Ablative radiation alone in stage I lung cancer produces an adaptive systemic immune response: insights from a prospective study

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ABSTRACT
Stereotactic ablative body radiation (SABR) delivers high rates of local control in early-stage non-small cell lung cancer (NSCLC); however, systemic immune effects are poorly understood. Here, we evaluate the early pathologic and immunologic effects of SABR. Blood/core-needle tumor biopsies were collected from six patients with stage I NSCLC before and 5–7 days after SABR (48 Gy/4 or 50 Gy/5 fractions). Serial blood was collected up to 1-year post-SABR. We used immunohistochemistry to evaluate pathological changes, immune-cell populations (CD8, FoxP3), and PD-L1/PD-1 expression within the tumor. We evaluated T-cell receptor (TCR) profile changes in the tumor using TCR sequencing. We used the MANAFEST (Mutation-Associated Neoantigen Functional Expansion of Specific T-cells) assay to detect peripheral neoantigen-specific T-cell responses and dynamics. At a median follow-up of 40 months, 83% of patients (n=5) were alive without tumor progression. Early post-SABR biopsies showed viable tumor and similar distribution of immune-cell populations as compared with baseline samples. Core-needle samples proved insufficient to detect population-level TCR-repertoire changes. Functionally, neoantigen-specific T-cells were detected in the blood prior to SABR. A subset of these patients had a transient increase in the frequency of neoantigen-specific T-cells between 1 week and 3–6 months after SABR. SABR alone could induce a delayed, transient neoantigen-specific T-cell immunologic response in patients with stage I NSCLC.

INTRODUCTION
Efforts to improve the effects of immune-checkpoint inhibitors (ICIs) with the addition of radiotherapy (RT) are ongoing among patients with non-small cell lung cancer (NSCLC). In select subgroups of patients with advanced NSCLC, the addition of stereotactic ablative body radiotherapy (SABR) to systemic therapy has shown promise in improving overall (OS) and progression-free survival (PFS). In seminal phase II trials, patients with oligo-metastatic NSCLC treated with SABR after receipt of first-line systemic therapy had an OS benefit compared with no SABR. SABR also improved PFS for patients with advanced NSCLC, who develop isolated areas of progressive disease on systemic therapy. In a study of patients with chemoradiation ICI-naïve advanced NSCLC, SABR-ICI provided no additive response to ICI alone, but generated a significant PFS benefit for patients with program-death ligand 1 (PD-L1) negative tumors, suggesting that SABR may convert an immunologically ‘cold’ tumor back to a more ICI-sensitive phenotype. Understanding the immunologic effects of SABR alone may provide opportunities for pairing with ICIs.

The effects of SABR alone on T-cell response in the tumor and the peripheral blood is limited, especially in patients with stage I NSCLC due to the hurdles of obtaining post-SABR biopsies. Preclinical studies suggest that SABR directly kills tumor cells, enhances tumor killing via vascular damage, increases immune stimulating cytokines, and provides tumor antigen immunogenic priming.

Here, we report a pilot translational study focused on examining immunologic changes induced by SABR alone in stage I tumors. Within the tumor, we aimed to describe the early pathologic changes and influx of key tumor infiltrating lymphocytes (TILs) in the tumor. Systemically, we evaluated if SABR induces peripheral activation of neoantigen-specific T-cells subsequent to ablative tumor antigen release that may potentiate downstream immunogenic cell death.
METHODS

Trial eligibility and study design
Trial eligibility included age ≥18 years, AJCC 8 stage I NSCLC (T1a-T2a N0 M0, ≤4 cm), with sufficient samples for tissue analyses. Patients gave informed consent to participate in this study before taking part. Patients received core-needle tumor biopsies for diagnosis and study sample collection. Pathologic mediastinal staging was performed at the discretion of the treating provider. Patients could not use oral steroids within 14 days before SABR, up to the time of the post-SABR biopsies.

Baseline blood (120 mL) was obtained for peripheral blood mononuclear cells (PBMCs) and plasma isolation. Patients received SABR, 48 Gy in four fractions or 50 Gy in five fractions. Prior to the second fraction, 20 mL whole blood was obtained. Post-SABR study tumor and 100 mL whole blood were obtained between 5 and 7 days after SABR completion. Blood (20–120 mL) was obtained 3, 6, 9, and 12 months in follow-up after SABR (online supplemental figure S1A). Additional peripheral blood was obtained 1 month after SABR for patients who gave additional consent.

Biospecimen acquisition and cryopreservation
Whole blood was collected in EDTA vacutainer tubes. PBMCs were isolated from whole blood via Ficoll gradient centrifugation and viably cryopreserved at –140°C. Tumor biopsies were also collected prior to SABR and within 1 week after SABR. Six core research biopsies were obtained, partitioning half of the samples to be submersed in 10% neutral buffered formalin to be formalin fixed or to be flash frozen. Formalin-fixed paraffin-embedded (FFPE) cores were sectioned, retaining a portion of fixed samples for whole exome sequencing (WES), pathologic assessment, and TCR sequencing (TCRseq).

Pathologic assessment
Reserved FFPE paired pre-SABR and post-SABR slides were used for pathologic assessment. Standard 4 μm H&E stained sections were evaluated histologically by a thoracic pathologist (PBI) for tumor content and background histologic changes. The following immunohistochemical stains were used: CD8, transcription factor forkhead box protein 3 (FoxP3), PD-L1, and programmed cell death protein 1 (PD-1). CD8 is a marker of cytotoxic T-cells, while FoxP3 is a transcription factor expressed in regulatory CD4+T-cells. PD-L1 is a key checkpoint-related cell surface marker, and PD-1 is a checkpoint protein on cell surface of B and T-cells that downregulates the immune system and promotes self-tolerance by suppressing the T-cell functions. All immunohistochemistry was performed on an automated stainer (Ventana Benchmark Ultra, Ventana Medical Systems, Tucson, Arizona, USA) using standard staining protocols validated for clinical use. A list of antibodies and detection methods are shown in online supplemental table S1A.

PD-L1 expression was assessed in viable tumor cells and the results were expressed as Tumor Proportion Score (TPS; percent of tumor cells that exhibit at least partial membranous staining of any intensity). Online supplemental table S1B describes the semiquantitative scoring system used for evaluating CD8, PD-1 and FOXP3 stains.

Whole exome sequencing
WES was performed on pretreatment tumor and matched normal DNA samples as previously described. Following microdissection, DNA was extracted from tumor tissue and matched peripheral blood using the Qiagen DNA FFPE and DNA blood mini kit, respectively (Qiagen, California, USA). Fragmented genomic DNA from tumor and normal samples used for Illumina TruSeq library construction (Illumina, San Diego, California, USA) and exonic and exonic regions were captured in solution using the Agilent SureSelect V.4 kit (Agilent, Santa Clara, California, USA). Paired-end sequencing (100 bp) was performed on Illumina HiSeq 2500 (Illumina). Somatic mutations were identified using the VariantDx custom software as previously described. For neoantigen prediction, exome data combined with each individual patient’s major histocompatibility complex (MHC) class I haplotype were applied in a neoantigen prediction platform as previously described (ImmunoSelect-R, Personal Genome Diagnostics, Baltimore, Maryland, USA).

T-cell stimulation with putative neoantigens
Sufficient tumor samples for WES and peripheral blood are both required to assess for potential antitumor neoantigen-specific T-cell response detected systemically in the peripheral blood. Peripheral blood T-cells were cultured with putative neoantigens as part of the MANAFEST (Mutation-Associated Neoantigen Functional Expansion of Specific T-cells) assay, as described previously with minor modifications. From each patient, 28–60 putative neoantigens were selected based on predicted MHC class I affinity and expression in the relevant tumor type and were synthesized (JPT Peptide Technologies). Peptides were combined into pools of 3–5 peptides per pool for a total of 9–20 pools per patient. T-cells were isolated from PBMC by negative selection (EasySep; STEMCELL Technologies) on day 0. The T-cell negative fraction was co-cultured with an equal number of T-cells in culture medium (IMDM/5% human serum with 50 μg/mL gentamycin) with 10 μg/mL of relevant neoantigen peptide pools. CEFX Ultra Superstim consisting of pools of CMV, EBV, and influenza MHC-I-restricted epitopes (CEF; JPT Peptide Technologies) at 10 μg/mL was used as a positive control, HIV-1 Gag ultra-peptide pool (JPT Peptide Technologies) as a negative control, or no peptide (to use as a reference for non-specific or background clonotypic expansion).

On days 3 and 7, half the medium was replaced with fresh medium having cytokines for a final concentration of 50 IU/mL IL-2 (Chiron), 25 ng/mL IL-7 (Miltenyi) and 25 ng/mL IL-15 (PeproTech) except for IL-2 concentration, which was 100 IU/mL on day 7. Cells were harvested on day 10, and CD8+ T-cells were isolated using a CD8+
negative enrichment kit (EasySep; STEMCELL Technologies). DNA was extracted from harvested CD8+ cells using the Qiamp micro-DNA kit according to the manufacturer’s instructions.

T-cell receptor (TCR) sequencing and identification of neoantigen-specific TCRs

TCRseq of extracted DNA from cultured CD8+ cells or serial PBMC was performed by the Johns Hopkins Fest and TCR Immunogenomics Core Facility (FTIC) using the Ampliseq for Illumina TCR short read assay. Data preprocessing was performed to eliminate non-productive TCR sequences and to align and trim the nucleotide sequences to obtain only the complementarity-determining region 3 (CDR3). Sequences not beginning with C or ending with F or W and having less than seven amino acids in the CDR3 were eliminated.

To identify neoantigen-specific clonotypes, processed data files from cultured CD8+ T cells were uploaded to our publicly available MANAFEST analysis web app (http://www.stat-apps.onc.jhmi.edu/FEST) for bioinformatic analysis. Clones were considered positive if they met the following criteria: (1) significant expansion (Fisher’s exact test with Benjamini–Hochberg correction for false discovery rate (FDR), p<0.05) compared with T-cells cultured with no peptide, (2) significant expansion compared with every other peptide-stimulated culture (FDR<0.05), (3) have an OR >5 compared with the no peptide control, and (4) present in at least 10% of the cultured wells to ensure adequate distribution among culture wells.

Statistical analysis

We performed descriptive statistical analysis for the clinical data. Differential abundance of TCR clonotypes at longitudinal peripheral blood timepoints was determined using Fisher’s exact test on TCR files down-sampled to the lowest library size within each patient.

RESULTS

Patient and treatment characteristics

Six patients with stage I NSCLC treated with SABR were enrolled. Median age was 75 years (range 66–83 years). All patients were former smokers (median 48 pack-year history, range 10–80). The majority of patients (n=4) had lung adenocarcinomas. Median tumor size by short axis was 1.6 cm (range 1 cm –2.1 cm, online supplemental table S1C). Post-SABR biopsy was tolerated well in all patients. At a median follow-up of 40 months (range 12–51 months), the majority of patients (n=5, 83%) had tumor control after SABR alone. One patient (ci1425) developed locoregional recurrence 13 months after completion of SABR, received only 2 cycles of chemoimmunotherapy due to declining performance status, and died from lung cancer 24 months after SABR ended (table 1). No patients developed grade 2+ toxicity.
The early pathologic impact of SABR on the tumor

Four patients had paired pre-SABR and post-SABR biopsies with tumor detected on the representative H&E stained sections. Cellular fibrotic stroma with variable number of inflammatory cells are highlighted by CD8, FOXP3 and PD-1 immunostains. The PD-L1 immunostain shows weak-to-moderate at least partial membranous labeling in 20%–30% of the tumor cells. (B) Neoantigen-specific T cell responses and peripheral clonal dynamics in patient ci1371. (i) MANAFEST was performed on peripheral blood from patient ci1371 to identify neoantigen-specific T cell receptor clonotypes. Data are shown as the frequency of each antigen-specific TCR after 10 days of culture with the positive peptide (solid bar), and in every other condition that was tested (transparent bars). Empty bars indicate that the clone was not detected in that culture condition. (ii) TCR sequencing was performed on peripheral blood T cells from patient ci1371. The frequency of each neoantigen-specific TCR clonotype is shown at each peripheral blood timepoint. (iii) The frequency of all clones that expanded between the 1 week and 3–6 month post-SABR timepoints among all peripheral blood T cells sequenced. MANAFEST, Mutation-Associated Neoantigen Functional Expansion of Specific T-cells; PD-1, programmed cell death protein 1; PD-L1, programmed-death ligand 1; SABR, stereotactic ablative body radiation; TCR, T-cell receptor.

Figure 1  (A) Early tumor response, presence of immune cell populations, and PD-L1/PD-1 expression with paired pre-SABR and post-SABR biopsies in patient ci1371 (images ×200). Tumor biopsy samples show viable squamous cell carcinoma on H&E stained sections. Cellular fibrotic stroma with variable number of inflammatory cells are highlighted by CD8, FOXP3 and PD-1 immunostains. The PD-L1 immunostain shows weak-to-moderate at least partial membranous labeling in 20%–30% of the tumor cells. (B) Neoantigen-specific T cell responses and peripheral clonal dynamics in patient ci1371. (i) MANAFEST was performed on peripheral blood from patient ci1371 to identify neoantigen-specific T cell receptor clonotypes. Data are shown as the frequency of each antigen-specific TCR after 10 days of culture with the positive peptide (solid bar), and in every other condition that was tested (transparent bars). Empty bars indicate that the clone was not detected in that culture condition. (ii) TCR sequencing was performed on peripheral blood T cells from patient ci1371. The frequency of each neoantigen-specific TCR clonotype is shown at each peripheral blood timepoint. (iii) The frequency of all clones that expanded between the 1 week and 3–6 month post-SABR timepoints among all peripheral blood T cells sequenced. MANAFEST, Mutation-Associated Neoantigen Functional Expansion of Specific T-cells; PD-1, programmed cell death protein 1; PD-L1, programmed-death ligand 1; SABR, stereotactic ablative body radiation; TCR, T-cell receptor.

shown minimal difference among the three paired biopsies (table 1 and figure 1A).

Paired pre-SABR and post-SABR core-needle samples were insufficient to study TCR-repertoire changes in the radiated tumor of patients Ci1371 and Ci1424.

The impact of SABR on peripheral neoantigen-specific T-cell responses

Our central hypothesis is that SABR induces peripheral activation of neoantigen-specific T-cells subsequent to antigen release. Four of the six patients had sufficient paired pre-SABR and post-SABR tumor and blood samples for analysis to identify antigen-specific TCRs in the blood (online supplemental table S1E,F). We queried between 28 and 60 putative neoantigens per patient (in pools of 3–5 peptides each) and detected neoantigen-specific CD8+T-cell responses in three of these patients.
In patient ci1371, five TCRs specific for one neoantigen each were detected (figure 1B). Patients ci1425 and ci1424 each had one TCR specific for one neoantigen (online supplemental figure S1B). No neoantigen-specific T-cell responses were detected in patient ci1488. We also performed bulk TCRseq on tumor biopsies when possible. At least one tumor biopsy yielded sufficient TCRseq information from all three patients in whom neoantigen-specific TCRs were detected; however, none of these TCRs were found in the tumor biopsies.

We next tested if SABR induces systemic T-cell activation. We performed bulk TCRseq on PBMC obtained before, during, and after SABR in the three patients with neoantigen-specific responses. In patient ci1371, three of five neoantigen-specific TCRs expanded in the periphery between 1 week and 3–6 months post-SABR (figure 1B(ii)). One of these clonotypes was detected before SABR, suggesting this expanded TCR was not from a naïve, circulating T-cell precursor but was instead already antigen-experienced at baseline. In patient ci1425, similar to patient ci1371, the neoantigen-specific TCR also expanded peripherally between 1 week and 3–6 months post-SABR (online supplemental figure S1B). In patient ci1424, the neoantigen-specific TCR was at too low of a frequency in the uncultured blood to be detected by bulk TCRseq at any of the serial timepoints. These findings indicate that neoantigen-specific T-cells can be detected in the peripheral blood of patients with very early stage lung cancers and that these T-cells undergo peripheral activation and expansion on SABR.

**Systemic, antigen-agnostic impact of SABR on T-cell dynamics**

While we were unable to perform MANAFEST on every patient due to limited tissue, we were able to perform bulk TCRseq of serial blood T-cells. We studied TCR clones that were dynamic after the 1-week timepoint, regardless of their antigen specificity. In five of the six patients, TCR clones expanded after completion of SABR (between the 1 week and 3–6 month timepoints; Figure 1B(iii) and online supplemental table S1G). We did not obtain blood at the 1 week and 3–6 month timepoints for the sixth patient, ci1488 (the only post-SABR PBMC timepoints from this patient were D+24 and D+390). Interestingly, the frequency of these dynamic clones did not change between pre-SABR and 1-week post-SABR timepoints nor between the 6 months to 1-year time points (figure 1B(iii) and online supplemental figure S1C), thus supporting the relative stability of these clones outside the 1 week to 3–6 month interval where we saw expansions of tumor-reactive T-cells. The dynamic clones were of variable frequency in the peripheral blood and a range of 7.7%–39.3% of these were detected in the tumor specimens. Paired pre-SABR and post-SABR biopsies were compared in patients ci1371 and ci1424, and of the clones that expanded in the periphery from ~1 week to 3–6 months, 5.0% and 14.3%, respectively, were also expanded in the post-SABR tumor relative to pre-SABR (online supplemental table S1G). Given this, we conclude that most peripherally expanded clones were non-dynamic in the tumors from these two patients. Taken together, these data support gradual and transient systemic activation of peripheral blood-derived neoantigen-specific and antigen-agnostic T-cells following SABR, with minimal impact on early T-cell infiltration of the radiated tumor lesion.

**DISCUSSION**

In this prospective study of patients with stage I NSCLC treated with SABR, we identify that an antitumor neoantigen-specific T-cell response is detected systemically in the blood, but is gradual and transient, developing 1 week to 3–6 months after SABR alone. These data suggest that there exists (1) the capacity for a systemic immune response to be induced even in small volume, early stage lung cancers with SABR and (2) there may be an optimal window for considering delivery of ICIs in relation to receipt of SABR to potentially maximally synergize an immune response.

In NSCLC, RT-induced responses have mainly been evaluated in the context of ICIs. A phase II study showed patients treated with neoadjuvant SABR and durvalumab had improved pathological response, with increase in MHC-I gene expression and antigen-presenting cells in the tumor, compared with durvalumab alone. In a phase II study of 39 patients with chemo-refractory advanced NSCLC, patients treated with RT with ipilimumab had 31% disease control. In one patient, dynamic peripheral T-cell clones changes at day+22 were associated with clinical responses and RT-increased expression of mutation-associated neoantigens recognized by T-cells. Studies also demonstrated improved immune response when RT is sequenced prior to, rather than after receipt of ICI, with a delayed systemic immune response following SABR and ICI. We observe that SABR alone, in the absence of concurrent or sequenced delivery of ICI, can induce an antitumor T-cell response in small-volume localized tumors. Based on our data, we speculate that irradiation of a small, solitary lesion can induce systemic circulation and possibly activation of tumor-reactive T cells.

This study is limited by the small patient numbers and tissue sample sizes, but adds to the data that SABR alone can incite a systemic adaptive unprimed immune response and is among the first data in stage I NSCLC to annotate that SABR alone primes an immune response. Our small tissue samples also limited our ability to assess if the peripheral T-cell expansions were from reactivation of memory T-cells or resulted from de novo priming of new T-cells, although our findings suggest it is likely a combination of both. Given these limitations, further studies with larger sample sizes are needed to clarify the dynamic changes of T cell clones in the peripheral blood and...
tumor after SABR, but our study provides a rationale to perform additional studies of the direct impact of SABR on systemic antitumor immunity. Better understanding on how SABR alone induces an immune response may allow optimal sequencing with ICIs to overcome acquired or innate resistance to ICIs.

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Patient consent for publication
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Ethics approval
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Supplemental material
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Figure S1a. Schematic figure of SABR, and blood and tissues collection. Created on BioRender.com.
Figure S1b. Neoantigen-specific T cell responses in early stage lung cancer patients treated with SABR. MANAFEST was performed on peripheral blood from patients ci1425 (A) and ci1424 (B) to identify neoantigen-specific T cell receptor clonotypes (left). Data are shown as the frequency of each antigen-specific TCR after 10 days of culture with the positive peptide (solid bar), and in every other condition that was tested (transparent bars). Empty bars indicate that the clone was not detected in that culture condition. TCR sequencing was also performed on uncultured peripheral blood T cells from ci1425. The frequency of each neoantigen-specific TCR clonotype is shown at each peripheral blood timepoint (A, right).
**Figure S1c. Peripheral dynamics of T cell clonotypes that expanded between one week and 3-6 months post-SABR.** TCR Vβ CDR3 sequencing was performed on peripheral blood T cells at serial timepoints before, during, and after SABR for patients ci1424, ci1425, ci1426, and ci1437. Datapoints represent TCR Vβ clones that significantly expanded (Fisher’s exact test, p<0.05) after the one week post-SABR timepoint. The frequency of these expanded clones is shown at all timepoints sequenced within a given patient. Data are shown as the frequency of the clone among all peripheral blood T cells sequenced at that timepoint.
**Figure S1a.** Schematic figure of SABR, and blood and tissues collection. Created on BioRender.com.
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