



A Randomized Trial of Combined PD-L1 and CTLA-4 Inhibition with Targeted Low-Dose or Hypofractionated Radiation for Patients with Metastatic Colorectal Cancer

Arta M. Monjazeb¹, Anita Giobbie-Hurder², Ana Lako³, Emily M. Thrash², Ryan C. Brennick², Katrina Z. Kao², Claire Manuszak², Ryan D. Gentzler⁴, Anteneh Tesfaye⁵, Salma K. Jabbour⁶, Olatunji B. Alese⁷, Osama E. Rahma^{2,3}, James M. Cleary^{2,3}, Elad Sharon⁸, Harvey J. Mamon^{2,3}, May Cho¹, Howard Streicher⁸, Helen X. Chen⁸, Mansoor M. Ahmed⁹, Adrian Mariño-Enríquez^{2,3}, Seunghye Kim-Schulze¹⁰, Sacha Gnjatic¹⁰, Emanuel Maverakis¹¹, Alina I. Marusina¹¹, Alexander A. Merleev¹¹, Mariano Severgnini², Kathleen L. Pfaff², James Lindsay², Jason L. Weirather², Srinika Ranasinghe², Alexander Spektor^{2,3}, Scott J. Rodig^{2,3}, F. Stephen Hodi^{2,3}, and Jonathan D. Schoenfeld^{2,3}

ABSTRACT

Purpose: Prospective human data are lacking regarding safety, efficacy, and immunologic impacts of different radiation doses administered with combined PD-L1/CTLA-4 blockade.

Patients and Methods: We performed a multicenter phase II study randomly assigning patients with metastatic microsatellite stable colorectal cancer to repeated low-dose fractionated radiation (LDFRT) or hypofractionated radiation (HFRT) with PD-L1/CTLA-4 inhibition. The primary endpoint was response outside the radiation field. Correlative samples were analyzed using multiplex immunofluorescence (IF), IHC, RNA/T-cell receptor (TCR) sequencing, cytometry by time-of-flight (CyTOF), and Olink.

Results: Eighteen patients were evaluable for response. Median lines of prior therapy were four (range, 1–7). Sixteen patients demonstrated toxicity potentially related to treatment (84%), and 8 patients had grade 3–4 toxicity (42%). Best response was stable disease in 1 patient with out-of-field tumor shrinkage. Median

overall survival was 3.8 months (90% confidence interval, 2.3–5.7 months). Correlative IF and RNA sequencing (RNA-seq) revealed increased infiltration of CD8⁺ and CD8⁺/PD-1⁺/Ki-67⁺ T cells in the radiation field after HFRT. LDFRT increased foci of micronuclei/primary nuclear rupture in two subjects. CyTOF and RNA-seq demonstrated significant declines in multiple circulating immune populations, particularly in patients receiving HFRT. TCR sequencing revealed treatment-associated changes in T-cell repertoire in the tumor and peripheral blood.

Conclusions: We demonstrate the feasibility and safety of adding LDFRT and HFRT to PD-L1/CTLA-4 blockade. Although the best response of stable disease does not support the use of concurrent PD-L1/CTLA-4 inhibition with HFRT or LDFRT in this population, biomarkers provide support that both LDFRT and HFRT impact the local immune microenvironment and systemic immunogenicity that can help guide future studies.

Introduction

Although immune checkpoint inhibition has led to significant clinical benefit in patients with microsatellite instable colorectal cancer

(1, 2), the majority of patients with microsatellite stable (MSS) disease have proven largely refractory to single-agent PD-1 blockade and other immunotherapies, including combined PD-1/CTLA-4 blockade (1, 2). Thus, strategies are needed to help engender antitumor immunity and increase response rates. Focal radiation demonstrates immune-stimulating effects in animal models and anecdotal clinical reports (3), including the potential ability to increase response to combined PD(L)-1 and CTLA-4 blockade (4).

We performed a randomized multi-center, phase II study with the goal of evaluating the safety and efficacy of two different radiation regimens given in combination with combined PD-L1/CTLA-4 blockade in patients with MSS colorectal cancer who had progressed on at least one line of prior systemic chemotherapy. A hypofractionated radiation (HFRT) regimen of 24 Gy given over three fractions was adapted from preclinical studies demonstrating positive immune effects in combination with immune checkpoint blockade (5), including in tumor types that were otherwise unresponsive to immune checkpoint therapy (4). A lower dose hyperfractionated arm of 0.5 Gy was administered twice daily for 2 days and then repeated during each of the first four cycles of therapy to investigate immune effects of a regimen similar to that used in preclinical models (6) and pilot clinical studies (7, 8), but unlikely to have pronounced cytotoxic effects directly or lead to significant toxicity. Correlative studies were performed on circulating blood and tumor biopsies to examine the immunologic impacts of these different regimens.

¹Department of Radiation Oncology, University of California Davis, Comprehensive Cancer Center, Sacramento, California. ²Dana-Farber Cancer Institute, Boston, Massachusetts. ³Brigham and Women's Hospital, Boston, Massachusetts. ⁴University of Virginia, Charlottesville, Virginia. ⁵Karmanos Cancer Institute/Wayne State University, Detroit, Michigan. ⁶Rutgers Robert Wood Johnson Medical School, New Brunswick, New Jersey. ⁷Winship Cancer Institute of Emory University, Atlanta, Georgia. ⁸Cancer Therapy Evaluation Program, NCI, Bethesda, Maryland. ⁹Radiation Research Program, NCI, Bethesda, Maryland. ¹⁰Mount Sinai School of Medicine, New York, New York. ¹¹Department of Dermatology, University of California Davis, School of Medicine, Sacramento, California.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corrected online July 29, 2021.

Corresponding Author: Jonathan D. Schoenfeld, Radiation Oncology, Dana-Farber Cancer Institute/Brigham and Women's Hospital, Dana-Farber Cancer Institute, Boston, MA 02115. Phone: 617-632-3591; E-mail: jdschoenfeld@partners.org

Clin Cancer Res 2021;27:2470–80

doi: 10.1158/1078-0432.CCR-20-4632

©2021 American Association for Cancer Research.

Translational Relevance

Microsatellite stable (MSS) colorectal cancers are resistant to immune checkpoint blockade with PD-1/L1 and/or CTLA-4 inhibitors. Focal radiotherapy has been demonstrated to affect anti-tumor immunity in preclinical models, but the impact of different radiation doses in human tumors is uncertain. We conducted a randomized phase II study evaluating the combination of the PD-L1 inhibitor durvalumab and the CTLA-4 inhibitor tremelimumab with either hypofractionated radiation (HFRT) or low-dose fractionated radiation (LDFRT) in patients with advanced MSS colorectal cancer. Despite tolerability of both regimens, HFRT or LDFRT given with combined PD-L1/CTLA-4 inhibition to patients with metastatic colorectal cancer did not lead to systemic response, but was associated with local and systemic immunologic changes, including T-cell infiltration, formation of micronuclei/primary nuclear rupture, and changes in circulating T-cell populations and T-cell diversity. These findings inform future trials to optimize radiation parameters to capitalize on immune stimulation.

Patients and Methods

Participants and eligibility

Patients with histologically confirmed metastatic MSS colorectal adenocarcinoma who had progressed on at least one line of chemotherapy were recruited to six centers in the NCI Experimental Therapeutics Clinical Trials Network (ETCTN) to ETCTN protocol 10021 (ClinicalTrials.gov identifier: NCT02888743). Investigators obtained written informed consent from each participant prior to enrollment. The research was conducted in accordance with the recognized ethical guidelines of the Declaration of Helsinki, CIOMS, Belmont Report, and U.S. Common Rule. Clinical study was performed after approval by a central institutional review board. Patients were aged ≥ 18 years and had adequate hepatic, bone marrow, and renal organ function and an Eastern Cooperative Oncology Group performance status of 0 or 1. All patients had measurable disease, including one liver lesion that could be targeted by radiation in the context of this trial and at least one other site of disease outside of the radiotherapy field for response assessment. To be eligible, the liver lesion to be irradiated within the context of this trial could not have been the target of previous radiotherapy. Subjects also needed at least one additional lesion outside of the radiation treatment field (more than one lesion permitted) that could be subsequently monitored for systemic (out-of-field) treatment response. Patients were confirmed to have MSS disease documented by either IHC that did not suggest loss of MLH-1, MSH-2, PMS2, or MSH6 or PCR testing that did not suggest microsatellite instability.

Procedures and screening

Patients were required to either undergo a fresh tumor biopsy for the purposes of screening or provide an archival tumor sample obtained less than 3 months prior to study enrollment. Baseline screening included a CT of the chest abdomen and pelvis for staging. All patients underwent CT-based planning for radiation of 1–2 lesions within the liver with intravenous contrast and internal tumor and organ motion management. 4D-CT planning and image-guided radiotherapy (IGRT) were mandatory for patients randomized to HFRT. The use of 4D-CT and IGRT was not mandated for subjects receiving low-dose

radiotherapy. Radiation was administered with an optional 0–1 cm clinical tumor volume margin to account for any uncertainty or microscopic disease and all patients were treated with a 5 mm planning tumor volume.

Study design and treatment

The primary objective of the trial was to determine the overall response rate in the two treatment arms according to RECIST v1.1 criteria (9) and excluding the lesions that had been irradiated. Patients were randomized 1:1 upon study entry to a treatment regimen consisting of the PD-L1 inhibitor durvalumab, administered at a fixed dose of 1,500 mg every 4 weeks for a maximum of 13 cycles, and the CTLA-4 inhibitor tremelimumab, administered at a fixed dose of 75 mg every 4 weeks for a maximum of four cycles, combined with either low-dose fractionated radiotherapy (LDFRT) or HFRT. The treatment regimens are shown in Supplementary Fig. S1. LDFRT consisted of a dose of 2 Gy administered in four fractions over 2 days repeated for the first four cycles of therapy (total dose 8 Gy). HFRT consisted of a total dose of 24 Gy administered in three 8 Gy fractions no more frequently than every other day during the first cycle of therapy only. Radiation was administered the week following durvalumab/tremelimumab administration. Patients were evaluated for response every 12 weeks after an initial restaging scan at 7–8 weeks. Best overall response was best RECIST response observed from the start of treatment until disease progression or recurrence.

Secondary endpoints included progression-free survival and overall survival. Overall survival was the time interval between study enrollment and death from any cause. For patients lost to follow-up or who had no documentation of death at the time of analysis, follow-up was censored at the date of last assessment of vital status. Progression-free survival was the time from enrollment to the earlier of objective disease progression or death. For patients without progression, follow-up was censored at the date of last adequate restaging, unless death occurred within 12 weeks following the date last known to be progression free, in which case the death was counted as a progression-free survival event.

Correlative blood samples were obtained prior to all study treatment and then again prior to cycle 2 of durvalumab/tremelimumab. On-treatment biopsies of irradiated lesions were obtained during week 7 or 8 (between cycles 2 and 3) and around the time of the first restaging scans (Supplementary Fig. S1). Toxicities were graded according to Common Terminology Criteria for Adverse Events v4.0 (10).

Statistical analysis

This phase II study was designed using two parallel, Simon optimal two-stage designs to identify combinations capable of effecting a systemic response outside of the radiation treatment field according to RECIST v1.1 criteria. The goal was to identify a promising rate of response of 20% (null rate, 5%), with a type I error of 5% and 80% power. Enrollment was stopped after 10 patients were enrolled in each arm in the first stage of the study according to prespecified stopping criteria looking for at least one objective response before proceeding to the second stage.

The distributions of overall and progression-free survival were summarized using the method of Kaplan–Meier and compared using log-rank tests.

Correlative studies

PD-L1 IHC, multiplex immunofluorescence (mIF), cytometry by time-of-flight (CyTOF), and Olink correlative studies were performed through the Cancer Immune Monitoring and Analysis Centers Immuno-Oncology Biomarkers Network using analytically validated and standardized platforms. Detailed standard operating procedures

are available at <https://cimac-network.org/assays/> and additional detail is provided in the Supplementary Materials and Methods and Supplementary Table S6. PD-L1 IHC was performed using the 9A11 antibody clone (Cell Signaling Technology) using the BOND Max by Leica Biosystems. PD-L1 H-score was determined by multiplying the percentage of tumor cells staining positive for PD-L1 by the intensity as determined on a 1+, 2+, 3+ scale. mIF used formalin-fixed, paraffin-embedded slides of the primary tumor stained using BOND RX automated stainer using published protocols (11–13).

Peripheral blood mononuclear cells (PBMC) were analyzed using CyTOF with reference sample spike-in and palladium-based mass tag cell barcoding of individual samples as described previously (14, 15). Serum cytokines were analyzed using Olink multiplex assay platform with Immuno-oncology Panel (Olink Bioscience), according to the manufacturer's instructions. The inflammatory panel includes 92 proteins associated with immune response. Additional detail is provided in the Supplementary Materials and Methods. Analyses of changes in circulating biomarkers over time were based on longitudinal mixed models with intervention arm, time, and the interaction of intervention as independent predictors. Biomarker studies were exploratory; there were no adjustments made for multiple comparisons.

RNA sequencing (RNA-seq) was performed as described previously and techniques and analyses are described in more detail in the Supplementary Materials and Methods (16). Differential expression *P* values were corrected for multiple testing using the FDR method. Genes with FDR-adjusted *P* < 0.05 and fold change >2 or <0.5 were considered differentially expressed. To calculate the number of expanded/contracted clones, we performed statistical analysis of each T-cell receptor (TCR) clones abundance for each patients before and after treatment using "fisher.test" R function. Clones which showed *P* value less than 0.05 in the test were counted as contracted or expanded.

Data and materials availability

All data associated with this study are present in the article or the Supplementary Materials and Methods.

Results

Treatment with combined PD-L1/CTLA-4 and low- or hypofractionated radiation was tolerated, but did not induce systemic responses

Twenty subjects were enrolled across six centers in the United States from August to November 2017, with 10 subjects randomized to each arm (Supplementary Fig. S2).

One patient subsequently withdrew before starting therapy and was excluded from all subsequent analyses. Baseline demographics are summarized in Supplementary Table S1. Subjects were well balanced with regards to gender and age. All patients had previously been treated with surgery and five had received prior radiotherapy to locations that were not subsequently irradiated on this trial. The median number of prior lines of therapy was four (range, 1–7). Sixteen patients had toxicity at least possibly related to therapy; there were 6 patients with grade 3 toxicities and 3 patients with grade 4 toxicities (Supplementary Table S2). The overall rate of grade 3–4 adverse events that were deemed at least possibly related to therapy was 0.42 [90% confidence interval (CI), 0.23–0.63]; these occurred in 3 of 10 patients who received HFRT (0.30; 90% exact CI, 0.09–0.61), and 5 of 9 patients treated with LDFRT (0.56; 90% CI, 0.25–0.83).

No objective responses outside of the radiation field were observed. Two patients were unevaluable for response (1 patient withdrew before treatment as above and another patient withdrew after treatment, but

before restaging with disease-related toxicity) and 1 patient demonstrated stable disease. The subject with stable disease had previously progressed on six prior lines of therapy and demonstrated a response in an unirradiated porta hepatis lymph node following HFRT and durvalumab/tremelimumab in the setting of decreasing overall tumor burden before being removed from study treatment after four cycles because of symptomatic new lesions (Supplementary Fig. S3). Median follow-up was 3.9 months. Median overall survival was 3.8 months (90% CI, 2.3–5.7 months) and progression-free survival was 1.7 months (90% CI, 1.5–1.8 months), with no significant differences between arms (Supplementary Fig. S4).

IHC and mIF revealed changes in T-cell and macrophage infiltration that were radiation dose dependent and micronuclei/primary nuclear rupture formation

Sixteen patients had baseline specimens for PD-L1 assessment. Baseline expression of PD-L1 was relatively minimal (tumor proportion score, TPS ≤ 5 and H-score ≤ 10 out of a maximum possible score of 300) in all patients. A TPS score of 5/H-score of 10 was seen in two subjects, including the subject who demonstrated an out-of-field response on trial. In addition, comparing matched baseline and on-treatment specimens, the change in PD-L1 expression was minimal (<2) or none among all five subjects with evaluable pre- and on-treatment samples.

mIF evaluated levels of CD4+, CD8+, PD-1+, and Ki-67-expressing cell populations (Fig. 1). Eighteen patients had tissue for baseline evaluation. Baseline levels were highly variable (CD8+ T-cell range, 11–107 cell/mm² and PD-1 cell range, 0–209 cells/mm²). The highest levels of tumor-infiltrating CD8+ and PD-1+ cells were observed in the hepatic lesion that was to be irradiated in the patient who subsequently demonstrated an out-of-field tumor response (Fig. 1A). There were no significant differences in baseline immune populations analyzed among patients who received prior radiation (*n* = 5) and others (*n* = 13), with a nonsignificant difference in baseline infiltration of CD4+ and CD8+ T cells (median CD8+/CD4+ T cells, 77 and 214 cells/mm² for patients who received prior radiation, and 21 and 1,122 cells/mm² for patients who did not receive prior radiation; *P* = 0.17 and *P* = 0.20, respectively). Evaluation of baseline and on-treatment samples in 5 patients demonstrated that the study treatment led to an increase in CD8+ T cells (*P* = 0.01 and *P* = 0.06), as well as CD8+/PD-1+/Ki-67+ T cells (*P* = 0.004 and *P* = 0.004) among 2 patients who received HFRT (Fig. 1B and C). There were no significant increases among LDFRT patients (*N* = 3).

Gene set variation analysis of RNA-seq data corroborated these findings. Specifically, there were no differences between treatment arms in the expression of T cell, CD8+ T cell, and Th-1 T-cell gene sets at baseline, but trends toward increases after HFRT in comparison with LFRT were noted (Supplementary Fig. S5). Interestingly, this trend was also observed for natural killer cells (Supplementary Fig. S5).

We also interrogated other immune populations and immune effects of radiation on these paired samples obtained from HFRT and LDFRT patients. Although there was interpatient variability, the ratio of M1/M2 macrophages in the tumor microenvironment decreased in 2 of 2 patients receiving HFRT and increased in all 3 patients treated with LDFRT (Fig. 2), suggesting an influence of radiation (RT) dose/fractionation on macrophage polarization. We also used immunofluorescence (IF) to identify colocalization of cGAS and DAPI outside of the nucleus to score micronuclei and cGAS with a corresponding nuclear DAPI defect in the nuclear rim to score primary nuclear ruptures (PNR, Fig. 3). These analyses demonstrated that 2 of 3 patients treated with LDFRT demonstrated increases in micronuclei/

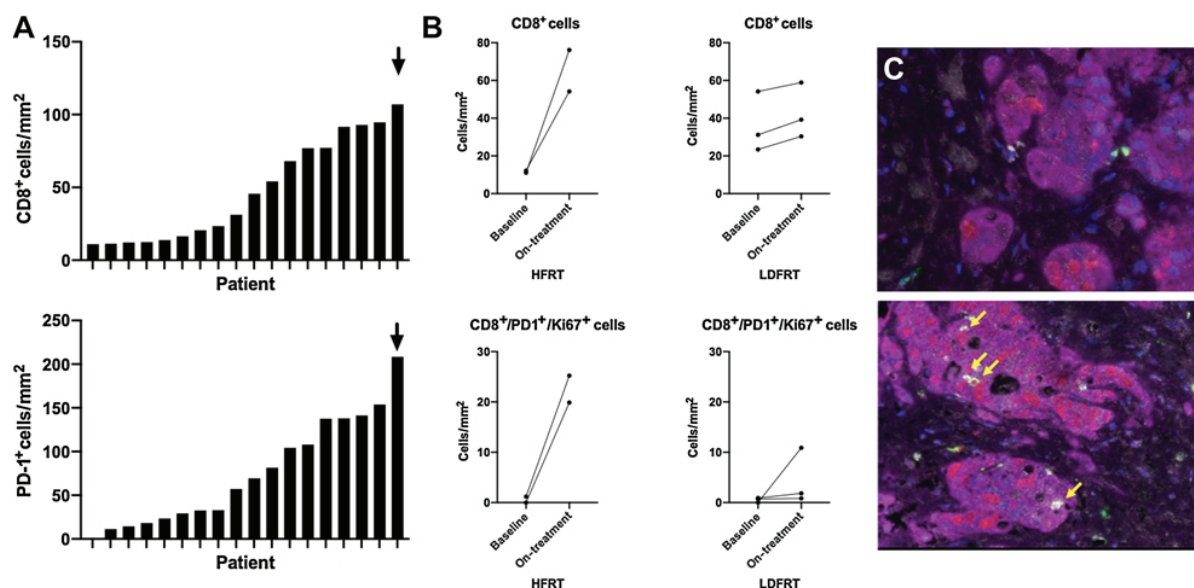


Figure 1.

T-cell populations in the tumor microenvironment. mIF evaluating expression of cyokeratin (purple), CD8 (white), PD-1 (green), and Ki67 (red) as shown on the right. **A**, Baseline variability in CD8⁺ and PD-1⁺ cell populations, with highest levels of both populations observed in the irradiated liver lesion from the subject who reported an out-of-field response. **B**, Changes in cell populations over the course of treatment in the HFRT and LDFRT arms (on-treatment samples obtained week 7–8). In **B**, for each patient, the variance at a timepoint was estimated by (number of frames × SEM²) and the variance of the difference (post-pre) was estimated by the sum of the timepoint variances. The SD of the difference was the square root of the variance. **C**, Baseline (top) and on-treatment (bottom) specimen from a patient treated with HFRT demonstrates increases in CD8⁺/PD-1⁺/Ki-67⁺ cells (yellow arrows).

PNR (3.7- and 5.5-fold increase; $P = 0.04$ and $P < 0.0001$, respectively), while numbers remained relatively stable or slightly decreased at the 7- to 8-week timepoint in the other LDFRT and 2 HDRT patients (0.76-, 0.83-, and 0.84-fold change; $P = 0.81$, $P = 0.54$, and $P = 0.68$, respectively).

Macrophage markers in the tumor microenvironment were further evaluated in RNA-seq data from paired tumor biopsy RNA-seq. Gene set variation analysis of data was evaluated for macrophage gene marker sets. In the HFRT cohorts, there was a significant increase in macrophage markers posttreatment, but no differences were observed in the LDFRT group (Supplementary Fig. S6). We additionally examined macrophage polarization genes *ARG1*, *NOS2*, *IL10*, and *CD163*. In the HFRT group, we also observed a significant decrease in *ARG1* posttreatment, but no significant differences were seen in *NOS2*, *IL10*, and *CD163* or in the LDFRT cohort.

Treatment-induced changes in systemic immune markers were also impacted by radiation dose and included declines in effector T-cell populations

Blood was collected prior to treatment and then in conjunction with cycle 2 in 19 patients. Initial analyses of overall impacts on white blood cell counts demonstrated minimal overall changes in both arms (Supplementary Table S3). There were nonsignificant declines in absolute lymphocytes counts in both arms that were more pronounced following HFRT (Fig. 4A). CyTOF was employed to further analyze specific cell populations. Results on matched PBMC samples from 12 patients revealed significant declines in multiple CD4⁺ and CD8⁺ cell populations following HFRT compared with LDFRT, including global CD3⁺CD4⁺ ($P = 0.004$), CD3⁺CD8⁺ ($P = 0.02$), and CD4⁺ and CD8⁺ T cells expressing markers of activation, including

CD8⁺CXCR3⁺ ($P = 0.003$), CD4⁺ICOS⁺ ($P = 0.02$), CD4⁺Lag3⁺ ($P = 0.03$), CD4⁺CXCR3⁺ ($P = 0.002$), CD4⁺41BB⁺ ($P = 0.003$), CD4⁺CRTH2⁺ ($P = 0.004$), CD4⁺GITR⁺ ($P = 0.03$), and CD4⁺OX40⁺ ($P = 0.003$; Fig. 4B and C). In contrast, T-cell populations generally remained stable or increased in patients who received LDFRT compared with HFRT, with increases in CD8⁺CD40L⁺ ($P = 0.002$) and decreases in TIGIT⁺ T regulatory cells (defined as CD3⁺CD19⁺CD4⁺CD8⁺CD127^{low}CD25⁺; $P = 0.0008$).

We used the Olink multiplex immune-oncology panel to interrogate changes induced by cycle 2 of treatment in 92 measured cytokines and chemokines as compared with pretreatment levels among matched samples from 13 patients. Of these, 34 demonstrated significant increases over the course of treatment in both arms (Supplementary Fig. S7). These included known mediators of antitumor immunity, such as IFN γ ($P = 0.01$), CCL20 ($P = 0.002$), CXCL-9, 10, 11 ($P = 0.001$, $P = 0.0001$, and $P = 0.030$, respectively), as well as others such as PD-L1 ($P < 0.0001$ HFRT and $P = 0.0006$ LDFRT).

Treatment was associated with differential expression of immune genes in the tumor microenvironment and peripheral blood

Next, we further examined associations between study therapy and gene expression, both within circulating PBMCs and in the tumor microenvironment, by performing RNA-seq of pre- and posttherapy samples obtained from 5 patients for whom sufficient tissue was available. When pre- to posttreatment samples were compared, there were 407 differentially expressed genes ($FDR \leq 0.05$ and fold change $\geq |2|$) in PBMCs (Fig. 5; Supplementary Table S4). Therapy-induced gene expression differences were also observed in the tumor

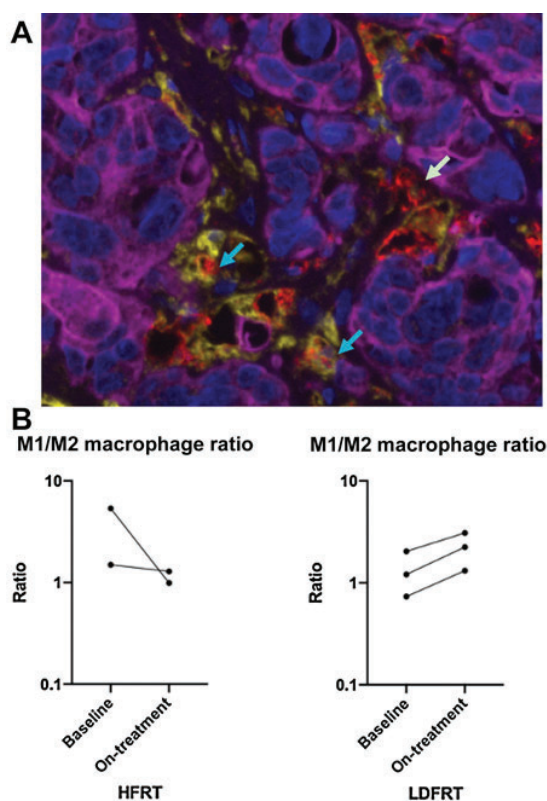


Figure 2.

Macrophage populations within the tumor microenvironment. **A**, mIF evaluating expression of cyokeratin (purple), DAPI (blue), CD68 (red), and CD163 (yellow) as shown. M1 macrophages demonstrate CD68 staining (white arrow), while M2 demonstrate CD68 and CD163 costaining (blue arrows). **B**, Changes in the ratio of M1 to M2 cell populations over the course of treatment (on-treatment samples obtained week 7–8) in the HFRT and LDFRT arms.

microenvironment with 445 differentially expressed genes (Supplementary Fig. S8; Supplementary Table S5). Principal component analysis (PCA) of tumor microenvironment gene expression data largely separated pre- and posttreatment biopsy specimens (Supplementary Fig. S8B). Examination of the four paired biopsy specimens by cluster analysis of the top 30 most variable expressed genes confirmed the results of the PCA, showing most samples clustering by pre- versus posttreatment (Supplementary Fig. S8C). We also examined the consensus molecular subtypes of these paired biopsies pre- and posttreatment (17) to ascertain whether therapy impacted the subtype. Three of four samples were CMS4 at baseline, the fourth sample could not be aligned with a subtype at baseline, but was most closely aligned with CMS4. All four samples remained CMS4 after treatment. Next, using gene ontology (GO) biological process pathway analysis, we examined the top 20 pathways statistically significantly altered by therapy (Supplementary Fig. S8D). Not surprisingly, many of these pathways reflected changes in cell metabolic processes and were likely related to gene expression changes in cancer cells and stroma. However, the third most highly significant pathway was regulation of adaptive immune response, suggesting that treatment was altering gene expression of immune pathways within the tumor microenvironment. Also, as outlined above (Supplementary Figs. S5 and S6),

gene expression analysis corroborated the changes in immune cell populations in the tumor microenvironment identified by IF. We further analyzed the paired biopsy samples on the basis of HFRT versus LDFRT treatment groups with two paired biopsies in each sample. While conclusions are limited by the small sample size, some interesting hypothesis generating findings were noted. In comparing HFRT versus LDFRT, there are 990 differentially expressed genes in the HFRT cohort and only 147 differentially expressed genes in the LDFRT, suggesting that HFRT + dual immune checkpoint inhibition (ICI) causes more gene expression changes in the tumor microenvironment than LDFRT + dual ICI. Evaluation of gene marker sets of different immune cell types in the tumor microenvironment using gene set variation analysis revealed no statistically significant therapy-induced changes in the gene marker sets for any immune cell type in the LDFRT or HFRT samples other than the changes already noted above (Supplementary Figs. S5 and S6).

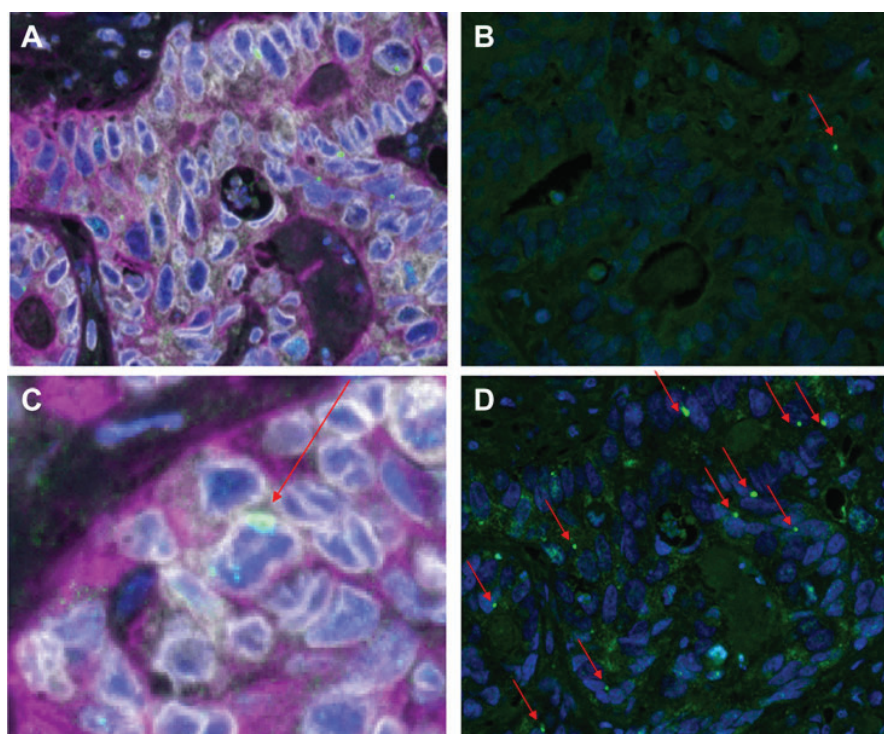
As both immune checkpoint inhibitors and radiotherapy have been reported to have their most pronounced effects on T cells, we next examined therapy-induced differences in expression of T-cell-related genes in circulating PBMCs. Hierarchical clustering based upon expression of T-cell activation genes (GO biological processes) revealed separation of pre- and post-therapy samples (Fig. 5B). Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways also identified T-cell signaling as a major pathway impacted by treatment (Fig. 5C), which was also evident by PCA of the antigen receptor-mediated signaling gene expression data (Fig. 5E). These data suggest therapy-induced differences in T-cell functionality, at least at a transcriptional level. Analysis of the individual T-cell activation genes revealed significant therapy-associated declines in gene expression (Fig. 5D). These findings are likely due to an overall posttreatment decline in T cells (Fig. 4A–C). Overall, these results corroborate the significant declines in T-cell populations observed by CyTOF analysis in Fig. 4.

Treatment was associated with alterations of the tumor-infiltrating and peripheral T-cell repertoires

As another means to evaluate treatment-associated immune alterations, we quantified the number of expanded and contracted clones in tumor and blood following therapy. CDR3 sequence analysis revealed a therapy-associated increase in the number of expanded compared with contracted T-cell clones in PBMCs. In contrast, T-cell expansions were also noted in the tumor microenvironment, but these were accompanied by a comparable number of T-cell contractions (Fig. 5G). To better visualize the T-cell clonal dynamics following therapy, we constructed galaxy plots of the PBMC and tumor microenvironment T-cell repertoires, in which the number of clones, anatomic location, and CDR3 sequence similarities were represented by the size, color, and location of the plotted circles, respectively (Fig. 5H). On these plots, expansions and contractions within the tumor and PBMCs can be visualized following treatment. Clusters within the plots are formed when unique T-cell clones have similar CDR3 TCR sequences, presumably because of shared antigen specificity. Thus, some of the new treatment-induced clonal expansions that appeared in the tumor had similar TCR sequences. The overlap between the tumor-infiltrating T-cell repertoire and the peripheral T-cell repertoire was also clearly evident, with each anatomic site having both shared and unique clones (Fig. 5H). The Shannon diversity of the T-cell repertoire pre- and posttherapy was then calculated to help characterize the treatment-associated changes. In the PBMC population, there was a significant decrease in Shannon diversity (Fig. 5I),

Figure 3.

mIF evaluating formation of micronuclei and PNRs. **A** and **C**, Samples were stained for cytokeratin (purple), DAPI (blue), cGAS (green), and Lamin B receptor (white) as shown in **A** (low power) and **C** (high power). **B**, Micronuclei and PNR(s) were scored by identifying colocalization of cGAS and DAPI outside the nucleus for micronuclei and cGAS with a DAPI defect in the nuclear rim of primary nuclei for PNRs (red arrow). Pretreatment (**B**) and posttreatment (**D**) specimens (on-treatment samples obtained week 7–8) demonstrate pronounced increase in micronuclei and foci of PNRs (red arrows) in two subjects.



which was likely due to the noted increase in T-cell expansions (Fig. 5G). Finally, pie charts were also constructed, which revealed similar treatment-induced alterations in the T-cell repertoire, with several new T-cell expansions appearing following therapy (Fig. 5J).

Discussion

In this phase II trial, we found that the PD-L1 inhibitor, durvalumab, and CTLA-4 inhibitor, tremelimumab, combined with either HFRT or LDFRT directed against 1–2 liver metastases was tolerated, with the spectrum and frequency of immune-related adverse events expected from combined PD-1/L1 and CTLA-4 blockade alone (2). No unexpected radiation-related adverse events were observed. These results demonstrate the feasibility of combining PD-L1/CTLA-4 blockade with focused liver-directed radiation for patients with metastatic colorectal cancer and other malignancy types.

Despite promising preclinical data demonstrating radiation can induce tumor-specific immune responses (18) and increased T-cell infiltration (6) and immune-mediated tumor cell death (19), clinical trials to date have demonstrated mixed results. Translation to the clinic has been hindered by uncertainty regarding the appropriate radiation dose to induce immune modulation (20). In preclinical models, a variety of different radiation dose/fractionation regimens have been demonstrated to induce IFN-stimulated genes via generation of micronuclei/PNR leading to cytosolic double-stranded DNA and activation of the cGAS/STING pathway (21–23). The dose threshold for this effect is uncertain, particularly in human tumors. This is a relevant clinical question, as higher radiation doses are not only associated with increased side effects, but may also lead to regulatory inhibition of the cGAS/STING pathway (24) and deleterious lymphopenia (25). Lower doses of radiation have also been associated with

other positive immune effects, in particular inducing a more favorable presence of M1 macrophages (6).

We did not observe objective responses outside of the radiation treatment field in either the HFRT and LDFRT arms, and median progression-free and overall survival was limited. These results are likely due, at least, in part, to the advanced nature of disease and extensive pretreatment among enrolled patients given the median number of prior lines of therapy was four (range, 1–7). In contrast to earlier, in patients with untreated colorectal cancer treated with immunotherapy alone prior to any other treatment (26), immune systems may be impaired by advanced tumor burden and prior treatment (27), and may thus be less likely to respond to combined immune checkpoint/radiation strategies. Indeed, combination radiation/immune checkpoint blockade treatment strategies may have more promise in earlier disease settings (28–31). This approach should be tested in patients with less extensive disease burden, or perhaps oligometastatic colorectal cancer disease to the liver, a setting in which all gross disease can potentially be targeted by ablative radiation treatment (31). In addition, we found relatively low PD-L1 expression across tumor specimens, with the highest expression of PD-L1 observed in the patient with evidence of an out-of-field response in a porta hepatis lymph node. Thus, this low PD-L1 expression may contribute to the lack of benefit. Overall, our results clearly demonstrated that at the selected radiation doses/schedules and in this particular clinical setting, focal radiation did not reverse resistance to anti-PD-L1/CTLA-4 in a tumor type resistant to PD(L)-1/CTLA-4-directed therapy.

We explored biomarkers, such as PD-L1 tumor expression and infiltrating CD8⁺ T cells, predictive of response to immune checkpoint blockade in other studies. In line with previous studies (1), PD-L1 expression was generally low or negative in this cohort of MSS

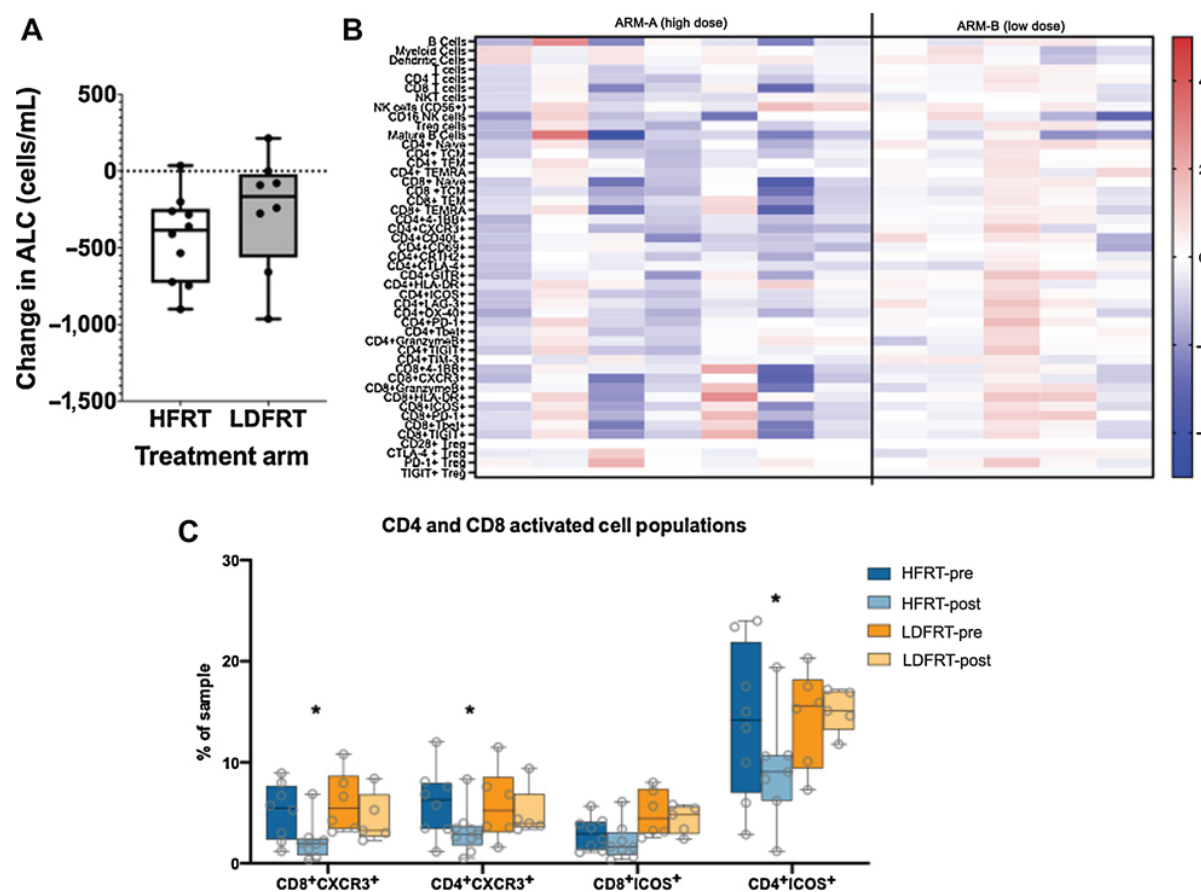


Figure 4.

Changes in circulating biomarkers between pretreatment and week 5 of treatment. **A**, Absolute lymphocyte count (ALC, cells/mL). Median, interquartile range, and maximum changes are plotted for both treatment arms. **B** and **C**, Fold change in cell populations over the course of treatment. Heatmap shows $\log_2$ fold changes for each patient. Significant changes ($P < 0.05$) are denoted with *.

colorectal cancer, and CD8⁺ T-cell infiltration was variable. We did observe the highest levels of PD-L1 expression, CD8⁺ T-cell infiltration, and PD-1⁺ cell infiltration in the pretreatment irradiated liver lesion of 1 patient with stable disease on study and a notable out-of-field response. Of interest, this patient had targeted next-generation tumor sequencing that revealed a POLE mutation of questionable functional significance [c.1784A>G (p.N595S), exon 16 in 91% of 343 reads]. POLE mutations have been associated with higher mutational burden in MSS colorectal cancers and may predispose for response to immune checkpoint blockade (32). Interestingly, we also observed a nonsignificant trend toward greater CD4⁺ and CD8⁺ T-cell infiltration in patients who previously received radiation treatment, but these comparisons were limited by small numbers in the prior radiation group.

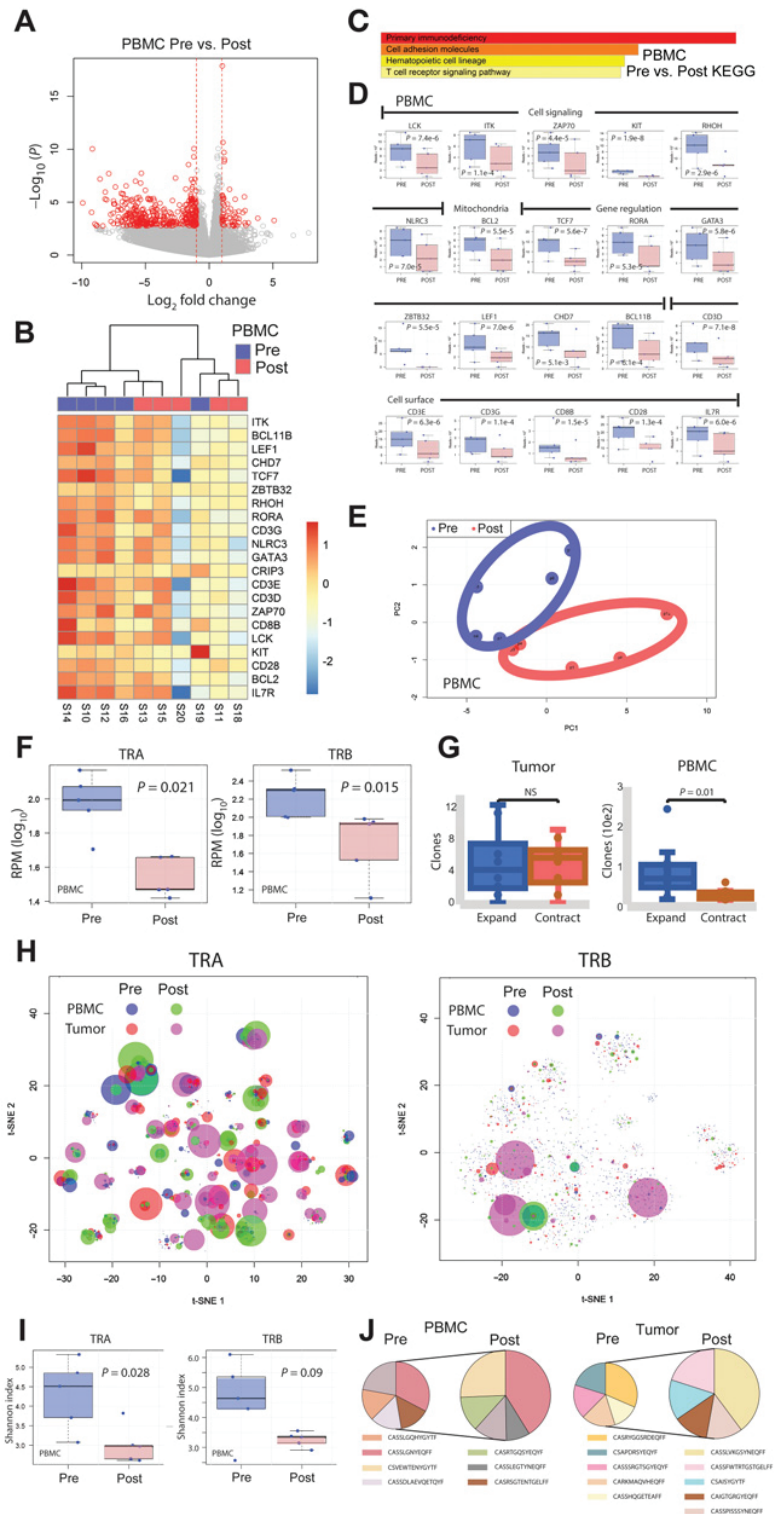
Despite the lack of systemic objective responses, correlative studies performed on matched tumor biopsies taken before and after therapy suggest that this combination therapy alters the tumor immune microenvironment. We observed increases in CD8⁺ and proliferating CD8⁺/PD1⁺/Ki-67⁺ T cells after HFRT in comparison with LDFRT. Gene expression analyses supported this finding. Higher dose radiation has been associated with immunogenic cell death with increased cell surface calreticulin and increased HMGB1 and ATP release (19)

that might be associated with greater levels of immune activation and increased T-cell infiltration over time.

Immunologic impacts of LDFRT in human tumors were more uncertain given these regimens are not standardly used in clinical practice and less likely to result in tumor cell death in the absence of systemic therapy. Within the local tumor microenvironment, we observed modest increases in CD8⁺ T-cell infiltration and variable changes in the CD8⁺/PD1⁺/Ki-67⁺ subset following LDFRT. However, consistent with preclinical data (6), LDFRT led to increases in the M1/M2 macrophage ratio in all 3 LDFRT patients as compared with none of the HFRT patients. In the HFRT patients, the M1/M2 macrophage ratio actually decreased, which was also consistent with preclinical reports that demonstrate an increase in M2 macrophages after RT at higher doses (pmid: 24992164 and 26946344). Gene expression data also showed an increase of macrophage gene marker sets posttherapy in the HFRT patients, but clear conclusions could not be drawn regarding expression changes of macrophage polarization genes. Drawing conclusions from these samples is difficult given the limited sample size and that RNA-seq was bulk RNA-seq that can limit detecting changes in cell types which are scarce in comparison with tumor and stromal cells. Intriguingly, we also observed pronounced increases in micronuclei/PNR with LDFRT. Previous studies have

Figure 5.

Immunotherapy-induced alterations of the T-cell repertoire. RNA-seq was performed on PBMCs before and after initiation of immunotherapy. **A**, Volcano plot of resulting gene expression data reveals numerous differentially expressed genes (outlined in red) post-therapy. Calculated Euclidean distances between T-cell activation genes (GO biological processes) were used to perform complete linkage clustering centered on $\log_2$ -transformed data. **B**, The resulting gene expression heatmap highlights gene expression differences in T-cell activation genes. **C**, KEGG pathway analysis reveals significant alterations in immune pathways following initiation of therapy, including the TCR signaling pathway. **D**, Expressions of T-cell activation genes (GO biological processes) are presented as box-and-whisker plots, where the top and bottom bars connected to each box indicate the boundaries of the normal distribution and the box edges mark the first and third quartile boundaries within each distribution. The dark horizontal line represents the median. Paired analysis P values were calculated using DESeq2. **E**, PCA of antigen receptor-mediated signaling gene expression data completely separates pre- and posttreatment samples. **F**, TCR reads per million ($\log_{10}$) reveal a decline in T cells after treatment. TCRminer was used to extract TCR reads from RNA-seq data, and P values were calculated using paired Student t test. **G**, MiXCR was used to identify unique complementarity-determining region 3 sequences from RNA-seq data, and the numbers of expanded and contracted clones per sample were graphed for pre- and posttreatment samples. **H**, To visualize how similar T-cell clones were to one another with respect to their CDR3 sequences, the number of individual clones present in a particular sample, the copy number of each clone, the expansion and contractions following therapy, and the overlap between the T-cell repertoire of the peripheral blood and the tumor-infiltrating cells, T-cell repertoire galaxy plots were constructed using a modified dimensionality reduction strategy (t-SNE). Separate plots were constructed for TCR alpha and TCR beta (TRB) chains. In these plots, the size of the circle represents the CDR3 copy number. The location of the circle represents the CDR3 sequence. Specifically, circles that are located far from one another have dissimilar CDR3 sequences, whereas circles that are located close to one another have similar CDR3 sequences. Circles that share a center point have identical CDR3 sequences. Finally, the color of the circle or datapoint represents the sample (blue, PBMC pretreatment; green, PBMC post-treatment; red, tumor pretreatment; and purple, tumor posttreatment). **I**, The Shannon diversity index was calculated for the peripheral blood T-cell repertoire pre- and posttreatment. **J**, As another way to visualize the alterations in the T-cell repertoire before and after treatment, pie charts were constructed. Different colors represent unique CDR3 beta sequences. The size of each colored wedge represents the copy number for that particular CDR3 sequence.



highlighted the importance of these structures in leading to cGAS/STING activation following radiation and subsequent immune response (22, 23). The ability of low-dose radiation to generate micronuclei/PNR in combination with immune checkpoint blockade in human tumors is intriguing and could be explored in future studies. The failure to observe micronuclei/PNR after HFRT may have been a result of the 7- to 8-week timepoint that was evaluated, as cells with multiple abnormal nuclear structures, including multiple micronuclei, chromosome bridges, and PNR, may have been selected against over time because of limited proliferation capacity.

Evaluation of circulating biomarkers demonstrated that both treatment regimens led to systemic immunologic changes. Olink serum profiling demonstrated that in both radiation immunotherapy arms there was evidence of increased IFN γ and related chemokines, such as CXCL-9, 10, and 11. We observed posttreatment changes in circulating PD-L1, and these were likely due to the ligand being stabilized as a result of durvalumab treatment. TCR sequencing identified some specific T-cell clones expanded in the tumor microenvironment, as well as the peripheral circulation, as has been observed following radiation and immune checkpoint blockade in a prior study evaluating the combination of ipilimumab and HFRT in non-small cell lung cancer (29). Both treatment regimens led to nonsignificant overall declines in absolute lymphocyte count that was more pronounced in patients that received HFRT, as would be expected from prior studies demonstrating the potential lymphopenia that can be induced by higher dose radiation (25). More unexpected were significant declines observed in multiple circulating activated T-cell subsets in patients treated with HFRT as compared with LDFRT, again supported by gene expression data. It is possible that these declines in circulating effector T-cell populations limited systemic response despite local immune activation. Interestingly, LDFRT led to declines in specific circulating populations of regulatory T cells.

Limitations of our study include the choice of two specific HFRT and LDFRT regimens and timing in relation to immune checkpoint blockade, and the decision to irradiate 1–2 metastatic liver lesions as opposed to more lesions or metastases elsewhere in the body. These aspects of our study design helped maintain homogeneity, but it is possible that different radiation timing or parameters may be more conducive to generating an effective systemic immune response. In terms of correlative data, limited numbers of study patients had matched pre- and post-biopsy tissue available for study, in part, because several patients had no viable tumor at the irradiated site at the time of repeat biopsy, making it difficult to discern local treatment effects. Therefore, the immune changes we observed following immune checkpoint blockade and both HFRT and LDFRT are exploratory, although the formation of micronuclei/PNR, increases in CD8⁺ T-cell subsets after HFRT, and increase in M1/M2 macrophage ratios are all concordant with preclinical data.

In summary, although the combination of HFRT and LDFRT did not lead to any unexpected toxicities, we did not observe objective responses outside the radiation treatment field, suggesting irradiating 1–2 lesions at these radiation dose/fractionations in combination with immune checkpoint blockade is not sufficient to mediate systemic antitumor immunity in patients with refractory colorectal cancer. We did observe local immunologic changes following both HFRT and LDFRT, although the nature and extent of changes appeared different between the two radiation schedules. Our biomarker findings generated interesting hypotheses for further testing. Future efforts should focus on increasing the positive immunologic impacts of focused radiotherapy, perhaps by irradiating more lesions, such as in the case

of oligometastatic or oligoprogressive disease (31), or perhaps by investigating LDFRT in earlier disease settings, while also attempting to minimize any potential detrimental immune effects of irradiation, such as radiation-induced declines in circulating activated T-cell populations.

Authors' Disclosures

A.M. Monjazez reports grants from NIH during the conduct of the study, as well as grants, personal fees, and nonfinancial support from Merck; grants and personal fees from BMS and Incyte; personal fees from AstraZeneca and Zosano; personal fees and nonfinancial support from Dynavax; grants from Genentech and EMD Serono; grants and nonfinancial support from Transgene; and other from Multiplex Thera outside the submitted work. A. Lako reports personal fees from Bristol Myers Squibb outside the submitted work. E.M. Thrash reports personal fees from Fluidigm Corporation during the conduct of the study and employment with Fluidigm Corporation. R.D. Gentzler reports grants and nonfinancial support from NCI during the conduct of the study, as well as grants and personal fees from Pfizer; personal fees from AstraZeneca, Blueprint Medicines, Rockpointe CME, Targeted Oncology, and OncLive; grants from Jounce Therapeutics, Takeda, Bristol Myers Squibb, Merck, Mirati, and Daiichi Sankyo; and grants and other from Helsinn Therapeutics outside the submitted work. S.K. Jabbour reports grants, personal fees, and nonfinancial support from Merck & Co; grants from NCI; and personal fees from IMX Medical and Syntactx during the conduct of the study, as well as grants from NCI outside the submitted work. O.B. Alese reports grants from NCI during the conduct of the study, other from AstraZeneca, and grants from Bristol Myers Squibb and GlaxoSmithKline outside the submitted work. O.E. Rahma reports personal fees from Sobi, Genentech, Bayer, GlaxoSmithKline, Imvax, Puretech, Maverick Therapeutics, Five Prime, and Merck outside the submitted work, as well as a patent for methods of using pembrolizumab and trebananib pending. J.M. Cleary reports grants from Merck and Tesaro, nonfinancial support from AstraZeneca and Esperas Pharma, and personal fees from BMS outside the submitted work. H.J. Mamon reports grants from Dana Farber Cancer Institute during the conduct of the study, as well as personal fees from Merck and other from UpToDate outside the submitted work. M. Cho reports personal fees from AstraZeneca outside the submitted work. S. Gnjjatic reports grants from NCI during the conduct of the study, as well as grants from Genentech, Regeneron, Bristol-Myers Squibb, Janssen R&D, Takeda, and Immune Design and personal fees from OncoMed and Merck outside the submitted work. A. Spektor reports grants from NCI and Burroughs-Wellcome Fund and personal fees from Janssen Pharmaceutical, Bayer Pharmaceuticals, and Astellas Pharma outside the submitted work. S.J. Rodig reports research funding from Merck, Bristol-Myers-Squibb, Affimed, and KITE/Gilead for research unrelated to this project. F.S. Hodi reports grants from NIH during the conduct of the study, as well as grants from Sanofi; personal fees from Merck, EMD Serono, Apricity, Aduro, Pionyr, Checkpoint, Surface, Compass, Torque, Rheos, Bicara, Psioxus, Genentech, Takeda, Eisai, Iovance, Bioentre, and Idera; and grants and personal fees from Novartis outside the submitted work; in addition, F.S. Hodi has patents for MICA-related disorders pending, licensed, and with royalties paid, tumor antigens and uses thereof issued, angiopoietin-2 biomarkers predictive of anti-immune checkpoint response pending, compositions and methods for identification, assessment, prevention, and treatment of melanoma using PD-L1 isoforms pending, therapeutic peptides pending, vaccine compositions and methods for restoring NKG2D pathway function against cancers pending, licensed, and with royalties paid, antibodies that bind to MHC class I polypeptide-related sequence A pending, licensed, and with royalties paid, and anti-galectin antibody biomarkers predictive of anti-immune checkpoint and anti-angiogenesis responses pending. J.D. Schoenfeld reports grants from NCI during the conduct of the study, as well as grants from Merck, Regeneron, and BMS; grants and personal fees from Debiopharm; personal fees from ACI, Kline & Spector PC, Heidell, Pittoni, Murphy and Bach, Catenion, LEK, TILOS, Astellas, STIMIT, and Pearson Doyle Mohre & Pastis; and personal fees and other from Immunitas outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

A.M. Monjazez: Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, writing—original draft, writing—review and editing. A. Giobbie-Hurder: Conceptualization, formal analysis, validation, visualization, writing—review and editing. A. Lako: Investigation, visualization, methodology, writing—review and editing. E.M. Thrash: Formal analysis, supervision, investigation, visualization, methodology, writing—review and editing. R.C. Brennick: Formal analysis, investigation, writing—review and editing.

K.Z. Kao: Investigation, methodology. **C. Manuszak:** Investigation, methodology. **R.D. Gentzler:** Investigation, writing–review and editing. **A. Tesfaye:** Investigation, writing–review and editing. **S.K. Jabbour:** Investigation, writing–review and editing. **O.B. Alese:** Investigation, writing–review and editing. **O.E. Rahma:** Investigation, writing–review and editing. **J.M. Cleary:** Conceptualization, investigation, writing–review and editing. **E. Sharon:** Conceptualization, funding acquisition, writing–review and editing. **H.J. Mamon:** Conceptualization, investigation, writing–review and editing. **M. Cho:** Formal analysis, investigation, writing–review and editing. **H. Streicher:** Conceptualization, data curation, writing–review and editing. **H.X. Chen:** Conceptualization, resources, supervision, funding acquisition, methodology, writing–review and editing. **M.M. Ahmed:** Conceptualization, resources, supervision, writing–review and editing. **A. Mariño-Enriquez:** Formal analysis, investigation, writing–review and editing. **S. Kim-Schulze:** Methodology, writing–review and editing. **S. Gnatic:** Investigation, methodology, writing–review and editing. **E. Maverakis:** Formal analysis, investigation, writing–review and editing. **A.I. Marusina:** Formal analysis, writing–review and editing. **A.A. Merleev:** Formal analysis, writing–review and editing. **M. Severgnini:** Resources, supervision, investigation, visualization, methodology, writing–review and editing. **K.L. Pfaff:** Resources, supervision, investigation, visualization, writing–review and editing. **J. Lindsay:** Data curation, formal analysis, writing–review and editing. **J.L. Weirather:** Formal analysis, visualization, methodology, writing–review and editing. **S. Ranasinghe:** Resources, data curation, supervision, funding acquisition, visualization, methodology, writing–review and editing. **A. Spektor:** Conceptualization, investigation, methodology. **S.J. Rodig:** Conceptualization, resources, supervision, writing–review and editing. **F.S. Hodi:** Conceptualization, supervision, funding acquisition, methodology, writing–review and editing. **J.D. Schoenfeld:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing.

Acknowledgments

The trial was conducted under auspices of the Experimental Therapeutics Clinical Trials Network and funded by UM1 CA186709 (to principal investigator, Geoffrey Shapiro), a Biomarker Supplement to UM1 CA186709 (to project leaders, J.D. Schoenfeld, S.J. Rodig, and F.S. Hodi), and Center for Immunology, Dana-Farber Cancer Institute. Scientific and financial support for the CIMAC-CIDC Network was provided through the NCI Cooperative Agreements U24CA224319 (to the Icahn School of Medicine at Mount Sinai CIMAC), U24CA224331 (to the Dana-Farber Cancer Institute CIMAC), and U24CA224316 (to the CIDC at Dana-Farber Cancer Institute). Additional support was made possible through the NCI CTIMS Contract HHSN261201600002C. Scientific and financial support for the PACT project was made possible through funding support provided to the FNHI by AbbVie Inc., Amgen Inc., Boehringer-Ingelheim Pharma GmbH & Co. KG., Bristol-Myers Squibb, Celgene Corporation, Genentech Inc, Gilead, GlaxoSmithKline plc, Janssen Pharmaceutical Companies of Johnson & Johnson, Novartis Institutes for Biomedical Research, Pfizer Inc., and Sanofi. The CIMAC-CIDC website is found at <https://cimac-network.org/>. We acknowledge Janice Russell and all participating patients and sites. We would also like to thank John Daley and his team at the Longwood Medical Area CyTOF Core at DFCI for their technical assistance.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 30, 2020; revised January 14, 2021; accepted February 5, 2021; published first February 10, 2021.

References

- Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 2015;372:2509–20.
- Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol* 2017;18:1182–91.
- Ngwa W, Irabor OC, Schoenfeld JD, Hesser J, Demaria S, Formenti SC. Using immunotherapy to boost the abscopal effect. *Nat Rev Cancer* 2018;18:313–22.
- Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature* 2015;520:373–7.
- Dewan MZ, Galloway AE, Kawashima N, Dewyngraert JK, Babb JS, Formenti SC, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res* 2009;15:5379–88.
- Klug F, Prakash H, Huber PE, Seibel T, Bender N, Halama N, et al. Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T cell immunotherapy. *Cancer Cell* 2013;24:589–602.
- Arnold SM, Regine WF, Ahmed MM, Valentino J, Spring P, Kudrimoti M, et al. Low-dose fractionated radiation as a chemopotentiator of neoadjuvant paclitaxel and carboplatin for locally advanced squamous cell carcinoma of the head and neck: results of a new treatment paradigm. *Int J Radiat Oncol Biol Phys* 2004;58:1411–7.
- Reiss KA, Herman JM, Zahurak M, Brade A, Dawson LA, Scardina A, et al. A phase I study of veliparib (ABT-888) in combination with low-dose fractionated whole abdominal radiation therapy in patients with advanced solid malignancies and peritoneal carcinomatosis. *Clin Cancer Res* 2015;21:68–76.
- Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45:228–47.
- National Cancer Institute. Division of Cancer Treatment & Diagnosis. Cancer therapy evaluation program. Available from: https://ctep.cancer.gov/protocoldeveloment/electronic_applications/ctc.htm#ctc_40.
- Griffin GK, Weirather JL, Roemer M, Lipschitz M, Kelley A, Chen PH, et al. Spatial signatures identify immune escape via PD-1 as a defining feature of T-cell/histiocyte-rich large B-cell lymphoma. *Blood* 2020 Sep 1 [Epub ahead of print].
- Patel SS, Weirather JL, Lipschitz M, Lako A, Chen PH, Griffin GK, et al. The microenvironmental niche in classic Hodgkin lymphoma is enriched for CTLA-4-positive T cells that are PD-1-negative. *Blood* 2019;134:2059–69.
- Carey CD, Gusenleitner D, Lipschitz M, Roemer MGM, Stack EC, Gjini E, et al. Topological analysis reveals a PD-L1-associated microenvironmental niche for Reed-Sternberg cells in Hodgkin lymphoma. *Blood* 2017;130:2420–30.
- Kleinsteuber K, Corleis B, Rashidi N, Nchinda N, Lisanti A, Cho JL, et al. Standardization and quality control for high-dimensional mass cytometry studies of human samples. *Cytometry A* 2016;89:903–13.
- Thrash EM, Kleinsteuber K, Hathaway ES, Nazzaro M, Haas E, Hodi FS, et al. High-throughput mass cytometry staining for immunophenotyping clinical samples. *STAR Protoc* 2020;100055.
- Dillen CA, Pinsker BL, Marusina AI, Merleev AA, Farber ON, Liu H, et al. Clonally expanded gammadelta T cells protect against *Staphylococcus aureus* skin reinfection. *J Clin Invest* 2018;128:1026–42.
- Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015;21:1350–6.
- Sharabi AB, Nirschl CJ, Kochel CM, Nirschl TR, Francica BJ, Velarde E, et al. Stereotactic radiation therapy augments antigen-specific PD-1-mediated anti-tumor immune responses via cross-presentation of tumor antigen. *Cancer Immunol Res* 2015;3:345–55.
- Golden EB, Frances D, Pellicciotta I, Demaria S, Helen Barcellos-Hoff M, Formenti SC. Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. *Oncoimmunology* 2014;3:e28518.
- Monjazeb AM, Schoenfeld JD. Radiation dose and checkpoint blockade immunotherapy: unanswered questions. *Lancet Oncol* 2016;17:e3–4.
- Deng L, Liang H, Xu M, Yang X, Burnette B, Arina A, et al. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity* 2014;41:843–52.

22. Harding SM, Benci JL, Irianto J, Discher DE, Minn AJ, Greenberg RA. Mitotic progression following DNA damage enables pattern recognition within micronuclei. *Nature* 2017;548:466–70.
23. Mackenzie KJ, Carroll P, Martin CA, Murina O, Fluteau A, Simpson DJ, et al. cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* 2017;548:461–5.
24. Vanpouille-Box C, Alard A, Aryankalayil MJ, Sarfraz Y, Diamond JM, Schneider RJ, et al. DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity. *Nat Commun* 2017;8:15618.
25. Pike LRG, Bang A, Mahal BA, Taylor A, Krishnan M, Spector A, et al. The impact of radiation therapy on lymphocyte count and survival in metastatic cancer patients receiving PD-1 immune checkpoint inhibitors. *Int J Radiat Oncol Biol Phys* 2019;103:142–51.
26. Chalabi M, Fanchi LF, Dijkstra KK, Van den Berg JG, Aalbers AG, Sikorska K, et al. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nat Med* 2020;26:566–76.
27. Huang AC, Postow MA, Orlowski RJ, Mick R, Bengsch B, Manne S, et al. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature* 2017;545:60–5.
28. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, et al. Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. *N Engl J Med* 2018;379:2342–50.
29. Formenti SC, Rudqvist NP, Golden E, Cooper B, Wennerberg E, Lhuillier C, et al. Radiotherapy induces responses of lung cancer to CTLA-4 blockade. *Nat Med* 2018;24:1845–51.
30. Theelen W, Peulen HMU, Lalezari F, van der Noort V, de Vries JF, Aerts J, et al. Effect of pembrolizumab after stereotactic body radiotherapy vs pembrolizumab alone on tumor response in patients with advanced non-small cell lung cancer: results of the PEMBRO-RT phase 2 randomized clinical trial. *JAMA Oncol* 2019;5:1276–82.
31. Bauml JM, Mick R, Ciunci C, Aggarwal C, Davis C, Evans T, et al. Pembrolizumab after completion of locally ablative therapy for oligo-metastatic non-small cell lung cancer: a phase 2 trial. *JAMA Oncol* 2019;5:1283–90.
32. Wang F, Zhao Q, Wang YN, Jin Y, He MM, Liu ZX, et al. Evaluation of POLE and POLD1 mutations as biomarkers for immunotherapy outcomes across multiple cancer types. *JAMA Oncol* 2019;5:1504–6.