Expression of LIGHT/TNFSF14 Combined with Vaccination against Human Papillomavirus Type 16 E7 Induces Significant Tumor Regression

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Abstract

LIGHT, a ligand for the lymphotixin-β receptor, establishes lymphoid-like tissues inside tumor sites and recruits naive T cells into the tumor. However, whether these infiltrating T cells are specific for tumor antigens is not known. We hypothesized that therapy with LIGHT can expand functional tumor-specific CD8+ T cells that can be boosted using HPV16E6E7-Venezuelan equine encephalitis virus replicon particles (HPV16-VRP) and that this combined therapy can eradicate human papillomavirus 16 (HPV16)–induced tumors. Our data show that forced expression of LIGHT in tumors results in an increase in expression of IFNγ and chemotactic cytokines such as interleukin-1α, MIG, and macrophage inflammatory protein-2 within the tumor and that this tumor microenvironment correlates with an increase in frequency of tumor-infiltrating CD8+ T cells. Forced expression of LIGHT also results in the expansion of functional T cells that recognize multiple tumor antigens, including HPV16 E7, and these T cells prevent the outgrowth of tumors on secondary challenge. Subsequent boosting of E7-specific T cells by vaccination with HPV16-VRP significantly increases their frequency in both the periphery and the tumor and leads to the eradication of large well-established tumors, for which either treatment alone is not successful. These data establish the safety of Ad-LIGHT as a therapeutic intervention in preclinical studies and suggest that patients with HPV16+ tumors may benefit from combined immunotherapy with LIGHT and antigen-specific vaccination. Cancer Res; 70(10); 3955–64. ©2010 AACR.

Introduction

The importance of the cellular immune system in controlling the pathogenesis of human papillomavirus (HPV) and associated cervical lesions in humans is very well established (1–7). In the case of HPV16-induced cancers, the most frequently targeted antigens are the E6 and E7 proteins because they are oncogenic and sustained expression is required for the maintenance of the cancerous phenotype. This blatant expression of foreign antigens in a tumor makes HPV a very attractive target for “proof-of-concept” studies in the development of therapeutic vaccines. Several therapeutic vaccines that target the E6 and/or E7 proteins have been developed over the last 15 years (reviewed in ref. 8). These include regimens based on peptides, proteins, plasmids, and viral vectors, each having its strengths and limitations. Of the viral vector–based approaches, Venezuelan equine encephalitis alphavirus replicon particles (VRP; ref. 9) are very promising. VRPs encoding several viral genes are immunogenic and protective (10–14). Previous studies have tested the antitumor efficacy of VRP containing the HPV16 E7 gene and have shown protective responses as well as some therapeutic efficacy for early-stage tumors (15). However, whether HPV16-VRP vaccination can induce regression of large tumors has not been evaluated. Increased frequency of tumor-specific lymphocytes after vaccination argues that vaccine interventions are doing their job, which is to induce tumor-specific T cells. However, the induced T cells are frequently ineffective in controlling tumor growth, likely due to lack of an inflammatory environment in the tumor. Altering the tumor to create a proinflammatory environment in situ that can sustain T-cell activation may lead to regression of large well-established tumors.

LIGHT, a ligand for LTβR and herpes virus entry mediator (HVEM), is predominantly expressed on activated dendritic cells (DC) and lymphocytes in the spleen (18). TNFSF14/LIGHT, a ligand for LTβR and herpes virus entry mediator (HVEM), is predominantly expressed on activated immune cells, especially on the surface of activated DCs and T cells (16, 20–23). Expression of LIGHT restores lymphoid structures
in lymphotoxin-β knockout mice, establishes lymphoid-like tissues inside tumor sites via its interactions with LTβR on stromal cells, induces intratumoral production of chemokines such as CCL21, and recruits naïve T cells into the tumor (16, 22–24). It coordinately induces activation and expansion of incoming T cells through HVEM, thereby functioning as a costimulatory molecule and generating stronger antitumor immunity (20, 21, 23, 24). Although enhanced T-cell infiltration is observed in LIGHT-expressing tumors, their specificity is unknown.

The C3.43 cell line is an aggressive derivative of the C3 line, which was transformed using a pRSVneo-derivative plasmid containing the HPV16 genome (25). The cell line expresses an H-2Db-bound peptide, E7(49–57) (RAHYNIVTF), and CTLs that recognize E7(49–57) are capable of eradicating HPV16-transformed cells in vitro and in vivo (25, 26). The ampitope (SSPVNSLRNVV) peptide is also a highly immunogenic H-2Db–restricted epitope that was encoded by a cryptic open reading frame in the antisense strand of the ampicillin resistance gene of the plasmid backbone, and vaccination with the ampitope peptide protects against challenge with C3-derived cell lines (27). Therefore, the ampitope peptide also functions as a tumor-specific antigen in this model. In the present study, we examined whether therapy with either HPV16-VRP or forced expression of LIGHT in large well-established HPV16+ tumors could induce regression and whether combined therapy had superior efficacy in inducing antitumor immunity that could eradicate tumors.

Materials and Methods

Mice and cell lines. Specific pathogen-free female C57BL/6 mice, 6 to 8 weeks old, were purchased from Taconic Farms. Tumor challenge studies were performed using the C3.43 cell line, an in vivo passaged derivative of the C3 HPV16+–transformed murine tumor cell line (25). C3.43 cells were clonally expanded in vitro from a progressively growing C3 tumor in vivo, and low–passage number working vials were frozen as seed stocks. C3.43 cells retained expression of the HPV16 E6 and E7 (by reverse transcription-PCR and Western blot), expressed similar levels of MHC class I molecules on the surface compared with the parental C3 line (by flow cytometry), and responded to prophylactic vaccination with various HPV16 E7–containing vaccines in vivo. C3.43 cells tested negative for Mycoplasma contamination (MycAlert Mycoplasma Detection kit, Cambrex, Inc.). Cells used for tumor challenge were cultured no longer than 2 weeks from original seed stocks before in vivo injection. C3.43 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum. All procedures were performed in accordance with institutional guidelines and approved by the University of Southern California Institutional Animal Care and Use Committee (USC IACUC).

Peptides. The HPV16 E7(49–57) RAHYNIVTF peptide (25), the SSPVNSLRNVV ampitope peptide (amino acids 49–59; ref. 27), and the AQMNRRDCL peptide from prostate stem cell antigen [PSCA(23–31); ref. 28] were synthesized at the University of Chicago (Chicago, IL) and purified by reverse-phase high-performance liquid chromatography (HPLC). Purity was assessed by analytic HPLC and determined to be >90% pure.

Tumor challenge and treatment. Groups of fifteen 8-week-old female C57BL/6 mice were challenged s.c. in the right flank with 5 × 10^6 C3.43 tumor cells in PBS. Throughout the duration of the experiments, tumor growth was monitored two to three times per week with a caliper in three dimensions. Mice were euthanized per USC IACUC guidelines when tumor volume exceeded 1,500 mm^3 or when ulcerated.

In experiments examining efficacy of therapeutic vaccination with HPV16-VRP (29), mice were vaccinated with 10^5 infectious units of VRP s.c. in 50 μL PBS on days 14 and 21 after tumor challenge, as boosting after 1 week is optimal for generating immune responses. In studies examining the effect of LIGHT, recombinant adenovirus carrying DNA encoding for the LIGHT gene (Ad-LIGHT) was injected intratumorally at 10^10 virus particles per mouse on day 14, and the second injection was done after an interval of 72 hours, which has been previously (30) optimized to prevent adenovirus-specific immune responses from neutralizing the delivery vector. Particles carrying either LacZ or green fluorescent protein (GFP) were used as controls (Ad-CTRL or VRP-CTRL). For combined therapy, mice were treated with adenovirus particles as described and vaccinated with 10^5 infectious units of either HPV16-VRP or GFP-VRP on days 24 and 31. No treatment-related toxicities were noted.

Tumor resection and rechallenge. Survival surgeries were performed under the guidance of an experienced veterinarian according to protocols approved by USC IACUC. Briefly, mice were anesthetized and tumors were surgically excised under sterile conditions. Skin flaps were closed using Nexa- bond tissue adhesive followed by suturing. Mice were rechallenged with 5 × 10^5 C3.43 tumor cells in the left flank and monitored until revival. Tumor growth and survival were monitored twice per week. Mice were euthanized per USC IACUC guidelines.

Isolating tumor-infiltrating lymphocytes. Tumor-infiltrating lymphocytes (TIL) were isolated as described previously (28, 31). Tumor tissues were cut into small pieces and digested in medium supplemented with 0.25% (w/v) dispase for 2 hours with continuous stirring at 37°C. The tumor digest was passed through a nylon mesh to obtain a single-cell suspension, washed with PBS, and analyzed by flow cytometry to determine the frequency of E7-specific T cells in the tumors.

Flow cytometry and tetramer staining. H-2Db tetramers containing the HPV16 E7(49–57) peptide were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). Single-cell suspension of tumors, which contain TILs, was obtained as described. CD8+ T cells were isolated from individual spleens and pooled lymph nodes using MACS cell sorting (Miltenyi). One million unsorted tumor cells or CD8-enriched splenocytes or CD8-enriched lymphocytes were incubated for 1 hour at 4°C with 0.5 μg/mL tetramer, 1:100 diluted anti-CD3 antibody, and 1:100 diluted anti-CD8 antibody (BD Pharmingen). Cells were
washed twice and fixed in 2% paraformaldehyde. At least 100,000 cells were acquired on the Beckman Coulter FC 500 flow cytometer and analyzed using the CXP software. Gating was done on CD8+ cells, and percentage of CD3+/CD8+/tetramer+ cells was determined.

**Enzyme-linked immunospot assay.** Functional tumor antigen–specific T cells specific for HPV16 E7(49–57) and the ampitope peptides were detected. The Db-binding PSCA(23–31) peptide was used to set background. Multiscreen HA plates (Millipore) were coated with 10 μg/mL anti-IFNγ antibody (BD Pharmingen). Plates were washed and blocked with culture medium. Splenocytes were added in 2-fold serial dilutions ranging from 5 × 10⁵ to 6.25 × 10⁴ cells per well in medium containing 5 IU interleukin (IL)-2 and 10 μg/mL peptide. After 40 hours, plates were washed and incubated with 1 μg/mL of biotinylated anti-IFNγ antibody. Washed plates were incubated with 100 μL of 1:4,000 diluted streptavidin–horseradish peroxidase (Sigma Chemical Co.) per well. Spots were developed using 3-amino-9-ethylcarbazole (Promega) for 5 minutes, and the reaction was stopped with water. Spots were counted using the Zeiss KS enzyme-linked immunospot (ELISPOT) system. Assays were performed in triplicate, and results were calculated as spot-forming cells per 10⁶ splenocytes after subtracting background.

**Measuring intratumoral cytokines.** Tumors were collected, weighed, and homogenized in PBS containing 2× Halt Protease Inhibitor Cocktail (Pierce). Supernatants were collected by centrifugation at 4°C. Cytokine and chemokine levels were quantified using the Milliplex Mouse Cytokine/Chemokine -Premixed 32 Plex (Millipore) and a Bio-Plex Suspension Array system following manufacturer’s instructions. Transforming growth factor b (TGFb) levels were quantified separately using a Milliplex TGFb kit according to the manufacturer’s instructions.

**Statistical analysis.** Each figure is representative of at least two independent experiments. Tumor growth, multiplex ELISA, ELISPOT, and flow cytometry data were analyzed by either a two-tailed Student’s t test or a one-way ANOVA when more than two groups were compared. Survival was analyzed by the log-rank test. All statistical analyses were performed using the GraphPad Prism version 4.0 software.

**Results**

**Therapy with HPV16-VRP does not induce regression of well-established C3.43 tumors.** To determine whether therapy of well-established HPV16+ tumors with HPV16-VRP could induce tumor regression, two groups of C57BL/6 mice
were challenged and tumors were allowed to grow for 14 days, until average tumor volume was >100 mm³, and vaccinated with HPV16-VRP. When compared with control-vaccinated mice, mice that were vaccinated with HPV16-VRP did not show statistically significant differences in either average tumor volume (P = 0.83, two-tailed t test; Fig. 1A) or survival (P = 0.38; Fig. 1B). To ensure that lack of efficacy of HPV16-VRP vaccination on survival was not due to a failure in inducing functional E7-specific T cells, the frequency and function of E7-specific CD8+ T cells were analyzed 1 week after final vaccination. Figure 1C shows that HPV16-VRP vaccination resulted in the expansion of E7-specific T cells in the spleens of tumor-bearing mice but not in the tumors of the same mice (P = 0.04, two-tailed t test). Figure 1D shows that the E7-specific T cells expanded in the spleen on vaccination with HPV16-VRP were able to secrete IFN-γ on stimulation with the E7(49–57) peptide (P = 0.004, two-tailed t test). Additionally, the cells were able to secrete both IL-2 and tumor necrosis factor α (TNFα) and proliferate in response to peptide stimulation (data not shown). Together, these data show that HPV16-VRP vaccination does not induce regression of large HPV16+ tumors because tumor-specific T cells are absent in the tumor despite the induction of strong functional immunity in the peripheral lymphoid tissue.

**Ad-LIGHT treatment leads to the expansion of E7-specific T cells and increases T-cell infiltration into tumors.** We hypothesized that altering the tumor microenvironment by overexpressing LIGHT may induce functional tumor-specific T cells that control tumor growth and increase the survival of tumor-bearing mice. To test this hypothesis, mice were challenged with tumor and treated with intratumoral injections of either Ad-CTRL or Ad-LIGHT. To determine whether expression of LIGHT induced the expansion of HPV16+ tumor-specific T cells, individual spleens and pooled lymph nodes from mice were assessed for frequency of E7-specific CD8+ T cells 1 week after final treatment. Figure 2A shows that the average frequency of CD3+CD8+ tetramer+ T cells in the draining inguinal lymph nodes from Ad-CTRL–treated mice was 0.09%, which is similar to naïve mice, and that treatment with Ad-LIGHT increased the average frequency of CD3+CD8+ tetramer+ T cells to 1.08%. Data comparing the frequency of CD3+CD8+ tetramer+ T cells in individual spleens from groups of five mice treated with either Ad-CTRL or Ad-LIGHT show that intratumoral injection of Ad-LIGHT significantly increased the frequency of E7-specific T cells ranging from 0.31% to 1.49% (P = 0.045, two-tailed t test; Fig. 2B). To determine whether intratumoral injection of Ad-LIGHT altered the tumor microenvironment to increase...
infiltration of CD8+ T cells into tumors, individual tumors were analyzed for CD8+ T cells by flow cytometry and for cytokine/chemokine expression by multiplex ELISA. Our data show that frequency of CD8+ TILs increases significantly in Ad-LIGHT–treated tumors compared with control-treated tumors (P = 0.002, two-tailed t test; Fig. 2C). As shown in Fig. 3, multiplex ELISA analysis showed a statistically significant increase in the intratumoral levels of IFNγ, IL-1α, macrophage inflammatory protein-2 (MIP-2), and vascular endothelial growth factor (VEGF) as well as a trend toward an increase in monokine induced by IFNγ (MIG/CXCL9). Similar trends were seen for IP-10 and granulocyte macrophage colony-stimulating factor (GM-CSF). Additionally, there was an increase in RANTES, TNFa, G-CSF, IL-1β, IL-2, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, KC, and LIX in the treated groups compared with no treatment (data not shown). However, there were no statistically significant differences between control-treated and Ad-LIGHT–treated mice. No differences in the intratumoral levels of IL-3, IL-4, IL-5, IL-7, IL-12p40, IL-17, ILF, MCP-1, and M-CSF were observed between any of the groups (data not shown). Finally, treatment with Ad-CTRL resulted in a decrease in intratumoral concentration of the immunosuppressive cytokine, TGFβ1, which was further decreased on expression of LIGHT. Together, these results show that intratumoral injection of Ad-LIGHT in tumors increases the concentration of IFNγ and chemotactant cytokines while reducing the concentration of immunosuppressive TGFβ1, which may lead to the observed increase in T-cell infiltration into tumors and the expansion of E7-specific T cells.

**LIGHT expression in HPV16+ tumors leads to increased survival.** Because treatment with Ad-LIGHT induced the expansion of E7-specific T cells, we sought to determine whether these T cells were functional. The number of splenic T cells secreting IFNγ in response to peptide stimulation was measured by ELISPOT 1 week after final treatment. Our data

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Forced expression of LIGHT in C3.43 tumors induces increased expression of IFNγ and chemokines. Three groups of five tumor-bearing mice were left either untreated or treated with Ad-CTRL or treated with Ad-LIGHT. On day 24, tumors were resected and homogenized. Intratumoral cytokines and chemokines were measured. Columns, cytokine/chemokine concentration per gram of tumor; bars, SD.
show that T cells from Ad-LIGHT–treated mice were able to secrete IFNγ on stimulation with the E7(49–57) peptide, whereas those treated with the control did not (P = 0.036, two-tailed t test; Fig. 4A). To assess whether therapy with Ad-LIGHT could induce functional T cells to multiple tumor antigens, we also assessed the T-cell response to the ampitope (SSPVNSLRNVV) peptide (27). Our data show that T cells from mice treated with Ad-LIGHT were also able to specifically secrete IFNγ when stimulated with the ampitope peptide, whereas control-treated mice did not (P = 0.041, two-tailed t test; Fig. 4B). We next sought to determine whether the functional tumor-specific T cells expanded by intratumoral expression of LIGHT could induce tumor regression. Our data show that Ad-LIGHT therapy partially controlled tumor growth (P = 0.02, two-tailed t test; Fig. 4C) and resulted in a statistically significant increase in the duration of survival of tumor-bearing mice compared with mice treated with control particles (P = 0.0002, log-rank test; Fig. 4D). Together, these data show that functional tumor-specific T cells induced by treatment with Ad-LIGHT partially controlled growth of HPV16 C3.43 tumor but did not induce complete tumor regression.

**Figure 4.** Forced expression of LIGHT in C3.43 tumors induces functional T cells that are specific for multiple tumor antigens and increases survival of tumor-bearing mice. Groups of five tumor-bearing mice were treated with either Ad-LIGHT or Ad-CTRL. IFNγ ELISpot assay was used to detect functional peptide-specific T cells in individual spleens. A, HPV16 E7 (49–57); B, ampitope (SSPVNSLRNVV) peptide. C and D, tumor growth was monitored in groups of 10 tumor-bearing mice treated with either Ad-LIGHT or Ad-CTRL. Points, average tumor volumes and survival against days after tumor challenge; bars, SE.

**Combined treatment with Ad-LIGHT followed by vaccination with HPV16-VRP induces regression of large HPV16+ tumors.** Because treatment of 14-day-old tumor with either HPV16-VRP alone or Ad-LIGHT alone did not result in tumor regression despite induction of tumor-specific immunity, we hypothesized that inducing E7-specific immunity via intratumoral...
further increased the frequency of E7-specific T cells in the combined treatment with both Ad-LIGHT and HPV16-VRP specific T cells compared with control-treated mice and that mice treated with either Ad-LIGHT alone or HPV16-VRP analysis of tumors and spleens with E7(49–57) tetramers showed that mice treated with either Ad-LIGHT alone or HPV16-VRP alone had increased frequencies of tumor-infiltrating E7-specific T cells compared with control-treated mice and that combined treatment with both Ad-LIGHT and HPV16-VRP further increased the frequency of E7-specific T cells in the tumors (P = 0.009, one-way ANOVA; Fig. 6A). IFNγ ELISPOT analysis revealed that mice treated with both Ad-LIGHT and HPV16-VRP had stronger functional tumor-specific T-cell responses compared with mice treated with Ad-LIGHT (P = 0.0001, one-way ANOVA; Fig. 6B), showing that Ad-LIGHT treatment followed by vaccination with HPV16-VRP increases the frequency of functional tumor-specific T cells. Figure 6C shows that treatment with HPV16-VRP alone did not affect tumor growth, whereas treatment with Ad-LIGHT resulted in partial control of tumor growth. Most importantly, the data show that combined treatment with Ad-LIGHT and HPV16-VRP reduced the average tumor volume compared with treatment with Ad-LIGHT alone (P = 0.09, one-way ANOVA; Fig. 6C). Furthermore, the data show that control-treated mice or mice treated with HPV16-VRP alone died by day 47, whereas 70% of mice treated with Ad-LIGHT and 70% of mice treated with both Ad-LIGHT and HPV16-VRP survived. By day 80, only 10% of mice treated with Ad-LIGHT survived, whereas 50% of mice treated with both Ad-LIGHT and HPV16-VRP were tumor-free (P = 0.0002, log-rank test; Fig. 6D). Together, these data support our hypothesis that intratumoral expression of Ad-LIGHT alters the tumor microenvironment by increasing the expression of proinflammatory and chemotactic cytokines/chemokines and that boosting LIGHT-induced E7-specific T cells with HPV16-VRP would increase the frequency of tumor-specific T cells, which leads to the regression of large well-established HPV16+ tumors.

Discussion

VRPs are excellent immunogens with numerous advantages over other vaccination platforms. Previously, our laboratory has shown that HPV16-VRP vaccination is protective and partially therapeutic when administered to treat small palpable tumors (15). In this study, we tested whether HPV16-VRP vaccination could eradicate large tumors that mimic more advanced stages of disease and show that treatment with HPV16-VRP alone does not induce regression. Our data show that HPV16-VRP vaccination can induce high frequencies of functional E7-specific T cells in the peripheral lymphoid organs of tumor-bearing hosts; however, the CD8+ T cells are absent within the tumors themselves. In essence, these data recapitulate observations from the multitude of clinical trials in patients with various stages of HPV16+ lesions that show an induction of HPV-specific immunity but have not been successful in terms of inducing objective clinical responses. Thus, inducing inflammation within the tumor may be critical for successful immunotherapy of HPV16+ lesions such as cervical cancer.

LIGHT/TNFSF14 is a molecule that promotes a variety of proinflammatory processes through its interaction with LTβR on stromal cells and HVEM on activated immune cells. In this study, we tested whether expression of LIGHT, delivered using adenoviruses, could lead to the expansion of tumor antigen–specific T cells and overcome tumor-mediated immune suppression by altering the tumor microenvironment. To do so, we used the C3.43 tumor challenge model in which tumor cells express HPV16 E7, a clinically relevant antigen, under its natural promoter. We first showed that using adenoviruses to deliver LIGHT to HPV16+ tumors resulted in an increased frequency of E7(49–57)–specific T cells in both the periphery and within the tumor. Treatment with Ad-LIGHT also resulted in increased proinflammatory Th1 cytokines/chemokines that recruit T cells, natural killer cells, neutrophils, and macrophages. Of these, we found a statistically significant increase in the concentrations of IFNγ, IL-1α, MIP-2, and MIG that was dependent on the expression of LIGHT. For other Th1 cytokines, expression seemed to increase on treatment with either Ad-CTRL or Ad-LIGHT, which suggests an adjuvant-like effect of adenovirus-mediated delivery. For molecules such as IL-15, IP-10, and MIP-1α, there was a large increase, and for IL-10, there was a large decrease in concentration on treatment with Ad-LIGHT compared with Ad-CTRL. However, the difference was not statistically significant. Additionally, there was a decrease in TGFβ1, an immunosuppressive cytokine that inhibits secretion and activity of proinflammatory cytokines such as IFNγ and TNFα. Previous studies have shown that LIGHT-LTβR signaling induces CCL21 production that colocalizes T cells and DCs (32). CCL21 may induce IFNγ expression, and IFNγ, which is also
induced by LIGHT-mediated signaling, is necessary for antitumor immunity. MIG and IP-10 are induced by IFN-γ, have antitumor properties, and are necessary for IFN-γ-mediated antitumor responses (33, 34). Our novel finding that the intratumoral expression of LIGHT results in increased expression of MIG and IP-10 fits with the role of IFN-γ and IFN-γ-induced chemokines in antitumor immunity. Remarkably, we found no increase in the expression of Th2 cytokines. Another interesting observation was the statistically significant increase in VEGF expression. A previous study has shown that VEGF can increase expression of LIGHT on macrophages (35); however, whether LIGHT can increase the expression of VEGF through the ligation of LTβR on endothelial cells has not been established thus far. The mechanism of LIGHT-mediated VEGF expression and its biological relevance remain to be understood.

In addition to the E7(49–57) peptide, C3.43 cells express the amputope peptide, which functions as the dominant tumor antigen (27). Our data show that intratumoral expression of LIGHT leads to increased expression of MIG and IP-10 fits with the role of IFN-γ and IFN-γ-induced chemokines in antitumor immunity. Remarkably, we found no increase in the expression of Th2 cytokines. Another interesting observation was the statistically significant increase in VEGF expression. A previous study has shown that VEGF can increase expression of LIGHT on macrophages (35); however, whether LIGHT can increase the expression of VEGF through the ligation of LTβR on endothelial cells has not been established thus far. The mechanism of LIGHT-mediated VEGF expression and its biological relevance remain to be understood.

In addition to the E7(49–57) peptide, C3.43 cells express the amputope peptide, which functions as the dominant tumor antigen (27). Our data show that intratumoral expression of LIGHT leads to the induction of functional T-cell responses to both antigens. More importantly, immune responses strongly correlated with control of tumor growth, although it did not result in tumor clearance. Thus, our results show that adenovirus-mediated delivery of LIGHT can induce the expansion of functional T cells specific to both dominant and subdominant tumor antigens, which provides a significant clinical advantage over antigen-specific therapies because T cells specific for the
dominant antigens may become tolerized by the tumor (36). Our data showing that LIGHT-induced immunity can significantly prevent the development of tumors on re-challenge at a distal site agrees with the study by Yu and colleagues (30), data from which support the notion that expression of LIGHT delivered using adenoviruses results in the prevention or clearance of metastasis. However, our study also suggests that the bulk of tumor and pace of tumor growth are important factors in the efficacy of therapeutic intervention with Ad-LIGHT. To evaluate whether improving the kinetics of the immune response could enhance the efficacy of treatment with LIGHT, we boosted the frequency of tumor antigen-specific T cells by HPV16-VRP vaccination and found a significant increase in functional E7-specific T cells after combined therapy compared with treatment with LIGHT alone. More importantly, combined treatment resulted in a significant increase in tumor-free survival. We also observed comparable frequency of E7-specific T cells infiltrating into the tumors on treatment with Ad-CTRL/HPV16-VRP, which could be a result of the increase in Th1-type cytokines in the tumors, but this did not have any effect on survival. This could be the result of treatment with HPV16-VRP beginning on day 24 in the Ad-CTRL/HPV16-VRP group, by which time tumor volume is significantly smaller in the LIGHT-treated groups. However, data in Figs. 2 and 4 show that treatment with Ad-CTRL alone does not yield functional immunity in either the periphery or within the tumor and does not affect tumor growth. This suggests that whereas kinetics of the immune response is important, LIGHT-mediated alteration of the tumor microenvi-

rnonment plays a significant role in activating tumor-specific T-cell immunity that is critical for achieving tumor-free survival.

In summary, our study provides the first evidence of LIGHT-mediated induction of T cells specific for a clinically relevant oncogenic protein both peripherally and within the tumor, the frequency of which can be further increased by vaccination. Moreover, immunity is induced against both dominant and subdominant antigens and results in the rejection of tumors on secondary challenge. Combined treatment of intratumoral injection of Ad-LIGHT and vaccination with HPV16-VRP has significantly greater efficacy in inducing tumor-specific immunity that leads to the eradication of large well-established HPV16+ tumors than either treatment alone. Therefore, this type of combination therapy may be of significant advantage to enhance tumor-specific immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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