Intravaginal immunization with HPV vectors induces tissue-resident CD8+ T cell responses

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The induction of persistent intraepithelial CD8+ T cell responses may be key to the development of vaccines against mucosally transmitted pathogens, particularly for sexually transmitted diseases. Here we investigated CD8+ T cell responses in the female mouse cervicovaginal mucosa after intravaginal immunization with human papillomavirus vectors (HPV pseudoviruses) that transiently expressed a model antigen, respiratory syncytial virus (RSV) M/M2, in cervicovaginal keratinocytes. An HPV intravaginal prime/boost with different HPV serotypes induced 10-fold more cervicovaginal antigen-specific CD8+ T cells than priming alone. Antigen-specific T cell numbers decreased only 2-fold after 6 months. Most genital antigen-specific CD8+ T cells were intra- or subepithelial, expressed αE-integrin CD103, produced IFN-γ and TNF-α, and displayed in vivo cytotoxicity. Using a sphingosine-1-phosphate analog (FTY720), we found that the primed CD8+ T cells proliferated in the cervicovaginal mucosa upon HPV intravaginal boost. Intravaginal HPV prime/boost reduced cervicovaginal viral titers 1,000-fold after intravaginal challenge with vaccinia virus expressing the CD8 epitope M2. In contrast, intramuscular prime/boost with an adenovirus type 5 vector induced a higher level of systemic CD8+ T cells but failed to induce intraepithelial CD103+CD8+ T cells or protect against recombinant vaccinia vaginal challenge. Thus, HPV vectors are attractive gene-delivery platforms for inducing durable intraepithelial cervicovaginal CD8+ T cell responses by promoting local proliferation and retention of primed antigen-specific CD8+ T cells.

Introduction

An important role of CD8+ T cells is to clear intracellular pathogens through the interaction between their T cell receptor and pathogen-derived peptides presented in the groove of the MHC I at the surface of infected cells (1, 2). Because CD8+ T cells are activated by cell-to-cell interactions, it is assumed that memory CD8+ T cells present at the site of infection are advantageous for early control of infections. The ability to direct vaccine-induced CD8+ T cell responses to the female cervicovaginal mucosa may be critical to the successful development of prophylactic vaccines against some sexually transmitted viral infections, such as HIV and herpes simplex virus (HSV), as well as the development of therapeutic vaccines against human papillomaviruses (HPV) and the intraepithelial neoplasia that they induce. However, the female cervicovaginal mucosa is generally considered a difficult site in which to induce an immune response (3), and, with the exception of replication-competent microbial vectors (which raise safety concerns; refs. 4, 5), there is little evidence for effective vaccination via the female cervicovaginal mucosa (6, 7). Efforts to induce genital CD8+ T cell responses have therefore focused on either systemic immunization or mucosal immunization at distant sites, particularly the upper respiratory tract.

Studies have shown that after systemic immunization or viral infection, CD8+ T cells spread to virtually all peripheral tissues, where they can subsequently differentiate into effector memory cells with increased survival potential and distinct phenotypes influenced by their microenvironment (8–10). Furthermore, several systemic immunization strategies using live viruses, replication-defective viral vectors, or protein antigens and adjuvant have been shown to induce CD8+ T cell responses in the cervicovaginal mucosa in addition to systemic CD8+ T cell responses (11–13). Nevertheless, T cell trafficking is clearly regulated at the site of induction through the expression of an array of homing molecules such as integrins, addressins, and chemokine receptors (14). For instance, the acquisition of a gut-homing phenotype, characterized by the expression of CCR9 and integrin αEβ7 by T cells after infection or immunization, is driven by the local environment, notably by local DCs, which may account for the preferential localization of effector CD8+ T cells at the site of viral infection or immunization (15–19). On the other hand, cervicovaginal T cells induced after vaginal Chlamydia infection display a different set of homing molecules compared with their intestinal counterpart and more closely resemble systemic T cells, as they express integrin β7, CCR5, and CXCR3 (20, 21). Other studies have shown that local immunization preferentially induces CD8+ T cell responses at the site of immunization, including the cervicovaginal mucosa, supporting the concept of anatomical compartmentalization of CD8+ T cell responses (22–25). In addition, the integrin αE(CD103)β7, a marker for intraepithelial lymphocytes, was shown to be upregulated by tissue-resident memory CD8+ T cells upon in situ antigen expression (26) and expressed by mucosal CD8+ T cells after viral infection (27, 28).

Together, these data suggest that local immunization may be better suited for the induction of local CD8+ T cells than a remote immunization regimen. However, to our knowledge there is no study that directly compares distant versus local mucosal immunization to determine whether the induced T cells reside in the epithelium, lamina propria, and/or the vasculature of the target mucosa. The generation of effector or effector memory T cells that home to
the genital tract epithelium may be particularly advantageous for combating infections that are initiated and/or maintained in cervicovaginal keratinocytes, such as HSV and HPV (29, 30). Most replication-defective viral vectors for genetic vaccination have been administered systemically and have successfully induced CD8+ T cell responses, but few of these vectors have been used for mucosal vaccination (31, 32). HPV pseudoviruses (PsV), composed of the viral L1 and L2 proteins and a double-stranded DNA pseudogenome, have several attractive features as vectors for genetic immunization of the female genital mucosa, which is the natural target tissue for many HPV types. They can encapsidate virtually any 6- to 8-kb circular target plasmid, and the target antigen(s) can be the only gene product(s) expressed (33). Titer of up to 1011 can easily be generated in tissue culture. Previous studies have shown that de novo expression of a reporter gene after intravaginal (Ivag) instillation of HPV PsVs in mice (34) and non-human primates (35, 36) requires mild disruption of the epithelium and can be routinely accomplished by pretreatment with an over-the-counter spermicide containing nonoxynol-9 (N-9), a non-ionic detergent. Expression of the transduced gene is transient and restricted to cervicovaginal keratinocytes. Thus, the vectors can be used to test the specific hypothesis that transient expression limited to “wounded” keratinocytes can effectively induce mucosal immune responses.

In an initial study, we showed that Ivag vaccination with HPV PsVs primed broad-based systemic and respiratory T and B cell responses against the M/M2 antigen from respiratory syncytial virus (RSV) (37). In the current study, we evaluated genital CD8+ T cell responses in mice after Ivag HPV PsV prime/boost immunization with M/M2-expressing vectors, using different HPV types in the prime and boost to overcome antibody-mediated neutralization of the vectors. The local versus distal mucosal and systemic responses were compared with those induced after delivery of the same vectors to the upper respiratory mucosa or induced by i.m. vaccination with standard adenvirus type 5 (Ad5) vectors, respectively. In addition, we investigated the phenotype and activity of the induced mucosal T cells, their localization within the cervicovaginal tissue, the site of their proliferation after primary or booster immunization, and the duration of the cervicovaginal CD8+ T cell response. Finally, the protection conferred by Ivag HPV PsV vaccination was assessed in an Ivag challenge with recombinant vaccinia virus (VV).

Results
Heterotypic HPV PsV prime/boost Ivag immunization overcomes type-specific antibody neutralization and enhances the frequency of CD8+ T cells in the cervicovaginal mucosa. HPV PsV immunization can induce high-titer type-specific neutralizing antibodies, which could compromise a strategy that uses the same HPV PsV for prime and boost immunization. To determine whether a prime/boost strategy that uses heterotypic PsVs could overcome this problem, we inoculated groups of 5 mice Ivag with 1 x 10^7 infectious units (IU) of HPV16 or HPV45 PsV encoding luciferase. Luciferase expression was monitored daily by measuring luciferase activity after Ivag luciferin instillation (expressed in photons/second [p/s]) in the genital area using a Xenogen IVIS imager. Following Ivag delivery of HPV16 or HPV45 luciferase PsV, luciferase expression peaked between days 2 and 3 and returned to baseline by day 5 for both serotypes (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI63287DS1). Inoculation with 1 μg HPV16 PsV elicited type-specific serum neutralizing antibodies (geometric mean titer [GMT] range, 150–1350) (Supplemental Figure 1B and Supplemental Methods). Ivag delivery of 5 x 10^7 IU HPV16 luciferase PsV conferred complete protection against a subsequent secondary challenge with 5 x 10^7 IU HPV16 luciferase PsV but did not affect secondary challenge with 5 x 10^7 IU HPV45 luciferase PsV (Figure 1A).

In other experiments, mice were immunized Ivag with 5 x 10^7 IU of an HPV16 PsV encapsidating a plasmid encoding M/M2 (a fusion protein of the RSV M and M2 protein) as a model antigen. Dominant CD8+ T cell responses directed against the K14-restricted peptide (SYIGSINNI) from the M2 protein were measured by flow cytometry using allophycocyanin-conjugated (APC-conjugated) K14M242-44 tetramer (K14M242-tetramer). Two weeks after a single Ivag immunization with HPV16-MM2 PsV, K14M242-tetramer+CD8+ T cells were readily detected in the cervicovaginal mucosa. However, upon a secondary immunization with HPV45-MM2 PsV, the number of K14M242-tetramer+CD8+ T cells per cervicovaginal mucosa increased 10-fold compared with single immunization (Figure 1B). In other experiments, mice were primed with HPV16-MM2 PsV and boosted with either HPV16-MM2 PsV or HPV45-MM2 PsV. Homologous prime/boost immunization did not result in an enhanced number of K14M242-tetramer+CD8+ T cells.
compared with N-9, suggesting that de novo antigen expression is required for the enhancement of genital CD8\(^+\) T cell responses (Supplemental Figure 2). These results underscore the advantage of heterotypic prime/boost immunization regimen to increase genital CD8\(^+\) T cell responses after HPV PsV Ivag immunization.

Prime/boost Ivag immunization regimen with HPV PsV induces intraepithelial CD8\(^+\) T cells in the cervicovaginal mucosa. To evaluate in more detail the CD8\(^+\) T cell response induced by the HPV PsV immunization, groups of 5–7 mice were primed Ivag with HPV16-MM2 PsV (5 × 10\(^7\) IU) and 1 month later were boosted Ivag with HPV45-MM2 PsV (5 × 10\(^7\) IU). Two weeks after the second immunization, the presence of K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T cells was measured by flow cytometry. Prime/boost Ivag immunization with HPV-MM2 PsV induced a 10-fold increase in the percentage of total CD8\(^+\) T cells (15.5%) of a cervicovaginal cell suspension compared with HPV-control PsV (1.57%), whereas the percentage of CD4\(^+\) T cells (15.5%) of a cervicovaginal cell suspension compared with HPV-MM2 PsV remained unchanged (Figure 2A). Remarkably, the majority of the cervicovaginal CD8\(^+\) T cells induced after Ivag immunization were specific for the immunodominant M282-90 epitope (Figure 2A). Thus, most of the increase in cervicovaginal CD8\(^+\) T cells observed after HPV-MM2 PsV immunization was attributable to the CD8\(^+\) T cell response against the K\(^{d}\)-restricted M282-90 epitope. This was confirmed by the analysis of the absolute number of cervicovaginal CD8\(^+\) T cells and K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T cells (Figure 2B).

Next, we used confocal microscopy to determine the localization in the cervicovaginal mucosa of the CD8\(^+\) T cells induced after HPV PsV Ivag immunization. Tissue sections were stained with laminin 332 antibody to delineate the basement membrane and with CD8 and CD3 antibodies to detect CD8\(^+\) T cells. To our surprise, the cervicovaginal mucosa of the control animals was essentially devoid of CD8\(^+\) T cells associated with the epithelium (Figure 3A). By contrast, the cervicovaginal mucosa of immunized animals displayed high numbers of CD8\(^+\) T cells lining, within, or beneath the epithelium (Figure 3A). Further staining of cervicovaginal tissue sections revealed that most intraepithelial CD8\(^+\) T cells expressed \(\alpha\)E-integrin (CD103), a cell surface marker often associated with bona fide intraepithelial T cells (Figure 3B).

Figure 2
HPV Ivag prime/boost immunization induces high numbers of cervicovaginal CD8\(^+\) T cells. Depo-Provera–treated mice (\(n = 5\)/group) were immunized with 5 × 10\(^7\) IU HPV16MM2 or HPV16 control (HPV-CTL) Ivag, and 1 month later, primed mice were immunized with 5 × 10\(^7\) IU HPV45MM2 or HPV45 control, respectively. Two weeks after the second immunization, cervicovaginal cell suspensions were analyzed by flow cytometry for the presence of K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T lymphocytes in HPVMM2-immunized and sham-treated animals. (A) CD8 and CD4 expression on gated vaginal cells with a lymphocyte morphology (contour plot) and K\(^{d}\)M282-tetramer binding on gated CD8\(^+\) T cells (histogram plot); the percentage of each gate and marker of the parent population is indicated in each plot. (B) Total CD8\(^+\) T lymphocytes and K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T lymphocytes per cervicovaginal mucosa. Data are representative of more than 3 experiments (*\(P < 0.05\), **\(P < 0.01\), Mann-Whitney U test).

Prime/boost Ivag immunization regimen with HPV PsV induces systemic CD8\(^+\) T cell responses. We further analyzed the tissue distribution of CD8\(^+\) T cell responses 2 weeks after HPV-MM2 PsV Ivag prime/boost immunization in the cervicovaginal mucosa, blood, spleen, and draining lymph nodes (Figure 4). In the cervicovaginal mucosa, 61% of CD8\(^+\) T cells were K\(^{d}\)M282-tetramer\(^+\), whereas the average frequency of K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T cells was much lower in blood (1.4%), spleen (1.3%), and ilio-sacral/inguinal lymph nodes (ILNs) (1.7%). These results indicate that HPV PsV prime/boost Ivag immunization can induce systemic CD8\(^+\) T cell responses but it preferentially induces cervicovaginal CD8\(^+\) T cells responses.

Ivag immunization with HPV pseudovirions induces durable effector-memory CD8\(^+\) T cell responses in the cervicovaginal mucosa and in lymphoid tissues. Durability of CD8\(^+\) T cell responses, especially in the cervicovaginal mucosa, is a critical parameter for many applications of vaccines against sexually transmitted infection. After prime/boost Ivag immunization with 5 × 10\(^7\) IU of HPV-MM2 PsV, we monitored the K\(^{d}\)M282-specific CD8\(^+\) T cell response in blood, spleen, ILN, and cervicovaginal mucosa from week 1 to week 14 after boost. In all organs tested, the K\(^{d}\)M282-specific CD8\(^+\) T cell responses peaked between weeks 1 and 2 (Figure 5A). At week 7, the K\(^{d}\)M282-specific CD8\(^+\) T cell response in the cervicovaginal mucosa had declined by only 15%, compared with week 1, whereas the response in blood had undergone a 2-fold decline (Figure 5A). At week 14, K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T cells in the genital tract had declined approximately 2-fold and still accounted for 24% of total CD8\(^+\) T cells in the cervicovaginal mucosa, whereas the frequency in blood was less than 0.5%. These results indicate that HPV PsV Ivag prime/boost immunization induces long-lasting CD8\(^+\) T cell responses in the cervicovaginal mucosa.

Next, we assessed the T cell memory phenotype induced after HPV PsV Ivag immunization. Previous studies have shown that effector, effector-memory, and central memory T cell populations can be distinguished based on their expression of IL-7 receptor (CD127) and L-selectin (CD62L), which reflects their differential migratory, survival, and protective potential (38–40). We used multi-parametric flow cytometry to analyze the expression of CD62L and CD127 by K\(^{d}\)M282-tetramer\(^+\)CD8\(^+\) T cells in the cer-
vicovaginal mucosa and blood from animals at week 2 to week 14 after booster immunization. In the cervicovaginal mucosa at the peak of the secondary response, KdM282-specific CD8+ T cells were distributed between CD62L-CD127+ (effector-memory) and CD62L-CD127− (effector) cells (Figure 5B). By week 14, the proportion of effector-memory KdM282-specific CD8+ T cells had increased, consistent with the known properties of short-lived effector T cells relative to effector-memory T cells (Figure 5B). At no time point were significant numbers of central memory KdM282-specific CD8+ T cells detected in the cervicovaginal mucosa, consistent with their propensity to localize in lymph nodes. In the blood, 2 weeks after booster immunization, the majority (70%) of KdM282-specific CD8+ T cells were effector-memory cells, whereas central memory and effector cells accounted for 7% and 15% of KdM282-specific CD8+ T cells, respectively (Figure 5B). These results indicate that Ivag immunization with HPV PsV induces predominantly long-lived effector-memory CD8+ T cells and that many of these cells reside at the side of inoculation in the cervicovaginal mucosa.

Ivag immunization with HPV pseudovirions induces CD8+ T cells able to produce multiple cytokines and have cytotoxic activity. Multiple functions (e.g., cytokine production and cytotoxicity) displayed by CD8+ T cells are usually associated with a response of better quality in the settings of viral infections, and such functions simultaneously displayed are considered as predictive markers of protective CD8+ T cells induced by vaccination (41). We evaluated the ability of KdM282-specific CD8+ T cells induced after HPV PsV Ivag immunization to produce IFN-γ, TNF-α, and IL-2 using intracellular cytokine staining after in vitro restimulation with M282–90 peptide. Two weeks after booster HPV PsV immunization, cell suspensions of cervicovaginal mucosa, spleen, and ILN were stimulated for 5 hours in vitro with 5 μM M282–90 or without peptide. After surface staining with CD3, CD4, and CD8 antibodies, we performed intracellular cytokine staining with IFN-γ, TNF-α, and IL-2 antibodies or isotype control antibodies. In the cervicovaginal mucosa, an average of 58% of CD8+ T cells produced at least 1 cytokine after M282–90 stimulation, which is concordant with the high percentage of KdM282-tetramer+CD8+ T cells detected in the cervicovaginal tissue (Figure 6). In the ILN and spleen, the average percentages of cytokine-producing CD8+ T cells were 0.5% and 1%, respectively, which correlates with the percentage of KdM282-tetramer+CD8+ T cells detected in each organ (Figure 6). In the cervicovaginal mucosa, 51% of CD8+ T cells produced IFN-γ, 47% produced TNF-α, and less than 3% of CD8+ T cells produced IL-2 (Figure 6A and Supplemental Figure 3). The percentage of peptide-stimulated CD8+ T cells producing 1, 2, or 3 cytokines from the cervicovaginal mucosa, spleen, and ILN was further analyzed and is represented in Figure 6B. In the genital tract, the majority of the cytokine-producing CD8+ T cells (61%) produced 2 cytokines, predominantly IFN-γ and TNF-α (Supplemental Figure 3). Single-cytokine-producing cells represented 35% of the total cytokine-producing cells; they produced IFN-γ or TNF-α but not IL-2 (Supplemental Figure 3).
Only 4% of cervicovaginal CD8+ T cells simultaneously produced all 3 cytokines. In contrast, ILN and spleen cytokine-producing CD8+ T cells were more evenly distributed among triple-, double-, and single-cytokine-producing profiles (Figure 6B). In the ILN and spleen, most double-cytokine-producing CD8+ T cells produced IFN-γ and TNF-α (Supplemental Figure 3), and most of the IL-2 was produced by triple-producing CD8+ T cells (Figure 6B and Supplemental Figure 3). The difference in cytokine production by the cervicovaginal CD8+ T cells versus secondary lymphoid organ CD8+ T cells may be due to the preferential induction of effector-memory CD8+ T cells in the cervicovaginal mucosa and the selective induction of central memory CD8+ T cells in secondary lymphoid organs after HPV PsV Ivag immunization.

In addition to cytokine production, we assessed the cytotoxic activity of the CD8+ T cells induced after HPV PsV immunization and measured in vivo the presence of bona fide CTLs against the M282–90 epitope. Cytotoxic activity was measured in vivo by injection of target cells, which consisted of a mixture of spleen cells that had been either pulsed with M282–90 peptide or mock treated and had been labeled, respectively, with high or low concentrations of CFSE. Target cells were injected simultaneously i.v. and subdermally in the vaginal wall, and 18 hours after injection KdM282-specific in vivo cytotoxicity was assessed by flow cytometry in cervicovaginal mucosa, ILN, and spleen cell suspensions. Two weeks after the booster immunization, immune mice displayed 74% specific lysis in the cells from the cervicovaginal mucosa, 65% in those from the spleen, and 69% in the ILN cells (Figure 6C). Together, these results indicate that HPV PsV Ivag prime/boost immunization induces polyfunctional CD8+ T cells and bona fide CTL in the cervicovaginal mucosa and in the systemic compartment.

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Naive CD8+ T cells are primed in the genital draining lymph node after HPV PsV Ivag immunization. Because, the Ivag prime/boost immunization regimen with HPV PsV is so potent at inducing genital CD8+ T cell responses, we first sought to determine whether CD8+ T cells were primed in the cervicovaginal mucosa or in the genital draining lymph node (Figure 7A). FTY720 promotes the retention of naive lymphocytes in the lymph nodes leading to severe lymphopenia in blood and has been a useful tool for analyzing the dynamics of T cell responses during infection or vaccination (42–44). Therefore, mice were treated with FTY720 in drinking water.
water from day 1 after immunization until the end of the experiment on day 14. As expected, FTY720 treatment abrogated the KdM282-tetramer+CD8+ T cell responses in the blood and spleen after a single HPV PsV Ivag immunization (Figure 7A). FTY720 treatment induced a 5-fold decrease in the number of KdM282-tetramer+CD8+ T cells compared with mock-treated mice in the cervicovaginal mucosa but did not completely abrogate the CD8+ T cell response (Figure 7A). Interestingly, analysis of the phenotype of KdM282-tetramer+CD8+ T cells in the cervicovaginal mucosa showed that in FTY720-treated animals, 81% of genital KdM282-tetramer+CD8+ T cells were effector cells, whereas in mock-treated animals, 61% of KdM282-tetramer+CD8+ T cells were memory cells (Supplemental Figure 4). In the draining lymph nodes, the expected increase in the number of KdM282-tetramer+CD8+ T cells in FTY720-treated animals compared with mock-treated mice was observed (Figure 7A). The phenotype of the KdM282-tetramer+CD8+ T cells in FTY720-treated as well as mock-treated animals was predominantly effector-memory (Supplemental Figure 4). These data suggest that ILNs are the primary site of priming of CD8+ T cell responses during HPV PsV immunization and that the CD8+ T cell response in spleen and in the cervicovaginal mucosa requires systemic circulation through the bloodstream after priming.

Primary CD8+ T cell responses are abrogated in the absence of CD4+ cells after a single HPV Ivag immunization. We also assessed the role of CD4+ T cells in the induction of primary CD8+ T cell response after a single HPV Ivag immunization. Mice received CD4-depleting antibody by i.p. injection every other day from 3 days prior to immunization until 9 days after the day of immunization. In preliminary experiments, we evaluated the efficacy of CD4+ T cell depletion, which was >90% in the genital tract and >95% in the lymph node (Supplemental Figure 5 and unpublished observations). In addition, neither plasmacytoid DCs nor conventional DCs were depleted by the CD4 antibody treatment, even though they have been shown to express CD4 in mice (ref. 45, Supplemental Figure 5, and unpublished observations). CD4 depletion did not affect the efficacy of HPV-mediated gene delivery in vivo (Supplemental Figure 5), but it completely abrogated the IgG response against the major protein (L1) of the HPV capsid (Supplemental Figure 6A). KdM282-specific CD8+ T cell responses were assessed in the vagina, ILN, and spleen on day 14 (Figure 7B). In vivo CD4 depletion completely abrogated KdM282-specific CD8+ T cell responses in the cervicovaginal tract and spleen (Figure 6B). However, residual KdM282-tetramer+CD8+ T cells were detected in the ILN (Figure 6B), and these cells exhibited predominantly a central memory phenotype (Supplemental Figure 6C). These data indicate that CD4+ T cells play a critical role in the induction of effector and effector-memory CD8+ T cell responses after HPV PsV immunization but that their role is at least partially dispensable for the induction of central memory CD8+ T cells in the ILN.

Secondary CD8+ T cell responses expand locally in the cervicovaginal mucosa and in the ILN upon secondary HPV PsV Ivag immunization. Because a single HPV PsV Ivag immunization induced a strong CD8+ T cell response in the cervicovaginal mucosa, we sought to analyze the behavior of resident effector-memory CD8+ T cells upon a secondary Ivag immunization with HPV PsV (Figure 8). We used FTY720 treatment to evaluate whether the dynamics of the secondary CD8+ T cell response would differ from the dynamics of the primary CD8+ T cell response after Ivag HPV PsV booster immunization (44). Mice were primed with HPV16 PsV Ivag in the absence of FTY720 and 1 month later received an Ivag HPV45 PsV booster immunization with or without FTY720 treatment from day 1 to day 14 after booster immunization. While CD8+ T cell responses were still detected in the cervicovaginal mucosa, blood, ILN, and spleen 1 month after priming, CD8+ T cell responses were, as expected, further enhanced after a booster immunization in mice not treated with FTY720 (Figure 8A). In spleen and blood, boosted animals that were FTY720 treated had CD8+ T cell responses...
that were similar to those in primed-only animals, indicating that FTY720 treatment abrogated secondary CD8+ T cell responses in these organs (Figure 8A). In the ILN of FTY720-treated animals, CD8+ T cell responses were increased compared with mock-treated animals, indicating that memory CD8+ T cells present in the lymph node could further expand locally upon secondary Ivag HPV PsV immunization (Figure 8A). In the cervicovaginal mucosa, FTY720 treatment during booster immunization only modestly reduced the secondary CD8+ T cell response by a non-significant 25% (Figure 8A), in marked contrast to the large reduction in cervicovaginal CD8+ T cells when FTY720 treatment was given during priming (Figure 7A). Remarkably, in FTY720-treated animals, secondary genital K/M282-specific CD8+ T cells were predominantly effector-memory cells, as was the case with mock-treated animals (Supplemental Figure 4), which suggest that FTY720 treatment did not alter the acquisition of this memory phenotype.

In some experiments, mice received both FTY720 and BrdU in drinking water from day 1 to day 14 after booster immunization to evaluate the proliferation in vivo of K/M282-specific CD8+ T cells. ILN, spleen, and cervicovaginal mucosa cell suspensions were obtained on day 14 after boosting, and surface staining was performed for detection of K/M282-tetramer+CD8+ T cells in FTY720- or mock-treated mice upon secondary immunization. Two weeks after boost, incorporation of BrdU by K/M282-tetramer+CD8+ T cells was measured by intracellular staining of cervicovaginal and ILN cell suspensions and further analyzed by flow cytometry. (B) Representative plot of BrdU and K/M282-tetramer staining of CD8+ T cells (percentage of CD8+ T cells in each quadrant is indicated). (C) Mean + SD percentage of BrdU relative to K/M282-tetramer+CD8+ T cells. Data are representative of 2 (A) and 3 (B) experiments (*P < 0.05, **P < 0.01, Kruskal-Wallis/Dunn's test).
Remarkably, in mock-treated mice, up to 80% of cervicovaginal KdM282-tetramer+CD8+ T cells had incorporated BrdU within 2 weeks after the booster immunization, whereas BrdU incorporation in nonspecific CD8+ T cells was modest (20%) and probably accounted for by homeostatic proliferation (Figure 8B). In the cervicovaginal mucosa of FTY720-treated mice, 80% of KdM282-tetramer+CD8+ T cells also incorporated BrdU (Figure 8B), indicating recent proliferation upon booster immunization. Because FTY720 treatment abrogated the circulation of KdM282-specific CD8+ T cells in blood, these data suggest that upon booster Ivag immunization, secondary CD8+ T cell responses can proliferate in the cervicovaginal mucosa in response to de novo antigen expression by genital keratinocytes.

HPV PsV prime/boost Ivag immunization induces more potent CD8+ T cell responses in the cervicovaginal mucosa than does prime/boost immunization at remote sites. We next compared genital CD8+ T cell responses in the cervicovaginal mucosa induced by HPV Ivag immunization versus other well-characterized routes of immunization. Intranasal immunization has been shown to induce CD8+ T cell responses in the upper respiratory tract as well as remote CD8+ T cell responses in the cervicovaginal mucosa (24). Efficient i.n. gene delivery of HPV PsV required mild disruption of the nasal epithelium using a lower dose of N-9 (0.2%). After i.n. prime/boost immunization with 5 x 10^7 IU HPV-MM2 PsV, 12% of cervicovaginal CD8+ T cells were KdM282-tetramer+, which was much lower than the frequency of KdM282-tetramer+CD8+ T cells (40%) after HPV PsV Ivag immunization (Figure 9A). The difference was even more striking when analyzed in terms of absolute numbers of cells, as Ivag prime/boost immunization induced 10 times more cervicovaginal KdM282-tetramer+CD8+ T cells than i.n. immunization. Conversely, i.n. prime/boost immunization induced a higher frequency of KdM282-tetramer+CD8+ T cells in the lungs than Ivag immunization (0.3% of CD8+ T cells) (Figure 9A).

Intramuscular immunization with adenoviral vectors has been reported to induce potent CD8+ T cell responses in the cervicovaginal mucosa (13, 46). Consistent with these data, i.m. prime/boost immunization with 5 x 10^6 PFU of Ad5-MM2 induced a strong CD8+ T cell response in the spleen and cervicovaginal mucosa, with 3.3% and 12.9% of KdM282-tetramer+CD8+ T cells, respectively (Figure 9B). After HPV PsV Ivag immunization, the frequency of KdM282-tetramer+CD8+ T cells (30%) was 2.5 times higher than after Ad5 intramuscular immunization in the cervicovaginal mucosa (Figure 9B). More strikingly, the absolute number of KdM282-tetramer+CD8+ T cells in the cervicovaginal mucosa after HPV PsV immunization was 10 times higher than after Ad5 intramuscular immunization (Figure 9B). In contrast, the M282–90–specific CD8+ T cell response in the spleen after HPV PsV Ivag immunization was less than half that induced by Ad5 intramuscular immunization (Figure 9B). Taken together, these data indicate that HPV PsV Ivag immunization is more effective than remote immunization regimens for the preferential induction of CD8+ T cell responses in the cervicovaginal mucosa.
HPV PsV Ivag, but not Ad5 i.m., prime/boost immunization induces high numbers of intraepithelial K2M282-tetramer CD8+ T cells with restricted CD103 expression in the cervicovaginal epithelium. As shown above, HPV PsV Ivag immunization induced high numbers of intraepithelial CD8+ T cells (Figure 3), and Ad5 i.m. immunization induced K2M282-tetramer CD8+ T cell responses in the cervicovaginal mucosa (Figure 9B). We therefore analyzed the distribution of CD8+ T cells in the cervicovaginal mucosa by immunofluorescence staining of tissue sections from mice prime/boost immunized Ivag or i.m. with HPV PsV or Ad5 vectors, respectively (Figure 10A). As previously noted, the cervicovaginal epithelium of sham-treated mice was devoid of CD8+ T cells, whereas HPV PsV Ivag prime/boost immunization induced numerous CD8+ T cells within or beneath the epithelium (Figure 10A). Strikingly, the cervicovaginal epithelium of mice prime/boost immunized i.m. with 5 × 106 PFU of Ad5-MM2 vector was devoid of CD8+ T cells (Figure 10A), despite the fact that K2M282-tetramer CD8+ T cells could be readily detected by flow cytometry. This surprising result suggests that i.m. immunization fails to induce intraepithelial T cells. The induced T cells detected by flow cytometry may instead be associated with the vasculature or remain deeper in the cervicovaginal tissue, and so are either lost during processing or not seen in the stained sections. We also compared expression of the CD103 integrin on K2M282-tetramer CD8+ T cells in the cervicovaginal mucosa, blood, and ILN 4 weeks after the booster doses (Figure 10B). After Ivag immunization, most K2M282-tetramer CD8+ T cells were CD103+ in the genital tract (80%), few were positive in the ILN (17%), and a negligible number were positive in blood (Figure 10B). Importantly, i.m. prime/boost induced a very different pattern of CD103 expression; it was detected on only 14% of the K2M282-tetramer CD8+ T cells in the cervicovaginal mucosa and was nearly absent in blood and ILN (Figure 10B). Together, these data suggest that CD103 is differentially upregulated on CD8+ T cells exposed to antigens expressed transiently in the genital tract and may account for the preferential intraepithelial localization of CD8+ T cells after Ivag but not after i.m. immunization.

HPV Ivag prime/boost immunization confers protection against recombinant vaccinia vaginal challenge. We further analyzed the potential of HPV Ivag prime/boost immunization to confer protection to a model infectious agent for genital infection, recombinant VV. VV typically infects stratified squamous epithelium and has been used previously to infect the vaginal mucosa (7, 22). We used a recombinant VV expressing the M282–90 epitope (rVV-M282–90) (47) to assess the protective potential of cervicovaginal M282–90–specific CD8+ T cells induced after HPV Ivag or Ad5 i.m. prime/boost immunization (Figure 11). Two months after the last immunization, mice received 105 PFU of rVV-M282–90 Ivag, and on day 3 after challenge, cervicovaginal mucosa was collected and homogenates assessed for rVV-M282–90 content by plaque assay. Recombinant VV-M282–90 titer did not differ significantly between sham-treated and HPV-Luc–treated mice, with rVV-M282–90 geometric mean titers of 1.6 × 106 and 2.5 × 106 PFU per organ, respectively (Figure 11). In contrast, HPV-MM2–immunized mice showed a statistically significant 1,000-fold-lower VV titer in cervicovaginal homogenate (2.1 × 104 PFU per organ) compared with control groups, implying that M282–90–specific CD8+ T cells played a key role in controlling VV replication in the vagina. Importantly, the Ad5-MM2 i.m. immunized mice displayed an average recombinant VV titer in the cervicovaginal mucosa of 1.3 × 105 PFU, a level that was 100-fold lower than that of sham-treated mice.
higher than that in the HPV-MM2–immunized mice and non-significantly lower than in the sham-immunized mice.

In separate experiments, we evaluated whether recognition of the M282–90 epitope by CD8+ T cells was required for protection. Sham-treated, HPV-Luc–, or HPV-MM2–immunized mice were challenged Ivag with 1 × 10^7 PFU of recombinant M282–90–VV. Three days after challenge, vaginal tissue was collected, and recombinant VV titers were determined by plaque assays. Results are expressed as PFU per vagina for individual mice (symbols) and geometric mean (horizontal bars). Data are representative of 3 experiments (*P < 0.05, **P < 0.01, Kruskal-Wallis/Dunn’s test).

**Discussion**

This study provides evidence that the female cervicovaginal mucosa can be an effective site for induction and boosting of effector memory CD8+ T cell responses using a non-replicating viral vector can be an effective site for induction and boosting of effector memory CD8+ T cell responses using a non-replicating viral vector. Some vaccination strategies have sought to increase the strength and durability of effector memory CD8+ T cell responses by inducing persistent antigen expression with live virus vectors (12). Our results demonstrate that even transient antigen expression can induce strong, durable responses. It is noteworthy that we did not observe an acute contraction phase in genital CD8+ T cells after Ivag vaccination. Our findings support accumulating evidence that intraepithelial CD8+ T cells can participate in the control of viral replication in the cervicovaginal mucosa.

This is the first study to our knowledge to demonstrate the preferential induction of large numbers of intraepithelial CD103+ CD8+ T cells (IELs) in the cervicovaginal mucosa after Ivag immunization with a non-replicating vector. Unfortunately, because of technical limitations inherent in the available KdM282-tetramers, we were unable to monitor in situ antigen-specific CD8+ T cells by M2-tetramer staining on tissue sections of the cervicovaginal mucosa. However, the paucity of preexisting CD8+ T cells in the cervicovaginal epithelium of young mice that have not been bred, and the fact that the exceptional increase in genital CD8+ T cells was attributable entirely to the expansion of KdM282-tetramer–positive CD8+ T cells as shown by flow cytometry, strongly imply that the intraepithelial CD8+ T cells induced after HPV PsV Ivag immunization are specific for the immunodominant epitope M282–90. The low number of cervicovaginal T cells prior to vaccination led us to focus on absolute numbers of T cells induced rather than on their relative percentage in the tissue, since the latter calculation can produce an overestimate of the response in tissues with low preexisting numbers of T cells.

Based on the large number of CD3+CD8+ T cells detected histologically in most tissue sections from vaccinated mice, it is our impression that the flow cytometry analysis substantially underestimates the true number of T cells induced in the cervicovaginal mucosa, which might result from the incomplete liberation of T cells from the tissue by our extraction procedures. In future studies, it will be important to determine whether higher numbers of preexisting IELs in the cervicovaginal epithelium inhibit induction of antigen-specific CD8+ T cells after Ivag HPV PsV vaccination and whether the pool of memory IEL CD8+ T cells can expand further, as previously reported for the pool of memory CD8+ T cells in PBMCs (54).

Some vaccination strategies have sought to increase the strength and durability of effector memory CD8+ T cell responses by inducing persistent antigen expression with live virus vectors (12). Our results demonstrate that even transient antigen expression can induce strong, durable responses. It is noteworthy that we did not observe an acute contraction phase in genital CD8+ T cells after Ivag vaccination. Our findings support accumulating evidence that intraepithelial T cells, especially effector memory cells, may in general be longer lived than systemic effector T cells, as befits their putative role as guardians of epithelial surfaces. It is unclear whether the marked acquisition of an effector memory phenotype observed in the genital CD8+ T cells over time is due to the evolu-
tion of the phenotype of individual cells or gradual replacement of effector with effector memory cells, for instance, by preferential survival or homeostatic proliferation.

The ability to preferentially induce large numbers of antigen-specific IELs by vaccination should facilitate future studies of this unique class of lymphocytes. Our characterization indicates that primary CD8+ T cells depend on CD4+ T cell help, which is consistent with their priming occurring in lymph nodes. However, the role of CD4+ T cells during at the booster immunization step remains to be assessed. The recruitment of circulating T cells to the genital mucosa has been previously described and requires CD4+ T cells as well as expression of CXCR3 by circulating memory CD8+ CD4+ T cells (55). Our FTY720 and BrdU experiments strongly implicate local mucosal proliferation during the secondary response to keratinocyte-expressed antigen, but it does not address the role of CD4+ T cells or whether inflammation-driven recruitment from the circulation was required. While this is, to our knowledge, the first evidence of this phenomenon in a mucosal tissue after vaccination, several recent studies have presented evidence that peripheral proliferation of primed CD8+ T cells in response to antigen can occur in other settings, for instance, in tumors or neuronal tissues (26, 56). In future studies, it will be important to determine whether the “wounded” keratinocytes, which can express costimulatory and MHC I molecules, can directly present antigen for in situ proliferation of CD8+ T cells or whether cross-presentation by professional APCs or membrane transfer from keratinocytes to DCs, referred to as cross-dressing, are involved in the recall of memory T cells in the cervicovaginal mucosa (57). In addition, the trafficking patterns of IELs within the epithelium and between the epithelium and other tissues are poorly understood, and this system might allow for their study.

There are numerous reports of the induction of CD8+ T cell responses in the cervicovaginal mucosa after systemic vaccination with non-replicating vectors (7, 11, 13, 46), and we confirmed these observations using our Ad5-MM2 vector in an intramuscular prime/boost regimen. However, this regimen was qualitatively and quantitatively inferior to the Ivag prime/boost immunization regimen. In spite of the relatively high numbers of cervicovaginal K\textsuperscript{d}M282-tetramer+CD8+ T cells measured by flow cytometry, there was no increase in IEL number after systemic prime/boost immunization, which raises the possibility that the T cells extracted from the cervicovaginal tissues after the systemic immunization procedures may have largely been retained within the mucosal vasculature or were loosely associated with the submucosa. The unique ability of HPV PsV Ivag vaccination, compared with Ad5 i.m. vaccination, to confer enhanced protection against a local viral challenge is associated with the unique induction of CD8 IELs and their upregulation of CD103. Intraepithelial memory CD8+ T cells would be able to respond to infection more promptly than their circulating counterparts, but one could also speculate that the expression of CD103 at the surface of cervicovaginal IELs and their upregulation of CD103. Intraepithelial memory CD8+ T cells would contribute to enhancing protection, as this integrin has been shown to participate in the cytotoxic activity of human tumor-infiltrating CD8+ T cells (58). Also, the fact that CD103 expression was restricted to K\textsuperscript{d}M282-tetramer+CD8+ T cells from the cervicovaginal mucosa and expressed up to 6 months after boost (our unpublished observations) suggests a role for the cervicovaginal microenvironment in CD103 induction. This conjecture is consistent with recent reports indicating that persistent tissue-resident memory T cells upregulate CD103 in peripheral tissues but not in other lymphoid organs (27, 59). Furthermore, the limited CD103 expression in the cervicovaginal CD8+ T cells induced after i.m. immunization may explain the absence of intraepithelial CD8+ T cells in i.m. immunized mice and suggests that de novo antigen expression in wounded keratinocytes is driving the upregulation of CD103.

The phenotypic and functional characterization of the genital CD8+ T cells induced after genital HPV PsV vaccination is consistent with their being functional effector memory cells with potential antiviral activity. First, most cells were polyfunctional, as they simultaneously secreted IFN-γ and TNF-α. Second, they exhibited in vivo cytotoxic activity against autologous cells pulsed with the immunodominant M282-90 peptide. Third, they were associated with a 1,000-fold reduction in the mean cervicovaginal mucosa viral load of recombinant VV expressing the M282-90 peptide after vaginal inoculation. It is noteworthy that this viral challenge model is rather stringent, since protection is entirely mediated by CD8+ T cells.

The ability to preferentially induce long-lived antigen-specific intraepithelial CD8+ T cells may be an attractive feature for a vaccine designed to prevent a viral infection that is initiated in the cervicovaginal mucosa such as HIV or HSV. Having sentinel T cells at the initial site of infection has been shown to lead to a more rapid response in the skin during HSV-1 infection and thereby control infection before it can spread systemically (57). Indeed, recruitment of systemic CD8+ T cells may take too long to effectively control the initial phase of infection, which is an important determinant of clinical outcome. Having local T cells can increase the local ratio of virus-specific CD8+ T cells to virus-infected cells and provide enhanced protection (29, 60). For application to HIV vaccines, it is noteworthy that our Ivag vaccination protocol did not increase the number of cervicovaginal mucosa CD4+ T helper cells, so it would not be expected to increase the pool of infection targets, which could thereby potentiate infection.

Transduced systemic antibodies and effector IELs ideally could act synergistically to prevent or control infection of the genital mucosa, and vaccines inducing both major limbs of the adaptive immune response should be considered. If the absence of genital IELs after systemic immunization of mice is predictive for humans, then the role of IELs should be critically evaluated in future HIV or HSV clinical trials of candidate vaccines (61, 62). Using HPV-encapsidated DNA or other viral vectors to locally induce effector CD8+ IELs should be a high-priority approach to evaluate difficult vaccine targets, such as HSV or HIV.

Vaccines that induce cervicovaginal mucosa IELs might also be considered for therapeutic vaccines, particularly against HSV herpetic lesions and HPV-induced intraepithelial neoplasia. Abatement of recurrent genital herpes lesions was recently correlated with the appearance of CD8+ T cells located between the nerve endings and the adjacent epithelial cells at the site of previous herpetic lesions (63). Generation of HSV-specific effector memory T cells at these sites by vaccination might have a similar effect. A marked recruitment of CD8+ T cells into the epithelium of HPV-induced cervical intraepithelial neoplasia was observed in lesions that subsequently regressed (30). In contrast, a T cell infiltrate in the submucosa, along with its exclusion from the overlying dysplastic epithelium, was observed in lesions that persisted. Induction of HPV-specific IELs by vaccination might overcome this apparent barrier to clearance.

Finally, the feasibility of translating our vaccination strategy to women should be considered. We have recently shown that our
research article

HPV–based vaccination strategy is not restricted to mice, with studies in non-human primates demonstrating efficient gene delivery and immunogenicity against SIV antigens in Depo-Provera–treated monkeys (35). The mice in this study were similarly progestrone treated to ensure that they had uniformly thin cervicovaginal epithelium and so relatively consistent vector infection. However, this treatment might be more critical in mouse studies than in human applications for two reasons. First, the thickness of the cervicovaginal epithelium appears to be more hormonally dependent in mice, with estrogen-dominated mice exhibiting highly keratinized squamous epithelium. The thickness of the squamous epithelium varies less across the menstrual cycle in women. Second, the simple columnar epithelium of the endocervix begins rather high up the endocervical channel in mice and is not readily exposed to N-9 and PsV unless the cervix is cannulated (34). In contrast, the endocervical epithelium is exposed in women, particularly in young women. It might be predicted that the simple columnar epithelium of the endocervix would be exceptionally susceptible to N-9 disruption and PsV infection, and this susceptibility to infection would not be expected to vary substantially across the menstrual cycle. It will be important to further determine in non-human primate models whether IELs can similarly be induced in the simple columnar epithelium of the endocervix throughout the menstrual cycle, particularly since the endocervix and/or the transformation zone between endo- and ectocervix may be especially susceptible to viral transmission.

The requirement for epithelial permeabilization or disruption in conjunction with vaccination might also raise safety concerns. For this reason, we purposely chose to use a disrupting agent that is an over-the-counter product containing N-9 (Conceptrol) that is approved for unregulated Ivag application in women. While N-9–containing spericides used repeatedly may increase susceptibility to some sexually transmitted infections, these agents could be acceptable for a limited number of applications as part of a vaccination protocol. Although the use of N-9–containing products was associated with an increased risk of HIV transmission (64), the epithelial disruption they induce is transient, and refraining from unprotected sexual activity for several days after their use as part of a vaccination regimen would likely mitigate this risk. Finally, the experience from clinical trials of topical microbicides against STDs provides a rational basis for developing a relatively simple gel-based vaccine for topical application.

Methods

Mice. Six- to 8-week-old female BALB/cAnCr and CB6F1/Cr mice were purchased from the Animal Production Program (NCI-Frederick) and housed and handled under specific pathogen–free conditions in the animal care facilities at the National Cancer Institute. Mice were housed and handled under specific pathogen–free conditions in the animal care facilities at the National Cancer Institute.

Viral vectors and viruses. HPV16 and HPV45 pseudovirions (PsV) containing a plasmid that encodes a fusion protein of the M and M2 protein of RSV-2 (pCMMC, NCBI AA866677 and AA866660) or firefly luciferase (pCLucf, NCBI AY738225) were generated as described previously (37, 65, 66). Briefly, 293TT cells were co-transfected with a plasmid encoding the M/M2 fusion protein or luciferase and a plasmid too large to self-package encoding the L1 and L2 protein of HPV type 16 (L1 and L2, EMBL 16L1h L2 AJ313179 AJ313180) or 45 (L1 and L2, NCBI DQ080002) (67). After maturation, HPV PsV were purified over an OptiPrep gradient; fractions L2 AJ313179 AJ313180) or 45 (L1 and L2, NCBI DQ080002) (67). After maturation, HPV PsV were purified over an OptiPrep gradient; fractions containing the encapsidated plasmid were pooled; and infectious titer was determined on the 293TT cells by measuring a coexpressed green fluorescent protein gene by flow cytometry and expressed as IU/ml.

A non-replicating adenoviral type 5 vector (Ad5) encoding the MM2 fusion protein was generated and produced by GenVec. The number of infectious particles (PFU) of Ad5 was measured by plaque assay using a helper cell line, and MM2 protein expression was measured by Western blot analysis in 293T cells infected with Ad5-MM2 (37).

Respiratory syncytial virus M2Δ41-50 and influenza NP157-165 recombinant vaccinia viruses were a gift from Brian Murphy (NIAID) and were generated, produced, and characterized essentially as previously described (47, 68). The titer of the recombinant VV was analyzed by plaque assay on BSC-1 cells and expressed as PFU/ml.

Immunization procedures and recombinant vaccinia challenge. For all immunization procedures, mice received 3 mg of medroxy-progesterone acetate (Depo-Provera, Pfizer) subcutaneously in a volume of 100 μl PBS 5 days prior to vaccination. Mice were immunized with HPV PsV Ivag as previously described (34, 37). Briefly, 5 hours before immunization, the vaginal epithelium was disrupted by instillation of a gel containing 4% N-9 (Conceptrol, Ortho Options) in a 50-μl volume. Mice were immunized with 20 μl of HPV PsV diluted in 2% carbosymethyl cellulose gel (medium viscosity, 400–800 cP at 25°C; Sigma-Aldrich).

For intranasal immunization, mice were pretreated in each nostril with 10 μl of 0.2% N-9 (Spectrum) diluted in PBS and 5 hours later received a volume of 5 μl HPV PsV (5 × 106 IU) in each nostril. For i.m. immunization, the Ad5 vector (1 × 109 or 5 × 107 PFU) was diluted in 50 μl PBS and injected in the quadriceps muscle.

For prime/boost mucosal immunization, mice were primed with 5 × 106 IU of HPV16 PsV and boosted 4 weeks later with 5 × 106 IU of HPV45 PsV in order to overcome type-specific antibody-mediated neutralization. For the single immunization regimen, mice were immunized with 1 × 106 IU of HPV16 PsV.

For Ivag recombinant vaccinia challenge, mice were given Ivag 1 × 107 PFU of recombinant VV diluted in 20 μl of 2% carbosymethyl cellulose, and 3 days after challenge the viral load was assessed in a vaginal homogenate by plaque assay and expressed as PFU per organ.

Preparation of cell suspensions. Mice were euthanized and organs were removed by dissection. Vaginal and lung cell suspensions were obtained by enzymatic dispersion in RPMI containing 2% FBS, collagenase (0.5 mg/ml; Roche Diagnostics), and DNase I (0.1 mg/ml; Roche Diagnostics) for 1 hour at 37°C while shaking. Vaginal cells were passed through a 70-μm cell strainer (BD). Lymph node and spleen cell suspensions were obtained after 20 minutes enzymatic dispersion at 37°C and were mechanically dispersed by teasing the organs on a 70-μm cell strainer (BD). Vaginal, spleen, and lymph node cell suspensions were washed in RPMI/FBS 2% and were freed from erythrocytes by treatment with ammonium chloride solution (ACK, Lonza).

Antibodies and tetramers. The following anti-mouse antibodies were used for cell surface staining for flow cytometry analysis: anti-NKp46 (29A1.4), anti-IA/IE (M5/114), anti-CD3 (clone 145-2C11), anti-PDCA1 (clone 927), anti-CD8 (clone 53-6.7), anti-CD103 (2E7), and anti-CD4 (RM4-5 and RM4-4) from BioLegend, anti-CD127 (SB/199, eBioscience), and anti-CD11c (HL3, BD Biosciences). APC-conjugated M2Δ41-50/H2-Kd-tetramer (KdM2Δ41-tetramer) was obtained from the NIH Tetramer Core Facility (Emory University).

Cell surface staining. All staining was performed in 96-well plates in a final volume of 100 μl PBS/2% FBS (FACS buffer) for 30 minutes at 4°C. To avoid nonspecific antibody binding through surface Fc receptor, all cells were preincubated with CD16/32 purified antibody (2.4G2, BioXcell). Samples were acquired directly from 96-well plates on a FACSCanto II flow cytometer with the HTS module (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

In vitro peptide stimulation and intracellular cytokine staining. Spleen, lymph node, and vaginal cells were incubated in RPMI/FBS 10%, 1-glutamine, sodium pyruvate, and β-mercaptoethanol supplemented with 1 μg/ml of Brefeldin A (GoGiPlug, BD Bioscience) for 5 hours at 37°C/5% CO2 in the
presence of M2Δαβ-90 peptide (GenScript) at 5 μg/mL in medium only or with PMA (5 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) as a positive control. Cells were washed and surface stained with anti-CD3, anti-CD4, and anti-CD8 antibodies, then fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) before staining for intracellular cytokine content using anti–TNF-α (MP6-XT22, BD Biosciences), anti–IL-2 (JES6-5H4, BioLegend), and anti–IFN-γ (XMG1.2, BD Biosciences) antibodies. Stained cells were analyzed by flow cytometry.

**Immunofluorescence confocal microscopy analysis.** Cervicavaginal tracts were dissected, washed in PBS, and snap frozen in tissue freezing medium (Tissue-Tek OCT, Sakura). Six-micrometer tissue cryosections were transferred onto glass slides and fixed for 10 minutes in cold 100% ethanol. Fixed tissue sections were kept at 4°C for at least 2 weeks. Tissues sections were blocked with CD16/CD32 antibody (2.4G2, BioXcell) and normal donkey serum, then stained with purified rabbit laminin 332 antibody (Abcam), followed by a second-step incubation with donkey anti-rabbit IgG–Alexa Fluor 594 (51), anti-CD8–Alexa Fluor 488 (BioLegend), and anti-CD3–Alexa Fluor 647 (BioLegend) diluted in 0.1% Brij58. Alternatively, sections were stained with CD103 or Armenian hamster IgG isotype control antibodies (BioLegend), followed by a second-step incubation with CD8–Alexa Fluor 488 and anti-Armenian hamster IgG–Alexa Fluor 594 (BioLegend). Confocal images were acquired at the Confocal Microscopy Core Facility, Center for Cancer Research, National Cancer Institute, NIH, with Zeiss ZEN software on a Zeiss LSM 510 Confocal system with a Zeiss Observer Z1 inverted microscope and 50-mW argon UV laser tuned to 364 nm, a 25-mW argon visible laser tuned to 488 nm, a 1-mW HeNe laser tuned to 543 nm, and a 5-mW HeNe laser tuned to 633 nm. A x40 Plan-Neofluar 1.3 NA oil immersion objective was used. Emission signals after sequential excitation of each fluorochrome were collected with a BP 385–470 filter, BP 505–530 filter, BP 560–615 filter, or LP 650 filter using individual photomultipliers. Images were analyzed using LSM image browser (Zeiss).

In vivo CD4+ T cell depletion. Mice were depleted of CD4+ T cells by i.p. injection of 100 μg anti-CD4 mAb (GK1.5, BioXcell) on days –3, –1, 2, 4, and 6 with respect to day 0 of immunization. Control mice were injected with a rat IgG2b isotype control mAb (LTF-2, BioXcell).

In vivo FTY720 treatment. Mice were treated with FTY720 (Cayman Chemical) in drinking water (4 μg/mL) from the day of immunization until the end of the experiment. In some experiments, FTY720 was administered during a single immunization regimen as described previously (42). For the prime/boost immunization regimen, FTY720 was administered at the time of boost only.

In vivo BrdU incorporation assay. Mice were given BrdU (Sigma-Aldrich) in drinking water at a concentration of 0.8 mg/mL. Drinking water was changed daily to overcome BrdU degradation. Intracellular staining for BrdU was performed using a BrdU Flow Kit (BD Biosciences) after surface staining for KI67/M2Δαβ-tetramer CD8+ T cells.

In vivo CTL assay. The in vivo CTL assay was performed as previously described (69, 70). Briefly, spleen cells from a naive BALB/c mouse were split into two fractions. The first fraction was labeled with 4 μM CFSE (CFSEβ) and pulsed with 1 μM RSV M2Δαβ-90 peptide (GenScript), and the other fraction was labeled with 0.4 μM CFSE (CFSEγ) and remained unpulsed. Equal numbers of cells from CFSEβ and CFSEγ fractions were pooled and were rested as for target cells. A total of 1.5 × 105 or 2 × 106 target cells were injected intravenously or directly in the vaginal mucosa, respectively. One day after transfer of the target cells, specific CTL activity was measured by flow cytometry in cell suspensions from the spleen, ILN, and cervicavaginal mucosa. Specific lysis was calculated as follows: (1 – [ratio of CFSEβ/CFSEγ cells in control mice divided by ratio of CFSEβ/CFSEγ cells in immunized mice]) × 100.

**Statistics.** The non-parametric Mann-Whitney U test was used for comparisons in experiment of 2 independent groups. For experiments involving more than 2 groups, ANOVA by Kruskal-Wallis followed by Dunn’s post-test was performed for selected pairwise comparisons. A P value less than or equal to 0.05 was considered significant.

**Study approval.** All animal experiments and procedures were carried out in accordance with National Cancer Institute guidelines and under NCI Animal Care and Use Committee–approved protocols.

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