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Supplementary Information for

Radiation-induced Neoantigen Broaden the Immunotherapeutic Window of Cancers with Low Mutational Burden

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Materials and Methods

Mice. Female WT and *Rag2*$^{-/-}$ mice on a 129S4 background were bred in animal barrier facility. Mice used in the study were between 8-12 weeks of age and maintained in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University in St. Louis.

Tumors. KP sarcoma cells used in this study were generated in the Tyler Jacks' lab in female 129S4 mice, as previously described. Irradiated KP sarcoma cells were generated using a cell irradiator to deliver 2, 4, or 9 Gy doses of ionizing radiation *in vitro.* All irradiated KP sarcoma cells were maintained *in vitro* in culture until viability reached >95%. Limited dilution cloning of the irradiated KP sarcoma cell lines were performed as previously described. KP.PADRE sarcoma cells used in this study were generated from the KP sarcoma cell line in which PADRE was ectopically expressed using the retroviral vector with GFP (RV-GFP), gifted from Dr. Kenneth Murphy, following digestion of the RV-GFP with BglII and XhoI.

Tumor cells were maintained *in vitro* in RPMI media (Hyclone) supplemented with 10% FCS (Hyclone). 1.0×10^6 tumour cells were injected subcutaneously. Tumour growth was monitored at least two times a week using a digital calliper. The mean of long and short diameters was used for tumour growth curves. Mice were euthanized when tumours were > 2 cm or severely ulcerated. No statistical methods were used to predetermine sample size. However, adequate sample size was chosen based on extensive previous work with this animal model. No randomization or blinding was performed. Ex vivo analyses were performed as previously described[25]. For detection of PD-L1 and MHC class I expression in vitro, tumor cells were treated with 300 U ml^{-1} murine IFN-γ for 48-72 hours unless otherwise indicated. Cell lines were authenticated by NGS in our lab and routinely tested for mycoplasma infection.

Tumor resection/rechallenge. $1x10^6$ tumor cells were injected subcutaneously. Tumor growth was monitored using a digital calliper. Tumors were resected following our IACUC protocol for surgical procedures prior to tumors reaching 1cm. Greater than 30 days following surgical resection mice were challenged with $1x10^6$ tumor cells on the opposite flank. Challenged tumors were monitored via calliper measurements and mice were euthanized when tumors were > 2 cm in size.

Antibodies. Antibodies used for comprehensive flow cytometry analysis of cell subsets in tumours are listed in a previous publication. Additionally, the following antibodies were used anti-H2-K^b (AF6-88.5), anti-H2-D^b (KH-95), Dead cells were stained with Po-Pro-1 or NIR (Invitrogen). For *in vivo* checkpoint blockade treatment we used rat IgG2a anti-PD-1 (RMP1-14) and rat IgG2a anti-CTLA-4 (9D9). Mouse IgG2a anti-human CD3 (OKT3) was used as a control. All antibodies were injected 200 µg i.p. per dose. Injections were on days 3, 6, and 9. *In vivo* CD4$^+$/CD8$^+$ cell depletion was performed as previously described[24]. Antibodies were purchased from Biolegend, BD Bioscience, Bio X Cell, and Leinco Technologies, or kindly gifted from Bristol-Myers Squibb.

Tetramer staining against radiation neoantigens. H-2kb and H-2Db monomers were purchased from the Center for Human Immunology and Immunotherapy Program (CHiiPs). Tetramerization of monomers was performed as described previously. Briefly, monomers containing a photocleavable peptide were saturated with short peptides containing the predicted epitope cores for the radiation antigens, and left under UV light for 1 hr. These radiation antigen monomers were tetramerized upon addition of streptavidin APC and streptavidin PE, so that all tetramer positive populations stained dual color.

ELISPOT. $CD8^+$ T cells were enriched from tumors using the Miltenyi mouse $CD8^+$ enrichment kit following manufacturer's protocols. 10,000 TIL-derived T cells were stimulated with 500,000 splenocytes isolated from naïve mice pulsed with 1μM 15-mer peptide. Cells were stimulated overnight in anti-murine IFNγ-coated ELIspot plates (Immunospot). Plates were developed following manufacturer's protocol and spots were quantified using a CTL ImmunoSpot S6 Universal machine and Professional 6.0.0 software.

Retroviral expression of radiation-induced neoantigens. The retroviral vector with GFP (RV-GFP) was gifted from Dr. Kenneth Murphy. Following digestion of the RV-GFP with BglII and XhoI, gene blocks containing 30-mer sequences encompassing the irradiation induced mutations were ligated into the vector. After 48 hours of retroviral production[24], the supernatant was subsequently used for transfection with tumour cells. Tumors were subsequently sorted for GFP expression to maintain populations all containing the radiation antigen of interest.

In vitro cytotoxicity assay. LDH ELISA kit ordered from Promega used to test the percent cytotoxicity based on release of LDH. Protocol and mathematical analysis done following the available protocol online. Splenocytes from KP.P.4G1 tumor bearing mice were isolated as effector cells, plated at $10x10^6$ and cocultured with naïve splenocytes peptide pulsed with all antigens identified in KP.P.4G1, individually.

cDNA-CapSeq and mutation calling. Following data generation and alignment of Illumina paired-end reads to the mouse reference genome sequence, we used our somatic variant analysis pipeline to call somatic variants from the comparison of cDNA-CapSeq data to matched normal exome data from the mouse in which the tumor was generated. Mutation calling was performed using a combination of three variant callers – Samtools[39,40], Sniper[41], and VarScan[42,43] as previously described[37]. Missense mutations were shortlisted based on specific coverage and variant allele fraction (VAF)-based filters. Firstly, to only target variants in the expressed genes, we restricted our subsequent analysis to genes with expression levels (in fragments per kilobase of exon per million reads mapped (FPKM)) values of > 1, and wherein we could identify evidence that the mutant allele was expressed. Secondly, we filtered out any variants with normal coverage <=5× and normal VAF of >=2%. Additionally, only variants with tumor coverage of >=10× with a VAF of >=25% were considered. Lastly, this filtered list of variants was visually inspected to ascertain the aligned reads in IGV as a means of reducing false positive mutations[37].

Inactivated tumor vaccines. For irradiated-tumor cell vaccines, single antigen expressing KP.PADRE tumor sarcoma cells were lethally irradiated with 10Gy and 500,000 cells were injected subcutaneously into 129S4 mice. KP.PADRE.4G1 challenge following vaccination occurred on the opposite flank. No statistical methods were used to determine group size. Tumor growth was measured by calipers and individual growth curves are represented as the average of two perpendicular diameters. Tumor measurements were performed blinded to treatment group. In accordance with our IACUC-approved protocol, maximal tumor diameter was 20 mm in one direction, and in no experiments was this limit exceeded.

Statistical analysis. Prism 6 (GraphPad Software, Inc) was used for statistical analysis. No samples or animals were excluded from the analysis. Comparison between samples were performed using an unpaired, two-tailed Student's t-test or one-way ANOVA followed by multiple comparison test. Welch's corrections were used when variances between groups were unequal. $p < 0.05$ was considered as statistically significant.

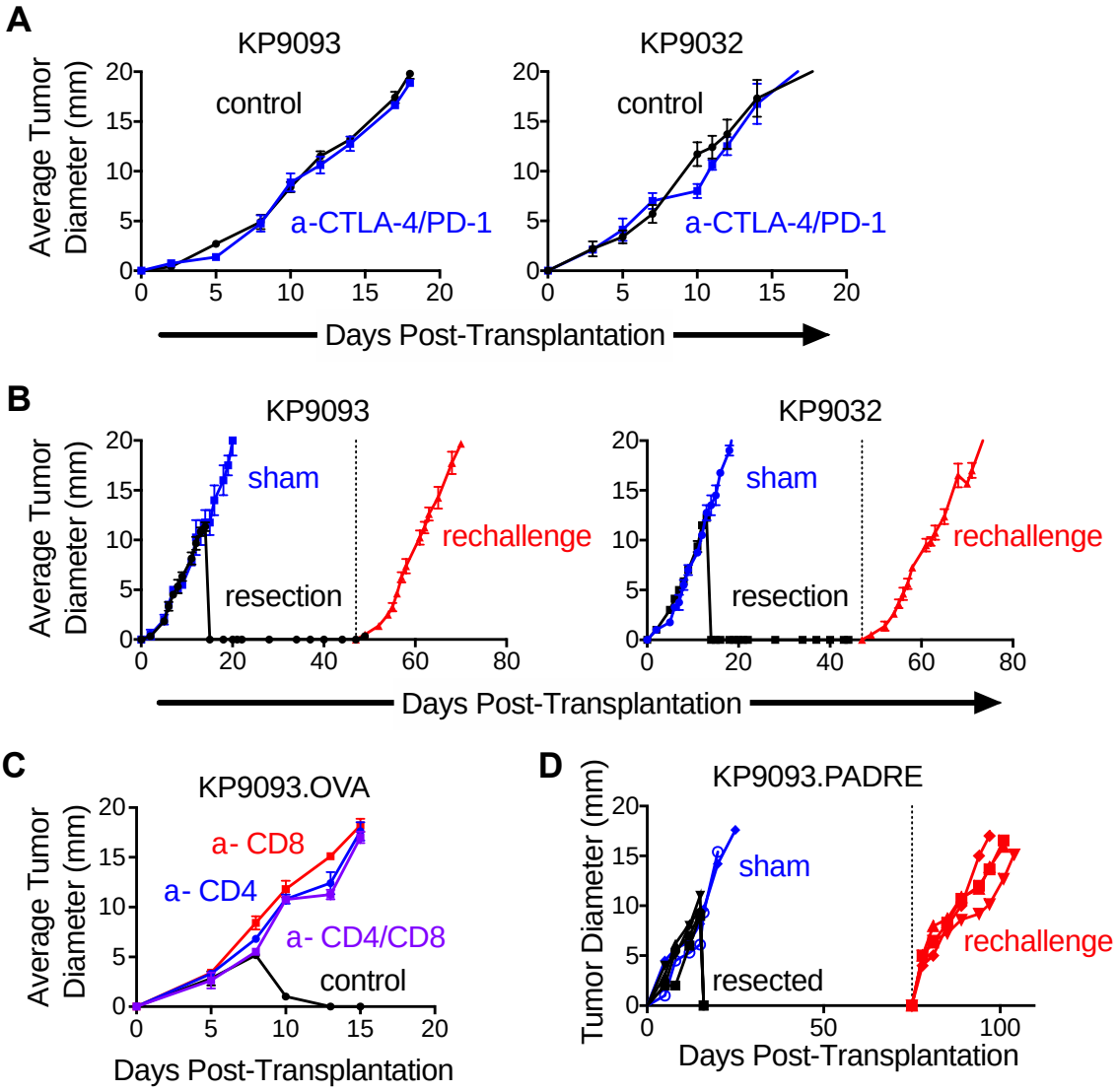

Fig. S1. Expression of full-length OVA but not PADRE is sufficient to drive rejection of nonimmunogenic oncogene-induced sarcoma cells. A, Tumor growth kinetics of parental KP9093 and KP9032 tumor cell lines in WT animals treated with control mAb (black) versus dual anti-CTLA-4/anti-PD1-treatment (blue). Representative data from multiple biologic repeats is shown as average tumor diameter ± SEM, n=5 per group. B, Mice were implanted with KP9093 or KP9032 tumor cell lines followed by sham surgery (blue) or surgical resection (black) of established tumors and rechallenge (red) with the same tumor cell line. Representative data is shown as average tumor diameter ± SEM, n≥3 per group. C, WT mice were implanted with KP9093 cells ectopically expressing full-length OVA and treated with control mAb (black), anti-CD8 depleting antibody (red), anti-CD4 depleting antibody (blue), or both anti-CD4 and anti-CD8 depleting antibodies (purple). Representative tumor growth curves show average tumor diameter ± SEM, n≥3 per group. D, WT mice were implanted with KP9093.PADRE cells followed by sham surgery (blue) or surgical resection (black) of established tumors and rechallenge (red) with the same tumor cell line. Representative data is shown as individual tumor growth curves, n=3 per group.

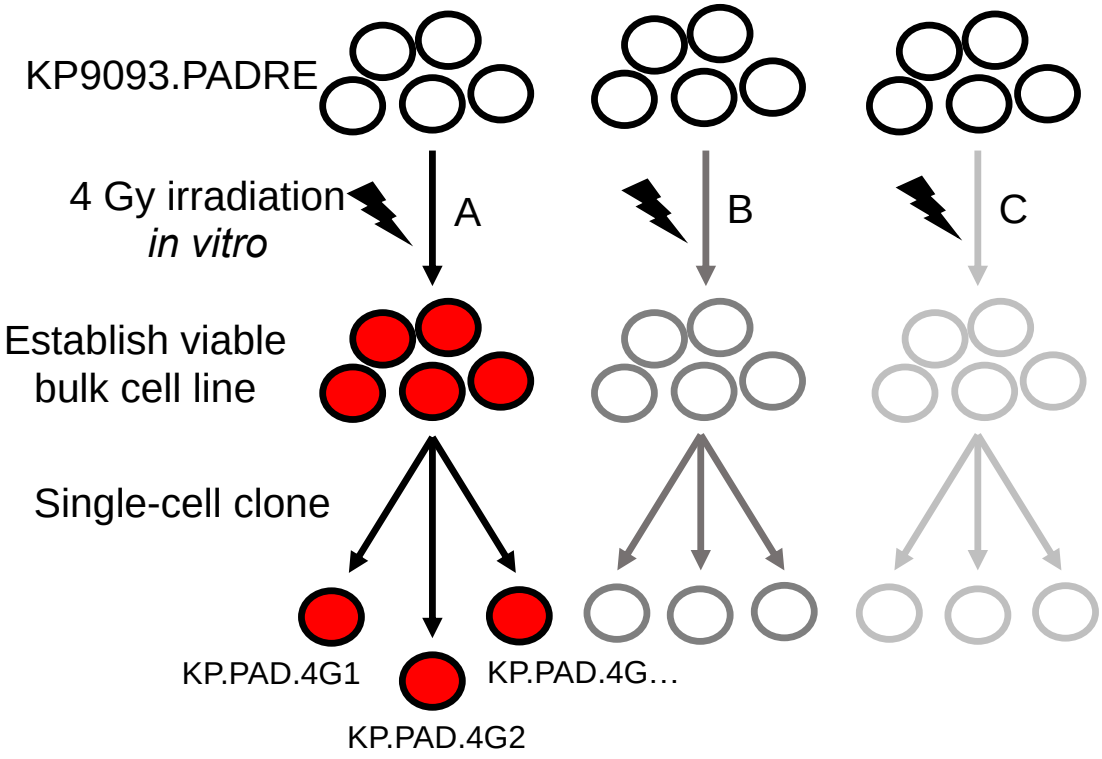

Fig. S2. Schematic of irradiated cell line generation: KP9093.PADRE cells were irradiated with 2, 4 or 9 Gy (4 Gy is shown as an example). Irradiation of the KP9093.PADRE line was performed during several, independent events. Following irradiation, viable bulk cell lines were established, and single cell cloning was performed to obtain multiple clonal lines from each irradiation event. The clones obtained from irradiation event A are mutationally/antigenically related to one another because they were derived from the same irradiated bulk cell population. The clones obtained from irradiation event C, for example, are mutationally/antigenically unrelated to the clones obtained from irradiation event A because they were derived from an independently irradiated bulk cell population.

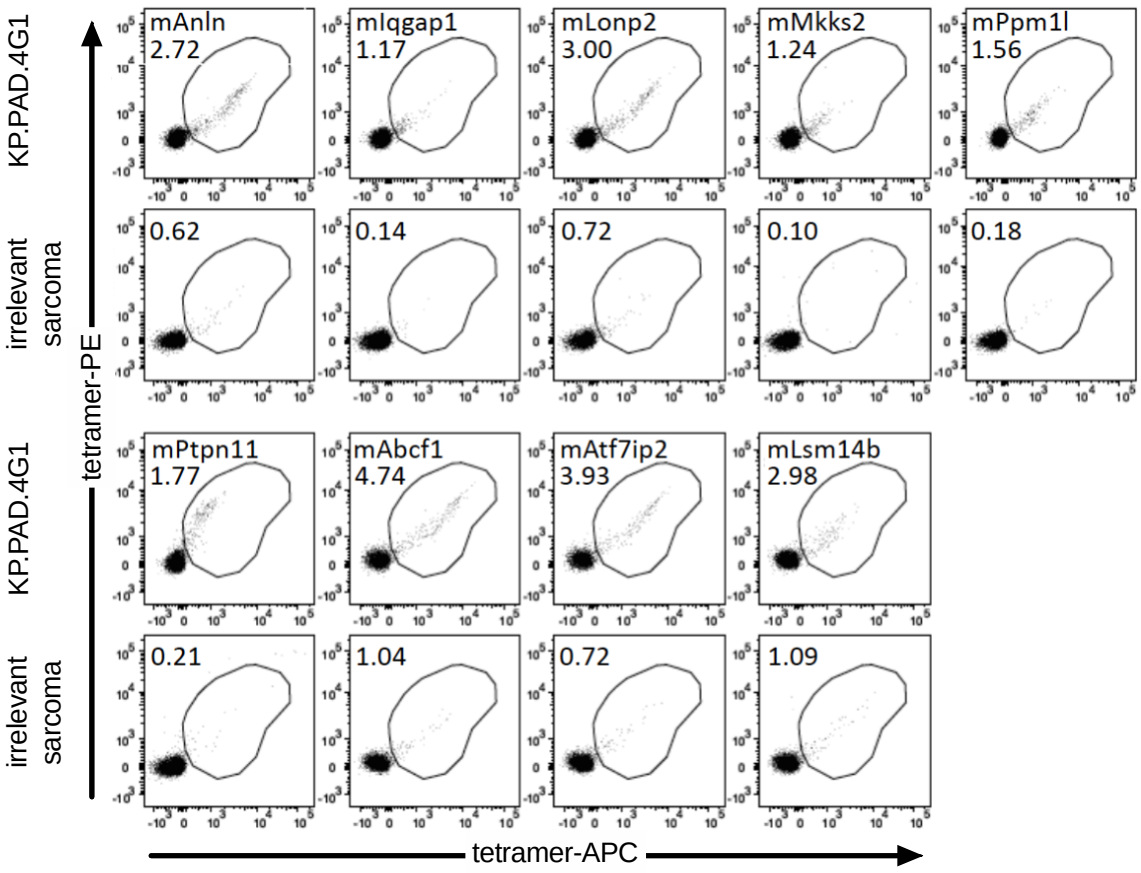

Fig. S3. Tumor cell clone KP.PAD.4G1 contains unique radiation-induced mutations that lead to de novo anti-tumor CD8+ T cell responses. TIL was harvested from KP.PAD.4G1 or irrelevant MCA-induced T3 tumors 11 days post transplantation and H-2-K^{b} p-MHCI tetramer staining was performed for the indicated radiation-induced mutations expressed in KP.PAD.4G1. Representative flow cytometry plots are shown. Numbers indicate the percentage of tetramer-positive cells previously gated on viable, Thy1.2^{+} CD8^{+} cells.