

Toll-like Receptor Agonist Imiquimod Facilitates Antigen-Specific CD8⁺ T-cell Accumulation in the Genital Tract Leading to Tumor Control through IFN γ

Ruey-Shyang Soong^{1,2,3}, Liwen Song^{1,4,5,6}, Janson Trieu⁷, Jayne Knoff¹, Liangmei He¹, Ya-Chea Tsai¹, Warner Huh⁸, Yung-Nien Chang⁹, Wen-Fang Cheng¹⁰, Richard B.S. Roden^{1,11,12}, T.-C. Wu^{1,11,12,13}, Cornelia L. Trimble^{1,11,12}, and Chien-Fu Hung^{1,12}

Abstract

Purpose: Imiquimod is a Toll-like receptor 7 agonist used topically to treat external genital warts and basal cell carcinoma. We examined the combination of topical imiquimod with intramuscular administration of CRT/E7, a therapeutic human papillomavirus (HPV) vaccine comprised of a naked DNA vector expressing calreticulin fused to HPV16 E7.

Experimental Design: Using an orthotopic HPV16 E6/E7⁺ syngeneic tumor, TC-1, as a model of high-grade cervical/vaginal/vulvar intraepithelial neoplasia, we assessed if combining CRT/E7 vaccination with cervicovaginal deposition of imiquimod could result in synergistic activities promoting immune-mediated tumor clearance.

Results: Imiquimod induced cervicovaginal accumulation of activated E7-specific CD8⁺ T cells elicited by CRT/E7 vaccination. Recruitment was not dependent upon the specificity of the activated CD8⁺ T cells, but was significantly reduced in mice lacking the IFN γ receptor. Intravaginal imiquimod deposition induced upregulation of CXCL9 and CXCL10 mRNA expression in the genital tract, which are produced in response to IFN γ receptor signaling and attract cells expressing their ligand, CXCR3. The T cells attracted by imiquimod to the cervicovaginal tract expressed CXCR3 as well as CD49a, an integrin involved in homing and retention of CD8⁺ T cells at mucosal sites. Our results indicate that intramuscular CRT/E7 vaccination in conjunction with intravaginal imiquimod deposition recruits antigen-specific CXCR3⁺ CD8⁺ T cells to the genital tract.

Conclusions: Several therapeutic HPV vaccination clinical trials using a spectrum of DNA vaccines, including vaccination in concert with cervical imiquimod, are ongoing. Our study identifies a mechanism by which these strategies could provide therapeutic benefit. Our findings support accumulating evidence that manipulation of the tumor microenvironment can enhance the therapeutic efficacy of strategies that induce tumor-specific T cells. *Clin Cancer Res*; 20(21); 5456–67. ©2014 AACR.

Introduction

Human papillomaviruses (HPV) are the primary etiologic agents for cervical cancer and subsets of vaginal, vulvar, and anal cancers and head and neck cancers (1). Approximately 90% of vaginal, vulvar, and anal cancers associated with

HPV are attributable to HPV16 (1). Although surgical treatment is effective and well tolerated for precancer and early cancer lesions of the cervix, a subset of treated patients are at risk for preterm delivery and/or cervical incompetence and premature rupture of membranes (2). Surgical treatment of

¹Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland. ²Department of General Surgery, Chang Gung Memorial Hospital at Keelung, Keelung City, Taiwan. ³College of Medicine, Chang Gung University, Taoyuan, Taiwan. ⁴School of Pharmacy, Fudan University, Shanghai, China. ⁵Department of Pharmacology and Toxicology, Shanghai Institute of Planned Parenthood Research, Shanghai, China. ⁶Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital of Tongji University, Shanghai, China. ⁷Johns Hopkins University, Baltimore, Maryland. ⁸Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama. ⁹Papivax LLC, Severna Park, Maryland. ¹⁰Department of Obstetrics and Gynecology, National Taiwan University College of Medicine, Taipei, Taiwan. ¹¹Department of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, Maryland. ¹²Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland. ¹³Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, Maryland.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

R.-S. Soong and L. Song contributed equally to this article.

C.L. Trimble and C.-F. Hung share senior authorship.

Corresponding Authors: Chien-Fu Hung, The Johns Hopkins University School of Medicine, 1550 Orleans Street, Baltimore, MD 21231. Phone: 410-502-8215; Fax: 443-287-4295; E-mail: chung2@jhmi.edu; and T.-C. Wu, Phone: 410-614-3899; Fax: 443-287-4295; E-mail: wutc@jhmi.edu

doi: 10.1158/1078-0432.CCR-14-0344

©2014 American Association for Cancer Research.

Translational Relevance

Peripheral therapeutic human papillomavirus (HPV) vaccination in combination with direct application of imiquimod on preinvasive lesions is under investigation clinically. Here, we used an orthotopic HPV16 E6/E7⁺ syngeneic murine tumor model, TC-1, to test if imiquimod enhanced the recruitment of antigen-specific CD8⁺ T cells generated by a naked DNA vaccine to the cervicovaginal tract. We found that imiquimod induced cervicovaginal accumulation of activated E7-specific CD8⁺ T cells elicited by DNA vaccination with a naked vector expressing calreticulin fused to HPV16 E7 (CRT/E7). Intravaginal imiquimod deposition led to accumulation of T cells in the cervicovaginal tract, which exhibited upregulation of the expression of the chemokine receptor CXCR3, and the tissue resident memory T cell marker CD49a. Our study has high translational relevance because imiquimod is currently FDA approved for treatment of both condyloma accuminata and basal cell carcinoma, and two therapeutic HPV vaccination clinical trials using CRT/E7 DNA vaccine are ongoing. Our findings support accumulating evidence that manipulation of the tumor microenvironment can enhance the therapeutic efficacy of strategies that induce tumor-specific T cells.

HPV-related vaginal, vulvar, and anal intraepithelial lesions, particularly multicentric and recurrent lesions, is associated with significant morbidity and high recurrence rates (3, 4). As such, it is important to develop effective alternative therapeutic methodologies to treat HPV-associated precancer lesions and cancers, particularly for the noncervical sites that are predominantly associated with HPV16.

Immunotherapy can potentially be used to treat HPV-associated disease. HPV oncoproteins E6 and E7 are required for the induction and maintenance of cellular transformation and are consistently coexpressed in all HPV-infected, but not normal cells. Therapeutic vaccines, through activation of the adaptive immune system, can elicit a specific immune response to target precancer and cancer cells while sparing normal cells (for review see ref. 5), particularly if targeting nonself proteins such as viral antigens. A long peptide-based therapeutic vaccine targeting HPV16 E6 and/or E7 showed partial efficacy against HPV16⁺ high-grade vulvar intraepithelial neoplasia (VIN; ref. 6). Although the E6 and E7 viral oncoproteins are weak antigens in the context of naturally elicited responses, vaccine-induced T-cell responses are capable of eliminating a subset of preinvasive lesions. Thus, approaches are needed to render therapeutic vaccination targeting HPV more immunogenic and effective in humans.

DNA vaccines targeting E6/E7 are promising because the antigen is produced intracellularly and can therefore enter the MHC class I pathway that is critical for the induction of cytotoxic T-cell responses. To circumvent the poor immu-

nogenicity of the HPV oncoproteins, we have developed an approach to enhance their immune presentation upon expression from a naked DNA vector. In this therapeutic HPV DNA vaccine, HPV16 E7 is linked to calreticulin (CRT/E7), a heat shock-related chaperone protein that enhances antigen processing and directs binding to, and activation of antigen-presenting cells (7). Vaccination of mice by intramuscular administration of CRT/E7 DNA generated potent systemic cell-mediated immune responses against HPV16 E7 and subcutaneous TC-1 tumor, a syngeneic model of advanced cervical cancer that expresses HPV16 E6 and E7 (8–17). A clinical grade version of this vaccine, pNGVL4a-CRT/E7 (detox), is being tested in patients with HPV16⁺ high-grade cervical intraepithelial neoplasia (CIN2/3), using different routes of administration (NCT00988559; ref. 18).

Studies of the naturally occurring systemic immune responses of patients with high-grade CIN indicate that HPV16-specific T-cell responses in peripheral blood did not predict lesion regression (19). Furthermore, in a phase I trial of a therapeutic HPV vaccine, systemic HPV-specific T-cell responses were again not correlated with lesion clearance (20). In contrast, evidence of local responses at the lesion site was associated with disease clearance, suggesting the importance of both eliciting an HPV immune response and appropriately targeting it to the lesion site, possibly by altering the local immune microenvironment (21). Therapeutic HPV vaccination in combination with imiquimod has been tested clinically in the setting of VIN (22), and is currently being tested in subjects with HPV16-associated CIN2/3 (NCT00788164; ref. 50). Because DNA vaccination reliably elicits detectable systemic immune responses to vaccine antigen in mice, we used the murine TC-1 model to determine if imiquimod could enhance the localization of induced antigen-specific T cells to the site of the lesion.

Application of an adjuvant locally can further enhance site-specific immunity. For example, in mice, intravaginal administration of the nontoxic B subunit of cholera toxin (CTB) linked to the model antigen ovalbumin (OVA) increased OVA-specific CD8⁺ T cells in the draining lymph nodes and genital mucosa (23). In addition, vaccination of non-human primates using Toll-like receptor (TLR) agonists as adjuvants generated stable antigen-specific CD8⁺ T cells (24). Finally, in a model in which OVA was expressed as a transgene on skin grafts, and grafted onto naïve recipients, the effector activity of adoptively transferred OVA-specific T cells was potentiated by application of imiquimod on the grafts (25).

Treatment of human squamous cancers with imiquimod has been shown to induce immunologic destruction of cancer cells (26). Treated tumors contained dense T-cell infiltrates colocalized with tumor cell apoptosis and tumor regression. In contrast, treatment of normal human skin induced activation of resident T cells, but had no effect on IFN γ , perforin, or granzyme, suggesting that the T cells infiltrating the squamous cancers were recruited from blood. Nonspecific immunotherapy by topical application

of imiquimod to HPV16+ high-grade VIN has shown significant therapeutic activity, but was less effective against large lesions (27). In addition, the combination of imiquimod with a therapeutic HPV fusion protein vaccine has been shown to increase the number of CD4⁺ and CD8⁺ T cells in VIN lesions (22).

Imiquimod activates the innate immune response through TLR7-MyD88-dependent signaling, by interacting with TLR7 expressed by myeloid dendritic cells (DC), plasmacytoid DCs, monocytes, and macrophages (28). TLR7 activation induces secretion of various proinflammatory cytokines and also enhances DC maturation and antigen presentation (28). Furthermore, TLR7 ligands, including imiquimod, have been shown to lead to plasmacytoid DC activation and generate a curative effect against a breast tumor model and a melanoma model (29, 30). In particular, imiquimod stimulates IFN γ production (31). IFN γ in turn induces the production of CXCL9 and CXCL10, chemokines that attract CXCR3⁺ T cells (for review see ref. 32). These studies suggest that the use of local adjuvants, such as imiquimod, may enhance recruitment of vaccine-induced T cells. In the current study, we examined whether deposition of imiquimod cream in the cervicovaginal tract following intramuscular CRT/E7 DNA vaccination could enhance attraction of the systemic E7-specific cellular immune response to the genital tract and therein facilitate the cure of mice bearing syngeneic TC-1 tumors implanted in the vaginal wall, a model of HPV16⁺ epithelial cancer.

Materials and Methods

Experimental mice and tumor cells

Six- to 8-week-old female C57BL/6 mice were purchased from the NCI. IFN γ receptor 1-deficient (*ifngr1^{tm1Agt}*) mice (IFN γ R^{-/-}) and CXCR3-deficient (B6.129P2-*Cxcr3^{tm1Dgen/J}*) mice (CXCR3^{-/-}) have been previously described (The Jackson Laboratory; ref. 33). Mice were housed in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

The tumor cell line used in our study was TC-1. TC-1 cells were subjected to RapidMAP (Taconic Farms) testing, a panel of PCR tests for rodent viruses, most recently in May 2011 with negative results. To trace the tumor growth *in vivo*, TC-1 transfected with Luciferase (TC-1-Luc) was generated by lentivirus stable transfection as previously described (34). Cells were cultured in RPMI 1640 medium containing 10% FBS, 2 mmol/L L-glutamine, 10% sodium pyruvate, 10% nonessential amino acids, and 100 pg/mL streptomycin in a humidified atmosphere of 5% CO₂/95% air at 37°C.

Luciferase-expressing E7-specific CD8⁺ T cell and OT-1 T-cell preparation and adoptive transfer in mice

E7-specific T cells were generated from splenocytes of E7-vaccinated mice and were stimulated with irradiated TC-1

cells and 10 IU IL2 weekly. To trace the E7-specific CD8 T cells *in vivo*, we have previously generated E7-specific cytotoxic T cells expressing luciferase (E7-Luc T cell; ref. 34). OT-1 T cells were generated from OT-1 Rag^{-/-} TCR transgenic mice from our laboratory as previously mentioned (35). T-cell adoptive transfer was performed by injecting 5 × 10⁵ T cells in 200 μ L PBS through the tail veins of mice.

DNA vaccine and drug treatments

The pNGVL4a-CRT-E7 (detox) plasmid was prepared by the NIH Rapid Access to Interventional Development (RAID) program. Imiquimod (Aldara) 5% cream was purchased from Taro Pharmaceutical Industries, Ltd. Mice were injected in the tibialis muscle of the shaved hind leg with a 28G syringe containing 40 μ g of DNA plasmid diluted in a total volume of 40 μ L of PBS. Each leg was injected with 20 μ g DNA vaccine. After the mice were under anesthesia with ketamine (75 mg/kg)/xylazine (20 mg/kg), 0.4 mg of imiquimod was applied in the vagina using a micropipette tip.

In vivo luciferase-based bioluminescence imaging

Luciferin (Sigma) was used to test for firefly luciferase activity *in vivo* (36). Mice were injected with the substrate luciferin 40 mg/kg intraperitoneally and sedated by inhaling isoflurane USP (Baxter International, Inc.). Bioluminescence of the mice was detected via the IVIS Imaging System 200 Series. The region of interest from displayed images was designated and quantified as total photon counts using Living Image 2.50 software (Xenogen).

Orthotopic cervical cancer model and treatment

Mice (5 per group) were challenged intravaginally with 2 × 10⁴ TC-1-Luc cells per mice using methods previously described (37). Briefly, 4 days before the tumor implantation, female mice were diestrus synchronized by injection of medroxyprogesterone (Greenstone LLC) s.c. (3 mg/mouse), and all vaginal procedures were performed upon isoflurane anesthesia. Mice were administered 4% nonoxynol-9 (N9; Igepal; Sigma) intravaginally one day before tumor challenge. On day one, the genital tracts were washed with PBS before injecting 2 × 10⁴ TC-1-Luc cells into the vaginal tract. Tumor growth was confirmed by IVIS 2000 system on day 7 before immunization began. Mice were vaccinated with 40 μ g of CRT-E7 DNA plasmid via intramuscular injection 3 times at 3-day intervals. Imiquimod 5% cream (0.4 mg) was deposited in the vagina on day 13 after the last boost vaccination and was subsequently applied every seven days. Mice were euthanized when they demonstrated stress or when they lost more than 20% of their body weight, based on animal care regulations.

Analysis of the immune cells in vaginal and tumor tissues

The genital tracts and tumor tissue of mice were dissected. Tissue samples were cut into small pieces and digested with 0.05 mg/mL collagenase I, 0.05 mg/mL collagenase IV, 0.025 mg/mL hyaluronidase IV, 0.25 mg/mL DNase I,

100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C for 60 minutes as previously described (17, 38). The tissue digest was then filtered through a 70-µm nylon filter mesh to remove undigested tissue fragments.

Flow cytometry analysis and intracellular cytokine staining to detect IFN γ secretion by E7-specific CD8 $^+$ T cells in the tumor microenvironment

H2-2D b tetramers labeled with phycoerythrin (PE) and complexed to the HPV-16 E7 (RAHYNIVTF) peptide were gifts from the NIH (39, 40). Single-cell suspended splenocytes, genital lymph nodes, and cervicovaginal cells from immunized and control mice were incubated in FACS buffer (0.5% BSA, 2 mmol/L EDTA, 0.1% NaN $_3$ in PBS). FITC-labeled anti-CD8a antibodies (BD 53-6.7) and PE-labeled tetramers were added and incubated at 4°C for 30 minutes. Samples were also stained with 7-AAD to exclude dead cells (BD). For analysis of the expression of integrin and chemokine receptors, CD8 $^+$ T cells were costained with anti-mouse CD103 APC-mAb (eBioscience 2E7), anti-mouse CD49a Alexa Fluor 647 (BD Ha3118) mAb, and CXCR3/CD183 APC-mAb (BD CXCR3-173). Cells were incubated with Fc blocker anti-CD16/CD32 (eBioscience) at 4°C for 5 minutes.

Cell surface marker staining for anti-mouse PE CD8 (BD RPA-T8) and intracellular cytokine staining for anti-mouse FITC IFN γ (BD XMG1.2) as well as FACS analysis were performed in the same conditions as those previously described (41). Lymphocytes extracted from tumor fragments were collected and incubated with 1 µg/mL of E7 peptide (RAHYNIVTF) as previously described (42) in 24-well plates for 8 hours. The number of IFN γ -secreting CD8 $^+$ T cells was analyzed by FACS analysis. All analyses were performed with Flowjo 10.1.

Quantitative real-time PCR

mRNA from vaginal tissue was extracted using Trizol after homogenization as previously described (43). First strand cDNA was synthesized by reverse transcription, according to the manufacturer's protocol (BioRad). Then, the first strain cDNA was used for quantitative real-time PCR using IQ SYBR Green (Invitrogen) on the MyiQ real-time detection system (BioRad) following the manufacturer's protocol. The forward and reverse primers are as follows: β -actin: sense, ACTGGGACGACATGGAGAAG, antisense, GGGG-TGTTGAAGGTCTCAA; TLR-7: sense, CCACAGGCTCAC-CCATACTTC, antisense, GGGATGTCCTAGCTGGTGACA; CXCR-9: sense, CAAATCCCTCAAAGACCTCAAAC, antisense, GATCTCCGTTCTTCACTGTAGC; CXCR-10: sense, TCATCCCTGCGAGCCTAT, antisense, CTTGATGGTCT-TAGATTCCGGAT, and IFN γ : sense, ACAATGAACGCTA-CACACTGCAT, antisense, TGGCAGTAACAGCCAGAAA-CA (23). Gene expression levels were normalized to β -actin housekeeping gene, and data were represented as fold differences by the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-Actin}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{induced}} - \Delta Ct_{\text{reference}}$ as previously mentioned (44).

Statistical analysis

All data are expressed as mean \pm SE where indicated. Comparisons between individual data points for intracellular cytokine staining with flow cytometric analysis and tumor treatment were made using the Student *t* test. In the tumor treatment experiments, the principal outcome of interest was duration until mice were sacrificed for ethical reasons (in stress, body weight lost is greater than 20%). The event time distributions for different mice were compared using the Kaplan–Meier method and the log-rank statistic by Prism 6 software. All *P* values <0.05 were considered significant.

Results

Imiquimod deposition in the vagina increased the population of E7-specific CD8 $^+$ T cells locally

First, to examine the impact of inflammation in the genital tract of non-tumor-bearing mice, we examined the systemic and local effects of imiquimod treatment on the cervicovaginal tract after vaccination. Mice were vaccinated with 40 µg pNGVL4a-CRT-E7 (detox) plasmid vaccine via intramuscular injection in the hind legs on days 1, 4, and 8. Imiquimod, or as a control PBS, was then applied to the vaginal tract of mice on day 15. Peripheral blood and vaginal tissue were collected on day 21. Mice treated with either imiquimod or PBS generated similar levels of E7-specific CD8 $^+$ T cells in the circulation (Fig. 1A and B). However, we observed that the genital tract of mice treated intravaginally with imiquimod displayed a 10-fold increase in total CD8 $^+$ T cells compared with mice treated with PBS (Fig. 1C). Furthermore, the number of E7-specific CD8 $^+$ T cells in the vaginal tissue was also dramatically increased (8.2-fold) in mice treated with imiquimod compared with those treated with PBS, but suggests that the enrichment of activated T cells is not antigen specific (Fig. 1D and E). This suggests that local imiquimod application enhances local replication of the CD8 $^+$ T cells and/or recruits them to the site of treatment.

Imiquimod application attracts luciferase-expressing E7-specific CD8 $^+$ T cells to the cervicovaginal tract

To determine if application of imiquimod results in the accumulation of luciferase-expressing E7-specific CD8 $^+$ T cells in the cervicovaginal tracts of treated mice, we applied imiquimod or PBS to the cervicovaginal tracts of mice and then adoptively transferred the E7-Luc CD8 $^+$ T cells (5×10^5 /mouse) via tail vein. After 48 hours, the entire genital tract was excised to measure bioluminescence intensity and thereby detect the presence of the luciferase-expressing E7-specific CD8 $^+$ T cells (Supplementary Fig. S1A and S1B). Supplementary Figure S1B shows that a significantly higher number of E7-Luc T cells were present in the cervicovaginal tracts of mice treated with imiquimod compared with those treated with PBS. These data indicate that the application of imiquimod results in the accumulation of activated antigen-specific CD8 $^+$ T cells from the systemic compartment into the genital tract.

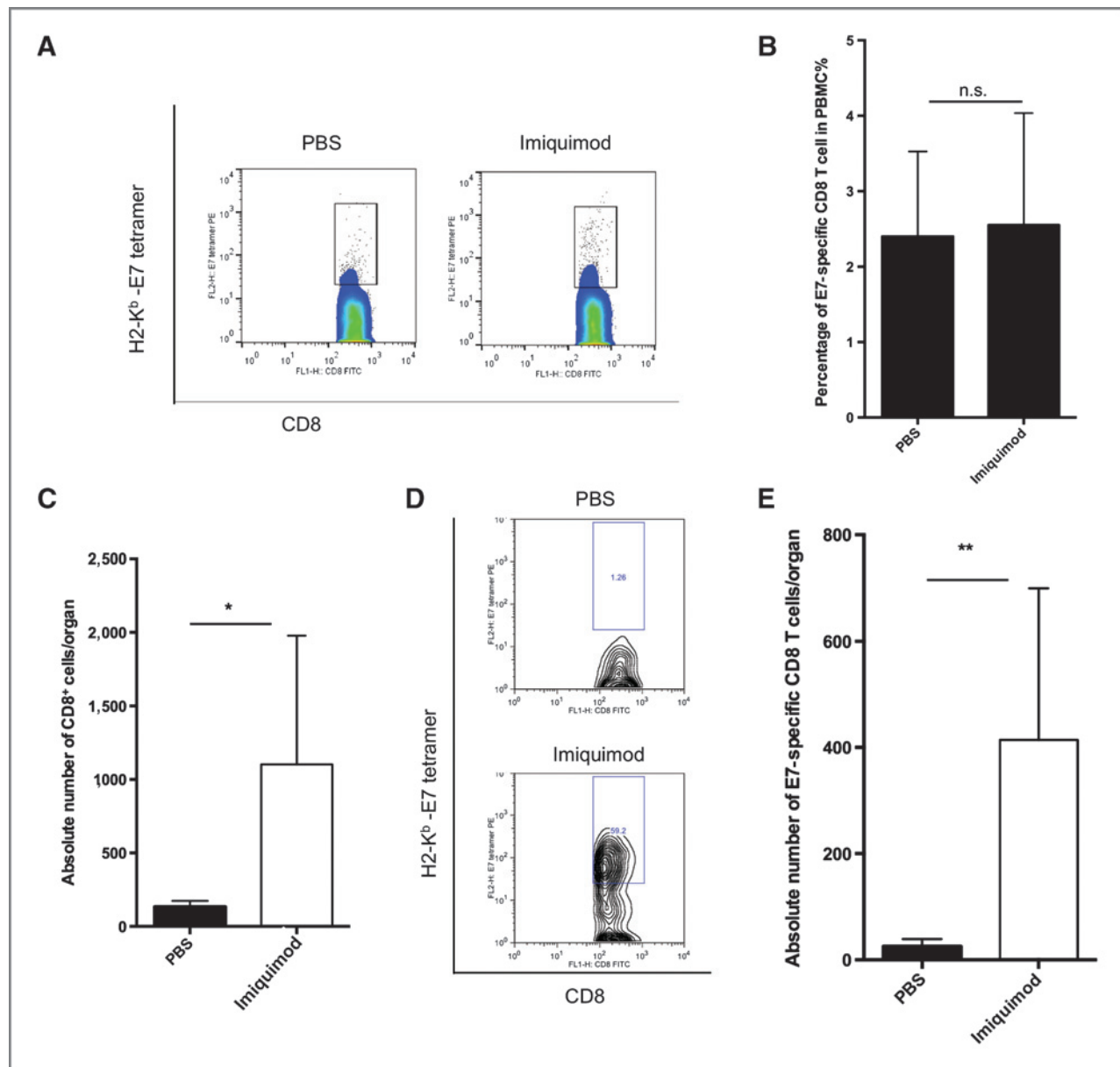


Figure 1. Deposition of imiquimod in the vagina attracts CD8⁺ T cells to the genital tract. Groups of mice were vaccinated twice with a DNA plasmid encoding pNGVL4a-CRT-E7 (40 μ g) by intramuscular injection. Mice were treated with or without imiquimod. Blood was drawn one week after vaccination, and cells were stained with anti-CD8 antibody, H2-K^b E7 tetramer, and 7-AAD (to exclude dead cells). A, representative flow cytometry analysis showing E7-specific CD8⁺ T cells in peripheral blood. B, bar graph depicting the percentage of E7-specific CD8⁺ T cells in circulation. C, PBS or imiquimod 5% cream was applied in the vaginal tract one week after systemic vaccination with pNGVL4a-CRT-E7. Genital tract tissue was excised and digested. Bar graph shows the absolute number of CD8⁺ T cells in the genital tracts of treated mice. D, representative flow cytometry. E, bar graph depicting the absolute number of E7-specific CD8⁺ T cells in the genital tracts of mice treated with or without imiquimod (*, $P < 0.05$; **, $P < 0.01$).

Systemic CRT-E7 DNA vaccination combined with local imiquimod application generated potent antitumor effects in the orthotropic cervical cancer model

Next, to test if the combination strategy of systemic vaccination with local imiquimod application could improve the treatment effect in an orthotropic genital mucosal tumor model, luciferase-expressing TC-1 tumor cells were grown in the vaginal wall. Mice bearing vaginal TC-1-luc tumors one week after inoculation were divided into four

groups: naïve, vaccination only (VAC), imiquimod only (IMQ), and vaccination plus imiquimod (VAC + IMQ). In the VAC + IMQ group, vaccination was administered a total of three times, followed by three administrations of imiquimod. The treatment schedule is illustrated in Fig. 2A.

As shown in Fig. 2B, the bioluminescence signals, indicating vaginal tumor load, in all groups of mice were not significantly different on day 7, and increased to a similar level by day 14. Subsequently, the signal dramatically

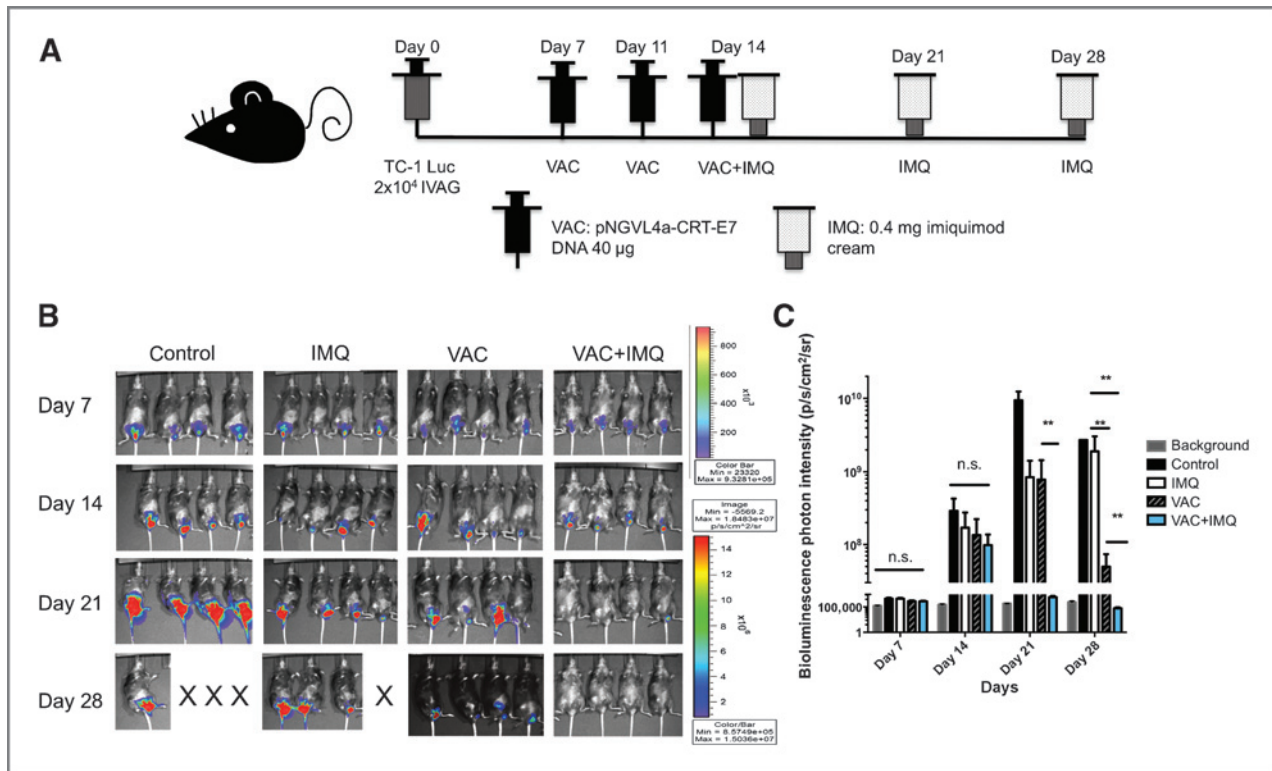


Figure 2. Systemic vaccination combined with local imiquimod application generated potent antitumor effects in the vaginal tract. A, tumor challenge and vaccination/imiquimod application schedule. Mice were treated with pNGVL4a-CRT-E7 vaccine alone (VAC), imiquimod alone (IMQ), or both (VAC + IMQ). B, bioluminescence imaging showing the TC-1 tumors following challenge with TC-1-Luc cells (5×10^7 /mouse) administered in the vagina. C, bar graph depicting bioluminescence intensity of the various treatment groups over time (**, $P < 0.01$).

decreased in the VAC+IMQ group by day 21. In contrast, the signals in the mice of the remaining groups continued to increase steadily, although the VAC only and IMQ only signals were about 10-fold lower than the control mice. Figure 2C shows that as early as day 21, the tumors of mice treated with DNA vaccine and imiquimod were significantly smaller than those of the other treatment groups. These results correlated with the survival rates of the groups of mice. Although mice in the VAC group and IMQ group showed improved survival compared with the control mice, mice in the VAC + IMQ group had significantly prolonged survival compared with both the VAC group ($P < 0.01$) and the IMQ group ($P < 0.01$; Supplementary Fig. S2A). All the mice in the VAC + IMQ group survived until day 70 without any sign of tumor recurrence. Furthermore, mice treated with DNA vaccination and imiquimod had significantly higher numbers of total CD8⁺ T cells and E7-specific CD8⁺ T cells in the tumor loci compared with those in the other treatment groups (Supplementary Fig. S2B and S2C). Here, the fold increase in E7-specific CD8⁺ T cells was greater than total CD8⁺ T-cell accumulation, suggesting preferential accumulation of the activated CD8⁺ T cells in the tumor because of the presence of cognate antigen. Furthermore, we found that mice that cleared TC-1 tumors in Supplementary Fig. S2A and were subsequently rechallenged with TC-1 tumor cells remained tumor free for at least 60 days (data not shown). Taken together, the data shown

in Fig. 2 and Supplementary Figs. S1 and S2 suggest that intravaginal application of imiquimod leads to the accumulation in the cervicovaginal tumor of systemic E7-specific CD8⁺ T cells generated by CRT-E7 DNA plasmid vaccination, resulting in potent antitumor effect against E7-expressing orthotopic tumors in the cervicovaginal tract.

Activation of antigen-specific CD8⁺ T cells in the cervicovaginal tract leads to accumulation of other antigen-specific CD8⁺ T cells in the cervicovaginal tract

We further explored whether the activation of antigen-specific CD8⁺ T cells in the cervicovaginal tract would create a suitable environment to attract other antigen-specific CD8⁺ T cells. Naïve C57BL/6 mice and tumor-free mice previously challenged with TC-1-Luc cells and effectively treated with CRT/E7 DNA vaccine and imiquimod (antigen-experienced mice) were used to further characterize the attraction of other antigen-specific CD8⁺ T cells to the cervicovaginal tract. As outlined in Fig. 3A, mice were administered with E7 peptide (RAHYNIVTF) in the cervicovaginal tract one day before adoptive transfer of OVA-specific CD8⁺ T cells (OT-1). OT-1 T cells (5×10^5) were labeled with carboxyfluorescein succinimidyl ester (CFSE) before injection into mice via the tail vein. Two days after adoptive transfer, splenocytes and vaginal tissue were collected, and CFSE-labeled OT-1 T cells were quantified by flow cytometry analysis. We observed similar numbers of OT-1

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/20/21/5456/2022305/5456.pdf by guest on 23 April 2025

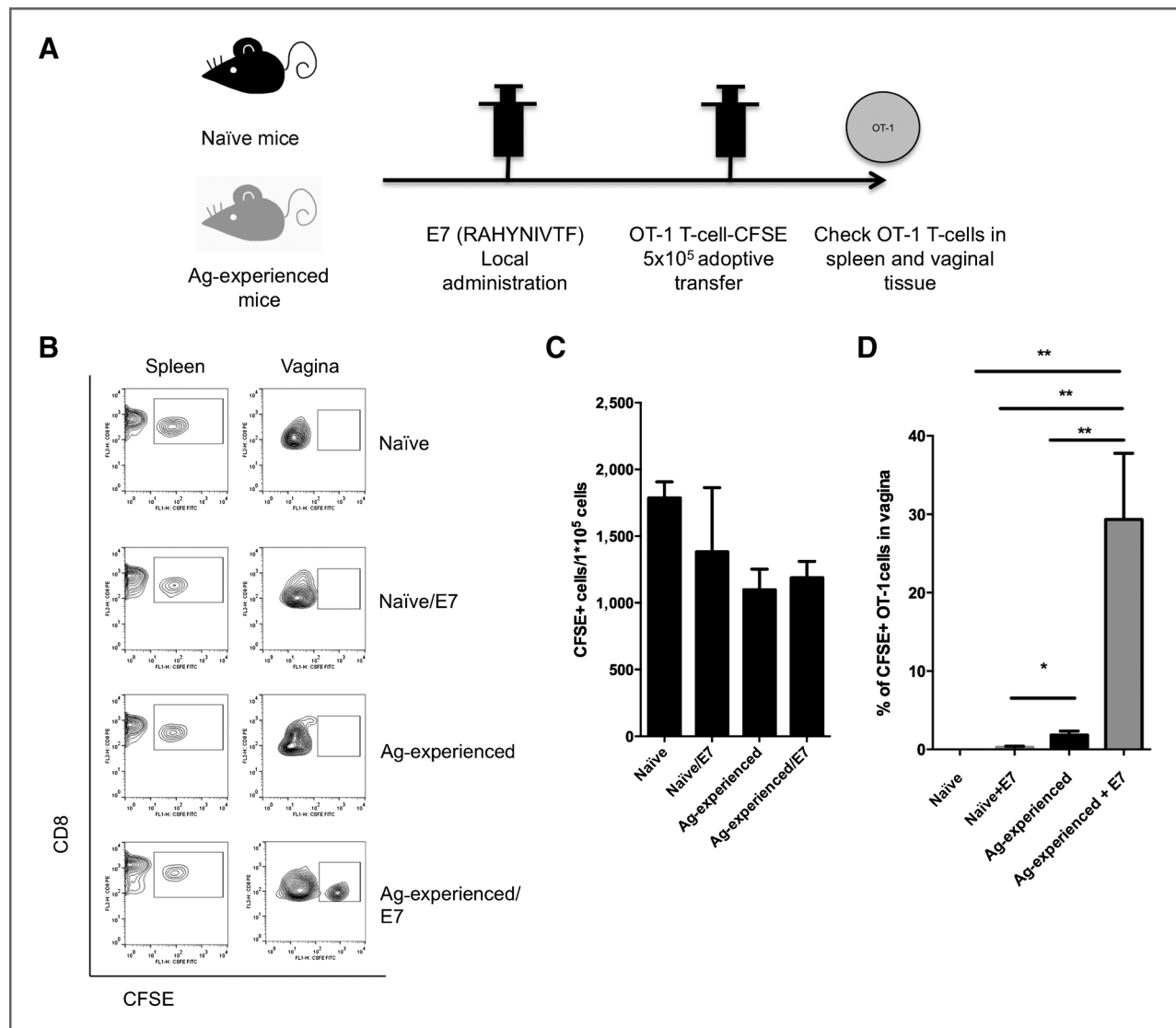


Figure 3. Antigen-experienced Trm cell accumulation in the vaginal area after antigen stimulation. A, illustration of assay. Briefly, E7 (RAHYNIVTF) peptide or PBS was administered locally followed by adoptive transfer of CFSE-labeled OT-1 CD8⁺ T cells. Two days after adoptive transfer, PBMCs and vaginal tissue were examined for CFSE-labeled OT-1 CD8⁺ T cells. B, representative flow cytometry. C, bar graph depicting the number of CFSE⁺ cells in the spleens of treated mice. D, bar graph depicting the percentage of CFSE⁺ cells in the genital tracts of treated mice (*, $P < 0.05$; **, $P < 0.01$).

T cells in the circulation after adoptive transfer in all treatment groups (Fig. 3B and C). A significantly greater number of OT-1 T cells accumulated in the vaginal tracts in antigen-experienced mice that received E7 peptide administration, but did not in antigen-experienced mice not receiving E7 peptide administration (Fig. 3B and D). These results suggest that local inflammation induced by the activation of antigen-specific CD8⁺ T cells can recruit activated CD8⁺ T cells, regardless of antigen specificity, to the vaginal tissue.

Imiquimod induced local expression of CXCL9 and CXCL10 leading to CXCR3⁺ cytotoxic T-cell accumulation in the cervicovaginal tract

Previous studies have shown that imiquimod application on the cervix elevated the expression of the chemokines

CXCL9 and CXCL10 in the local tissue in murine and non-human primate models (34–36). To characterize the expression of CXCL9 and CXCL10 mRNA, we performed qRT-PCR using mRNA derived from the cervicovaginal tracts following local imiquimod treatment. Treatment with PBS was used as a control for comparison. As shown in Fig. 4A, we found that the CXCL9 and CXCL10 mRNA expression was significantly higher in imiquimod-treated vaginal tissue compared with PBS-treated tissue. Because CXCR3, the receptor for both CXCL9 and CXCL10, is expressed on T cells and functions to induce T-cell migration to tissues displaying danger signals (32), we also examined the expression of CXCR3 on CD8⁺ T cells isolated from the cervicovaginal tract. Treatment with imiquimod induced a significantly increased number of CXCR3-expressing CD8⁺

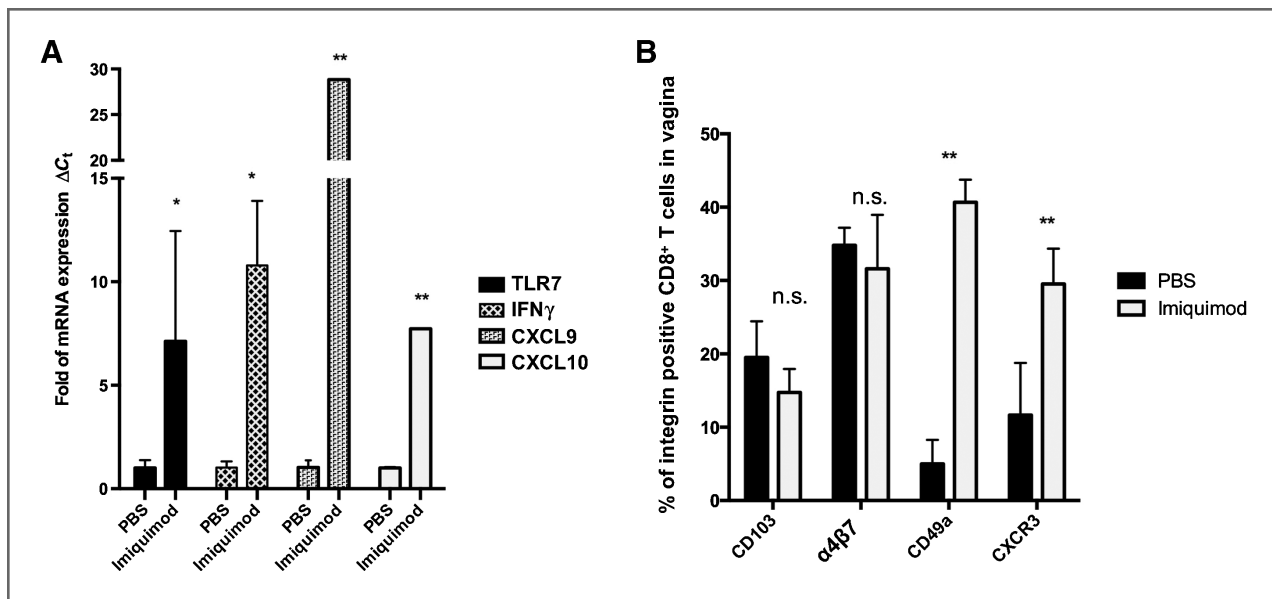


Figure 4. Expression of CXCL9 and CXCL10 following imiquimod treatment and characterization of the CD8⁺ T-cell accumulation in the cervicovaginal tract. A, expression levels of TLR7, IFN γ , CXCL9, and CXCL10 were evaluated from mRNA extracted from vaginal tissue treated with PBS or imiquimod by qRT-PCR. Gene expression levels were normalized to β -actin housekeeping gene, and data were represented as fold differences. B, surface integrin markers of cervicovaginal CD8⁺ T cells were screened (*, $P < 0.05$; **, $P < 0.01$).

T cells recovered from the genital tract as compared with treatment with PBS (Fig. 4B). In addition, we compared the expression of surface markers associated with T-cell migration to lesions, CD103, $\alpha 4\beta 7$, and CD49a, on genital tract T cells of control mice versus those treated with imiquimod (45, 46). Figure 4B shows that in imiquimod-treated mice, there was a significantly higher proportion of CD49a⁺ T cells in the cervicovaginal tract compared with mice treated with PBS.

IFN γ signaling is important for both CXCL9/10 expression and accumulation of antigen-specific CD8⁺ T cells in the cervicovaginal tract following imiquimod treatment

IFN γ signaling induces CXCL9/10 expression (33). In our study, we found that IFN γ expression was upregulated after local imiquimod treatment (Fig. 4A). This implies that IFN γ may be a key cytokine governing CXCL9/10 upregulation and the attraction of the activated CD8⁺ T-cell immune response after imiquimod application. To determine if the IFN γ signal pathway is important for the upregulated expression of CXCL9/10 in the cervicovaginal tract, we compared the genital tracts of IFN γ receptor knockout mice (IFN γ R^{-/-}) with wild-type (WT) mice following imiquimod treatment for their levels of TLR7, IFN γ , CXCL9, and CXCL10 mRNA. We found that the expression levels of IFN γ , CXCL9, and CXCL10 were significantly reduced in IFN γ R^{-/-} mice compared with WT mice (Fig. 5A). In comparison, the expression level of TLR7 was not significantly different between IFN γ R^{-/-} mice and WT mice.

To demonstrate that IFN γ is important for the accumulation of antigen-specific CD8⁺ T cells in the cervicovaginal

tract following imiquimod treatment, we compared the number of E7-specific CD8⁺ T cells in the cervicovaginal tracts of WT and IFN γ R^{-/-} mice following DNA vaccination and local imiquimod application. As shown in Fig. 5B, although the numbers of E7-specific CD8⁺ T cells in peripheral blood mononuclear cells (PBMC) were similar between WT and IFN γ R^{-/-} mice, there were significantly fewer CD8⁺ T cells (Fig. 5C) and E7-specific CD8⁺ T cells in the cervicovaginal areas of IFN γ R^{-/-} mice compared with WT mice (Fig. 5D and E). Taken together, these data suggest that the IFN γ signal pathway plays an important role in triggering CXCL9/10 chemokine expression and the accumulation of E7-specific CD8⁺ T cells in the cervicovaginal tract following imiquimod treatment.

CXCR3 is crucial for antigen-specific CD8⁺ T-cell accumulation in imiquimod-treated cervicovaginal tract

In Fig. 4B, we found that CXCR3⁺ CD8⁺ T cells accumulated in imiquimod-treated cervicovaginal tissue. To test the importance of CXCR3 expression on the CD8⁺ T cells for accumulation in the genital tract upon imiquimod treatment, we compared the number of CD8⁺ T cells in WT mice and CXCR3^{-/-} mice. All mice were vaccinated systemically with CRT-E7 DNA followed by treatment with imiquimod in the cervicovaginal tract. As shown in Fig. 6, we found that in the cervicovaginal tract, the total number of CD8⁺ T cells and E7-specific CD8⁺ T cells were significantly reduced in CXCR3^{-/-} mice compared with Wt mice. Taken together, these data suggest that the expression of CXCR3 on CD8⁺ T cells is crucial for the presence of antigen-experienced cytotoxic T cells in CXCL9/10-expressing tissue following local imiquimod application.

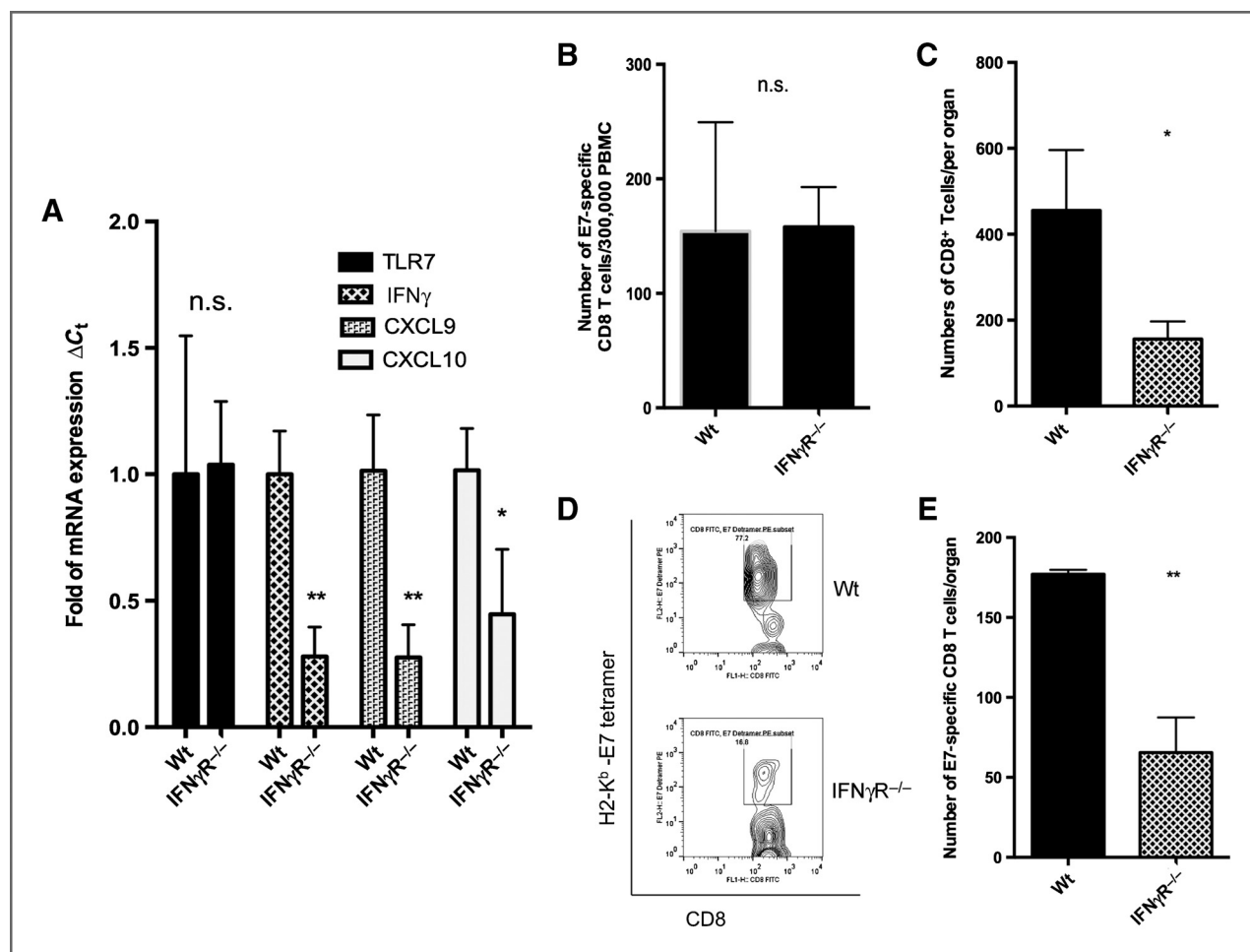


Figure 5. Recruitment of T cells from circulation to treatment location in IFN γ R^{-/-} mice. A, mRNA was extracted from the imiquimod-treated vaginal tissue of Wt mice and IFN γ R^{-/-} mice. The expression levels of TLR7, IFN γ , CXCL9, and CXCL10 were detected by qRT-PCR and are depicted in the bar graph. B, bar graph showing the number of E7-specific CD8⁺ T cells per 3×10^5 PBMCs. C, total number of CD8⁺ T cells in the genital tracts of mice. D, representative flow cytometry and bar graph (E) of E7-specific CD8⁺ T cells in vaginal tissue (*, $P < 0.05$; **, $P < 0.01$).

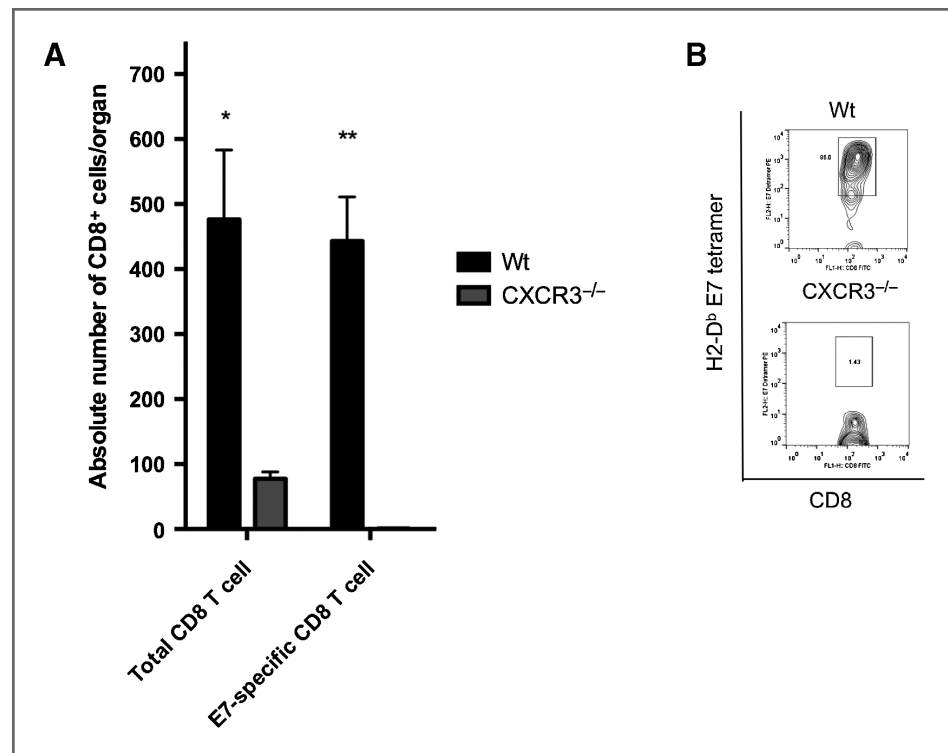
Discussion

The current study examined the effects of imiquimod application following CRT/E7 DNA vaccine administration on antigen-specific CD8⁺ T-cell-mediated immune responses and antitumor effects. Following imiquimod treatment, the vaginal tissue of mice exhibited a significant increase in local E7-specific CD8⁺ T cells. Furthermore, after adoptive transfer of activated E7-Luc T cells, imiquimod treatment attracted them to the cervicovaginal tracts of mice. Mice treated with CRT/E7 DNA vaccine followed by intravaginal imiquimod deposition, compared with either treatment alone, generated synergistic antitumor effects and dramatically improved survival. Imiquimod application induced local CXCL9/10 expression, and antigen-specific T cells that accumulate in the cervicovaginal tract following imiquimod application express CXCR3 as well as the tissue homing integrin CD49a. Finally, we show that the IFN γ signal pathway is important for the expression of CXCL9/10 as well as the

accumulation of antigen-specific CD8⁺ T cells in the cervicovaginal tract following imiquimod treatment.

Here, we observed that cervicovaginal application of imiquimod following CRT/E7 DNA vaccination resulted in the accumulation of antigen-specific CD8⁺ T cells in the cervicovaginal tract. Several mechanisms may account for our observations. For example, imiquimod may facilitate the migration of antigen-specific CD8⁺ T cells from the circulation to the point of imiquimod application in the cervicovaginal tract. Our data are consistent with this concept. In our study, we observed that IFN γ is essential for the accumulation of antigen-specific CD8⁺ T cells in the cervicovaginal tract (Fig. 5). In addition, we have observed that CXCL9/10 are upregulated following imiquimod application (Fig. 4). It has been shown that CXCL9/10 can be induced by IFN γ and play an important role for attracting CXCR3⁺ T cells to the tissue (for review, see ref. 32). Thus, the local application of imiquimod can activate the IFN γ and IFN γ receptor pathway and induce CXCL9/10

Figure 6. Characterization of CD8⁺ T-cell migration in CXCR3 knockout mice following vaginal deposition of imiquimod. WT and CXCR3 knockout mice were vaccinated with pNGVL4a-CRT-E7 and treated with imiquimod in the cervicovaginal tract. A, bar graph showing the absolute number of CD8⁺ T cells number in the cervicovaginal tracts of WT and CXCR3^{-/-} mice. *, $P < 0.05$; **, $P < 0.001$. B, representative flow cytometry analysis of E7-specific CD8⁺ T cells in the cervicovaginal tracts of WT and CXCR3^{-/-} mice.



expression, resulting in the migration of CXCR3⁺ CD8⁺ T cells to the cervicovaginal tract. Alternatively, expansion of the antigen-specific CD8⁺ T cells at the tissue location or extravasation of activated T cells from the imiquimod-treated area may also contribute to the significantly increased number of antigen-specific CD8⁺ T cells following imiquimod application.

Antigen-specific T cells accumulating in the cervicovaginal tract after intravaginal imiquimod application expressed CD49a (Fig. 4B), an integrin associated with mucosal homing and retention. Expression on E7-specific CD8⁺ T cells is induced following intranasal mucosal vaccination (45). Previous data show that VLA-1 enables not only homing and retention of antigen-specific CD8⁺ T cells in tissue, but also survival (45, 47, 48). Taken together, these data suggest that vaccination with CRT/E7 DNA and topical imiquimod application may together promote the generation of mucosal-homing E7-specific CD8⁺ T cells that are capable of localizing to mucosal tissues, infiltrating mucosal tumors, and eliciting potent antitumor effects.

In humans, therapeutic vaccination targeting HPV E6 and E7 in subjects with HPV16⁺ high-grade cervical dysplasia elicits dense, clonally expanded T-cell infiltrates in the lesion site, which are associated with a molecular signal of immune activation (49). In subjects with residual lesions, evidence of immune activation was increased in both the epithelial and the stromal compartment, whereas the intensity of Foxp3⁺ infiltrates was not affected. Although the final results from this ongoing clinical trial are not yet available, insights related to our study are anticipated, as subjects are currently undergoing therapeutic vaccination in concert

with direct application of imiquimod to the lesion (50). To determine whether the results of the current study can be extended to human VIN, additional considerations will need to be taken into account. For example, elimination of VIN lesions and prevention of recurrence will likely require effector cells to concentrate at or pass through the basement membrane to target neoplastic cells that express the virus-derived antigen target. Furthermore, it will be important to determine whether our regimen also attracts other cells, in addition to CD8⁺ T cells, to the treated area including antagonistic cell populations.

It will be important to determine the optimal regimen of local imiquimod application in relation to a therapeutic HPV vaccination regimen. We have created numerous therapeutic HPV vaccines and are testing these vaccines using various regimens (e.g., DNA prime-vaccinia boost; ref. 50) and administration methods (e.g., electroporation; ref. 51). A clinical trial testing different routes of vaccination in subjects with HPV16⁺ CIN2/3 is ongoing (18). In humans, administration of DNA vaccination with electroporation elicits significant vaccine-specific T-cell responses that are detectable without prolonged *ex vivo* manipulation (52). Once the most potent vaccines and regimens for the generation of systemic HPV antigen-specific immune responses have been identified, it will be essential to coordinate these interventions with a better understanding of the timing of imiquimod relative to vaccination, to maximize activity in the tissue. Appropriate use of imiquimod in conjunction with a potent vaccine should result in enhanced recruitment of vaccine-induced T cells and increased therapeutic antileision effects.

Our example of using imiquimod to generate T-cell-mediated antitumor effects in an accessible tissue presents the opportunity to use other TLR ligands that may be capable of generating even stronger T-cell recruitment. Currently, in addition to imiquimod, two TLR agonists are FDA approved for use in patients with cancer: the TLR4 agonist monophosphoryl lipid A and the TLR2/4 agonist bacillus Calmette-Guérin. Also of interest is the TLR7/8 agonist, resiquimod, which, like imiquimod, is an imidazoquinoline, and has been shown to have antitumor effects (for review, see ref. 53). TLR9 and TLR3 agonists are also being developed commercially for application in oncology and against viral infections (for review, see ref. 54). A recent study by Domingos-Pereira and colleagues (55) demonstrated that intravaginal administration of a TLR9 or TLR3 agonist after s.c. E7 peptide vaccination increases local E7-specific CD8 T cells and rejection of TC-1 tumors cells at the genital mucosa. Future studies to compare these TLR ligands should lead to the identification of TLR ligands that will be suitable for further clinical translation.

In summary, we found that following therapeutic CRT/E7 DNA vaccination in an orthotopic murine model, local imiquimod application resulted in the accumulation of antigen-specific CXCR3⁺ CD8⁺ T cells in the cervicovaginal tracts in both tumor-bearing and in non-tumor-bearing mice. In this model, this phenomenon was dependent on the IFN γ pathway and local CXCL9/10 expression. Our findings support accumulating evidence that manipulation

of components of the tumor microenvironment can enhance the therapeutic efficacy of tumor-specific T cells.

Disclosure of Potential Conflicts of Interest

R.B.S. Roden and T.-C. Wu have ownership interest (including patents) in and are consultant/advisory board members for Papivax. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R.-S. Soong, W. Huh, Y.-N. Chang, W.-F. Cheng, R.B.S. Roden, T.-C. Wu, C.L. Trimble, C.-F. Hung

Development of methodology: R.-S. Soong, L. Song, Y.-C. Tsai, W.-F. Cheng, T.-C. Wu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.-S. Soong, L. Song, L. He, T.-C. Wu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.-S. Soong, L. Song, W. Huh, W.-F. Cheng, T.-C. Wu, C.L. Trimble

Writing, review, and/or revision of the manuscript: R.-S. Soong, L. Song, J. Trieu, J. Knoff, W. Huh, R.B.S. Roden, T.-C. Wu, C.L. Trimble, C.-F. Hung

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Song, T.-C. Wu, C.-F. Hung

Study supervision: R.B.S. Roden, T.-C. Wu

Grant Support

This work was supported by the NIH/NCI Cervical Cancer SPORE P50CA098252, Head and Neck Cancer SPORE P50 CA-DE019032, R43CA174436, 2R01CA114425-06, and R01CA142691 grants.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 10, 2014; revised April 24, 2014; accepted April 28, 2014; published OnlineFirst June 3, 2014.

References

- Chaturvedi AK. Beyond cervical cancer: burden of other HPV-related cancers among men and women. *J Adolesc Health* 2010;46:S20-6.
- Armarnik S, Sheiner E, Piura B, Meirovitz M, Zlotnik A, Levy A. Obstetric outcome following cervical conization. *Arch Gynecol Obstet* 2011; 283:765-9.
- Cardosi RJ, Bomalaski JJ, Hoffman MS. Diagnosis and management of vulvar and vaginal intraepithelial neoplasia. *Obstet Gynecol Clin North Am* 2001;28:685-702.
- Abbasakoor F, Boulos PB. Anal intraepithelial neoplasia. *Br J Surg* 2005;92:277-90.
- Su JH, Wu A, Scotney E, Ma B, Monie A, Hung CF, et al. Immunotherapy for cervical cancer: research status and clinical potential. *BioDrugs* 2010;24:109-29.
- Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361:1838-47.
- Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M, et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001; 108:669-78.
- Chuang CM, Monie A, Hung CF, Wu TC. Treatment with imiquimod enhances antitumor immunity induced by therapeutic HPV DNA vaccination. *J Biomed Sci* 2010;17:32.
- Kim JW, Hung CF, Juang J, He L, Kim TW, Armstrong DK, et al. Comparison of HPV DNA vaccines employing intracellular targeting strategies. *Gene Ther* 2004;11:1011-8.
- Peng S, Trimble C, He L, Tsai YC, Lin CT, Boyd DA, et al. Characterization of HLA-A2-restricted HPV-16 E7-specific CD8(+) T-cell immune responses induced by DNA vaccines in HLA-A2 transgenic mice. *Gene Ther* 2006;13:67-77.
- Tseng CW, Monie A, Wu CY, Huang B, Wang MC, Hung CF, et al. Treatment with proteasome inhibitor bortezomib enhances antigen-specific CD8+ T-cell-mediated antitumor immunity induced by DNA vaccination. *J Mol Med (Berl)* 2008;86:899-908.
- Tseng CW, Monie A, Trimble C, Alvarez RD, Huh WK, Buchsbaum DJ, et al. Combination of treatment with death receptor 5-specific antibody with therapeutic HPV DNA vaccination generates enhanced therapeutic anti-tumor effects. *Vaccine* 2008;26:4314-9.
- Best SR, Peng S, Juang CM, Hung CF, Hannaman D, Saunders JR, et al. Administration of HPV DNA vaccine via electroporation elicits the strongest CD8+ T cell immune responses compared to intramuscular injection and intradermal gene gun delivery. *Vaccine* 2009;27:5450-9.
- Tseng CW, Trimble C, Zeng Q, Monie A, Alvarez RD, Huh WK, et al. Low-dose radiation enhances therapeutic HPV DNA vaccination in tumor-bearing hosts. *Cancer Immunol Immunother* 2009;58:737-48.
- Kim D, Hung CF, Wu TC, Park YM. DNA vaccine with alpha-galactosylceramide at prime phase enhances anti-tumor immunity after boosting with antigen-expressing dendritic cells. *Vaccine* 2010;28:7297-305.
- Peng S, Monie A, Pang X, Hung CF, Wu TC. Vascular disrupting agent DMXAA enhances the antitumor effects generated by therapeutic HPV DNA vaccines. *J Biomed Sci* 2011;18:21.
- Peng S, Lyford-Pike S, Akpeng B, Wu A, Hung CF, Hannaman D, et al. Low-dose cyclophosphamide administered as daily or single dose enhances the antitumor effects of a therapeutic HPV vaccine. *Cancer Immunol Immunother* 2013;62:171-82.
- Center SKCC. A Pilot Study of pNVL4a-CRT/E7 (Detox) for the treatment of patients with HPV16+ cervical intraepithelial neoplasia 2/3 (CIN2/3). In: *ClinicalTrials.gov* [Internet]. Bethesda, MD: National Library of Medicine (US); 2009 [cited 2014 Feb 10]. Available from: <http://clinicaltrials.gov/show/NCT00988559> MLM Identifier: NCT00988559.
- Trimble CL, Peng S, Thoburn C, Kos F, Wu TC. Naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN2/3. *Cancer Immunol Immunother* 2010;59:799-803.

20. Trimble CL, Peng S, Kos F, Gravitt P, Viscidi R, Sugar E, et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin Cancer Res* 2009;15:361-7.
21. Trimble CL, Clark RA, Thoburn C, Hanson NC, Tassello J, Frosina D, et al. Human papillomavirus 16-associated cervical intraepithelial neoplasia in humans excludes CD8 T cells from dysplastic epithelium. *J Immunol* 2010;185:7107-14.
22. Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL, et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulvar intraepithelial neoplasia. *Br J Cancer* 2010;102:1129-36.
23. Luci C, Hervouet C, Rousseau D, Holmgren J, Czerkinsky C, Anjuere F. Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin. *J Immunol* 2006;176:2749-57.
24. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Miles AP, Saul A, et al. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 2006;203:1249-58.
25. Fiorenza S, Kenna TJ, Comerford I, McColl S, Steptoe RJ, Leggatt GR, et al. A combination of local inflammation and central memory T cells potentiates immunotherapy in the skin. *J Immunol* 2012;189:5622-31.
26. Huang SJ, Hijnen D, Murphy GF, Kupper TS, Calarese AW, Mollet IG, et al. Imiquimod enhances IFN-gamma production and effector function of T cells infiltrating human squamous cell carcinomas of the skin. *J Invest Dermatol* 2009;129:2676-85.
27. van Seters M, van Beurden M, ten Kate FJ, Beckmann I, Ewing PC, Eijkemans MJ, et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. *N Engl J Med* 2008;358:1465-73.
28. Adams S, Kozhaya L, Martiniuk F, Meng TC, Chiriboga L, Liebes L, et al. Topical TLR7 agonist imiquimod can induce immune-mediated rejection of skin metastases in patients with breast cancer. *Clin Cancer Res* 2012;18:6748-57.
29. Le Mercier I, Poujol D, Sanlaville A, Sisirak V, Gobert M, Durand I, et al. Tumor promotion by intratumoral plasmacytoid dendritic cells is reversed by TLR7 ligand treatment. *Cancer Res* 2013;73:4629-40.
30. Kalb ML, Glaser A, Stary G, Koszik F, Stingl G. TRAIL(+) human plasmacytoid dendritic cells kill tumor cells in vitro: mechanisms of imiquimod- and IFN-alpha-mediated antitumor reactivity. *J Immunol* 2012;188:1583-91.
31. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* 2002;3:196-200.
32. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 2011;89:207-15.
33. Pak-Wittel MA, Yang L, Sojka DK, Rivenbark JG, Yokoyama WM. Interferon-gamma mediates chemokine-dependent recruitment of natural killer cells during viral infection. *Proc Natl Acad Sci U S A* 2013;110:E50-9.
34. Kim D, Hung CF, Wu TC. Monitoring the trafficking of adoptively transferred antigen-specific CD8-positive T cells in vivo, using non-invasive luminescence imaging. *Hum Gene Ther* 2007;18:575-88.
35. Peng S, Monie A, Kang TH, Hung CF, Roden R, Wu TC. Efficient delivery of DNA vaccines using human papillomavirus pseudovirions. *Gene Ther* 2010;17:1453-64.
36. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther* 2005;11:435-43.
37. Zeng Q, Peng S, Monie A, Yang M, Pang X, Hung CF, et al. Control of cervicovaginal HPV-16 E7-expressing tumors by the combination of therapeutic HPV vaccination and vascular disrupting agents. *Hum Gene Ther* 2011;22:809-19.
38. Decrausaz L, Goncalves AR, Domingos-Pereira S, Pythoud C, Stehle JC, Schiller J, et al. A novel mucosal orthotopic murine model of human papillomavirus-associated genital cancers. *Int J Cancer* 2011;128:2105-13.
39. Lamikanra A, Pan ZK, Isaacs SN, Wu TC, Paterson Y. Regression of established human papillomavirus type 16 (HPV-16) immortalized tumors in vivo by vaccinia viruses expressing different forms of HPV-16 E7 correlates with enhanced CD8(+) T-cell responses that home to the tumor site. *J Virol* 2001;75:9654-64.
40. Lee SY, Kang TH, Knoff J, Huang Z, Soong RS, Alvarez RD, et al. Intratumoral injection of therapeutic HPV vaccinia vaccine following cisplatin enhances HPV-specific antitumor effects. *Cancer Immunol Immunother* 2013;62:1175-85.
41. Soong RS, Trieu J, Lee SY, He L, Tsai YC, Wu TC, et al. Xenogeneic human p53 DNA vaccination by electroporation breaks immune tolerance to control murine tumors expressing mouse p53. *PLoS ONE* 2013;8:e56912.
42. Kim TW, Hung CF, Ling M, Juang J, He L, Hardwick JM, et al. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. *J Clin Invest* 2003;112:109-17.
43. Hung CF, Tsai YC, He L, Wu TC. Control of mesothelin-expressing ovarian cancer using adoptive transfer of mesothelin peptide-specific CD8+ T cells. *Gene Ther* 2007;14:921-9.
44. Gunn L, Ding C, Liu M, Ma Y, Qi C, Cai Y, et al. Opposing roles for complement component C5a in tumor progression and the tumor microenvironment. *J Immunol* 2012;189:2985-94.
45. Sandoval F, Terme M, Nizard M, Badoual C, Bureau MF, Freyburger L, et al. Mucosal imprinting of vaccine-induced CD8(+) T cells is crucial to inhibit the growth of mucosal tumors. *Sci Transl Med* 2013;5:172ra20.
46. Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. *Nat Rev Immunol* 2013;13:309-20.
47. Ray SJ, Franki SN, Pierce RH, Dimitrova S, Kotliansky V, Sprague AG, et al. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* 2004;20:167-79.
48. Richter MV, Topham DJ. The alpha1beta1 integrin and TNF receptor II protect airway CD8+ effector T cells from apoptosis during influenza infection. *J Immunol* 2007;179:5054-63.
49. Maldonado L, Teague JE, Morrow MP, Jotova I, Wu TC, Wang C, et al. Intramuscular therapeutic vaccination targeting HPV16 induces T cell responses that localize in mucosal lesions. *Sci Transl Med* 2014;6:221ra13.
50. Center SKCC. A Phase I efficacy and safety study of HPV16-specific therapeutic DNA-vaccinia vaccination in combination with topical imiquimod, in patients with HPV16+ high grade cervical dysplasia (CIN3). In: *ClinicalTrials.gov* [Internet]. Bethesda, MD: National Library of Medicine (US); 2008 [cited 2014 Feb 10]. Available from: <http://clinicaltrials.gov/show/NCT00788164> MLM Identifier: NCT00788164.
51. Center SKCC. A phase I clinical trial assessing the safety and feasibility of administration of pNGVL4a-CRT/E7(Detox) DNA vaccine using the intramuscular TriGridTM delivery system in combination with cyclophosphamide in HPV-16 associated head and neck cancer patients. In: *ClinicalTrials.gov* [Internet]. Bethesda, MD: National Library of Medicine (US); 2011 [cited 2014 Feb 10]. Available from: <http://clinicaltrials.gov/show/NCT01493154> MLM Identifier: NCT01493154.
52. Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC, et al. Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. *Sci Transl Med* 2012;4:155ra38.
53. Meyer T, Surber C, French LE, Stockfleth E. Resiquimod, a topical drug for viral skin lesions and skin cancer. *Expert Opin Investig Drugs* 2013;22:149-59.
54. Gearing AJ. Targeting toll-like receptors for drug development: a summary of commercial approaches. *Immunol Cell Biol* 2007;85:490-4.
55. Domingos-Pereira S, Decrausaz L, Derre L, Bobst M, Romero P, Schiller JT, et al. Intravaginal TLR agonists increase local vaccine-specific CD8 T cells and human papillomavirus-associated genital-tumor regression in mice. *Mucosal Immunol* 2013;6:393-404.