Toll like receptor agonist imiquimod facilitates antigen-specific CD8+ T cell accumulation in the genital tract leading to tumor control through interferon-γ

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Translational Relevance: Peripheral therapeutic 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, that exhibited upregulation of the expression of the chemokine receptor CXCR3, and the tissue resident memory T cell (Trm) marker CD49a. Our study has high translational relevance because imiquimod is currently FDA approved for treatment of both condyloma acuminata 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.
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 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 IFNγ receptor. Intravaginal imiquimod deposition induced upregulation of CXCL9 and CXCL10 mRNA expression in the genital tract, which are produced in response to IFNγ 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.
Introduction

Human papillomaviruses (HPVs) 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). While 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 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 non-cervical 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 co-expressed 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 (5)), particularly if targeting non-self proteins such as viral antigens. A long peptide-based therapeutic vaccine targeting HPV16 E6 and/or E7 showed partial efficacy against HPV16+ high grade VIN (6). While 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 immunogenicity 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
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 vulvar intraepithelial neoplasia (22), and is currently being tested in subjects with HPV16-associated CIN2/3 (NCT00788164, (50)(22). Since 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 OVA increased OVA-specific CD8+ T cells in the draining lymph nodes and genital mucosa (23). Additionally, 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. Non-specific immunotherapy by topical application of imiquimod to
HPV16+ high-grade vulvar intraepithelial neoplasia (VIN) has shown significant therapeutic activity, but was less effective against large lesions (27). Additionally, 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 (DCs), 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 (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**

6- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). IFN-γ receptor 1-deficient (ifngr1<sup>tm1Agt</sup>) mice (IFNγR<sup>-/-</sup>) and CXCR3-deficient (B6.129P2-Cxcr3<sup>3<sub>imlDgen</sub></sup>/J) mice (CXCR3<sup>-/-</sup>) have been previously described (Jackson Laboratories) (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, Rensselaer, NY) testing, a panel of PCR tests for rodent viruses, most recently in May 2011 with negative results. In order 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 RPMI1640 medium containing 10% FBS, 2mM L-glutamine, 10% sodium pyruvate, 10% non-essential amino acids, and 100µg/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 interleukin-2 (IL-2) weekly. In order 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) (34). OT-1 T cells were generated from OT-1 Rag-/- TCR transgenic mice from our lab as previously mentioned (35). T cell adoptive transfer was performed by injecting 5x10⁵ T cells in 200µL 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 (Haifa Bay, Israel). Mice were injected in the tibialis muscle of the shaved hind leg with a 28G syringe containing 40µg of DNA plasmid diluted in a total volume of 40μL of PBS. Each leg was injected with 20µg DNA vaccine. After the mice were under anesthesia with ketamine (75mg/kg)/xylazine (20mg/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 40mg/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 2x10⁴ TC-1-Luc cells/mice using methods previously described (37). Briefly, four days before the tumor implantation, female mice were diestrus synchronized by injection of medroxyprogesterone (Greenstone LLC,
NJ) s.c. (3mg/mouse), and all vaginal procedures 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 2x10⁴ 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 40µg of CRT-E7 DNA plasmid via intramuscular injection 3 times at 3-day intervals. 0.4 mg of imiquimod 5% cream 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 mM EDTA, 0.1% NaN₃ in PBS). FITC-labeled anti-CD8a antibodies (BD 53-6.7) and PE-labeled tetramers were added and incubated at 4°C for 30 min. 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 co-stained 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 min.

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 FACScan 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
(RAHYINVTF) as previously described (42) in 24-well plates for 8 hours. The number of IFN-γ-secreting CD8+ T cells was analyzed by FACScan cytometry. 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 for β-actin sense-ACTGGGACGACATGGAGAAG, antisense-GGGGTGTTGAAGGTCTCAAA, TLR-7 sense-CCACAGGCTCACCCATACTTC, antisense-GGGATGTCCTAGCTGGTGACA, CXCR-9 sense-CAAATCCCTCAAAGACCTCAAAC, antisense-GATCTCCGTTCTTCACTGTAGC, CXCR-10 sense-TCATCCCTGCGAGCCTAT, antisense-CTTGATGGTCTTATTCCGGAT, IFN-γ sense-AACAATGAACGCTACACACTGCAT, antisense-TGGCAGTAACAGCCAGAAACA, and β-Actin sense-ACTGGGACGACATGGAGAAG, antisense-GGGGTGTTGAAGGTCTCAAA (23). Gene expression levels were normalized to β-Actin housekeeping gene, and data were represented as fold differences by the 2^-ΔΔCt method, where ΔCt = Ct_target gene - Ct_β-Actin and ΔΔCt = ΔCt_induced - ΔCt_reference, as previously mentioned (44).

Statistical analysis
All data are expressed as mean ± S.E. where indicated. Comparisons between individual data points for intracellular cytokine staining with flow cytometric analysis and tumor treatment were made using Student’s 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 40µg 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 (Figures 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 (Figure 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 to those treated with PBS, but suggests that the enrichment of activated T cells is not antigen-specific (Figure 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

In order 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 (5x10^5/mouse) via tail vein. After 48 hours, the entire genital tract was excised in order to measure bioluminescence intensity and thereby detect presence of the luciferase-expressing E7-specific CD8+ T cells (Supplemental Figure 1A, B). Supplemental Figure 1B shows that a significantly higher number of E7-Luc T cells were present in the cervicovaginal tracts of mice treated with imiquimod compared to those treated with PBS. This data indicates that the application of imiquimod results in the accumulation of activated antigen-specific CD8+ T cells from the systemic compartment into the genital tract.

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 growing 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 Figure 2A.

As shown in Figure 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 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 to the control mice, mice in the VAC+IMQ group had significantly prolonged survival compared to both the VAC group (p<0.01) and the IMQ group (p<0.01) (Supplemental Figure 2A). All the mice in 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 to those in the other treatment groups (Supplemental Figure 2B and C). 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 Supplemental Figure 2A 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 Figure 2 and Supplemental Figures 1 and 2 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 Figure 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). 5x10^5 OT-1 T cells were labeled with 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 T cells in the circulation after adoptive transfer in all treatment groups (Figure 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 (Figure 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). In order 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 Figure 4A, we found that the CXCL9 and CXCL10 mRNA expression was significantly higher in imiquimod treated vaginal tissue compared to 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+ T cells recovered from the genital tract as compared to treatment with PBS (Figure 4B). Additionally, we compared the expression of surface markers associated with T cell migration to lesions, CD103, α4β7, 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 to mice treated with PBS.

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

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

In order to demonstrate that IFNγ 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 IFNγR -/- mice following DNA vaccination and local imiquimod application. As shown in Figure 5B, while the numbers of E7-specific CD8+ T cells in PBMCs were similar between Wt and IFNγR -/- mice, there were significantly fewer CD8+ T cells (Figure 5C) and E7-specific CD8+ T cells in the cervicovaginal areas of IFNγR -/- mice compared to Wt mice (Figure 5D and E). Taken together, these data suggest that the IFNγ 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 Figure 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 Figure 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 to 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.

**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 to 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 IFNγ 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 IFNγ is essential for the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract (see Figure 5). In addition, we have observed that CXCL9/10 are upregulated following imiquimod application (Figure 4). It has been shown that CXCL9/10 can be induced by IFNγ and play an important role for attracting CXCR3+ T cells to the tissue (for review, see (32)). Thus, the local application of imiquimod can activate the IFNγ and IFNγ receptor pathway and induce CXCL9/10 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 (Figure 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 shows 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, while 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). In order 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 (50)) and administration methods (e.g. electroporation (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 anti-lesion 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 cancer patients; the TLR4 agonist monophosphoryl lipid A (MPL), and the TLR2/4 agonist bacillus Calmette–Guérin (BCG). 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 (53)). TLR9 and TLR3 agonists are also being developed commercially for application in oncology and against viral infections (for review see (54)). A recent study by Domingos-Pereira et al 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 (55). 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 IFNγ 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.
References


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**Figure Legend**

**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 μg) 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-Kb 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).

**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 (VA), imiquimod alone (IM), or both (VA+IM). (B) Bioluminescence imaging showing the TC-1 tumors following challenge with TC-1-Luc cells (5x10⁴/mouse) administered in the vagina. (C) Bar graph depicting bioluminescence intensity of the various treatment groups over time (**p<0.01).

**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).

**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, IFNγ, 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).

Figure 5. Recruitment of T cells from circulation to treatment location in IFNγR-/- mice. (A) mRNA was extracted from the imiquimod-treated vaginal tissue of Wt mice and IFNγR -/- mice. The expression levels of TLR7, IFNγ, CXCL9, and CXCL10 were detected by qRTPCR and are depicted in the bar graph. (B) Bar graph showing the number of E7-specific CD8+ T cells per 3x10^5 PBMCs. (C) Total number of CD8+ T cells in the genital tracts of mice. (D) Representative flow cytometry and (E) bar graph of E7-specific CD8+ T cells in vaginal tissue (*p<0.05; **p<0.01).

Figure 6. Characterization of CD8+ T cell migration in CXCR3 knockout mice following vaginal deposition of imiquimod. Wild type 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. (B) Representative flow cytometry analysis of E7-specific CD8+ T cells in the cervicovaginal tracts of Wt and CXCR3-/- mice.
Figure 1

A

H2-Kb-E7 tetramer

PBS

Imiquimod

CD8

B

Percentage of E7-specific CD8 T cell in PBMC%

PBS

Imiquimod

n.s.

C

Absolute number of CD8+ cells/organ

PBS

Imiquimod

D

H2-Kb-E7 tetramer

PBS

Imiquimod

CD8

E

Absolute number of E7-specific CD8 T cells/organ

PBS

Imiquimod

**
Figure 3

A


naive mice

Ag experienced mice

E7 (RAHYNVTF)
Local administration
OT-1 T-cell-CFSE
5x10^5 adoptive transfer
Check OT-1 T-cells in spleen and vaginal tissue

B

spleen
Vagina

Naïve
Naïve/E7
Ag experienced
Ag experienced/E7

CD8

CFSE

C

Naive
Naive/E7
Ag experienced
Ag experienced/E7

CFSE+ cells/1x10^5 cells

D

% of CFSE+ OT-1 cells in vagina

naive
naive/E7
Ag experienced
Ag experienced/E7
Ag experienced + E7

**
**
*

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Figure 5

A

Fold of mRNA expression $\Delta Ct$

B

Number of $E7$ specific CD8 T cells/500,000 PBMC

C

Numbers of CD8+ T cells/per organ

D

H2-Kb - E7 tetramer

E

Number of $E7$ specific CD8 T cells/organ

CD8
Figure 6

A

**Absolute number of CD8+ cells/organ**

- Total CD8 T cell
- E7-specific CD8 T cell

**Wt**

**CXCR3/−**

B

- **Wt**
- **CXCR3/−**

- H2-D\(^B\) E7 tetramer
- CD8
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