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Targeting the Genital Tract Mucosa with a Lipopeptide/Recombinant Adenovirus Prime/Boost Vaccine Induces Potent and Long-Lasting CD8+ T Cell Immunity against Herpes: Importance of MyD88

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Targeting of the mucosal immune system of the genital tract with subunit vaccines has failed to induce potent and durable local CD8+ T cell immunity, which is crucial for protection against many sexually transmitted viral pathogens, including HSV type 2 (HSV-2), which causes genital herpes. In this study, we aimed to investigate the potential of a novel lipopeptide/adenovirus type 5 (Lipo/rAdv5) prime/boost mucosal vaccine for induction of CD8+ T cell immunity to protect the female genital tract from herpes. The lipopeptide vaccine and the rAdv5 vaccine express the immunodominant HSV-2 CD8+ T cell epitope (gB498–505), and both were delivered intravaginally in the progesterone-induced B6 mouse model of genital herpes. Compared with mice immunized exhibiting cytotoxic activity, were associated with protection (p < 0.005) after intravaginal HSV-2 challenge. Polyfunctional CD8+ T cells, producing IFN-γ, TNF-α, and IL-2 and exhibiting cytotoxic activity, were associated with protection (p < 0.005). The protective CD8+ T cell response was significantly compromised in the absence of the adapter MyD88 (p = 0.0001). Taken together, these findings indicate that targeting of the vaginal mucosa with a Lipo/rAdv5 prime/boost vaccine elicits a potent, MyD88-dependent, and long-lasting mucosal CD8+ T cell protective immunity against sexually transmitted herpes infection and disease. The Journal of Immunology, 2012, 189: 4496–4509.

Development of a mucosal immunization approach that generates long-lasting local effector and memory CD8+ T cell populations in the genital tract and its draining lymph nodes is likely to be essential in achieving immediate and sustained protective immunity against many sexually transmitted viral (STV) pathogens that use the genital mucosa as a portal of entry into their human hosts (1–4). Evidence from both animal models (5, 6) and humans (7) suggests that successful control of many STV infections, such as HIV-1 (which causes AIDS) and HSV type 2 (HSV-2; which causes genital herpes) is associated with the presence of sustained local CD8+ T cells within the genital tract draining lymph nodes (GT-DLN) and the vaginal mucosal tissues (4, 8–11). Increasing evidence demonstrates a substantial link between the epidemics of sexually transmitted HIV-1 and HSV-2 infection (12). However, no subunit vaccine strategy delivered parenterally has generated demonstrable high-level and sustainable protective CD8+ T cell immunity against either infection in clinical trials (3, 13, 14). A successful immunization through the mucosal route, such as intravaginally, appears critical but remains a roadblock due to the robust mucosal epithelial barrier and the inability to overcome mucosal tolerance by many subunit vaccines (4). Lipopeptides (peptide epitopes linked to a fatty acid) are promising mucosal vaccines that induce protective CD8+ T cells against many STV pathogens for which induction of cytotoxic CD8+ T cells and IFN-γ signaling is critical (reviewed in Ref. 1). We recently demonstrated that a prototype herpes lipopeptide vaccine expressing the immunodominant H2b-restricted CD8+ T cell epitope from HSV-2 gB (HSV-2 gB498–505) (15), delivered intravaginally, induced HSV-specific protective CD8+ T cell responses (6). However, these CD8+ T cell responses were rather moderate and were only transiently protective against genital herpes (6). An emerging and promising mucosal immunization approach to elicit potent CD8+ T cell responses is to use recombinant viruses (16). Several studies have recently used replication-defective adenovirus vectors to induce potent local viral-specific CD8+ T cells in GT-DLN and to mobilize them quickly into the vaginal mucosal tissues (8, 16, 17).
In the current study, we hypothesize that a prime/boost vaccination regimen, which consists of priming CD8+ T cell responses with a lipopeptide vaccine and boosting them with an adenovirus vector-based vaccine, would result in strong and long-lasting protective CD8+ T cell immunity against genital herpes. We used the replication-defective recombinant adenovirus serotype 5 (rAdv5) vector based on its many attractive features: 1) its remarkable ability to significantly boost mucosal CD8+ T cell responses that are primed by a subunit vaccine (e.g., peptide and DNA) (reviewed in Ref. 18); 2) its ability to elicit a considerably potent and long-lasting pathogen-specific CD8+ T cell response in humans (19); and 3) its natural tropism for mucosal tissues (20–22), which makes it an ideal Ag delivery system for intravaginal vaccination.

Although some progress has been made in defining the cellular mechanisms of the immunogenicity of lipopeptide and recombinant adenovirus (rAdv)-based vaccines, relatively little is known about the underlying innate molecular pathways. Mucosal delivery of lipopeptide and rAdv vaccines potentially recruits many of the 13 known mammalian TLR pathways (6). Each TLR has TIR domains that engage two main intracellular signaling pathways 1): the MyD88 pathway; and 2) the TIR-domain-containing adapter inducing IFN-β (TRIF) pathway (23). Because most TLRs recruit the MyD88 pathway (6, 24), the current study is focused on exploring whether the MyD88 pathway would be required for the generation of protective HSV-specific CD8+ T cell responses by the lipopeptide/rAdv5 vaccine (Lipo/rAdv5) after an intravaginal immunization. The results show that 1) priming with a lipopeptide and boosting with an rAdv5 vector, both expressing the same HSV-2 gB 498–505 epitope and both delivered intravaginally, induced a much more potent local mucosal HSV-specific IFN-γ-producing CD8+ T cell response than the homologous lipopeptide/ lipopeptide (Lipo/Lipo) vaccine (p = 0.05); 2) Lipo/rAdv5 prime/ boost mucosal vaccine elicits long-lasting CD8+ T cells, with a faster kinetic of mobilization, than the Lipo/Lipo vaccine; 3) the HSV-specific CD8+ T cell responses induced by the Lipo/rAdv5 prime/boost mucosal vaccine are associated with protection against genital herpes infection and disease; 4) the HSV-specific CD8+ T cell responses induced by the Lipo/rAdv5 prime/boost mucosal vaccine persisted up to 8 mo postimmunization; and 5) the MyD88 pathway plays a pivotal role in the HSV-specific CD8+ T cell response and protection induced after intravaginal immunization with the Lipo/rAdv5 vaccine. Altogether, these findings lay the foundation for an accessible and durable mucosal prime/boost T cell-based vaccine approach to reduce genital herpes and presumably other sexually transmitted pathogens.

Materials and Methods

Mice

MyD88-deficient mice (MyD88−/− mice), 4–5 wk old, were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) and were on the C57BL/6 background. Female C57BL/6 (B6) mice, 4–5 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal studies conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Viruses and cell lines

Triple plaque-purified 333 HSV-2 was prepared, as described previously (6). The live attenuated thymidine kinase-deficient HSV-2 [HSV-2 TK−/−] was provided by Dr. James R. Smiley and Dr. Lynda A. Morrison. Triple plaque-purified HSV-2 was prepared as we described previously (25). Rabbit skin (RS) cells, used to prepare virus stocks and to culture virus from vaginal swabs, were grown in Eagle’s MEM supplemented with 5% FCS (Invitrogen, Grand Island, NY). Heat-killed virus was made by heating virus solution at 100°C for 5 min. HSV inactivation was confirmed by the inability to produce plaques when tested on Vero cells, as we described (1).

A recombinant adenovirus type 5 (rAdv5) expressing the H-2Kb-restricted, HSV type 1 (HSV-1)/2–cross-reactive CTL recognition epitope, HSV glycoprotein B residues 498 to 505 (SSIEFARL) (gB498–505), was constructed. Briefly, the DNA sequence (PB1) encoding the H2Kb SSIEFARL CD8+ CTL target peptide (HSV-gB498–505) was synthesized and cloned into pShuttle-CMV (Stratagene, Santa Clara, CA) vector between KpnI and XbaI sites. The transfer pShuttle-CMV-PB1 plasmid DNA was linearized with Pmel, gel purified, and then transformed into Bl5193-Ad1. In Vitro material (transfected pAdEasy-1) carrying the plasmid DNA was electroporated to generate recombinant adenovirus plasmid pAdEasy-1-PB1. The recombinant pAdEasy-1-PB1 plasmid DNA was linearized with Pael and transfected into 293 cells to produce recombinant adenovirus Adv5.CMV-PB1 that will express the HSV-gB498–505 CD8+ T cell epitope by CMV promoter. The Adv5.CMV-PB1 recombinant adenovirus was plaque-purified and amplified to the titer of 108 PFU/ml. The virus stock was aliquoted and stored at −80°C.

Synthesis and assembly of lipopeptide vaccines

The H-2Kb SSIEFARL CD8+ CTL target peptide (HSV-gB498–505) (27) and the [dA][K][Ch][VAAWTLLK][dA][Alh][C] Pan DR peptide (PADRE) (26) were synthesized either individually or as PADRE-CTL chimeric epitopes using Fmoc chemistry, with PyBOP/HOBt activation, as we described previously (26, 28). The parental peptides were washed three times with dimethylformamide (DMF) and treated twice with 2% hydrazine in DMF. After additional washings (×6) with DMF, hydrazine-reactive acetyl peptides were cleaved using trifluoroacetic acid (TFA)/tri-isopropylsilane/H2O (95:2.5:2.5) and the resultant peptides precipitated in cold ether. The peptides were then washed (×3) with cold ether and analyzed by MS and HPLC. Purification of the peptides was performed using a Gilson HPLC and Vydac C18 columns (2.2 × 25 cm or, for larger amounts of crude peptide, 5 × 25 cm). The analysis was performed using Vydac C18, 5-μm, 0.46 × 25 cm columns, with a gradient of 2% per minute of water, 0.1% TFA, 95% acetonitrile, and 0.1% TFA. Once peptides were >95% purity rate, they were lyophilized. Mass spectrometric analysis was performed by MDS/Scieni QStar XL mass spectrometer equipped with a nanospray source. Final QC included collision-inducation dissociation MS/MS experiments using nitrogen as the collision gas, which confirm the mass and number of amino acids of the synthesized peptides. As a positive control, mice were inoculated intravaginally with 5 × 103 of the rAdv5 vaccine, both delivered intravaginally in sterile PBS on day 0 and 21 (Figs. 1a, 2A, 3A, 4A, 5A, and 6A). As a negative control, mice were primed intravaginally with the irrelevant OVA257–264 lipopeptide and boosted with an empty Adv5 vector (mock-immunized mice). As a positive control, mice were inoculated intravaginally with 5 × 106 PFU of the HSV-2 TK−/− virus, as previously described (6, 29) (Figs. 3A, 4A, 5A, 5B, and 6A). We previously found that i.n. injection of 0.5 mg Depo-Provera four times, instead of 2 mg one time, was safer and better in the synchronizing of the estrus cycle of mice. Ten days after the final immunization, each group of mice was i.p. injected daily with four doses of...
0.5 mg Depo-Provera in 100 μl sterile PBS. Mice were then challenged intravaginally, on day 14, with either 5 × 10^6 PFU (= 200 × LD₅₀ for survival analysis) or 5 × 10^7 PFU (for virus titers and disease analysis) of HSV-2 (strain 333).

**Immunohistochemistry**

Mice were euthanized, and the vaginal mucosal tissues were collected and fixed with 2% paraformaldehyde. After overnight fixation, the vaginal mucosa samples were cut into small longitudinal bands. Then, samples were blocked with anti-FcRg Ab (US Biological) at a dilution of 1:100 and in goat serum/PBS overnight. The anti-CD8 Ab conjugated to FITC at a dilution of 1:100 and 14.3 mM DAPI (Molecular Probes, Invitrogen) were applied overnight at 4°C. Then, samples were mounted in 50% glycerol/PBS. Confocal microscopy was performed with a laser confocal and multiphoton microscope system with a conventional laser confocal microscope (Zeiss LSM 510 META; Zeiss, Jena, Germany) equipped with a femto-second titanium laser (Chameleon; Coherent, Santa Clara, CA).

**Isolation of genital tract mucosal lymphocytes**

Lymphocytes were isolated from the female genital tract (GT) mucosal tissues and treated with calcium- and magnesium-free PBS for whole-body perfusion prior to tissue harvest, as previously described (30). The female GT included the ovaries, fallopian tubes, uterus, and vagina (5, 6). GT tissues were digested after a 2-h treatment with 2.5 mg/ml collagenase type A (cat. no. 1088 785; Roche) and 5 U/ml DNase I (cat. no. 104 159; Roche) suspended in RPMI 1640 with 5% FBS, penicillin-streptomycin, and HEPES. Mucosal GT tissues were pooled from five mice/immunization group and washed in 10 ml tissue culture medium. Cells to perform replicates of each subsequent experiment (IFN-γ ELISPOT) and to allow for accurate measurement of immune responses in each immunization group. The average yield of cells per mouse was 4 × 10⁶ cells/female reproductive tract.

**Cytokine assays**

Two weeks after the second immunization, mice were euthanized, the iliac lymph nodes (5, 6) draining the GT (GT-DLN) or GT mucosal tissues and treated with calcium- and magnesium-free PBS for whole-body perfusion prior to tissue harvest, as previously described (30). The female GT included the ovaries, fallopian tubes, uterus, and vagina (5, 6). GT tissues were digested after a 2-h treatment with 2.5 mg/ml collagenase type A (cat. no. 1088 785; Roche) and 5 U/ml DNase I (cat. no. 104 159; Roche) suspended in RPMI 1640 with 5% FBS, penicillin-streptomycin, and HEPES. Mucosal GT tissues were pooled from five mice/immunization group and washed in 10 ml tissue culture medium. Cells to perform replicates of each subsequent experiment (IFN-γ ELISPOT) and to allow for accurate measurement of immune responses in each immunization group. The average yield of cells per mouse was 4 × 10⁶ cells/female reproductive tract.

**IFN-γ ELISPOT assay**

GT-DLN or GT mucosal cells were cultured in 24-well plates for 5 d in a humidified 5% CO₂ atmosphere with HSV-gB 498–505 peptide alone (10 μg/ml), the irrelevant OVA 257–264 CD8+ T cell peptide (10 μg/ml), or autologous HSV-2-infected stimulator cells, and subsequently were analyzed in an IFN-γ ELISPOT assay. Functional T cell recognition IFN-γ ELISPOT assays were performed with the mouse IFN-γ ELISPOT mAb pair (BD Pharmingen). Briefly, on day 4, 96-well MultiScreen-IP plates were coated overnight at 4°C with 100 μl (1:250) of anti–IFN-γ capture mAb. Plates were then blocked with RPMI 1640 medium supplemented with 10% FCS for 2 h at room temperature. Then, 5 × 10⁵ cells/well were added in triplicate to mAb-coated plates and incubated for 18–24 h at 37°C, 5% CO₂. Plates were then washed with PBS and supplemented with a detection peroxidase-labeled Ab followed by a substrate according to the manufacturer’s instructions. The developed spots were counted under a light microscope. Controls included cells cultured in medium in the absence of peptide stimulation. The frequency of IFN-γ SFCs in control wells (typically <10 IFN-γ SFCs/10⁶ cells) was subtracted from the frequency of IFN-γ SFCs detected in the peptide-stimulated cells for calculation of Ag-specific IFN-γ responses.

**Flow cytometry**

Standard flow cytometry was used, as we previously described (6, 31), to assess surface expression of various markers using the following mAbs directly conjugated with either PE or FITC: PE–CD4, FITC–CD8, PE–HSV-gB tetramer (Pharmingen, San Diego, CA). IgG isotype-matched control Abs were used in all experiments. After staining, cells were washed and fixed in 1% buffered paraformaldehyde before being acquired on a Becton Dickinson FACS Calibur (Mountain View, CA). Gating was done on live, single cells, and for each sample, 20,000 events were acquired on a FACSCalibur and analyzed with CellQuest software on an integrated Macintosh G4 (Becton Dickinson, San Jose, CA).

**Tetramer assay**

gB₄₉₈–⁵₀₅ tetramers were prepared and used as described (6). A total of 0.1 to 0.2 μg of PE-labeled gB₄₉₈–⁵₀₅ tetramer complexes with APC-labeled anti-mouse CD8 (Ly-2; Caltag, South San Francisco, CA) mAb was used to identify gB₄₉₈–⁵₀₅-specific CD8+ T cells. Samples were analyzed with tetrameric gB₄₉₈–⁵₀₅ tetramer complexes for the percentages of CD8+ T cells by two-color flow cytometry with a FACSCalibur (Becton Dickinson) system.

**CTL activity**

DTL-gB₄₉₈–⁵₀₅-derived immune CD8+ T cells (5 × 10⁶) were restimulated in vitro with 1 × 10⁵ HSV-gB₄₉₈–⁵₀₅ target peptide (5 μg/ml)-pulsed, syngeneic, irradiated, T cell-depleted splenocytes (2500 rad from a 137Cs source) and 1 × 10⁶ irradiated EL4 cells (3000 rad from a 137Cs source), as we previously described (6). CTL activity was assessed by a standard 4-h ⁵¹Cr release assay against EL4 target cells loaded with 10 μM HSV-gB₄₉₈–⁵₀₅ target peptide or infected with heat-inactivated HSV-2 (MOI = 3), as previously described (6). After 5 d of culture, effector CD8+ cells were mixed at 1, 3, 10, 30, or 100 E:T ratios with ⁵¹Cr-labeled EL4 cells for 4 h. Maximum release of ⁵¹Cr was determined by adding 5% Triton X-100 to ³⁵Cr-labeled EL4 cells. Spontaneous release (<10% of total release) was determined by incubating target EL4 cells with medium alone. The percent of specific lysis was calculated as follows: 100 × [(experimental release – spontaneous release)/(maximum release – spontaneous release)].

**Monitoring virus replication in vaginal tissue**

Two weeks after the final immunization, mice were treated with progesterone (Depo-Provera) to synchronize the ovarian cycle and increase susceptibility to herpes infection, and then received an intravaginal HSV-2 challenge. The infection of the GT in the progesterone-treated mouse model appears to be similar to the initial infection in humans, the main difference being that the susceptible epithelial cells in mice are present in both vagina and cervix whereas they are mainly in the cervix of humans (32). An inoculum of 5 × 10⁶ PFU HSV-2 (333) or 5 × 10⁷ PFU (≈ 200 × LD₅₀ for survival analysis) in 10 μl tissue culture medium was placed into the vaginal canal of immunized and control mice. To quantify vaginal HSV-2, the vaginal canal of immunized and control mice were swabbed once daily (days 1–10 postinfection) with a Dacron swab and each swab placed in a 75-mm culture tube containing 0.5 ml media. Aliquots (100 μl) of 10-fold serial dilutions were placed on confluent monolayer of RS cells in 6-well plates, incubated at 37°C for 1 h, and overlaid with medium containing 1% methylcellulose. The plates were incubated at 37°C for 3 d, stained with 1% crystal violet, and the viral plaques were counted.

**Detection of latent virus in dorsal root ganglia**

Equal numbers of mice in each group surviving 30 d postinfection were euthanized and their dorsal root ganglia (DRG) removed and individually explanted onto RS cell monolayer in RPMI medium. The culture was monitored for 10 d for the presence of infectious virus.

**CD8+ depletion in vitro**

CD8+ lymphocytes were depleted from GT-DLN and mucosal samples with magnetically activated cell sorting CD8α (Ly-2; Caltag, South San Francisco, CA), following the protocol provided with the microbeads. Briefly, single-cell suspensions were incubated with microbeads for 20 min at 4°C. The cells were then washed with 5 ml PBS, 0.5% FBS, and 2 mM EDTA. The pellet was then resuspended in 500 μl wash buffer and placed onto a prewetted MS+ selection column (Miltenyi) in the separator. After the separation, the column was washed three times, and the negatively selected cells were washed and pelleted before being counted and adjusted to a proper concentration for the ELISPOT assay.

**Statistical analysis**

Data for each assay were compared by ANOVA and Student t test using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences between the groups were identified by ANOVA and multiple comparison procedures, as we previously described (6). Data are expressed as the mean ± SD. Results were considered to be statistically significant at p < 0.05.
Results

Lipo/rAdv5 prime/boost vaccine induced potent and long-lasting HSV-specific IFN-γ–producing CD8+ T cells detected in both GT-DLN and in the vaginal mucosa

We first performed a dose-response study in B6 mice of the lipopeptide vaccine using 50, 100, or 200 μg as well as of the rAdv5 vaccine using 107, 5 × 107, or 108 PFU, both delivered intravaginally in adjuvant-free saline on days 0 and 21. All three doses induced a similar magnitude of T cell responses in GT-DLN (data not shown). There were no obvious vaccine-related severe side effects with either the lipopeptide or the rAdv5 vaccine at any of the three doses tested, as evaluated by weight loss or vaginal lesions (data not shown). Accordingly, all subsequent experiments were carried out using the middle dose of 100 μg for the lipopeptide vaccine and 5 × 107 PFU for the rAdv5 vaccine.

Three groups of female B6 mice (n = 10) were immunized intravaginally with 1) the lipopeptide in adjuvant-free saline on day 0 and 5 × 107 PFU rAdv5 in saline on day 21 (Lipo/rAdv5 prime/boost mucosal vaccine) or 2) 100 μg of the lipopeptide in saline on days 0 and 21 (homologous Lipo/Lipo vaccine) or 3) the irrelevant OVA257–264 lipopeptide on day 0 and 5 × 107 PFU of an empty Adv5 vector in saline on day 21 (mock vaccine). An illustration of the immunization scheme and subsequent immunological experiment timeline is shown in Fig. 1A. The iliaca and inguinal lymph nodes draining the genital tract (5, 6) (designated as GT-DLN) and the vaginal mucosa (VM) were harvested on day 35 and day 261 (i.e., ~8 mo after the final immunization). The induced IFN-γ–producing CD8+ T cells against autologous H2b EL4 cells loaded with the HSV-gB498–505 target peptide (Fig. 1B, 1C) or EL4 cells infected with HSV-2 (Fig. 1D, 1E) were evaluated in GT-DLN and VM by a standard ELISPOT assay, as we previously described (6). Both the Lipo/rAdv5 prime/boost and the Lipo/Lipo vaccines induced significant HSV- and gB498–505–specific IFN-γ–producing CD8+ T cell responses compared with mock vaccine 35 d postimmunization in both GT-DLN and VM (p < 0.005, Fig. 1B–E). However, CD8+ T cell responses induced by the Lipo/Lipo vaccine were rather moderate compared with those induced by the Lipo/rAdv5. Immunoassaying of vaginal mucosal tissue from progesterone-treated immunized mice confirmed an abundance of CD8+ T cells in the vaginal epithelium of the Lipo/rAdv5 group compared with the Lipo/Lipo group (Fig. 1F). When assessed on day 261, only the Lipo/rAdv5 vaccinated mice had significant HSV- and gB498–505–specific IFN-γ–producing CD8+ T cell responses compared with mock-immunized mice (p < 0.05). However, no significant HSV- and gB498–505–specific IFN-γ–producing CD8+ T cell responses were detected in the Lipo/Lipo vaccinated mice (p > 0.05). As expected, no significant T cell responses were seen against H2b EL4 cells loaded with the irrelevant OVA257–264 target peptide in either the Lipo/rAdv5 prime/boost or the Lipo/Lipo vaccinated mice, demonstrating the specificity of CD8+ T cell responses induced by either type of vaccine (data not shown). The CD8+ T cell responses induced by either vaccine were abrogated after blockage with anti-CD8 mAb but not with anti-CD4 mAb (data not shown). Altogether, these results suggest that Lipo/rAdv5 prime/boost vaccine induced potent and long-lasting HSV-specific IFN-γ–producing CD8+ T cells in both GT-DLN and VM.

Lipo/rAdv5 prime/boost mucosal vaccine elicits long-lasting CD8+ CTLs with fast kinetics of mobilization compared with the Lipo/Lipo vaccine

Three groups of female B6 mice (n = 40) were immunized intravaginally with 1) the lipopeptide in adjuvant-free saline on day 0 and 5 × 107 PFU rAdv5 in saline on day 21 (Lipo/rAdv5 prime/boost mucosal vaccine) or 2) 100 μg of the lipopeptide in saline on days 0 and 21 (homologous Lipo/Lipo vaccine) or 3) the irrelevant OVA257–264 lipopeptide on day 0 and 5 × 107 PFU of an
empty Adv5 vector in saline on day 21 (mock vaccine). An illustration of the immunization scheme and subsequent CTL experiment timeline is shown in Fig. 2A. To better analyze the kinetics of CD8+ T cell responses induced by each vaccine; time points were expanded by harvesting GT-DLN on days 31, 41, 51, and 261 (i.e., 10, 20, 30, and 240 d postimmunization). Five mice were used per time point in each group. The cell suspensions were restimulated in vitro with UV-inactivated HSV-2 for 5 d, and CD8+ CTL responses were evaluated in a standard Cr51 assay against autologous H2b EL4 cells loaded with the HSV-gB498–505 target peptide (Fig. 2B, upper panel) or against target cells transfected with VVgB, a vaccinia virus expressing gB (Fig. 2B, lower panel). VVgB was used to ascertain that the induced CD8+ T cells were able to recognize the native epitope processed endogenously, not just the artificial synthetic gB498–505 peptide epitope. Significantly higher CTL responses were detected against HSV-gB498–505 on days 20, 30, and 240 postimmunization in the Lipo/rAdv5 prime/boost vaccine group compared with the Lipo/Lipo group (Fig. 2B, upper panel, p = 0.03, one-way ANOVA test). However, higher CTL responses were detected against target cells transfected with VVgB in the Lipo/rAdv5 prime/boost vaccine group only on days 30 and 240 postimmunization (p = 0.05, one-way ANOVA test, Fig. 2B, lower panel). As expected, no CTL responses were detected in the mock-immunized control group at any time point. The difference in the CTL responses between the Lipo/rAdv5 and the Lipo/Lipo was even more significant on day 240, suggesting that the Lipo/rAdv5 prime/boost mucosal vaccine may induce more sustainable memory CD8+ CTL responses than the homologous Lipo/Lipo. The gB498–505- and HSV-specific CTL responses were abrogated after blockage with anti-CD8 mAb but not with anti-CD4 mAb (data not shown). Together, these results 1) indicate a clear difference in the kinetics with which CD8+ CTL are mobilized after Lipo/Lipo versus Lipo/rAdv5 prime/boost mucosal vaccination, with the later mobilizing CD8+ T cells faster than the former; 2) confirm the sustainability of the CD8+ T cell responses induced by the Lipo/rAdv5 prime/boost vaccine that last 8 mo after the final immunization; and 3) suggest that the Lipo/rAdv5 prime/boost mucosal vaccine may induce memory CD8+ T cells that are polyfunctional (i.e., produce IFN-γ and have a cytotoxic activity).

**High and sustained frequency of HSV-specific CD8+ T cells induced by Lipo/rAdv5 prime/boost mucosal vaccine**

Three groups of female B6 mice (n = 40) were immunized intravaginally with 1) the lipopeptide in adjuvant-free saline on
day 0 and 5 × 10^7 PFU rAdv5 in saline on day 21 (Lipo/rAdv5 prime/boost mucosal vaccine) or 2) 100 µg of the lipopeptide in saline on days 0 and 21 (homologous Lipo/Lipo vaccine) or 3) the live attenuated HSV-2 TK^−^ on day 21, as we previously described (6) (positive control), as illustrated in Fig. 3A. GT-DLN were harvested on days 10, 20, 30, and 240 postimmunization from Lipo/rAdv5, Lipo, and HSV-2 TK^−^ immunized mice (n = 5 for each time point), and cell suspensions were restimulated in vitro with UV-inactivated HSV-2 and then stained with HSV-gB498–505/H2-Kb tetramer followed by an mAb specific to mouse CD8. To obtain an objective enumeration of HSV-gB498–505-specific CD8^+ T cells induced by the Lipo/rAdv5 prime/boost versus the homologous Lipo/Lipo vaccine, we used the MHC tetramer-staining assay, which provides a quantitative measure of the frequency of gB498–505-specific CD8^+ T cells. The specificity of the HSV-gB498–505 epitope in combination with H2-Kb MHC H chain modified for tetramer formation has been established (33). As shown in Fig. 3B, a significantly higher percentage of CD8^+HSV-gB498–505/H2-Kb tetramer^+ T cells were detected on days 10, 20, 30, and 240 postimmunization in the Lipo/rAdv5 vaccine group compared with the Lipo/Lipo group (p = 0.03, one-way ANOVA test). The difference in the percentage of CD8^+HSV-gB498–505/H2-Kb tetramer^+ T cells detected between the Lipo/rAdv5 and the Lipo/Lipo groups was highest on days 30 and 240. Significantly increased numbers of CD3^+CD8^+ T cells (Fig. 3C, 3D) and more HSV-gB498–505-specific CD8^+ T cells (Fig. 3E, 3F) were detected in the GT-DLN of Lipo/rAdv5 immunized mice compared with the Lipo/Lipo mice, both on 30 and 240 d postimmunization. Similarly, significantly higher numbers of CD3^+CD8^+ T cells (Fig. 3G, 3H) and of HSV-gB498–505-specific CD8^+ T cells (Fig. 3I, 3J) were detected in the VM of the Lipo/rAdv5 group (p < 0.05, one-way ANOVA test). As expected, the thymidine kinase mutant HSV-2 (HSV-2 TK^−^) also induced a high number and percentage of CD8^+HSV-gB498–505/H2-Kb tetramer^+ T cells. Altogether, these results 1) confirm that the Lipo/rAdv5 prime/boost mucosal vaccine induced the highest number of HSV-specific CD8^+ T cells with different kinetics of mobilization and 2) suggest that the Lipo/rAdv5 prime/boost mucosal vaccine induced stronger and more sustained memory CD8^+ T cells in both GT-DLN and VM than its homologous Lipo/Lipo vaccine.

Next, we compared the profile of cytokines produced by CD8^+ T cells induced by the Lipo/rAdv5 vaccine versus the homologous Lipo/Lipo vaccine. Thirty days after the second immunization, GT-DLN—derived CD8^+ T cells were isolated from five mice in each group and restimulated in vitro with gB498–505 peptide-pulsed irradiated autologous spleen cells for 72 h. The amount of IFN-γ, TNF-α, and IL-2 released in culture media was determined in a specific sandwich ELISA. The Lipo/rAdv5 vaccine induced significantly higher amounts of IFN-γ, TNF-α, and IL-2 compared with the Lipo/Lipo vaccine (p < 0.05, one-way ANOVA test, Fig. 3K). A similar level of cytokines was detected in the Lipo/rAdv5 group and the HSV-2 TK^−^ positive control group. Altogether, these data 1) indicate that intravaginal immunization with Lipo/rAdv5 prime/boost vaccine induced a higher and sustained frequency of HSV-specific CD8^+ T cells than the homologous Lipo/Lipo vaccine and 2) suggest that the Lipo/rAdv5 prime/boost mucosal vaccine induced a polyfunctionality of CD8^+ T cell responses, with a type 1 pattern of cytokines and a cytotoxic function.

The Lipo/rAdv5 prime/boost mucosal vaccine induced better protection against genital herpes than its homologous Lipo/Lipo vaccine

Because immunization with the Lipo/rAdv5 prime/boost mucosal vaccine induced stronger HSV-specific CD8^+ T cell responses than the homologous Lipo/Lipo vaccine, it was of interest to determine whether it could also better protect against genital herpes. Four groups of 20 age-matched B6 mice were immunized intravaginally with the Lipo/rAdv5 prime/boost vaccine, with the homologous Lipo/Lipo, with the irrelevant OVA257–264 lipopeptide/“empty” Adv (mock-immunized negative control), or with HSV-2 TK^−^ positive control (Fig. 4A). Ten days after the final immunization, all mice were treated daily with four doses of Depo-Provera to synchronize the ovarian cycle and increase susceptibility to herpes infection. The mice were divided into two groups of 10 and were then challenged intravaginally on day 35 (i.e., 14 d postimmunization), either with 5 × 10^7 PFU (200 × LD50 for survival analysis) or with 5 × 10^4 PFU (for virus titers and disease analysis) of HSV-2 (strain 333), as we previously described (6). Mice immunized with the Lipo/rAdv5 prime/boost vaccine showed 100% survival compared with mice immunized with the homologous Lipo/Lipo (only 50% of mice survived 30 d postchallenge; p < 0.005, one-way ANOVA test) and with mock-vaccinated mice (0 survived 20 d postchallenge; p < 0.005, one-way ANOVA test) (Fig. 4B). As expected, mice immunized with HSV-2 TK^−^ vaccine also showed little mortality with 80% of mice surviving 30 d postchallenge (positive control). The pathology scores observed in the Lipo/rAdv5 prime/boost group were also much lower than all three other groups (p < 0.005 for all, one-way ANOVA test) (Fig. 4C). Additionally, viral titers measured in the vaginal washes from days 5, 7, 9, and 11 post-infection showed that the Lipo/rAdv5 prime/boost vaccine group had significantly lower viral loads than the other groups (Fig. 4D, p < 0.005 for all, Student t test, only day 11 shown). Virus clearance in the VM was within 5–7 d in the Lipo/rAdv5 prime/boost mucosal vaccine groups compared with 9 and 11 d in the homologous Lipo/Lipo group and in the mock-vaccinated group, respectively (data not shown).

The effect of these vaccinations on virus reactivation from latency was examined by ex vivo explant cultivation of DRG harvested on day 30 postinfection (Fig. 4E). The presence of reactivated virus was monitored from explanted DRG for 10 d. Because it is unlikely that vaccination prior to initial infection would suppress detection of virus reactivating from explanted DRG, these types of assays are assumed to produce an estimate of the relative ability of the vaccines to inhibit establishment of latency. Virus reactivation was detected from significantly fewer DRG of Lipo/rAdv5 immunized mice compared with the Lipo/Lipo group (Fig. 4F, p < 0.004, t test). Thus, Lipo/rAdv5 prime/boost mucosal vaccination appeared to reduce significantly the amount of virus that reactivated from DRG.

Altogether, the above results indicate that after intravaginal HSV-2 challenge, Lipo/rAdv5 immunized mice had significantly lower virus titers at both the GT (the site of viral entry) and DRG (the site of latency). Lipo/rAdv5 immunization also decreased overt signs of genital herpes disease. Finally, Lipo/rAdv5 immunized mice did not succumb to lethal infection compared with Lipo/Lipo immunized mice (p < 0.005).

The long-lasting CD8^+ T cell-dependent protective immunity induced by the Lipo/rAdv5 prime/boost mucosal vaccine

To assess the protective efficacy of Lipo/rAdv5 vaccine during the memory phase, 60 d after the final immunization (i.e., on day 81), 10 mice in each of the four groups described earlier were challenged intravaginally with 5 × 10^7 PFU HSV-2 (Fig. 5A). Although all the HSV-2 TK^−^ immunized mice survived lethal infection (positive control), 90% of the Lipo/rAdv5 immunized mice had less death rate, whereas only 40% of Lipo/Lipo immunized mice survived (Fig. 5B). Linear regression analysis of all
FIGURE 3. Higher percentages and increased numbers of HSV-gB498–505-specific CD8+ T cells induced by the Lipo/rAdv5 prime/boost compared with its Lipo/Lipo homologous mucosal vaccine. (A) Groups of female B6 mice (n = 40) were immunized intravaginally with lipopeptide on day 0 and 5 × 10^7 PFU rAdv5 in saline on day 21 (Lipo/rAdv5 prime/boost mucosal vaccine), with 100 μg lipopeptide in saline on days 0 and 21 (homologous Lipo/Lipo vaccine), or with the live attenuated HSV-2 TK(−) on day 21, as we previously described (6) (positive control). (B) On days 10, 20, 30, and 240 postimmunization, GT-DLN were harvested (five mice per each time point), and derived T cells were stimulated in vitro with UV-inactivated virus pulsed APCs for 5 d. Stimulated HSV-gB498–505-specific CD8+ T cells were then stained with a PE-labeled anti-mouse CD8+ mAb followed by an FITC-labeled HSV-gB498–505/H2-Kb tetramer. Cells were then analyzed using a FACSCalibur with a total of 2 × 10^5 events collected for each point. The percentages of CD8+/tetramer+ cells are determined for each time point. Shown is mean ± SD of the results obtained in five mice/group. Each bar is representative of the mean ± SE of results from five mice. Data for each group were repeated twice and compared by ANOVA test and multiple comparison procedures (Tukey) to determine differences between groups, as we previously described (6). A p value of 0.03 indicates significant differences between Lipo/Lipo and Lipo/rAdv5 immunized groups (one-way ANOVA). (C–F) Numbers of total CD3+CD8+ T cells (C, D) and HSV-gB498–505-specific CD8+ T cells (E, F) detected in the GT-DLN on days 30 and 240 postimmunization. (G–J) Numbers of total CD3+CD8+ T cells (G, H) and HSV-gB498–505-specific CD8+ T cells (I, J) detected in the VM on days 30 and 240 postimmunization. (K) Profile of HSV-gB498–505-specific cytokine produced by CD8+ T cells after Lipo/Lipo and Lipo/rAdv5 immunizations. GT-DLN were harvested 30 d after the second immunization, and GT-DLN–derived CD8+ T cells were stimulated in vitro with target gB498–505 peptide-loaded H2b irradiated splenocytes for 72 d at 37°C in 5% CO2. The amounts of IFN-γ, TNF-α, and IL-2 secreted into the culture media were determined in a specific sandwich ELISA, according to the manufacturer’s instructions. Shown is cytotoxic activity and profiles obtained in a group of five mice. The data are representative of two independent experiments, and the bars represent SD between the experiments. *p < 0.05 (one-way ANOVA comparing the amount of cytokine levels between Lipo/Lipo and Lipo/rAdv5 groups).
experimental groups indicated that high IFN-γ-producing CD8+ T cell responses correlated positively with the survival (Fig. 5C). The linear regression was also found indicating the correlation between CD8+ T cell response in the VM and survival ($R^2 = 0.7836; p < 0.0001$). To investigate the roles of CD4+ and CD8+ T cell subsets in the protection, Lipo/rAdv5 immunized mice were depleted for either CD4+ or CD8+ T cell subsets before intra-vaginal HSV challenge, as we previously described (6) (Fig. 5D). CD8+ T cell-depleted mice lost their protection against death compared with nondepleted mice. In contrast, depletion of CD4+ T cells had little effect on survival in Lipo/rAdv5 immunized mice (Fig. 5D). Sixty days after immunization, 10 additional mice in each of the four groups above were challenged intravaginally with $5 \times 10^6$ PFU HSV-2, and both the virus replication and disease was followed during the memory phase of the responses (i.e., days 81–110). The pathology scores were 0 to 1 in both the Lipo/rAdv5 prime/boost group and the HSV-2 TK$^{-/}$ group compared with a score of 2–3 in the Lipo/Lipo group and a score of 2–5 in the mock group. As expected, viral titers measured in the vaginal washes on day 71 postinfection were significantly lower in the Lipo/rAdv5 prime/boost vaccine group compared with the other groups ($p < 0.005$ for all, $t$ test). Additionally, when DRG were harvested on day 110, there were less than 10% of reactivated ganglia in the Lipo/rAdv5 and TK$^{-/}$ groups compared with 25–30% in the Lipo/Lipo group and 75–90% in the mock group ($p < 0.005$ for all, one-way ANOVA test). Altogether, these results suggest that 1) in the Lipo/rAdv5 prime/boost mucosal vaccine system, virus-specific CD8+ T cells were involved in protective immunity to genital herpes; and 2) both long-lasting CD8+ T cell responses and better protection were observed in mice immunized with Lipo/rAdv5 vaccine, suggesting that the combination of lipopeptide and viral vectors is beneficial for the induction of an effective long-term protective immunity against genital herpes.

**Absence of MyD88 abolishes HSV-specific CD8+ T cell response and protection induced by the Lipo/rAdv5 prime/boost mucosal vaccine**

To examine the contribution of MyD88—a critical adapter protein shared by most TLRs (23)—in the immunogenicity of the Lipo/rAdv5 vaccine delivered intravaginally, groups of age-matched female MyD88$^{-/-}$ mice ($n = 20$) and wild-type parental B6 mice ($n = 20$) were immunized intravaginally with the Lipo/rAdv5 vaccine or with HSV-2 TK$^{-/}$ (positive control) (Fig. 6A). Fourteen days after the second immunization, VM tissues (Fig. 6B) and GT-DLN (Fig. 6C) were harvested from 10 mice and their cell suspension analyzed for HSV-specific IFN-γ-producing CD8+ T cell responses by ELISPOT assay. The other 10 mice were treated daily, starting 10 d postimmunization, with four doses of
Depo-Provera to synchronize the ovarian cycle and increase susceptibility to herpes infection and were then challenged intra-vaginally 14 d postimmunization with 5 × 10⁶ PFU HSV-2 as described earlier. B6 mice developed significantly more HSV-specific IFN-γ–producing CD8+ T cells than MyD88−/− mice, as detected in VM (Fig. 6B) and GT-DLN (Fig. 6C) (p = 0.001 for both VM and GT-DLN, one-way ANOVA test). As expected, the apparent similar trend in HSV-2 TK−/− immunized mice did not reach significance suggesting that immunity induced by HSV-2 TK−/− was independent of MyD88 (data not shown). These results are consistent with the role of MyD88 pathway signaling triggered by interactions of LipolAdv5 vaccine with TLRs (34).

Finally, MyD88−/− mice immunized with the LipolAdv5 prime/boost mucosal immunization had increased death rate compared with wild-type B6 mice (Fig. 6D). Thus, absence of MyD88 significantly abolished both the HSV-specific CD8+ T cell response and protection induced after intravaginal immunization with the LipolAdv5 vaccine (p = 0.001). As expected, wild-type and MyD88−/− mice immunized with HSV-2 TK−/− had similar reduced virus titers and survivals (data not shown). Together, these results indicate a pivotal role of the MyD88 pathway in the immunogenicity of LipolAdv5 vaccine after intravaginal immunization.

**Discussion**

A mucosal immunization regimen that induces potent CD8+ T cells in the GT and GT-DLN is necessary for protection against STV pathogens, such as HSV-2. Surprisingly, compared with other mucosal tissues, induction of local CD8+ T cell immunity to protect the genital mucosa from STV infection and pathogenicity has received much less attention. To our knowledge, the current study describes for the first time a LipolAdv5 prime/boost mucosal immunization strategy that induces a robust HSV-specific CD8+ T cell-dependent protective immunity against genital herpes. Viral replication in the GT was significantly lower in the LipolAdv5 vaccine group compared with the LipolLipol vaccine group. Moreover, after the HSV-2 challenge, mice immunized intravaginally with the LipolAdv5 prime/boost mucosal vaccine showed less overt signs of genital herpes disease and did not
succumb to lethal infection compared with mice immunized with the homologous Lipo/Lipo vaccine. The CD8+ T cell responses elicited by the Lipo/rAdv5 vaccine were completely abolished in MyD88−/− deficient mice (n = 10) were immunized intravaginally with Lipo/rAdv5. The CD8+ T cell responses persisted in the GT-DLN for up to 8 mo post-immunization. These long-lasting CD8+ memory T cells may play a critical role in immune surveillance and could rapidly respond and expand after sensing the virus to prevent genital herpes. The findings underscore the potential of Lipo/rAdv5 prime/boost mucosal immunization as an efficient vaccination strategy for inducing genital CD8+ T cell immunity and should impact the development of vaccines against other STV diseases.

Understanding which innate molecular pathways are involved in the induction and maintenance of mucosal CD8+ T cell immunity is key to future successful clinical vaccines against STV infections. TLRs have TIR intracellular domains that engage two main signaling pathways via the TIR-containing adapters: MyD88 and TRIF. Most of the 13 known mammalian TLRs recruit MyD88, with the exception of TLR3, which recruits TRIF, and of TLR4, which recruits both MyD88 and TRIF. One of the goals of the current study was to examine the role of MyD88 pathway in the immunogenicity and protective efficacy of the Lipo/rAdv5 prime/boost mucosal vaccine. Compared to wild-type B6 mice, MyD88−/− mice developed significantly lower HSV-specific CD8+ T cell responses after intravaginal immunization with the Lipo/rAdv5 prime/boost mucosal vaccine. This result suggests a MyD88-dependent mechanism in the T cell protective immunity at the female GT. Not every mucosal immunization strategy results in MyD88-dependent protective immunity against genital herpes. Harandi and colleagues (35) recently reported that intranasal immunization with HSV-2 gD in combination with β-GalCer mucosal adjuvant elicited MyD88-independent T cell immunity that protected from an otherwise lethal vaginal HSV-2 challenge. Although the role of MyD88 pathway in the mucosal CD8+ T cell immunogenicity of lipopeptides (6) and rAdv5 vectors has been previously reported (23), its role in the T cell immunity at the female GT after a Lipo/rAdv5 prime/boost intravaginal administration has never been reported. Thus, the current study is the first to our knowledge to demonstrate that CD8+ T cell-mediated protective immunity elicited by a Lipo/rAdv5 prime/boost intravaginal vaccine against genital herpes was completely abolished in MyD88−/− mice. It is likely that
stimulation of the MyD88-dependent transcriptional program contributes to the success of the Lipo/rAd5 prime/boost mucosal vaccine. However, our findings do not exclude the involvement of other intracellular signaling pathways in the immunogenicity and protective efficacy of the Lipo/rAd5 prime/boost mucosal vaccine. The TRIF pathway may be particularly involved, as TLR3, which exclusively engages TRIF (23), has been recently reported as important in inducing HSV-specific CD8+ T cells (36). Thus, investigation of other intracellular pathways underlying TLR signaling after intravaginal immunization with the Lipo/rAd5 vaccine is currently being pursued in our laboratory. Because MyD88 is a critical adapter protein shared by at least six TLRs (i.e., TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9) (23), it remains to be determined whether the potent immunogenicity and protective efficacy induced by the Lipo/rAd5 prime/boost mucosal vaccine is abrogated in TLR2−/−, TLR4−/−, TLR5−/−, TLR6−/−, TLR7−/−, and TLR9−/− deficient mice (23). These studies will be the subject of future reports.

The Lipo/rAd5 prime/boost mucosal vaccine delivered intravaginally stimulated potent and sustained HSV-specific CD8+ T cell responses, detected not only in the GT-DLN but also in the VM. Clear differences were apparent in the kinetics with which CD8+ T cells are mobilized after Lipo/Lipo versus Lipo/rAd5 prime/boost mucosal vaccination. The success of Lipo/rAd5 prime/boost mucosal vaccine is also highlighted by its ability to induce and facilitate the mobilization and establishment of local effector memory CD8+ T cells in the genital mucosal tissue (VM) by apparently promoting their migration. We found a cluster of IFN-γ–producing CD8+ T cells in the VM of Lipo/rAd5 vaccinated mice, suggesting an establishment of memory cell foci in the genital mucosa (data not shown). These clusters of CD8+ T cells appear to migrate to the GV-DLN to vaginal mucosa, as the vaginal submucosa does not contain MALT in the steady state (30). Several explanations are possible for the difference in mobilization and kinetics of CD8+ T cells by Lipo/Lipo versus Lipo/rAd5 prime/boost mucosal vaccines. Generally, effector memory T cells circulate throughout the peripheral tissues, such as the VM, whereas central memory T cells reside in the secondary lymphoid tissues, such as GT-DLN. Thus, regardless of the site of Ag encounter, HSV-specific memory CD8+ T cells must be found in various tissues, including the VM and GT-DLN. However, peripheral tissue distribution of memory CD8+ T cells occurs mainly after immunization with live replicating vectors, such as rAd5. The mucosa of the vaginal canal is drained by several lymph nodes, including the common iliac, interilical, external iliac, and inguinal femoral lymph nodes (in descending order, designated in this report as GT-DLN) (reviewed in Ref. 4). It is likely that the intravaginal immunogenicity of Lipo/rAd5 occurs through vaginal submucosal dendritic cells efficiently taking up the rAd5 vaccine and migrating to the GT-DLN, where they present the gB498–505 peptide to cognate CD8+ T cells. The proportion of CD8+ T cells after lipopeptide priming is greater, and these expand more prominently after heterologous rAd5 boost. Because all the mechanisms above are not mutually exclusive, it is possible that they all play a role in our immunization scheme, but additional experiments will be needed to assess both the relative proportion of each mechanism in the observed immunogenicity.

We recently demonstrated the requirement of CD4+ T cell help for efficient priming and maintenance of HSV-specific CD8+ T cells (6, 26). Others have shown that CD4+ T cells are required to pave the way for efficient migration of memory CD8+ T cells into restricted tissues, such as the VM (37). Thus, epitope vaccines based on the generation of only systemic CD8+ T cell immunity are likely to fail because helpless CD8+ T cells may not be sufficient for entry into the infected VM tissue. For these reasons, we designed our lipopeptide mucosal prime vaccine to include both the HSV-gB498–505 CD8+ T cell epitope and the PADRE that expresses a universal CD4+ Th epitope (6, 26). Besides the HSV-gB498–505-specific CD8+ T cell response, the rAd5 also boosted substantial CD4+ T cells that likely help in the maintenance and migration of effector CD8+ T cells into infected tissue (17, 38, 39). Future studies will determine whether Lipo/rAd5 vaccine–induced CD4+ T cells respond to local chemokines, enter the VM tissue, and induce a subsequent wave of chemokines that would enable HSV-specific CD8+ memory T cell populations to enter the VM tissue, as has been shown in other systems (37), and the results will be the subject of future reports.

HSV-2 is one of the most common STV infections (6). After rectal, vaginal, or penile exposure to HSV-2, the virus replicates in the mucosal epithelial cells, which leads eventually to rectal or genital herpes lesions (31). The global prevalence of seropositive individuals, 15 years and older, is estimated to be at least 60 million within the United States and well over 540 million worldwide (40, 41) with a greater frequency of infection in women (6). Despite the availability of many intervention strategies, such as sexual behavior education, barrier methods, and the costly guanine nucleoside anti-viral drug therapies (e.g., acyclovir and derivatives), controlling the spread of genital herpes remains a challenge (1, 6, 41). The current medical opinion is that an effective clinical vaccine would constitute the best approach to protect the human population from genital herpes (40, 41). Such a vaccine would likely have the greatest impact in both developed and underdeveloped regions of the world (40, 41). To date, however, no clinical vaccine for the prevention or treatment of genital herpes is available (22). Direct experiments in animal models (5, 6, 42) and indirect evidence in humans (7) suggests that successful control of herpes infection is associated with induction of robust and polyfunctional CD8+ T cells within the vaginal submucosal tissues (9–11). Our current inability to efficiently deliver Ags to stimulate strong local mucosal HSV-specific CD8+ T cell responses remains a critical roadblock in the development of an effective vaccine. The current study is the first to our knowledge to show that a combination of the two vaccine formulations (i.e., a lipopeptide and an adenovirus) in a prime/boost Lipo/rAd5 mucosal vaccine strategy, administered intravaginally, induced robust, sustained, and polyfunctional CD8+ T cells locally in the GT-DLN and VM, characterized by cytotoxic activity and co-production of IFN-γ, TNF-α, and IL-2. Both migrating and tissue-resident memory T cells have been implicated in long-term peripheral protective immunity, especially at the nonlymphoid mucocutaneous tissues, such as the VM, which serve as virus entry points into the body (43). In the current study, a correlation was found between the numbers of VM-resident HSV-specific CD8+ T cells and protection against vaginal infections. A linear regression analysis also indicated that high numbers of VM-resident IFN-γ–producing CD8+ T cell responses correlated positively with the survival (R² = 0.7836; p < 0.0001, Fig. 5C). These results are in agreement with recent reports that tissue-resident memory (TRM) CD8+ T cells, those that survived in nonlymphoid tissues, may provide a more potent protective immunity against HSV than circulating memory CD8+ T cells (9–11). Various anti-herpes TRM subsets may make distinct contributions to the observed protective immunity at the inflamed VM, as has been reported in other systems (43). Whether a unique subset of anti-herpes TRM CD8+ T cells (e.g., the nonmigratory CD103+CD8+ TRM cell subset) induced by the heterologous Lipo/rAd5 immunization or after the acute HSV-2 challenge persisted for an extended period of time in the VM tissue and/or in the latently
infected sensory DRG will be determined in future phenotypic and functional kinetic studies.

HSV-2 infects the GT and then establishes latency in sensory neurons of the DRG. Although suboptimal “inherent” CD8+ T cells are detected in sensory ganglia and appeared to provide an immune surveillance of the infected neurons, these cells cannot clear latent virus. It is generally assumed that prevention of HSV-2 reactivation from sensory ganglia requires a therapeutic vaccine that will boost a more vigorous and/or a different virus-specific CD8+ T cell response. This is because the quality and/or the magnitude of “inherent” CD8+ T cell responses resulting from natural infection are not sufficient enough to reduce virus reactivation and recurrent genital herpes in symptomatic individuals (1, 40, 41, 44, 45). After HSV-2 genital challenge, our Lipo/rAdv5 prime/boost mucosal vaccination appeared to reduce significantly the amount of virus that reactivated ex vivo from DRG. Protection was mediated by CD8+ T cells, as determined by CD8-depletion studies and greater protection correlating with cytotoxic CD8+ IFN-γ-producing cells in both the GT-DLN and VM. As expected, mice immunized with thymidine kinase mutant HSV-2 TK−, which is incapable of reactivation in the sacral DRG, also show little reactivation from the DRG (positive control). The cluster of IFN-γ-producing CD8+ T cells found in the VM of Lipo/rAdv5 vaccinated B6 mice is likely important for providing an immediate response after reactivation of HSV-2 from DRG. In humans, memory CD8+ T cells are rapidly recruited after reactivation of infection and persist adjacent to peripheral nerve endings at the dermal–epidermal junction for more than 60 d after reactivation and healing (46). Importantly, subsequent virus reactivation at the site where CD8+ T cells are present did not result in lesion formation, indicating that HSV-2–specific CD8+ T cells at the site of genital herpesvirus lesions control local viral replication. Therefore, localized mucosal memory T cell populations seem to provide superior control of viral infection compared with circulating memory T cells. Our hope is that a therapeutic genital herpes prime/boost Lipo/rAdv5 vaccine that would increase the number and the function of HSV-specific CD8+ T cells in sensory ganglia of latently infected hosts will significantly decrease HSV-2 spontaneous reactivation (as measured by shedding in GT) and recurrent genital herpetic disease. This will be the subject of future investigation in an HSV-2 latently infected guinea pig model, which, like humans, develops spontaneous reactivation that leads to recurrent genital herpetic disease (reviewed in Ref. 13).

The morbidity and socioeconomic burden associated with genital herpes, as well as the alarming relationship between genital herpes and HIV susceptibility, transmission, and acquisition, highlight the need for the development of an effective vaccine. Several vaccine strategies have been introduced in the past two decades (1, 41). A clinical vaccine trial using protein-in-adjuvant HSV-2 vaccine (gD) delivered parenterally in women from so-called discordant couples (in which one partner is infected and the other is not) reported in 2002 a limited success (13, 20, 21). When delivered intramuscularly, that protein-in-adjuvant vaccine induced transient immunity against HSV-2 disease in women who were seronegative for both HSV-1 and HSV-2 (20–22). However, that vaccine failed to protect HSV-2 seropositive women or seropositive men, even though good neutralizing Ab responses were elicited (20, 21). A more recent clinical vaccine trial reported in 2012, based on the same glycoprotein gD vaccine, showed efficacy in preventing HSV-1, but not in preventing HSV-2, genital herpes (20, 21). The reason for this discrepancy remains to be determined. A common conclusion could be drawn from these clinical trials (20, 21), together with our recently reported preclinical studies using the established murine model of intravaginal immunization (6, 31), that T cell-based mucosal vaccines may be protective against genital herpes. However, despite intensive research, the progress toward a T cell-based vaccine still faces major hurdles including the lack of an efficient Ag delivery system that would safely induce potent and sustained mucosal T cell immunity in and around the GT mucosa. To our knowledge, the current study is one of the first to demonstrate that intravaginal immunization with a Lipo/rAdv5 prime/boost mucosal vaccine induced potent, sustained, and polyfunctional HSV-specific CD8+ T cells in the GT-DLN that were associated with a reduction in virus replication in the GT and protection from most overt signs of genital herpes disease.

CD8+ T cell cytotoxicity and IFN-γ are thought to be central effector functions in the control of genital herpes (6, 29, 47, 48), acting directly or indirectly on resident cells, including parenchymal cells (49), or facilitating recruitment of effector CD8+ T cells into the inflamed vaginal tissue (50). To obtain the most accurate picture of CD8+ T cell responses elicited by the Lipo/rAdv5 prime/boost vaccine compared with the HSV-2 TK− positive control vaccine, we used multiple immunological assays including 1) visualization of induced CD8+ T cells in inflamed VM, using immunostaining assay; 2) quantification of CD8+ T cell cytotoxic function in Cr51 release assay; 3) determination of the frequency of induced CD8+ T cells by tetramer/FACS assay; and 4) detection of multiple cytokines production by ELISA and IFN-γ ELISpot assays. It is interesting that the high frequency of HSV-specific CD8+ T cells in the Lipo/rAdv5 group did not translate into higher amounts of cytokines production compared with the HSV-2 TK− group. Nevertheless, it is not unusual that a vaccine may induced more T cell proliferation results in a high frequency of CD8+ T cells without exhibiting one of the immunological functions above (e.g., cytokine production, cytotoxic activity, etc.) (6, 31). The opposite is also true: a CD8+ T cell can produce cytokines or exhibit cytotoxic function without extensive proliferation.

The concept of using recombinant viral-vector vaccines to deliver an unrelated viral Ag was developed more than 25 y ago (44). Recombinant adenoviruses are promising and safe vectors due to their capacity to elicit potent CD8+ T cell responses to unrelated T cell epitopes (reviewed in Refs. 8 and 51). rAd5 vector-based vaccines have been recently tested successfully in animal models, and several clinical trials are currently ongoing in the United States [(8, 51); see also http://clinicaltrials.gov/]. In this study, we show that intravaginal priming with a lipopeptide and boosting with recombinant rAd5v expressing the same HSV-2 gB CD8+ T cell epitope significantly reduce HSV-2 replication in the GT. A critical roadblock in the development of an ocular herpes therapeutic vaccine is our current inability safely and efficiently to deliver the Ag to stimulate the ocular mucosal immune system and to boost protective HSV-specific CD8+ T cells in latently infected trigeminal ganglia (TG) that will then prevent or, at least, reduce attempts of virus reactivation before infectious viral particles reach the eye to cause potentially blinding ocular disease. The knowledge gained from the current study will undoubtedly be useful to guide the development of a future ocular mucosal herpes vaccine. On the basis of our and other recent reports showing that intranasal immunizations efficiently induce T cell responses in remote HSV reactivation/replcication sites (i.e., VM, eye, and TG) (52–54), a logical extension of the current findings would be to apply ocularly or intranasally the Lipo/rAdv5 prime/boost vaccine and determine whether it would induce long-lasting protective CD8+ T cell responses in the eye and TG. However, we should keep in mind the high frequency of human anti-Adv5 neutralizing Abs in the developing world, which will likely limit the immunogenicity and clinical utility of Adv5-vector based vaccines (17,
38, 39). Thus, the ability of rAdv5 to boost potent HSV-specific CD8+ T cell responses induced in a mouse model by a lipopeptide demonstrated in this study may not be extrapolated to humans. Nevertheless, to our knowledge, this study constitutes a first proof-of-principle showing a strong immunogenicity of a prime/boost mucosal vaccine strategy using an rAdv vector. A future candidate Lipo/rAdv5 prime/boost clinical herpes vaccine will probably have to be constructed using an rAdv vector derived from an adenovirus serotype that is rare in human populations and distinct from the Adv5 serotype, such as rAdv26 or rAdv35, which are serologically distinct from rAdv5 and do not infect the same type of cells as Adv5 (13). Neither Adv35 nor Adv26 are affected by anti-Adv5 immunity, and rAdv35 is not affected by anti-Adv6 immunity (55). A recent study by a Pfizer group showed weak correlation between IFN-γ-producing T cell responses induced by gB and/or gD protein-in-adjuvant vaccines and protection against genital herpes in mice and guinea pigs (56). In this context, we should emphasize our recent findings that most HSV proteins, including gB and gD, contain “asymptomatic” protective epitope but may also contain “symptomatic” nonprotective T cell epitopes that rather exacerbate genital herpes disease (reviewed in Refs. 1, 40). “Asymptomatic” epitopes are those that are exclusively recognized by T cells from HSV-2 seropositive but asymptomatic individuals. An epitope-based vaccine that incorporates only “asymptomatic” CD8+ T cell epitopes while excluding “symptomatic” epitopes is logically expected to be more protective and preferable to whole protein vaccines that contain “symptomatic” epitopes that may be a source of an exacerbating disease (6, 45, 57).

In conclusion, this study 1) demonstrates the ability of a novel Lipo/rAdv5 mucosal vaccine regimen to induce higher CD8+ T cell responses not only at a higher magnitude but also with an increase in quality level; and 2) indicates that the protective efficacy of this formulation is associated with the induction of local and sustained polyfunctional CD8+ T cell responses not only at a higher magnitude but also with an increase in quality level; and 2) indicates that the protective efficacy of this formulation is associated with the induction of local and sustained polyfunctional CD8+ T cell responses.

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