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Enhanced cancer radiotherapy through immunosuppressive stromal cell destruction in tumors

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Abstract

Purpose—Radiation therapy (RT) 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, RT 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 RT will lead to the priming of antigen-specific cytotoxic T lymphocyte (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.

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Results—We show that by combining RT and targeted antigenic peptide delivery to the tumor, the adjuvant effect generated by RT itself was sufficient to elicit the priming and expansion of antigen-specific CTLs, through the type I interferon dependent pathway, leading to synergistic therapeutic antitumor effects compared to 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 HPV vaccines.

Conclusions—These data serve as an important foundation for the future clinical translation of RT combined with a clinically tested therapeutic HPV vaccine for the control of HPV-associated cancers.

Keywords

radiotherapy; immunotherapy; tumor microenvironment; myeloid derived suppressor cells

Introduction

Radiation therapy (RT) 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). RT achieves its therapeutic effect by inducing tumor cell death. However, a secondary effect of RT is the local antigen release from necrotic tumor cells, which can stimulate systemic antitumor immune responses (5–8). In addition to the release of tumor antigens into the microenvironment, there is extracellular release of damage-associated molecular patterns (DAMPs), such as HMGB1, S100 protein, heat shock proteins, CRT, DNA and ATP (6, 7). Many of these DAMPs, as well as pro-inflammatory 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 provides a rationale for combining RT 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 RT 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 accumulation of CD11b+ myeloid cells including CD11b+Gr1+ myeloid derived suppressor cells (MDSCs) and CD11b+F4/80+ tumor associated macrophages (TAMs) (13–16). Given the infiltration of these cell types in the post-RT microenvironment, there is an opportunity to target these myeloid infiltrates following RT to restore CD8+ T cell responsiveness and antitumor responses.

In the current study, we hypothesized that by locally injecting antigenic peptide into the irradiated tumor, not only would we be able to prime antigen-specific CD8+ T cells through the adjuvant effect of RT, but also load the myeloid cells with antigen, rendering them susceptible to killing by the vaccine-induced antigen-specific CD8+ T cells. In order to test this hypothesis, it is important to identify a tumor model that demonstrates MDSCs as a major source of immunosuppression. Previously, it has been shown that MDSCs contribute to the immunosuppressive status of HPV E7-expressing TC-1 tumors and selective depletion of MDSCs in TC-1 tumors results in potent antitumor effects (17, 18). Thus, TC-1 is a suitable model for testing our hypothesis. With the TC-1 tumor model (19), we show that by locally injecting E7 antigenic peptide without adjuvant into the irradiated tumor, the secondary adjuvant effect generated by RT itself is sufficient to elicit a potent antigen-specific CD8+ T cell immune response, which is able to control both local and systemic TC-1 tumor growth. In addition, the injected E7 or ovalbumin (OVA) antigenic peptides can

be processed by CD11b⁺ myeloid cells, making them susceptible to CD8⁺ T cell-mediated killing, and thus modulates the tumor microenvironment to be favorable for better tumor control. The clinical applications of the treatment regimen in human cancers are discussed.

Materials and Methods

Mice

6- to 8-week-old female C57BL/6 and nude BALB/c mice were obtained from the National Cancer Institute. TLR4 knockout mice and B6.Cg-*foxn1*^{nu}/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IFNAR knockout mice were a kind gift from Dr. G. Cheng (University of California, Los Angeles, CA), and HLA-A*0201/D^d (AAD) transgenic female C57BL/6 mice, 6–8 weeks of age, were kindly provided by Dr. Victor Engelhard at the University of Virginia Health Sciences Center (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 has been described previously (19). TC-1 cells were subjected to RapidMAP (Taconic Farms, Rensselaer, NY) testing, a panel of PCR tests for rodent viruses, most recently in May 2011 with negative results. 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 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential 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/D^d (AAD) transgenic mice (n = 4–10). Fourteen or fifteen days later, (when tumor diameter was approximately 0.5 cm), mice were treated with RT at a dose of 14 Gy/mouse (using a Shepherd Mark I irradiator) in combination with intratumor (i.t.) injection of 50 µg E7 peptide (aa 43–62). A total of 5 E7 injections were administered, starting on day 15 with 3-day intervals. Notably, the injected E7 peptide (aa 43–62) contains an H-2D^b-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 i.t. injected with 40 µL TA-HPV once on the day of RT. In TA-CIN (24) treated groups, mice were injected with 25 µg TA-CIN protein at 5 day intervals, for a total of 3 treatments. Mice were monitored for tumor growth by visual inspection and palpation every three 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 s.c. with 1×10^5 TC-1 cells each and treated as described above. Mice were then inoculated intravenously with 1×10^5 TC-1 cells each on day 14 after s.c. tumor challenge. The number of pulmonary tumor nodules was quantified on day 35.

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

C57BL/6 mice were inoculated s.c. with 1×10^5 TC-1 cells each and treated with local RT and intratumoral vaccination as described above. Mice were then inoculated intravenously with 1×10^5 TC-1 cells each on day 14 after s.c. 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 s.c. with 1×10^5 TC-1 cells per mouse and treated with radiation and E7 peptide (50 μ g) according to the regimen described above. In the CD8+ T cell depletion group, 100 μ g/mouse of anti-CD8 antibody (clone 2.43) was delivered via i.p. injection on the same day as RT, as well as 3 and 6 days after RT.

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^d (AAD) transgenic mice (n = 4-5) were treated as described above in the tumor treatment experiments. Two weeks after RT, splenocytes, draining lymph nodes (DLN) and peripheral blood mononuclear cells (PBMCs) were isolated from the mice and characterized for the presence of antigen-specific CD8+ T cells. All samples were pre-treated with CD16/CD32 FcR blocker (BD Biosciences, San Jose, CA) before staining. For tetramer staining, PE-labeled H-2D^b tetramers containing HPV-16 E7 aa 49-57 peptide (RAHYNIVTF) (Beckman Coulter) were used for the analysis of E7-specific CD8+ T cells (26). Allophycocyanin (APC)-labeled H-2K^b 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 RT and 6×10^5 pooled PBMCs from each group were incubated with 1 μ g/ml E7 peptide or NS1 peptide (AIMDKNIIL), together with GolgiPlug (1000 $\times$) (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 RT, tumors were harvested, chopped into 2–3 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 μ g/ml streptomycin) at 37 °C for 1 hour before passage through a cell strainer. Cells were washed and enriched for lymphocytes by LymphoprepTM (AXIS-SHIELD) separation. After washing with PBS twice, cells were pre-treated 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-2D^b tetramer or OVA peptide (SIINFYKL) loaded H-2K^b tetramer and analyzed by flow cytometry.

CD11c+ DC migration into lymph nodes

TC-1 tumor-bearing mice were treated with or without RT as described above and administered i.t. with 50 μ g 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 RT. Cells were stained with APC-labeled anti-CD11c antibody and PE-labeled anti-ICAM-1, -CD80, or -CD86 antibody (BD Pharmingen).

Tumor-infiltrating CD11b⁺ myeloid cell populations

To analyze the post-RT myeloid-cell-infiltrates, TC-1 tumor bearing mice (n=3) were treated with RT. Three days after RT, tumors were harvested, chopped into 2–3 mm pieces and digested with digestion buffer at 37 °C for 1 hour before passage through cell strainer. Cells were washed twice with 1 $\times$ PBS, pre-treated 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 RT combined with i.t. peptide injection, groups of TC-1 tumor bearing mice (n = 4-5) were

treated as described in the tumor treatment experiment. Two weeks after RT, tumors were harvested, processed into single cells as mentioned above and stained with the antibodies listed above along with PE-labeled Ly-6G (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-2K^b) on CD11b⁺ myeloid cells, TC-1 tumor bearing mice (n=3) were treated with RT at a dose of 14 Gy/mouse and 50 µg of OVA peptide (aa 241-270) was i.t. injected 3 days after RT. One day later, tumors were harvested, processed into single cells and pre-treated 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 SIINFYKL-H-2K^b 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 RT and injected with OVA peptide as mentioned. The treated tumors were harvested one day after OVA peptide injection, processed into single cells and co-cultured with 1×10^6 activated OT-1 T cells for one day. Cells were collected, washed, stained with anti-CD11b, -Gr-1, -F4/80 and PE-labeled anti-active caspase 3 antibodies (eBioscience), and analyzed by flow cytometry.

Tumor treatment experiments in nude mice with T cell adoptive transfer

TC-1 tumor cells (1×10^5 per mouse) were inoculated s.c. into nude BALB/c mice and nude C57BL/6 B6.Cg-*foxn1*^{nu}/J mice (n=5). Fifteen days after tumor inoculation, tumors were irradiated with 14 Gy/mouse in combination with i.t. injection of 50 µg E7 peptide (aa 43-62) every 3 days from the day of RT until the mice were sacrificed. One day after RT, 2×10^6 activated E7-specific CD8⁺ T cells (21) were adoptively transferred 3 times at one week intervals via intravenous tail vein injection. Mice were monitored for tumor growth by visual inspection and palpation every three days.

Statistical analysis

The data presented in this study are representative of 3 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. Individual data points were compared using Student's t-test. The event-time distributions for different mice were estimated using the Kaplan-Meier method and compared by 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 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 RT results in potent antitumor immune responses

First, we assessed the anti-tumor effect generated by intratumoral E7 vaccination following RT using the HPV-16 E7-expressing TC-1 murine tumor model (19). The treatment schedule included local RT followed by either intratumoral E7 peptide (aa 43-62) vaccination or vaccination into the contralateral subcutaneous (s.c.) tissue, as outlined in

Figure 1A. The combination of RT 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 to either RT alone or the combination of RT and s.c. administration of E7 peptide (Figure 1B). Additionally, three weeks after RT, seven out of ten mice receiving the combined treatment had complete tumor regression and remained tumor free up to 60 days after completing RT. Long term tumor protection is further illustrated by the fact that these mice remained tumor free after s.c. re-challenge with TC-1 tumor cells (Supplementary Figure 2). In order to demonstrate whether the local RT 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 Figure 1C, local RT 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. This data indicates that local RT in conjunction with local E7 vaccination is able to create systemic immunity against E7-expressing tumors at distant sites. In order 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 ($P=0.036$) (Figure 1D). Furthermore, in order 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 Figure 3). Taken together our data indicate that the combination of intratumoral vaccination with E7 peptide following RT is capable of improving local and systemic control of tumors in the absence of any adjuvant.

RT 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 RT and intratumoral E7 peptide vaccination. In tumor-bearing mice treated with RT followed by intratumoral E7 peptide vaccination, a significantly higher number of E7-specific CD8⁺ T cells were present in PBMCs, DLNs and splenocytes, compared to other treatment groups ($P<0.05$ for each comparison) (Figure 2A, Supplementary Figure 4). E7-specific TILs were also statistically higher in mice receiving combination treatment as compared to other groups ($P<0.001$) (Figure 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 ($P<0.001$) (Figure 2B). Thus, these data indicate that RT 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 RT facilitates the development of an adaptive immune response in mice treated with RT followed by intratumoral E7 peptide vaccination. We administered FITC-labeled E7 peptide into the tumors of TC-1 tumor-bearing mice with or without RT and isolated DLNs from the treated mice. We found an increased number of FITC⁺ DCs in the DLNs of mice treated with RT compared to mice vaccinated without RT ($P=0.009$) (Figure 2D). Furthermore, the FITC⁺ DCs had a more mature phenotype characterized by higher mean expression of the co-stimulatory molecules CD80, CD86 and ICAM-1 relative to FITC⁻ DCs (Figure 2D). These data indicate that RT 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 RT and intratumoral E7 peptide vaccination is mediated through the type I interferon and TLR4 pathways

We further explored the molecular signaling pathways underlying the priming of an adaptive E7-specific immune response. After the combination of RT and intratumoral E7 vaccination, CD8⁺ T cells from both transgenic mice deficient in type I interferon receptor (IFNAR^{-/-}) and mice deficient in TLR4 (TLR4^{-/-}) mounted weaker systemic and local E7-specific immune responses compared to wild type mice ($P<0.001$) (Figure 3A and B). IFNAR^{-/-} mice also had weaker antitumor immune responses, characterized by poor tumor control ($P=0.035$) (Figure 3C). Interestingly, IFNAR^{-/-} mice had significantly reduced survival as compared to wild type mice, whereas survival in TLR4^{-/-} mice was comparable to wild type (Figure 3D). This data suggests that the type I interferon pathway is important for mounting adaptive CD8⁺ T cell immune responses after combination treatment with RT and intratumoral E7 peptide vaccination treatment. We also evaluated the antitumor response generated in mice deficient in TLR9 (TLR9^{-/-}) following combination treatment with RT and intratumoral E7 peptide vaccination. We did not find a significant difference as compared to wild type mice (Supplementary Figure 5) suggesting that TLR9 does not contribute significantly to tumor control after RT and antigenic peptide administration.

Combination RT 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 RT. Therefore, we first assessed the presence of CD11b⁺ myeloid cells in the tumor infiltrate after RT 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 to non-irradiated tumors ($P=0.015$) (Figure 4A). In addition, RT resulted in a significant enrichment of the major CD11b⁺ myeloid cell subsets CD11b⁺Gr-1⁺ MDSCs and CD11b⁺F4/80⁺ TAMs in the tumor microenvironment ($P<0.05$) (Figure 4B). The enrichment of CD11b⁺ myeloid cells after RT was most pronounced in the tumor, but could also be detected in the spleen and DLN (Supplementary Figure 6). 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 K^b-restricted CTL epitope. When OVA was administered intratumorally following RT, 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 (Figure 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, OT-1 T cells. We incubated OT-1 T cells with CD11b⁺ cells derived from tumors of TC-1 tumor-bearing mice treated with RT either alone or with intratumoral OVA peptide vaccination. As shown in Figure 4C, the number of caspase 3⁺ cells in mice treated with intratumoral OVA peptide vaccination was significantly higher compared to 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 Figure 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 to TAMs from mice not vaccinated intratumorally with OVA (Supplementary Figure 7A). Next, we determined whether the

tumor-infiltrating CD11b⁺ myeloid cells were indeed reduced in number following RT 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 RT either alone or with intratumoral E7 antigenic peptide vaccination. As shown in Figure 4D, we observed a significant reduction in the frequency of tumor-infiltrating CD11b⁺ myeloid cells from mice treated with combination RT and intratumor vaccination with E7 peptide compared to mice receiving RT 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 Figure 4D, MDSCs were significantly reduced in tumors treated with RT and intratumoral peptide vaccination compared to tumors treated with monotherapy, either RT or E7 peptide ($P<0.01$). When subsets of MDSCs were further characterized, Ly6G⁺Ly6c^{low} granulocytic MDSCs (G-MDSCs) were found to be the dominant population affected (Supplementary Figure 8). We did not observe a significant reduction in the tumor-infiltrating CD11b⁺ F4/80⁺ TAMs in mice treated with RT with intratumoral E7 peptide vaccination compared to tumors treated with RT alone (Supplementary Figure 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 RT 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 RT and intratumoral vaccination with antigenic peptide contributes to the antitumor effect. Since the E7 peptide (aa 43-62) we used contains an H-2D^b 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-*foxn1*^{nu}/J mice (H-2D^b) and not those in TC-1 tumor-bearing nude BALB/c mice (H-2D^d). With adoptive transfer of E7-specific CD8⁺ T cells, we observed that B6.Cg-*foxn1*^{nu}/J mice carrying the H-2D^b MHC class I molecules had significantly better tumor control as compared to the nude BALB/c mice ($P=0.015$) (Figure 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 employed 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⁺ stromal cells of TC-1 tumor-bearing HLA-A2 transgenic mice (29). Following the combination treatment of RT and intratumoral NS1 peptide vaccination, NS1-specific CD8⁺ T cells were enriched and tumor control was enhanced (Figure 5B and C). Taken together, our data suggest that *in vivo* killing of the tumor stromal cells, including CD11b⁺ myeloid cells, by antigen-specific CD8⁺ T cells further contributes to the antitumor effect achieved with the combination of RT and intratumoral peptide vaccination.

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

Since 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 non-specific peptide vaccinations. Therefore, we vaccinated with a non-TC-1 tumor related antigen, the OVA peptide (29). As shown in Supplementary Figure 9A and 9B, OVA-specific CD8⁺ T cells were significantly enriched after combination treatment compared to 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 Figure 9C), which correlated with an enhanced antitumor effect ($P=0.036$) (Supplementary Figure 9D). Thus, these data indicate that intratumoral vaccination with non-tumor related antigenic peptides following RT 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 RT with a variety of clinically tested HPV vaccines results in improved antitumor effects compared to RT alone

In order to extend the potential clinical translation of the treatment methodology beyond peptide vaccines, we treated TC-1 tumor-bearing mice with RT 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 show that mice treated with RT followed by intratumoral TA-HPV administration have decreased tumor growth and enhanced survival compared to those treated with RT alone. Furthermore, as shown in Figure 6B and D, TC-1 tumor bearing mice treated with RT combined with intratumoral TA-CIN generated enhanced antitumor effects and prolonged survival compared to mice treated with RT alone. This data indicates that RT 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 RT, with immunotherapy. We demonstrate that treatment of TC-1 tumor-bearing mice with RT 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 to vaccination alone, RT alone, or RT followed by s.c. 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 RT 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 RT, 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 (Figure 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 to 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 RT, there is a better antitumor effect when antigen-specific CD8⁺ T cells are capable of recognizing and killing tumor infiltrating myeloid stromal cells (Figure 5A, Figure 6, Supplementary Figure 7). Additionally, 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 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 RT (Figure 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 to CD11b⁺ F4/80⁺ TAMs under the same conditions (Figure 4C, Supplementary Figure 6A). Consistent with this finding, a greater reduction of MDSCs between treatment with RT and RT combined with peptide vaccination was observed compared to TAMs (Figure 4D, Supplementary Figure 6B). Taken together, our findings indicate that MDSCs are more susceptible than TAMs to T cell-mediated killing. Our observation is consistent with previous reported by Sinha et al that MDSCs are susceptible to T cell-mediated killing (48). Some potential mechanisms may account for this observation. For example, the expression level of anti-apoptotic 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 RT have been targeted in several therapeutic attempts in order 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 RT has been previously reported by other investigators. For example, Zhang et al. have previously reported that RT 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 employing 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 et al. Furthermore, our data indicate that the intratumoral vaccination with antigen significantly expands tumor antigen-specific CD8+ T cells compared to irradiation alone, without vaccination. Therefore, our data represents a significant strategy to expand tumor antigen-specific CD8+ T cells through active immunization as compared to the approach described by Zhang et al. 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 interferon signaling. We observed that although both TLR4 deficient mice and type I interferon receptor deficient mice had reduced generation of antigen-specific CD8+ T cells after RT, type I interferon pathway deficient mice were observed to have a more drastically inhibited antitumor effect. Our observation is consistent with a previous study by Burnette et al (52). They reported that type I interferon is important for RT-induced antitumor effects. However, Burnette's study did not describe the same approach as that which we employ here. Specifically, they did not employ any vaccination strategy. Nevertheless, we similarly observed that type I interferon is important for the expansion of T cells and antitumor effects generated by local RT and intratumoral vaccination (Figure 4).

The observation of different degrees of impact by the knockout of the TLR4 pathway and the type I interferon 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 expressed on TAMs has been shown to induce NF- κ B 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 interferon 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 vulval intraepithelial neoplasia (55, 56) and 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). Additionally, 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 RT.

Although we successfully applied our regimen to treat tumor-bearing humanized HLA-A2 transgenic mice, indicating its potential for treating human tumors, in order to further extend this concept for clinical translation, a few issues will need to be addressed. Since 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, since 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Radiation therapy (RT) 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 RT and targeted antigenic peptide delivery to the tumor. We found that the adjuvant effect generated by RT 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 RT could be combined with intratumorally injected clinically tested therapeutic HPV vaccines to decrease tumor size and improve survival in treated mice compared to RT alone. These data serve as an important foundation for future clinical translation using RT combined with a clinically tested therapeutic HPV vaccine for the control of cancer.

Figure 1. Characterization of antitumor effect in tumor-bearing mice treated with radiation and E7 peptide

(A) Schematic diagram of treatment regimens. C57BL/6 mice (5 per group) were injected with 1×10^5 TC-1 cells subcutaneously, irradiated with 14 Gy/mouse and then vaccinated with E7 peptide for a total of 5 times with 3-day intervals. (B) Top: characterization of tumor growth in groups of treated mice ($n=5$). Left panel shows tumor size over time ($*P=0.026$ & $**P=0.029$). Bottom: Kaplan-Meier survival analysis of tumor-bearing mice ($n=8-10$) in different treatment groups. Representative data from duplicated experiments ($**P<0.001$) (C) Characterization of systemic antitumor effect against TC-1 tumor following local treatment. C57BL/6 mice were inoculated s.c. with 1×10^5 TC-1 cells each and treated as outlined in (A). Mice were then inoculated intravenously with 1×10^5 TC-1 cells each on day 14 after s.c. tumor challenge. The number of pulmonary tumor nodules was quantified on day 35. Box and whisker plot depicts the number of pulmonary nodules in each group after treatment. The box represents IQR, the horizontal line in the box represents the median, and the vertical line with margin represents the maximum and minimum percentiles. (D) *In vivo* CD8⁺ T cell depletion experiment. TC-1 tumor-bearing mice ($n=5$) were treated with radiation followed by i.t. vaccination with E7 peptide, with or without anti-CD8 antibody. Tumor size was plotted over time ($*P=0.036$).

Figure 2. Characterization of antigen-specific immune responses in tumor bearing mice treated with radiation and E7 peptide

(A) Left: groups of TC-1 tumor bearing mice (n=5) were treated as described in Figure 1A. Two weeks after RT, splenocytes, DLNs and PBMCs were isolated and characterized for the presence of E7-specific CD8⁺ T cells using flow cytometry. Right: Box and Whisker plot depicting the percentage of E7 tetramer-positive CD8⁺ cells among total CD8⁺ cells (mean $\pm$ S.D.) (* P <0.05). The box represents IQR, the horizontal line in the box the median, vertical lines with margin represent maximum and minimum percentiles. (B) Left: Tumor infiltrating lymphocytes (TILs) isolated from treated tumors were characterized for the presence of E7-specific CD8⁺ T cells 2 weeks after RT using flow cytometry. Right: bar graph depicting the percentage of E7-specific CD8⁺ T cells in TILs (mean $\pm$ S.D) (* P <0.001). (C) To characterize E7 antigen-loaded CD11c⁺ DCs in the DLN, i.e. vaccination with FITC-E7 peptide was administered to TC-1 tumor-bearing mice on the same day of RT. Left panel shows representative flow cytometry analysis demonstrating the number of FITC-E7 peptide-loaded CD11c⁺ cells in the DLN. Right panel depicts the mean percentage of FITC positive cells in CD11c⁺ cells in the DLNs (* P =0.009). (D) Flow cytometry to characterize the expression of CD80, CD86 and ICAM-1 in CD11c⁺ cells with or without uptake of FITC-E7 antigen in DLNs from tumor bearing mice treated with RT. Left panel shows representative flow cytometry analysis. Right panel shows representative bar graph depicting the mean fluorescence intensity (MFI) of CD80, CD86 and ICAM-1 in CD11c⁺ cells.

Figure 3. Characterization of the contribution of type I interferon and TLR4 pathways in the E7 antigen-specific CD8⁺ T cell response

(A) WT C57BL/6 mice, IFNAR^{-/-} mice and TLR4^{-/-} mice bearing TC-1 tumors were treated with local RT and intratumor vaccination with E7 peptide using the regimen outlined in Figure 1A. Flow cytometry analysis demonstrating E7-specific CD8⁺ T cells in PBMCs and TILs using E7 peptide-loaded H-2D^b tetramer staining. (B) Left: Box and Whisker plot depicting the percentage of E7-specific CD8⁺ cells among total CD8⁺ PBMCs. The box represents IQR, the horizontal line in the box the median, vertical lines with margin represents the maximum and minimum percentiles. Right: bar graph depicting the percentage of CD8⁺ T cells of total TILs. E7-specific CD8⁺ T cells in TILs are indicated by darker shading (mean $\pm$ S.D) (* P <0.001). (C) TC-1 tumor-bearing WT C57BL/6 mice, IFNAR^{-/-} mice and TLR4^{-/-} mice (n=5) were treated as described in Figure 1A. Tumor growth was characterized by measuring tumor size over time (* P =0.035). (D) Kaplan-Meier survival analysis of TC-1 tumor-bearing WT, IFNAR^{-/-} and TLR4^{-/-} mice treated as described in Figure 1A.

Figure 4. Characterization of CD11b⁺ myeloid cells in the tumor microenvironment after radiation and antigenic peptide vaccination

(A) Flow cytometry analysis. Left panel is a histogram and right panel is a bar graph showing the presence of CD11b⁺ myeloid cells in TC-1 tumors (mean $\pm$ S.D.) (* $P=0.015$) (B) Left: characterization of the CD11b⁺ myeloid cell subsets in tumor infiltrates showing the percentage of CD11b⁺ myeloid cell subsets in total live cells isolated from tumors. Top: representative flow cytometry. Bottom: histogram (mean $\pm$ S.D.) (* $P<0.05$). Right: characterization of OVA peptide loading on the MHC class I molecule (H-2K^b) on CD11b⁺ myeloid cell subsets from tumors following RT and i.t. vaccination with OVA peptide by flow cytometry. The presentation of OVA peptide through K^b MHC class I molecules was determined using PE-labeled OVA-K^b-specific monoclonal antibody 25-D1.16 (eBioscience). Dark gray histogram represents the isotype control. (C) Top: characterization of OT-1 T cell-mediated apoptosis of CD11b⁺ myeloid cells from irradiated tumors with i.t. vaccination with OVA peptide by flow cytometry analysis to demonstrate active caspase 3 expression in CD11b⁺ myeloid cells (mean $\pm$ S.D.) (* $P=0.043$). Bottom: characterization of the OT-1 T cell-mediated apoptosis of CD11b⁺ Gr-1⁺ MDSCs from irradiated tumors with or without i.t. vaccination with OVA peptide to demonstrate OT-1 T cell-mediated apoptosis of MDSCs by active caspase 3 staining followed by flow cytometry analysis (mean $\pm$ S.D.) (* $P=0.015$). (D) Left: flow cytometry analysis to characterize the percentage of CD11b⁺ cells in total live cells from TC-1 tumors treated as described in Figure 1A (mean $\pm$ S.D.) (* $P<0.05$). Right: top is representative flow cytometry analysis and bottom is bar graph of CD11b⁺ Gr-1⁺ MDSC in tumors after treatment (mean $\pm$ S.D.) (* $P<0.01$).

Figure 5. Characterization of the antitumor effect facilitated by CD8+ T cell-mediated killing of stromal cells

(A) Characterization of TC-1 tumor growth in nude-BALB/c mice or nude-C57BL/6 B6.Cg-*foxn1*^{nu}/J mice treated with RT and intratumor vaccination with E7 peptide as described in Figure 1A. Adoptive transfer of E7-specific CD8+ T cells was initiated one day after RT and repeated twice at one week intervals. Line graph depicts tumor growth over time (mean $\pm$ S.D) (* $P=0.015$). (B) and (C) Characterization of antigen-specific CD8+ T cell immune response and antitumor effects in TC-1 tumor-bearing HLA-A2 transgenic mice treated by RT with i.t. vaccination with NS1 peptide. (B) Intracellular IFN- γ staining followed by flow cytometry analysis to demonstrate NS1 peptide-specific CD8+ T cells in PBMCs. PBMCs isolated from TC-1 tumor-bearing HLA-A2 transgenic mice that received RT and i.t. vaccination with NS1 peptide as described in Figure 1A. Upper panel is a representative flow cytometry analysis and lower panel shows bar graph depicting the percentage of NS1-specific CD8+ T cells among total T cells (mean $\pm$ S.D.). (C) *In vivo* tumor treatment experiment. TC-1 tumor-bearing HLA A2 transgenic mice (5 per group) were treated with RT followed by i.t. vaccination with NS1 as described in Figure 1A. Tumor size was measured and plotted over time (mean $\pm$ SD)(* $P=0.038$).

* All mice that survived past day 40 were tumor-free

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 1×10^5 TC-1 cells subcutaneously and treated 14 days later (tumor size approximately 0.5 cm in diameter) with RT at a dose of 14 Gy/mouse combined with i.t. administration of clinically tested TA-HPV or TA-CIN vaccine. TA-HPV was i.t. injected at a dose of 40 μ L on the day of RT. TA-CIN was given at a dose of 25 μ g and injected on the day of RT and every 5 days after for a total of 3 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.