A vaccine strategy that protects against genital herpes by establishing local memory T cells

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Most successful existing vaccines rely on neutralizing antibodies, which may not require specific anatomical localization of B cells. However, efficacious vaccines that rely on T cells for protection have been difficult to develop, as robust systemic memory T-cell responses do not necessarily correlate with host protection1. In peripheral sites, tissue-resident memory T cells provide superior protection compared to circulating memory T cells2,3. Here we describe a simple and non-inflammatory vaccine strategy that enables the establishment of a protective memory T-cell pool within peripheral tissue. The female genital tract, which is a portal of entry for sexually transmitted infections, is an immunologically restrictive tissue that prevents entry of activated T cells in the absence of inflammation or infection4. To overcome this obstacle, we developed a vaccine strategy that we term ‘prime and pull’ to establish local tissue-resident memory T cells at a site of potential viral exposure. This approach relies on two steps: conventional parenteral vaccination to elicit systemic T-cell responses (prime), followed by recruitment of activated T cells by means of topical chemokine application to the restrictive genital tract (pull), where such T cells establish a long-term niche and mediate protective immunity. In mice, prime and pull protocol reduces the spread of infectious herpes simplex virus 2 into the sensory neurons and prevents development of clinical disease. These results reveal a promising vaccination strategy against herpes simplex virus 2, and potentially against other sexually transmitted infections such as human immunodeficiency virus.

Viral sexually transmitted infections (STIs) such as human immunodeficiency virus 1 (HIV-1) and herpes simplex virus 2 (HSV-2) account for considerable morbidity and mortality around the world. Strong preclinical evidence for the role of T cells in controlling viral STIs has led to the design of prophylactic vaccines that elicit systemic cellular immunity, and yet these vaccines have not been efficacious5,6. Although systemic memory T cells can migrate freely through organs such as the spleen and liver, other parts of the body such as the intestines, lung airways, central nervous system, skin and vagina are restrictive for memory T-cell entry5. In the latter tissues, inflammation or infection is often required to permit entry of circulating activated T cells to establish a tissue-resident memory T-cell pool that is separate from the circulating pool5,6. Given that side effects of inflammation in the reproductive tissue may preclude the use of a live prophylactic vaccine given vaginally, we investigated an alternative approach to recruit virus-specific T cells into the vaginal mucosa without inducing local inflammation or infection.

After genital HSV-2 infection, chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL10 expression is induced by interferon-γ secreted by CD4+ T cells and mediates recruitment of effector CD8+ T cells to the infected tissue via the chemokine receptor CXCR3 (ref. 4). CXCR3 is expressed by both effector T-helper 1 (Tem1) cells and activated CD8+ T cells, as well as other cell types5. Thus, we proposed that the topical application of chemokines CXCL9 and CXCL10 might be sufficient to recruit effector T cells to the vagina in the absence of infection. To test this hypothesis we used T-cell antigen receptor transgenic CD8+ T cells that recognize an epitope within the HSV glycoprotein B (gBT-1)6 to track the HSV-2 specific CD8 T-cell population. Naïve female C57BL/6 mice were transplanted with 106 congenically marked gBT-1 CD8+ T cells and immunized subcutaneously with an attenuated strain of HSV-2 that lacks thymidine kinase (TK− HSV-2)7 (Fig. 1a). As expected, this route of immunization resulted in minimal migration of activated CD8+ T cells into the vagina (Fig. 1b, c). To recruit or ‘pull’ activated HSV-specific CD8+ T cells, the chemokines CXCL9 and CXCL10 were topically applied to the vaginal cavity of subcutaneously immunized mice (Fig. 1a). Another group of mice was immunized intravaginally with TK− HSV-2, which served as a positive control for maximal CD8+ T-cell recruitment to the vagina (Fig. 1b, c). At day 6 post infection, all three treatment groups exhibited primary CD8+ T-cell responses of similar magnitudes, as indicated by the numbers and percentages of systemic gBT-1 CD8+ T cells found in the spleen (Fig. 1b, c). However, the number and percentage of gBT-1 CD8+ T cells in the vagina were significantly higher in mice treated with the chemokine pull (subcutaneous immunization plus pull) compared to the control subcutaneously immunized mice (Fig. 1b, c). Furthermore, the action of the chemokine pull was restricted to the genital mucosa, as gBT-1 CD8+ T-cell recruitment to the vagina-draining iliac lymph nodes was limited (Fig. 1c). Activated CD4+ T cells were also strongly recruited to the vagina by the chemokine pull (Fig. 1d). Antigen in the vagina was not responsible for the recruitment, as HSV-2 genomic DNA was absent from the genital tract after subcutaneous immunization (Supplementary Fig. 1). To mimic a vaccination scenario more closely, we also tested whether endogenous virus-specific T cells could be recruited by prime and pull. Like gBT-1 CD8+ T cells, the systemic endogenous HSV-specific CD8+ T-cell response was similar in all immunized groups (Supplementary Fig. 2a). However, significantly greater numbers of HSV-specific CD8+ T cells as well as CD4+ T cells were present in the genital tracts of mice treated with the chemokine pull as compared to subcutaneous immunization alone (Supplementary Fig. 2). Thus, these data show that the prime and pull method is capable of recruiting a large number of parenterally primed T cells to the genital tract with a single topical application of chemokines.

To assess the possible inflammatory consequences of topical chemokine application to the vagina, we examined the presence of innate inflammatory cells after the chemokine pull. Other cell types, including natural killer cells and plasmacytoid dendritic cells (pDCs) express CXCR3 (ref. 9). However, no significant increase in the number of pDCs, natural killer cells, granulocytes, dendritic cells, monocytes, macrophages and monocyte-derived dendritic cells was elicited by the chemokine treatment (subcutaneously immunized plus pull) compared to the subcutaneously immunized control (Supplementary Fig. 3). These data indicated that topical chemokines do not induce appreciable recruitment of natural killer cells or pDCs to the vagina and that effector T cells are selectively recruited during prime and pull without inducing a general inflammatory response.

During genital HSV infection, CD4+ T cells act as a pioneering population for the migration of virus-specific CD8+ T cells by inducing the
Figure 1 | Effector T cells are recruited to the vagina by topical chemokine treatment. a, Experimental schematic. Donor gBT-I CD8 T-cell recipients were not immunized (naive), or were immunized either intravaginally (ivag) or subcutaneously (s.c.) with TK HSV-2. Five days post infection, subcutaneously immunized mice were treated vaginally with either the chemokines CXCL9 and CXCL10 (pull) or PBS. b, The frequency of donor gBT-I CD8 T cells in the vagina 1 day post pull in the indicated tissues (ILN, iliac lymph nodes). Plots are gated on total CD8 T cells and numbers indicate per cent gBT-I (CD45.1+). c, The number of donor gBT-I CD8 T cells 1 day post pull in the indicated tissues. d, The number of CD4+ T cells 1 day post pull in the vagina. c, d, Numbers in graphs indicate fold difference in T-cell number for subcutaneously immunized compared with subcutaneously immunized plus pull. Statistical significance was determined by two-tailed unpaired Student’s t-test. Data are pooled from 2–7 independent experiments (n = 6–21 per group). All error bars show s.e.m. and *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P < 0.0001 throughout all figures (individual P values are found in the Supplementary Information).

production of critical chemokines within the tissue4. To determine whether the recruitment of gBT-I CD8+ T cells to the genital tract was similarly dependent on CD4+ T-cell help during prime and pull, subcutaneously immunized mice were injected with a CD4-depleting antibody on day 3 and day 5 post infection to preserve normal CD8+ T-cell priming12, and then treated with the chemokine pull (Supplementary Fig. 4a). In CD4+ T-cell-depleted mice (Supplementary Fig. 4b), both systemic gBT-I CD8+ T-cell numbers and migration to the vagina were unaffected (Supplementary Fig. 4c, d), indicating that recruitment of effector CD8+ T cells to the vagina after chemokine treatment bypasses the requirement for CD4+ T-cell help.

CXCR3 is upregulated on T cells upon activation and remains high through the effector and memory stages8. Having demonstrated that CXCL9 and CXCL10 could recruit CXCR3+ effector T cells to the vagina, we next examined the efficacy of the chemokine pull at different stages of T-cell priming. After subcutaneous TK HSV-2 immunization, CXCR3 was upregulated on both gBT-I CD8+ T cells and CD4+ T cells throughout the response (Fig. 2a), suggesting that both effector and memory T cells should be capable of responding to the chemokine pull. Previous reports have shown that early effector CD8+ T cells had an increased ability to migrate to peripheral tissues3, so we next determined whether the timing of chemokine pull dictated the efficacy of T-cell recruitment to the genital tract. When subcutaneously immunized mice were treated with the chemokine pull at the effector (day 5), contraction (day 15) and memory (day 28) phase of the T-cell response13, we found that the chemokine pull was most effective at recruiting antigen-specific CD8+ T cells during the effector (day 5) phase, which correlated with the increased number and percentage of systemic gBT-I CD8+ T cells (Fig. 2b). Despite similar CXCR3 expression (Fig. 2a), memory gBT-I CD8 T cells were not present in the tissue after pull when treated during the memory phase (day 28) (Fig. 2b). We speculate that this might be due to altered homing patterns14 and the reduced number and percentage of gBT-I CD8 T cells in circulation at the memory time point. Recruitment of CD44+CD44+ T cells

Figure 2 | Chemokine pull is specific for highly activated effector T cells. Mice were subcutaneously immunized and given chemokines or PBS at day 5, 15 or 28 post infection and analysed 1 day post pull. a, CXCR3 expression on donor gBT-I CD8+ T cells or CD44+CD4+ T cells from the spleen 1 day post pull from subcutaneously immunized mice (open black) and subcutaneously immunized plus pull (open red). Shaded histograms are CD44+CD8+ or total CD4+ T cells. b, The gBT-I CD8+ T cell number in the vagina (left) or spleen (middle) and frequency in the spleen (right) were examined 1 day post pull. c, The number of CD44+CD4+ T cells in the vagina 1 day post pull on the indicated days post infection. d, The number of endogenous CD44+CD8+ T cells in the vagina 1 day post pull on the indicated days post infection. b–d, Statistical significance was determined by two-tailed unpaired Student’s t-test. Data are pooled from 2–3 independent experiments; n = 6–18 per group.
(Fig. 2c) and endogenous CD8$^+$ T cells (Fig. 2d) followed a similar pattern. Collectively, these data indicate that the chemokine pull is most effective at recruiting recently activated effector CD8$^+$ T cells that are circulating at high frequency, establishing a specific time frame within which the chemokine pull should be administered after priming.

For the prime and pull approach to be an effective vaccine strategy, pathogen-specific T cells must be retained within the tissue for an extended time and establish a pool of memory cells. To determine whether the effector gBT-I CD8$^+$ T cells recruited into the vagina after prime and pull were capable of establishing a long-term population of memory CD8$^+$ T cells, we examined the presence of gBT-I CD8$^+$ T cells 4 weeks after the chemokine pull. The number of systemic memory gBT-I CD8$^+$ T cells, although decreased compared to day 1 post pull (Fig. 1c) due to contraction of the T-cell response, was similar regardless of immunization route or treatment (Fig. 3a). However, a significantly greater number and percentage of memory gBT-I CD8$^+$ T cells was present in the genital tract of subcutaneously immunized mice treated with the chemokine (subcutaneously immunized plus pull) compared to chemokine untreated mice (subcutaneously immunized) (Fig. 3a, b). Despite significant recruitment during the effector phase (Fig. 1d), CD4$^+$ T cells were not retained within the vagina long term (Fig. 3c), reminiscent of CD4$^+$ T-cell behaviour after dermal HSV-1 infection in which the cells leave the site of infection to mediate immunosurveillance. Thus, CD4$^+$ T cells may require additional signals, such as those generated during HSV-2 infection, to be retained long term within the vagina.

To investigate the stability of this tissue-resident population of memory gBT-I CD8$^+$ T cells, we also examined T-cell numbers at 12 weeks post pull. Donor gBT-I CD8$^+$ T-cell numbers in the vagina were significantly higher after prime and pull than after subcutaneous immunization alone (Fig. 3d). Furthermore, the number of memory gBT-I CD8$^+$ T cells did not decline between 4 weeks and 12 weeks (Fig. 3d), suggesting that this tissue-resident population was stable and retained long term. CD4$^+$ T-cell numbers in the vagina remained low at week 12 after prime and pull, and were comparable to numbers detected at week 4 (Fig. 3e). Thus, a single chemokine pull given to mice during the effector phase is sufficient to establish a long-term population of tissue-resident memory CD8$^+$ T cells, but not CD4$^+$ T cells, within the vagina.

Tissue-resident memory T cells are effective in mediating immunity against local infections. HSV-2 spreads from its initial replication site at the epithelium to the innervating neurons, and subsequently establishes latency within the dorsal root ganglia (DRG). Reactivation from latency leads to viral shedding and formation of genital lesions that are commonly associated with genital herpes. Thus, preventing the spread of virus from the mucosal epithelium to the DRG is key in preventing disease and transmission of the virus. As a single chemokine pull administered after subcutaneous immunization is capable of establishing a population of tissue-resident memory CD8$^+$ T cells within the vagina long term, we next examined whether the prime and pull strategy would provide enhanced immunity against genital HSV-2 infection. Mice were challenged intravaginally with a lethal dose of wild-type HSV-2 4 weeks after prime and pull and monitored for disease and survival. Notably, mice treated with the chemokine pull (subcutaneously immunized plus pull) lost significantly less weight than either the non-immunized or subcutaneously immunized controls (Fig. 4a). Furthermore, prime and pull almost completely prevented the development of clinical symptoms, which were observed in both non-immunized and subcutaneously immunized controls (Fig. 4b). Accordingly, mice treated with the chemokine pull had a 100% survival rate compared to the 36.3% survival rate of the subcutaneously immunized control (Fig. 4c).

Upon challenge with wild-type HSV-2 4 weeks post pull, mice immunized and chemokine-treated in the absence of T-cell antigen receptor transgenic CD8$^+$ T cells were also significantly protected from weight loss (Supplementary Fig. 5a) and clinical disease (Supplementary Fig. 5b), although we did not observe a significant difference in survival rate (Supplementary Fig. 5c). Anti-HSV antibody titres in the vagina were not significantly different between subcutaneously immunized controls and chemokine-treated mice (Supplementary Fig. 6), suggesting that the control of viral challenge was
Our study demonstrates that after conventional vaccination to generate a systemic T-cell population (prime), a single topical treatment with chemokines applied vaginally (pull) can provide superior protection against genital herpes by decreasing the spread of virus from the mucosal epithelia into the neurons. Importantly, protection of neurons from HSV-2 infection by prime and pull may decrease reactivation and viral shedding, which may reduce disease and transmission. Although the exact role of T cells in controlling neuronal HSV-2 infection after prime and pull is not yet clear, we speculate that the local HSV-specific T cells may help to control entry of virus at the neuronal endings, or promote blockade of viral replication once inside the neurons. Furthermore, other studies have demonstrated that T cells recruited to the genitral tract by inflammation alone can decrease viral replication at the mucosal surface\textsuperscript{19}, suggesting that control of infection at the site of entry may be possible by optimizing prime and pull. Thus, in addition to preventing reactivation of latent HSV\textsuperscript{20}, virus-specific memory T cells may be mobilized to control neuronal viral infection during primary infection. Although topical application in the genital tract of Toll-like receptor ligands such as imiquimod have been shown to be effective as a therapeutic approach\textsuperscript{21}, they may not be ideal vaccine candidates as they seem to be effective for only a short time after application and function through the induction of pro-inflammatory cytokines\textsuperscript{22}. The novel prime and pull vaccine strategy described here provides an alternative to direct immunization of the genital tract, and establishes robust, long-term immunity with minimal local inflammation.

Cellular immunity is critical in mediating protection against viral STIs such as HSV-2 and HIV-1 (ref. 23). Both viruses enter through the genital mucosa, begin local replication and then spread to other tissues\textsuperscript{23}. Although our data highlight the role of prime and pull in controlling viral spread to the peripheral nervous system, this vaccination approach is not necessarily restricted to neurotropic viruses. HIV-1 enters the genital mucosa and invades the draining lymph node, from which systemic dissemination of the virus occurs\textsuperscript{23}. In its current form, prime and pull establishes tissue-resident memory CD8\textsuperscript{+} T cells but not CD4\textsuperscript{+} T cells. Given that a single HIV-1 virion can establish infection in humans\textsuperscript{23}, local memory CD8\textsuperscript{+} T cells may be key to protection against HIV-1 (ref. 23) by reducing replication and dissemination of the founder virus, while the absence of local CD4\textsuperscript{+} T cells could limit the availability of immediate target cells. Beyond viral infections, prime and pull could be applied to improve recruitment of immune cells to other restrictive microenvironments such as solid tumours. Effective immunotherapy can be hindered by either decreased or inappropriate expression of chemokines at the tumour tissue, leading to minimal migration of immune cells\textsuperscript{24}. Delivery of appropriate chemokines to the tumour tissue after immunization could enhance recruitment of tumour-specific T cells and augment the efficacy of immunotherapies. Although the protocol we present pairs the pull with a suboptimal subcutaneous immunization, we propose that the prime and pull strategy could be used in conjunction with more effective immunizations\textsuperscript{5} to enhance protection. Ultimately, the ability to boost recruitment of T cells and establish resident T-cell populations in peripheral tissues restrictive for lymphocyte homing will aid not only in the prevention but also in the treatment of a wide variety of diseases.

**METHODS SUMMARY**

Adoptive transfers, infections and T-cell depletion. CD8\textsuperscript{+} T cells (10\textsuperscript{6}) from the spleens of naïve CD45.1\textsuperscript{+} gB\textsuperscript{T-} TCR transgenic mice\textsuperscript{25} were adoptively
transferred into Depo-Provera (GE Healthcare) treated25, naive 6-week-old C57BL/6 recipients (National Cancer Institute). Recipients were immunized intravaginally or subcutaneously in the neck ruff with 10^5 or 10^6 plaque forming units (PFU) of 186TKΔkpn HSV-2 (TK- HSV-2)Δg, respectively. Some mice were treated twice with 200 μg anti-CD4 antibody (GK1.5) intraperitoneally to deplete CD4^+ T cells. Five days post-immunization, subcutaneously immunized mice were vaginally swabbed with a Calginate swab (Fisher) and either PBS or a solution of CXCL9 and CXCL10 (3 μg each, Peprotech) in PBS was delivered via pipette tip into the vagina. For 4-week challenges, mice were infected intravaginally with 5,000 PFU of wild-type HSV–2 186 syn+ (ref. 26). For 10–12 week challenges, mice were treated again with Depo-Provera 9–10 weeks before challenge.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Mice. Female 6-week-old C57BL/6 mice were purchased from the National Cancer Institute. gBT-I T cell antigen receptor (TCR) transgenic mice specific for the glycoprotein B epitope gB(498–505) were provided by F. R. Carbone and W. R. Heath and bred in our facility to C57BL/6-Ly5.2Cr mice (CD45.1⁺). All procedures used in this study complied with federal and institutional policies of the Yale Animal Care and Use committee.

Adoptive transfers and infections. Spleens were collected from naïve CD45.1⁺ gBT-I TCR transgenic mice and CD8⁺ T cells were magnetically purified by CD8x microbeads or CD8²⁺ T-cell isolation kits (Miltenyi Biotec). Donor cells (10⁵) gBT-I CD8⁺ T cells were adaptively transferred into Depo-Provera-treated (GE Healthcare), 7–8-week-old C57BL/6 recipients retro-orbitally. Mice were then immunized intravaginally or subcutaneously with 5,000 PFU wild-type HSV-2 (TK⁻ HSV-2) respectively. At 5 days post-infection, the vaginal cavity of mice was swabbed with a Calginate swab (Fisher) and either PBS or a solution of CXCL9 and CXCL10 (3 μg each, Peprotech) in PBS was delivered via pipette tip into the vagina. Where indicated, C57BL/6 mice that did not receive gBT-I cells were primed and pulled in a similar manner. Some subcutaneously immunized mice were intraperitoneally injected with 200 μg anti-CD4 (GK1.5) antibody at day 3 and 5 post infection to deplete CD4⁺ T cells. For the 4-week challenge, non-immunized or previously immunized mice at the indicated time points were infected intravaginally with 5,000 PFU wild-type HSV-2 186 syn⁻. Challenges given at 10–12 weeks post pull were treated with Depo-Provera for a second time 1–2 weeks post pull (9–10 weeks before challenge) before infection with 5,000 PFU wild-type HSV-2 186 syn⁻.

Flow cytometry. At various time points, single cell suspensions from the spleen, lungs, vagina and iliac lymph nodes were prepared for analysis as described. Briefly, lungs were digested with collagenase D (Roche). Vaginas were treated with Dispase (Stratagene). DNA purified from TK⁺ HSV-2 (TK⁻ HSV-2) respectively. At 5 days post-infection, the vaginal cavity of mice was swabbed with a Calginate swab (Fisher) and either PBS or a solution of CXCL9 and CXCL10 (3 μg each, Peprotech) in PBS was delivered via pipette tip into the vagina. Where indicated, C57BL/6 mice that did not receive gBT-I cells were primed and pulled in a similar manner. Some subcutaneously immunized mice were intraperitoneally injected with 200 μg anti-CD4 (GK1.5) antibody at day 3 and 5 post infection to deplete CD4⁺ T cells. For the 4-week challenge, non-immunized or previously immunized mice at the indicated time points were infected intravaginally with 5,000 PFU wild-type HSV-2 186 syn⁻. Challenges given at 10–12 weeks post pull were treated with Depo-Provera for a second time 1–2 weeks post pull (9–10 weeks before challenge) before infection with 5,000 PFU wild-type HSV-2 186 syn⁻.

Measurement of viral titres, weight and disease scores. Vaginal secretions were collected 5 days post challenge using PBS and Calginate swabs. Lumbar and sacral dorsal root ganglia (DRG) were collected at days 6–7 post challenge as described. DRG were homogenized using a motorized pestle (VWR). Titres from vaginal and DRG samples were measured on Vero cell monolayers as previously described. Weight loss was measured daily and normalized to body weight on day 0 of challenge. Disease was monitored daily and scored as follows: (0) no disease; (1) genital inflammation; (2) genital lesions and hair loss; (3) hunched posture and ruffled fur; (4) hind limb paralysis; and (5) premorbid. Mice were euthanized before reaching the moribund state due to humane concerns.

Detection of HSV-2 antigen by quantitative PCR. Mice were immunized subcutaneously or intravaginally and were killed at day 5 post infection. Vaginal tissue was collected and genomic DNA was extracted as previously described. Briefly, tissue was homogenized in a salt homogenizer buffer using a motorized pestle. Proteinase K and SDS were added to samples and incubated overnight at 55 °C. After addition of a sodium chloride solution, samples were centrifuged and supernatants were transferred to new tubes. Isopropanol was added to the supernatants and incubated at 20 °C for 1 h. DNA was pelleted by centrifugation, washed with ethanol and resuspended in H₂O. HSV-2 was measured with primers detecting glycoprotein B (gB) (Forward: 5’-AGACCAGGGCGCGCTGATC-3’; reverse: 5’-GGGCTGGAGGCTGCTGTAG-3’). Quantitative polymerase chain reaction (Stratagene) DNA purified from TK⁻ HSV-2 was used as standard to calculate PFU equivalents. Measurement of HSV-specific antibody titres. Vaginal secretions were collected from mice with PBS and Calginate swabs 4 weeks post pull. HSV-specific immunoglobulin-G (IgG) was measured by ELISA assay as previously described. Known quantities of anti-HSV gB monoclonal antibody (SS10 mouse IgG) kindly provided by G. Cohen and R. Eisenberg was used as a standard.

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