Therapeutic effects of ablative radiation on local tumor require CD8+ T cells:
Changing strategies for cancer treatment

Youjin Lee1*, Sogyong L. Auh1*, Yugang Wang1, Byron Burnette1, Yang Wang1, Yuru Meng2, Michael Beckett2, Rohit Sharma3, Robert Chin1, Tony Tu1, Ralph R. Weichselbaum2†, and Yang-Xin Fu1†

1Department of Pathology, University of Chicago, Chicago, IL 60637, 2Department of Radiation and Cellular Oncology, The Ludwig Center for Metastasis Research, University of Chicago, Chicago, IL 60637, 3 Department of Surgery, University of Chicago, Chicago, IL 60637

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: (rrw@radonc.bsd.uchicago.edu) (R.R.W.); (yfu@uchicago.edu) (Y.-X.F.). These authors contributed equally to this work.

Running Title: Therapeutic effect of radiation mediated immunity

Key word: ablative radiation, chemotherapy; CTL; immunotherapy; radiotherapy
Summary
Patients with locally advanced cancer or distant metastasis frequently receive prolonged treatment with chemotherapy and/or fractionated radiotherapy (RT). Despite the initial clinical response, treatment resistance frequently develops and cure in these patients is uncommon. Developments in radiotherapy technology allow for the use of high dose (or ablative) RT to target local tumors, with limited damage to the surrounding normal tissue. We report that reduction of tumor burden following ablative RT depends largely on T cell responses. Ablative RT dramatically increases T cell priming in draining lymphoid tissues, leading to reduction/eradication of the primary tumor or distant metastasis in a CD8$^+$ T cell dependent fashion. We further demonstrate that ablative RT-initiated immune responses and tumor reduction are abrogated by conventional fractionated RT or adjuvant chemotherapy, but greatly amplified by local immunotherapy. Our study challenges the rationale for current radio/chemotherapy strategies and highlights the importance of immune activation in preventing tumor relapse. Our findings emphasize the need for new strategies that not only reduce tumor burden but also enhance the role of anti-tumor immunity.
Introduction

The rationale for radiotherapy (RT) is based on inducing lethal DNA damage to tumor cells or tumor associated stroma. Cancer patients often receive fractionated RT at low doses (1.5-3 Gy) that are administered daily over weeks, frequently in combination with chemotherapy. RT has traditionally been viewed as immunosuppressive, but studies published in recent years have suggested that the effects of RT on the immune system are complex. Lymphocyte radiosensitivity is well established, but whether varying doses or delivery methods can differentially target naïve, effector or regulatory T cell populations and/or alternative regulatory molecules, is still unclear. While studies have investigated the potential immunomodulatory effects of localized RT on tumors, there have been conflicting reports as to whether these responses promote or interfere with tumor reduction.

A recent study showed that radiation can trigger signals that stimulate toll-like receptor 4 on dendritic cells (DC), suggesting another dimension of immune modulation by RT. Other studies reported a direct effect of radiation on tumors by either modifying the phenotype of tumor cells to render them more susceptible to vaccine-mediated T-cell killing, or altering the tumor microenvironment to promote greater infiltration of immune effector cells. A large tumor burden often creates severe suppression that prevents effective immune intervention. It has been proposed that local RT can induce a sufficient reduction in tumor burden to allow for therapeutic intervention by immunotherapy, such as vaccination or blockade of inhibitory molecules on immune cells. Whether RT can also generate significant cytotoxic T lymphocytes (CTL) to kill metastasis has not been well documented.

Advances in radiotherapy technology allow for the use of ablative RT to be delivered very precisely to small tumors. For example, stereotactic body RT (SBRT) takes advantage of the technological advancements in image guidance and radiation dose delivery in order to direct...
ablative doses to tumors with acceptable toxicity. Phase I/II trials have provided evidence that the potent doses delivered with SBRT may provide results that rival surgery for some localized primary tumors and has efficacy in the oligometastatic setting. The initial clinical reports indicate that the use of ablative RT is associated with better survival than conventional fractionated treatment, but the mechanisms remain unclear. Using an animal model, we unexpectedly observed that ablative RT (15-25 Gy x1) alone, generates strong enough CD8+ T cell dependent immunity to lead to tumor reduction, reduced relapse of primary tumor, and even eradication of metastasis in some settings. The clinical implication of this study is that some current strategies employing fractionated radiotherapy or chemotherapy may limit RT-mediated immunity and increase relapse over time. Indeed, we also demonstrate that ablative RT-mediated immunity is erased by current conventional chemotherapy and prolonged fractionated radiotherapy, resulting in early relapse due to inadvertent suppression of protective immune responses. On the other hand, ablative RT followed by proper immunotherapy can synergistically overcome the tumor barriers and generate more cytotoxic T cells that circulate systemically to eradicate micro-metastasis. Therefore, we should revisit our current strategies and develop new approaches that can reduce tumor burden while boosting protective immunity.
Materials and methods:

Mice, cell line, and reagents C57BL/6, Nude, B6/Rag, OTI, and Balb/c mice were purchased from Jackson Laboratory at 6-7 weeks old. 2C TCR- transgenic mice were provided by Jianzhu Chen, MIT, Cambridge, MA, and maintained in the specific pathogen free (SPF) facility at the University of Chicago. For all experiments, mice were between the ages of 6-16 weeks of age, bred under SPF conditions and used in accordance to the animal experimental guidelines set by and with the approval of the Institute of Animal Care and Use Committee (IACUC). 4T1 is a 6-thioguanine-resistant cell line derived from spontaneous mammary carcinoma\textsuperscript{19}. B16 lines were obtained by ATCC and maintained Fu and Weichselbaum. B16-SIY melanoma cells and anti–2C TCR (1B2) antibody were obtained from Tom Gajewski (The University of Chicago). B16-CCR7 melanoma cell was generously provided by Sam T. Hwang (National Cancer Institute)\textsuperscript{20}. The human lung tumor cell line A549 was purchased from ATCC. All other antibodies for FACS were purchased from BD Biosciences. The generation of ad-LIGHT was described previously\textsuperscript{21}.

Harvesting of mouse lymphoid DC For DC harvest for FACS, draining lymph nodes (DLN) and spleen (SP) were digested with 1.5 mg/ml collagenase and 100 µg/ml DNase for 20 min at 37°C and then gently pipetted in the presence of 0.01 M EDTA for 1 min. Single-cell suspensions were stained and analyzed by flow cytometry on a FACSCanto (BD Biosciences).

Adoptive transfer of T cells LN cells and splenocytes were isolated from 2C TCR Tg mice. A total of 2 x 10\textsuperscript{6} 2C cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) then adoptively transferred intravenously (i.v.) into B16-SIY tumor bearing C57BL/6 mice as
described previously\textsuperscript{21,22}. Cells were isolated from the inguinal LNs (DLNs), SP, or tumors at the time indicated. CFSE dilution was evaluated as described before\textsuperscript{21,22}.

**TCR tetramer and FACS staining** For tetramer staining, tumor, DLN and SP were excised from mouse, chopped, and collagenase digested (1.5 mg/ml) for 20 min in shaking incubator at 37°C. Single cell suspensions of cells were incubated with 2.4G2 to block antibody binding to the Fc receptors, CD11c\textsuperscript{+}-APC, 1 \textmu g SIY-K\textsuperscript{b}-specific m67 TCR tetramer-PE, and mAb CD11b\textsuperscript{+}-PerCP-Cy5.5. Samples were analyzed on a FACSCanto (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc.). The m67 ab was a generous gift from David Kranz (University of Illinois) and Hans Schreiber (University of Chicago).

**Local tumor irradiation and systemic chemotherapy** Mice were irradiated using an x-ray generator (PCM 1000; Pantak) at the doses indicated by each experiment. Each mouse was protected with a lead cover with only tumor exposed, allowing local irradiation. For systemic chemotherapy, tumor-bearing mice were injected intraperitoneally (i.p.) with 20 mg/kg paclitaxel (Ameristat Pharmaceuticals) for 4T1 bearing mice and 200 mg/kg dacarbazine (Bedford Laboratories) for B16 bearing mice.

**Tumor injection, treatments, and evaluation of metastases by colonogenic assay** Cultured cancer cells were trypsinized, washed with media, and injected subcutaneous (s.c.) on the back. Tumor size was determined at 3-4 day intervals. Tumor volumes were measured along three orthogonal axes (\(a, b,\) and \(c\)) and calculated as tumor volume = \(abc/2\). The tumor nodules were inoculated with indicated amount of ad-LIGHT or ad-control virus intratumorally. For surgical excision of primary 4T1 and B16-CCR7 tumors, mice were anesthetized, and tumors were resected with sterilized instruments. A colonogenic assay was used to evaluate metastases in 4T1
and B16-CCR7 tumors as previously described\textsuperscript{19}. Briefly, lungs for 4T1 tumor or DLN for B16-CCR7 were collected, chopped and dissociated in DMEM supplemented with 10\% FCS containing 1.5 mg/ml collagenase type D (Sigma-Aldrich) for 20 min in 37°C shaking incubator. Single cell suspensions were plated at various dilutions in media supplemented with 10\% FCS and selection drug. 4T1 is resistant to 6-thioguanine (60 mM) and B16-CCR7 is resistant to G418 (0.7 mg/ml). Individual colonies representing micrometastases were counted after 5–10 days\textsuperscript{19}.

**Statistical analysis** Statistics were done using an unpaired student two-tailed $t$ test. Error bars represent standard deviations. For survival curves statistics were done using the log rank (Mantel-Cox) test.
Results

Ablative RT induces strong T cell responses leading to tumor rejection

B16 melanoma is well established to be a highly aggressive, rapidly growing, poorly immunogenic, radio-resistant tumor and also known to resist various treatments including immunotherapies\textsuperscript{14,23}. Intriguingly, we observed that after ablative RT (20 Gy x1) B16 tumors show significant regression in wt mice (Fig. 1A) and an increase of infiltrating T cells to the tumor microenvironment and the lymphoid tissues 1-2 weeks after treatment (data not shown). This raised the possibility that the sensitivity to RT observed \textit{in vivo} was T cell-mediated. Therefore, nude mice (T cell deficient) were used to determine the role of T cells in RT-mediated B16 tumor reduction. Impressively, the tumor remained radio-resistant to ablative RT in the absence of T cells (Fig. 1A). Similar results were observed in B and T cell deficient B6/Rag\textsuperscript{-/-} mice (data not shown). These findings reveal that the therapeutic effect of ablative RT requires T cells.

We wondered whether RT-mediated regression could be influenced by the immunogenicity of the tumor. To test this, we introduced a K\textsuperscript{b}-binding peptide SIYRYYGL (SIY) into B16 cells (B16-SIY) and tumors remained very aggressive in both wt and nude mice (Fig. 1B). We demonstrate that a single dose of RT alone is sufficient to completely reject B16-SIY tumors in 9/10 mice, while in nude mice, the tumors grew progressively, rapidly killing the host in 9/9 mice (Fig. 1B). To test whether CD8\textsuperscript{+} cells, the major killer T cells, are essential for RT-mediated tumor reduction, we treated wt mice bearing established B16 tumors with ablative RT in conjunction with antibody-mediated CD8\textsuperscript{+} T cell depletion. The therapeutic effect of RT was largely diminished and survival decreased by more than 75 percent in the absence of CD8\textsuperscript{+} T cells (Fig. 1C, 1D). With CD8\textsuperscript{+} cell depletion, the tumors become radioresistant. The depletion
of NK cells by anti-NK1.1 does not increase resistance of the tumor (data not shown). These results suggest that ablative RT can elicit a strong CTL response accountable for the reduction or even eradication of established tumor.

**RT matures DC for priming of Ag-specific cells.**

It is unclear how localized ablative RT generates such a strong T cell response that mediates tumor regression since it has been shown that by 14 days after implantation, immunity against B16 is lost and immunosuppression becomes dominant. Potential immune stimulatory effects of localized RT on tumors have been reported to be involved in various phases of the immune response. To address whether high dose of RT reenergizes the priming phase of naïve T cells, naïve CD8⁺ 2C transgenic T cells, that recognize the SIY antigen, were carboxyfluorescein succinimidyl ester (CFSE) labeled then adoptively transferred into B16-SIY tumor bearing mice that received high dose of RT on local tumor. The degree of CFSE dilution was determined on CFSE⁺1B2⁺CD8⁺ cells in the draining lymph node (DLN) and spleen of RT or no-RT mice 4-5 days post adoptive transfer. Nominal proliferation was detected in the DLN of the non irradiated tumor bearing group but impressively, transferred Ag-specific naïve T cells demonstrated robust priming in the DLNs of the localized RT group (Fig. 2A). To test whether the increase of priming occurs not only in non-self antigens (such as SIY) but also shared self-antigens, T cells from pmel mice specific for endogenous gp100, an antigen expressed on normal melanocytes and the majority of malignant melanomas, were used and also found to exhibit robust proliferation in the DLN (data not shown). Therefore, large single dose RT on the tumor can alter the tumor microenvironment from that of immune suppressive to immune activating,
ultimately resulting in vigorous priming and the expansion of effector T cells with both low and high affinity to antigens.

To determine whether increased priming in the RT group is attributed to reduced T cell suppression, improved cytokine milieu, increased maturation of DC, or increased cross presentation of endogenously acquired tumor derived SIY peptide, we harvested tumor, DLN, and spleen after local RT and stained for antigen presenting cells (APC) expressing SIY peptide using a unique TCR tetramer\textsuperscript{24}. This tetramer binds to SIY peptide presented by major histocompatibility complex (MHC) class I molecules, thereby allowing us to determine whether SIY\textsuperscript{+} DCs are increased in tumor or DLN after RT. The results indicate that 5 days post RT, there was an increase in SIY peptide presenting CD11c\textsuperscript{+} cells in the DLN (Fig 2B). This increase could be due to residential DCs in the DLN picking up free-floating antigens or RT-activated DCs inside the tumor that picked up antigens and then migrated to the DLN. To test this, we harvested DCs from tumor tissues and found increased intratumoral CD11c\textsuperscript{+}TCR tetramer\textsuperscript{+} cells and that they increased their ability to stimulate T cells after local RT (data not shown). Increased T cell priming in DLN suggests that high dose RT might activate DCs inside the tumor which then promotes maturation and migration to the DLN to present antigens to awaiting T cells. To determine whether local RT on the tumor can promote maturation of myeloid DC (mDCs) in the DLN, we analyzed the level of MHC class II on mDCs post RT. Within 48 hours post local RT, there was an increased percentage of MHC class II on mDCs, but not plasmacytoid DC (pDC) compared to no RT (Fig 2C and data not shown). Together, ablative RT on the tumor can activate and mature DCs to subsequently facilitate better T cell priming.
Radiation-initiated immunity and anti-tumor effects can be suppressed by either chemotherapy or fractionated radiation

As chemotherapy is often combined with RT in clinical practice, our results raised concern about the potential immunosuppressive effects of prolonged chemotherapy on RT-mediated immunity. Indeed, chemotherapy given as adjuvant after localized radiotherapy significantly hindered tumor regression and promoted melanoma outgrowth (Fig. 3A). One of the goals of adjuvant chemotherapy after surgery and radiation is to reduce subclinical metastasis. In the 4T1 breast tumor model, the influence of RT-mediated tumor regression was less pronounced in regards to the primary tumor (Fig. 3B) yet impressively, lung metastases with ablative RT were completely eliminated (Fig. 3C). The prolonged effect of ablative RT on metastasis suggests a likely immune effect at distal sites more than local ablative effect. Indeed, distant metastasis is dramatically increased with CD8-depletion (data not shown). Unexpectedly, the addition of chemotherapy (paclitaxel) actually increased the number of tumor colonies and exclusively erased the suppressive effect of ablative RT on metastasis (Fig. 3C). To test whether the negating effect of chemotherapy (dacarbazine) was directly on CD8+ T cell priming, naïve CFSE labeled 2C cells were adoptively transferred into B16-SIY tumor bearing mice that received RT on day 0, chemotherapy on day 2 post transfer, and were then sacrificed on day 4 to determine the degree of T cell priming in the DLN and the spleen. Our results illustrate that the addition of chemotherapy to the RT group does indeed abolish priming of CD8+ T cells and chemotherapy given alone does not elicit CTL proliferation (Fig. 3D). Therefore, it is possible that many types of chemotherapy regimens may forestall CTL generation, especially when followed by local RT, leading to the increased incidence of recurrent tumor.
Our study also raised concern about fractionated RT, another potentially immunosuppressive conventional treatment. Fractionated RT delivers low daily doses of radiation to the tumor over weeks, in contrast to ablative hypofractionated radiation which employs 1-5 larger doses usually administered in 1-7 days. To test the relative effectiveness of these treatment schemes in immune competent models, we designed two protocols delivering the same total RT dose: 5 Gy x4 over two weeks or single 20 Gy. Surprisingly, even though the 5 Gy x4 treated mice initially responded to RT, over time tumors relapsed in a manner analogous to the CD8 depleted 20 Gy RT condition (Fig. 3E). However, in the absence of lymphocytes, B6/Rag\(^{-/-}\) showed comparable progressive growth of B16-SIY tumor irrespective of being treated with ablative RT dose of 20 Gy or low dose of 5 Gy x4. Conversely, wt mice showed considerable delay in 100% cases (26/26 mice) and even complete tumor regression in 35% (9/26 mice) when given 20 Gy, but had nominal therapeutic impact with 5 Gy x4 (0/15). It is possible that fractionated low dose radiotherapy may continually kill off infiltrating effector T cells over time, leading to early relapse or recurrence. It is also possible that the dose of 5 Gy x4 may not be equivalent to one dose of 20 Gy for direct tumor cell killing. It is important to note that the repair of sublethal damage or proliferation between doses may have accounted for a portion of the inferior tumor control in the fractionated treatment groups. However, we found tumor outgrowth to be comparable to 5 Gy x4 even when RT was extended to seven times (data not shown). It is difficult to determine the relative magnitude of these direct effects vs. indirect effect via activation of anti-tumor immunity, but based on the data above it is highly likely that anti-tumor immunity significantly contributes to the superior response induced by one dose of 20 Gy. Nonetheless these findings suggest that the current standard practice of fractionated RT may
hinder RT-initiated anti-tumor immunity, resulting in an early relapse of tumor growth or recurrence at both local and distant sites.

**RT on human tumor also causes immune-mediated rejection: a new immune xenograft model for preclinical study**

Human tumors might respond to various anti-cancer treatments differently from murine tumors. The most commonly used model for preclinical testing of anti-cancer agents before clinical trials involves xenografts of human tumors into the immunodeficient nude mouse as required by the US food and drug administration (FDA). In fact, many of our assumptions about the behavior of human tumors *in vivo* are derived from these types of xenograft models. Our current study now questions whether such models can provide comprehensive evaluation of treatments in the absence of T cells. To overcome the current problem, we developed a novel xenograft model whereby the immune response to RT can be assessed. It is estimated that there are at least 80 mutated antigens per growing tumor, some of which can be presented to T cells in each patient. Each antigenic epitope has 20-200 specific T cells per host. Therefore, there are 300-3,000 tumor reactive T cells in each immunocompetent host. Transfer of such small numbers of T cells into B6/Rag−/− mice can result in rapid homeostatic proliferation that might artificially activate T cells and reject tumor. To overcome this limitation, our new immune xenograft model integrates a sufficient number of non-responsive T cells to limit homeostatic proliferation of responsive T cells in B6/Rag−/− mice. CD8+ T cells from MHC class I restricted OVA specific T cell receptor transgenic mice (OT-I) contain 97-98% OVA specific T cells that cannot respond to antigens from human tumor but can still effectively inhibit homeostatic proliferation. The remaining 2-3% non-OT-I T cells have the potential to recognize antigens
from human tumor. We injected human lung tumor A549 cells subcutaneously into B6/Rag\(^{-}\) mice. After tumors were established for 4 weeks, mice were transferred with \(2 \times 10^6\) total LN cells (300-3,000 tumor reactive versus one million non-reactive cells from OT-I transgenic mice), which approximates the number of tumor reactive T cells in human patients. Three days later, mice received localized 20 Gy to the tumor. Interestingly, tumor growth was inhibited only when RT was administered in the presence of CD8\(^+\) T cells, and failed when RT was given alone or with adoptive transfer of T cells alone (Fig. 3F). These data suggest that the immune stimulating effects of RT are also applicable to human tumors and accordingly, convey the need to revisit conclusions based on models that utilized immunodeficient mice.

**Local immunotherapy can amplify radiation-initiated immunity to eradicate disseminated metastasis**

Our study opens the avenue for new strategies, such as RT and immunotherapy, to synergize anti-tumor effects. Homologous to lymphotoxin, exhibits inducible expression, competes with herpes virus glycoprotein D for HVEM on T cells (LIGHT) is a tumor-necrosis factor superfamily member that is a ligand of stromal cell-expressed lymphotoxin beta receptor (LT\(\beta\)R) and T cell-expressed herpes viral entry mediator (HVEM). LIGHT has been shown to greatly enhance host responses to progressively growing tumor \(^{21,22}\). Therefore we tested whether targeting tumor with ad-LIGHT after a suboptimal dose of RT could amplify radiation-initiated immunity to control metastasis. Mice bearing established 4T1 or B16-CCR7 tumors, which are both spontaneously metastatic tumor lines, received two consecutive doses of 12 Gy followed by intratumoral injections of relatively low dose ad-LIGHT, concomitant with the second dose of radiation. No metastatic colonies in lung or DLN were detected on day 35 only
in the RT + ad-LIGHT group (Fig. 4). Impressively, most mice (86%) treated with RT and ad-LIGHT showed prolonged survival (> 100 days) while all mice treated with either radiation or ad-LIGHT died in < 60 days (data not shown). Together these data demonstrate that RT in combination with immunotherapy can better control metastasis than either single treatment.

Discussion

Recently, enabled by technological advances in targeting of radiotherapy, there has been an increased interest in utilizing 1-3 high doses of radiation in contrast to low dose fractionation. Initially applied to arteriovenous malformations, benign brain tumors and brain metastasis (referred to as radiosurgery), there are now clinical trials applying this concept to extracranial targets. Recently, it has been suggested that high-dose single-fraction RT achieves better local control than would be predicted, implicating alternative mechanisms beyond direct killing of tumor cells 28. It has been suggested that damage to the tumor-associated endothelium contributes to the superior local anti-tumor effect of high-dose radiation 29. We have recently observed that stereotactic body radiation therapy (SBRT) effectively targets ablative doses of RT to isolated oligometastasis 17,18. Most patients with oligometastasis die in 6 months due to the lack of treatment and rapid progression of tumor. Our clinical data showed that better survival of patients with oligometastasis is closely associated with higher dose of RT: 1/6 (15%) of patients progressed with 12 Gy x3, 4/19 (20%) with 10 Gy x3, while 19/31 (60%) progressed in 6 months when treated with 8 Gy x3 of RT. In this study, we have now revealed the essential role of the immune response in tumor reduction in modified SBRT, shifting the goal of targeting tumors beyond local control towards generating systemic immunity for the eradication of distant
metastases. Further clinical trials are urgently needed to study the role of T cells, chemotherapy, and immunotherapy in SBRT-mediated control of oligometastasis.

Considering many cancer patients are under immune suppression or will be treated by immunosuppressive drugs before RT, the current ongoing trials using high dose radiation might underestimate its potency. Many cancer patients with potential metastasis routinely receive prolonged cycles of chemotherapy and conventional/prolonged RT. This combination therapy, whether delivered concurrently or sequentially, is aimed at direct cytotoxic reduction of tumor cells. Indeed, studies have shown that radiation used in conjunction with chemotherapy can synergistically reduce tumor burden in \textit{in vitro} cultures and \textit{in vivo} using immune-deficient xenograft models. Our results have revealed that the use of certain immunosuppressive adjuvant chemotherapy actually erases radiation-initiated T cell priming, challenging the rationale of some current combinations. Studies of radiotherapy and chemotherapy using standard lymphocyte deficient xenograft models or immune suppressive patients fail to consider an integral effect of the immune response, which might cater to misleading interpretations and conclusions as well as overestimate the therapeutic effect of radio/chemotherapy in immunocompetent hosts. Therefore, our newly generated immune xenograft animal model will allow evaluation of the effect of RT in various types of human tumor in the presence of immune system.

In summary, RT disrupts physical and immunological barriers, introduces danger signaling, increases DC cross-presentation of tumor antigen, and possibly reverses T cell unresponsiveness in tumor bearing hosts, leading to the rejection of local and distal tumors. Our study reveals critical insight into the immune-mediated therapeutic effect of RT, potential mobilization of immune response against established tumor, and challenges current intensive radio/chemotherapy protocols. It raises the possibility that while immunotherapy is a viable
alternative, current conventional cancer treatment strategies may cause attenuating effects on the immune system. Data analyzed in the context of immune-suppressed patients for various clinical trials may undermine the potency of immune responses against cancer and lead to misguided interpretation of the results. Therefore, our study unveils a paradigm shift in combined modality treatment of cancer and opens new strategies to mobilize the host immune system and potentially cure patients with metastasis.
Acknowledgements

We thank Drs David M. Kranz for the TCR tetramer and Bin Zhang for technical assistance, and Drs Hans Schreiber, Tom Gajewski, and Ping Yu for critical reading. This research was in part supported by US National Institutes of Health grants AI062026, CA115540 and CA97296 to Y.X.F, CA111423 to R.R.W. and a grant from the Ludwig Foundation to R.R.W. and Y.X.F.

S.L.A. is part of the Medical Scientist Training Program at the University of Chicago and is supported by a Medical Scientist National Research Service Award (5 T32 GM07281).

Youjin Lee and Sogyong L. Auh designed and performed most experiments as well as wrote the paper; Yugang Wang and Yang Wang performed human xenograft model; Yuru Meng and Michael Beckett have done experiments on nude mice; Rohit Sharma, Byron Burnette, Robert Chin, and Tony Tu performed some experiments; Ralph R. Weichselbaum and Yang-Xin Fu organized, designed, and wrote the paper.

Statement

The authors have declared that no conflict of interest exists.
References

**Figure legends**

**Fig.1. Immunodeficiency abrogates the anti-tumor effect of RT.** A) Wt C57BL/6 or nude mice (n= 10) were injected with $2 \times 10^6$ B16 melanoma cells and treated 7 days later with 20 Gy. Radiation group in wt but not in nude mice showed significant smaller tumor size (\(**p=0.002\) at day 10 post RT). B) Wt or nude mice (n=8-12) were injected with $2 \times 10^5$ B16-SIY and treated 10 days later with 25 Gy. Radiation group showed significant smaller tumor size (\(***p=0.0002\) on day 12 post RT). Similar trend of the inhibition was also detected with single 20 Gy. 60% Wt mice were cured while 100% nude mice die with 20 Gy. C) Tumor growth curve and D) survival for Wt mice injected with $1 \times 10^5$ B16 and treated on d14 with 15 Gy given on d0, d1 and d2 (post RT). 200 ug/mouse anti-CD8 antibody was administered on d0, d4, d8 post RT (n=5-9/group). After RT plus depletion of CD8, the size of tumor increased significantly from RT alone (\(***p=0.0073\) at d14). Survival increased after RT \(***p=0.0001\), but with CD8 depletion survival was significantly reduced \(***p=0.0009\). \(*p<0.05, **p<0.01, ***p<0.001\). Similar experiments were repeated three times (A-D).

**Fig. 2. RT promotes priming of Ag-specific cells.** A) $5 \times 10^5$ B16-SIY tumor cells were subcutaneously injected into the lower back of C57BL/6 (n=8-9/group). 14 days after tumor challenge, mice received localized RT (20 Gy) on the tumors and were transferred i.v. with CFSE labeled naïve 2C cells. 4-5 days post adoptive transfer, mice were sacrificed for analysis of DLN and spleen. The degree of CFSE dilution via FACS was determined by gating on 1B2+CD8+ lymphocyte population. The RT group has more proliferative T cells than no RT group (\(***p<0.0001\)). B) $5 \times 10^5$ B16-SIY tumor cells were subcutaneously injected into the lower back of C57BL/6 (n=5-6/group). 14 days after tumor challenge, mice received local RT
(20 Gy) on the tumors and were sacrificed 5 days later for tetramer+ cell analysis. DLN and spleen were harvested and collagenase digested, then stained for FACS. Cells were gated on CD11c+ cells. Similar experiments were repeated two times. The RT group has more positive cells than no RT group (**p=0.0008). C) 2x10^5 B16 tumor cells were subcutaneously injected into the lower back of C57BL/6 mice (n=4-6/group). Fourteen days after tumor challenge, mice received localized RT (20 Gy) on the tumors and were analyzed 48 hrs later. DLN was isolated, collagenase digested (1.5 mg/ml), then stained for FACS. Cells were gated on CD11c+ cells. Mean + SD for no RT group was 6.8 + 4 while RT group was 14.6 + 2. Similar experiments were repeated at least two times.

Fig 3. Chemotherapy diminishes the effect of radiation-mediated eradication of metastases and T cell priming. A) 2x10^5 B16-CCR7 cells were subcutaneously injected and on d14, 15, and 16, mice received 15 Gy. On d7 and d14 post RT, 200 mg/kg dacarbazine (also for human melanoma) was administered i.p. Radiation group showed a significant smaller tumor size (**p=0.0006 at day 13 post RT). Additional dacarbazine after RT led to significant regrowth (**p=0.0075 at day 26 post RT, *p=0.015 d32 post RT (n=3-5). B) Tumor growth curve. 1x10^5 4T1 tumor cells were injected and on d15, 16, and 17 mice received 15 Gy. On d7 and d14 post RT, 20 mg/kg paclitaxel was administered i.p. Radiation group showed significantly smaller tumor size (**p=0.008 at day 23) (n=4-9 per group). C) Metastasis assay. 1x10^5 4T1 tumor cells were subcutaneously injected and on d12, 13, and 14 Balb/c mice received local RT of 15 Gy. The tumors were removed on day 21. On d7 and d12 post RT, 20 mg/kg paclitaxel was administered i.p. No colonies were detected after radiation while addition of chemotherapy
completely eliminated the effect of radiation (n=4-5/group). D) 5x10^5 B16-SIY melanoma cells were injected subcutaneously. On d17 mice were transferred with 2x10^6 CFSE labeled 2C cells and locally RT with 20 Gy. 200 mg/kg dacarbazine i.p. was given two days post adoptive transfer. DLN and spleen were harvested on d21 for analysis. E) 5x10^5 B16-SIY melanoma cells were injected subcutaneously. Mice received local tumor RT of 20 Gy x1 or 5 Gy x4. Single treatment 200 μg/mouse of anti-CD8 antibody was administered on d0, d4, d8, d12 post RT. Repeated treatment of radiation showed significant regrowth of tumor mass *p=0.03 at d25 (n=4-6). F) 8 x10^6 Human lung tumor A549 cells were subcutaneously injected into B6/Rag^-/- mice and 4 weeks later, the mice were transferred with 2x10^6 LN cells from OT-I transgenic mice. Three days later, mice received 20 Gy of local RT. RT (p=0.48) or T cells (p=0.3) alone showed no significant differences from no treatment group while the radiation + T cell group showed significantly smaller tumor size (*p=0.018 at d60). Similar experiments were repeated at least two times (A-F).

**Fig 4. The synergy of RT plus ad-LIGHT immunotherapy eradicates distant metastases.**

4T1 tumor: Balb/c mice were subcutaneously injected with 1x10^5 cells on the lower back. Mice received local RT (12 Gy) on d14 and d15 and intratumoral injection with ad-control (2x10^10 vp) or ad-LIGHT (2x10^10 vp) on d15 and d16 (n=24-41 pooled from five experiments). B16-CCR7 tumor: C57BL/6 mice were subcutaneously injected with 1x10^5 cells on the lower back. Mice received local RT (12 Gy) on d14 and d15 and intratumoral injection with ad-control (2x10^10 vp) or ad-LIGHT (2x10^10 vp) on d15, d16 and d17 (n=6-9 pooled from two experiments). On d25 post tumor injection, tumors were surgically removed. Mice were sacrificed on d35 for tumor
colonogenic assay (n=204-5/group). No colonies were detected in combination group in both types of tumor. Similar experiments were repeated three times.
Figure 1
Figure 3
Figure 3
Figure 4
Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment

Youjin Lee, Sogyong L. Auh, Yugang Wang, Byron Burnette, Yang Wang, Yuru Meng, Michael Beckett, Rohit Sharma, Robert Chin, Tony Tu, Ralph R. Weichselbaum and Yang-Xin Fu