Dose– and time-dependent systemic immune modulation by stereotactic radiotherapy in early-stage lung cancer

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Abstract

Importance:

There is an unmet need to understand the immunomodulatory mechanisms of stereotactic body radiotherapy (SBRT) to effectively enhance responses to immunotherapy, while reducing tumor burden.

Objective:

To evaluate the impact of ablative SBRT on systemic immunity in patients with early-stage NSCLC.

Design:

The LAPIS trial accrued patients between 2016-2021, with a median follow-up of 31 months. This primary analysis was based on data that were current as of December 14, 2021.

Setting:

Single center prospective clinical trial.

Participants:

Fifty-six patients with early-stage inoperable NSCLC patients treated with SBRT in different dose/schedules were enrolled, of whom 50 were evaluable.

Intervention(s) (for clinical trials) or Exposure(s) (for observational studies):

We used immune profiling of peripheral blood at first SBRT fraction (baseline), during and at the end of SBRT as well as at first (FU1) and second (FU2) follow-up (six weeks and another 4.5 months after the last SBRT fraction, respectively).

Main Outcome(s) and Measure(s):

The pre-specified primary endpoint was increase (yes/no) in circulating $CD8^+$ CTL counts at FU1 compared to pre-treatment, and secondary endpoints included changes in other T-cell subsets at all time-points.

Results:

Fifty patients with early-stage NSCLC (median age 70 years, male 68%, female 32%) were evaluated. The absolute counts of circulating $CD8^+$ CTLs at FU1 increased only in 21% of the patients (not significant), but there was a statistically significant increase in the proportion of proliferating $CD4^+$ and $CD8^+$ T-cells immediately after SBRT (at the end of the treatment, p £ 0.01), also in the subsets expressing PD-1, containing tumor-specific T-cells (p £ 0.001). These effects were significant only in the subset of patients receiving 10Gy or less per SBRT fraction (n=25, p £ 0.05). At 2 and 4 years, OS rate was 75% and 51%,

respectively, and progression-free survival (PFS) was 56% and 25%, respectively. A longer PFS was associated with an absolute increase of the $CD8^+$ CTLs at FU1 compared to pre-treatment values (p=.04).

Conclusions and Relevance:

SBRT induces a significant expansion in subsets of circulating effector T-cells immediately post-treatment and support the testing of lower doses per fraction for SBRT prior to or combined with immunotherapy.

Trial Registration:

The study was registered accordingly in the German trials registry (DRKS 00011266).

https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00011266

Key Points

Question: How to achieve activation of anti-tumor cytotoxic T lymphocyte (CTL) responses using SBRT in combination with immune checkpoint blockade, and what are the optimal radiation dose (ablative versus non-ablative), fractionation schedule and treatment sequence in combination with ICB?

Findings: In the LAPIS trial, 50 patients received SBRT in different dose/schedules, thus allowing studies of systemic immune responses. Study data showed significant increases in the fractions of expanding Ki-67^+CD8^+ and Ki-67^+CD4^+ T-cell subsets at the end of SBRT. T-cell expansion occurred in both PD-1^+ and PD1^- Ki-67^+CD8^+ CTL fractions, and only in patients treated with less than 10 Gy per fraction.

Meaning: Our results show that expansion of tumor-specific lymphocytes can be induced after ablative SBRT, but only when using doses or 10Gy or less per fraction and support the testing of lower doses per fraction for SBRT prior to or with ICB in early-stage or oligometastatic NSCLC.

Main Text

Stereotactic body radiotherapy (SBRT) is a key treatment modality for early stage and oligo-metastatic non-small cell lung cancer (NSCLC)[1, 2]. SBRT induces DNA double strand breaks, leading to cell killing, and may also modulate systemic immunity. Understanding the immunomodulatory mechanisms of SBRT may have a significant impact on the strategies for combining immune checkpoint blockade (ICB) and SBRT.

CD8+ cytotoxic T lymphocytes (CTLs) can mount responses against many human cancer types, but are often insufficient to eradicate tumors, as they become exhausted[3-5]. ICBs may reduce CTL exhaustion, while SBRT may further promote systemic immune activation. But how to achieve activation of anti-tumor CTL responses using SBRT and what the optimal radiation dose (ablative versus non-ablative),

fractionation schedule and treatment sequence in combination with ICB are, remain outstanding questions.

We evaluated the impact of ablative SBRT on systemic immunity in patients with early stage NSCLC in a prospective study. We used immune profiling of peripheral blood by longitudinal assessment at first SBRT fraction (baseline), during and at the end of SBRT as well as at first (FU1) and second (FU2) follow-up (six weeks and another 4 and a half months after the last SBRT fraction, respectively). The pre-specified primary endpoint was increase (yes/no) in circulating $CD8^+$ CTL counts at FU1 compared to pre-treatment, and secondary endpoints included changes in other T-cell subsets at all time-points. Study accrued 56 early-stage NSCLC patients between 2016-2021, of whom 50 were evaluable (4 dropped out and 2 withdrew consent). Patients and treatment characteristics are shown in **eTable 1**.

The absolute counts of circulating $CD8^+$ CTLs at FU1 compared to baseline increased only in 21% of the patients (not significant). Moreover, there was a significant decrease in the mean absolute counts of $CD8^+$ CTLs and $CD4^+$ T-cells at all time-points compared to pre-treatment values (**Figure 1A, B**). These data show that there is a significant lymphodepletion during and after SBRT, despite the smaller irradiated volumes and no nodal irradiation.

We then sought to examine the changes in Ki-67, a marker of cellular proliferation and T-cell reinvigoration, expressed by cycling or recently divided cells[4, 6, 7]. Interestingly, the proportion of proliferating $CD4^+$ and $CD8^+$ T-cells among peripheral blood lymphocytes ($CD3^+$ T-cells) significantly increased immediately after SBRT (at the end of the treatment) (**Figure 1C**). These increases occurred in the proliferating $CD4^+$ and $CD8^+$ T-cell subsets expressing PD-1, containing tumor-specific T-cells[8, 9], but also in the $PD\text{-}1^-$ subsets (**Figure 1D, E**). Moreover, median fluorescence intensity of PD-1 immunostaining was also higher at the end of treatment in the $CD8^+$ and $CD4^+$ T-cells, indicating an increased expression level for this activation marker (**Figure 1F**). Additionally, the proportion of T-cells expressing the activation markers IFN-γ and IL-17A was increased during and after SBRT (**Figure 1G-I**).

Overall, there was a significant decrease in the number of naïve and memory $CD8^+$ and $CD4^+$ T-cell subpopulations after SBRT compared to pre-treatment values (**eFigure 1A-B**). Nevertheless, the fractions of $CD8^+$ and $CD4^+$ T-cells expressing inducible costimulatory (ICOS) increased at FU1 (**eFigure 1C**). Regulatory T cells (Treg), which are considered more radioresistant than other lymphocyte subsets, showed a significant decrease at FU1 (**eFigure 1D**). Similarly, myeloid-derived suppressor cells (MDSC), which can modulate tumor progression[10], decreased at post-treatment time-points (**eFigure 1E**). TIM3 and CTLA-4 expression was detected only on a small minority of circulating T-cells, indicating that most circulating T-cells were not terminally exhausted after SBRT (**eFigure 1F-G**). All results are summarized in **eTable 2.**

As high radiation doses per fraction seem to attenuate immunogenicity[11], we stratifyed for dose per fraction using as cut-off 10Gy. Patients treated with 10Gy or less (n=25) showed significant increases in

proportion of proliferating $CD8^+$ and $CD4^+$ T-cells compared to pre-treatment values, but we detected no changes in patients who received more than 10Gy per fraction (n=19) (**Figure 2A**). The same results were obtained for the proliferating $PD1^+$ and $PD1^-$ $CD8^+$ T-cell fractions (**Figure 2B**).

Clinically, with a median follow-up of 31 months, median overall survival (OS) was not reached. At 2 and 4 years, OS rate was 75% and 51%, respectively, and progression-free survival (PFS) was 56% and 25%, respectively, with a median PFS of 36 months (**eFigure 2**). Only one patient developed local progression with a regional and distant progression, four patients developed distant metastases (8%), 3 patients regional and distant metastases (6%) and nine patients developed a regional recurrence (8%). There was no correlation between the biological effective dose (BED) and OS (hazard ratio per Gy [HR]=0.99, 95%CI: 0.98-1.01, p=0.4) or PFS ([HR]=0.99, 95% CI: 0.98-1.01, p=0.2). Moreover, there was no difference in outcomes between patients treated with more than 3 fractions versus those who received three fractions (OS: [HR]=1.50, 95%CIs: 0.56-3.99, p=0.4; PFS: [HR]=1.60, 95%CIs: 0.71-3.38, p=0.3). Thus, we found no signal of superior efficacy based on BED or dose per fraction. However, in an exploratory analysis, we found that a longer PFS was associated with an absolute increase of the $CD8^+$ CTLs at FU1 compared to pre-treatment values (p=0.043, log-rank test, **eFigure 2C**).

Taken together, these data show that SBRT can lead to lymphopenia in early-stage NSCLC, despite the smaller irradiated volumes. Of note, an absolute increase in circulating $CD8^+$ CTLs at follow-up compared to pre-treatment values was associated with longer PFS. Interestingly, SBRT-induced lymphopenia was associated with increased T-cell proliferation, which included tumor-specific T-cells[12]. Use of ablative SBRT has been reported to decrease the inhibitory signals from the tumor, reduce T-cell exhaustion and promote T-cell activation[13, 14]. In patients with treated with ICBs clinical failure was due to an imbalance between T-cell reinvigoration and tumor burden [4]. The magnitude of T-cells reinvigoration in relation to pre-treatment tumor burden correlated with clinical response[4]. Moreover, the Ki-67 response in the PD-1^+CD8^+ T-cell subset peaked at 3-4 weeks after initiating ICB treatment[4, 7], while in our study the Ki-67 response in the PD-1^+CD8^+ subset peaked at the end of SBRT. Optimal integration of ICB with SBRT should take into consideration these T-cell responses.

The increased proliferation of circulating $CD8^+$ and $CD4^+$ T-cells in patients treated with more than 10 Gy per fraction could be due to immunogenic cancer cell death. In pre-clinical studies cytoplasmic leakage of DNA after 8-12Gy was detected by cGAS/STING and activated primordial viral response pathways leading to production of type I IFN activation, while at higher doses there was a reduction in the IFN response and T-cell priming leading in lack of synergy with ICB[11]. This concept was tested in a study, in which sub-ablative total doses of 3x8 Gy were used for the combination with ICBs showing a prolongation of survival but did not meet the pre-specified endpoints[15]. Our results show that both systemic immune modulation and reduction of tumor burden can be achieved when using ablative SBRT with less than 10Gy per fraction.

Our study has limitations. Due to the different duration of SBRT regimens, post-treatment evaluations were not time matched.

In conclusion, our study shows that SBRT alone can significantly increase the fraction of proliferating $CD4^+$ and $CD8^+$ T cells, most prominently at the end of treatment and only when using 10Gy or less per fraction. These data have direct implications for the optimal integration of ICBs with SBRT in NSCLC and potentially other malignancies.

Online Materials And Methods

The prospective study LAPIS was conducted in the Department of Radiation Oncology, University Medical Center Freiburg, Germany and in the Department of Radiation Oncology, Massachusetts General Hospital (MGH) and Harvard Medical School Boston, USA per the Declaration of Helsinki. The study was registered accordingly in the German trials registry (DRKS 00011266). All patients gave written informed consent according to institutional and federal guidelines. The institutional ethics committees approved the study protocol (EK 38/16, Freiburg and MGH IRB Agreement #:2016D009860). The prospective LA-PI-S trial enrolled patients with primary or recurrent non-metastatic lung cancer (n = 50) and liver cancer (n = 50) as well as patients with oligometastatic/oligoprogressive lung or liver metastases treated with SBRT without (lung n = 50, liver n = 50) or in combination with immunomodulating treatments (lung: n = 50, liver: n = 50). According to the protocol, each subgroup was analyzed separately. Herein we present the results of the circulating immune cell profiling of patients with early-stage non-small cell lung cancer (NSCLC) treated with SBRT by longitudinal assessment at first SBRT fraction (baseline), during and at the end of SBRT as well as at first (FU1) and second (FU2) follow up (six weeks and another 3 months after the last SBRT fraction respectively). Patients with inoperable stage I-II judged to be suitable for SBRT by interdisciplinary consensus were enrolled in the study. All patients were previously staged with a ^{18}F-FDG PET. Patients under systemic treatment, treatment with corticosteroids or other immunosuppressive drugs as well as patients with previous radiotherapy within the last 3 months were deemed ineligible.

Treatment planning and treatment delivery

Patients were immobilized in supine position with a customised vacuum cushion system and received a 4D/CT or a 4D/PET-CT. Patients with peripheral tumors received 3 x 18.75 Gy to the D50% such that 95% of the PTV received a minimum of 45 Gy, (3x15 Gy, 80% of the nominal dose) and a dose maximum between 110 and 120%. Depending on the proximity to the central bronchial system and the thoracic wall, a total dose of 50 Gy in 5 fractions of 10 Gy or 60 Gy in 8 fractions of 7.5 Gy for central tumors or 66 Gy in 12 fractions for ultra-central tumors. Dose prescription was chosen such that 95% of the PTV received at least the nominal fraction dose, and 99% of the PTV receives a minimum of 90% of the nominal dose. The dose maximum within the PTV should not be less than 110%, nor should it exceed 120% of the prescribed dose. Aim was to apply a minimum biological effective dose of 100Gy. Treatment was given on alternate days.

Response to treatment was assessed at the same time points according to the Response Evaluation Criteria in Solid Tumors (RECIST) by means of thoracic CT and/or 18F-FDG PET/CT, the latter being mandatory in case of suspected disease progression.

Blood samples were collected by venipuncture prior to treatment (Baseline), 1 day after (During), at the end (End), at the 1st follow-up (FU1: six weeks after the end of SBRT) and at the 2nd follow-up (FU2: 3 months after FU1). PBMCs were isolated and frozen until use. Samples were available from 27–42 patients at each time-point. Missing samples were either not collected or had insufficient cells for all analyses. All results are summarized in **eTable 2.**

Flow cytometry

PBMC samples were thawed and washed in RPMI 1640 media. Samples were then resuspended in RPMI 1640 media and filtrated through a 30 µm preseparation filter (Miltenyi Biotec). Cells were then counted, and live death staining was done using Zombie Red Fixable Viability stain (BioLegend), according to the manufacturer instructions. For detection of surface markers, cells were incubated with a mixture of antibodies for 20´at 4°C. For detection of intracellular antigens samples were fixed and permeabilized using the FoxP3 Fixation/Permeabilisation Kit from eBioscience. For in vitro restimulation of PBMCs 10^6 cells/ml were incubated in RPMI 1640 media with PMA (50 ng/ml), Ionomycin (1µg/ml) and BFA (1:1000) for 5h. Thereafter cells were stained for Zombie Red (BioLegend) for cell death exclusion and then surface markers. Cells were then fixed with IC Fixation buffer (eBioscience) and stained for intracellular markers, 30´at room temperature. Cells were stained in 4 different multicolor panels: MDSCs: HLA-DR-AF700 (L243), CD11b-PE (ICRF44), CD33-APC (P67.6); cytokines: CD3-FITC (OKT3), CD4-BV510 (OKT4), CD8-APC (HIT8a), IFNγ–BV421 (B27), IL-17A-PE (BL168); Treg and activation markers: CD3-FITC, CD8-APC, CD4-BV510, CD25-PE-Cy7 (BC96), CD127-PE (A019D5), ICOS-PerCP-Cy5.5 (C398.4), FoxP3-BV421 (206D); T cell proliferation and exhaustion markers: CD3-FITC, CD4-BV510, CD8-APC, PD-1-PE-Cy7 (EH12.1), Tim3-PE (F38-2E2), CTLA-4-BV605 (BNI3), CD45RA-PerCP-Cy5.5 (HI100), CCR7-AF700 (G043H7), Ki67-BV421 (Ki-67). All antibodies were purchased from BioLegend except PD-1-PE-Cy7, which was obtained from BD Biosciences. Analysis was performed on a Cytoflex S flow cytometer (Beckman Coulter). The gating strategy is shown in the online only information (**eFigures 3** and **4**).

Statistical Analysis

The study is planned to include n = 50 patients with NSCLC (reported here), with pulmonary metastases, primary liver cancer and with hepatic metastases, respectively. This target sample size of was derived based on feasibility considerations and the following considerations of statistical power. For the primary endpoint, the null hypothesis was that the probability of an increase (yes/no) of CD8 + counts six weeks after treatment compared to baseline, P (increase), is less than or equal to 50% (50% corresponds to no change from baseline to six weeks and to a median post : pre CD8 + count ratio = 1, lower percentages correspond to a decrease and to a median post : pre CD8 + count ratio < 1). The alternative hypothesis was P (increase) > 50%. According to STPLAN (Version 4.5), an exact one-sided binomial test at significance level 5% would have at least 80% power to reject the null hypothesis if the true P(increase) is

68.5% or greater. The null hypothesis would be rejected if at least 32 out of 50 patients experienced an increase. The exact significance level is 3.25% (STPLAN version 4.5).

Dynamic changes in the plasma level of blood biomarkers were examined using a matched paired mixed effect analysis with a Geisser-Greenhouse correction at four post-baseline time-points compared to baseline, respectively (**eTable 2**).

Overall survival (OS) and progression free survival (PFS) were calculated from the start of SBRT and estimated according to the Kaplan–Meier method. For event-free patients, the observations for OS were censored at the date of last contact, and for PFS and FFLP at the time of the last imaging or death.

For investigation of correlation between parameters which were significantly changed at FU1 or FU2 compared to baseline, PFS was calculated from FU1 or FU2 in the patients still at risk, respectively.

The impact of dose and fractionation on OS and PFS was estimated using a Cox regression. The impact of different blood biomarkers on PFS was investigated using a log-rank test. Data in figures are presented as box and whisker plots, with the center line representing the median, while the whiskers represent minimum/maximum values.

Differences were considered statistically significant when * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. All p values are two-sided. Due to the exploratory nature of this analysis, we applied no corrections for multiple testing. Statistical analyses were performed using Prism (Prism V.8, GraphPad Software) and SPSS (IBM, SPSS, v27).

Data availability

All data generated or analyzed are included in this published article and its supplementary information files and raw data are available from the corresponding author upon reasonable request, to respect patient confidentiality.

Declarations

Author Contributions: Drs. Gkika, Duda, and Grosu had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Concept and design: Gkika, Duda, Grosu.

Acquisition, analysis, or interpretation of data: All authors.

Drafting of the manuscript: Gkika, Firat, Duda, Grosu.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Gkika, statistical analysis consultation of Graf

Obtained funding: Grosu.

Administrative, technical, or material support: Grosu.

Supervision: Duda, Grosu.

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Figures

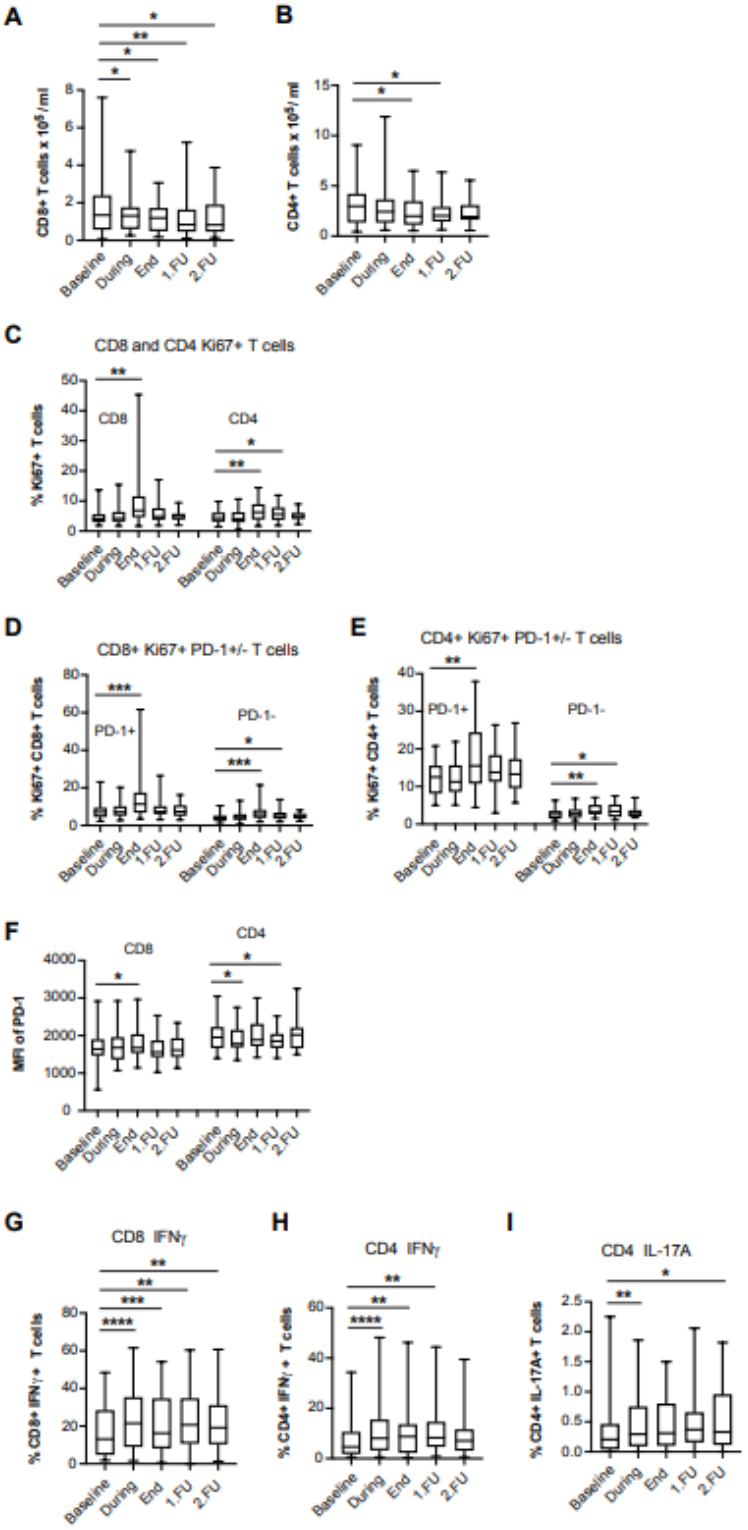

Figure 1

SBRT causes lymphodepletion but induces proliferation of circulating CD8$^+$ and CD4$^+$ T cells after treatment in early-stage NSCLC patients. (**A**) Absolute CD8$^+$ T cell counts. (**B**) Absolute CD4$^+$ T cell counts. (**C**) Fraction of Ki67$^+$ CD8 and CD4 T cells. (**D**) Fraction of Ki67$^+$ PD-1$^+$/ PD-1$^-$ CD8 T cells. (**E**) Fraction of Ki67$^+$ PD-1$^+$/ PD-1$^-$ CD4 T cells. (**F**) Median fluorescence intensity (MFI) of PD-1 expression

on CD8 and CD4 T cells. (**G**) Expression of IFN-g in CD8 T cells. (**H**) Expression of IFN-g in CD4 T cells. (**I**) Expression of IL-17A in CD4 T cells. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 from matched paired mixed effect analysis, two-sided.

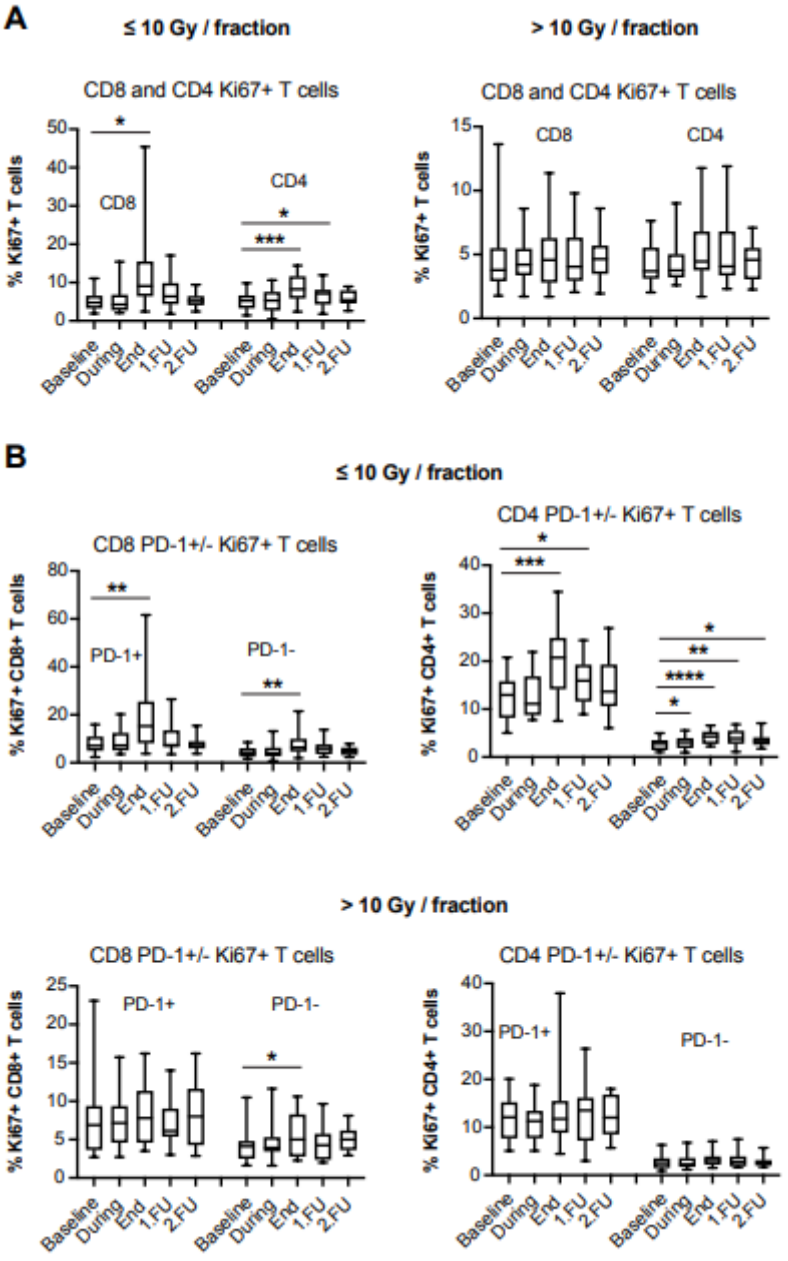

Figure 2

SBRT has dose-dependent effects on proliferation of CD8 and CD4 T cells in NSCLC patients. (**A**) Fraction of Ki67$^+$ CD8 and CD4 T cells after SBRT doses ≤ 10 Gy (left) and > 10 Gy (right). (**B**) Fraction of Ki67$^+$ PD-1$^+$/ PD-1$^-$ CD8 and CD4 T cells after SBRT doses ≤ 10 Gy (upper) and > 10 Gy (lower).* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 from matched paired mixed effect analysis, two-sided.

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