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Editor's Summary

Let's Work Together

Despite decades of research into causes and potential therapies for cancer, cancers still account for almost 13% of all deaths every year. It is becoming increasingly clear that monotherapies are not the answer, and combining drugs to improve efficacy and prevent resistance is becoming the norm. However, care must be taken when combining even drugs already in the clinic —two treatments may not necessarily be better than one and may even cause harm. Thus, there is a need for rationally designed combination therapy. Here, Seung *et al.* conduct a phase 1 trial on one such rationally combined therapy —interleukin-2 (IL-2) and stereotactic body radiation therapy (SBRT).

IL-2, an immune activator, has been long used in the clinic either as a single-agent immunotherapy or in combination with various drugs for melanoma and renal cell carcinoma, with limited success. Here, the authors combine high-dose IL-2 with targeted radiation therapy based on clinical observation of enhanced efficacy in patients as well as the still to be proven hypothesis that radiation damage induces tumor antigen release and microenvironment changes that should enhance the immune-activating effects of IL-2. They found that the combination therapy was safe, and, albeit in a small number of patients, appeared to have improved efficacy over IL-2 alone. Intriguingly, they found a greater frequency of proliferating early effector memory T cells in the peripheral blood of these patients. Although studies with more patients and more detailed mechanistic follow-up must be performed, this study suggests that the rational combination of SBRT and IL-2 may improve upon current therapies for metastatic melanoma and renal cell carcinoma.

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TUMOR RADIOTHERAPY

Phase 1 Study of Stereotactic Body Radiotherapy and Interleukin-2: Tumor and Immunological Responses

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Preclinical models suggest that focal high-dose radiation can make tumors more immunogenic. We performed a pilot study of stereotactic body radiation therapy (SBRT) followed by high-dose interleukin-2 (IL-2) to assess safety and tumor response rate and perform exploratory immune monitoring studies. Patients with metastatic melanoma or renal cell carcinoma (RCC) who had received no previous medical therapy for metastatic disease were eligible. Patients received one, two, or three doses of SBRT (20 Gy per fraction) with the last dose administered 3 days before starting IL-2. IL-2 (600,000 IU per kilogram by means of intravenous bolus infusion) was given every 8 hours for a maximum of 14 doses with a second cycle after a 2-week rest. Patients with regressing disease received up to six IL-2 cycles. Twelve patients were included in the intent-to-treat analysis, and 11 completed treatment per the study design. Response Evaluation Criteria in Solid Tumors criteria were used to assess overall response in nonirradiated target lesions. Eight of 12 patients (66.6%) achieved a complete (CR) or partial response (PR) (1 CR and 7 PR). Six of the patients with PR on computed tomography had a CR by positron emission tomography imaging. Five of seven (71.4%) patients with melanoma had a PR or CR, and three of five (60%) with RCC had a PR. Immune monitoring showed a statistically significantly greater frequency of proliferating $CD4^+$ T cells with an early activated effector memory phenotype ($CD3^+CD4^+Ki67^+CD25^+FoxP3^-CCR7^-CD45RA^-CD27^+CD28^{+/-}$) in the peripheral blood of responding patients. SBRT and IL-2 can be administered safely. Because the response rate in patients with melanoma was significantly higher than expected on the basis of historical data, we believe that the combination and investigation of $CD4^+$ effector memory T cells as a predictor of response warrant further study.

INTRODUCTION

Interleukin-2 (IL-2)–based immunotherapy has been used for decades to treat patients with metastatic melanoma and renal cancer with occasional, but limited, success. There have also been extensive clinical and preclinical studies combining IL-2 with other biological response modifiers and chemotherapy, but there have been few studies combining radiation and IL-2. We report here on the clinical and preliminary immunological observations of combining high-dose per fraction radiation and IL-2. The first publication describing high-dose IL-2 for patients with metastatic melanoma appeared in 1985, and a subsequent manuscript with 270 patients reported a complete response (CR) rate of 6% and a partial response (PR) rate of 10%, with a median duration of response greater than 40 months (*1*, *2*). More than 70% of patients achieving a CR and about 15% of those achieving a PR were alive and without recurrence at 15 years. In the last year, there have been several advances in melanoma treatment including the recent U.S. Food and Drug Administration (FDA) approval of ipilimumab based on a phase 3 study showing a 4-month increase in median survival (*3*, *4*). Vemurafenib, a specific BRAF inhibitor now also FDA-approved, appears to induce tumor regression in up to 70% of patients having the BRAFv600E mutation (*5*, *6*); however, complete regressions with these new agents are infrequent. Durability of response, which is the main attribute of IL-2–based therapy, has not been validated for ipilimumab or vemurafenib.

IL-2 is also effective in metastatic renal cell carcinoma (RCC); CR and PR rates are 7 and 8%, respectively, with a median duration of response of 54 months (*7*). More than 70% of patients achieving a CR are alive and disease-free with more than 10 years of follow-up at the last analysis conducted in 2000 (*8*, *9*). There are also new agents for the treatment of RCC including tyrosine kinase inhibitors, antibodies to vascular endothelial growth factor, and mammalian target of rapamycin inhibitors, which, although they may show a superior response rate to IL-2, most responses are partial and transient with a median duration of response of about 11 months. In contrast to high-dose IL-2, it appears that none of the patients treated with these newer agents are cured (*10*). Thus, IL-2 remains the only treatment for either melanoma or RCC with a probability for cure, albeit in a small percentage of patients.

Preclinical studies indicate that exposure of tumor cells to high-dose radiation can augment the release of inflammatory cytokines and up-regulate expression of major histocompatibility complex (MHC) class I, B7.1, and Fas/CD95 (*11*–*16*). Tumor cells injured by radiation can also release damage-associated molecular patterns such as HMGB1 that can trigger a Toll-like receptor 4 (TLR4)–dependent cognate immune response (*17*). High-dose per fraction radiation also increases tumor-infiltrating activated $CD8^+$ T cells and has been associated with enhanced tumor control at distant sites when combined with immunomodulatory agents in preclinical studies (*18*–*20*).

We have observed that melanoma or RCC patients who had radiation for urgent palliation in the week before IL-2 had a surprisingly high systemic response rate. To test the hypothesis that focal high-dose

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radiation to a metastatic tumor could be administered safely in conjunction with high-dose IL-2, we performed a phase 1 trial of stereotactic body radiation therapy (SBRT) and IL-2 in which the primary objective was to determine the maximum tolerated dose (MTD). Secondary objectives were to determine local control of SBRT-treated lesions and systemic disease response to IL-2 and to assess immunomodulatory effects.

RESULTS

Patient characteristics

Twelve patients were enrolled from May 18, 2009, through June 28, 2010. Table 1 summarizes their characteristics and SBRT treatments before IL-2. Patient 3 was included as part of the intent-to-treat analysis of response, but was replaced in the study because he did not receive treatment as defined in the protocol due to an unrelated cardiac event (see below).

Overall response after IL-2

Figure 1 shows a waterfall plot of best overall response after SBRT and IL-2 of the target lesions not treated with SBRT. There were one complete and seven partial regressions by Response Evaluation Criteria in Solid Tumors (RECIST) criteria for an overall response rate in the intent-to-treat analysis of 66%. Six patients with residual radiographic abnormalities on computed tomography (CT) were CRs by positron emission tomography (PET) imaging with no abnormal fluorodeoxyglucose (FDG) uptake in the residual radiographic abnormalities on CT. Five of the seven patients with melanoma were PET CRs (71% response rate; 95% confidence interval, 29 to 96%). Our 71% response rate is statistically significant because its 95% confidence interval does not include the historical response rate of IL-2 monotherapy in melanoma of 16% (*2*) ($P = 0.05$ with a power of 80%) (Table 1).

Three of five (60%) patients with RCC had a PR; one patient was a PET CR. One individual with RCC developed a new metastatic lesion at the time of the first assessment in a peri-aortic lymph node, but had subsequent regression of the lesion about 1 year after IL-2 without any other medical or radiation therapy, and is included as one of the PRs. Table 2 summarizes CT and PET responses. There was no relationship between the SBRT dose and overall response and no relationship between disease burden and response. Figure 2 shows PET imaging in a patient with melanoma who received three SBRT doses to two liver metastatic deposits and six IL-2 cycles.

Of the eight responding patients, six have maintained their response with a median follow-up of 480 days. One patient with melanoma developed a brain metastasis that required treatment with radiation and surgery. In retrospect, the brain lesion was present before SBRT and IL-2 started but was interpreted at the time as a vascular malformation. PET

Table 1. Summary of patient characteristics, sites treated with SBRT, and IL-2 cycles. Lactate dehydrogenase (LDH) values are given only for patients with melanoma. The duration of response is from the date of initial treatment through the most recent imaging available at the time of preparing the manuscript. RLL, right lower lobe; LLL, left lower lobe; RUL, right upper lobe; RML, right middle lobe; L Hilum, left hilum; R Hilum, right hilum.

Patient	Sex	Age (years)	Performance status	Baseline LDH	Cohort	Histology	SBRT site (max diameter, cm)	Sum of target lesions at baseline (cm)	IL-2 cycles	Duration of response (days)	Best response by PET/CT
1	M	64	0	251	1	Melanoma	1 Mediastinum (6.1)	27.4	6	745+	CR
2	M	59	0	148	1	Melanoma	1 RLL (1.2)	3.8	6	381	CR
3	M	61	0	—	1	Renal	1 L Hilum (2.7)	7.7	2	61	PD
4	M	62	1	—	1	Renal	1 LLL (2.4)	23.2	4	543+	CR
5	M	61	0	—	2	Renal	1 R Hilum (1.0)	2	2	61	PD
6	F	64	0	165	2	Melanoma	1 RUL (0.5) 1 LLL (0.7)	4.1	6	530+	CR
7	M	61	0	192	2	Melanoma	1 RML (1.8)	5	6	577+	CR
8	M	65	1	144	3	Melanoma	1 RLL (2.1)	7	2	62	PD
9	M	51	0	135	3	Melanoma	1 Hepatic (1.4) 1 Hepatic (1.4)	7.5	2	60	PD
10	F	64	0	—	3	Renal	1 RUL (1.0) 1 RLL (2.1)	1.0	2	422+	PR*
11	M	61	1	1087	3	Melanoma	1 Hepatic (3.6) 1 Hepatic (3.5)	24.3	6	399+	CR
12	M	61	0	—	3	Renal	2 RLL (1.5) 1 LLL (2.1)	8.6	6	362	PD

*Patient had a new lymph node metastasis after IL-2 that regressed spontaneously without other medical treatment.

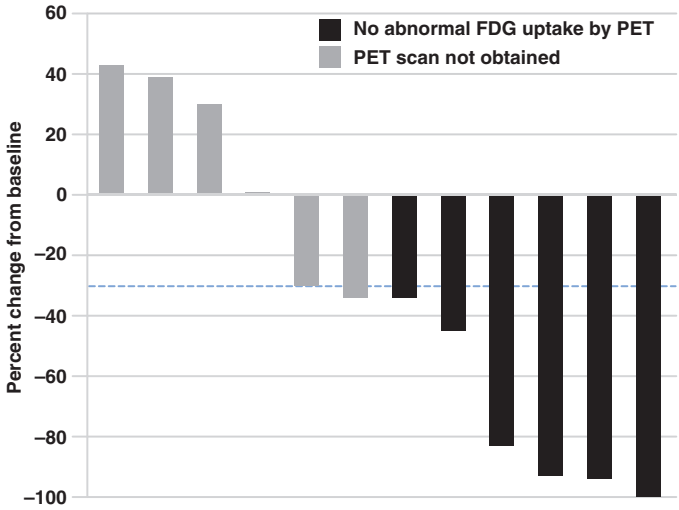

Fig. 1. Waterfall plot of best tumor response by RECIST criteria of all target lesions not treated with SBRT. Each bar represents the response of an individual patient. A dashed line is placed at 30% to indicate the minimum regression of tumor to qualify for a PR by RECIST criteria of target lesions.

Table 2. Comparison of CT and PET imaging. The five responding melanoma patients and one patient with renal cancer had PET imaging. For the patients who had CT imaging only, their response (partial, stable, or progression) was carried over to the PET column to have the same number of patients in the calculation of overall response.

	CT (%)	PET (%)
Complete response (CR)	1 (8.4)	6 (50)
Partial response (PR)	7 (58.3)	2 (16.7)
Stable disease	1 (8.4)	1 (8.4)
Progressive disease	3 (25)	3 (25)
Overall response (CR + PR)	8 (66.7)	8 (66.7)
Response by disease		
Melanoma (n = 7)	CR 1 (14.3)	CR 5 (71.4)
	PR 4 (57.1)	PR (0)
Renal cancer (n = 5)	CR 0 (0)	CR 1 (20)
	PR 3 (60)	PR 2 (40)

imaging in this patient showed no FDG-avid visceral metastatic sites about 745 days after IL-2.

Response of lesions treated with SBRT

All lesions treated with SBRT regressed and none have recurred. SBRT-induced dose-dependent radiographic changes in the area of treatment were observed (Fig. 3); this has also been described by others (*21*, *22*). CT scan changes were dose-dependent and made the interpretation of local control difficult; however, no lesion treated with SBRT showed significantly increased FDG uptake on PET.

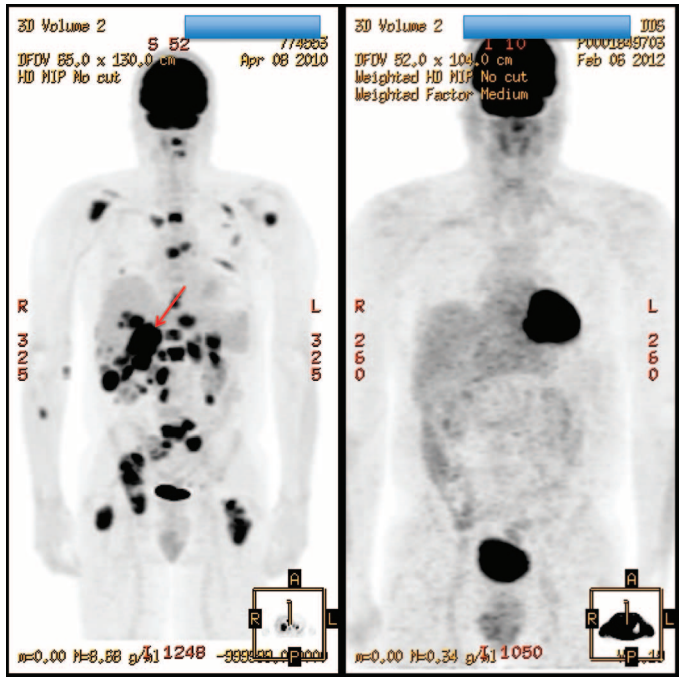

Fig. 2. Before and after PET imaging in a patient with widely metastatic melanoma. Two liver lesions were treated with SBRT.

Toxicities

There were no dose-limiting adverse events attributable to SBRT; thus, an MTD of SBRT was not reached. Anticipated toxicities from IL-2 were observed and included hypotension requiring vasopressor support, pulmonary capillary leak with hypoxemia, fever, rigors, myalgias, arthralgias, pruritis, erythematous rash, diarrhea, nausea, electrolyte abnormalities, elevations of hepatocellular enzymes, azotemia, peripheral neuropathy, and mental status changes. These toxicities resolved after completion of IL-2. The median numbers of IL-2 doses for the first and second cycles of therapy were 11 and 10, respectively, which is identical to the median numbers of IL-2 doses tolerated historically by other patients in our Biotherapy Program who did not receive SBRT. There was no significant change in pulmonary function testing, clinical manifestations of pulmonary capillary leak, or chest radiographs during IL-2 administration after SBRT (table S1).

Patient 3 (metastatic RCC) developed paroxysmal atrial fibrillation (PAF) after SBRT, but before IL-2 started. In retrospect, this patient had a history of PAF, although he was in sinus rhythm during pre-study assessment. The PAF was not caused by SBRT because there are no published reports of this in the medical literature and the radiation port did not include the cardiac muscle or the vagal nerve. After pharmacologic conversion to sinus rhythm, he went on to receive IL-2, although delayed by 3 days (6 days after the last SBRT dose). The PAF did not recur during IL-2. This patient had disease progression and was included in the analysis of response even though he did not receive treatment on the planned schedule. Another patient with metastatic RCC with regressing pulmonary, hepatic, and lymph node metastases developed a hemorrhagic stroke during cycle 4 IL-2. The patient made a full recovery from the stroke and has no FDG-avid lesions on PET. IL-2 was not administered after the stroke.

Retrospective evaluation of patients who received IL-2 and high-dose per fraction radiation

We have maintained a database on patients receiving high-dose IL-2 since 1997 that encompasses more than 1000 admissions to our Biotherapy Program. Eight melanoma patients and two RCC patients had hypofractionated radiation therapy for palliation of symptoms the week before starting IL-2. Three patients had stereotactic radiosurgery for brain metastases, three had treatment of painful bone metastases, and four had pulmonary lesions treated to relieve bronchial obstruction. Nonirradiated lesions responded (PR = 2 and CR = 3) in five of the eight patients with melanoma for an overall response rate of 62.5%. Both patients with RCC had PRs by RECIST with minor persistent radiographic abnormalities. All of the responding patients are alive, have not had progression of malignancy, and have not required other cancer therapy.

Immunological monitoring

Responding patients showed a significantly higher frequency of proliferating FoxP3$^-$, Ki67$^+$ CD4$^+$ T cells with an early activated effector memory (T_{EM}) phenotype (CD25$^+$, CCR7$^-$, CD45RA$^-$, CD27$^+$, CD28$^{+/-}$) at baseline ($P = 0.021$) that was maintained during treatment at day 8 ($P = 0.036$) and day 15 ($P = 0.036$) of cycle 1 (Fig. 4). Nonproliferating Ki67$^-$CD4$^+$ T cells with the same early nonactivated T_{EM} phenotype signature were not different in responder versus nonresponder patients at baseline or at any time during treatment. Similarly, proliferating CD8$^+$ T_{EM} phenotype (CD25$^-$, CCR7$^-$, CD45RA$^+$, CD27$^+$, CD28$^+$ characterized by the retention of CD45RA expression, and previously designated T_{EMRA}) (*23*) showed higher frequencies in responding patients at baseline ($P = 0.021$), day 8 ($P = 0.021$), and day 15 ($P = 0.036$) of therapy (Fig. 4). Notably, there was no significant difference in the average frequency of proliferating regulatory T cells (T_{regs}) between responders and nonresponders at baseline (12.7% versus 11.7%; $P = 0.4$), day 8 (74.2% versus 64.2%; $P = 0.1$), and day 15 (7.8% versus 6.1%; $P = 0.15$).

DISCUSSION

We performed a pilot study that was designed to assess the safety, tumor responses, and exploratory immunological studies of SBRT and IL-2 immunotherapy. The combination of SBRT and IL-2 resulted in tumor regression in a significantly greater fraction of patients with melanoma than expected on the basis of published studies of IL-2 monotherapy. We acknowledge the small sample size; however, the proportion of melanoma patients responding

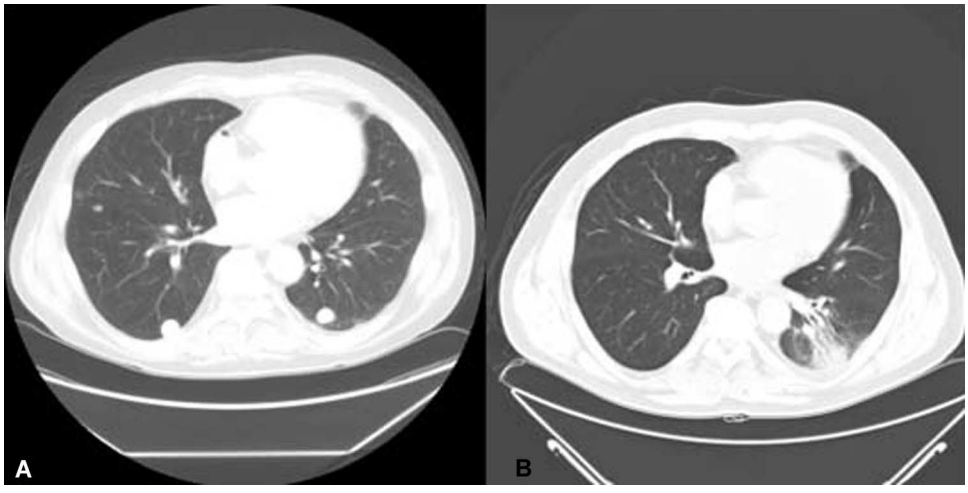

Fig. 3. (**A** and **B**) Before (A) and after (B) CT images in a patient with metastatic renal cancer. In (B), fibrotic changes are seen in the posterior left lung from SBRT (arrows). Two nodules in the right lung were not treated with SBRT and regressed after IL-2.

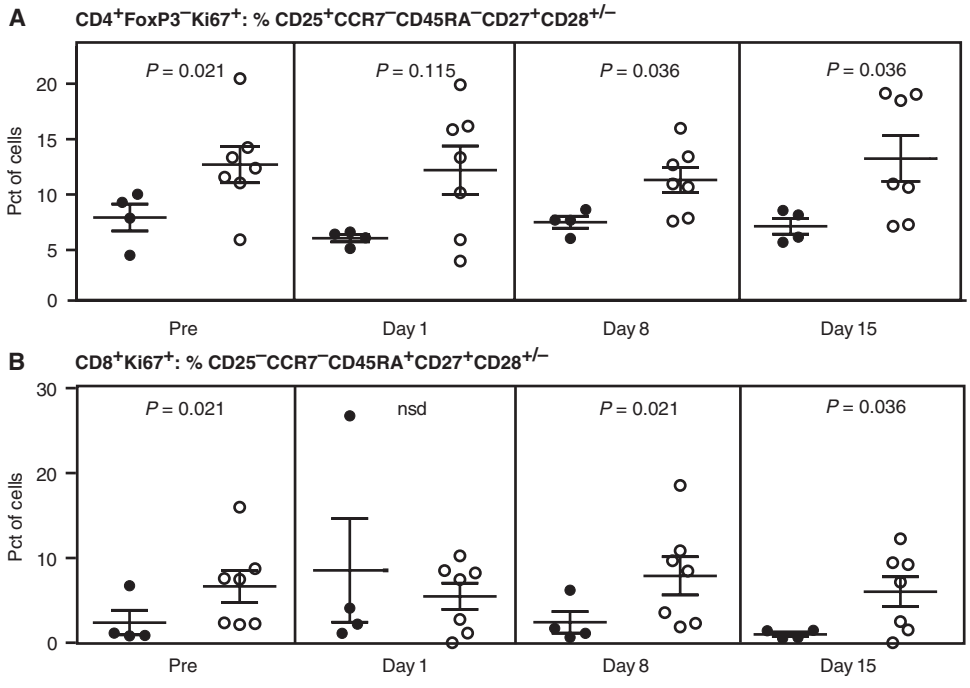

Fig. 4. PBMCs from pretreatment and from samples collected 3 days after SBRT (just before day 1 of IL-2 treatment). Days 8 and 15 of cycle 1 IL-2 were analyzed using a 10-color flow cytometry mAb staining panel (CD3/CD4/CD8/Ki67/FoxP3/CD25/CCR7/CD45RA/CD27/CD28). Scatter plots show mean (±SEM) results for individual patients grouped by clinical response, with nonresponders indicated by filled circles and responders represented by open circles. (**A** and **B**) Responders show significantly higher frequencies of proliferating (Ki67$^+$) CD4$^+$/FoxP3$^-$ early activated T_{EM} phenotype (CD25$^+$/CCR7$^-$/CD45RA$^-$/CD27$^+$/CD28$^{+/-}$) at pretreatment, day 8, and day 15 compared to nonresponding patients (A), and statistically higher frequencies of proliferating (Ki67$^+$) CD8$^+$ early unactivated T_{EMRA} phenotype (CD25$^-$/CCR7$^-$/CD45RA$^+$/CD27$^+$/CD28$^+$) at pretreatment, day 8, and day 15 (Wilcoxon rank-sum test, one-sided) (B).

to SBRT and IL-2 is at a level comparable to the 73% overall response rate reported for patients with melanoma receiving the BRAF inhibitor vemurafenib (*5*). A higher than expected response rate was also seen in patients with RCC and should be explored by studying a larger number of patients. Four of the five melanoma patients had residual radiographic abnormalities on CT imaging that were not FDG-avid on PET (one patient with melanoma had a CR on both PET and CT), and have generally continued to regress on CT for a year or more after treatment. These response kinetics are similar to those described in some patients treated with ipilimumab (*3*, *4*) and may represent ongoing T cell–mediated destruction of the melanoma. PET imaging has not been extensively evaluated as a monitoring strategy after immunotherapy. We have incorporated PET imaging in an ongoing randomized clinical trial of SBRT + IL-2 versus IL-2 and other studies in which we are combining radiation and immunotherapy to validate the clinical value of PET in this setting. SBRT and high-dose IL-2 can be administered safely to patients with metastatic melanoma and RCC. There was no association between the SBRT dose and response, an MTD for radiation was not reached, and excessive toxicity in the irradiated organ was not observed.

This report is one of the few that show significantly enhanced clinical effects of IL-2 immunotherapy when combined with another agent or modality. Many investigators have attempted to enhance the efficacy of IL-2 by combining it with chemotherapy, vaccines, cytokines, tumor-infiltrating lymphocyte (TIL) infusions, and monoclonal antibodies (mAbs) (*24*–*27*). TIL in conjunction with total body radiation, nonmyeloablative chemotherapy, and IL-2 does enhance response rates in melanoma patients, but is expensive and technically difficult. TIL is still an experimental approach and has not been adopted into general clinical practice. Radiation has been administered in conjunction with IL-2, but no enhancement of the IL-2 response rate was observed (*28*). Fourteen melanoma and 12 RCC patients were treated with 500 cGy twice daily to a total of 10 to 20 Gy over 1 to 2 days and 2 to 24 hours before starting IL-2. There are several reasons why this radiation and IL-2 regimen may have been ineffective. Field sizes from this era were often large, possibly negating any synergistic effect because of excess bystander irradiation (*29*). There may be a threshold radiation dose required to enhance immunogenicity. Lee *et al.* demonstrated that $CD8^+$ T cells exhibited more effective antitumor responses after 20-Gy compared to 5-Gy radiation fractions (*30*). Most of the preclinical studies demonstrating the immune-enhancing effects of radiation used fraction sizes of >5 Gy (*12*–*14*, *16*, *18*, *19*). In our study, the response of tumors treated with SBRT was 100% compared to 7% (2 of 28) in the trial reviewed above (*28*). The higher dose fractions and superior tumor targeting may explain the higher response rate of SBRT and IL-2 in our study. If our initial clinical results are confirmed, then the administration of SBRT and IL-2 will be much more broadly applicable therapy with curative potential to offer melanoma patients compared to TIL-based therapy, and is not restricted to a particular mutation profile as is the case with vemurafenib and other targeted therapies, which do not appear to have curative potential.

Exploratory immunological studies were performed to generate hypotheses about the interaction between SBRT and IL-2. In contrast to others (*31*), we did not observe decreases in the percentage of circulating T_{regs} in patients responding to IL-2. The higher frequency of proliferating $CD4^+$ and $CD8^+$ early T_{EM} phenotypes before and during therapy suggests that responding patients may have already formed an endogenous memory response to their tumor cells that was amplified during treatment. These early T_{EM} ($CD4^+$) and T_{EMRA} ($CD8^+$) subpopulations have not been described previously in patients receiving IL-2 monotherapy. A prospective clinical trial is under way to validate the response rate and the observation that a higher percentage of early T_{EM} at baseline predicts response to IL-2 or SBRT + IL-2. Although we do not know the antigen(s) to which the proliferating T cells were responding and did not perform an extensive analysis of antigen-specific responses, we found MART-1 antigen–specific $CD8^+$ T cells in one of three patients tested after treatment with SBRT + IL-2. A much more comprehensive analysis of tumor antigen–specific T cell responses in the randomized trial currently under way will be performed to determine whether proliferating early T_{EM} subsets are melanoma tumor antigen–specific.

Although we do not yet understand the mechanism through which SBRT may enhance the response to IL-2, this work is hypothesis-generating. Our hypothesis is that radiation increases tumor antigen release and changes the tumor microenvironment such that the immune effects of IL-2 are significantly more effective in melanoma and perhaps RCC. A randomized clinical trial comparing SBRT and IL-2 versus IL-2 alone is under way in melanoma to confirm the response rate and obtain more data about early memory T cell subsets and response, and will also include tumor biopsy in selected patients to gain more insight about the tumor microenvironment after SBRT.

MATERIALS AND METHODS

A single-institution phase 1 study was conducted at the Providence Portland Medical Center (PPMC). The main eligibility criteria were patients >18 years old; Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1; histological confirmation of metastatic melanoma or RCC; at least one metastatic lesion amenable to SBRT in the lung, mediastinum, or liver; and at least one other metastatic site not treated with SBRT. Cardiopulmonary status sufficient to tolerate high-dose IL-2 was required (*32*), and patients had essentially normal hematologic, hepatic, and renal function before treatment. Exclusion criteria included having no metastatic site amenable to SBRT, active infection, previous radiation to sites proposed for SBRT, and need for chronic steroids or active autoimmune disease. Signed informed consent was obtained before enrollment. The study was approved by the Providence Health System Regional Institutional Review Board, Oregon.

SBRT planning was performed with a four-dimensional CT scan with the patient in the treatment position immobilized with the BodyFIX (Elekta). The internal target volume was delineated on the planning CT, and a 3- to 5-mm planning target volume margin was used. An intensity-modulated radiation therapy treatment plan with 6-MV photons was generated using Pinnacle v.9.0 software (Philips Medical Systems) on the basis of tumor location and geometry. The target was localized with cone beam CT before each treatment, which was delivered on the Synergy S (Elekta) machine. A minimum of one and a maximum of three lesions were treated with SBRT. A maximum diameter of 7 cm was allowed for each SBRT target lesion. All patients were treated by two of the authors (S.K.S. and M.C.).

Patients were assigned to cohorts consecutively. In cohort 1, a single 20-Gy radiation fraction was administered on the Friday before IL-2; in cohort 2, two 20-Gy fractions were administered on the Wednesday and Friday before IL-2; and in cohort 3, three 20-Gy fractions were administered on the Monday, Wednesday, and Friday

before IL-2. The dose per fraction was reduced to 16 Gy in patients whose SBRT target was in the "central location" in the thorax (*33*). SBRT was only given before the first IL-2 cycle.

IL-2 (Prometheus Pharmaceuticals) treatment began on the Monday after the last radiation treatment and was administered at 600,000 IU per kilogram by means of intravenous bolus infusion given every 8 hours × 14 planned doses with an additional cycle given after a 16-day hiatus (two cycles = one course of IL-2). Imaging was obtained after each course, and patients with tumor regression could receive up to three courses. We used PPMC Biotherapy Program guidelines for IL-2 management, which are a modification of published IL-2 dosing rules (*32*). Pulmonary function testing was obtained at baseline and on day 1 of each IL-2 cycle in course 1 to assess for pulmonary toxicity. Chest radiographs were obtained on days 1, 3, and 5 of the first two IL-2 cycles to ascertain whether IL-2–induced capillary leak was exacerbated by SBRT.

This protocol used a modified version of the RECIST (*34*). The overall response assessment included all measurable and nonmeasurable target lesions except the lesions treated by SBRT, which were assessed separately. Both CT and PET imaging were used to assess response. A radiologist (A.B.) who did not know the patients' cohort assignment or clinical response assessment reviewed the PET images.

Immune monitoring studies

Peripheral blood mononuclear cells (PBMCs) from cryopreserved Ficoll-separated blood were obtained before treatment and on days 1, 8, and 15 of the first cycle of IL-2. All samples were stained with mAbs specific for cell surface epitopes, fixed and permeabilized, and then stained internally for Ki67 and FoxP3 expression. The fluorescent antibodies used were as follows: CD3 Alexa Fluor 700 (clone OKT3; eBioscience), CD4 eFluor (clone OKT4; eBioscience), CD8 V500 (clone RTA-P8; BD Horizon), CD28 peridinin chlorophyll protein (PerCP)–Cy5.5 (clone CD28.2; eBioscience), CD27 allophycocyanin (APC)–H7 (clone M-T271; BD Pharmingen), CD45RA Qdot 605 (clone MEM-56; Invitrogen), CCR7 phycoerythrin (PE)–Cy7 (clone 3D12; BD Pharmingen), and CD25 APC (clone 4E3; Miltenyi Biotec). Thawed PBMCs were washed and stained with all eight surface mAbs for 30 min at 4°C. Cells were then washed twice and treated with the fixation/permeabilization concentrate and diluent (eBioscience) using the eBioscience protocol. Permeabilized cells were washed twice and stained with FoxP3 PE (clone PCH101; eBioscience) and Ki67 fluorescein isothiocyanate (FITC) (clone B56; BD Pharmingen) for 30 min at 4°C; cells were washed and flow cytometry was performed immediately. On the basis of differential staining patterns, the 10-parameter mAb panel delineated memory and effector subpopulations using markers for CCR7, CD45RA, CD27, CD28, T_{reg} (CD25, FoxP3) subsets, and proliferation status (Ki67) simultaneously on a T cell background (CD3, CD4, and CD8). Forward scatter (FSC) area versus FSC height gating was used to eliminate doublets, and a standard FSC versus SSC lymphocyte gate and a $CD3^+$ gate were used to select viable T cells for analysis. Cells were then further segregated into $CD3^+$, $CD4^+$ ($CD8^-$), and $CD3^+$, $CD8^+$ ($CD4^-$) subpopulations. These populations were further interrogated using the remaining seven markers to determine their subphenotypes and proliferation status. Activated T_{EM} cells were defined as $FoxP3^-$, $CCR7^-$, $CD45RA^-$, $CD27^+$, $CD28^{+/-}$. Early T_{EM} cells were defined as $CD25^-$, $CCR7^-$, $CD45RA^+$, $CD27^+$, $CD28^+$.

Data and statistical analysis

Data were acquired in FCS format using BD FACSDiva v6 on an LSRII and analyzed using WinList 6.0 Software. Compensation was performed with single-color staining controls, and regions were set using fluorescence-minus-one FMO controls. Subphenotype frequencies were determined using the FCOM analysis tool in WinList as described previously (*35*). FCOM subphenotype arrays were generated for six different preselected "parent" T cell populations ($CD4^+FoxP3^+Ki67^+$, $CD4^+FoxP3^+Ki67^-$, $CD4^+FoxP3^-Ki67^+$, $CD4^+FoxP3^-Ki67^-$, $CD8^+FoxP3^{null}Ki67^+$, and $CD8^+FoxP3^{null}Ki67^-$). Exhaustive expansion analysis (*36*) was used to characterize the expression patterns of five variable parameters (that is, CCR7, CD45RA, CD25, CD27, and CD28) and to generate 243 subphenotypes for each of the parent populations. We then selected subphenotypes that constituted greater than 2% of the preselected parent population and also showed statistically significant differences in expression between the responding and the nonresponding patients. The Clopper-Pearson method was used to determine the confidence interval for comparing the response rate in this study to published response rates in high-dose IL-2.

SUPPLEMENTARY MATERIALS

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Table S1. Assessment of pulmonary toxicity during IL-2.

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