

# Radiotherapy and CD40 Activation Separately Augment Immunity to Checkpoint Blockade in Cancer



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## Abstract

Immunotherapy in pancreatic ductal adenocarcinoma (PDA) remains a difficult clinical problem despite success in other disease types with immune checkpoint blockade (ICB) and chimeric antigen receptor T-cell therapy. Mechanisms driving immunosuppression and poor T-cell infiltration in PDA are incompletely understood. Here, we use genetically engineered mouse models of PDA that recapitulate hallmarks of human disease to demonstrate that CD40 pathway activation is required for clinical response to radiotherapy and ICB with  $\alpha$ CTLA-4 and  $\alpha$ PD-1. The combination of an agonist  $\alpha$ CD40 antibody, radiotherapy, and dual ICB eradicated irradiated and unirradiated (i.e., abscopal) tumors, generating long-term immunity. Response required T cells and also short-lived myeloid cells and was dependent on the long noncoding RNA myeloid regulator *Morbid*. Using unbiased random forest machine learning, we built unique, contextual signa-

tures for each therapeutic component, revealing that (i) radiotherapy triggers an early proinflammatory stimulus, ablating existing intratumoral T cells and upregulating MHC class I and CD86 on antigen-presenting cells, (ii)  $\alpha$ CD40 causes a systemic and intratumoral reorganization of the myeloid compartment, and (iii) ICB increases intratumoral T-cell infiltration and improves the CD8 T-cell:regulatory T-cell ratio. Thus,  $\alpha$ CD40 and radiotherapy nonredundantly augment antitumor immunity in PDA, which is otherwise refractory to ICB, providing a clear rationale for clinical evaluation.

**Significance:** Radiotherapy and  $\alpha$ CD40 disrupt key links between innate and adaptive immunity, ameliorating resistance to immune checkpoint blockade in pancreatic cancer via multiple cellular mechanisms. *Cancer Res*; 78(15); 4282–91. ©2018 AACR.

## Introduction

Despite the success of cancer immunotherapy in many disease types, pancreatic ductal adenocarcinoma (PDA) is notably unresponsive to immune checkpoint blockade (ICB) with  $\alpha$ PD1 and/or  $\alpha$ CTLA4 (1–4). PDA is projected to become the second leading cause of cancer-related deaths in the United States by 2030, with a 5-year overall survival of less than 10% (5, 6). Despite advances in cancer therapy, the overall mortality for PDA remains high, which is attributable to late diagnosis, early met-

astatic spread, and poor response to therapy (7–9). Thus, novel treatment strategies are needed to improve outcomes.

Unlike other cancers that are responsive to ICB (e.g., melanoma), the PDA tumor microenvironment harbors dense desmoplasia and an immunosuppressive infiltrate that typically excludes CD8 T cells and represents a site of acquired immune privilege (10–14). Whereas the total predicted load of classically defined neoepitopes does not correlate with cytolytic immune activity in PDA (15), the presence of "high-quality" tumor neoepitopes and CD8 infiltration positively correlates with long-term survival in resectable patients (16, 17). Thus, novel therapeutic strategies should be aimed at overcoming these mechanisms of resistance to promote a more favorable tumor microenvironment and antitumor response.

Understanding the potential of combining ICB with chemotherapy, radiotherapy, and/or immunotherapy is therefore a high priority in PDA. Radiotherapy, first understood to act locally via DNA damage, is also immunomodulatory (18). Using a preclinical mouse model of metastatic melanoma, we previously demonstrated that combining radiotherapy with  $\alpha$ PD1 and  $\alpha$ CTLA4 ICB improved response through distinct mechanisms, and we correlated these findings in a phase I/II clinical trial (19). In PDA, we and others have demonstrated that  $\alpha$ CD40 agonist can activate antitumor myeloid cells and, in combination with gemcitabine and nab-paclitaxel,  $\alpha$ CD40 triggers T-cell immunity in PDA mouse models (20–22). Thus, we hypothesized that combining radiotherapy,  $\alpha$ CD40, and ICB would generate immunity in PDA via multiple, nonredundant cellular mechanisms.

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Here, we used the genetically engineered KrasLSL<sup>G12D</sup>-Trp53LSL<sup>R172H</sup>-Pdx1-Cre (KPC) mouse model that recapitulates the key genetic, stromal, and immunosuppressive features of human PDA (10, 23). The KPC model was used to determine the contribution to response of each therapeutic component (radiotherapy,  $\alpha$ CD40, and  $\alpha$ CTLA4/ $\alpha$ PD1 ICB). Our results indicate that ICB alone is ineffective, but the addition of radiotherapy and  $\alpha$ CD40 overrides resistance via short-lived myeloid cells and CD4/8 T cells in a manner independent of canonical innate activation pathways. Furthermore, using unbiased machine learning to understand the immune response, we identify that each therapeutic component nonredundantly alters the tumor microenvironment.

## Materials and Methods

### Cell lines and tissue culture

Mouse KPC pancreatic cancer cell line 4662 (KPC.4662) was derived from single-cell suspensions of PDA tissue from KPC mice as described previously (24), and the C57BL/6 background of KPC mice was confirmed using DartMouse (Geisel School of Medicine at Dartmouth, Hanover, NH) as described previously (21). B16-F10 was obtained from ATCC. Cell lines were determined to be pathogen free using the Infectious Microbe PCR Amplification Test and authenticated by the Research Animal Diagnostic Laboratory at the University of Missouri (Columbia, MO). Cell lines were used within 3 weeks of being thawed. KPC.4662 and B16-F10 cells were cultured at 37°C in DMEM containing 10% heat-inactivated FBS (Gemini Bio Products), 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mmol/L L-glutamine.

### Mouse strains

Murine models were on the C57BL/6J background and were obtained from The Jackson Laboratory and/or bred at the University of Pennsylvania (Philadelphia, PA; see Supplementary Methods). Mice were 5 to 8 weeks old at the time of tumor injection and strains were a mixture of male and female. All mice were housed under pathogen-free conditions at the University of Pennsylvania. Animal protocols were approved by the Institute of Animal Care and Use Committee at the University of Pennsylvania.

### Tumor models

KPC.4662 and B16-F10 cells at approximately 80% confluency and >90% viability were prepared for injection. For subcutaneous tumors, mice were injected with  $4 \times 10^5$  KPC.4662 cells or  $5 \times 10^5$  B16-F10 cells on days 0 and 2 on the right and left flanks, respectively. Cells were injected in 100  $\mu$ L DMEM or, for B16-F10, 50% growth factor-reduced Matrigel (BD Biosciences). For orthotopic tumors, mice were injected with  $1.25 \times 10^5$  KPC.4662 cells in 25  $\mu$ L of DMEM.

### Orthotopic model

A transverse 10-mm laparotomy incision was made in anesthetized mice and KPC.4662 cells were injected into the thickest portion of the pancreas. Visual blebbing confirmed successful implantation. Tumor cells were injected with 2  $\mu$ L of lipiodol (Guerbet; gift from Gregory Nadolskim and Michael Soulen, University of Pennsylvania), an oil-based, radiopaque contrast agent that allowed for tumor site visualization via CT. Flow

cytometric analysis of tumor immune infiltrate confirmed that lipiodol had no immunomodulatory properties. Following tumor cell injection, a cotton swab was placed over the injection site for 1 minute to limit cell leakage into the peritoneal cavity. Gross dissection of the peritoneum 16 days after tumor injection showed intact pancreatic tumors with no peritoneal studding. Disintegrating Polymend MT 5-0 sutures were used to perform a double layer closure of the peritoneal cavity and epidermal layer. Mice were monitored after surgery and given a dose of 0.1 mg/kg buprenorphine SR (ZooPharm) intraperitoneally every 4 to 6 hours for 72 hours.

### KrasLSL<sup>G12D</sup>-Trp53LSL<sup>R172H</sup>-Pdx1-Cre model

KPC mice were evaluated for spontaneous tumor development via ultrasound every week using a Vevo 2100 Imaging System with 55 MHz MicroScan Transducer (Visual Sonics). Mice with tumors  $\geq 30$  mm<sup>3</sup> were enrolled within 24 hours of baseline imaging using blocked randomization, and tumors were visualized for radiotherapy via laparotomy and lipiodol injection as described for the orthotopic model. Mice were designated as responders if tumor progression was <25% compared with baseline 14 days after initiation of therapy.

### Tumor measurements

Electronic calipers were used to measure subcutaneous tumor diameter. Tumor volumes were calculated as: (length \* width<sup>2</sup> \* 0.52). Length was defined as the longest dimension and width was defined as perpendicular to length. Orthotopic pancreatic tumors were measured every 2 weeks using a Vevo 2100 Imaging System with 55 MHz MicroScan Transducer (Visual Sonics). Contours were drawn on tumor images to calculate tumor volumes using Visual Sonics software.

### Radiotherapy

All radiotherapy was delivered using the Small Animal Radiation Research Platform (SARRP; XStrahl) as described previously (19). A dose of 20 Gy was directly targeted to the tumor using a 0.5 cm<sup>2</sup> collimator rotated along a 180-degree arc during delivery to minimize exposure of surrounding normal tissue.

### In vivo antibodies

*In vivo* antibodies were injected intraperitoneally at 200  $\mu$ g per dose, with the exception of  $\alpha$ CD40 that was administered at 100  $\mu$ g per dose and include:  $\alpha$ CD4 (GK1.5),  $\alpha$ CD40 (FGK4.5),  $\alpha$ CD8 (2.43),  $\alpha$ CTLA-4 (9H10),  $\alpha$ Ly6C (Monts 1),  $\alpha$ IFNAR-1 (MAR1-5A3),  $\alpha$ PD-1 (RMP1-14), and  $\alpha$ IgG2A control (2A3).  $\alpha$ CTLA-4 and  $\alpha$ PD-1 were administered on days 5, 8, and 11 after tumor injection.  $\alpha$ CD40 was administered on day 11.  $\alpha$ IFNAR-1,  $\alpha$ CD4, and  $\alpha$ CD8 were administered on days -2, 0, and every 3 days thereafter.  $\alpha$ Ly6C was administered on days 5, 6, 8, 11, and 13. All *in vivo* antibodies were purchased from Bio X Cell and verified to be endotoxin free.

### Flow cytometry

Flow cytometric studies were performed on tumors, tumor-draining lymph nodes (TDLN), and spleen. Tumor, spleen, and lymph node single-cell suspensions were prepared by mechanical dissociation through a 70- $\mu$ m cell strainer. Prior to mechanical dissociation, pancreatic tumors were incubated for 45 minutes in 1 mg/mL collagenase V in DMEM (Thermo Fisher Scientific) at

37°C, and spleen samples were incubated in ACK lysis buffer (Life Technologies) for 3 minutes. Single-cell suspensions were then washed twice with PBS and enumerated (Beckman Coulter Counter Z2) before preparation for analysis. Cell surface staining for flow cytometry was performed for 30 minutes in PBS with 2% FBS, and intracellular staining was performed using a Fixation/Permeabilization Kit (eBioscience) according to the manufacturer's instructions. Live cells were identified using a Live/Dead Fixable Dead Cell Stain Kit (Life Technologies). Samples were run on a LSRII flow cytometer (BD Biosciences). Cell number was calculated as: (tissue cell number \* percent live cells/tissue grams)

### Histopathology

For histopathologic analysis, tissues were fixed in zinc formalin, embedded in paraffin and 4 to 5  $\mu\text{m}$  sections were stained with the following primary antibodies: mCD8a (Dianova DIA-808, 1:200) and SMA (Abcam ab5394, 1:200). Staining was performed on a Bond Max automated staining system (Leica Biosystems) using the Bond Intense R Staining Kit (Leica Biosystems DS9263). The standard protocol was followed with the following exceptions: primary antibody incubation was extended to 1 hour at room temperature and Avidin Biotin Blocking (Vector Labs SP-2001) and Peptide Blocking (DAKO X0909) were added. Antigen retrieval was performed with E2 (mCD8a) or E1 (SMA) retrieval solution (Leica Biosystems) for 20 minutes. Sections were stained with the following secondary antibodies: biotinylated anti-rat (mCD8a, Vector BA-4001, 1:200) or anti-rabbit (SMA, Vector BA-1000, 1:200.) After staining, slides were rinsed, dehydrated through a series of ascending concentrations of ethanol and xylene, and then coverslipped. For SMA score, the percentages of stromal cells positive for SMA were quantified in five fields under low magnification ( $\times 100$ ). Staining intensity was graded on a scale of 0 to 3 compared with the total stromal area, with 0 indicating 0%, 1 indicating  $\leq 33\%$ , 2 indicating 33% to 66%, and 3 indicating  $> 66\%$ .

### Quantification and statistical analysis

**Sample size.** Sample sizes were estimated based on pilot experiments. Control mice were estimated to have an average tumor volume of 0.4  $\text{cm}^3$  at day 18. Treated mice were assumed to have 50% less average tumor volume when compared with control mice. Sigma was estimated to be 1.5. Using a 0.80 power at the 0.05 alpha level, we chose a sample size of 5 mice. Mice were randomly assigned a treatment or control group. For experiments where the treatment effect was expected to be small and/or weak, two independent researchers with one blinded to the treatment groups performed caliper tumor measurements.

**Tumor kinetics.** Differences in tumor growth were determined by a linear mixed-effects model using the *lmerTest* (version 2.0-33) and *lsmeans* (least-squares means method, version 2.26-3) R packages. R (<https://www.r-project.org>) was version 3.4.2. For plots of mean tumor size by treatment group, time points across experiments that differed by less than 48 hours were pooled. To avoid bias from dropout, time points were not graphed if the number of mice remaining alive was  $\leq 65\%$  of the maximum group size. Statistical analysis was performed using all data, and correction for multiple comparisons was performed by calculating the FDR (Benjamini-Hochberg, R version 3.4.3)

**Random forest analysis.** Random forest analysis is a multivariable nonparametric ensemble partitioning tree method for modeling classification, regression, or survival problems (25, 26), and was performed as described previously (19). This machine learning approach is used to determine how well input variables predict a response variable of interest. During bootstrapping, two thirds of samples were used to train each tree and remaining (out-of-bag, OOB) samples were used for cross-validation and forest-related estimates. Missing values were imputed and the following tuning parameters were used:  $n\text{tree} = 3000$ ;  $\text{nodesize} = 2$ ;  $\text{nsplit} = 10$ ;  $\text{mtry} = (\text{input variable number})^{3/4}$ . A Gini index splitting rule was used for classification, and no iterative training or parameter optimization was performed.

Variables were then ranked by minimal depth (MD), a dimensionless statistic that measures variable predictiveness in tree-based models (27). For a variable  $x$ , MD is the shortest distance between the root node of a tree and the parent node of a maximal subtree. A maximal subtree is the largest subtree whose root node splits on  $x$ . Smaller MD values indicate greater predictiveness, that is, greater importance to the overall ability of the model to correctly classify OOB samples.

Overall model performance was determined using OOB sample classification accuracy. Relative stability was determined using the normalized Brier score. Each analysis was externally cross-validated over 50 iterations using resampling without replacement (28). Overall accuracy, geometric mean accuracy, and input variable MD was then compared with bootstrapped control data containing a scrambled response variable, and significance was determined via FDR generated by Student  $t$  test or ANOVA with Tukey HSD. All control data with scrambled response variables were nonpredictive.

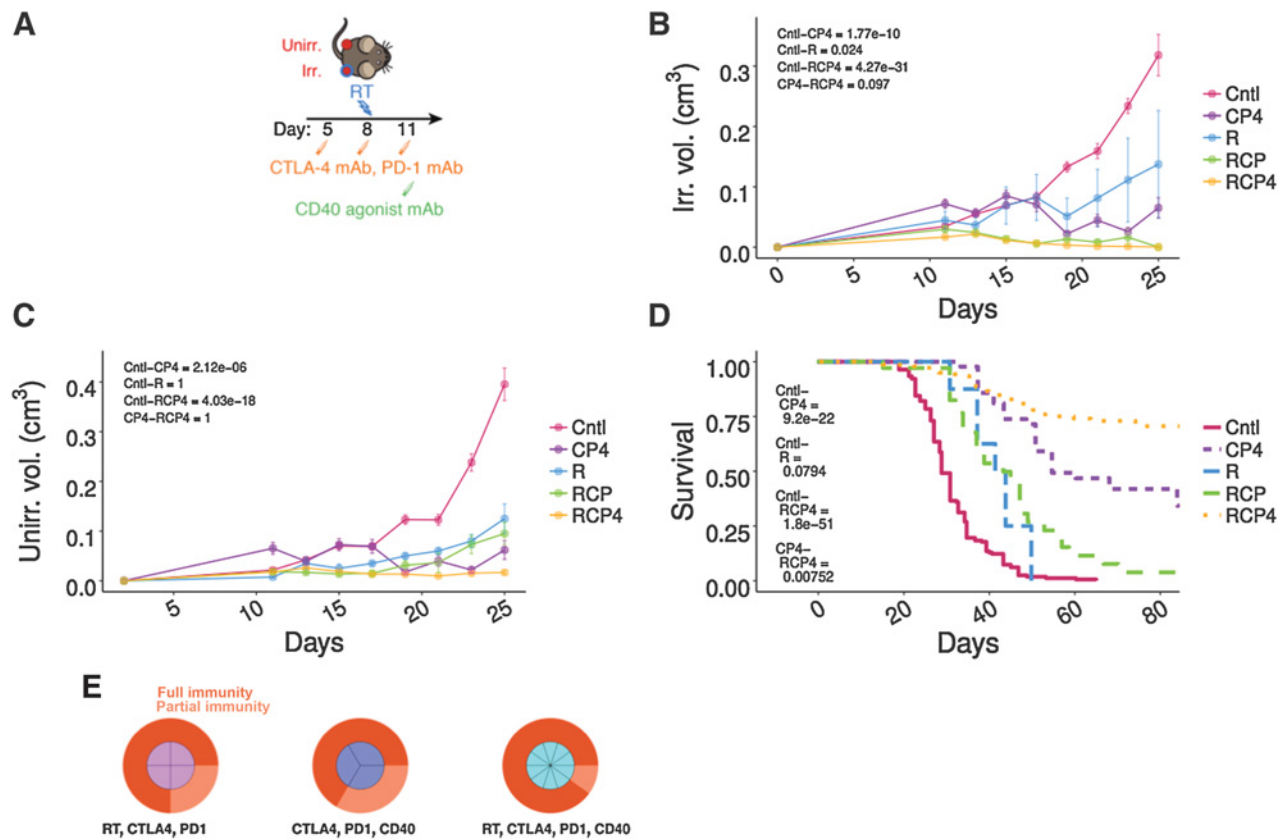
**Kaplan-Meier survival analysis.** Survival was recorded as the number of days from tumor injection until an event. An event was defined as tumor-related death or euthanasia due to protocol-specified tumor burden. Overall  $P$  values were calculated using the log-rank test (*survival* R package, version 2.42-0), and *post hoc* testing was performed using the *survMisc* (*Pairwise survdiff* method) R package (version 0.5.4). Analyses were performed on all data. Correction for multiple comparisons was performed by calculating the FDR (Benjamini-Hochberg).

**Additional analysis software.** Flow cytometric data were gated using Flowjo (version 10.3). The following R packages were used: *cowplot* (<https://CRAN.R-project.org/package=cowplot>, version 0.6.3); *figure layouts*, *data.table* (<http://r-datatable.com>, version 1.9.6); *tabular data analysis*, *ggplot2* (version 2.1.0); *general plots*, *pheatmap* (<https://CRAN.R-project.org/package=pheatmap>, version 1.0.8); *heatmaps*, *stats* (version 3.3.2); tests for significance.

## Results

### Radiotherapy and $\alpha\text{CD40}$ override PDA resistance to $\alpha\text{CTLA-4}/\alpha\text{PD-1}$ ICB

To determine the ability of radiotherapy,  $\alpha\text{CD40}$  agonist mAb, and  $\alpha\text{CTLA-4}/\alpha\text{PD-1}$  ICB (designated "RCP4") to mediate anti-tumor responses in PDA, we used a "two-tumor" subcutaneous KPC.4662 tumor model as described previously (Fig. 1A; ref. 19). Briefly, KPC.4662 cells were injected into both flanks and tumor-bearing mice were treated with *in vivo* antibodies intraperitoneally and/or 20 Gy radiation to only one tumor (the irradiated tumor).



**Figure 1.**

Radiotherapy and  $\alpha$ CD40 override resistance to  $\alpha$ CTLA-4/ $\alpha$ PD-1 ICB in PDA. **A**, Experimental design. Mice were injected subcutaneously on each flank on days 0 and 2 with KPC.4662 PDA cells, followed by treatment with radiotherapy,  $\alpha$ CD40, and/or ICB ( $\alpha$ CTLA-4 and  $\alpha$ PD-1). Radiotherapy (20 Gy) was delivered on day 8 to the right-sided tumor (irradiated tumor), ICB was administered on days 5, 8, and 11, and  $\alpha$ CD40 was administered on day 11. **B** and **C**, Mean growth kinetics of irradiated (**B**) and unirradiated (**C**) tumor following treatment. **D**, Overall survival. Data are from two to six independent experiments each with 5 to 10 mice per group. Growth curves, mean  $\pm$  SEM. Significance was determined via mixed linear effect model, followed by least-squares means (**B** and **C**) or log-rank (**D**) and is corrected for multiple comparisons. **E**, Mice with no evidence of disease were rechallenged with KPC.4662 PDA cells on both flanks 75 to 100 days after initial tumor injection. The outcomes of rechallenge [either full immunity (i.e., no tumor) or partial immunity (i.e., delayed tumor growth)] are shown per initial treatment.

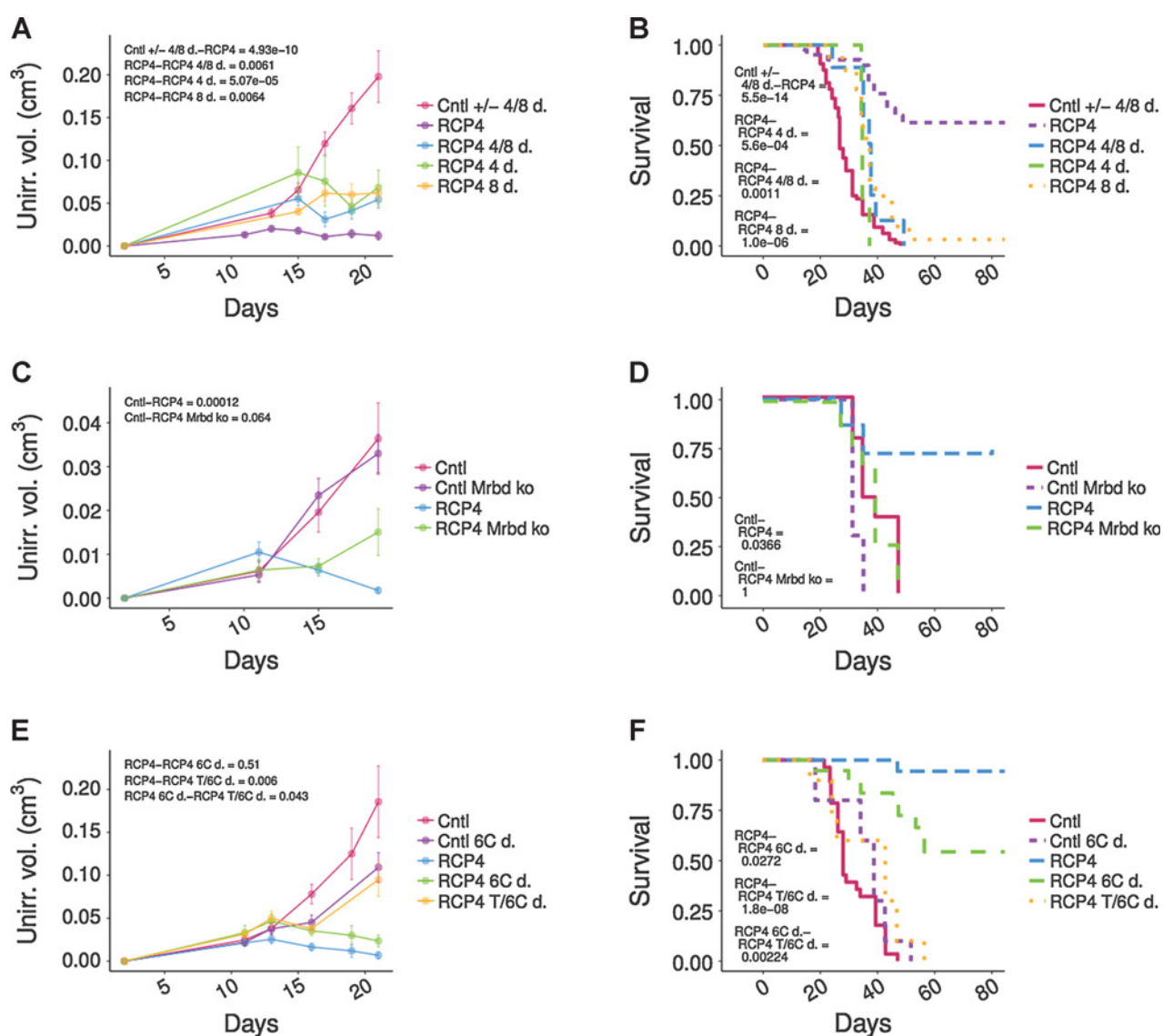
We found that radiotherapy alone (designated "R") or in combination with ICB (designated "RCP")  $\pm$   $\alpha$ CD40 (RCP4) resulted in reduction of irradiated tumor growth, with 91% of radiotherapy-treated mice across groups achieving complete regression at this tumor site (Fig. 1B). However, radiotherapy alone (R) or in combination with ICB (RCP) did not affect the growth of the unirradiated tumor or survival in these treatment groups (Fig. 1C and D; Supplementary Fig. S1A). The addition of  $\alpha$ CD40 to ICB (CP4) decreased growth of both the irradiated and unirradiated tumors and the effect was greatest when it was combined with both radiotherapy and ICB (RCP4; Fig. 1B and C; Supplementary Fig. S1A). Rates of long-term survival mirrored these observations: 0% in control mice compared with 17%, 50%, and 70% of mice treated with RCP, CP4, and RCP4, respectively (Fig. 1D). Combination therapy with RCP4 resulted in protective immunity upon rechallenge with bilateral KPC.4662 tumors in 9/10 RCP4-treated mice (Fig. 1E). In contrast, the addition of  $\alpha$ CD40 to radiotherapy and ICB did not improve response in mice challenged with B16-F10 melanoma cells following the same experimental approach (Supplementary Fig. S1B-S1D). Thus, RCP4 augments antitumor immunity in ICB-refractory PDA,

eradicating both index and unirradiated tumors in a majority of mice.  $\alpha$ CD40 agonist mAb sensitizes the immune response to radiotherapy and ICB in PDA in a tumor-specific fashion.

### T and myeloid cells distinctly contribute to antitumor immunity

We next hypothesized that the RCP4-mediated antitumor immune response is T cell dependent. To assess this hypothesis, T cells were depleted with  $\alpha$ CD4 and/or  $\alpha$ CD8 and tumor-bearing mice were treated with RCP4. Both CD4 and CD8 T-cell depletion abrogated response to RCP4, resulting in greater unirradiated tumor burden and worse survival (Fig. 2A and B). However, we noted that an initial tumor growth stabilization still occurred despite T-cell depletion, suggesting a potential T-cell-independent mechanism of response (Supplementary Fig. S2A).

To assess the hypothesis that myeloid cells were required for the T-cell-independent tumor regression we observed after RCP4, we evaluated response in mice lacking the long noncoding RNA (lncRNA) myeloid RNA regulator of Bim-induced death (*Morbidity*). *Morbidity* is required for survival of short-lived myeloid cells via regulation of the proapoptotic gene *Bcl2l11* (29). Mice

**Figure 2.**

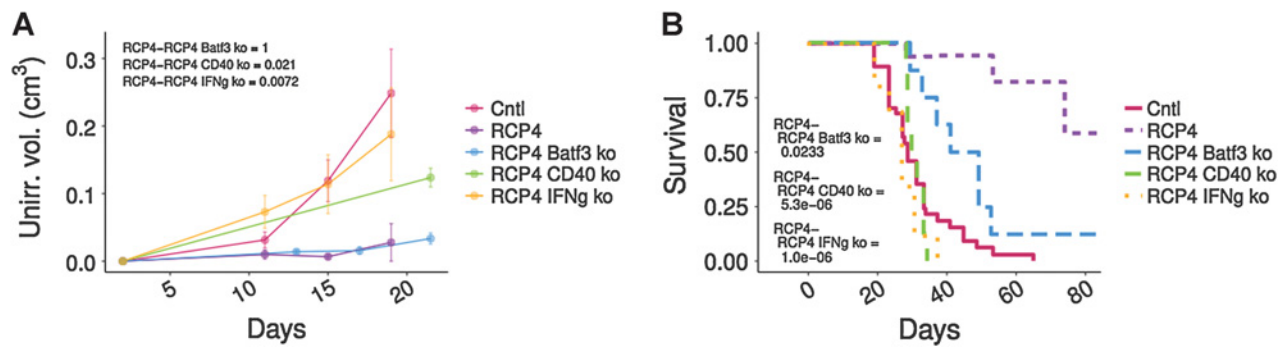
T and myeloid cells contribute distinctly to antitumor immunity. **A** and **B**, T-cell-depleting antibodies were administered every 3 days beginning on day -2 and mice treated as in Fig. 1A. Shown is the mean growth kinetics of the unirradiated tumor (**A**) and corresponding survival (**B**). **C** and **D**, *Morrbid* KO mice treated as in Fig. 1A. Shown is mean growth kinetics of the unirradiated tumor (**C**) and corresponding survival (**D**). **E** and **F**, Ly6C-depleting antibody was administered on days 5, 6, 8, 11, and 13 and mice treated as in Fig. 1A. Shown is the mean growth kinetics of the unirradiated tumor (**E**) and corresponding survival (**F**). 4 d., CD4 T-cell depletion; 8 d., CD8 T-cell depletion; 4 d./8 d., CD4 + CD8 T-cell depletion; 6C d., Ly6C depletion; and T/6C d., CD4 + CD8 T cell + Ly6C depletion. Data are from two to four independent experiments, each with 5 to 10 mice per group. Growth curves, mean  $\pm$  SEM. Significance was determined via mixed linear effect model, followed by least-squares means (**A**, **C**, and **E**) or log-rank (**B**, **D**, and **F**) and is corrected for multiple comparisons.

lacking *Morrbid* have greatly reduced populations of neutrophils, eosinophils, and Ly6C<sup>hi</sup> myeloid cells, but normal numbers of other myeloid and lymphoid populations (29). *Morrbid*-knockout mice challenged with tumor and treated with RCP4 exhibited rapid growth in the unirradiated tumor with complete loss of response to therapy (Fig. 2C; Supplementary Fig. S2B), and no survival advantage (Fig. 2D). Consistent with this finding, the addition of  $\alpha$ Ly6C-depleting antibody (30) to RCP4 also negatively impacted survival due to unirradiated tumor outgrowth occurring after day 20 (Fig. 2E and F; Supplementary Fig. S2C). Concomitant  $\alpha$ Ly6C and  $\alpha$ CD4/ $\alpha$ CD8 administration

completely abrogated the RCP4 antitumor response. Thus, CD4 and CD8 T cells as well as *Morrbid*-dependent short-lived myeloid cells cooperate for optimal response to combination radiotherapy, ICB, and  $\alpha$ CD40.

#### Response is dependent on CD40, IFN $\gamma$ , and Batf3 but bypasses canonical activation pathways

To investigate host-dependent mediators of response to RCP4, we treated tumor-bearing CD40-knockout mice, IFN $\gamma$ -knockout mice, and Batf3-knockout (which lack cross-presenting CD8 $\alpha^+$  dendritic cells). Antitumor response and long-term survival were



**Figure 3.**

Radiotherapy, ICB, and  $\alpha$ CD40 PDA response is dependent on CD40, Batf3, and IFN $\gamma$ . **A** and **B**, Batf3 KO, CD40 KO, or IFN $\gamma$  KO mice were treated as per Fig. 1A. Shown is the mean growth kinetics of the unirradiated tumor (**A**) and corresponding survival (**B**). Growth curves, mean  $\pm$  SEM. No differences in tumor kinetics or survival existed between untreated mice (WT and knockouts); thus, all untreated mice were pooled for clarity (designated "Cntl"). Significance was determined via mixed linear effect model, followed by least-squares means (**A**) or log-rank (**B**) and is corrected for multiple comparisons.

abrogated in CD40 and IFN $\gamma$ -knockout mice (Fig. 3A and B; Supplementary Fig. S2D). In Batf3-knockout mice, tumors initially responded to RCP4 treatment; however, this was not sustained past day 22 and there was no effect on overall survival. Given the requirement of CD40 for B-cell responses (31), we also investigated whether response to RCP4 is B cell dependent. In mice lacking immunoglobulin heavy constant gamma 1 ( $\mu$ MT), or in mice lacking B-cell activation factor and thus mature B220<sup>+</sup> B cells (Tnfsf13b<sup>tm1Msc</sup>), we observed RCP4-mediated antitumor responses and overall survival that matched RCP4-treated wild-type mice (Supplementary Fig. S2E and S2F). Thus, B cells do not appear to be required.

We next assessed whether canonical innate activation pathways were required for response to RCP4, evaluating TLR4/MyD88, stimulator of IFN genes (STING), and IFN $\alpha$ /IFN $\beta$ , which have been shown to be critical for antitumor immunity (32–35). We found that mice lacking these pathways had fully intact antitumor immunity and survival (Supplementary Fig. S3A–S3D). Mice treated with IFNAR-blocking antibody, to exclude the possibility of cancer cell-autonomous type I IFN signaling, also showed no deficits in response. Thus, RCP4 antitumor immunity is dependent on host CD40, Batf3, and IFN $\gamma$  and is independent of B cells and canonical innate immune activation pathways.

#### Nonredundant immune contributions of radiotherapy, ICB, and CD40 to antitumor response

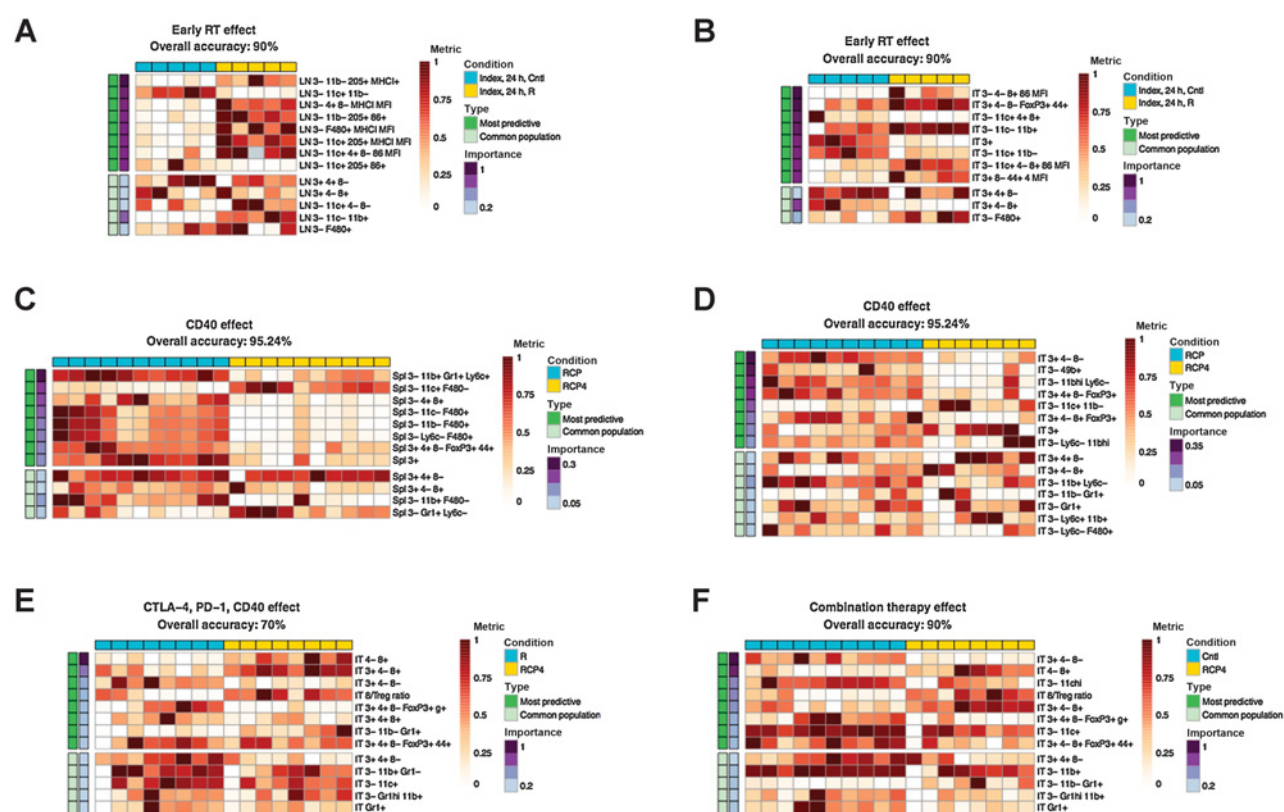
We investigated the myeloid and T-cell compartments during the time course of RCP4 treatment to understand the contribution of each therapeutic component. To identify important immune metrics, we used random forest machine learning as described previously (19, 36). Random forest is a multivariable, nonparametric ensemble partitioning tree method applicable to classification problems (25). We used RF to assess immune measurements in tumor, TDLN, and spleen at 24 hours, 72 hours, and 1 week post-radiotherapy, which allowed us to model the effect of immune metrics over multiple treatment conditions and time points. We measured 23 immune markers across these permutations, resulting in more than 1,000 measurements. First, we determined whether immune metrics could jointly classify mice into treatment groups with high accuracy. Second, we determined which immune metrics were most important to classification. Advantages of this approach compared with other methods are a

lack of supervision, excellent discriminatory ability in high dimensional space, and resistance to noisy or interrelated input variables, missing data, and overfitting.

Random forest analysis demonstrated that the effect of radiotherapy is best modeled 24 hours post-radiotherapy in TDLN and the irradiated tumor (Fig. 4A and B). In TDLN, the top predictors of radiotherapy response were increased MHC class I/CD86 by percentage or mean fluorescence intensity principally on DEC205<sup>+</sup> dendritic cells (Fig. 4A, top, and Supplementary Fig. S4). Changes in total T and myeloid TDLN populations were not predictive (Fig. 4A, bottom). Within the irradiated tumor, increased intratumoral CD11b<sup>+</sup> myeloid cells and decreased total and CD8<sup>+</sup>, but not CD4<sup>+</sup>, T cells were predictive (Fig. 4B, top vs. bottom and Supplementary Fig. S4). In both TDLN and irradiated tumor, total CD11c<sup>+</sup> antigen-presenting cells (APC) were decreased after radiotherapy in most mice. These effects of radiotherapy were confined to the irradiated tumor and corresponding TDLN at 24 hours post-radiotherapy with no difference at 72 hours post-radiotherapy at these sites. No immune metrics could distinguish CP4 compared with RCP4 in the unirradiated tumor at any time point (overall model accuracy  $\leq$ 60%, data not shown).

We next modeled the effect of  $\alpha$ CD40 in the context of radiotherapy and ICB at peak tumor regression (i.e., one week post-radiotherapy), detecting substantial systemic and intratumoral immune reorganization due to  $\alpha$ CD40 (Fig. 4C and D). Systemically, a shift in splenic myeloid cells predictive of  $\alpha$ CD40 consisted of decreased Ly6C<sup>hi</sup> and F4/80<sup>+</sup> myeloid cells (Fig. 4C, top, and Supplementary Fig. S5). Decreased splenic total CD3 T cells were also a top predictor, with no predictive changes in proportion of CD4 or CD8 T cells (Fig. 4C, bottom). The most predictive immune metrics in the tumor were decreases in CD4<sup>+</sup>CD8<sup>+</sup> T cells, CD49b<sup>+</sup> non-T cells, and regulatory T cells (Treg), and increases in CD11c<sup>+</sup> APCs and total T cells (Fig. 4D, top, and Supplementary Fig. S5). Similar to the spleen, the intratumoral ratio of CD4 to CD8 T cells was unaffected (Fig. 4D, bottom).

Random forest analysis further revealed that the addition of ICB and  $\alpha$ CD40 to radiotherapy could be predicted by favorable reconstitution of the T-cell compartment at peak tumor regression one week post-radiotherapy in the unirradiated tumor (Fig. 4E). Most predictive was an increase in intratumoral CD8



**Figure 4.** Radiotherapy, ICB, and  $\alpha$ CD40 nonredundantly modify tumor immune infiltrate. **A–F**, Random forest analysis (RF) of tumor infiltrate shows distinct component contributions to immunity. RF is a multivariable, nonparametric ensemble partitioning tree method for modeling classification. This approach is used to model the importance of flow cytometry metrics to classification by treatment group in an unsupervised, robust manner. Heatmaps show flow cytometry metrics normalized by row. Each box is an individual mouse. Rows are ordered from top to bottom by importance to RF model predictiveness, expressed as minimal depth distance from threshold (see Materials and Methods). The top heatmap in each panel shows the most important metrics to classification, and the bottom heatmap shows the importance of common immune metrics. Columns are hierarchically clustered within treatment groups. Mice were treated as described in Fig. 1 and tissue was harvested and analyzed via flow cytometry. Phenotypes listed indicate the proportion of the parent population, grandparent population (g), or mean fluorescence intensity, pregated on live, CD45<sup>+</sup> cells unless otherwise indicated. LN, tumor-draining lymph node; IT, intratumoral immune infiltrate; Spl, spleen. **A** and **B**, RF model for radiotherapy compared with control 24 hours after radiotherapy in tumor-draining lymph node (**A**) and irradiated tumor (**B**). **C** and **D**, RF model for addition of  $\alpha$ CD40 at peak tumor regression (1 week after radiotherapy) in spleen (**C**) and orthotopic tumor (**D**). **E**, RF model for addition of ICB and  $\alpha$ CD40 at peak regression in the unirradiated tumor. **F**, RF model for combination therapy with radiotherapy,  $\alpha$ CD40, and ICB at peak unirradiated tumor regression. See Supplementary Fig. S7 for immune changes over time and Supplementary Figs. S4–S6 for corresponding boxplots of flow cytometry metrics shown.

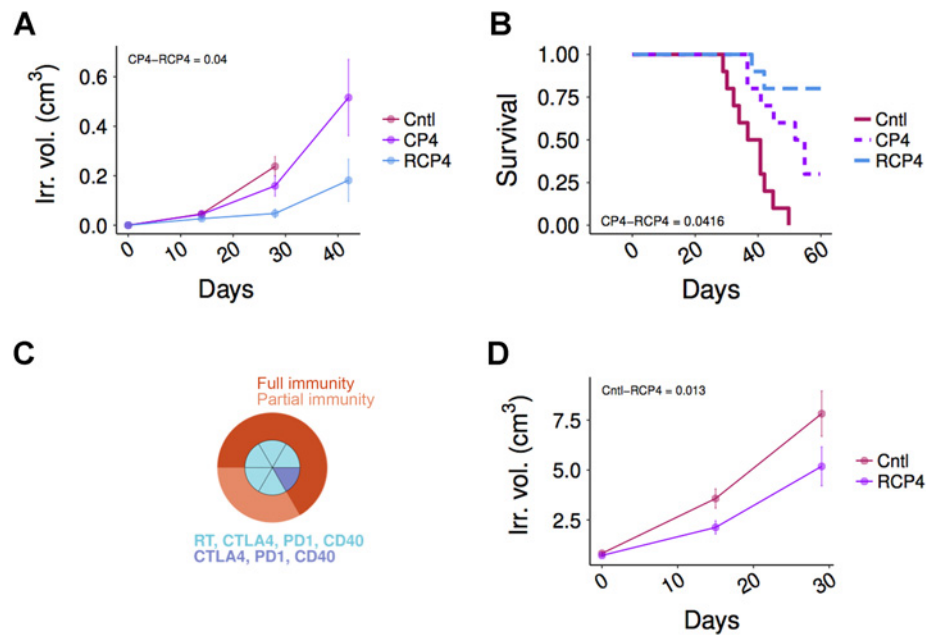
T cells, expressed as either a proportion of all CD45<sup>+</sup> leukocytes or CD3 T cells (Fig. 4E, top, and Supplementary Fig. S6). In addition, there is a corresponding decreased proportion of CD4<sup>+</sup>CD8<sup>+</sup> T cells as well as increased CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells, whereas changes in total CD4 T cells, CD11b<sup>+</sup> myeloid, or CD11c<sup>+</sup> APCs were not predictive (Fig. 4E, bottom).

Our unbiased assessment of immune changes following RCP4 compared with control mice surprisingly revealed that a decrease in intratumoral CD4<sup>+</sup>CD8<sup>+</sup> T cells was the single best predictor of whether mice received therapy (Fig. 4F, top, and Supplementary Fig. S6). Together with a corresponding increase in CD8 T cells and CD8/Treg ratio in the unirradiated tumor, 90% of RCP4-treated mice are correctly classified one week post-radiotherapy. Reduced prevalence of CD11c<sup>+</sup> APCs and Tregs as a percentage of all T cells were also top predictors. Furthermore, at 1 week postradiotherapy, no other alterations in intratumoral myeloid subsets were predictive (Fig. 4F, bottom). When we evaluated immune changes in RCP4-treated mice over time, we observed

reduced intratumoral APCs and increased T cells, mirroring predictive changes between control and RCP4-treated mice (Supplementary Fig. S7A and S7B). In summary, radiotherapy triggers an early proinflammatory stimulus, ablating existing CD8 T cells and upregulating antigen presentation machinery in the irradiated tumor and TDLN 24 hours post-radiotherapy. At peak tumor regression,  $\alpha$ CD40 causes systemic myeloid compartment reorganization that is identified in both the tumor and spleen, while ICB increases intratumoral T-cell infiltration, thereby improving the CD8/Treg ratio.

#### Combination therapy attenuates growth of orthotopic and *de novo* PDA tumors

To determine whether anatomic location impacts response, we also investigated RCP4 in orthotopic and *de novo* KPC PDA models. The KPC model manifests some of the hallmarks of human PDA such as minimal nonsynonymous mutations, dense desmoplasia, recruitment of tumor-promoting myeloid and Treg,

**Figure 5.**

Radiotherapy, ICB, and  $\alpha$ CD40 improve response of orthotopic KPC.4662 and *de novo* KPC tumors. **A–C**, Mice were injected with a single KPC.4662 orthotopic pancreatic tumor on day 0, followed by treatment with ICB (days 6, 9, and 12) and  $\alpha$ CD40 (day 12)  $\pm$  20 Gy radiotherapy (day 9). Shown is the mean tumor growth kinetics (**A**) and corresponding overall survival (**B**). **C**, Mice with no evidence of disease were rechallenged with subcutaneously KPC.4662 PDA cells 75 to 100 days after initial tumor injection. The outcomes of rechallenge [either full immunity (i.e., no tumor) or partial immunity (i.e., delayed tumor growth)] are shown per initial treatment. **D**, KPC mice with spontaneous tumors  $\geq 30$  mm<sup>3</sup> were treated as in **A**. Shown is the mean tumor growth kinetics. Data are from two independent experiments, each having 5 to 10 mice per group. Tumor growth curves, mean  $\pm$  SEM. Significance was determined via mixed linear effect model, followed by least-squares means (**A** and **D**) or log-rank (**B**) and is corrected for multiple comparisons.

and minimal CD8 T-cell infiltrate (10, 23). Briefly, mice bearing a single orthotopic pancreatic tumor (surgically injected or spontaneous in the orthotopic and KPC models, respectively) were treated with ICB,  $\alpha$ CD40, and/or radiotherapy as per the regimen described previously in Fig. 1A and tumor volume was documented using ultrasound. In the orthotopic model,  $\alpha$ CD40 in combination with ICB (CP4) decreased tumor growth (Fig. 5A) and increased overall survival (Fig. 5B) in comparison with control. However, the greatest response occurred with the addition of radiotherapy to ICB and  $\alpha$ CD40 (RCP4), which resulted in a significant reduction in tumor burden (Fig. 5A) and improvement in overall survival (Fig. 5B) in comparison with both control and CP4-treated mice. Treatment was durable, generating protective immunity to subcutaneous rechallenge in 5/6 RCP4-treated mice with initial complete responses (Fig. 5C). Thus, RCP4 generates potent antitumor immunity in the pancreas, and radiotherapy and  $\alpha$ CD40 are required to maximize response.

We next assessed response in the *de novo* KPC model, finding that RCP4 resulted in significant inhibition of tumor growth compared with control mice (Fig. 5D). Two weeks after treatment with RCP4, we observed stable disease in 7 of 29 KPC mice treated with RCP4 (24%), compared with 0 of 22 control mice. Thus, at the primary site of disease in the pancreas, RCP4 results in higher rates of complete response in the orthotopic model and reduced tumor growth in the *de novo* KPC tumors.

Although there were significant responses in both the orthotopic and *de novo* KPC models, they were not as robust as those in the subcutaneous model. To identify possible causes, we examined intratumoral CD8 T cells and stroma in both untreated

controls and RCP4-treated mice. There were significantly more infiltrating CD8 T cells in the untreated controls in the subcutaneous model in comparison with the orthotopic and KPC models (Supplementary Fig. S8A). With RCP4 treatment, the number of infiltrating CD8 T cells significantly increased in subcutaneous and orthotopic tumors, but the response was less robust in KPC tumors. There were comparable levels of SMA in all untreated control tumors, which decreased proportionally with RCP4 treatment (Supplementary Fig. S8B.)

## Discussion

PDA remains refractory to conventional and immune approaches, with combination chemotherapy only modestly improving survival (3, 8, 9, 37). Here, using genetically engineered mouse models of PDA that recapitulate this clinical challenge, we demonstrate that the combination of radiotherapy, ICB, and  $\alpha$ CD40 generates potent antitumor T-cell immunity. Although effective in other models, combination radiotherapy and ICB was insufficient in PDA. We found here that  $\alpha$ CD40 was critical to achieving an antitumor effect, but the greatest benefit occurred with the combination of ICB, radiotherapy, and  $\alpha$ CD40. Using random forest immune analysis, we found that (i)  $\alpha$ CD40 uniquely reorganizes and activates the myeloid and APC compartment, (ii) radiotherapy nonredundantly ablates existing intratumoral T cells and upregulates MHC class I/CD86, and (iii) ICB increases the prevalence of intratumoral CD8 T cells (Supplementary Fig. S9.) Antitumor immunity was dependent on host CD40, Batf3, and IFN $\gamma$ , and did not require innate



immune activation pathways such as STING. Thus, our findings provide a rationale for the clinical evaluation of combination therapy with radiotherapy, ICB, and  $\alpha$ CD40 in PDA.

RF immune analysis revealed that radiotherapy and  $\alpha$ CD40 nonredundantly alter immune dynamics during response. In addition to upregulation of antigen presentation machinery, irradiated tumors became void of total T cells, especially CD8 T cells, 24 hours after irradiation, consistent with the high radiosensitivity of most T cells. In contrast, intratumoral CD8 T cells in the unirradiated tumor increased at peak tumor regression with RCP4. The early post-radiotherapy T-cell depletion we observed, and the lack of any detectable immune signature for radiotherapy in unirradiated tumor after 72 hours, suggests that radiotherapy may modify adaptive immunity by "creating space" for an influx of cytolytic T cells. In contrast to local radiotherapy effects, in the context of radiotherapy and ICB,  $\alpha$ CD40 resulted in dramatic fluctuation of CD11b<sup>+</sup> myeloid and CD11c<sup>+</sup> APC populations in tumor and spleen during peak tumor regression. Thus, radiotherapy and  $\alpha$ CD40 have qualitatively and temporally distinct effects on innate and adaptive immune populations. Moreover, the effect of ICB was also unique, resulting in increased prevalence of intratumoral CD8 T cells, and combination RCP4 ultimately culminates in increased intratumoral CD8 T cell/Treg ratio during peak tumor regression.

CD40 agonism and ICB differ fundamentally in pharmacodynamic effects, but both are IFN $\gamma$  dependent.  $\alpha$ CD40 is well described to activate multiple cell types that contribute to antitumor immunity, influencing the immune response proximal to T-cell priming (31). We hypothesize that CD40 activation in combination with radiotherapy, chemotherapy, or vaccines may be able to trigger antitumor T cells in tumors otherwise immunologically "cold" and thereby sensitize for response to ICB. Both CD40 agonism and ICB are dependent on IFN $\gamma$ ; in the context of  $\alpha$ CD40, IFN $\gamma$  redirects inflammatory monocytes to secrete matrix metalloproteinases that digest PDA tumor ECM (38). Thus, IFN $\gamma$  may be required for T-cell infiltration into tumors in addition to myeloid cell-mediated antitumor activity, consistent with the dependency of RCP4 response on IFN $\gamma$  that we observe.

Our results suggest that CD40 agonists may be able to reprogram Ly6C myeloid cells from tumor-promoting to tumor-suppressive. Previous work in KPC PDA has shown that radiotherapy can induce both immunosuppressive macrophages that inhibit T cells and Ly6C<sup>hi</sup> myeloid cells that promote neovascularization (39, 40). Here, we show that in combination with radiotherapy and ICB, CD40 agonist response is dependent on the lncRNA myeloid regulator *Morbid* and Ly6C<sup>hi</sup> myeloid cells. These findings suggest that radiotherapy-induced, tumor-promoting myeloid populations may be reprogrammable via CD40 signaling. Furthermore, *Morbid*-knockout mice can be used as a cell type-specific tool to investigate the complex role of myeloid cells in antitumor responses. We have previously demonstrated that  $\alpha$ CD40 can induce tumor regression via macrophages when used as monotherapy and trigger T-cell immunity when used in combination with ICB and chemotherapy (20–22). The complete loss of RCP4 response in *Morbid*-knockout mice suggests that short-lived myeloid cells may be critical for both myeloid and T cell-induced immunity with combination therapy.

Our unsupervised random forest immune analysis unexpectedly identified a decrease in intratumoral CD4<sup>+</sup>CD8<sup>+</sup> T cells as the best predictor of whether mice received RCP4 compared with control. This finding provides a strong rationale for the use of

unsupervised approaches in high dimensional immune analysis to reveal novel mechanistic insights. CD4<sup>+</sup>CD8<sup>+</sup> T cells are likely to be  $\gamma\delta$  T cells, which may have an immunosuppressive role in PDA (41).  $\gamma\delta$  T cells are highly prevalent in PDA and promote pancreatic oncogenesis by inhibiting  $\alpha\beta$  T cells via immune checkpoint receptor ligation (41). Thus, reduction in prevalence of  $\gamma\delta$  T cells with RCP4 is consistent with RCP4 converting the tumor microenvironment from suppressive to stimulatory.

Overcoming immunosuppressive barriers in PDA is likely to require coordinated targeting of innate and adaptive immunity. We show here that radiotherapy and  $\alpha$ CD40 are key links between these systems, ameliorating resistance to ICB in PDA via multiple cellular mechanisms. Clinical trials combining radiotherapy +  $\alpha$ CD40 (NCT03165994) and  $\alpha$ CD40 + ICB (NCT03123783, NCT01103635, NCT02706353, NCT02304393, NCT03214250) are underway, including in PDA (38, 42). Our results provide rationale for combining radiotherapy,  $\alpha$ CD40, and ICB in human PDA.

### Disclosure of Potential Conflicts of Interest

R.H. Vonderheide reports receiving a commercial research grant from Lilly and other commercial research support from Janssen. No potential conflicts of interest were disclosed by the other authors.

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