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Intravaginal Administration of Fc-Fused IL7 Suppresses the Cervicovaginal Tumor by Recruiting HPV DNA Vaccine-Induced CD8 T Cells

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Abstract

Purpose: The induction of tissue-localized virus-specific CD8 T-cell response is essential for the development of an effective therapeutic vaccine against genital diseases, such as cervical cancer and genital herpes. Here, we aimed to elucidate the immunologic role of IL7 in the induction of mucosal cellular immunity.

Experimental Design: IL7 was engineered through Fc fusion to enhance mucosal delivery across the genital epithelial barrier. The immunomodulatory role of IL7 was evaluated by monitoring the kinetics of various immune cells and measuring the expression of chemokines and cytokines after intravaginal administration of Fc-fused IL7 (IL7-Fc). The antitumor effects of intramuscular human papillomavirus (HPV) DNA vaccine or topical IL7-Fc alone or in a combinational regimen on mice survival were compared using a orthotopic cervical cancer model.

Results: Intravaginal treatment of IL7-Fc, but not native IL7, induces upregulation of chemokines (CXCL10, CCL3, CCL4, and CCL5), cytokines (IFNγ, TNFα, IL6, and IL1β), and an adhesion molecule (VCAM-1) in the genital tract, leading to the recruitment of several leukocytes, including CD4, CD8, γδ T cells, and dendritic cells. Importantly, in this murine cervical cancer model, topical administration of IL7-Fc after intramuscular HPV DNA vaccination increases the number of HPV-specific CD8 T cells in the genital mucosa, but not in the spleen, leading to stronger antitumor activity than the HPV DNA vaccine alone.

Conclusions: Our findings provide an important insight into the immunomodulatory role of IL7-Fc via topical application and the design of therapeutic vaccine regimen that induces effective genital–mucosal CD8 T-cell responses. *Clin Cancer Res; 22(23); 5898–908. ©2016 AACR.*

Introduction

Cervical cancer is one of the most common causes of cancer-related deaths in women worldwide. Almost all cases are caused by infection with high-risk types of human papillomavirus (HPV; ref. 1). Among them, HPV16 and -18 account for about 70% to 75% of patients with cervical cancer (2). Clonal expansion of persistently infected cells leads to a premalignant cervical intraepithelial neoplasia (CIN), which then gradually transforms into invasive cancer (3). Although the prophylactic HPV vaccines can efficiently prevent HPV infection, they do not have therapeutic effects against preexisting infection and HPV-induced lesions (4). The most common treatment for CIN3 is surgical excision, which is associated with pregnancy-related complications and a 10% recurrence rate (5). More seriously, the mortality rate of cervical cancer after conventional treatment is more than 50% (6). Thus, there is an urgent need for an effective nonsurgical therapeutic approach to eradicate HPV-associated CIN and cervical cancer.

HPV-specific CD8 T-cell responses have been suggested to play a dominant role in the resolution of HPV infection (7, 8), and therapeutic vaccines are now being designed to induce a robust, yet specific, T-cell response. To date, several different types of therapeutic vaccines have been evaluated in patients with high-grade CIN (CIN2/3; ref. 3). However, considering the spontaneous regression rate of CIN2/3 (11%–30%), vaccines with a clinical response rate of 11% to 49.5% have shown limited success in improving the overall response rate (9–11). Although most of these vaccines increase the vaccine-induced blood CD8 T-cell response, systemic CD8 T-cell response did not specifically correlate with clinical outcome. The limited efficacy of therapeutic vaccines was related to inadequate T-cell trafficking to mucosal dysplastic area (12). Thus, current studies have highlighted the crucial role of local cellular immunity for therapeutic intervention (13–15). Recently, we have shown that electroporation-delivered immunization with a novel HPV DNA vaccine elicited strong

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

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Translational Relevance

Systemic HPV-specific CD8 T-cell response does not specifically correlate with disease clearance. However, clinical trial data for assessing the therapeutic efficacy of HPV vaccine suggest that high levels of intralesional CD8 T cells are associated with beneficial outcomes. Thus, the induction of vaccine-induced T-cell responses in the mucosal lesion is crucial for developing an effective therapeutic vaccine. In this study, intramuscular HPV DNA immunization in combination with topical IL7-Fc administration increases the recruitment of vaccine-induced CD8 T cells into genital tract, providing synergistic antitumor activity against established cervicovaginal tumor in mice. This study suggests that topical treatment of IL7-Fc is well tolerated and safe and that the combination regimen of IL7-Fc and HPV DNA vaccine (GX188E) is synergistic, potentially supporting further studies in humans. This study demonstrates a novel strategy for the use of IL7-Fc as a safe mucosal immunomodulator for inducing local cellular immunity against mucosal tumor and infectious disease.

HPV-specific CD8 T-cell responses in most patients with CIN3. Specifically, the clinical study showed that seven of the nine patients (78%) displayed complete regression of their neoplastic lesion. However, this clinical study did not elucidate the induction of tissue-localized HPV-specific CD8 T-cell response and its correlation with clinical outcomes (7). Thus, targeting vaccine-induced T-cell responses to the genital lesion should be important in the development of an effective therapeutic vaccine against the HPV-related genital disease.

Several groups have been developing treatment regimen to recruit HPV-specific T cells into the genital lesion. Local administration of toll-like receptor (TLR) 7 and 9 agonists, imiquimod or CpG, after parenteral HPV16 E7 vaccination has been shown to induce accumulation of E7-specific CD8 T cells in the genital tract and regression of genital tumors (16, 17). However, in humans, imiquimod use can induce severe adverse events, including, but not limited to, acute and severe local inflammation and ulceration (18, 19), while treatment of CpG requires repeated injections due to short-lived efficacy (17). In addition, some studies have demonstrated the ability of cytokines, such as IL2 and IL15, to serve as mucosal vaccine adjuvants in animal models to enhance the therapeutic efficacy (20, 21). However, these cytokines induced antibody-secreting plasma cells rather than CD8 T-cell responses in the mucosal tissues. Therefore, there is still a clinical need to develop a safe and highly effective therapeutic regimen for inducing local CD8 T-cell response against genital lesion.

IL7 is known to be an essential factor in the proliferation and homeostasis of T cells. Exogenous IL7 treatment with various types of vaccines enhanced the cytolytic activity of CD8 T cells (22) and the generation of long-lasting memory T cells (23), indicating that IL7 might be an ideal vaccine adjuvant to improve CD8 T-cell response to vaccine antigens. However, these studies typically focused on the function of IL7 in the lymphatic system, while its role in the induction of CD8 T-cell response at the genital tract has not been reported previously.

The neonatal Fc receptor (FcRn) is present in mucosal epithelia (lung, intestine, and genital mucosa) and is involved in the transport of IgG antibodies across the epithelial barrier (24). Studies have shown that the FcRn/IgG axis could be used to enhance the transport of mucosally introduced Fc-fused proteins (25, 26). Thus, the approach of delivering the desired cytokine through an Fc fusion may provide a valuable tool for investigating the immunomodulatory role of cytokine in mucosal tissue.

In this study and for the first time, we have elucidated the immunomodulatory role of Fc-fused IL7 (IL7-Fc) in the genital tract. After intravaginal administration, IL7-Fc more efficiently crossed the genital epithelial barrier in an FcRn-dependent manner than native IL7. We also demonstrated that intravaginal IL7-Fc administration significantly augmented recruitment of CD4, CD8, and γδ T cells, as well as dendritic cells (DC) in the genital tract, presumably due to induction of various chemokines, cytokines, and an adhesion molecule. More importantly, topical IL7-Fc administration following intramuscular HPV DNA immunization enhanced the accumulation of vaccine-induced CD8 T cells in the cervicovaginal (CV) tissue, which correlated well with genital tumor suppression. Taken together, these results suggest that the topical IL7-Fc application with systemic vaccination is a potentially effective therapeutic strategy for not only enhancing tissue-localized CD8 T-cell response but also eradicating virus-induced premalignant and malignant mucosal lesions.

Materials and Methods

Animal

Female C57BL/6 mice, 8 to 10 weeks of age, were purchased from The Jackson Laboratory. All animals were housed under specific pathogen-free conditions in the animal care facility in Pohang University of Science and Technology (Pohang, Gyeongbuk, Republic of Korea). The procedures of animal experiments were performed in accordance with the NIH (Bethesda, MD) guidelines for mouse experiments. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

Preparation and treatment

The codon-optimized human IL7 (27) and G-CSF gene were individually fused with a hybrid Fc-fragment, which contains the upper CH2 domain of IgD, and the last CH2 and CH3 domains of IgG4 (28). The schematic structure of Fc-fused IL7 is presented in Supplementary Fig. S1. Chinese hamster ovary (CHO) cells were stably transfected with a plasmid encoding IL7-Fc or G-CSF-Fc, and proteins were prepared as described previously (29). Recombinant human IL7 (rIL7), purified from HEK 293E cells, was purchased from BioLegend. Detailed descriptions of cytokine treatment, FTY720 treatment, HPV DNA vaccine (GX188) immunization, and electroporation are provided in Supplementary Materials and Methods.

Distribution and retention of fluorescence-conjugated IL7-Fc in the genital tract

IL7-Fc was coupled with Cy-5.5 monoreactive NHS ester according to the manufacturer's instructions (GE Healthcare). Eluted proteins were desalted and concentrated by using centrifugal filter devices (Merck Millipore), and protein concentration of the dye-labeled IL7-Fc was measured using an anti-human IL7 ELISA set (SouthernBiotech). Diestrus mice were intravaginally administered with an equivalent signal intensity of Cy-5.5–conjuganted IL7-Fc (1 mg/kg) or unconjugated Cy-5.5 in PBS. Further details are provided in Supplementary Materials and Methods.

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Quantification of cytokines and chemokines in the CV cells

To measure the levels of the chemokines and cytokines in CV cell culture supernatant, 1×10^6 CV cells were maintained in RPMI1640, supplemented with 10% FBS (HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) for 2 days prior to supernatant collection. The levels of chemokines and cytokines were estimated with a MILLIPLEX MAP Mouse Cytokine/Chemokine Kit (Millipore).

Immunohistochemical staining and quantification of fluorescence intensity

Tissues were embedded in optimal cutting temperature compound (Tissue-Tek), frozen in liquid nitrogen. Frozen blocks were cut into 7-μm thick sections, fixed in acetone, followed by staining with anti-CD31 (MEC13.3, BioLegend) and VCAM-1 (429 (MVCAM.A), BioLegend). For the quantification of fluorescence intensity, a previously described method was employed (30). Further details are provided in Supplementary Materials and Methods.

Splenocytes and CV cell preparation

Spleen and CV tissue were surgically excised using sterile technique. The splenocytes were obtained by gentle mechanical disruption. For the preparation of CV cells, the uterine horns were removed from the genital tract, and CV tissue was minced and digested with 1 mg/mL collagenase D (Roche) and 0.5 mg/mL DNase (Sigma-Aldrich). The cells were passed through a 40-μm strainer (BD), washed, and resuspended with RPMI1640 containing 10% FBS and antibiotics.

Flow cytometry analysis

Cells were stained with the appropriate combination of the monoclonal antibodies (mAbs). Detailed information of antibodies and tetramer staining method is provided in Supplementary Materials and Methods.

In vivo tumor experiments

The TC-1/luc cell line, TC-1 cotransformed with the HPV16 E6, E7 gene and transfected with a luciferase gene, was kindly provided by Professor T.-C. Wu (Johns Hopkins Medical Institutions, Baltimore, MD; ref. 31). Each diestrus mouse was intravaginally inoculated with 1×10^5 TC-1/luc cells by using the methods described previously (32). Further details are provided in Supplementary Materials and Methods.

Statistical analysis

A two-tailed paired Student *t* test was used to evaluate the statistical difference between the two experimental groups. For *in vivo* tumor experiments, differences in survival rates between groups were determined by a log-rank test using the Prism 5.0 software (GraphPad).

Results

FcRn-mediated transcytosis of Fc-fused IL7 in genital tract

As FcRn enhances Fc-mediated transcytosis across genital epithelial cells, we first examined whether IL7-Fc could be efficiently delivered into genital tract tissues. After mice were treated with topical Cy-5.5–conjugated IL7-Fc (Cy5.5–IL7-Fc) or unconjugated Cy-5.5 (Cy5.5), we determined the distribution and the amount of the fluorochrome by using quantitative fluorescence imaging. The intensity of Cy5.5–IL7-Fc significantly increased 1 day after treatment and was observed until day 7 in the CV tissue. In particular, the signal intensity in CV tissue of Cy5.5–IL7-Fc treated mice was 6- or 4.5-fold higher than that of Cy5.5 alone treated mice at 1 or 7 days after treatment, respectively. Moreover, the fluorescent signal was detected at measurable levels in various anogenital tissues (i.e., cervix-vagina, uterus, ovary, and rectum) of Cy5.5–IL7-Fc treated mice, but not in the Cy5.5-treated mice (Fig. 1A). Thus, the Fc fusion approach can enhance mucosal epithelial barrier penetration at the local level and the retention of IL7 in genital tract after topical treatment. Interestingly, a higher level of fluorescence was observed not only in the genital tract tissues but also in the liver, kidney, and spleen in mice treated with Cy5.5–IL7-Fc than those treated with Cy5.5 at 1 and 7 days after treatment, indicating that transcytosed IL7-Fc can circulate throughout the body (Fig. 1A). To confirm whether intravaginal (IVAG) administration of IL7-Fc penetrates mucosal epithelial lining and allows systemic circulation, we measured the serum concentration of IL7 after mice were intravaginally treated with either rIL7 or IL7-Fc. Mice treated with IL7-Fc, but not rIL7, exhibited significantly increased level of IL7 compared with PBS control (Supplementary Fig. S2). These data are in close agreement with the previous study that demonstrated an increased level of IgG in the serum of wild-type mice but not of FcRn-KO mice after IVAG injection, indicating that FcRn in mucosa epithelium can mediate transcytosis of Fc-fused protein in the genital tract (26). Taken together, Fc fusion to IL7 markedly improves the genital epithelial barrier transcytosis after local administration.

Substantial increase of T-cell infiltration in genital tract after local application of IL7-Fc

As IL7 is known to have multiple functional roles in regulating a variety of immune cells (23, 33, 34), we analyzed the change of leukocyte populations in the CV tissue after topical IL7-Fc treatment. Local delivery increased the absolute numbers of CD4 and CD8 T cells (Table 1; Fig. 1B). This increase of genital tract T cells peaked at day 7 after IL7-Fc treatment and gradually decreased to the baseline levels by day 14 (Table 1). Moreover, the number of CD4 or CD8 T cells was significantly increased by about 20-fold or 10-fold, respectively, 7 days after IL7-Fc administration compared with the baseline levels (Table 1). Of note, the absolute numbers of $CD44^{high}CD62L^{low}$ effector CD4 and CD8 T cells were significantly increased at day 7 and waned in a similar pattern to the total CD4 and CD8 T cells over time (Table 1; Fig. 1B), whereas the number of T cells did not change after topical rIL7 treatment (Fig. 1B). These results of T-cell kinetic profile and immunomodulatory effect agree with our recent study on the T-cell immunomodulatory effect of topical applied IL7-Fc in the lungs (35). Interestingly, the majority of the CD4 T cells were found in the submucosa, whereas CD8 T cells preferentially localized in the epithelium after IVAG treatment of IL7-Fc (Supplementary Fig. S3). Overall, topical IL7-Fc delivery causes the infiltration of CD4 and CD8 T cells, which display distinct migration patterns in the genital tract.

Intravaginal administration of IL7-Fc also increased the absolute numbers of γδ T cell and monocyte-derived DC ($CD11b^+CD11c^+MHCII^+$) at day 3. Although slightly numerically superior, in the CV tissue of IL7-Fc–treated mice, the conventional genital DCs ($CD11b^-CD11c^+MHCII^+$) were not significantly different from the baseline levels at day 3. The number of γδ T cells, conventional DCs, and monocyte-derived DCs also peaked at day 7, at which they increased up to about 45-, 6-, and 8-fold,

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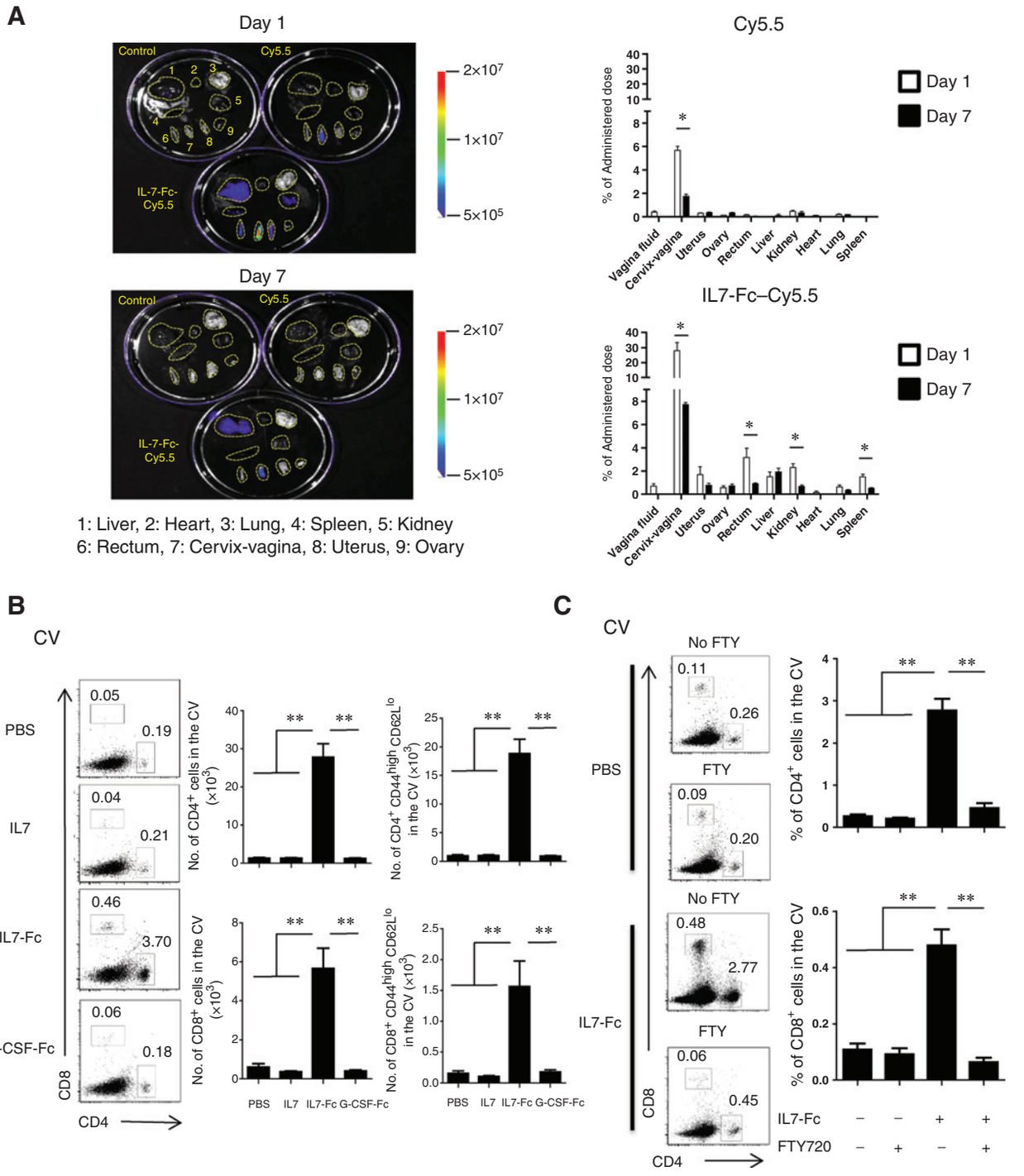

Figure 1.

FcRn-mediated transcytosis of IL7-Fc and recruitment of T cells in CV tissue by intravaginal administration of IL7-Fc, but not IL7 and G-CSF-Fc. **A,** C57BL/6 wild-type mice ($n = 3$/group) were intravaginally administered with Cy-5.5 or Cy-5.5–conjugated IL7-Fc. Fluorescent images (left) and bar graph depicting fluorescence intensity (right) of the various organs at day 1 and 7 are shown. **B,** Mice ($n = 6$/group) were treated with PBS, IL7, IL7-Fc, or G-CSF-Fc. After 7 days, CD4 and CD8 T cells in the CV tissue were analyzed by flow cytometry. Left, data are representative dot plots of T cells in CV tissue; middle, absolute number of CD4 or CD8 T cell counts; right, absolute numbers of $CD62L^{lo}CD44^{high}$ subsets in CD4 or CD8 T-cell populations. **C,** Mice ($n = 4$/group) were treated intravaginally with PBS or IL7-Fc. One day later, FTY720 was administered twice at 3-day intervals. CD4 and CD8 T cells in CV tissue were analyzed by flow cytometry at day 7. Representative dot pots (left) and frequency (right) of T cells in CV tissue. Data, shown as means ± SEMs, are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$ by Student t test.

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Table 1. Kinetics of CV leukocytes after intravaginal IL7-Fc treatment

	Absolute cell number after IL7-Fc treatment				
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Total CD4 T cells ($\times 10^3$)	2.86 ± 0.49	12.76 ± 0.53[a]	51.51 ± 9.18[a]	3.33 ± 0.77	2.57 ± 0.44
$CD62L^{lo}$ $CD44^{high}$ CD4 T cells ($\times 10^3$)	2.21 ± 0.31	10.26 ± 0.68[a]	35.06 ± 7.03[a]	2.51 ± 0.72	2.13 ± 0.41
Total CD8 T cells ($\times 10^3$)	0.49 ± 0.08	1.65 ± 0.18[a]	6.21 ± 0.76[a]	0.65 ± 0.17	0.84 ± 0.30
$CD62L^{lo}$ $CD44^{high}$ CD8 T cells ($\times 10^3$)	0.11 ± 0.01	0.64 ± 0.11[a]	1.96 ± 0.29[a]	0.23 ± 0.10	0.27 ± 0.14
γδ T cells ($\times 10^3$)	0.61 ± 0.14	2.40 ± 0.30[a]	28.58 ± 3.88[a]	2.05 ± 0.56[a]	1.80 ± 0.07[a]
Conventional DC ($\times 10^3$)	0.33 ± 0.07	0.48 ± 0.09	2.15 ± 0.31[a]	1.02 ± 0.12[a]	0.56 ± 0.04
Monocyte-derived DC ($\times 10^3$)	4.78 ± 0.28	10.15 ± 0.83[a]	38.89 ± 2.10[a]	14.66 ± 2.16[a]	5.64 ± 1.03

NOTE: Mice ($n = 3$/group) were intravaginally administered with IL7-Fc at 0, 3, 7, 14, and 21 days prior to sacrifice. Absolute numbers of leukocytes in the CV tissue were calculated on the basis of the percentage of total cell number with flow cytometry. Data, shown as means ± SEMs, are representative of two independent experiments.

[a]$P < 0.05$ by Student t test compared with cell numbers on day 0.

respectively, compared with the baseline levels. Interestingly, a higher cell count was still observed until day 21 for γδ T cells and day 14 for conventional and monocyte-derived DC. Thus, these data indicate that IVAG application of IL7-Fc induces local accumulation of T cells and DCs (Table 1).

As the infiltration of various immune cells may contribute to bystander tissue damage (18, 19), we assessed the toxicity of five repeated weekly treatments of IL7-Fc administered in different dosages (0, 0.8, 3, and 8 mg/kg/dose) in rats (Supplementary Table S1). There were no adverse events in terms of anatomic pathology of CV tissues (Supplementary Table S1A) and vaginal irritation scores (Supplementary Table S1B), indicating that topical IL7-Fc administration was well tolerated and safe in the genital tract.

To clarify whether the immunomodulatory activity of IL7-Fc is specific to the activity of IL7, we compared the frequency of CD4 and CD8 T cells after topical administration of irrelevant Fc-fused cytokine, G-CSF (G-CSF-Fc). As shown in Fig. 1B, the absolute numbers of genital T cells in mice treated with G-CSF-Fc were not significantly different from that of PBS-treated mice. In addition, Fc-fragment alone also did not induce T-cell accumulation in the genital tract (Supplementary Fig. S4). Importantly, treatment of anti-IL7Rα antibody abrogated the infiltration of T cells, suggesting that T cell–modulatory activity of IL7-Fc in CV tissue might be mediated by IL7R (Supplementary Fig. S5). Taken together, IL7, but not the Fc-fragment, has the critical role in the accumulation of T cells at CV by topical IL7-Fc treatment.

Next, we investigated whether the increased T cells were recruited from systemic circulation or proliferated in the local area. Mice that were given IL7-Fc were treated with FTY720, which is known to block T-cell migration from lymph nodes. As shown in Fig. 1C, FTY720 treatment significantly abrogated the number of CD4 and CD8 T cells, indicating that IVAG IL7-Fc treatment recruits CD4 and CD8 T cells from systemic circulation into CV tissues.

To verify whether the delivery route of IL7-Fc was important for T-cell recruitment in CV tissues, mice were treated with IL7-Fc either through intravaginal or subcutaneous routes. A higher level of serum IL7 was observed in mice treated subcutaneously compared with those treated intravaginally (Supplementary Fig. S6A), whereas the extent of CD4 or CD8 T-cell accumulation was greater by IVAG administration than subcutaneous injection (Supplementary Fig. S6B and S6C). Thus, these data suggest that changes in the immune environment of the genital tract induced by local IL7-Fc administration are critical for T-cell recruitment.

Increase of chemokines, cytokines, and an adhesion molecule expression in the genital tract by topical IL7-Fc treatment

Chemokines and cytokines have crucial roles in the recruitment of immune cells into the genital tract tissue. Chemoattractants, such as CXCL10, CCL3, CCL4, and CCL5, as well as cytokines (IFNγ, TNFα, IL6, and IL1β) are well-known soluble factors for the recruitment of leukocytes into the genital tract (36). Thus, we evaluated the effect of IL7-Fc treatment on the expression of these chemokines and cytokines in the CV cells. Remarkably, chemokines, such as ligands for CXCR3 (CXCL10), CCR1 and CCR3 (CCL5), and CCR5 (CCL3, CCL4, CCL5), and cytokines (IFNγ, TNFα, IL6, and IL1β) were significantly increased after IL7-Fc treatment. Overall, chemokines and cytokines were upregulated at day 1 and peaked at day 3, to then decline rapidly to baseline levels by day 7 after IL7-Fc administration (Fig. 2A and B). As these chemokines and cytokines are known to induce the influx of various immune cells, such as T cells, immature DCs, monocytes, into the inflamed tissue (36, 37), the enhanced infiltration of T cells by IL7-Fc treatment (Table 1; Fig. 1B) might be mediated by the induction of these soluble factors. In addition to the chemokines and cytokines, the selective expression of cell adhesion molecules on the local vascular endothelium is crucial for the migration of T cells into the peripheral tissues. Therefore, we examined the expression of the representative adhesion molecule, VCAM-1, in the CV after IVAG treatment of IL7-Fc. As shown in Fig. 2C, IL7-Fc treatment, but not PBS, substantially induced VCAM-1 expression on the local vascular endothelium. Collectively, these results suggest that migration of leukocytes from blood to genital tract may be facilitated by the expression of adhesion molecules as well as the expression of different chemokines and cytokines in the genital tract after IL7-Fc administration.

Increased local accumulation of HPV DNA vaccine-induced CD8 T cells by intravaginal administration of IL7-Fc

To investigate whether IVAG treatment of IL7-Fc can be an effective therapeutic vaccine regimen for inducing antigen-specific CD8 T cells at the genital tract tissue, mice were vaccinated with GX188E, a novel HPV therapeutic vaccine (7), twice at a 7-day interval, and IL7-Fc was intravaginally administered at the last vaccination point. One week after treatment, HPV E7-specific CD8 T cells in spleen or CV cells were enumerated by tetramer staining with TetE7 (H-$2D^b$–restricted $E7_{49\text{-}57}$ peptide-loaded tetramer) or TetL1 (irrelevant HPV16 $L1_{165\text{-}173}$ peptide-loaded tetramer as a negative control). A similar number of HPV16 E7-specific CD8 T cells were induced in the spleen regardless of IL7-Fc administration. As CXCR3 expression on CD8 T cells is an important factor of T-cell recruitment into

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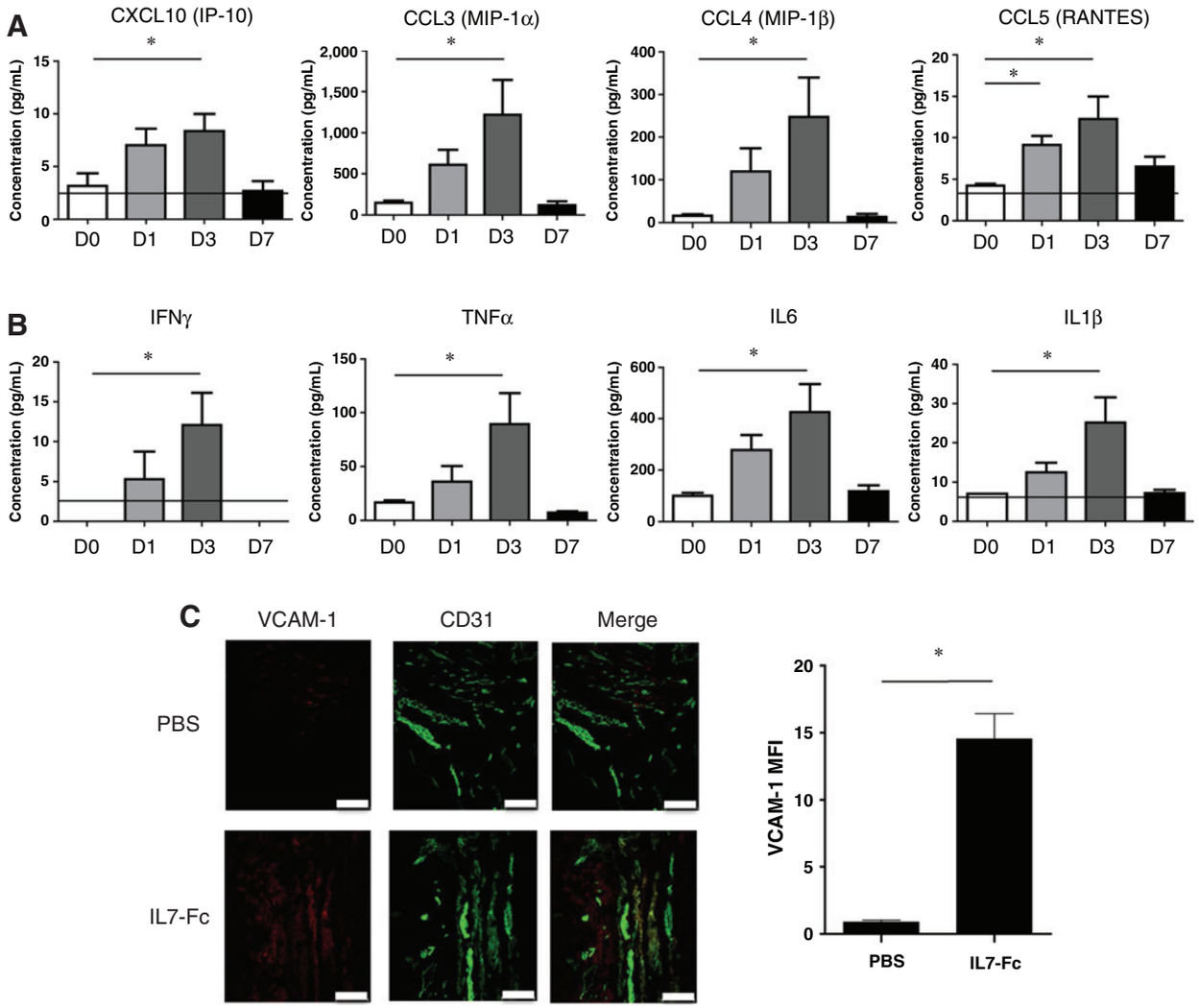

Figure 2.

Induction of various chemokines and cytokines in the CV cells and VCAM-1 on local vascular endothelium after topical treatment of IL7-Fc. Mice ($n = 4$/group) were intravaginally administered with IL7-Fc and sacrificed to harvest CV tissue at indicated days after application. The level of chemokines and cytokines in the CV cell culture supernatant was analyzed by ELISA. The concentrations of chemokines (CXCL10, CCL3, CCL4, and CCL5; **A**) and cytokines (IFNγ, TNFα, IL6, and IL1β; **B**) are shown. Horizontal line, limit of detection. **C,** Microscopic image of CV tissue as stained for VCAM-1 (red) and CD31 (green; left) and mean fluorescence intensity (MFI) of VCAM-1 on $CD31^+$ vessels (right) in mice 7 days after PBS or IL7-Fc treatment. Scale bars, 100 μm. Data, shown as means ± SEMs, are representative of two independent experiments. *, $P < 0.05$ by Student t test.

inflamed tissue (38), we also analyzed the level of CXCR3-expressing HPV16 E7-specific CD8 T cells harvested from the spleen. The absolute number of $CXCR3^+$HPV16 E7-specific CD8 T cells in the spleen seemed to be lower in mice treated with GX188E plus IL7-Fc compared with GX188E alone (Fig. 3A). On the other hand, a combination of GX188E with IL7-Fc significantly increased the number of HPV16 E7-specific CD8 T cells to a greater extent than GX188E alone in the CV tissue. Mice treated with GX188E and IL7-Fc also displayed a significantly higher number of $CXCR3^+$HPV16 E7-specific CD8 T cells in CV tissue compared with those treated with DNA vaccine alone (Fig. 3B). The relatively lower number of $CXCR3^+$HPV16 E7-specific CD8 T cells in spleen may be due to the migration of vaccine-induced T cells into the CV tissue by the locally expressed chemoattractants. Taken together, these data show that IVAG treatment of IL7-Fc with parenteral HPV DNA vaccination enhances the accumulation of vaccine antigen-specific CD8 T cells in the CV.

Enhanced antitumor effects induced by HPV DNA vaccination in the orthotopic murine cervical cancer model by topical treatment of IL7-Fc

We next investigated whether topical treatment of IL7-Fc could further enhance the antitumor efficacy of HPV DNA vaccination. To this end, mice were intravaginally challenged with luciferase-expressing TC-1 tumor cells (TC-1/luc) and treated with GX188E and IL7-Fc 7 days after tumor inoculation (Fig. 4A). When we measured tumor burden by examining bioluminescence signal

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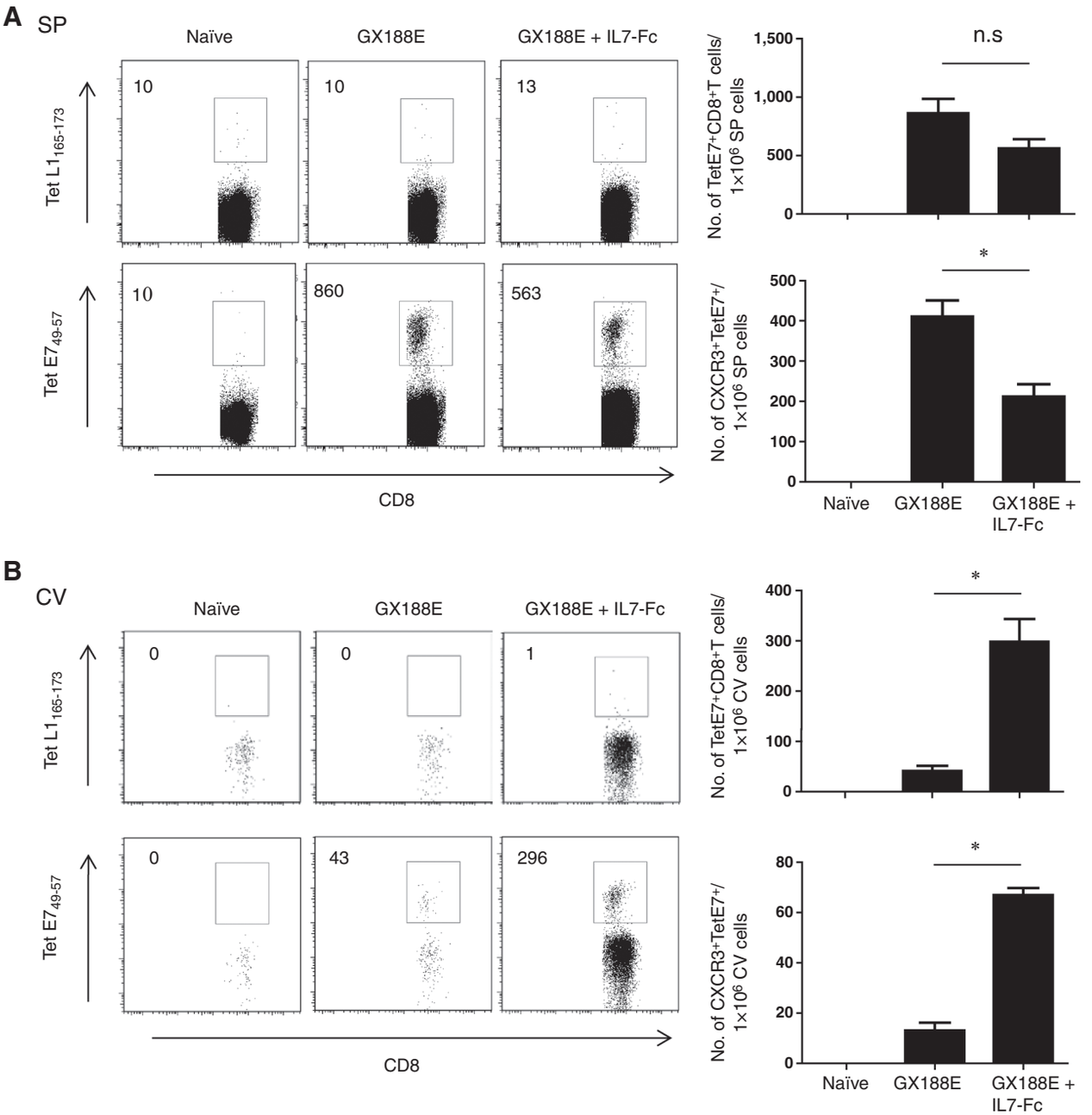

Figure 3.

Increase of HPV DNA vaccine-induced E7-specific CD8 T cells in CV tissue, but not in spleen, by topical administration of IL7-Fc. Mice ($n = 4$/group) were vaccinated with GX188E DNA via intramuscular injection. After 7 days, mice were boosted with GX188E DNA in combination with or without intravaginal IL7-Fc application. One week after the second immunization, spleen and cervicovaginal tissue were harvested and analyzed by flow cytometry. **A,** Representative dot plots (left) and absolute number of $TetE7_{49\text{-}57}{}^{+}CD8$ T cells (top right) and absolute number of $CXCR3^{+}TetE7_{49\text{-}57}{}^{+}CD8$ T cells (bottom right) in the spleen. n.s., not significant. **B,** Representative dot plots (left) and absolute number of $TetE7_{49\text{-}57}{}^{+}CD8$ T cells (top right) and absolute number of $CXCR3^{+}TetE7_{49\text{-}57}{}^{+}CD8$ T cells (bottom right) in the CV tissue. Data, shown as means ± SEMs, are representative of three independent experiments. *, $P < 0.05$ by Student t test.

intensities and survival rate, untreated mice showed rapid tumor growth and succumbed rapidly after tumor challenge. In contrast, IL7-Fc alone showed delayed tumor growth rate compared with untreated mice, and significant antitumor effect was shown in mice treated with GX188E alone. Most notably, mice cotreated with GX188E vaccine and IL7-Fc exhibited markedly delayed tumor growth and enhanced survival rate even compared with those treated with GX188E alone (Fig. 4B and C). To further determine the role of CD8 T cells in combinatory antitumor therapeutic efficacy, vaccine plus IL7-Fc adjuvant-treated

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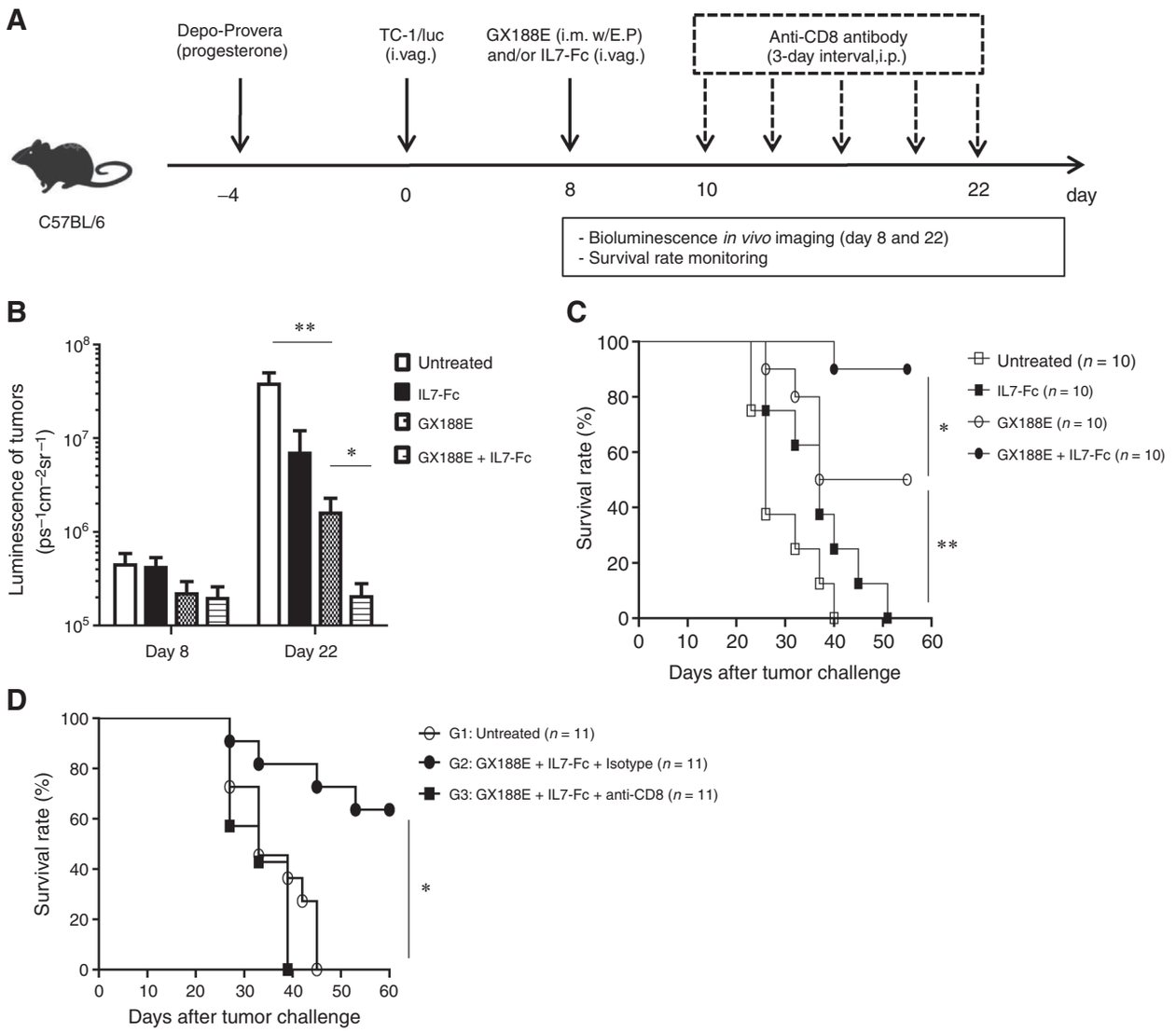

Figure 4.

Correlation of HPV DNA vaccine-induced CD8 T cells with therapeutic activity against cervical cancer. **A,** Schematic diagram of tumor challenge and treatment schedule. Mice were intravaginally (i.vag.) injected with 1×10^5 TC-1/luc cells. After 7 days, mice harboring genital tumor were detected by luminescence ($ps^{-1}cm^{-2}sr^{-1}$). GX188E DNA was intramuscularly (i.m.) injected into mice with or without intravaginal IL7-Fc administration. i.p., intraperitoneal. **B,** Bar graph depicting bioluminescence intensity of tumor-bearing mice on day 8 and day 22. *, $P < 0.05$; **, $P < 0.01$ by Student t test. **C,** Percentage of survival in each group as a function of time is represented. *, $P < 0.05$ (GX188E vs. GX188E + IL7-Fc); **, $P < 0.01$ (untreated vs. GX188E) by log-rank (Mantel–Cox) test. **D,** Seven days after tumor challenge, mice were treated with GX188E DNA and IL7-Fc. After 2 days, mice received isotype (rat-IgG2b), anti-CD8 antibodies at 3-day intervals for 22 days after tumor challenge. Percentage of survival in each group upon time is represented. *, $P < 0.05$ (GX188E + IL7-Fc + isotype vs. GX188E + IL7-Fc + anti-CD8) by log-rank (Mantel–Cox) test. Data, shown as means ± SEMs, are representative of three independent experiments.

mice were injected with either isotype or CD8 T cell–depleting antibodies after tumor challenge. As shown in Fig. 4D, mucosal tumor control was abolished in CD8 T cell–depleted mice, indicating that CD8 T cells have a crucial role in the augmented antitumor activity by HPV DNA vaccine with IL7-Fc.

Discussion

Here, we have demonstrated that Fc-fused IL7 can be efficiently delivered across the genital epithelial barrier in an FcRn-dependent manner after topical delivery. Importantly, after intramuscular HPV DNA vaccination to generate a systemic CD8 T-cell response, a single intravaginal application of IL7-Fc can provide superior antitumor activity against orthotopic cervical cancer, likely by increasing the recruitment of vaccine-induced CD8 T cells into the genital tract. These results provide a novel insight into the role of IL7 as a vaccine adjuvant and highlight several clinical implications. First, this study defines, for the first time, the immunomodulatory role of IL7 in the genital tract by Fc fusion approach to enhance mucosal delivery. This study also showed

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that local IL7-Fc administration markedly increased the recruitment of various leukocytes through the upregulation of adhesion molecule, as well as numerous chemokines, and cytokines at the mucosal site. Second, topical IL7-Fc treatment can elicit better migration of HPV DNA vaccine-induced CD8 T cells into genital tract compared with vaccine alone, which correlated with tumor control. Thus, this combined regimen potentially improves the efficacy of therapeutic DNA vaccine by inducing mucosal cellular immunity and can also be an important first step in providing a pathway to design effective therapy for curing patients with HPV-related genital lesions. Finally, as FcRn has been also detected in the other mucosal tissue, such as lung and intestine, our findings give a rational strategy for inducing immune response, particularly T-cell response, in other mucosal tissues and protection from a variety of infectious mucosal diseases and cancers.

This study also showed that Fc-fused IL7 more efficiently crossed the genital epithelial layer than rIL7 via topical administration (Supplementary Fig. S2). In our previous study, the serum IL7 level was comparable in mice treated with either IL7-Fc or rIL7 immediately after intraperitoneal injection, and the *in vivo* IL7-Fc level declined more slowly than rIL7 due to the prolonged circulating half-life by Fc fusion (27). In contrast, in this study, the serum IL7 level was initially undetectable (Supplementary Fig. S2), suggesting that Fc fusion to IL7 is more important for crossing the genital epithelium rather than half-life extension in mucosal delivery. In addition, topical administration of IL7-Fc induced T-cell accumulation in the genital tract (Fig. 1B), wherein genital CD4 and CD8 T cells were each localized in the submucosa and epithelium, respectively (Supplementary Fig. S3). This result is supported by a previous observation that the localization patterns of CD4 and CD8 T cells are distinct in the genital tract (39). Notably, tissue-localized CD4 T cells were clustered in the vaginal submucosa and required for the migration of CD8 T cells into the epithelium through the secretion of cytokine and induction of the local chemokine gradient (40, 41). Moreover, we demonstrated that local treatment of IL7-Fc elicited expression of various soluble mediators in the tissue, such as chemokines, cytokines, as well as a cell adhesion molecule (VCAM-1) on vascular endothelium in the genital tract (Fig. 2). These results are in agreement with previous reports that circulating CD8 T cells are rapidly recruited to the genital tract after local expression of inflammatory chemokines (30, 42). We also showed that treatment of FTY720 abrogated the accumulation of T cells in the CV tissue (Fig. 1C). Taken together, it is likely that immunomodulatory activities of topical IL7-Fc treatment induce recruitment of T cells from systemic circulation rather than the proliferation of locally resident T-cell population, presumably due to the expression of tissue localization signals in the genital mucosa that seem to be critical for T-cell recruitment.

Topical administration of IL7-Fc treatment attracted activated T cells and also innate immune cells, including conventional DCs and monocyte-derived DCs. Notably, a significant increase of γδ T cells in genital tract tissues was also observed (Table 1). Given that the immunomodulatory effects of IL7 were induced by an IL7Rα (CD127)–dependent manner (Supplementary Fig. S5), IL7R-expressing cells such as αβ (CD4, CD8) and γδ T cells that participate in the mucosal immunity might be involved in the recruitment of various leukocytes. Although αβ and γδ T cells augment the cytotoxic response and express a similar set of cytokines upon activation, γδ T cells play critical roles in immediate host defense because γδ T cells expand more rapidly than αβ T cells (43, 44). Consistently, in topical IL7-Fc–treated mice, the extent of expansion in γδ T cell (46.8-fold) was more remarkable than that of CD4 or CD8 T cell (18.1-fold or 12.7-fold, respectively) 7 days after treatment compared with baseline levels (Table 1). Because of γδ T-cell capacities, a topical application of IL7-Fc could confer partial protection to genital tumor (Fig. 4C). Moreover, γδ T cells secrete cytokines and chemokines, including IFNγ, CCL3, CCL4, and CCL5 (45, 46). Secretion of IFNγ in the reproductive tract induces the expression of VCAM-1 on local vascular endothelium, which leads to the recruitment of systemically circulating T cells (42). In addition, the expression of CXCL10, which is largely IFNγ dependent, is chemotactic for $CXCR3^+$ T cells and important for antitumor activity (47). In this regard, local treatment of IL7-Fc could presumably induce the expression of CXCL10 in CV tissue (Fig. 2A) and attract $CXCR3^+$ CD8 T cells into the genital tract (Fig. 3B). Furthermore, considering that CCL3 and CCL4, and CCL5 have been known to act as chemoattractants for DCs (48), they may mediate DC recruitment into the genital tract after local IL7-Fc application. Thus, γδ T cells as an initial responder may orchestrate mucosal immunity after intravaginal IL7-Fc treatment by secreting chemokines and cytokines at the genital mucosa, and inducing adhesion molecules in the local vasculature, leading to the recruitment of several leukocytes into the genital tract.

In our previous study, we found that Fc-fused IL7 could alter pulmonary immune environment after intranasal treatment (35). In addition, this study showed that FcRn in the lung was indispensable for transcytosis and immunomodulation of IL7-Fc, which is consistent with this study's results of immunomodulatory activity in the genital tract. Similar kinetics of immune cell recruitment, including CD4, CD8, and γδ T cells, has also shown in the respiratory tract after local administration. This phenomenon might be mediated by similar IL7-responsive cells residing in both the lung and genital tract. Interestingly, the lung-retentive polyclonal CD4 T cells, which were induced by IL7-Fc alone, had prophylactic effects against influenza infection (35), whereas tumor-specific CD8 T cells induced by IL7-Fc and HPV DNA vaccine played an essential role for therapeutic effects in the mucosal tumor suppression. Overall, topical IL7-Fc administration into FcRn-expressing mucosal tissues induces T-cell modulation, suggesting that the topical IL7-Fc application can be used as a mucosal adjuvant to confer protective and therapeutic immunity against virus infection at mucosal tissues.

Imiquimod, one of the known agents against genital diseases, increases the number of CD8 T cells in neoplastic lesion of the vulvar area through TLR7-mediated enhancement of innate immune responses, including secretion of cytokines (19). In addition, imiquimod facilitates HPV DNA vaccine-induced CD8 T-cell accumulation in the genital tract, leading to tumor control in a mouse cervical cancer model (16). However, in patients, severe local inflammations and ulcerations caused by excessive production of proinflammatory cytokines with repeated topical treatments limit the therapeutic potential and wider adoption (18, 19). Although proinflammatory responses were transiently induced by the topical IL7-Fc delivery, repeated injections did not cause observable adverse effects in anatomic pathology or vaginal irritation (Supplementary Table S1). Another safety concern in the human application of IL7-Fc

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can be immunogenicity, from an immune response against Fc component (49). When we measured for antidrug antibody (ADA) in sera of the IL7-Fc–treated cynomolgus monkeys, ADA was detected in subcutaneously treated monkeys without affecting patterns of pharmacokinetics and pharmacodynamics between ADA-positive and -negative monkeys, suggesting that ADA dose not neutralize the activity of IL7-Fc in monkeys (data not shown). We assume that the main factor for ADA induction in monkeys may account for the amino acid difference of IL7 and Fc-fragment between humans and monkeys. In fact, other Fc-fused proteins (EPO-Fc, G-CSF-Fc, and hGH-Fc), which are fused with same Fc-fragment, also generated ADA in monkeys. Importantly, these proteins do not induce immunogenicity-related adverse events and ADA production in a total of 531 human volunteers in phase I/IIa clinical trials (EPO-Fc: NCT02291991/NCT02044653, G-CSF-Fc: NCT01951027/EudraCT 2015-002693-20, and hGH-Fc: EudraCT 2013-002771-18/EudraCT 2014-002698-13 and EudraCT 2015-001939-21). Furthermore, the commercialized Fc-fused proteins, including abatacept (CTLA4-IgG1) and etanercept (TNFR-IgG1), induce ADA in humans, but these antibodies do not affect the drug efficacy (50). Taken together, although it will be necessary to address the immunogenicity issues of IL7-Fc in future study, we speculate that the application of IL7-Fc in humans may be safe and does not cause immunogenicity-related problems.

Surgical treatment of HPV-related intraepithelial lesion does not permanently eradicate HPV and is associated with high recurrence rates (51). Thus, development of effective immunotherapy is important to prevent recurrence. IL7 as a vaccine adjuvant plays critical roles in the transition of effector cells to memory CD8 T cells and enhances the generation of vaccine-induced long-term memory CD8 T cells (23). This study suggests that vaccine-specific memory CD8 T cells induced by IL7 might be more potent than those induced by vaccine alone in preventing reinfection. Thus, even though it is not clear whether IL7-Fc can enhance the generation of memory CD8 T cells in the genital tract, it gives rise to the possibility that these T cells may prevent recurrent genital diseases, such as CINs and genital herpes.

In conclusion, our findings provide a novel role of IL7 in promoting cellular immunity in the genital mucosa. In addition, topical IL7-Fc administration along with HPV DNA vaccine is a safe and potent strategy for enhancing therapeutic efficacy against HPV-related genital disease. Furthermore, this study can promote a series of future investigations on the use of IL7-Fc as a mucosal modulator for treating other mucosal cancers and infectious diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.W. Choi, M.C. Kang, H. Namkoong, D.-H. Choi, S.-W. Lee, Y.C. Sung, H.-T. Jin

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.W. Choi, M.C. Kang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.W. Choi, M.C. Kang, Y. Park, S.-W. Lee, H.-T. Jin

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Clinical Cancer Research

Intravaginal Administration of Fc-Fused IL7 Suppresses the Cervicovaginal Tumor by Recruiting HPV DNA Vaccine-Induced CD8 T Cells

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