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Radiotherapy cooperates with IL15 to induce antitumor immune responses

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Abstract

Focal radiotherapy can promote cross-presentation of tumor antigens to T cells, but by itself it is insufficient to induce therapeutically effective T-cell responses. The common gamma-chain cytokine IL15 promotes and sustains the proliferation and effector function of CD8⁺ T cells, but has limited activity against poorly immunogenic tumors that do not elicit significant spontaneous T-cell responses. Here, we show that radiotherapy and subcutaneous IL15 had complementary effects and induced CD8⁺ T cell-mediated tumor regression and long-term protective memory responses in two mouse carcinoma models unresponsive to IL15 alone. Mechanistically, radiotherapy-induced IFN type I production and Batf3-dependent conventional dendritic cells type 1 (cDC1s) were required for priming of tumor-specific CD8⁺ T cells and for the therapeutic effect of the combination. IL15 cooperated with radiotherapy to activate and recruit cDC1s to the tumor. IL15 alone and in complex with a hybrid molecule containing the IL15 α receptor have been tested in early phase clinical trials in cancer patients and demonstrated good tolerability, especially when given subcutaneously. Expansion of NK cells and CD8⁺ T cells were noted, without clear clinical activity, suggesting further testing of IL15 as a component of a combinatorial treatment with other agents. Our results provide the rationale for testing combinations of IL15 with radiotherapy in the clinic.

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Keywords

Breast cancer; CD8⁺ T cells; dendritic cells; IFN type I; interleukin-15; radiotherapy

Introduction

The common gamma-chain cytokines interleukin (IL)2 and IL15 are primary regulators of T-cell homeostasis and thus have been considered prime candidates for increasing T-cell levels and function in cancer patients (1). IL2 was the first immunotherapy to be approved for metastatic melanoma and renal cell carcinoma due to its ability to elicit durable responses in a small percentage of patients (2). However, IL2 is effective only at high doses, which are associated with serious toxicities, limiting its use. Similarly to IL2, IL15 signals via a common intermediate affinity receptor complex consisting of γ_c (CD132) and β (CD122) subunits expressed mainly in CD8⁺ T cells and NK cells and stimulates the proliferation of activated and memory CD8⁺ T cells and the proliferation and activation of NK cells (3). In contrast to IL2, IL15 does not stimulate the expansion and maintenance of regulatory T cells (Tregs) and does not cause activation-induced T-cell death (4), making it an ideal candidate for increasing and sustaining antitumor CD8⁺ T- and NK-cell responses (1). Differences between the α chain of the IL2 and IL15 receptor (IL15R), which is expressed on the target lymphocytes for IL2 but on antigen-presenting cells (APCs) for IL15, underlie the biological differences between these two cytokines (4).

IL15 has shown antitumor effects in preclinical mouse tumor models (3,5) and has been tested in patients. In a first in human trial, recombinant human IL15 (rhIL15) administered intravenously (i.v.) elicits changes in circulating T and NK cells with some evidence of tumor response. However, the activity of IL15 as single agent was limited by its short half-life and dose-limiting toxicity related to massive cytokine secretion shortly after i.v. bolus infusion (6). In contrast, IL15 administered subcutaneously (s.c.) has shown good tolerability, while inducing significant NK and CD8⁺ T-cell expansion (7), supporting the subcutaneous administration route in studies testing the benefits of IL15 in combination with other treatments.

The requirement that IL15 is trans-presented to NK and T cells by IL15R α chain-positive APCs hinders its activity in tumors with low infiltration by APCs. To overcome this barrier, IL15 has been engineered as a complex with a modified IL15R α chain that binds directly to T and NK cells, achieving an up to 50-fold increase in activity (8). Among the IL15 complexes in development, a promising agent is ALT-803, which contains two molecules of an optimized IL15 “superagonist” and two molecules of the IL15 α receptor “sushi” domain fused to a dimeric human IgG1 Fc (9). Results of clinical testing indicate that subcutaneous administration of ALT-803 induces a modest expansion of CD8⁺ T cells and a more pronounced expansion of NK cells with minimal toxicity (10). Feasibility of combining ALT-803 with anti-PD-1 in lung cancer has been reported, and studies are ongoing to test its efficacy (11).

Our group and others have shown that radiation induces cancer cell intrinsic interferon type I (IFN-I) production mediated by accumulation of cytosolic DNA that activates the cyclic

GMP-AMP synthase (cGAS)/stimulation of interferon genes (STING) pathway (12,13). This response is abrogated by shRNA-mediated downregulation of cGAS expression in the cancer cells, and is optimally induced by radiation given in a hypo-fractionated regimen of 8GyX3, whereas a single dose of 20Gy does not lead to activation of IFN-I (12). IFN-I has been previously shown to be critical for the recruitment of conventional dendritic cells type 1 (cDC1s), a subset of DCs specialized in cross-presenting tumor antigens to CD8⁺ T cells (14-16), into tumors. Activation of systemic antitumor CD8⁺ T-cell responses by focal tumor radiotherapy requires concomitant blockade of CTLA-4 to allow for the expansion of a diverse repertoire of antitumor T cells (12,17).

Here, we hypothesized that radiation-induced IFN-I could synergize with subcutaneously delivered IL15 by recruiting DCs that bind and trans-present IL15 to T cells (18). We also wanted to test if the improved expansion of CD8⁺ T cells in the presence of IL15 could generate effective antitumor CD8⁺ T-cell responses in the absence of CTLA-4 blockade. To test this hypothesis, we utilized three mouse carcinoma models, the TSA mammary carcinoma and MCA38 colorectal carcinoma, which we have extensively characterized in terms of their production of IFN-I in response to radiation (12), and the lung carcinoma LLC1. Our data show that although IL15 by itself did not have any effect on tumor growth, it synergized with radiotherapy given at 8GyX3, but not at 20Gy, in inducing durable regression of the irradiated tumor, abscopal responses, and long-term survival of the TSA tumor-bearing mice. Similar results were obtained with MCA38, although no synergy was observed in the LLC1 model. Further studies revealed that radiation-induced IFN-I, cDC1s, and CD8⁺ T cells were required for the therapeutic synergy of radiotherapy and IL15. Overall, these data provide the rationale for future testing of radiotherapy with IL15 in the clinic.

Materials and Methods

Cells and reagents

BALB/c-derived mouse TSA mammary carcinoma cell line was obtained from Dr. Lollini (19) and authenticated by IDEXX Bioresearch (Columbia, MO, USA) in 2019. C57BL/6-derived mouse MCA38 colorectal carcinoma cells line was obtained from Dr. Frey (20) in 2009 and authenticated by IDEXX Bioresearch in 2016. Both cells were grown in complete medium consisting of DMEM (Invitrogen) supplemented with L-glutamine (2 mol/L), penicillin (100 U/mL), streptomycin (100 µg/mL), 2-mercaptoethanol (2.5×10^{-5} mol/L), and 10% fetal bovine serum (Invitrogen). Lewis lung carcinoma (LLC1) cells were obtained from ATCC in 2020 and maintained in DMEM containing 4mM L-glutamine, glucose (4500 mg/L), 1mM sodium pyruvate, sodium bicarbonate (1500 mg/L), and 10% FBS. Cells were routinely screened for *Mycoplasma* (LookOut® Mycoplasma PCR Detection kit, Sigma-Aldrich). For *in vivo* experiments, minimally passaged stock cells were freshly thawed and split once prior to implantation. Recombinant human IL15 was kindly provided by the Cancer Therapy Evaluation Program (NCI). InVivoMAB anti-mouse CD8a (Clone 53-6.7, used for CD8⁺ T-cell depletion), rat IgG2a isotype control, anti-mouse IFNAR-1 (Clone MAR1-5A3), and mouse IgG1 isotype control were all purchased from BioXCell (Lebanon, NH).

Animals and *in vivo* treatment

Six to eight-week old wild-type female BALB/c mice and male and female C57BL/6 mice were obtained from Taconic (Germantown, NY). BALB/c *Batf3*^{-/-} mice purchased from Jackson Labs were bred in-house to obtain sufficient numbers of 6-8 week old females used for tumor growth experiments. In experiments utilizing *Batf3*^{-/-} mice, age-matched naïve BALB/c female mice were simultaneously implanted with TSA tumor for comparison. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine. Mice were subcutaneously (s.c.) inoculated with 10⁵ cells (for TSA and LLC1 lines) or 5x10⁵ MCA38 cells and assigned randomly to treatment groups thirteen days later when tumors were 5-6 mm in average diameter. Ionizing radiation was delivered exclusively to the tumor using the Small Animal Radiation Research Platform (SARRP Xstrahl Ltd, Surrey, UK) in three doses of 8 Gy each on days 12, 13, and 14 post tumor implantation, as previously described (12). Briefly, all mice were anesthetized using avertin (240 mg/kg), and animals assigned to radiation were placed on a dedicated tray and positioned so that only the area of the tumor to be irradiated lies directly on the sharply delineated spot created by a focused beam using a 10x10mm collimator. IL15 was given peri-tumorally (5 µg in 50 µL PBS) starting on the first day of radiation and maintained daily for up to 10 days. This IL15 dose was chosen based on prior work showing that it was effective in mice (5). CD8-depleting antibody (200 µg/mouse) was given i.p. starting three days before radiotherapy and maintained weekly. Anti-IFNAR-1 (400 µg/mouse) was given i.p. every other day starting on the first day of radiation. Tumors were measured every 2–3 days until death or sacrifice when tumor dimensions exceeded 5% of body weight or if animals showed signs of significant pain or distress due to tumor size. Perpendicular tumor diameters were measured with a Vernier caliper, and total tumor volume calculated as: longer diameter x shorter diameter² x 0.52. In some experiments, mice that rejected the tumor completely after treatment and remained tumor-free for at least 100 days were inoculated in the contralateral flank with 10⁶ viable TSA cells and tumor growth monitored. A group of naïve mice was similarly challenged as control. For abscopal studies, TSA cells were injected in both flanks two days apart as previously described (21), and only one tumor was treated with three doses of 8 Gy each on days 12, 13, and 14 post tumor implantation. IL15 was given peri-tumorally only around the irradiated tumor.

Immunostaining

Freshly excised tumors were fixed in 4% paraformaldehyde for 1 hour prior to an overnight incubation with 30% sucrose. After several washes in PBS, tumors were frozen in optimum cutting temperature (OCT) medium and stored at -80°C. 5-micron sections were cut, incubated with 0.1% Tween-20 and 0.01% Triton-X100 for 20 minutes prior to blocking with 4% bovine serum albumin/PBS. Sections were stained with anti-mouse CD8-PE (Clone 53-6.7, BioLegend, San Diego, CA) applied at a 1:100 dilution in 1% BSA for 2 hours and washed three times. Slides were then counterstained with 4', 6-diamidino-2-phenylindole (Sigma), mounted with Vectashield, and imaged on a Nikon Eclipse 800 deconvolution microscope at 40x magnification. Cells with membranous PE staining and nuclear DAPI staining were counted from at least three randomly selected fields by 2 independent observers.

Flow cytometry

TSA tumors were excised and digested using mouse Tumor Dissociation Kit (Cat 130-096-730, Miltenyi Biotec) prepared per manufacturer's instructions and ran on a Miltenyi gentleMACS Octo Dissociator with Heaters using manufacturer's recommended pre-set program (37C_m_TDK1). The resulting cell suspensions were filtered using a 40-micron cell strainer, and red blood cells were lysed. Samples were counted, and stained with fixable Zombie Aqua (BioLegend) to exclude dead cells. All samples were incubated with purified anti-mouse CD16/32 (Fc block) for 15 minutes at room temperature prior to staining. The following anti-mouse antibodies, all purchased from BioLegend, were used for flow staining in the indicated dilutions: CD103 APC-Cy7 Clone 2E7 (1:25), CD45 APC-Cy7 Clone 30-F11 (1:400), CD11c Biotin Clone N418 (1:400), CD3 BV421 Clone 145-2C11(1:50), CD11c BV421 Clone N418 (1:100), CD8 FITC Clone 53-6.7 (1:100), CD11c PE Clone N418 (1:100), CD80 PE 16-10A1 (1:50), Streptavidin PECy7 (1:400), CD86 PECy7 Clone GL-1 (1:100), CD137 PerCP-Cy5.5 Clone 4B4-1 (1:50), CD40 APC Clone 3/23 (1:50), CD25 PE/Cy5 Clone PC61 (1:100), PD-1 APC Clone 29F.1A12 (1:100), CD86 BV421 Clone GL-1 (1:50). Flow data was acquired using a MACSQuant Analyzer 10, Data were collectively analyzed using FlowJo version 10.1 (Tree Star) and a multi-step gating strategy applied to identify immune cells (Supplementary Fig. S1).

Ex vivo IFN γ production

0.5×10^6 cells from tumor-draining lymph nodes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 μ g/mL), and 2-mercapthoethanol (2.5×10^{-5} mol/L) in a 48-well plate. Naïve BALB/c mice provided the source for 3×10^6 irradiated (12 Gy) feeder splenocytes pre-loaded with tumor-associated immunodominant antigen AH-1-A5 (SPSYAYHQF)(22) or irrelevant peptide pMCMV (YPHFMPNTNL) used at a final concentration of 1 μ g/mL (Genscript, Piscataway, NJ). Supernatants were collected after 48 hours, and secreted IFN γ was measured using Mouse IFN-gamma Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). As per manufacturer's protocol, 50 μ L of undiluted supernatant, standards, and recombinant mouse IFN- γ (positive control) were assayed in triplicate wells. Optical densities were determined on a FlexStation 3 plate reader (Molecular Devices, San Jose, CA) set at 450 nm with 540 nm subtraction and standard curve generated using a log/log curve-fit.

Type I interferon ELISA and quantitative real-time polymerase chain reaction

10^5 cells (TSA, MC38, or LLC1) were seeded in 6-well plates and treated with 8Gy radiation for three consecutive days. Cell-free supernatants were collected after 24 hours and IFN β measured using VeriKine-HS Mouse Interferon beta ELISA Kit (PBL Assay Science, Piscataway, NJ). As per manufacturer's instructions, 50 μ L of undiluted supernatant and standards and recombinant mouse IFN β (positive control) were assayed in triplicate wells. Optical densities were determined using a FlexStation 3 plate reader (Molecular Devices, San Jose, CA) set at 450 nm and standard curve generated using a 4-parameter fit. Concentrations were normalized by the number of viable cells counted on an automated bright-field Cellometer AutoT4 (Nexcelom, Lawrence, MA).

Total RNA was extracted using the RNEasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol and quantified using a Nanodrop™2000 (ThermoFisher Scientific). RNA reverse transcription and expression analysis were performed using iTaq™ Universal Probes One Step kit (Biorad) using 50 ng of total RNA in triplicate reactions. All TaqMan gene expression assays (PCR primers pair plus labelled probe) were purchased from Thermo Fisher Scientific, and their design selected to be exon-spanning. Selection of housekeeping genes *Rpl19* (60S ribosomal protein L19; Mm01606039_g1-VIC) or *Tbp* (TATA-binding protein; Mm01277042_m1-VIC) were based on expression stability after radiation treatment. *Mb21d1* (cGAS _ Mm00557695_m1-FAM), *Mx1* (Mm00487796_m1), *Cxcl10* (Mm00445235_m1-FAM), and *Ccl4* (Mm00443111_m1-FAM) TaqMan expression assays were used. Triplicate reactions were run using 900nM of each primer and 250nM of the corresponding probe on a Applied Biosystem 7500 Real-Time PCR machine (ThermoFisher Scientific). For gene expression analysis, only C_T values below 35 were considered. Relative gene expression was calculated using the 2^{-C_T} method.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v8. To determine significant differences in tumor volumes among treatment groups, RM two-way Analysis of Variance (ANOVA) with Tukey correction for multiple comparisons was utilized. Quantitative differences in cell populations were determined using one-way ANOVA with Holm-Sidak's post-test correction for samples with single pooled variance. Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to detect significant differences in lung metastases control among treatment groups. Differences in survival curves were compared using log-rank (Mantel-Cox) test with correction for multiple pair-wise comparisons. The Kaplan-Meier method was used to estimate median survival times and the log cumulative hazard transformation was used to derive 95% confidence limits for median survival in each arm. All reported p values are two-sided, and statistical significance defined as p<0.05.

Results

Focal radiotherapy and subcutaneous IL15 generates effective and durable responses

The TSA mouse mammary carcinoma is a poorly immunogenic tumor that does not respond to immune checkpoint blockade monotherapy (12,21). Focal tumor radiotherapy using an optimally immunogenic fractionated regimen of 8GyX3 promotes CD8⁺ T cell-mediated tumor rejection, but only when used in combination with CTLA-4 blockade, whereas radiotherapy alone causes tumor growth delay but does not lead to long-term survival (12). Thus, we used this tumor model to test the efficacy of IL15 alone and in combination with radiotherapy. We chose to deliver IL15 subcutaneously because this is the preferred route of administration in the clinic (6,7). As expected, TSA tumors treated with 8GyX3 radiotherapy remained stable for five days and then outgrowth was seen (Figure 1A-B). IL15 alone had no effect on tumor growth but significantly improved tumor response when used with radiotherapy. Mouse survival was also significantly enhanced (Figure 1C) and 3 of 11 mice treated with radiotherapy+IL15 remained tumor-free for >100 days and rejected a challenge with a tumorigenic inoculum of TSA cells, demonstrating a long-lasting protective

immunity (Figure 1D). These results indicated that IL15 increased the efficacy of radiotherapy, likely by promoting antitumor T-cell responses.

To determine if IL15 enhances the response to radiotherapy in other tumors models, colorectal MCA38 and lung LLC1 carcinomas were implanted into syngeneic C57BL/6 mice, followed by treatment as above. IL15 alone did not have any significant effect but increased tumor control and survival of MCA38 tumor-bearing mice when combined with radiotherapy (Figure 2A-B). In contrast, although LLC1 tumors responded to radiotherapy, the addition of IL15 did not further enhance tumor control or survival (Figure 2C-D). To understand the reasons for this differential response, we analyzed the ability of the three tumor cell lines to produce IFN β in response to 8GyX3 radiation. TSA and MCA38 cells significantly produced IFN β into the supernatant, whereas LLC1 did not (Figure 2E). Analysis of the expression of the gene encoding cGAS (*Mb21d1*), which is required for production of IFN β in response to radiation (12), revealed extremely low expression in LLC1 cells, which was not affected by radiation (Figure 2F), explaining the inability of LLC1 cells to make IFN β . Expression of IFN-I stimulated genes *Mx1*, *Cxcl10*, and *Ccl4* was also upregulated by radiation in TSA and MCA38 cells but not LLC1 cells (Supplementary Fig. S2).

Given these results, we next asked if IL15 could improve tumor control with a radiation dose of 20GyX1, which does not induce IFN β production by TSA cells (12). We also tested the ability of the combination radiotherapy+IL15 to induce regression of an established synchronous tumor that did not receive radiation (abscopal response). In the presence of IL15, radiotherapy given at 8GyX3 improved control of the irradiated tumor and induced an abscopal response, whereas 20GyX1 did not (Figure 3A-B), further supporting the key role of IFN type I induction in the synergy between radiotherapy and IL15.

Combination therapy enhances CD103⁺ DC infiltration and CD8⁺ T-cell priming

IFN-I produced in the tumor in response to radiotherapy is required to enhance tumor infiltration by cDC1s and priming of CD8⁺ T cells (12,23). Thus, we analyzed tumors for the presence of cDC1s (Figure 4A), which are best defined by expression of the marker CD103 (24). This analysis revealed that radiotherapy alone, used at 8GyX3, induced a significant increase in cDC1s (Figure 4B). IL15 alone did not have any effect on cDC1 infiltration but significantly increased the number of cDC1s recruited by radiotherapy (Figure 4B). Expression of costimulatory molecules CD80 and CD86, as well as CD40, by cDC1s was also increased by combination radiotherapy+IL15 (Figure 4C-E).

To interrogate whether radiotherapy+IL15 induced priming of tumor-specific CD8⁺ T cells, cells from tumor-draining lymph nodes (TDLNs) were tested *ex vivo* for the ability to produce IFN γ in response to the tumor-specific CD8⁺ T-cell epitope AH1-A5 (12). Only mice treated with Radiotherapy+IL15 showed significant production of IFN γ (Figure 4F), indicating that IL15 was indeed able to promote the activation and expansion of tumor-specific CD8⁺ T cells when combined with radiotherapy.

Batf3-dependent cDC1s are essential for the therapeutic synergy of radiotherapy and IL15

To determine if Batf3-dependent cDC1s are required for the therapeutic effect of the combination treatment, TSA cells were implanted into *Batf3*^{-/-} mice, and mice were treated with radiotherapy alone or in combination with IL15 as described in Figure 1A. IL15 alone did not have any effect on tumor progression in *Batf3*^{-/-} mice (Supplementary Figure S3). Untreated and irradiated TSA tumors implanted in *Batf3*^{-/-} mice were on average 25% bigger at day 26 compared to those in wild-type (WT) mice but survival was comparable (Figure 5A-B). In contrast, tumor rejection and long-term survival achieved by radiotherapy +IL15 in WT mice was abrogated in *Batf3*-deficient mice (Figure 5A-B). Overall, these results demonstrated that cDC1s were essential for the development of antitumor CD8⁺ T-cell responses elicited by the combination of radiotherapy with IL15.

To investigate this further, TSA tumor-bearing mice were treated as above and tumors analyzed on day 18, when the tumor growth curves of mice treated with radiotherapy alone and radiotherapy+IL15 began to separate. Treated mice showed increased CD8⁺ T-cell infiltration into tumors, with the largest increase seen in tumors of mice treated with radiotherapy+IL15 (Figure 6A). A larger fraction of the intratumoral CD8⁺ T cells were positive for expression of the co-stimulatory receptor CD137 (Figure 6B), while CD25 and PD-1 expression was not increased (Figure 6B-D).

Next, to determine if CD8⁺ T cells were required for the therapeutic effect of combination radiotherapy+IL15, mice were depleted of CD8⁺ T cells and followed for tumor response. We confirmed 99.8% reduction of intratumoral CD8⁺ T cells using the depletion protocol described in Methods. Depletion of CD8⁺ T cells abrogated the complete tumor rejection achieved in mice treated with radiotherapy+IL15 (Figure 6E), demonstrating a critical role for CD8⁺ T cells in this response. The above data suggested that IL15 was sufficient to induce an expansion of non-specific intratumoral CD8⁺ T cells and expanded tumor-specific CD8⁺ T cells primed by radiotherapy.

Tumor control with radiotherapy+IL15 treatment requires intact IFN type I signaling

We next studied the direct contribution of IFN-I signaling on the therapeutic efficacy of radiotherapy+IL15. To block IFN-I signaling *in vivo*, TSA tumor-bearing mice treated with radiotherapy with and without IL15 were concurrently given an antibody blocking the IFN α / β receptor 1 (IFNAR)(Figure 7A). IFNAR blockade had no effect on local tumor control achieved by radiotherapy alone but completely abrogated the benefits of combination radiotherapy+IL15 (Figure 7B). Analysis of tumor-infiltrating cDC1s revealed that IFNAR blockade reduced radiotherapy-induced infiltration of cDC1s in the tumor, an effect that was significant in mice treated with radiotherapy+IL15 (Figure 7C). This was accompanied by a significant reduction in CD8⁺ T cells (Supplementary Figure S4). Overall, these results demonstrated an essential role of radiation-induced IFN-I in the therapeutic synergy of radiotherapy and IL15.

Discussion

Immune checkpoint blockade (ICB) has revolutionized cancer treatment by demonstrating that targeting critical immunosuppressive receptors can unleash powerful and often durable antitumor T-cell responses (25). A complementary approach is to promote and sustain T-cell activation, proliferation, and effector function using cytokines that play a central role in these processes. IL15 functional profile makes it a prime candidate for this role (1,3). However, single agent IL15 has not shown much antitumor activity, even when used as a superagonist in complex with the IL15 α receptor, and it is being investigated in combination with ICB (6,10,11). Here we showed that IL15 synergizes with radiotherapy to induce immune-mediated tumor rejection, abscopal responses, and long-term protective immunological memory in mouse carcinoma models. Mechanistically, IL15 cooperated with radiotherapy to increase cDC1 infiltration into the tumor, their expression of costimulatory molecules, and their priming of tumor-specific CD8⁺ T cells. The induction of cancer cell-intrinsic IFN-I by radiotherapy was essential for the therapeutic synergy with IL15, and mice bearing LLC1, a lung carcinoma unable to mount this response, did not benefit from the combination. We found that LLC1 cells expressed extremely low *Mb21d1*, which explains their inability to activate the IFN-I response induced by radiation via the cGAS/STING pathway. Epigenetic silencing of cGAS and/or STING has been reported in many tumors (26), and our data predict that it would preclude the synergy of radiotherapy and IL15.

DCs deliver stimulatory signals to IL-15R $\beta\gamma$ -expressing NK and CD8⁺ T cells by trans-presentation of IL15 bound to IL15R α (27,28). Thus, radiotherapy-induced cDC1 recruitment is required to enable the activity of IL15, an interpretation supported by the abrogation of the therapeutic synergy between IL15 and radiotherapy in *Batf3*-deficient mice. Prior work in mice bearing CT26 colorectal carcinoma shows that administration of IL15 alone has a therapeutic effect and leads to increased expression of PD-1 on CD8⁺ T cells (5). In contrast, we did not find increased expression of PD-1 on CD8⁺ T cells infiltrating TSA tumors treated with IL15 alone or in combination with radiotherapy. On the other hand, expression of the costimulatory receptor CD137/4-1BB was upregulated by IL15 on CD8⁺ T cells. CD137 ligation promotes CD8⁺ T-cell proliferation and effector functions (29). These findings suggest that a rational combination to further improve the therapeutic effect of radiotherapy+IL15 may include agonistic anti-CD137, a hypothesis that will require further testing. rhIL-15 is currently being tested in combination with ipilimumab and nivolumab in metastatic or treatment-refractory solid cancers (NCT03388632). The addition of radiotherapy could enhance the likelihood of response in this setting. The combination of radiotherapy and rhIL15 could also be investigated in patients who do not tolerate the toxicity associated with ICB.

In summary, these studies demonstrated cooperative effects of subcutaneously delivered IL15 and focal hypofractionated radiotherapy in inducing durable antitumor immune responses against carcinomas refractory to IL15 alone. The low toxicity of subcutaneous IL15 and its ability to promote durable antitumor CD8⁺ T-cell responses support testing this combination treatment in the clinic as a strategy to immunize the patients against their own tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Peritumoral IL15 potentiates radiotherapy-induced tumor control and leads to durable protective immunity.

(A) Treatment schema. BALB/c mice were injected s.c. with TSA cells on day 0. Radiotherapy (RT) was given in three fractions of 8 Gy on days 12, 13, and 14. IL15 (or PBS in Control mice) was administered s.c. peri-tumorally daily for 10 days starting on day 12. (B) Tumor growth over time (n=5 mice/group). Mean tumor volume $\pm$ SD on day 21 = 851 $\pm$ 110 mm³ (control), 749 $\pm$ 236 (IL15 alone), 408 $\pm$ 251 (RT), and 82 $\pm$ 151 (RT+IL15); *p<0.05, **p<0.005, 2-way ANOVA. (C) Effect of treatment (n=10-11 mice/group) on survival. **p<0.005, ****p<0.0001, Log-rank test. (D) Tumor-free mice (n=3) from the RT +IL15 treatment group were re-challenged after 100 days with viable TSA cells and monitored for tumor development. Naïve mice (n=5) were used as controls.

Figure 2. Therapeutic benefit of peritumoral IL15 correlates with cancer cell intrinsic IFN-I response to radiation.

(A-B) C57BL/6 mice bearing subcutaneous MCA38 tumors were treated with radiotherapy (RT) and/or IL15 as described in Figure 1A, (n=7-8 mice/group). (A) Tumor growth overtime. Mean tumor volume±SD on day 25 = 784±286 mm³ (control), 633±184 (IL-15 alone), 189±75 (RT), and 69±93 (RT+IL15); **p<0.005, 2-way ANOVA. (B) Survival of mice in (A), **p<0.005, Log-rank test. (C-D) C57BL/6 mice bearing subcutaneous LLC1 tumors were treated with RT and/or IL15 as described in Figure 1A, (n=7-8 mice/group). (C) Tumor growth overtime. Mean tumor volume±SD on day 19 = 842±400 mm³ (control),

603±378 (IL15 alone), 342±155 (RT), and 263±127 (RT+IL15); * p <0.05, 2-way ANOVA. **(D)** Survival of mice in (C), ** p <0.005, Log-rank test. **(E)** Concentration of IFN β in supernatant of untreated or irradiated TSA, MCA38, and LLC1 cells 24 hours after the last dose of 8GyX3 radiation. Data shows concentrations combined from two independent experiments (n=6 biological replicates). Error bars indicate the mean±SD values for each treatment group, ** p <0.005, *** p <0.0005, T-test. **(F)** Expression of *Mb21d1* and housekeeping gene *Tbp* measured by quantitative RT-PCR in untreated or irradiated TSA, MCA38, and LLC1 cells 24 hours after last dose of radiation. Triplicate C_T values obtained from 3 biological replicates per group are plotted and mean values represented by bars. Data is representative of 3 independent experiments.

Figure 3. IL15 synergizes with radiotherapy given at 8GyX3, but not 20Gy single dose, and induces abscopal responses.

Mice bearing bilateral TSA tumors received no treatment (CONTROL), or were treated with focal RT to one of the tumors, given either as a single 20Gy dose (20GyX1) or in three daily 8Gy doses (8GyX3) starting on day 12. The other tumor remained untreated outside of the radiation field. Mice in each radiation group received either PBS or IL15 administered s.c., only around the irradiated tumor, daily for 10 days starting on day 12. Growth over time of the (A) irradiated, and (B) non-irradiated (abscopal) tumors. N=7-9 mice/group, *p<0.05, **p<0.005, ***p<0.0005, 2-way ANOVA.

Figure 4. IL15 enhances radiotherapy-driven cDC1 recruitment to tumors and priming of tumor-specific CD8⁺ T cells.

BALB/c mice were treated as in Figure 1A (n=5 mice/group). Tumors and tumor-draining lymph nodes (TDLNs) were collected on day 18 for analysis. **(A)** Representative plots of tumor-infiltrating CD103⁺CD11c⁺ cells. Samples from dissociated tumors were gated on viable CD45⁺ cells. **(B)** Percentage of CD103⁺ cells among CD11c⁺ DCs in TSA tumors of mice treated as indicated. RT: radiotherapy. Each symbol represents one animal, and bars represent mean±SD. **(C-E)** Expression of activation markers **(C)** CD80, **(D)** CD86, and **(E)** CD40 on viable CD11c⁺CD103⁺ DCs. Data are shown as mean±SD of mean fluorescence intensity (MFI). **(F)** TDLNs were collected on day 18 and stimulated for 48 hours with the tumor CD8 epitope AH1A5 or an irrelevant pMCMV peptide. IFN-γ concentration is shown

as mean $\pm$ SD after subtraction of the background in control wells stimulated with pMCMV. Each symbol represents one animal. *p<0.05, **p<0.005, ***p<0.0005, 1-way ANOVA. Data are representative of two independent experiments.

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Figure 5. Batf3-dependent DCs are indispensable for tumor rejection in mice treated with radiotherapy+IL15.

Wild-type (WT) and *Batf3*^{-/-} mice were inoculated with TSA cells and treated with RT ±IL15 as described in Figure 1A (n=6-8 mice/treatment group/strain). Animals were followed for (A) tumor growth, shown as mean tumor volume±SD, over time, *p<0.05, 2-way ANOVA and (B) survival, ***p<0.001, Log-rank test.

Figure 6. Tumor control by radiotherapy+IL15 is dependent on CD8⁺ T cells.

Wild-type (WT) mice (n=3/treatment group) were inoculated with TSA cells on day 0 and given radiotherapy (RT) and/or peritumoral s.c. IL15 as described in Figure 1A. On day 18, tumors were excised for (A) immunostaining of CD8⁺ T cells. Each symbol represents mean count obtained from 3 fields/tumor for each mouse. (B-D) In a separate experiment, cell suspensions were prepared from tumors (n=9/group) harvested at day 18 and analyzed by flow cytometry. To obtain sufficient material, each sample was prepared by pooling tumors from 3 mice. CD8⁺ T cells were analyzed for the expression of activation markers (B) CD137/4-1BB, (C) CD25, and (D) PD-1. *p<0.05, **p<0.005, 1-way ANOVA. Data is

representative of two independent experiments. **(A-D)** Bars represent mean values $\pm$ SD for each treatment group. **(E)** TSA tumor-bearing mice were untreated or treated with RT+IL15 as in Figure 1A (n=10 mice/group). Half of the mice in each group received anti-CD8 (Clone 2.43) or isotype control antibody. Tumor growth/regression over time was monitored and expressed as mean tumor volume $\pm$ SD at indicated timepoints. **p<0.005, ***p<0.0005, 2-way ANOVA.

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Figure 7. Therapeutic synergy of radiotherapy+IL15 requires IFN-I signaling.

(A) Treatment schema. TSA tumor-bearing mice were treated with radiotherapy (RT) and peritumoral PBS or IL15. Some animals were additionally treated with anti-IFNAR (or isotype control) given i.p. every other day starting at day 12. (B) Tumor weight was measured 18 days after tumor inoculation. (C) Tumor-infiltrating cDC1s were identified as a percentage of CD11c⁺ DCs. Each dot represents one animal (n=5-8 mice per treatment group). Bars represent mean±SD, *p<0.05, ***p<0.0005, 2-way ANOVA. Only one experiment was performed.