Enhanced Cancer Radiotherapy through Immunosuppressive Stromal Cell Destruction in Tumors

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Abstract

Purpose: Radiotherapy kills cancer cells by causing DNA damage, and stimulates a systemic antitumor immune response by releasing tumor antigen and endogenous adjuvant within the tumor microenvironment. However, radiotherapy also induces the recruitment of immunosuppressive myeloid cells, which can interfere with the antitumor immune responses elicited by apoptotic tumor cells. We hypothesized that local delivery of vaccine following radiotherapy will lead to the priming of antigen-specific CTL immune responses and render immunosuppressive myeloid cells susceptible to killing by the activated CTLs.

Experimental Design: Using several antigenic systems, we tested whether intratumoral injection of antigenic peptide/protein in irradiated tumors would be able to prime CTLs as well as load myeloid cells with antigen, rendering them susceptible to antigen-specific CTL killing.

Results: We show that by combining radiotherapy and targeted antigenic peptide delivery to the tumor, the adjuvant effect generated by radiotherapy itself was sufficient to elicit the priming and expansion of antigen-specific CTLs, through the type I IFN-dependent pathway, leading to synergistic therapeutic antitumor effects compared with either treatment alone. In addition, using two different types of transgenic mice, we demonstrated that CTL-mediated killing of stromal cells in tumors by our approach is important for tumor control. Finally, we confirmed the efficacy of this approach in our preclinical model using two clinically tested therapeutic human papilloma virus (HPV) vaccines.

Conclusions: These data serve as an important foundation for the future clinical translation of radiotherapy combined with a clinically tested therapeutic HPV vaccine for the control of HPV-associated cancers. Clin Cancer Res; 20(3); 644–57. ©2013 AACR.

Introduction

Radiotherapy is an effective and widely used treatment modality for various solid cancers, including breast cancer, prostate cancer, cervical cancer, and head and neck cancer (1–4). Radiotherapy achieves its therapeutic effect by inducing tumor cell death. However, a secondary effect of radiotherapy is the local antigen release from necrotic tumor cells, which can stimulate systemic antitumor immune responses (5–8). In addition, the release of tumor antigens into the microenvironment, there is extracellular release of damage-associated molecular patterns (DAMP), such as HMGB1, S100 protein, HSP, CRT, DNA, and ATP (6, 7). Many of these DAMPs, as well as proinflammatory cytokines, act as adjuvants to promote DC recruitment, antigen uptake, DC maturation, and antigen cross-presentation. The recruitment of antigen-presenting cells and local secretion of immunostimulatory adjuvants into the microenvironment provide a rationale for combining radiotherapy with local delivery of vaccines to generate antigen-specific immunity.

As a physiologic mechanism of tissue protection from inflammatory destruction, the profound inflammatory response induced by radiotherapy can be dampened through the local recruitment of immunosuppressive bone marrow-derived stromal cells, mostly CD11b+ myeloid cells (9–12). In fact, cancer progression has been associated with the...
Radiotherapy is a widely used treatment for various solid cancers, but in addition to its antitumor effects, it induces the recruitment of immunosuppressive cells. To counteract this effect, we used a combination treatment consisting of radiotherapy and targeted antigenic peptide delivery to the tumor. We found that the adjuvant effect generated by radiotherapy was sufficient for intratumoral injection of a vaccine, without additional adjuvant, to elicit the priming and expansion of antigen-specific CTLs. This led to CTL-mediated killing of the immunosuppressive stromal cells in tumors, resulting in synergistic therapeutic antitumor effects. Furthermore, we show that radiotherapy could be combined with intratumorally injected clinically tested therapeutic human papilloma virus (HPV) vaccines to decrease tumor size and improve survival in treated mice compared with radiotherapy alone. These data serve as an important foundation for future clinical translation using radiotherapy combined with a clinically tested therapeutic HPV vaccine for the control of cancer.

**Materials and Methods**

**Mice**
Six- to 8-week-old female C57BL/6 and nude BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). TLR4 knockout mice and B6.Cg-fomm1nu/nu mice were obtained from The Jackson Laboratory. IFNAR knockout mice were a kind gift from Dr. G. Cheng (University of California, Los Angeles, CA), and HLA-A*0201/Dd transgenic female C57BL/6 mice, 6 to 8 weeks of age, were kindly provided by Dr. Victor Engelhard at the University of Virginia Health Sciences Center (Charlottesville, VA; ref. 20).

**Cells**
We previously created the TC-1 tumor model by transformation of primary lung epithelial cells from C57BL/6 mice with active Ras together with HPV-16 E6 and E7 oncogenes, the production and maintenance of which have been described previously (19). TC-1 cells were subjected to RapidMAP (Taconic Farms) testing, a panel of PCR tests for rodent viruses, most recently in May 2011 with negative results. The E7-specific CD8+ cells used for adoptive transfer are from an E7-specific CD8+ T-cell line that has been previously described (21). Cells were cultured at 37°C with 5% CO2 in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 2 mmol/L nonessential amino acids, and 50 U/mL penicillin/streptomycin.

**Tumor treatment experiments**
TC-1 tumor cells (1 × 10^5 per mouse) were inoculated subcutaneously (s.c.) into C57BL/6, TLR4 knockout, IFNAR knockout, nude BALB/c, and HLA-A*0201/Dd (AAD) transgenic mice (n = 4–10). Fourteen or 15 days later, (when tumor diameter was approximately 0.5 cm), mice were treated with radiotherapy at a dose of 14 Gy/mouse (using a Shepherd Mark 1 irradiator) in combination with intratumor (i.t.) injection of 50 μg E7 peptide (aa 43-62). A total of five E7 injections were administered, starting on day 15 with 3-day intervals. Notably, the injected E7 peptide (aa 43-62) contains an H-2Db–restricted E7 epitope (aa 49-57) and can be loaded onto MHC class I molecules after processing. In OVA peptide (aa 241-270) and NS1 peptide (aa 122-130) treatment groups, peptide (50 μg) was injected every 3 days starting on the day of radiation until the mice were sacrificed. Isotonic saline was used as control. In TA-HPV (22, 23)-treated groups, mice were intratumorally injected with 40 μL TA-HPV once on the day of radiotherapy. In TA-CIN (24)-treated groups, mice were injected with 25 μg TA-CIN protein at 5-day intervals, for a total of three treatments. Mice were monitored for tumor growth by visual inspection and palpation every 3 days. Tumor volumes were calculated as: volume = (width)^2 × length/2. To assess tumor burden in the lung hematogenous spread model, C57BL/6 mice were inoculated subcutaneously with 1 × 10^7 TC-1 cells

Translational Relevance
Radiotherapy is a widely used treatment for various solid cancers, but in addition to its antitumor effects, it induces the recruitment of immunosuppressive cells. To counteract this effect, we used a combination treatment consisting of radiotherapy and targeted antigenic peptide delivery to the tumor. We found that the adjuvant effect generated by radiotherapy was sufficient for intratumoral injection of a vaccine, without additional adjuvant, to elicit the priming and expansion of antigen-specific CTLs. This led to CTL-mediated killing of the immunosuppressive stromal cells in tumors, resulting in synergistic therapeutic antitumor effects. Furthermore, we show that radiotherapy could be combined with intratumorally injected clinically tested therapeutic human papilloma virus (HPV) vaccines to decrease tumor size and improve survival in treated mice compared with radiotherapy alone. These data serve as an important foundation for future clinical translation using radiotherapy combined with a clinically tested therapeutic HPV vaccine for the control of cancer.
each and treated as described above. Mice were then inoculated intravenously with $1 \times 10^5$ TC-1 cells each on day 14 after subcutaneous tumor challenge. The number of pulmonary tumor nodules was quantified on day 35.

For the control of tumors at a distant site following local radiotherapy and E7 peptide vaccination

C57BL/6 mice were inoculated subcutaneously with $1 \times 10^5$ TC-1 cells each and treated with local radiotherapy and intratumoral vaccination as described above. Mice were then inoculated intravenously with $1 \times 10^5$ TC-1 cells each on day 14 after subcutaneous tumor challenge. The number of pulmonary tumor nodules was quantified on day 35 using methods described previously (25).

In vivo antibody depletion experiments

C57BL/6 mice ($n = 5$) were inoculated subcutaneously with $1 \times 10^5$ TC-1 cells per mouse and treated with radiation and E7 peptide (50 mg) according to the regimen described above. In the CD8$^+$ T-cell depletion group, 100 mg/mouse of anti-CD8 antibody (clone 2.43) was delivered via intraperitoneal injection on the same day as radiotherapy, as well as 3 and 6 days after radiotherapy.

Surface tetramer, intracellular cytokine staining, and flow cytometry

TC-1 tumor-bearing C57BL/6 mice, TLR4 knockout mice, IFNAR knockout mice, and HLA-A$^*$0201/D$^*$ (AAD) transgenic mice ($n = 4–5$) were treated as described above in the tumor treatment experiments. Two weeks after radiotherapy, splenocytes, draining lymph nodes (DLN), and peripheral blood mononuclear cells (PBMC) were isolated from the mice and characterized for the presence of antigen-specific CD8$^+$ T cells. All samples were pretreated with CD16/CD32 FcR blocker (BD Biosciences) before staining. For tetramer staining, phycoerythrin (PE)-labeled H-2Db tetramers containing HPV-16 E7 aa 49-57 peptide (RAHY-NIVTF; Beckman Coulter) were used for the analysis of E7-specific CD8$^+$ T cells (26). Allophycocyanin (APC)-labeled H-2Kb tetramers containing OVA peptide (SIINFEKL; Beckman Coulter) were used for the analysis of OVA-specific CD8$^+$ T cells. For intracellular cytokine staining, PBMCs were harvested 2 weeks after radiotherapy, and $6 \times 10^5$ pooled PBMCs from each group were incubated with 1 mg/mL E7 peptide or NS1 peptide (AIMDKNIIL), together with GolgiPlugs ($\times 1,000$; BD Biosciences) for 16 hours. Cells were then harvested and mixed with monoclonal antibodies against CD8 and IFN-γ as previously described (27). Samples were acquired on a FACSCalibur device using CellQuest Pro software (BD Pharmingen) and analyzed by FlowJo software.

Analysis of tumor-infiltrating antigen-specific CD8$^+$ T-cell populations

Groups of TC-1 tumor-bearing mice ($n = 4–5$) were treated as described above. Two weeks after radiotherapy, tumors were harvested, chopped into 2 to 3 mm pieces, and digested with digestion buffer (0.25 mg/mL collagenase I and IV, 0.12 mg/mL hyaluronidase IV, 0.25 mg/mL DNase I, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37°C for 1 hour before passage through a cell strainer. Cells were washed and enriched for lymphocytes by Lymphoprep (AXIS-SHIELD) separation. After washing with PBS twice, cells were pretreated with anti-CD16/CD32 FcR blocker (BD Pharmingen) and stained with FITC-labeled anti-CD8 antibody, PE-labeled E7 peptide (aa 49-57)-loaded H-2Db tetramer, or OVA peptide (SIINFEKL)-loaded H-2Kb tetramer and analyzed by flow cytometry.

CD11c$^+$ DC migration into lymph nodes

TC-1 tumor-bearing mice were treated with or without radiotherapy as described above and administered intratumorally with 50 μg of FITC-labeled E7 peptide (aa 43-62) on the same day. The DLNs of treated mice were isolated and processed into single cells for analysis 40 hours after radiotherapy. Cells were stained with APC-labeled anti-CD11c antibody and PE-labeled anti-ICAM-1, -CD80, or CD11c antibody and PE-labeled anti-ICAM-1, -CD80, or -CD86 antibody (BD Pharmingen). Tumor-infiltrating CD11b$^+$ myeloid cell populations

To analyze the post-radiotherapy myeloid-cell-infiltrates, TC-1 tumor-bearing mice ($n = 3$) were treated with radiotherapy. Three days after radiotherapy, tumors were harvested, chopped into 2 to 3 mm pieces, and digested with digestion buffer at 37°C for 1 hour before passage through a cell strainer. Cells were washed twice with 1× PBS, pretreated with anti-CD16/CD32 FcR blocker (BD Pharmingen), and stained with APC-labeled CD11c (BD Pharmingen), FITC-labeled anti-CD11b, APC-labeled anti-Gr-1, and PE-Cy5-labeled anti-F4/80 antibody (eBioscience), and analyzed by flow cytometry. To analyze tumor-infiltrating CD11b$^+$ myeloid cells after radiotherapy combined with intratumoral peptide injection, groups of TC-1 tumor-bearing mice ($n = 4–5$) were treated as described in the tumor treatment experiment. Two weeks after radiotherapy, tumors were harvested, processed into single cells as mentioned above, stained with the antibodies listed above along with PE-labeled Ly-6C (BD Pharmingen) and biotinylated Ly-6C (BD Pharmingen) antibody with streptavidin-perCP, and analyzed by flow cytometry.

MHC class I molecule loading of antigenic peptide and cell apoptosis

To characterize the loading of OVA peptide on MHC class I molecules (H-2Kb) on CD11b$^+$ myeloid cells, TC-1 tumor-bearing mice ($n = 3$) were treated with radiotherapy at a dose of 14 Gy/mouse and 50 μg of OVA peptide (aa 241-270) was intratumorally injected 3 days after radiotherapy. One day later, tumors were harvested, processed into single cells, and pretreated with anti-CD16/CD32 FcR blocker (BD Pharmingen) as mentioned above. Cells were washed and stained with anti-CD11b, -CD11c, -Gr-1, -F4/80, and SIINFEKL-H-2Kb-specific (eBioscience clone: eBio25-D1.16) antibodies and analyzed by flow cytometry. To analyze the OT-1–mediated apoptosis of CD11b$^+$ myeloid cells, TC-1 tumor-bearing mice ($n = 3$) were treated with radiotherapy.
and injected with OVA peptide as mentioned. The treated tumors were harvested one day after OVA peptide injection, processed into single cells, and cocultured with \(1 \times 10^6\) activated OT-1 T cells for one day. Cells were collected, washed, stained with anti-CD11b, -Gr-1, -F4/80, and PE-labeled antiactive caspase-3 antibodies (eBioscience), and analyzed by flow cytometry.

**Tumor treatment experiments in nude mice with T-cell adoptive transfer**

TC-1 tumor cells (\(1 \times 10^5\) per mouse) were inoculated subcutaneously into nude BALB/c mice and nude C57BL/6 B6.Cg-Foxn1nu/J mice (\(n = 5\)). Fifteen days after tumor inoculation, tumors were irradiated with 14 Gy/mouse in combination with intratumoral injection of 50 \(\mu\)g E7 peptide (aa 43-62) every 3 days from the day of radiotherapy until the mice were sacrificed. One day after radiotherapy, \(2 \times 10^6\) activated E7-specific CD8\(^+\) T cells (21) were adoptively transferred three times at one week intervals via intravenous tail vein injection. Mice were monitored for tumor growth by visual inspection and palpation every 3 days.

**Statistical analysis**

The data presented in this study are representative of three independent experiments. Descriptive statistics such as mean and SD are reported. The number of samples in each group for any given experiment was at least 3. Results for flow cytometry analysis and tumor treatment experiments were evaluated by one-way ANOVA and the Tukey–Kramer test. The event-time distributions for different mice were estimated using the Kaplan–Meier method and compared with the log-rank test. All \(P\) values <0.05 were considered significant.

**Study approval**

All animal procedures were performed under protocols approved by the Johns Hopkins Institutional (Baltimore, MA) Animal Care and Use Committee and in accordance with recommendations for the proper use and care of laboratory animals.

**Results**

**Intratumoral vaccination with E7 peptide following radiotherapy results in potent antitumor immune response**

First, we assessed the antitumor effect generated by intratumoral E7 vaccination following radiotherapy using the HPV-16 E7-expressing TC-1 murine tumor model (19). The treatment schedule included local radiotherapy followed by either intratumoral E7 peptide (aa 43-62) vaccination or vaccination into the contralateral subcutaneous tissue, as outlined in Fig. 1A. The combination of radiotherapy followed by intratumoral vaccination of E7 had the best antitumor response as measured by tumor growth (\(P < 0.03\)) and long-term survival (\(P < 0.001\)) compared with either radiotherapy alone or the combination of radiotherapy and subcutaneous administration of E7 peptide (Fig. 1B). In addition, 3 weeks after radiotherapy, seven out of ten mice receiving the combined treatment had complete tumor regression and remained tumor free up to 60 days after completing radiotherapy. Long-term tumor protection is further illustrated by the fact that these mice remained tumor free after subcutaneous rechallenge with TC-1 tumor cells (Supplementary Fig. S2). To demonstrate whether the local radiotherapy and intratumoral E7 vaccination could lead to control of the TC-1 tumor at a distant site, we injected TC-1 cells via the tail vein to create tumor nodules in the lung (25). As shown in Fig. 1C, local radiotherapy and intratumoral E7 vaccination created systemic immunity against TC-1 tumors by demonstrating a significant reduction in TC-1 tumor nodules in the lung. In comparison, irradiation or E7 vaccination alone failed to generate systemic antitumor effects against TC-1 tumors at a distant site. These data indicate that local radiotherapy in conjunction with local E7 vaccination is able to create systemic immunity against E7-expressing tumors at distant sites. To clarify which effector T cells contributed to the antitumor effect, we depleted CD8\(^+\) T cells and found that CD8\(^+\) T-cell depletion resulted in significant tumor growth (\(P = 0.036\); Fig. 1D). Furthermore, to demonstrate whether the current therapeutic strategy could be extended to other HPV-16 E7-expressing tumor models, we adopted a different E7-expressing tumor model, WF3 (28). Similarly, we observed significant antitumor effects against WF3 using our therapeutic strategy (Supplementary Fig. S3). Taken together, our data indicate that the combination of intratumoral vaccination with E7 peptide following radiotherapy is capable of improving local and systemic control of tumors in the absence of any adjuvant.

**Radiotherapy in conjunction with intratumoral vaccination with E7 peptide generates E7 peptide-loaded CD11b\(^+\) CD11c\(^+\) dendritic cells in the DLN, which can activate E7-specific CD8\(^+\) T cells**

We next sought to determine the effector T-cell population contributing to the antitumor effect induced by radiotherapy and intratumoral E7 peptide vaccination. In tumor-bearing mice treated with radiotherapy followed by intratumoral E7 peptide vaccination, a significantly higher number of E7-specific CD8\(^+\) T cells were present in PBMCs, DLNs, and splenocytes, compared with other treatment groups (\(P < 0.05\) for each comparison; Fig. 2A and Supplementary Fig. S4). E7-specific tumor-infiltrating lymphocytes (TIL) were also statistically higher in mice receiving combination treatment as compared with other groups (\(P < 0.001\); Fig. 2B). Not only were there more CD8\(^+\) T cells found in the tumor infiltrates, but >40% of total tumor infiltrating CD8\(^+\) T cells were specific for E7 (\(P < 0.001\); Fig. 2B). Thus, these data indicate that radiotherapy followed by intratumoral injection of E7 peptide can elicit a local as well as systemic expansion of antigen-specific CD8\(^+\) T cells, which likely contributes to the antitumor effect.

Next, we explored the mechanisms by which radiotherapy facilitates the development of an adaptive immune
response in mice treated with radiotherapy followed by intratumoral E7 peptide vaccination. We administered FITC-labeled E7 peptide into the tumors of TC-1 tumor-bearing mice with or without radiotherapy and isolated DLNs from the treated mice. We found an increased number of FITC⁺ DCs in the DLNs of mice treated with radiotherapy compared with mice vaccinated without radiotherapy (\( P = 0.009 \); Fig. 2D). Furthermore, the FITC⁺ DCs had a more mature phenotype characterized by higher mean expression of the costimulatory molecules CD80, CD86, and ICAM-1 relative to FITC⁻ DCs (Fig. 2D). These data indicate that radiotherapy can induce the maturation of antigen-loaded DCs and migration to DLNs where they would be able to prime and expand tumor-specific CD8⁺ T cells.

Antigen-specific T-cell response following radiotherapy and intratumoral E7 peptide vaccination is mediated through the type I IFN and TLR4 pathways

We further explored the molecular signaling pathways underlying the priming of an adaptive E7-specific immune response. After the combination of radiotherapy and intratumoral E7 vaccination, CD8⁺ T cells from both transgenic mice deficient in type I IFN receptor (IFNAR⁻⁻) and mice deficient in TLR4 (TLR4⁻⁻) mounted weaker systemic and local E7-specific immune responses compared with wild-type (WT) mice (\( P < 0.001 \); Fig. 3A and B). IFNAR⁻⁻ mice also had weaker antitumor immune responses, characterized by poor tumor control (\( P = 0.035 \); Fig. 3C). Interestingly, IFNAR⁻⁻ mice had significantly reduced survival as compared with WT mice.
whereas survival in TLR4−/− mice was comparable with WT (Fig. 3D). These data suggest that the type I IFN pathway is important for mounting adaptive CD8+ T-cell immune responses after combination treatment with radiotherapy and intratumoral E7 peptide vaccination treatment. We also evaluated the anti-tumor response generated in mice deficient in TLR9 (TLR9−/−) following combination treatment with radiotherapy and intratumoral E7 peptide vaccination. We did not find a significant difference as compared with WT mice (Supplementary Fig. S5), suggesting that TLR9 does not contribute significantly to tumor control after radiotherapy and antigenic peptide administration.

Combination radiotherapy and intratumoral peptide vaccination results in MHC class I peptide loading of CD11b+ immunosuppressive myeloid cells, which are susceptible to killing by antigen-specific CD8+ T cells

It has been reported that CD11b+ myeloid cells are recruited into the microenvironment after radiotherapy. Therefore, we first assessed the presence of CD11b+ myeloid cells in the tumor infiltrate after radiotherapy in our tumor model. We found a 3-fold accumulation of CD11b+ myeloid cells in irradiated tumor tissue (up to 30% of total cells in the tumor) compared with nonirradiated tumors (P = 0.015; Fig. 4A). In addition, radiotherapy resulted in a
significantly enriched the major CD11b+ myeloid cell subsets CD11b+Gr-1+ MDSCs and CD11b+F4/80+ TAMs in the tumor microenvironment (P < 0.05; Fig. 4B). The enrichment of CD11b+ myeloid cells after radiotherapy was most pronounced in the tumor, but could also be detected in the spleen and DLN (Supplementary Fig. S6). To characterize whether CD11b+ myeloid cells were able to process and present antigen following intratumoral vaccination with the antigen, we used long OVA peptide (aa 241-270), which contains a Kb-restricted CTL epitope. When OVA was administered intratumorally following radiotherapy, we found that only CD11b+ myeloid cells from the tumors, but not CD11b- cells, were able to process and present the OVA CTL epitope through MHC class I molecules. This included all three subsets of CD11b+ myeloid cells: TAMs, MDSCs, and DCs (Fig. 4B).

Next, we determined whether the OVA peptide-loaded CD11b+ myeloid cells from TC-1 tumors could be recognized by effector OVA-specific CD8+ T cells. We incubated OT-1 T cells with CD11b+ cells derived from tumors of TC-1 tumor-bearing mice treated with radiotherapy either alone or with intratumoral OVA peptide vaccination. As shown in Fig. 4C, the number of caspase-3+ cells in mice treated with intratumoral OVA peptide vaccination was significantly higher compared with mice not vaccinated with OVA peptide (P = 0.043). Because CD11b+ cells include MDSCs and TAMs, which are important immunosuppressive cells in the tumor microenvironment, we further characterized whether these subsets of CD11b+ cells could uptake, process, and present OVA to the OT-1 T cells. As shown in Fig. 4C, tumor-infiltrating MDSCs expressed caspase-3, an apoptotic marker, suggesting that they were able to present the OVA peptide to OT-1 T cells. In comparison, the subset of tumor-infiltrating CD11b+ F4/80+ TAMs isolated from mice vaccinated intratumorally with OVA did not show a difference in resistance to cell-mediated killing, when incubated with OT-1 T cells, compared with
Characterized the number of tumor-infiltrating CD11b+ myeloid cells in TC-1 tumors (mean ± SD; *P < 0.015). B, left: characterization of the CD11b+CD11c+CD11b+Gr-1+CD11c+CD11b+Flt3+MDSC in tumors after treatment (mean ± SD; *P < 0.05). Right: characterization of OT-1 T-cell-mediated apoptosis of CD11b+ myeloid cells from TC-1 tumors treated as described in Fig. 1A (mean ± SD; *P < 0.01). C, top: characterization of OT-1 T-cell-mediated apoptosis of CD11b+ irradiated tumors with intratumoral vaccination with OVA peptide by flow cytometry analysis. A histogram (left) and a bar graph (right) showing the presence of CD11b+ myeloid cells in TC-1 tumors (mean ± SD; *P < 0.05). D, left: flow cytometry analysis to characterize the percentage of CD11b+ cells in total live cells from TC-1 tumors treated as described in Fig. 1A (mean ± SD; *P < 0.05). Right: top is representative flow cytometry analysis and bottom is bar graph of CD11b+ Gr-1+ MDSC in tumors after treatment (mean ± SD; *P < 0.01).

TAMs from mice not vaccinated intratumorally with OVA (Supplementary Fig. S7A). Next, we determined whether the tumor-infiltrating CD11b+ myeloid cells were indeed reduced in number following radiotherapy and intratumoral antigenic peptide vaccination due to apoptotic cell death mediated by antigen-specific CD8+ T cells. We characterized the number of tumor-infiltrating CD11b+ myeloid cells from TC-1 tumor-bearing mice treated with radiotherapy either alone or with intratumoral E7 antigenic peptide vaccination. As shown in Fig. 4D, MDSCs were significantly reduced in tumors treated with radiotherapy and intratumoral peptide vaccination compared with tumors treated with monotherapy, either radiotherapy or E7 peptide (P < 0.01). When subsets of MDSCs were further characterized, Ly6G+Ly6Clow granulocytic MDSCs (G-MDSCs) were found to be the dominant population affected with combination radiotherapy and intratumor vaccination with E7 peptide compared with mice receiving radiotherapy alone (P < 0.05). We further characterized the number of tumor-infiltrating CD11b+Gr-1+ MDSCs from TC-1 tumor-bearing mice receiving various treatments. As shown in Fig. 4D, MDSCs were significantly reduced in tumors treated with radiotherapy and intratumoral peptide vaccination compared with tumors treated with monotherapy, either radiotherapy or E7 peptide (P < 0.01). When subsets of MDSCs were further characterized, Ly6G+Ly6Clow granulocytic MDSCs (G-MDSCs) were found to be the dominant population affected...
(Supplementary Fig. S8). We did not observe a significant reduction in the tumor-infiltrating CD11b+ F4/80+ TAMs in mice treated with radiotherapy with intratumoral E7 peptide vaccination compared with tumors treated with radiotherapy alone (Supplementary Fig. 6B). Taken together, these data indicate that antigen-specific CD8+ T cells can target peptide-loaded CD11b+ myeloid cell infiltrates, resulting in the death of MDSCs, which can subsequently reduce the immunosuppressive cells within the tumor microenvironment after the combination treatment of radiotherapy and intratumoral peptide vaccination.

**CTL-mediated killing of antigenic peptide-loaded MDSCs within the tumor microenvironment contributes to the antitumor effect**

Next, we assessed whether CD8+ T-cell–mediated killing of peptide-loaded stromal cells in tumors treated with radiotherapy and intratumoral vaccination with antigenic peptides contributes to the antitumor effect. Because of the E7 peptide (aa 43-62) we used contains an H-2Db MHC class I restricted CTL epitope (aa 49-57), it can only be processed and presented by the myeloid cells in TC-1 tumor-bearing B6.Cg-floxfoxn1nu/J mice (H-2Db) and not those in TC-1 tumor-bearing nude BALB/c mice (H-2Dd). With adoptive transfer of E7-specific CD8+ T cells, we observed that B6.Cg-floxfoxn1nu/J mice carrying the H-2Db MHC class I molecules had significantly better tumor control as compared with the nude BALB/c mice (P = 0.015; Fig. 5A).

To further explore the importance of tumor control through antigen-specific CD8+ T-cell–mediated killing of stromal cells (including CD11b+ myeloid cells), we used HLA-A2 transgenic mice and influenza NS1 peptide as a model system. The NS1 peptide (aa 122-130) is a human HLA-A2 transgenic mice and influenza NS1 peptide as a model system. The NS1 peptide (aa 122-130) is a human HLA-A2.1–restricted CTL epitope that can only be presented on the HLA-A2 molecules had significantly better tumor control as compared with the nude BALB/c mice (P = 0.015; Fig. 5A).

To further explore the importance of tumor control through antigen-specific CD8+ T-cell–mediated killing of stromal cells, we used HLA-A2 transgenic mice and influenza NS1 peptide as a model system. The NS1 peptide (aa 122-130) is a human HLA-A2.1–restricted CTL epitope that can only be presented on the HLA-A2 molecules had significantly better tumor control as compared with the nude BALB/c mice (P = 0.015; Fig. 5A).

With adoptive transfer of E7-specific CD8+ T cells, we observed that B6.Cg-floxfoxn1nu/J mice carrying the H-2Db MHC class I molecules had significantly better tumor control as compared with the nude BALB/c mice (P = 0.015; Fig. 5A).
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Figure 6. Evaluation of antitumor effect after radiation combined with intratumoral administration of clinically tested vaccine. In vivo tumor growth experiment. Groups of WT C57BL/6 mice (n = 5) were injected with 1 x 10^7 TC-1 cells subcutaneously and treated 14 days later (tumor size ~0.5 cm in diameter) with radiotherapy at a dose of 14 Gy/mouse combined with intratumoral administration of clinically tested TA-HPV or TA-CIN vaccine. TA-HPV was intratumorally injected at a dose of 40 μl on the day of radiotherapy. TA-CIN was given at a dose of 25 μg and injected on the day of radiotherapy and every 5 days after for a total of three times. A and B, tumor growth was characterized by tumor size plotted over time. C and D, Kaplan–Meier survival analysis of tumor-bearing mice in different treatment groups.

Antitumor effects achieved with local radiotherapy and intratumoral antigenic peptide vaccination can be independent of tumor peptide specificity

Because most tumors do not have well-defined tumor-specific antigens that can be easily targeted for immune control, we determined whether the combined treatment strategy could be extended to nonspecific peptide vaccinations. Therefore, we vaccinated with a non-TC-1 tumor-related antigen, the OVA peptide (29). As shown in Supplementary Fig. S9A and S9B, OVA-specific CD8^+ T cells were significantly enriched after combination treatment compared with other treatment groups (P < 0.05). In addition, the increased OVA-specific CD8^+ T cells resulted in a significant reduction in the tumor-infiltrating CD11b^+ Gr-1^+ myeloid cells (P < 0.05; Supplementary Fig. S9C), which correlated with an enhanced antitumor effect (P = 0.036; Supplementary Fig. S9D). Thus, these data indicate that intratumoral vaccination with nontumor-related antigenic peptides following radiotherapy can induce potent antigen-specific CD8^+ T cells, resulting in the killing of tumor-infiltrating immunosuppressive myeloid cells loaded with antigenic peptide, which can be independent of tumor specificity. Therefore, the overall reduction in this immunosuppressive population results in a favorable immunogenic microenvironment, which can enhance antitumor effects.

Combination radiotherapy with a variety of clinically tested HPV vaccines results in improved antitumor effects compared with radiotherapy alone

To extend the potential clinical translation of the treatment methodology beyond peptide vaccines, we treated TC-1 tumor-bearing mice with radiotherapy followed by intratumoral injection of a clinically tested HPV vaccine, either TA-HPV or TA-CIN. TA-HPV is based on the Wyeth strain of vaccinia virus and carries modified E6 and E7 genes from HPV types 16 and 18 (22, 23). TA-CIN is composed of HPV-16 L2, E6, and E7 in a single tandem fusion protein (24). Figure 6A and C shows that mice treated with radiotherapy followed by intratumoral TA-HPV administration have decreased tumor growth and enhanced survival compared with those treated with radiotherapy alone. Furthermore, as shown in Fig. 6B and D, TC-1 tumor-bearing mice treated with radiotherapy combined with intratumoral TA-CIN generated enhanced antitumor effects and prolonged survival compared with mice treated with radiotherapy alone. These data indicate that radiotherapy combined with a clinically tested therapeutic HPV vaccine may be an effective strategy for HPV-associated tumor control. Further contributing to the clinical translatability of this treatment, both TA-CIN and TA-HPV are applicable to a wide patient population as the antigens included in the vaccines are full-length proteins and therefore, not subject to MHC class restriction like short peptide-based vaccines. Other potentially suitable therapeutic HPV vaccines that are not subject to MHC class I restriction and can be applied to the current strategy include overlapping peptides, which cover the whole length of E6 and E7 (30, 31).
Discussion

Our study provides a novel strategy to combine standard cancer treatment modalities, such as radiotherapy, with immunotherapy. We demonstrate that treatment of TC-1 tumor-bearing mice with radiotherapy followed by intratumoral vaccination with HPV E7 peptide generates a potent E7-specific CD8^+ T-cell immune response, which translates in vivo into a strong antitumor effect and improved survival compared with vaccination alone, radiotherapy alone, or radiotherapy followed by subcutaneous vaccination with E7 peptide. Furthermore, this treatment regimen induces an immunogenic tumor microenvironment through the targeted elimination of immunosuppressive CD11b^+ myeloid cells, specifically CD11b^+ Gr-1^+ MDSCs. Our study serves as a foundation for future clinical translation.

Prior studies have evaluated the combination of radiotherapy with a variety of immunotherapies, including exogenous cytokine delivery, TLR stimulation, intratumoral DC injection, vaccination, or adoptive transfer of antigen-specific CD8^+ T cells (5, 32–39). In our treatment regimen, we took advantage of the adjuvant effect associated with radiotherapy, specifically, the local recruitment of myeloid-derived antigen presenting cells into the tumor microenvironment. Through the intratumoral administration of immunogenic antigens such as HPV E7 peptide and non-tolerized, high-affinity exogenous antigenic peptides, OVA, and NS1, the infiltrating MDSCs were loaded with the introduced antigen in high density. This high quantity of antigen-loaded antigen presenting cells was able to generate antigen-specific CD8^+ T cells, which eradicated not only the tumor cells, but also generated a favorable tumor microenvironment through the elimination of tumor-supporting stromal cells (Figs. 5B and 6A).

There are multiple advantages to addressing the immunosuppressive microenvironment through the targeting of tumor stromal cells. First, the antitumor effect produced by antigen-specific immunity targeting the tumor alone may be limited by various immune escape mechanisms utilized by the tumor (40, 41). In contrast with the genetic variance and instability of tumor cells, stromal cells are considerably more stable, thus making them less likely to evade CD8^+ T-cell killing. Also, myeloid cells have a strong ability to take up and process exogenous antigens for cross-presentation on MHC molecules allowing for CD8^+ T-cell recognition. Thus, by delivering a high density of peptides to the tumor, we selectively tagged the local myeloid cells for T-cell destruction. Finally, myeloid stromal cells support tumor progression by promoting tumor invasiveness and angiogenesis, by providing essential signals and by negatively modulating the tumor microenvironment (9–12). Therefore, killing myeloid cells in the tumor by CD8^+ T cells eliminates a major support system of the tumor, leading to a better outcome. In the present study, we have shown that after radiotherapy, there is a better antitumor effect when antigen-specific CD8^+ T cells are capable of recognizing and killing tumor-infiltrating myeloid stromal cells (Figs. 5A and 6 and Supplementary Fig. S7). In addition, our results have been corroborated by recent evidence demonstrating that the destruction of stromal cells within tumors by CD8^+ T cells is essential to eradicate well-established, large solid tumors (42–47). Because our method targets the myeloid stromal cells and bypasses the dependence on tumor antigen, it may be extended to enhance the treatment of tumors that do not have a well-defined tumor antigen.

Here, we studied different subsets of myeloid cells and found that despite the their equal ability to process and present the antigen, their susceptibilities to CD8^+ T-cell-mediated killing were different. We found that DCs, TAMs, and MDSCs were able to efficiently cross-present antigenic peptides on MHC class I complexes after radiotherapy (Fig. 4B). Although all of the myeloid cells were able to load antigen, not all subsets could be lysed by antigen-specific CD8^+ T cells at the same efficiency. Increased cell apoptosis was observed in antigen-loaded CD11b^+ Gr-1^+ MDSCs when they encountered antigen-specific CD8^+ T cells compared with CD11b^+ F4/80^+ TAMs under the same conditions (Fig. 4C and Supplementary Fig. S6A). Consistent with this finding, a greater reduction of MDSCs between treatment with radiotherapy and radiotherapy combined with peptide vaccination was observed compared with TAMs (Fig. 4D and Supplementary Fig. S6B). Taken together, our findings indicate that MDSCs are more susceptible than TAMs to T-cell-mediated killing. Our observation is consistent with previous report by Sinha and colleagues that MDSCs are susceptible to T-cell–mediated killing (48). Some potential mechanisms may account for this observation. For example, the expression level of antiapoptotic proteins may be different among these CD11b^+ cells in the tumor microenvironment. Further studies will provide insight into the potential mechanisms accounting for the differential susceptibility to T-cell–mediated killing of the various subsets of CD11b^+ cells in tumor stroma.

Previously, myeloid cells recruited to the tumor microenvironment after radiotherapy have been targeted in several therapeutic attempts to achieve better antitumor effects and to prevent the deadly recurrence of tumors. Success was reported in experiments blocking the recruitment of CD11b^+ myeloid cells to the irradiated tumor and directly depleting myeloid cells (10, 11, 47, 49, 50). However, most of these approaches result in global myeloid cell depletion and systemic blockage of myeloid cell migration, which raises concerns of interrupting the homeostatic functions of these cells in various organs resulting in toxicities (51). In our approach, we reduce the concern for the unfavorable systemic effects by selectively marking the myeloid cells within the tumor.

The targeted killing of stromal CD11b^+ cells in the tumor microenvironment following radiotherapy has been previously reported by other investigators. For example, Zhang and colleagues have previously reported that radiotherapy can lead to the uptake of tumor antigen by CD11b^+ stromal cells, rendering them susceptible to...
T-cell–mediated killing using an adoptive T-cell transfer system (47). The current study represents an innovative strategy using intratumor vaccination to trigger potent antitumor effects mediated by tumor antigen-specific T cells without the requirement of adoptive transfer of antigen-specific CD8^+ T cells, as described in the publication by Zhang and colleagues. Furthermore, our data indicate that the intratumoral vaccination with antigen significantly expands tumor antigen-specific CD8^+ T cells compared with irradiation alone, without vaccination. Therefore, our data represent a significant strategy to expand tumor antigen-specific CD8^+ T cells through active immunization as compared with the approach described by Zhang and colleagues. Our regimen avoids the need for expensive and patient-specific in vitro T-cell expansion process required in adoptive T-cell transfer.

Antigen-specific CD8^+ T-cell expansion is essential for tumor control in our treatment regimen, and it is promoted by various pathways, including TLR4 and type I IFN signaling. We observed that although both TLR4-deficient mice and type I IFN receptor-deficient mice had reduced generation of antigen-specific CD8^+ T cells after radiotherapy, type I IFN pathway-deficient mice were observed to have a more drastically inhibited antitumor effect. Our observation is consistent with a previous study by Burnette and colleagues (52). They reported that type I IFN is important for radiotherapy-induced antitumor effects. However, Burnette study did not describe the same approach as that which we use here. Specifically, they did not use any vaccination strategy. Nevertheless, we similarly observed that type I IFN is important for the expansion of T cells and antitumor effects generated by local radiotherapy and intratumoral vaccination (Fig. 4).

The observation of different degrees of impact by the knockout of the TLR4 pathway and the type I IFN receptor might be related to differing receptor distribution in the cells and downstream signaling, and may be further complicated by additional players such as myeloid cells, TAMs, and MDSCs. For example, TLR4 expression on TAMs has been shown to induce NF-kB activity in tumor cells to promote tumor progression (53). Thus, by knocking down TLR4, we not only reduced the quantity of antitumor CD8^+ T cells, but we also removed signals essential for tumor growth, resulting in better tumor control. Furthermore, it is known that TLR4 signaling is important to induce T-cell apoptosis by Gr-1^+ CD11b^+ F4/80^+ cells (54). Hence, knocking out TLR4 allows tumor-infiltrating CD8^+ T cells to survive and carry out effector functions. As a result, TLR4-deficient mice may have a different signaling profile in the tumor microenvironment compared with type I IFN knockout mice, which potentially contributes to tumor regression despite a reduction in CD8^+ T cells.

Our study utilized two clinically tested therapeutic HPV vaccines, TA-HPV and TA-CIN. TA-HPV has been examined in various clinical trials in healthy volunteers (55), patients with cervical intraepithelial neoplasia (23, 55), and patients with vaginal and vulvar intraepithelial neoplasia (55, 56) and has been shown to be safe with only mild to moderate toxicity. Furthermore, a proportion of patients with stage Ib or IIA cervical cancer developed HPV-specific CD8^+ T-cell responses and developed HPV-specific antibodies (23). TA-CIN has been clinically tested in healthy volunteers and shown to be safe and effective in generating T-cell immunity against HPV-16 E6 and E7 (57). In addition, TA-CIN also has been tested in combination with a topical immunomodulator, imiquimod (58) in patients with vulvar intraepithelial neoplasia. Several prime/boost regimens have been used to test TA-CIN in combination with TA-HPV in patients with anogenital intraepithelial neoplasia (59, 60) and vulvar intraepithelial neoplasia (61). Both of these therapeutic HPV vaccines have been tested extensively in clinical trials and proven to be safe. TA-HPV and TA-CIN are promising vaccine candidates for future clinical trials of our treatment approach to enhance the antitumor effects in combination with radiotherapy.

Although we successfully applied our regimen to treat tumor-bearing humanized HLA-A2 transgenic mice, indicating its potential for treating human tumors, to further extend this concept for clinical translation, a few issues will need to be addressed. Because the therapy used in this study includes intratumor vaccination with antigenic peptide, it may be limited in the case of inaccessible tumors. This, however, may be resolved by modifying the delivered peptide to include tumor homing or tumor environment targeting aptamers. For example, CD13 ligand has been shown to be capable of delivering the antigenic peptide to tumor loci (62, 63) and elicit an antigen-specific antitumor response (64). Furthermore, because the efficacy of locally delivered peptide relies on the loading of the peptides onto the MHC class I molecules, the applications of MHC-restricted peptides may be limited to only a proportion of the population due to the polymorphism of MHC class I alleles. To overcome this obstacle, the regimen could utilize peptides containing multiple MHC class I epitopes, whole proteins, or synthetic overlapping peptides covering the entire sequence of the antigen of interest. Taken together, future studies are warranted to advance this regimen toward clinical translation.

Disclosure of Potential Conflicts of Interest
R.B.S. Roden has ownership interest (including patents) in Sanofi Pasteur, Shantha Biotechnics, GlaxoSmithKline, PanVax, and Papivax. No potential conflicts of interest were disclosed by the other authors.

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Grant Support
This work was supported by the NIH/National Cancer Institute Cervical Cancer SPORE P50CA098252, Head and Neck Cancer SPORE P50DE019302, Z01CA141425-06, and R43CA174436 grants.

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Received May 15, 2013; revised September 26, 2013; accepted November 5, 2013; published OnlineFirst December 3, 2013.

References


Enhanced Cancer Radiotherapy through Immunosuppressive Stromal Cell Destruction in Tumors

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1334

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