Intrinsic tumor resistance to CAR T cells is a dynamic transcriptional state that is exploitable with low-dose radiation

Alexander B. Kim,[1] Ssu-Yu Chou,[1] Solomon Kang,[1] Eric Kwon,[1] Matthew Inkman,[1] Jeff Szymanski,[1] Neal Andruska,[1] Cian Colgan,[1] Jin Zhang,[1] Joanna C. Yang,[1] Nathan Singh,[2] and Carl J. DeSelm[1]

[1]Department of Radiation Oncology and [2]Department of Medicine, Washington University School of Medicine, St. Louis, MO

Key Points

- Tumor quality, reflected by distinct transcriptional states, predicts responses to CAR T-cell therapy but is dynamic.

- Low-dose radiation positively manipulates tumor quality to sensitize resistant leukemia cells to CAR T cells and improve overall survival.

Chimeric antigen receptor (CAR) T-cell therapy represents a major advancement for hematologic malignancies, with some patients achieving long-term remission. However, the majority of treated patients still die of their disease. A consistent predictor of response is tumor quantity, wherein a higher disease burden before CAR T-cell therapy portends a worse prognosis. Focal radiation to bulky sites of the disease can decrease tumor quantity before CAR T-cell therapy, but whether this strategy improves survival is unknown. We find that substantially reducing systemic tumor quantity using high-dose radiation to areas of bulky disease, which is commonly done clinically, is less impactful on overall survival in mice achieved by CAR T cells than targeting all sites of disease with low-dose total tumor irradiation (TTI) before CAR T-cell therapy. This finding highlights another predictor of response, tumor quality, the intrinsic resistance of an individual patient's tumor cells to CAR T-cell killing. Little is known about whether or how an individual tumor's intrinsic resistance may change under different circumstances. We find a transcriptional "death receptor score" that reflects a tumor's intrinsic sensitivity to CAR T cells can be temporarily increased by low-dose TTI, and the timing of this transcriptional change correlates with improved in vivo leukemia control by an otherwise limited number of CAR T cells. This suggests an actionable method for potentially improving outcomes in patients predicted to respond poorly to this promising therapy and highlights that intrinsic tumor attributes may be equally or more important predictors of CAR T-cell response as tumor burden.

Introduction

Chimeric antigen receptor (CAR) T cells represent a major innovation in treating advanced hematologic malignancies. The outcomes are often dichotomous in that this therapy either works very well and the patient experiences a lasting response or even cure[1,2] or the therapy fails, in which case the patient generally dies of their disease. There are predictors for who will respond well and who will not,[3,4] and numerous groups are attempting to optimize the CAR T-cell products themselves, but currently there is no way to immediately improve the outcome for the group of patients predicted to relapse or progress.

The full-text version of this article contains a data supplement.

Clinical predictors of poor response consistently reflect high baseline tumor burden, such as high total metabolic tumor volume or more extranodal sites of disease,[5] or minimal residual disease positivity.[1,6] Patients currently receive CAR T cells because their cancer has progressed through standard-of-care chemotherapies, so optimal disease reduction with further chemotherapy before CAR T-cell delivery is often not possible. Radiation therapy may still be used focally in high doses to eliminate bulky tumor deposits that have been unresponsive to other therapies, thus increasing the CAR T cell to tumor ratio, which would be expected to improve the chance of successful tumor elimination.[7] Low doses of radiation may affect the transcriptional profile of tumor or normal tissues without killing them,[8] but this strategy is not currently used therapeutically because direct tumor killing is minimal. Whether tumor debulking with high-dose focal RT before CAR T-cell therapy actually improves survival has not been tested; furthermore, whether low-dose RT designed to impact the transcriptional profile of tumor or host may impact subsequent CAR T-cell therapeutic outcomes is unknown. Because it is often difficult to predict in vivo efficacy based on in vitro CAR T-cell results, we examine these questions in vivo in an attempt to define a process both for selecting patients who will not respond and for intervening to improve their chances of long-term success.

Methods

Cell culture

NIH-3T3 (ATCC cat# CRL-1658, RRID: CVCL_0594) cell line was obtained from ATCC and cultured with Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals). NALM6 (ATCC cat# CRL-3273, RRID: CVCL_UJ05) cell line was obtained from ATCC and cultured in RPMI-1640 (Invitrogen, Waltham, MA) supplemented with 10% FBS (Atlas Biologicals), 10 mM HEPES (Invitrogen), L-glutamine 2 mM (Invitrogen), NEAA 1× (Invitrogen), 0.55 mM β-mercaptoethanol, and 1 mM sodium pyruvate (Invitrogen). NALM6 cells were transduced with firefly luciferase-green fluorescent protein (GFP) to allow in vivo tumor burden imaging. CD19$^{-/-}$ NAML6 generation was described previously[9] in brief using the gRNA sequence CTAGTGGTGAAGGTGGAAGG, followed by single-cell sorting and further assessment using flow cytometry and deep sequencing of the knockout site. p53$^{-/-}$ NALM6 was obtained commercially (Horizon HD cat# 115-049, RRID: CVCL_HE12). BID$^{-/-}$ NALM6 were a generous gift from Nathan Singh. All cells were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

Primary T cells were obtained from buffy coats or peripheral blood collection from healthy volunteer donors. Peripheral blood mononuclear cells were isolated by density gradient centrifugation, and cells were then stimulated with 2 μg/mL phytohemagglutinin-L (Sigma) in RPMI-1640 medium supplemented with 10% FBS. Forty-eight hours after activation, T cells were transduced with retroviral supernatants by centrifugation on retronectin (Takara)-coated plates. The medium was changed 1 day after transduction. Cells were then replated every 2 days at 10^6 cells per mL with 50 U mL^{-1} IL-2 (Miltenyi Biotech).

Vector constructs

19 to 28ζ CARs contain the SJ25C1 CD19–specific scFv fragment.[10] Control CAR T cells contained an anti-lewisA (irrelevant antigen) scFv followed by 28ζ.[11] CAR complementary DNAs (cDNAs) were cloned in a γ-retroviral vector. All constructs were prepared using standard molecular biology techniques. Viral supernatants were prepared using standard techniques as previously described.[12]

Mouse tumor model

Male or female NOD.Cg-Prkdc^{scid}Il2rg^{tmWjl}/SzJ (NSG) mice 8 to 12 weeks of age (Jackson Laboratory cat# 005557, RRID: IMSR_JAX:005557) were used under a protocol approved by the Washington University or MSKCC Institutional Animal Care and Use Committee, according to all relevant animal use guidelines and ethical regulations. In all experiments except Figure 3, a total of 0.5 × 10^6 firefly luciferase-GFP NALM6 cells were administered intravenously (IV) by tail vein injection (day −4). Four days later, mice were randomized to experimental (CD19-targeting) or control (LewisA-targeting) CAR T cells. CAR T cells were administered at 1 × 10^4 live CAR+ T cells in 100 microliters intravenously by tail vein injection (day 0), unless otherwise indicated. If RT was delivered, it was delivered the day before CAR T-cell administration. In Figure 3, 0.75 × 10^6 firefly luciferase-GFP NALM6 cells were administered in each mouse to better discern differences in tumor burden under the different treatment regimens. All in vivo assays were performed with bulk transduced CAR T cells, and only T-cell transductions containing 60% to 95% CAR positivity rate were used in experiments. Day 1 vs day 7 RT as well as focal vs total body RT with wild-type or p53- deficient mice were all performed as part of the same large experiment, allowing crosscomparison of groups. Tumor burden was measured using Bioluminescence imaging with the Xenogen IVIS Imaging System (Xenogen). Living Image software (Xenogen) was used to analyze acquired bioluminescence data.

Flow cytometry

All antibodies were titrated. CAR expression was measured with Alexa-Fluor-647–conjugated goat anti-mouse Fab (Jackson ImmunoResearch cat#115-607-003, RRID: AB_2338931). The following antibodies were used: CD19–PE (clone SJ25C1 BD Biosciences cat# 340364, RRID: 400018) or CD19–BUV395 (clone SJ25C1, BD Biosciences cat# 563551, RRID: AB_2738272), CD19–BV510 (clone SJ25C1, Biolegend cat#363020, RRID: AB_2564229), CD3–BUV737 (clone UCHT, BD Biosciences cat# 612750, RRID: RRID:AB_2870081), BUV-395CD4 (BD Biosciences cat# 563552, RRID:AB_2738273), and APC-cy7-CD8 (BD Biosciences cat# 557834, RRID:AB_396892). CountBright beads (Invitrogen cat# C36950) were used to determine the absolute number of cells according to the manufacturer's protocol. 7-AAD or DAPI was used to exclude dead cells. Fc Receptor–Binding Inhibitor Antibody Human (eBioscience cat# 14-9161-73, RRID: AB_468582) was used to block Fc receptors. In vitro viability after RT was assessed using Zombie NIR (Biolegend cat# 423106) and Annexin V PerCP-Cy5.5 (Biolegend cat# 640936) according to the manufacturer's protocols. Data were collected using BD LSR-II, BD LSR-Fortessa, and Cytek Northern Lights cytometers. Data were analyzed with FlowJo Software (Treestar/BD Biosciences, RRID: SCR_008520). Cell sorting was performed using a BD FACSAria cell sorter.

Irradiation

Animal irradiation was performed using a small animal irradiator (Precision X-Rad 225) with open jaws in the anterior-posterior

direction to deliver a dose of 1.8 Gy to the entire animal. In experiments targeting only 1 leg, the entire animal minus the leg was shielded with a 5 mm lead shield to deliver a dose of 20 Gy in 5 fractions (4 Gy each) twice daily separated by at least 6 hours per fraction.

RNA sequencing (RNA-seq) and analysis

Total RNA was isolated from 5 M Nalm6 cells by lysing with Trizol Reagent (Invitrogen) followed by extraction with 24:1 chloroform:isoamyl alcohol mixture (Sigma Aldrich, St. Louis, MO). After separation by centrifugation, the aqueous phase was frozen in 100% isopropanol overnight, and the RNA was then precipitated and washed twice with 75% ethanol. RNA yield was quantified using an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). From each isolate, 10 μg of RNA was treated with 2 units of DNAse I for 10 minutes at 37°C and purified on a Monarch RNA Cleanup Spin Column (New England Biolabs, Ipswich, MA), all according to the manufacturer's instructions.

RNA integrity number was determined using Bioanalyzer or 4200 Tapestation (Agilent, Santa Clara, CA). Library preparation was performed with 500 ng to 1 μg of total RNA using the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA) according to the manufacturer's instructions except as noted. Ribosomal RNA was blocked using FastSelect reagents (Qiagen, Germantown, MD) during cDNA synthesis. After fragmentation, RNA was reverse transcribed to yield cDNA using SuperScript III RT enzyme and random hexamers per the manufacturer's instructions (Life Technologies, Waltham, MA). Second-strand synthesis and adapter ligation was then performed per the manufacturer's instructions. Finally, fragments were sequenced on a NovaSeq-6000 (Illumina) using paired-end reads extending 150 bases.

Sequencing reads were mapped to the GRCh38/hg38 genome assembly using STAR (version 2.5.1a, RRID: SCR_004463) with ENSEMBL release 76 gene annotations. Quantification was performed using featureCounts (v2.0.1), and counts were normalized for library sampling depth using size factor normalization using DESeq2 (RRID: SCR_015687). The resulting logCPM values were then z score normalized.

DR score determination and gene set enrichment analysis

A death receptor (DR) signature score was calculated from each sample as the mean of the normalized expression for each gene of the set.[3] Visualization was performed using the R package ggplot2 (RRID: SCR_014601).

Gene set enrichment analysis[13] was conducted between Nalm6 samples 24 hours after irradiation with 1.8 Gy (n = 5) and those that received sham treatment (n = 8). We analyzed TMM-normalized[14] gene expression data using gene set enrichment analysis (GSEA) v2.2.0 and the Hallmark gene sets v6.1,[15] which we amended to include the DR signaling pathway genes as an additional gene set.

Evaluation of clinical specimens

Bone marrow (BM) specimens were collected from the patients enrolled in 3 multicenter, single-arm, open-label clinical trials (patients were not randomized and treatment was not blinded) (NCT02435849/ELIANA, NCT02228096/ENSIGN and NCT02030847) as described previously.[3] Briefly, the study protocols and their amendments were approved by the Institutional Review Board at each participating institution, and informed consent obtained at the time of study enrollment allowed for RNA-seq analysis of all patient samples to be evaluated. For the purposes of this analysis, patients were categorized as achieving complete response (CR) or nonresponders. CRs were defined as patients who achieved either complete remission or complete remission with incomplete hematologic recovery within 3 months and stayed in complete remission for at least 1 year after CAR T-cell infusion.

Statistics

All experimental data are presented as mean ± standard error of the mean. No statistical methods were used to predetermine the sample size. The means of 2 independent groups were compared using an unpaired, two-tailed t test. Survival analyses were performed using the Kaplan-Meier and Log-rank tests. Statistical analysis was performed using GraphPad Prism 7 and 9 software (RRID: SCR_002798). All in vivo studies were performed using 5 to 8 mice per group. RNA-seq was performed on 5 to 8 samples per treatment group of independently maintained, treated, and processed tumor cells.

Results

Low-dose total body irradiation significantly improves response to suboptimal CAR T-cell therapy

To test whether a potentially clinically relevant low dose of radiation delivered to all sites of disease before CAR T-cell therapy impacts the ultimate efficacy of otherwise suboptimal CAR T-cell therapy, we treated mice with established tumor with low- dose RT (1.8 Gy in a single fraction) followed by an inadequate dose of 10 000 CAR T cells 1 day after RT (Figure 1A). Nalm6 tumor was used, which is a B-cell ALL initially isolated from a 19-year-old male, for which CD19-targeted CAR T-cells are well characterized. This dose of CAR T cells alone was far below than required to achieve a complete response (CR), as expected from prior dose-adjusting studies using the same model in which the dose of 400 000 CAR T cells lead to ~50% CR.[16] However, when given after low-dose RT, the otherwise inadequate CAR T-cell dose achieved 100% CR and overall survival (Figure 1B-D). The same dose of nontargeted CAR T cells after low-dose RT had no benefit, as did RT alone. The magnitude of this phenotype was unexpected and encouraged us to delve further into characterizing this observation.

Low-dose RT's benefit before CAR T-cell therapy is not entirely because of direct tumor killing

First, we hypothesized that although this RT dose is relatively low, B-cell malignancies are still highly sensitive to RT-induced death and thus the benefit may be owing to a substantial amount of tumor debulking. It is commonly observed that the effector-target ratio (ie, the number of CAR T cells relative to the number of tumor cells) is important for CAR T-cell efficacy in vitro, and in patients, a higher disease burden is consistently a poor prognostic factor.[1,5,6] Thus, a logical approach to improving CAR T-cell efficacy is tumor debulking, resulting in fewer tumor cells needing to be killed by each CAR T cell. RT at higher doses is in fact often delivered to the
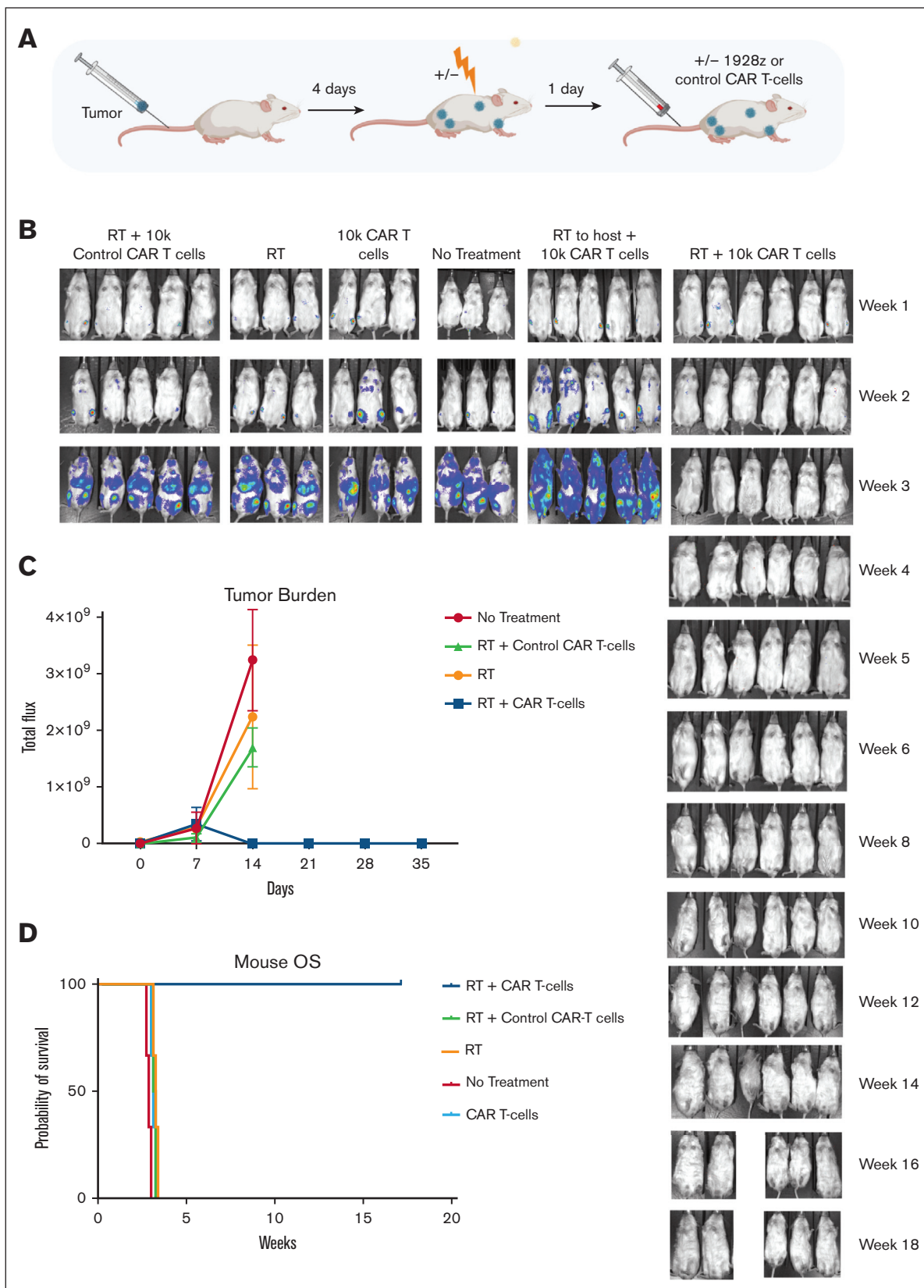
Figure 1. Low-dose RT improves survival of mice treated with CAR T cells. (A) Schematic diagram of treatment delivery. (B) Bioluminescent imaging (BLI) comparing the outcomes of mice treated with the indicated conditions, quantified in (C) with overall survival charted in (D). 10 000 CAR T cells were used in these studies.
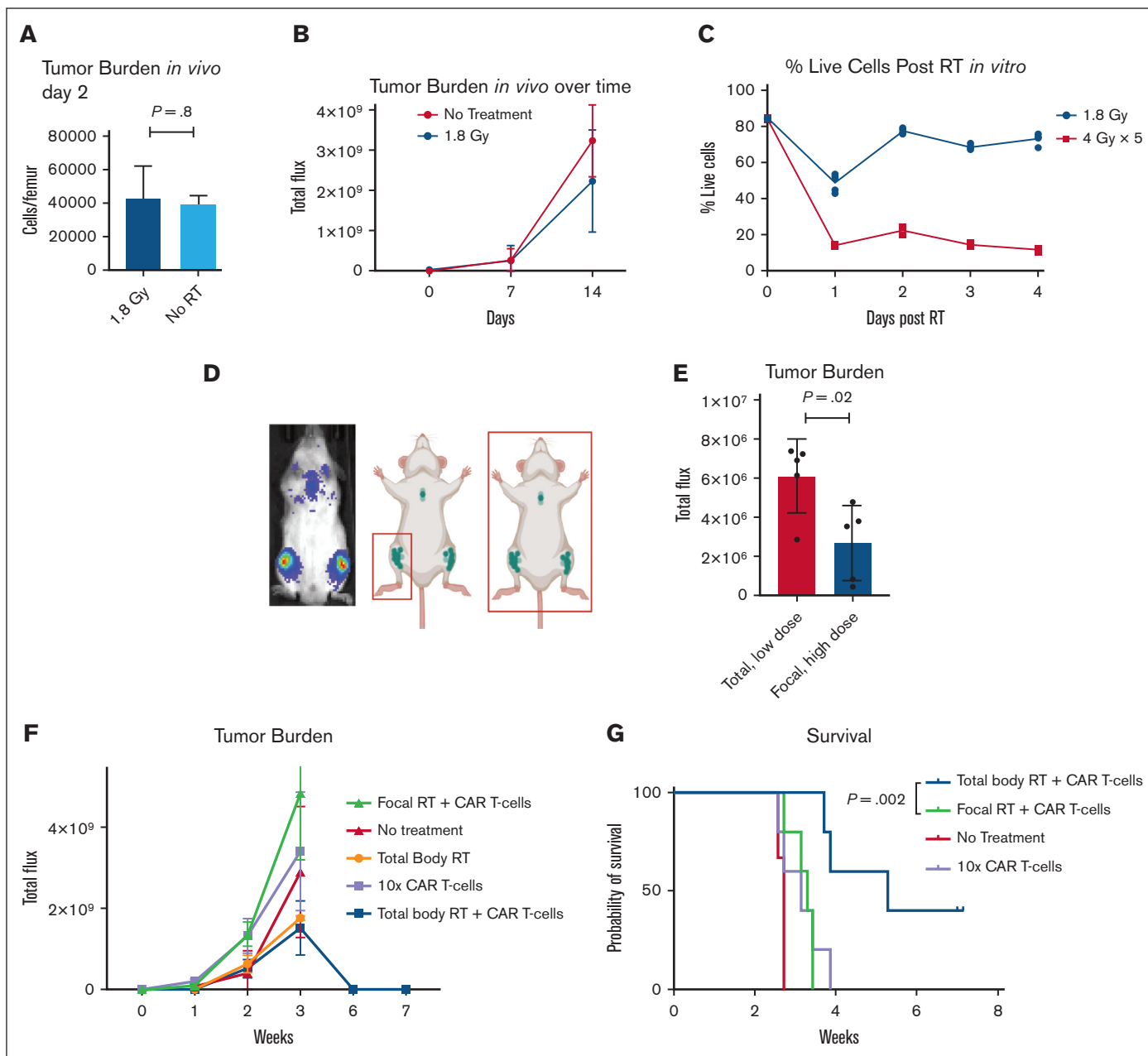
Figure 2. Systemic low-dose radiation is less debulking than focal high-dose radiation but results in significantly improved response and survival with CAR T cells. (A) Two days after the low-dose (1.8 Gy in 1 fraction) RT or sham procedure, mice were euthanized and the total number of human CD19+ cells were quantified per femur using flow cytometry. (B) Bioluminescence imaging was used to quantify F. Luciferase-expressing tumor cells in vivo over time, after low-dose RT or no treatment. (C) The precise cytotoxic effects of low- vs high- (20 Gy in 4 fractions) dose RT were quantified in vitro over time using flow cytometry, with live cells defined as Zombie NIR negative Annexin-V negative. (D) Bioluminescence imaging of tumor-bearing mice showing systemic tumor distribution (left), and the radiation fields used for focal or systemic tumor targeting (right). (E) Total body tumor burden 1 day after completing systemic (aka total body) low-dose vs focal "debulking" high-dose radiation. Total body tumor burden (F) and overall survival (G) of mice treated with low-dose CAR T cells after the various indicated conditioning regimens. 10 000 CAR T cells were used in these studies.

areas of bulky disease with the intent of eliminating targetable disease sites in conjunction with CAR T cells.[7] To first explicitly characterize the sensitivity of these tumor cells to RT, we performed an in vivo analysis of tumor cell number 2 days after low-dose total body RT (Figure 2A), as well as bioluminescence imaging of mice treated with or without low-dose RT (Figure 2B)

over time. These studies failed to detect an obvious decrease in tumor burden after low-dose RT. In vitro assessment of tumor cell death showed ~20% cell death immediately following low-dose RT, whereas a higher dose of 20 Gy in 5 fractions (subsequently referred to as "high dose" RT) induced ~90% cell death (Figure 2C). To further assess the precise role of RT-induced tumor

debulking before CAR T-cell therapy, we tested these 2 doses in vivo. Because high-dose RT cannot be given to all organs safely, we chose to target 1 leg with high-dose RT because in this tumor model, the femoral bone is a major site of disease (Figure 2D). We treated another cohort of tumor-bearing mice with low-dose RT to the entire body (Figure 2D). The systemic tumor burden measured using bioluminescent imaging the day after RT demonstrated that focal higher-dose RT resulted in a greater reduction in disease throughout the body than low-dose RT to the entire body (Figure 2E). Low-dose CAR T cells were then delivered IV. Although mice receiving focal high-dose RT had a lower systemic tumor burden on the day of CAR treatment, eventually disease relapsed, and these mice did not maintain long-term responses or achieve a significant survival advantage in the end. However, mice that received low-dose RT to the entire body, despite having higher baseline systemic tumor burden responded more dramatically to CAR T-cell therapy, with nearly half of them achieving a CR (Figure 2F-G). This was surprising because currently clinically, patients are treated with high-dose radiation to focal sites of bulky disease, rather than low-dose radiation to all sites because the (unproven) presumption is that effectively killing (>90%) one major area of disease is more beneficial than a poorly tumoricidal dose to all sites of the disease. However, this study contradicts this notion in the context of CAR T cells, as mice that received low-dose total body RT rather than higher-dose focal RT ultimately achieved improved tumor response that led to a significant long-term survival benefit. Low-dose total body RT followed by CAR T cells also resulted in a significantly better response and survival than total body RT alone or CAR T cells alone. Even 10 times as many CAR T cells alone were insufficient to induce a CR in this model, consistent with prior studies,[16] demonstrating the degree to which low-dose RT improved CAR T-cell efficacy in this model (Figure 2F-G). Thus, low-dose total body irradiation appears more beneficial than focal higher-dose treatment, and the benefit is not explained simply by a reduction in tumor quantity.

The benefit of low-dose total body irradiation before CAR T-cell therapy is not dependent on host engraftment effects

In addition to directly impacting the tumor, total body RT has long been used to create an environment conducive to donor cell engraftment and expansion[17] and may lead to other effects on the host, which could explain the improved CAR T-cell efficacy in these studies. Thus, we wondered whether the benefit of low-dose total body/total tumor irradiation (TTI) before CAR T-cell therapy resulted from the direct effects on the tumor or from host effects that indirectly promote CAR T-cell efficacy. To address this question, we established the following 3 treatment groups: the first group was irradiated with low-dose total body RT immediately followed by tumor injection so that the mice were exposed to RT to potentially facilitate CAR T-cell engraftment but the tumor cells remained RT-free; the second group was injected with tumor cells that were irradiated in the syringe directly before injection, so the mice were spared while the tumor irradiated; and the third group in which the tumor and mice were both exposed to low-dose RT just before tumor injection (Figure 3A). The following day, all mice were injected with the same number of CAR T cells. Surprisingly, irradiating the tumor alone before CAR T-cell treatment had the same impact on disease progression as irradiating both the tumor and

mice, whereas irradiating the mice alone before CAR T-cell therapy resulted in substantially greater tumor progression over time (Figure 3B). This suggests that the beneficial effects of low-dose RT before CAR T-cell treatment, at least in the early phases we are studying, are more because of the direct tumor effects than the indirect effects on the host. Thus, we refer to this method of CAR T-cell preconditioning as TTI because it is the direct effect of radiation on the tumor rather than the body or host that is critical.

Synergy of low-dose TTI is CAR target-antigen–dependent

Having determined that the low-dose TTI before CAR T-cell therapy improves outcomes primarily through direct tumor effects that do not correlate with direct tumor killing, we sought to uncover the underlying mechanism. We first hypothesized that low-dose TTI may induce the expression of surface receptors that facilitate CAR T-cell–mediated killing. CAR T cells can kill through a variety of mechanisms, including granzyme-perforin release in response to CAR target recognition, as well as TRAIL and Fas ligand,[18] which recognize their cognate receptors and induce apoptosis in cells that may or may not express the CAR target.[11] We first examined the expression levels of the target molecule CD19 and found it to be unchanged by radiation (supplemental Figure 1). Low-dose RT has been shown to induce the expression of apoptotic receptors that may facilitate the killing of tumor cells independent of direct CAR target expression.[11] We hypothesized if this mechanism was responsible for enhanced CAR T-cell efficacy, then low-dose TTI may allow for the elimination of tumor with heterogeneous CAR target expression. To test this, established mice with leukemia in which 5% of the cells were CD19$^{-/-}$ were delivered total tumor RT followed by low-dose CAR T cells. No mice survived these conditions, indicating that TTI does not facilitate clinically meaningful target-independent CAR T-cell tumor elimination in this model of disseminated leukemia (supplemental Figure 2).

RNA-seq reveals tumor responds to low-dose RT with both DNA repair and apoptosis pathway induction

To better characterize and understand the tumor-intrinsic effects of low-dose RT, we performed RNA-seq on independent unirradiated vs low-dose (1.8 Gy) irradiated samples, 24 hours after the irradiation. GSEA showed the P53 pathway to be the most highly enriched pathway after low-dose RT (Figure 4A). P53 is well known to be induced by radiation-induced DNA damage and mediates DNA repair, cell cycle arrest, and apoptosis, among other cellular responses.[19] Despite this radiation dose not being highly tumoricidal, the apoptosis pathway was also significantly induced, consistent with the concept that apoptosis can still be reversed up to a certain threshold of apoptotic molecule activation (Figure 4A).

Low-dose RT sensitivity to CAR T-cell killing is p53-independent

Because the p53 pathway was most significantly induced by low-dose RT, we hypothesized that p53 may be responsible for the beneficial effects of low-dose TTI before CAR T-cell therapy. To test this, we injected p53$^{-/-}$ or wild-type tumor cells intravenously, followed 4 days later by low-dose TTI and then low-dose CAR T
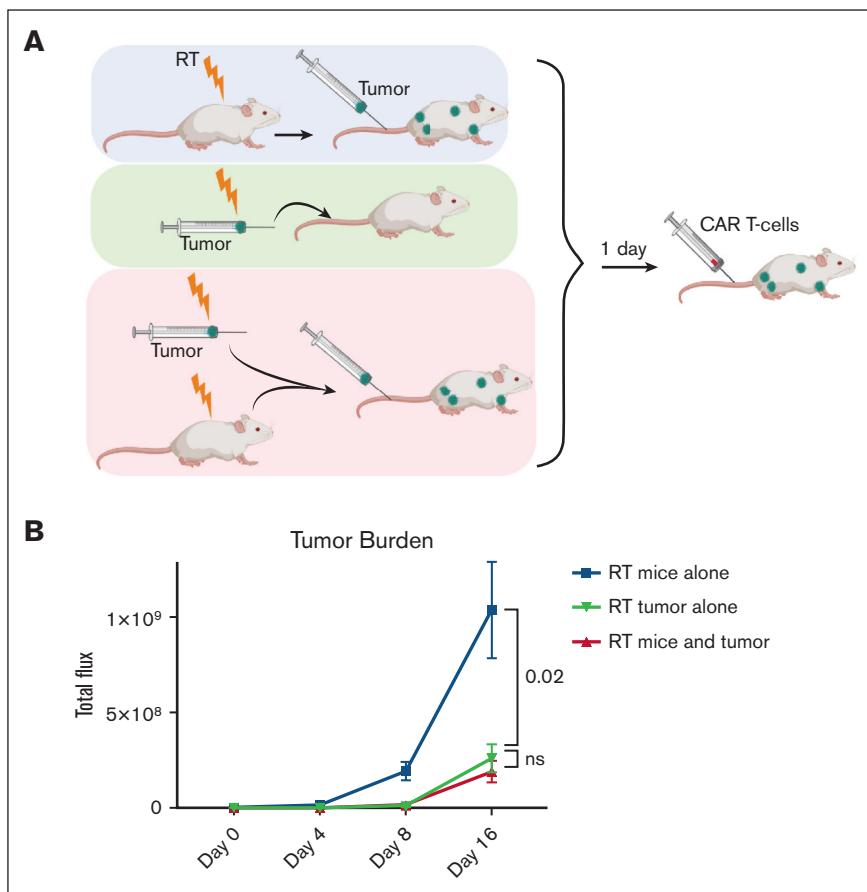
Figure 3. The benefit of low-dose total body irradiation before CAR T-cell therapy is primarily owing to direct tumor rather than host effects. (A) Schematic diagram of treatment delivery with low-dose RT being given to the mice alone (top), the tumor alone (middle), or the tumor and mice (bottom) separately followed by CAR T-cell therapy. (B) Bioluminescence imaging showing tumor burden over time of mice in the indicated treatment group. 10 000 CAR T cells were used in these studies, with 50% more tumor cells than Figures 1 and 2 to better assess differences in efficacy.

cells. We were surprised to observe no correlation between p53 status and response to CAR T cells after low-dose TTI, as both knockout and wild-type tumors benefited similarly, both in terms of tumor burden (supplemental Figure 3A) and survival (supplemental Figure 3B). Mice bearing p53 knockout tumors, despite being known for their resistance to therapeutic levels of RT,[20] exhibited an equivalent response to CAR T-cell therapy after low-dose TTI (supplemental Figure 3A-B). These data suggest that low-dose TTI sensitizes tumor cells to CAR T-cell killing independent of p53 status.

Low-dose RT significantly increases the DR score

RNA-seq analysis identified the apoptosis pathway as a significantly induced pathway after low-dose RT, but this apoptosis pathway contains a wide array of 130 genes. A previous unbiased tumor genome-wide CRISPR screen that identified intrinsic mediators of resistance to CAR T cells found a strong association between sequenced guides in the tumor and apoptosis pathway signaling; notably all sequenced guide targets associated with the apoptosis gene set belonged to the DR–driven apoptotic signaling pathway, and this pathway was then shown to be a key regulator as well as predictor of primary resistance to CD19 targeting CAR T cells in ALL.[3] Although the DR pathway was named for molecules that are downstream of DRs, the majority of these molecules are also downstream of granzyme B/perforin,[21] which is a major mechanism by which CAR T cells kill their targets. Thus, the DR score effectively encompasses key apoptotic molecules used by

CAR T cells to kill their target through both granzyme/perforin and DRs. We incorporated the DR pathway into our GSEA analysis and found it was also one of the most highly induced pathways by low-dose RT (Figure 4A). Although the DR pathway was previously identified as a tumor-intrinsic property that predicts baseline response to CAR T-cell killing, we found a significant increase in the DR score after low- dose RT. It indicated that rather than being a fixed property, the tumor-intrinsic DR score can actually be modulated by sublethal, low-dose RT (Figure 4B-C), suggesting inherent tumor resistance to CAR T cells may also be a dynamic and quantifiable property.

Efficacy related to the timing of RT and CAR T-cell treatment correlates with DR score changes

We hypothesized that the mechanism by which low-dose TTI enhances CAR T-cell efficacy may be related to its ability to temporarily induce a more proapoptotic state of the tumor cell, which is reflected by the DR score. Unfortunately, owing to the numerous proapoptotic molecules induced by RT, definitively proving this hypothesis is impossible by simply knocking out or over-expressing a single gene. Thus, we resorted to association data by temporally assessing the DR score and response to CAR T cells after low-dose RT. Seven days after low-dose RT, we found the DR score had returned to baseline (Figure 5A-B). To test whether the DR score's return to baseline is associated with decreased clinical efficacy at this time point, we treated tumor-bearing mice with low-dose TTI 4 days after tumor injection, then
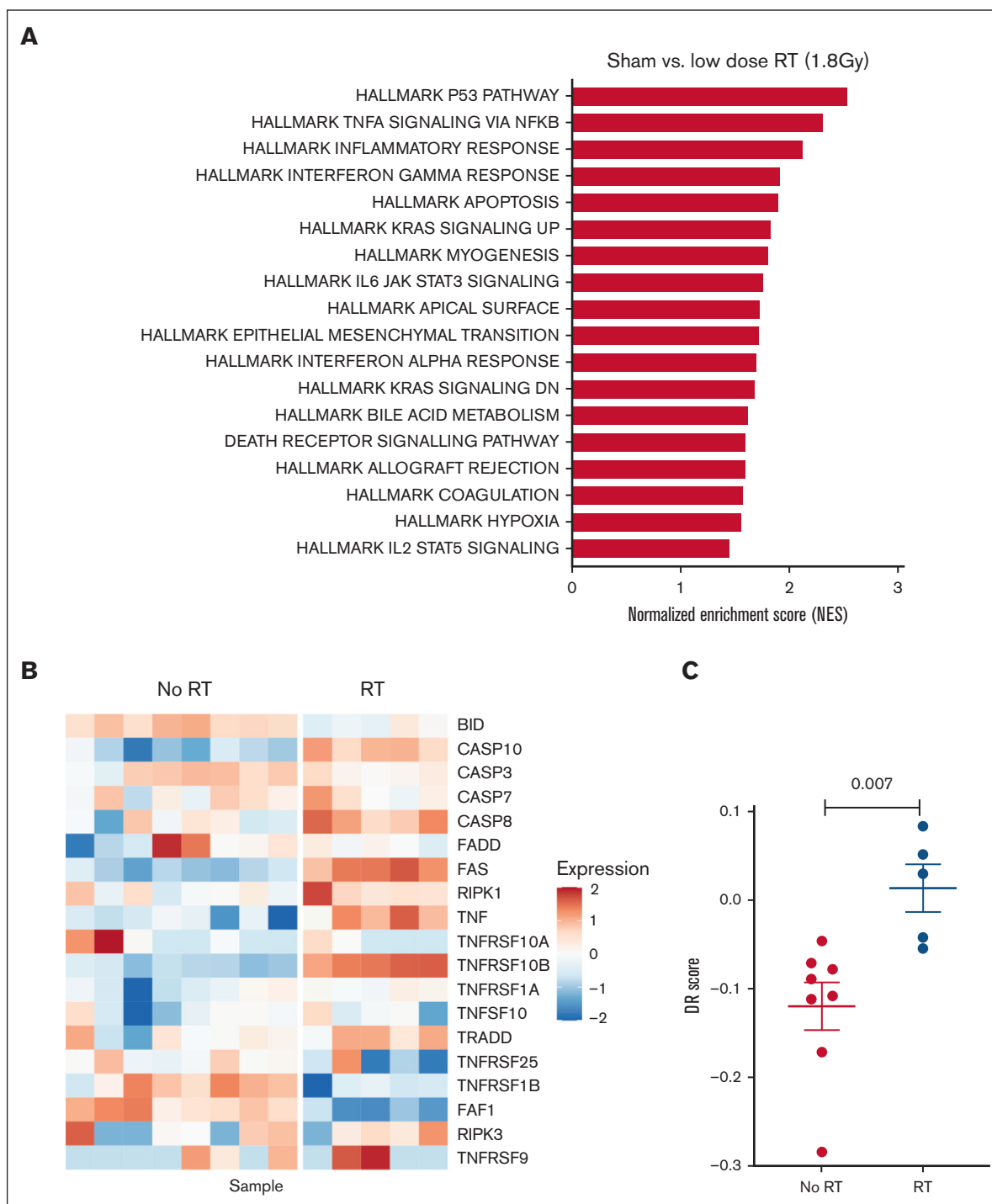
Figure 4. The DR score is significantly increased in tumor cells after low-dose radiation. Nalm6 samples were cultured independently and treated with either low-dose radiation (1.8 Gy, n = 5) or sham (0 Gy, n = 8) treatment, followed 24 hours later by RNA-seq, GSEA, and DR score determination. (A) GSEA of hallmark gene sets and the DR score gene set enriched in 1.8 Gy low-dose radiation compared with sham-treated Nalm6 cells. False discovery rate <5%. (B) Individual samples (columns) and genes (rows) comprising the DR score are shown as a heatmap. (C) DR score charted by treatment condition.

treated them with low-dose CAR T cells either 1 or 7 days later. As observed previously, treatment with CAR T cells 1 day after TTI resulted in significant reductions in tumor burden and improved animal survival, whereas treatment with CAR T cells 7 days after low-dose TTI imparted no therapeutic benefit (Figure 5C-D),

mirroring changes observed in the DR score at these timepoints. One difficulty in setting up this experiment is that no matter how the experiment is designed, treating on different days inevitably results in different tumor burden at the time of treatment, so it is impossible to compare the same E:T ratio in vivo. Owing to the complexity of
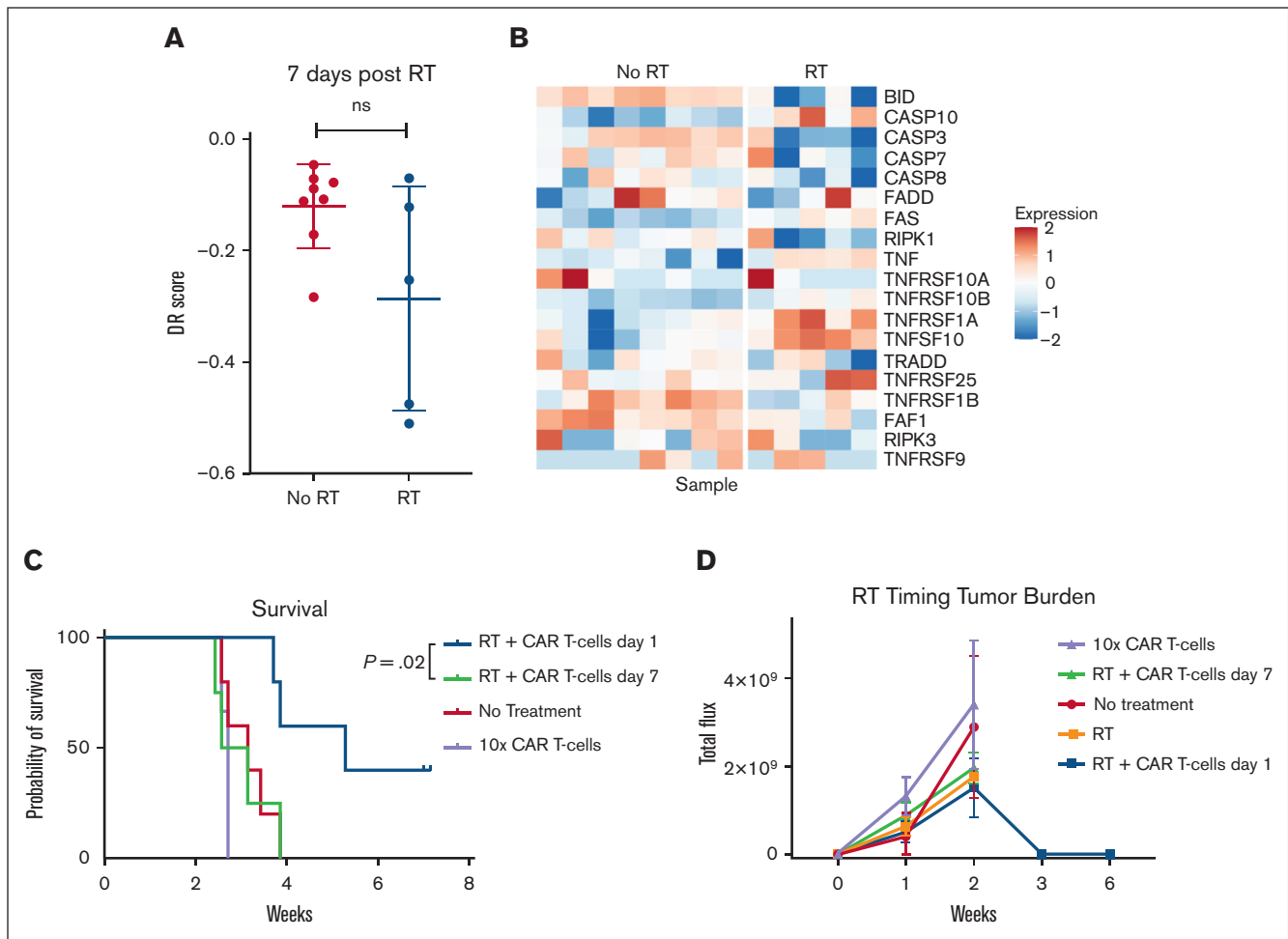
Figure 5. The DR score is dynamic within an individual tumor, its level at the time of CAR T-cell treatment correlates with overall survival and tumor response.
(A) DR score was compared between sham (0 Gy) and low-dose radiation (1.8 Gy) 7 days after the treatment. (B) heatmap of individual samples (columns) and genes (rows) comprising the DR score. (C) Mice injected with Nalm6 tumor cells were treated with low-dose RT on day 3 followed by low-dose CAR T cells either 1 or 7 days later, corresponding with the timepoints of RNA-seq from Figures 5A and 6A. Overall survival (C) and tumor burden (D) of mice in the indicated treatment group shown over time.

both CAR T-cell and RT dynamics in vivo, we also were unconvinced that simpler in vitro models would accurately reflect the in vivo scenario in this context. Thus, although the mechanism is unclear, a definitive interpretation of these in vivo experiments is that delaying CAR T-cell treatment by even 1 week after providing bridging radiation therapy results in a significant loss of survival benefit. This is clinically important because the average patient is treated with CAR T cells weeks after receiving the bridging radiation therapy.[22-24]

Low-dose RT raises poor DR scores to the level of excellent patient responders

Based on our observation that RT-driven alterations in DR score correlate with responses to CAR T-cell therapy in mice, we hypothesized that similar alterations in DR score may correlate with survival in patients. Thus, we independently assessed the DR scores of leukemic cells before and after low-dose RT using RNA-seq and defined a low score as the average baseline score or below, a high score as the average post-RT score or above, and an

intermediate (medium) score that included everything in between the average unirradiated and the irradiated tumor score (Figure 6A). To examine these scores in a clinical context, we examined previously published patient data from 2 multicenter clinical trials of tisagenlecleucel for relapsed pediatric ALL (ClinicalTrials.gov identifiers NCT02435849 and NCT02228096, previously analyzed in[3]), in which all available leukemia-infiltrated BM samples collected before the treatment from patients who had achieved durable CR (CR for >1 year, n = 17) or who did not respond to treatment and had no evidence of CD19 antigen loss (nonresponder, n = 8) were processed for RNA-seq before CAR T-cell therapy.[3] We analyzed the DR score as in the study by Singh et al[3] and plotted the survival of these patients by a low, medium, or high DR score as defined by average pre or post-RT levels (Figure 6B-C). When stratified in this way, we found that patients with a low score (defined by our baseline tumor cells analysis without any RT exposure) exhibited poor long-term survival of ~15%. Patients with pre-CAR–T-cell scores of either medium or high DR (defined by our analysis of tumor cells postlow-dose RT) experienced a long-term overall survival of >85% (Figure 6). These
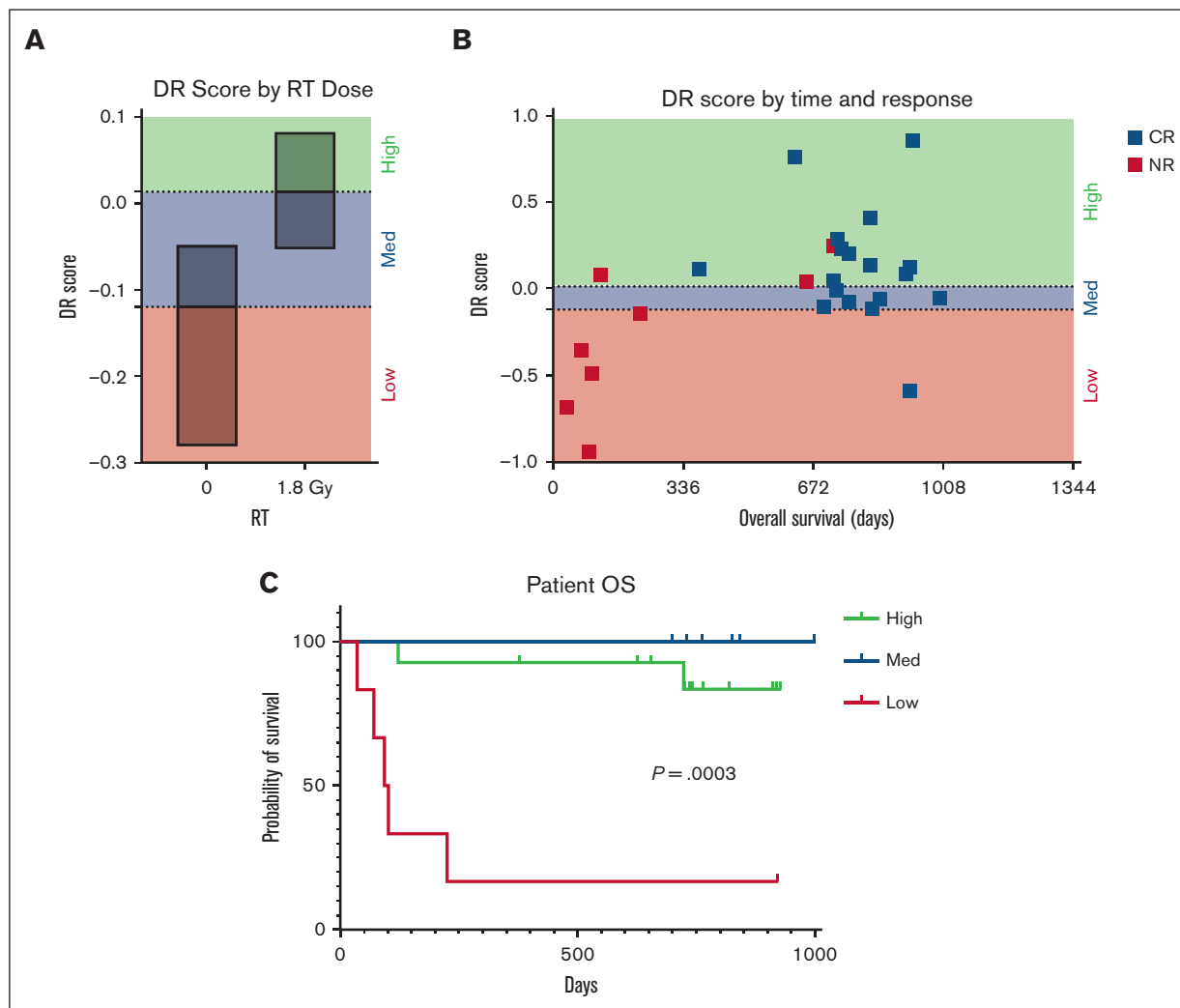
Figure 6. Low-dose RT elevates tumor DR scores to levels exhibited by excellent patient survivors. (A) DR score of tumor cells subjected to sham (0 Gy RT) or low-dose (1.8 Gy) RT grouped into the following 3 categories: low DR score (the average baseline, unirradiated sample), high DR score (the average DR score 1 day after low-dose RT), and medium DR score (between low and high). (B) Overall survival of patients based on the DR grouping in panel A charted over time with nonresponder (NR) and complete responder (CR) time of death or last follow-up charted in red and blue squares, respectively. (C) Overall survival of patients broken down by RT-defined low, med, and high DR scores from panel A.

patients' survival curves happen to correlate well with our preclinical RT studies with the same simple common thread being DR score, but this comparison is only exploratory. In summary, a low DR score, which reflects a state of tumor-intrinsic resistance to CAR T cells, can be raised by low-dose RT, and in separately analyzed patient samples, these differences in scores highly correlate with response and survival.

To examine whether low-dose RT's effect on the DR pathway extends beyond the ALL tumor used in this study and specifically to B-cell lymphoma, we examined 2 additional cell lines, OCI-Ly10 and Raji. OCI-Ly10 is a diffuse large B-cell lymphoma cell line established from the BM of a 44-year-old man with relapsed disease. Raji is a B-cell non-Hodgkin lymphoma (NHL) (specifically, Burkitt lymphoma) derived from an 11-year-old male. Three independently maintained cultures of each were irradiated with low-dose (1.8 Gy) RT and the DR score was determined before and

after RT. We found that the DR score significantly increases in these lymphoma samples after low-dose RT (supplemental Figure 4), suggesting low-dose RT may have the ability to raise the DR score in a range of B-cell malignancies.

Discussion

Determining who will respond to CAR T-cell therapy and who will relapse and understanding how to prevent an individual patient's mechanism of relapse or progression, remains a large challenge on which the lives of most patients who undergo CAR T-cell therapy depend. Tumor-intrinsic properties are important dictators of whether a tumor cell will be efficiently killed by a CAR T cell, and this impacts the ultimate efficacy and survival of treated patients. Solid and liquid tumors may possess different tumor-intrinsic determinants of CAR T-cell resistance.[25] In leukemia, the levels of proapoptotic molecules, collectively called the DR score, predict

the ability of CAR T cells to eliminate tumor in vitro, in vivo, and in patients.[3] Because this score is a tumor-intrinsic property, we assumed it would be a stable characteristic of the particular tumor. However, we show that the DR score can be modulated by low-dose, sublethal irradiation and may also be modulated by other potential preconditioning therapies. Low-dose TTI preconditioning, which raises a low DR tumor to an intermediate or high level, can bring mice to 100% CR and survival in response to an otherwise inadequate dose of CAR T cells. Furthermore, the survival difference of these mice mirrors retrospective patient outcomes with baseline low or medium/high DR scores, which have a 5-year survival of <20% vs >85%, respectively.

The implication that a readily determinable tumor-intrinsic property that predicts survival after CAR T-cell therapy can be modulated before the therapy provides an avenue for identifying in advance who is unlikely to respond to CAR T-cell therapy and provides a clinically actionable mechanism to attempt to improve response and survival in this subgroup. First, it will need to be determined whether low-dose RT increases the DR score in patients. If it does, it will be interesting to assess whether the patients identified to have a low DR score, who then undergo low-dose TTI before CAR T-cell therapy, will have an ultimate survival that correlates with their new DR score at the time of treatment as is suggested by the mouse data or whether their survival will still be more consistent with their baseline DR score.

Targeting focal areas of bulky disease with radiation before CAR T-cell therapy is the current most common clinical practice[22-24] because high-dose radiation cannot generally be safely delivered to all sites of disseminated disease. We find that targeting an area of bulky disease in mice with disseminated disease results in significantly increased reduction in tumor volume as expected yet a significantly worse survival compared with targeting all areas of disease with a much lower RT dose before the CAR T-cell delivery. In these studies, pretreatment tumor quality is a more important determinant than pretreatment tumor volume in response to CAR T cells. Thus, it may be beneficial to pursue strategies that target all areas of disease with lower doses in future clinical practice. A clinical trial is currently underway testing whether targeting all tumor (TTI) before CAR T-cell therapy is safe and effective (NCT05574114).

Most of this work was done with a B-cell ALL tumor line, largely because we wanted to model truly disseminated and advanced disease, but RT is more commonly used with CAR T cells in lymphoma, particularly in NHL in the setting of salvage therapy. Thus, we tested 2 NHL cell lines for the ability of low-dose RT to increase the DR score and found that it does by a similar magnitude as the leukemia line. Although it is rational to expect that both B-cell leukemia and lymphoma are killed by similar mechanisms, whether the DR score predicts response to CAR T cells in patients with lymphoma as well remains to be examined.

When the tumor is disseminated systemically and the entire body is irradiated before the CAR T-cell therapy, it is difficult to discern whether the ensuing effects are due to a response of the tumor, host, or both. Our experiments in which tumor, mouse, or both were irradiated separately before CAR T-cell treatment suggest that the beneficial effect of low-dose RT is entirely because of the effects on the tumor, as irradiating the tumor plus the host had no further benefit over tumor alone. Thus, we prefer the term TTI rather than total body irradiation, as the consequential target in this context is tumor cells

rather than the healthy body. It is possible that for tumors that take longer to eradicate or CAR T cells that take longer to expand (eg, BBz vs 28z), low-dose RT–mediated CAR T-cell engraftment may result in additional host-related beneficial effects over time.

It is important to note that in addition to efficacy considerations, the use of RT before CAR T-cell therapy may ameliorate CAR T-cell–related toxicity through cytoreduction, for which focal RT can be very effective. For example, in patients with NHL, bridging focal RT before CAR T-cell therapy significantly improved established high-risk parameters of post-CAR-T therapy progression, including in-field median metabolic tumor volume, maximum positron emission tomography (PET) standard uptake value, target tumor diameter, and lactate dehydrogenase.[13] CAR T-cell toxicity, such as immune effector cell-associated neurotoxicity syndrome and cytokine release syndrome, is difficult to quantify in mice and was not assessed in these studies, but optimal RT dose, timing, and field size for maximizing CAR T-cell efficacy may or may not be the same as for minimizing CAR T-cell toxicity.

Current "bridging" radiation strategies, in which patients are treated with RT before receiving their CAR T cells, generally receive radiation within 1 month of completing CAR T-cell infusion.[22-24] Our data suggest that to take advantage of synergistic DR pathway induction by low doses of RT, CAR T-cell delivery should ideally occur very soon after RT, such as the following day.

The effect of low-dose radiation temporarily increasing the proapoptotic state of the tumor and resulting in greater tumor killing may also affect the CAR T cells, which we did not study. However, we know that tumor cells deficient of key proapoptotic molecules that are more resistant to CAR T-cells lead to CAR T-cell dysfunction, such that the same CAR T cells eventually become less capable of killing normally sensitive tumor cells.[3]

Bystander CAR T-cell killing, in which an activated CAR T cell kills its target cell as well as neighboring tumor cells that may or may not express the CAR target antigen, may be difficult to achieve in liquid tumors such as ALL where tumor cells are physically more dispersed. Consistent with this, in contrast to mice bearing antigen-positive tumor treated with TTI followed by CAR T cells in which >50% of mice survived, the presence of only 5% antigen-negative ALL tumor cells was enough to result in 100% death from disease, whether or not the tumors were exposed to low-dose TTI before CAR T-cell therapy, indicating that the major benefit of low-dose TTI in this leukemia model in immunodeficient mice is target antigen-dependent.

A major limitation of these studies is that we do not have the ability to genetically express the array of apoptotic members of the DR pathway at constant levels to achieve a defined DR score within a tumor population. This would be required to determine whether the direct modulation of the DR pathway is mechanistically responsible for the effects of low-dose RT. Without this, our mechanistic results are limited to correlations. Other caveats of these studies include the exclusive use of immunodeficient mice; immunocompetent systems may have additional complicating factors. However, we do definitively show that (1) the DR score, which quantifies levels of apoptotic molecules that mediate the intrinsic resistance of leukemia cells to CAR T-cell killing, can be increased by low-dose radiation; (2) low-dose total body irradiation 1 day before the otherwise inadequate CAR T-cell therapy significantly improves survival in vivo through a direct effect on the tumor (thus advocating

for TTI); (3) low-dose TTI has more impact on long-term survival after CAR T-cell therapy than focal high-dose RT, despite having less systemic direct tumor killing benefit; and (4) delaying CAR T-cell therapy from 1 day to 1 week after TTI results in worse survival, suggesting that the timing is important.

Authorship

Contribution: C.J.D. conceptualized the experiments and wrote the manuscript; A.B.K., S.-Y.C., S.K., E.K., and N.A. performed mouse and in vitro experiments; C.C. performed RNA extractions; J.Z., J.S., and M.I. performed RNA-seq analyses; and N.S. and J.C.Y. provided experimental and manuscript feedback.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: A.B.K., 0000-0002-6425-4566; S.-Y.C., 0000-0002-7496-5185; M.I., 0000-0001-5490-2374; J.Z., 0000-0002-9117-2793; C.J.D., 0000-0001-7645-5798.

Correspondence: Carl J. DeSelm, Department of Radiation Oncology, Bursky Center for Human Immunology and Immunotherapy, Washington University School of Medicine, 660 S Euclid Ave, Campus Box 8224, St. Louis, MO 63110; email: deselmc@wustl.edu.

References

1. Park JH, Bienenfeld A, Orlow SJ, Nagler AR. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med*. 2018;19(3): 449-455.

2. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018; 378(5):439-448.

3. Singh N, Lee YG, Shestova O, et al. Impaired death receptor signaling in leukemia causes antigen-independent resistance by inducing CAR T-cell dysfunction. *Cancer Discov*. 2020;10(4):552-567.

4. Jain MD, Ziccheddu B, Coughlin CA, et al. Whole-genome sequencing reveals complex genomic features underlying anti-CD19 CAR T-cell treatment failures in lymphoma. *Blood*. 2022;140(5):491-503.

5. Vercellino L, Di Blasi R, Kanoun S, et al. Predictive factors of early progression after CAR T-cell therapy in relapsed/refractory diffuse large B-cell lymphoma. *Blood Adv*. 2020;4(22):5607-5615.

6. Jacoby E, Bielorai B, Hutt D, et al. Parameters of long-term response with CD28-based CD19 chimaeric antigen receptor-modified T cells in children and young adults with B-acute lymphoblastic leukaemia. *Br J Haematol*. 2022;197(4):475-481.

7. DeSelm C. The current and future role of radiation therapy in the era of CAR T-cell salvage. *Br J Radiol*. 2021;94(1127):20210098.

8. Dahl H, Eide DM, Tengs T, et al. Perturbed transcriptional profiles after chronic low dose rate radiation in mice. *PLoS One*. 2021;16(8): e0256667.

9. Hamieh M, Dobrin A, Cabriolu A, et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. *Nature*. 2019;568(7750): 112-116.

10. Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet*. 2015;385(9967):517-528.

11. DeSelm C, Palomba ML, Yahalom J, et al. Low-dose radiation conditioning enables CAR T cells to mitigate antigen escape. *Mol Ther*. 2018;26(11): 2542-2552.

12. Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. 2017; 543(7643):113-117.

13. Hubbeling H, Silverman EA, Michaud L, et al. Bridging radiation rapidly and effectively cytoreduces high-risk relapsed/refractory aggressive B cell lymphomas prior to chimeric antigen receptor T cell therapy. *Transplant Cell Ther*. 2023;29(4):259.e1.

14. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.

15. Liberzon A, Birger C, Thorvaldsdottir H, et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst*. 2015;1(6):417-425.

16. Zhao Z, Condomines M, van der Stegen SJ, et al. Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. *Cancer Cell*. 2015;28(4):415-428.

17. Tomita Y, Sachs DH, Sykes M. Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood*. 1994;83(4):939-948.

18. Benmebarek MR, Karches CH, Cadilha BL, Lesch S, Endres S, Kobold S. Killing mechanisms of chimeric antigen receptor (CAR) T cells. *Int J Mol Sci*. 2019;20(6):1283.

19. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*. 1993; 362(6423):847-849.

20. Lowe SW, Bodis S, McClatchey A, et al. p53 status and the efficacy of cancer therapy in vivo. *Science*. 1994;266(5186):807-810.

21. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;35(4):495-516.

22. Sim AJ, Jain MD, Figura NB, et al. Radiation therapy as a bridging strategy for CAR T cell therapy with axicabtagene ciloleucel in diffuse large B-cell lymphoma. *Int J Radiat Oncol Biol Phys*. 2019;105(5):1012-1021.

23. Pinnix CC, Gunther JR, Dabaja BS, et al. Bridging therapy prior to axicabtagene ciloleucel for relapsed/refractory large B-cell lymphoma. *Blood Adv*. 2020;4(13):2871-2883.

24. Wright CM, LaRiviere MJ, Baron JA, et al. Bridging radiation therapy prior to commercial chimeric antigen receptor T-cell therapy for relapsed/refractory aggressive B-cell lymphoma. *Int J Radiat Oncol Biol Phys*. 2020;108(1):178-188.

25. Larson RC, Kann MC, Bailey SR, et al. CAR T cell killing requires the IFNgammaR pathway in solid but not liquid tumours. *Nature*. 2022;604(7906): 563-570.