boost protocol, the level of Gag-specific CD8+ T cells in spleen was greatly reduced (2.1–5.8%) while highly exaggerated numbers of Ag-specific CD8+ T cells accumulated in vaginal tissue (21 to 62%). This was particularly evident after ivag administration of both vectors (ivag/ivag). The ivag/ivag regimen elicited strong Gag-specific responses in other peripheral tissues as well (30 and 40% in lung and liver, respectively; data not shown), but vagina showed the highest frequency of these Ag-specific T cells.

CD8 T cell effector function after rLm-gag(ivag)/rAd5-gag(ivag)

We next determined whether the Gag-specific CD8+ T cells generated in response to the ivag/ivag regimen expressed normal effector activities. Although the proportion of Gag-specific CD8+ T cells in spleen following the sequential mucosal vaccinations was reduced compared with that seen following the ig/im protocol, those Gag-specific T cells that were induced showed a high frequency of IFN-γ expression (80.8% of Gag-tetramer+CD8+ T cells were IFN-γ+; data not shown), similar to that seen after the ig/im protocol. The same examination for cytokine expression of Ag-specific T cells in vaginal lamina propria revealed significant redistribution to vagina of Gag-specific CD8+ T cells and given as mean values, except for the vaginal samples, which were combined before analysis. Similar results were obtained in one repeat of this experiment.

FIGURE 4. Bacterial translocation across the vaginal epithelium and immunity generated after ivag infection by *L. monocytogenes*. A, Wild-type *L. monocytogenes* (4 × 10^6 CFU in 20 μl) was applied to the surface of the vaginal mucosa of sedated mice over a 1-h period. Organs indicated were assessed on subsequent days in groups of mice (n = 3) for the presence of viable bacteria by plating dilutions of homogenized tissues on brain heart infusion agar (BHI; Difco) containing 20 μg/ml streptomycin. DLNs refers to the combined inguinal and iliac draining lymph nodes of vagina. Data are presented as mean CFU per organ ± SEM. Dotted line shows the threshold of detection of bacteria. B, rLm-gag (5 × 10^6 CFU in 20 μl) was applied to the vaginal mucosa of mice (n = 3), and at day 9 selected tissues were assessed for the induction of Gag-tetramer+CD8+ T cells after gating on CD8+ T cells. The numbers indicate the percentage of Gag-tetramer+CD8+ T cells within the boxed areas relative to total CD8+ T cells and given as mean values, except for the vaginal samples, which were combined before analysis. Similar results were obtained in one repeat of this experiment.

FIGURE 5. Redistribution to vagina of Gag-specific CD8+ T cells after ivag rLm-gag/rAd5-gag prime-boost vaccination. Groups of mice (n = 3 to 5) were immunized one time with rLm-gag by the oral (ig, 1.5 × 10^9 CFU), intrarectal (ir, 1.5 × 10^9 CFU), or ivag (5 × 10^9 CFU) routes of infection followed 5 wk later either by a single im boost with 5 × 10^8 vps rAd5-gag (A) or a single ivag boost (ivag) with 1 × 10^9 vps rAd5-gag (B). Samples were taken from spleen and vagina 6 days after the boost. The plots shown are gated on CD8+ T cells and analyzed for CD11a expression on H-2Kd-HIV-gag-tetramer+ cells. The numbers shown are the percentage of Gag-specific CD8+ T cells (in the boxed areas) relative to total CD8+ T cells, and represent the mean values for each group of mice. In all cases, the SEM was <10% of the mean values shown. Gag-specific CD8+ T cells in spleen following mucosal or systemic boosts by rAd5-gag were compared by paired Student’s t test and showed a very significant decline after mucosal boost, with p = 0.001. The percentage of Gag-specific CD8+ T cells in all naive samples was <0.6%; in rLm-gag-only memory mice it was 1% or less in spleen and vagina by all routes; in rAd5-gag-only effector mice it was <0.5% (ivag) or <4% (im) in spleen, and <4% (ivag) or <8% (im) in vagina. Portions of this experiment were repeated at least four times with similar results.
a lower proportion of IFN-γ-expressing cells (31.3% of Gag-tetramer−/CD11a+/CD8+ T cells were IFN-γ+; data not shown). Some other studies of cytokine expression by Ag-specific CD8+ T cells in peripheral mucosal tissues have also shown modest levels of expression (42, 43).

To assess additional functional activities in mice 8 days after the ivag/ivag protocol, several tissues including vagina, its draining iliac lymph node, and spleen were assayed for in vivo cytolytic activity (Fig. 6A). Vaginal tissues showed high cytolytic activity (75 and 98%, effector function at 2 and 16 h, respectively), as did lymph nodes and spleen. To determine whether this cytolytic activity was indeed dependent on the function of CD8 T cells, ivag/ivag-immunized mice were depleted of either CD4 or CD8 T cells by the injection of depleting rat anti-mouse mAbs (Fig. 6B).

Whereas sham-immunized mice showed no cytolytic activity in their spleens or in the draining iliac lymph nodes, immunized mice injected with control rat Ig showed 84–91% cytolysis of Gag-peptide-labeled targets in these tissues at 3 h after target cell injection. Depletion of CD8 T cells resulted in the loss of most of this activity whereas depletion of CD4 T cells had little effect, in agreement with earlier studies of this immune function (44). These results indicate that Gag-specific CD8 T cells, induced by Gag-expressing vaccine vectors but not the empty vectors, are responsible for the in vivo activity.

CD8 T cell memory after rLm-gag(ivag)/rAd5-gag(ivag)

Following resolution of an infection, the initial burst of effector T cells normally contracts by 90% or more, leaving behind a small pool of memory T cells (45). Protection against future infection demands the presence of such cells, and it was thus important to determine what fraction of the high number of effector T cells seen in vagina was retained as memory. At both 5 wk and 5 months after the ivag prime-boost protocol, the Gag-specific memory CD8+ T cell populations in vagina, spleen, and other tissues were examined. As shown in Table I, the vagina at 5 wk after vaccination possessed 25% Gag-specific CD8+ T cells, having retained 44% of its day-8 effector level. In spleen, liver, and lung, the fraction retained in memory was 36, 33, and 25%, respectively, of the effector day-8 levels of CD8+ T cells. A cohort of mice was also examined 5 mo after the ivag prime-boost protocol. By this time,
the fraction of Gag-specific memory CD8+ T cells in vagina had dropped further to 10.6% of total CD8+ T cells, which represented 18% of its effector level at day 8 after the prime-boost protocol (Table I). The proportion of Ag-specific CD8 T cells in spleen had fallen to 1.7% of total CD8+ T cells.

The phenotype of the bulk of the 5-wk vaginal Gag-specific memory CD8+ T cells was CD26L-*, like the vaginal effector cells, with only 7% exhibiting the CD62L* marker (Table I). In the spleen at 5 wk, a large fraction of the memory T cells (37%) had returned to CD62L-. By 5 mo after immunization, 13% of the vaginal Gag-specific memory CD8+ T cells had become CD62L-. All of these cells exhibited the CD44+ marker, and 75% were also CD127+. In spleen, the CD62L- fraction had risen to 62%, and all of these Gag-specific T cells exhibited the CD44+ marker, with 75% also showing CD127+. Thus the vast majority of vaginal memory CD8 T cells had an effector memory T cell (TEm) phenotype (CD62L-CD44+CD127+), while an increasing fraction in spleen assumed properties of a central memory T cell (T ECM) (CD62L-CD44+CD127+).

To examine the functional capacity of the week five vaginal memory T cells, we assessed the in vivo cytolytic activity of these memory mice. The vagina and its draining lymph node, even when assayed at 2 h after target cell transfer, continued to show cytolytic activity (69 and 44% cytolytic activity, respectively) (Fig. 6A). The spleen showed somewhat lower activity. Although this assay is not quantitative, not having an easily defined E:T ratio, the cytolytic activity seen in spleen correlated with the lower frequency of tetramer-positive memory CD8+ T cells in that tissue.

The recall response of the memory T cells was examined by infection of the wk 5 memory animals with rVac-gag. Despite the existing high level of memory cells in the vagina at this time (22.4% Gag-tetramer+ CD8+ T cells) (Fig. 7A), the virus challenged elicited a doubling in the size of this pool to 41.1%, possibly by direct expansion of the TEm cells. Most importantly, whereas ivag infection of naive mice led to high accumulations of vaccinia virus in ovaries and oviduct (the favored replication site after ivag infection; Ref. 34), ivag immunized mice were fully protected, allowing little or no virus replication in these tissues (Fig. 7B).

### Discussion

Heterologous live vectors have only rarely been used together to affect immune protection (30, 46), although such a prime-boost protocol might generate stronger and more diverse immune responses than possible with either agent alone or by using a DNA vaccine to prime either agent. In this report we demonstrate for the first time that attenuated forms of the two live mucosal vectors, L. monocytogenes and adenovirus type 5, can be paired in a heterologous prime-boost protocol to elicit robust cellular immunity in vagina and other tissues. L. monocytogenes, a common oral intracellular pathogen (47), delivers Ags via both the MHC class I and class II pathways to induce strong CTL and CD4 T cell help (48). Human serotype 5 adenovirus, a ubiquitous virus that commonly infects many humans by age 10, infects many cell types (49) and is being tested as a systemic live vector against SIV (50).

Used together in a prime-boost protocol, these agents produced superior immune responses, as shown for example after an oral Listeria prime followed by a systemic Ad5 boost, in the induction of high levels of Ag-specific CD8+ T cells in both spleen and vagina (Fig. 1, A–C), in the cytokine expression of these Ag-specific cells (Fig. 2, A and B), and in their in vivo cytolytic activity (Fig. 2C) and protective efficacy against vaccinia virus challenge (Fig. 3).

Mucosal and systemic infections by a variety of pathogens leads to wide dissemination of effector and memory CD4 and CD8 T cells to all peripheral nonlymphoid tissues (22, 35, 51). However, several studies have suggested that regional priming of T cells can lead to the imprinting of an “area code” that allows the selective recruitment of T cell subsets to the priming site (52). For example, skin infection by vaccinia virus at the base of a mouse tail leads to CD8 T cell activation in the draining inguinal lymph nodes and

<table>
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<th>Sample Phenotype</th>
<th>Vagina</th>
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<th>Liver</th>
<th>Lung</th>
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<td>CD62L*</td>
<td>73.0 ± 4.1</td>
<td>95.0 ± 2.0</td>
<td>81.0 ± 3.4</td>
<td>89.0 ± 3.8</td>
</tr>
<tr>
<td>Effector (day 8)</td>
<td>57.4 ± 3.0</td>
<td>6.7 ± 0.2</td>
<td>9.7 ± 1.1</td>
<td>17.7 ± 2.5</td>
</tr>
<tr>
<td>Gag-tetramer**</td>
<td>3.0 ± 0.4</td>
<td>15.0 ± 1.7</td>
<td>10.0 ± 2.0</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>CD62L*</td>
<td>7.0 ± 0.9</td>
<td>37.0 ± 5.3</td>
<td>27.0 ± 1.8</td>
<td>19.0 ± 2.1</td>
</tr>
<tr>
<td>Memory (wk 5)</td>
<td>25.0 ± 1.7</td>
<td>2.4 ± 0.5</td>
<td>3.2 ± 0.1</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Gag-tetramer**</td>
<td>7.0 ± 0.9</td>
<td>37.0 ± 5.3</td>
<td>27.0 ± 1.8</td>
<td>19.0 ± 2.1</td>
</tr>
<tr>
<td>CD62L*</td>
<td>10.6 ± 1.5</td>
<td>1.7 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Memory (month 5)</td>
<td>13.0 ± 1.8</td>
<td>62.0 ± 4.9</td>
<td>62.0 ± 4.9</td>
<td>62.0 ± 4.9</td>
</tr>
</tbody>
</table>

*Groups of 8–10 mice were infected (or not infected) with attenuated Lm-gag (5 × 10^7 CFU in 20 μl) by deposition into the vaginal canal. Four weeks later the animals were boosted with rAd5-gag (1 × 10^10 vps in 10 μl). Eight days (Effector) or 5 wk or 5 months later (Memory), lymphocytes from the indicated tissues were isolated and stained for FACS analysis. The numbers represent the percentage of Gag-specific CD11a+CD8+ T cells relative to total CD8+ T cells.

**The fraction of Gag-tetramer+ CD8+ T cells that were positive for the CD62L marker.

**The fraction of Gag-tetramer+ CD8+ T cells that were positive for the CD62L marker. All of these cells were also CD44+ (see Results).
up-regulation of T cell skin-homing molecules and their preferential infiltration into tail skin after infection (24). Likewise, intracranial injection of tumor cells leads to activation of tumor-specific CD8 T cells in the draining cervical lymph nodes and their preferential entry into the brain (23). The immune system may target effector T cells to the site of pathogen encounter to arrest an infection in situ but protects distant tissues against dissemination of the agent by also superimposing a more promiscuous T cell migration behavior. In this study, the number of protective T cells in vaginal lamina propria was greatly enhanced by sequential vaccination at that site with the two mucosal agents.

In addition to its usual oral route of infection, L. monocytogenes can also infect mice after intrarectal, intranasal, or intratracheal administration (38–40). In the current study, L. monocytogenes was found to cross the mucosal barrier of the vagina, travel hematogenously to other tissues, and generate Ag-specific CD8+ T cells in spleen, liver, lungs, and vagina (Fig. 4, A and B). Adenovirus had previously been shown to infect the mouse vagina and induce a CD8 T cell response (41). Therefore, these two agents were paired in a prime-boost protocol in an effort to enhance localized mucosal T cell immunity in vagina. The results revealed that while introduction of a Listeria prime by any mucosal route followed by a systemic im boost with Ad5 elicited strong vaginal responses mainly independent of the route of Listeria infection (Fig. 5A), the administration of Ad5 ivag elicited stronger and more striking vaginal responses, particularly after the Lm(ivag)/Ad(ivag) protocol (Fig. 5B). As a result of this sequential ivag vaccination protocol, the number of Gag-specific CD8 T cells in the animals because even in the ivag/ivag spleen, where Ag-specific T cells showed a reduced frequency (compare Fig. 5, A and B), there were as many as 5 × 10^9 Gag-specific CD8 T cells.

The efficacy of vaccines depends on their generating a pool of potent, long-lived memory T cells ready to expand rapidly to express killing functions upon re-exposure to Ag. Therefore, it was important to know whether the effector cells generated by the novel ivag/ivag protocol could transition into a population of long-lived vaginal memory cells. Table I shows that sizable pools of memory cells were found in all tissues examined at 5 wk after the ivag Lm prime followed by an ivag Ad5 boost. At 5 mo the vagina and spleen continued to harbor large numbers of Gag-specific CD8+ memory T cells. These large memory pools are not surprising, because memory frequency correlates with effector burst size (53) that in turn reflects Ag dose, duration of Ag exposure, level of activation of the naive T cells, and inflammatory signals at the time of Ag exposure (54–57), all likely to be high after a Listeria prime/Ad5 boost protocol. Indeed, there is some indication (58) that replication-incompetent adenovirus may continue to express Ag at its inoculation site long after vector administration.

The memory T cells in vagina were predominantly of the effector memory phenotype (Table I). The nature of memory cells most desirable for protective efficacy is currently a subject of debate (59). Vaginal T(EM) should provide acute protection against heterosexual HIV transmission, because these T cells reside at the site of virus entry, respond rapidly to Ag, and thus constitute an effective first line of defense (60). However, pools of T(EM) cells are likely to be necessary for their greater regenerative capacity, assuring preservation of anti-HIV T cell clonotypes for continuing protection against future infection (61). As indicated above, the large repository of Gag-specific T cells in spleen, mainly T(EM), following the ivag/ivag protocol can thus provide a continuous supply of effector T (T(E)) and T(EM) cells (59) to the vagina and other mucosal sites upon challenge. Reducing Ag levels by use of lower doses of vectors during the prime-boost vaccination protocol could further alter the ratio of these memory cell types and possibly enhance the T(EM) response (56, 57).

The vaccination model described here is predicated not on preventing infection but on promptly arresting an infection once viral gene expression has begun. The ivag/ivag route of administration for L. monocytogenes and adenovirus type 5 generated very high levels of effector and memory T cells in vaginal tissue. However, the use of Listeria as an oral or intrarectal vaccine along with a systemic boost by adenovirus should not be dismissed, as it produced robust responses both systemically and mucosally. Although a large fraction of the CD8 T cells in vaginal tissue following these prime-boost protocols are HIV-specific, the absolute number of T cells in vagina is low. Nonetheless, the initial founder population of HIV-infected cells after mucosal infections is also small and could conceivably be controlled by local CTLs. A model for such protection is the in
vivo cytosis shown in Fig. 2C and Fig. 6. It is important to note, finally, that the ivag vaccination protocol would be prohibited in pregnant women and would preclude unprotected intercourse until the infections and resulting inflammation have subsided and memory has been established.

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Disclosures

The authors have no financial conflict of interest.

References


