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Radiation therapy (RT), a critical modality in the treatment of lung cancer, induces direct tumor cell death and augments tumor-specific immunity. However, despite initial tumor control, most patients suffer from locoregional relapse and/or metastatic disease following RT. The use of immunotherapy in non–small-cell lung cancer (NSCLC) could potentially change this outcome by enhancing the effects of RT. Here, we report significant (up to 70% volume reduction of the target lesion) and durable (up to 12 weeks) tumor regressions in conditional Kras-driven genetically engineered mouse models (GEMMs) of NSCLC treated with radiotherapy and a programmed cell death 1 antibody (αPD-1). However, while αPD-1 therapy was beneficial when combined with RT in radiation-naive tumors, αPD-1 therapy had no antineoplastic efficacy in RT-relapsed tumors and further induced T cell inhibitory markers in this setting. Furthermore, there was differential efficacy of αPD-1 plus RT among Kras-driven GEMMs, with additional loss of the tumor suppressor serine/threonine kinase 11/liver kinase B1 (Stk11/Lkb1) resulting in no synergistic efficacy. Taken together, our data provide evidence for a close interaction among RT, T cells, and the PD-1/PD-L1 axis and underscore the rationale for clinical combinatorial therapy with immune modulators and radiotherapy.

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Grit S. Herter-Sprie,1,2,3 Shohei Koyama,1,2,4 Houari Korideck,5,6 Josephine Hai,1,2,3 Jiehui Deng,1,2,3 Yvonne Y. Li,1,2,3 Kevin A. Buczkowski,1,2,3 Aaron K. Grant,7 Soumya Ullas,8 Kevin Rhee,1,2,3 Jillian D. Cavanaugh,1,2,3 Neermala Poudel Neupane,1,2,3 Camilla L. Christensen,1,2,3 Jan M. Herter,9 G. Mike Makrigiorgos,5,6 F. Stephen Hodi,1,2 Gordon J. Freeman,1,2,4 Glenn Dranoff,1,2,4 Peter S. Hammerman,1,2,3 Alec C. Kimmelman,6,10 and Kwok-Kin Wong1,2,4,6

1Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. 2Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. 3Lowe Center for Thoracic Oncology, 4Cancer Vaccine Center, 5Division of Medical Physics and Biophysics, and 6Department of Radiation Oncology, Dana-Farber Cancer Institute, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA. 7Division of MRI Research, Department of Radiology, and 8Longwood Small Animal Imaging Facility, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA. 9Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA. 10Division of Genomic Stability and DNA Repair, Dana-Farber Cancer Institute, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA. 11Belfer Institute for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

Introduction

Despite recent advances in chemotherapy and molecular targeted therapy, lung cancer remains the leading cause of cancer-related deaths in the United States (1). Radiation therapy (RT) is a common nonsurgical treatment in the management of patients with non–small-cell lung carcinomas (NSCLC) that reduces disease recurrence and improves overall survival (2). The cytoreductive effectiveness of RT is a result of generating DNA damage, modulation of signal transduction, and promoting an inflamed tumor microenvironment attracting the host’s immune system to the tumor site. However, the role of the inflammatory response still remains a double-edged sword. This inflammation can trigger innate and adaptive immune responses leading to tumor regression, but it can also enable tumor cells to develop multiple resistance mechanisms facilitating tumor immune escape (3). Recently, promising clinical antitumor effects have been reported with the incorporation of agents targeting the programmed cell death 1 (also known as CD279)/programmed death-ligand 1 (also known as CD274) (PD-1/PD-L1) immune checkpoint in NSCLC; however, there is a need to understand how to combine checkpoint blockade with other treatments, such as local therapy with RT (4, 5). Specifically, the recent work by Rizvi et al. showed increased therapeutic efficacy of anti–PD-1 therapy with pembrolizumab in NSCLC when these tumors had higher

Radiation therapy (RT), a critical modality in the treatment of lung cancer, induces direct tumor cell death and augments tumor-specific immunity. However, despite initial tumor control, most patients suffer from locoregional relapse and/or metastatic disease following RT. The use of immunotherapy in non–small–cell lung cancer (NSCLC) could potentially change this outcome by enhancing the effects of RT. Here, we report significant (up to 70% volume reduction of the target lesion) and durable (up to 12 weeks) tumor regressions in conditional Kras-driven genetically engineered mouse models (GEMMs) of NSCLC treated with radiotherapy and a programmed cell death 1 antibody (αPD-1). However, while αPD-1 therapy was beneficial when combined with RT in radiation-naïve tumors, αPD-1 therapy had no antineoplastic efficacy in RT-relapsed tumors and further induced T cell inhibitory markers in this setting. Furthermore, there was differential efficacy of αPD-1 plus RT among Kras-driven GEMMs, with additional loss of the tumor suppressor serine/threonine kinase 11/liver kinase B1 (Stk11/Lkb1) resulting in no synergistic efficacy. Taken together, our data provide evidence for a close interaction among RT, T cells, and the PD-1/PD-L1 axis and underscore the rationale for clinical combinatorial therapy with immune modulators and radiotherapy.
nonsynonymous mutation burden (6). Thus, identification and modulation of the key immunoregulatory molecules within the tumor microenvironment are warranted to improve patient-tailored multimodal treatment options for NSCLC.

We and others previously demonstrated that molecular alterations commonly found in NSCLC have distinct effects on the therapeutic response to antineoplastic treatment regimens (7–10). The recent study by Skoulidis and colleagues demonstrated that co-occurring genetic alterations in serine/threonine kinase 11/ liver kinase B1 (STK11/LKB1), TP53, and CDKN2A/B (cyclin-dependent kinase inhibitor 2A/B) characterize 3 major subgroups of KRAS-mutant lung adenocarcinoma (10). KRAS/TP53 tumors were associated with higher levels of somatic mutations, inflammatory markers, and immune checkpoint effector molecules and improved relapse-free survival. However, KRAS/LKB1 tumors were biologically and therapeutically different: prominent aspects of these tumors were high rates of Kelch-like ECH-associated protein 1–inactivating (KEAP1-inactivating) mutations and low levels of immune markers. An analysis exerted by Schabath et al. on 442 tumors of lung adenocarcinoma patients confirmed the differential biological association of STK11 and TP53 in KRAS-mutant tumors (11). In line with these findings, our own preclinical analysis revealed that loss of the tumor suppressor Stk11/Lkb1 in Kras-mutated tumors induced marked changes within the tumor immune microenvironment in comparison to Kras-mutated tumors specified by accumulation of tumor-associated neutrophils, increased expression of T cell exhaustion markers, and secretion of tumor-promoting cytokines (12). Furthermore, these tumors were not responsive to αPD-1 blockade. Therefore, we speculate that similar genotype-dependent differences may affect therapeutic sensitivity to combinatorial treatment regimens of RT and immunotherapies and might require thorough testing for identification of optimal immune-modulating agents. In order to systematically address this question, we utilized our recently developed preclinical platform for image-guided radiotherapy with two different genetically engineered mouse models (GEMMs) of Kras-driven tumorigenesis, as these allow for autochthonous tumor formation surrounded by a normal tissue microenvironment in an immunocompetent animal (8).

In early-stage disease, as an alternative to surgery, RT is delivered using a focused, targeted approach (stereotactic radiation), sparing vulnerable healthy tissue and draining lymph nodes. This is of critical importance, as the latter are essential in mounting an effective immune response, but only if left functional after RT (13). Thus, despite the local application of stereotactic radiosurgery, the associated immunological effects are elicited systemically and might act on distant metastasis, leading to a phenomenon called “the abscopal effect” (14). Indeed, case reports have demonstrated spontaneous regression of NSCLC metastases outside of the irradiated field (primary adenocarcinoma in the right lower lobe) 12 months after completion of stereotactic body radiotherapy. Although the clinical case presented by Golden et al. describes similar findings in a patient with metastatic NSCLC, it remains unclear whether the additional application of ipilimumab, a cytotoxic T-lymphocyte-associated protein 4 antibody (αCTLA-4, also known as CD152), augments the antitumor immunity (15). Interestingly, only after application of local RT and subsequent maintenance therapy with ipilimumab, a melanoma patient with prior disease progression on ipilimumab treatment demonstrated a remarkable tumor reduction, most likely mediated by an abscopal effect (16, 17).

In this study, we demonstrate that RT leads to an adaptive upregulation of tumor cell PD-L1 expression and concomitant administration of αPD-1 antibody generates effective antitumor immunity and long-term tumor control in Kras-driven murine NSCLC (mNSCLC). Importantly, we provide further evidence that αPD-1 therapy may be best used with radiation in the primary setting, as RT-recurrent tumors do not respond to αPD-1 treatment. We also observe that the underlying molecular alterations markedly influence the treatment response, as Kras/Lkb1-mutated tumors are minimally affected by the combination therapy.

**Results**

Synergistic efficacy of RT and PD-1 blockade in Kras-driven mNSCLC. To evaluate the therapeutic efficacy of RT and αPD-1 treatment, we utilized our recently developed preclinical platform for image-guided radiotherapy with lung cancer-relevant GEMMs (8). Application of precise targeted RT is of particular significance to avoid the impact of RT-induced lymphocyte killing or the exacerbation of out-of-field inflammation. In this study, we utilized our previously tested RT regimen, which consists of 17 Gy in 2 fractions of 8.5 Gy, given on 2 consecutive days approximately 24 hours apart (8.5 Gy × 2). While the translation of radiation doses and fractionation schemes used in humans to mice is difficult and not very well studied (8, 18), prior work has shown that multi-fraction RT is more effective than single-fraction RT when combined with CTLA-4 checkpoint blockade in syngeneic tumor models (19). Additionally, we used a previously described
Radiobiologic effects on the immune tumor microenvironment. We next sought to determine how RT affects the quantity and subsequent recruitment of tumor-associated immune cell populations. Due to the limited literature on leucocyte behavior elicited by RT and in comparable models, we analyzed the immune cell infiltrates at two time points after RT, 24 and 96 hours (Figure 2A). Using flow cytometry, we analyzed the total numbers of tumor-infiltrating lymphoid (Figure 2B, top) and myeloid cell populations (Figure 2B, bottom) before and after RT. We detected a significant decline in all lymphoid populations 24 hours after RT, indicating their radiosensitivity (total T cells $P < 0.0001$, CD8$^+$ T cells $P = 0.0004$, CD4$^+$ T cells $P < 0.0001$, B cells $P = 0.0001$, NK cells $P = 0.0147$; Figure 2B, top, and Figure 2C). Investigation of cell counts at 96 hours after RT showed that B, NK, and T cell subpopulations began to increase, albeit only NK cells were no longer significantly diminished compared with values from naive tumors. Thus, despite high doses of RT, all analyzed lymphoid populations are capable of repopulating the tumor microenvironment 96 hours after RT. Several clinical reports suggest that a high density of tumor-infiltrating lymphocytes is associated with favorable overall prognosis in patients with cancer (26–28) and that lower percentages of Tregs correlate with improved outcomes (29, 30). Analysis of the Tregs in $Kras$ tumors after RT showed a 65% decrease in population, which remained partially stable after 3 days (unirradiated vs. 24 hours $P < 0.0001$, unirradiated vs. 96 hours $P < 0.0001$; Figure 2C). As a consequence, we observed that
Figure 1. Blockade of the PD-1/PD-L1 axis potentiates the antitumor efficacy of RT in Kras-mutant murine NSCLC. (A) Schematic representation of combinatorial treatment schedule. (B) Representative MRI images of a Kras-driven tumor (one row represents 4 mm of lung) treated with RT and PD-1 blockade at different time points (baseline, 4 weeks, and 12 weeks after treatment initiation). H, heart (circled in red); T, tumor (circled with a white dotted line). (C) Tumor volume kinetics after dual treatment with RT and αPD-1. Each line represents one mouse (n = 10). (D) Treatment response to RT, αPD-1, or a combination of both is depicted. Data from unirradiated and RT cohort were previously published (8). Numbers below time points indicate amount of mice evaluated per group. (D–F) Data are represented as mean ± SEM. P values were calculated using 1-way ANOVA with Tukey’s multiple comparisons test. **P < 0.01. °This control group had to be discontinued due to extensive tumor burden or death. As fewer than 3 RT-treated mice were alive at this time point, statistical analysis was omitted. (E) Kaplan-Meier survival analysis of study cohorts presented in D calculated from treatment initiation. (F) Kaplan-Meier progression-free survival analysis of study cohorts presented in D calculated from treatment initiation. (E and F) P values were calculated using log-rank test corrected for multiple comparisons. A P value of less than 0.0167 (Bonferroni-corrected threshold) was considered statistically significant. *P < 0.05. d, days; w, weeks; RT, radiation therapy; PD-L1, programmed death-ligand 1; PD-1, programmed cell death 1.
the CD8+ /Treg ratio increased 96 hours after RT. To further demonstrate a beneficial interaction between RT, T cells, and the PD-1/PD-L1 axis, we analyzed PD-L1 expression on Kras tumor cells and demonstrated its upregulation (Figure 2D). Furthermore, we showed stable expression of PD-1 on CD4+ and CD8+ T cells upon RT (Figure 2E).

Myeloid cells, such as macrophages, neutrophils, DCs, eosinophils, and monocytes, are considered to be more radioresistant in contrast to lymphoid cells (31, 32). Our present study partly confirms these findings (Figure 2B, bottom). Tumor-associated neutrophils (TAN; CD11b+Ly-6G+), eosinophils (CD11b+Ly-6G–SiglecF+CCR3+), and inflammatory monocytes (CD11b+Ly-6G SiglecF–Ly-6Chi) showed no marked differences in number 24 and 96 hours after RT. In contrast, tumor-associated macrophages (TAM; CD11c+CD-11b–CD103–) and CD103+ DCs displayed a significant decline after two fractions of RT.

Taken together, our data thus suggest that RT induces favorable changes within the tumor microenvironment — augmented CD8+/Treg ratio, rise in PD-L1 expression on Kras tumor cells, and unchanged expression of PD-1 on tumor-associated T cells — for additional blockade of the PD-1/PD-L1 axis.

**Immune phenotyping of Kras tumors progressed after RT.** The promising antineoplastic synergy of concurrent RT and αPD-1 treatment in mNSCLC led us to the question whether RT-relapsed Kras tumors

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**Figure 2. Phenotyping of tumor-associated immune cell populations after RT in Kras-mutant murine NSCLC.** (A) Schematic representation of short-term analysis time points. (B) Representative flow cytometry data (live/single/total CD45+ cells). Total numbers of tumor-infiltrating lymphoid cells (top) and myeloid cells (bottom) of unirradiated tumors or shortly (24 and 96 hours) after RT. (C) Total number of tumor-associated Tregs at indicated time points. CD8+/Treg ratio was calculated from B and C. (D) PD-L1 expression of EpCAM+ tumor cells unirradiated and 24 hours after RT. Each dot represents one mouse (n = 4). (E) Expression of inhibitory T cell markers on CD8+ and CD4+ T cells from unirradiated and RT-treated tumors. Representative data are shown with 5 (unirradiated), 5 (24 hours after RT), and 4 (96 hours after RT) mice (B, C, and E). Data are represented as mean ± SEM. P values were calculated using 1-way ANOVA with Tukey’s multiple comparisons test. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. d, days; h, hours; B, B cells; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; Eos, eosinophils; IM, inflammatory monocytes; EpCAM, epithelial cell adhesion molecule; Lag3, lymphocyte-activation gene 3; Tim3, T cell immunoglobulin and mucin-domain containing-3; Ctla4, cytotoxic T-lymphocyte–associated protein 4.
would also show treatment response to PD-1 blockade and thereby offer another therapeutic option for patients with recurrent NSCLC. In a first attempt to address this question, we examined the tumor-associated immune cell infiltrates 20–22 weeks after RT when mice were close to reaching euthanasia criteria (the cohort of aged mice is described in the legend for Figure 2) (Figure 3). As unirradiated Kras mice only survive up to 8 weeks after tumor identification, we analyzed the immune cell populations at this time point. The cell counts of all immune populations were slightly reduced compared with those of unirradiated Kras tumors, aside from TAM. Although several preclinical and clinical reports have demonstrated that RT is associated with subsequent recruitment of primarily M2 TAM (33–37), we only found increased numbers of TAM in half of the tumors. M2 macrophages, in contrast to the M1 phenotype, have been shown to promote tumor growth and suppress antitumor immunity, especially towards T cells (38). Detailed analysis of the T cell compartment in all RT-refractory tumor samples showed a small reduction in Tregs and an accompanying CD8+/Treg ratio drop due to a concomitant diminution of CD8+ T cells (Figure 3C). Besides a decreased expression of lymphocyte-activation gene 3 (Lag3, also Tim3, T cell immunoglobulin and mucin-domain containing-3; Ctla4, cytotoxic T-lymphocyte-associated protein 4).
as CD223) on CD8+ T cells, further inhibitory T cell markers on CD4+ and CD8+ T cells did not change significantly in these RT-recurrent tumors (Figure 3D). In accordance with these findings, we further observed that the correlation of TAM and the CD8+/CD4+ ratio significantly changed between the unirradiated and RT-refractory treatment group: in unirradiated tumors there was a strong positive correlation between the two variables ($r = 0.74$, $P = 0.0935$) and a very strong negative correlation in RT-refractory tumors ($r = -0.85$, $P = 0.069$) ($P = 0.016$, test for difference of 2 independent correlation coefficients; Supplemental Table 2). Similar significant changes were found for the association between neutrophils and B cells ($P = 0.0001$) and neutrophils and eosinophils ($P = 0.0054$, test for difference of 2 independent correlation coefficients; Supplemental Table 1), respectively.

Given our observation that PD-1 expression on CD4+ and CD8+ T cells remains constant in RT-naive and RT-relapsed tumors, we expected that the latter might respond to PD-1 blockade.

**RT-refractory Kras tumors do not respond to PD-1 blockade treatment.** In order to investigate whether progressed Kras tumors after RT respond to αPD-1 treatment, we designed another preclinical trial. Using non-

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**Figure 4. Phenotyping of tumor-associated immune cell populations in RT-refractory αPD-1-treated Kras-mutant murine NSCLC.**

(A) Schematic representation of treatment schedule. αPD-1 treatment was initiated once tumor recurrence was detected (11–15 weeks after RT). (B) Tumor volume kinetics of RT-relapsed tumors treated with αPD-1. Each line represents one mouse ($n = 4$). Arrows indicate the beginning of αPD-1 treatment. (C) Representative flow cytometry data (live/single/total CD45+ cells). Total numbers of tumor-infiltrating lymphoid cells and myeloid cells of RT-refractory or RT-refractory αPD-1-treated tumors. (D) Total number of tumor-associated Tregs at indicated time points. CD8+/Treg ratio was calculated from C and D. (E) Expression of inhibitory T cell markers on CD8+ and CD4+ T cells of RT-recurrent and RT-recurrent αPD-1-treated tumors. Representative data are shown conducted with 4 RT-relapsed and 4 RT-relapsed αPD-1-treated mice (C–E). Data are represented as mean ± SEM. $P$ values were calculated using 2-tailed Student’s $t$ test. **$P < 0.01$; *$P < 0.05$. d, days; w, weeks; RT, radiation therapy; PD-1, programmed cell death 1; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; Lag3, lymphocyte-activation gene 3; Tim3, T cell immunoglobulin and mucin-domain containing 3; Ctla4, cytotoxic T-lymphocyte-associated protein 4.
invasive MRI, we determined the time point of tumor regrowth after RT to initiate αPD-1 therapy (Figure 4A). However, none of the mice with recurrent tumors after RT responded to αPD-1 antibody treatment; instead, they showed continuous tumor growth (Figure 4B). Subsequent examination of tumor-associated immune cell populations in RT-relapsed tumors treated with αPD-1 revealed a marked reduction of B cells, NK cells, and CD103+ DCs compared with RT-recurrent tumors without αPD-1 treatment (Figure 4C). Addition of αPD-1 also decreased the number of TAM and mediated a small increase in TAN. Additionally, we observed an influx of CD8+ T cells, promoting an improved, albeit nonsignificant, CD8+/Treg ratio (Figure 4D). Surprisingly, the levels of the investigated inhibitory T cell markers Lag3, T cell immunoglobulin and mucin-domain containing-3; Tim3, cytotoxic T-lymphocyte–associated protein 4.

Figure 5. Phenotyping of tumor-associated immune cell populations after RT, αPD-1, and combination therapy in Kras/Lkb1-mutant murine NSCLC. (A) Treatment response of unirradiated (n = 4) and RT- (n = 7), αPD-1- (n = 4), and RT+αPD-1-treated (n = 7) mice at 2 weeks. Each dot represents one mouse. Data of the RT cohort was partly published previously (8). (B) Representative flow cytometry data (live/single/total CD45+ cells). Total numbers of tumor-infiltrating lymphoid cells and myeloid cells of tumors from indicated treatment groups. (C) Total number of tumor-associated Tregs of indicated treatment groups. CD8+/Treg ratio was calculated from B and C. (D) Expression of inhibitory T cell markers on CD8+ and CD4+ T cells of indicated treatment groups. PD-1 expression could not be measured in αPD-1-treated tumors due to antibody binding. Representative data are shown conducted with 3 unirradiated and 3 RT-treated, 3 αPD-1-treated, and 4 dual-treated mice (B–D). Data are represented as mean ± SEM. P values were calculated using 1-way ANOVA with Tukey’s multiple comparisons test. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. RT, radiation therapy; PD-1, programmed cell death 1; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; Lag3, lymphocyte-activation gene 3; Tim3, T cell immunoglobulin and mucin-domain containing-3; Ctl4a, cytotoxic T-lymphocyte–associated protein 4.
M2 macrophages as previously shown (38). Hence, we interrogated our data set of murine cancer immune genes for M1- and M2-associated genes but observed no significant trends (Supplemental Figure 2B).

In conclusion, our data suggest that αPD-1 application in RT-relapsed Kras tumors is not sufficient to stop tumor growth but induces increased expression of inhibitory T cell markers upon treatment.

RT and αPD-1 treatment are less effective in Kras/Lkb1-mutant NSCLC. We next sought to determine whether the synergistic therapeutic effect of RT and PD-1 blockade would yield a similar tumor response in Kras-driven mNSCLC, with concomitant loss of the tumor suppressor Lkb1 (also known as Stk11). Inactivating mutations of STK11/LKB1 are found in up to 30% of KRAS-mutated lung adenocarcinomas (39–41). Preclinical and clinical studies demonstrate that KRAS/LKB1-mutant neoplasms are more aggressive resulting in enhanced metastasis and reduced survival (41–43). Furthermore, we reported that the drug sensitivity of Kras-driven mNSCLC is dramatically impacted depending on the additional loss of either Lkb1 or Trp53 (7). More recently, we observed that Lkb1 inactivation in mNSCLC causes significant changes within the tumor microenvironment by mediating accumulation of TAN and reduction of tumor-infiltrating lymphocytes, increased expression of T cell inhibitory markers, and secretion of tumor-promoting cytokines (12). Additionally, we found that Lkb1 alteration is negatively correlated with PD-L1 expression on murine and human tumor cells. However, modulation of the inflammatory microenvironment by either IL-6–neutralizing antibody or antibody-mediated neutrophil depletion reverted the immune-associated phenotype and had therapeutic efficacy. Hence, we speculated that RT might exert similar immunomodulatory effects on the Lkb1-deficient tumor microenvironment. In comparison to Kras mice, we previously observed that Kras/Lkb1 mice have more rapid tumor growth kinetics when evaluated in our single lung nodule model (8). Despite careful intrathoracic application of Adeno-Cre and close clinical monitoring, we were unable to follow treatment response in these mice for longer periods due to rapid tumor growth. Mice treated with either RT or αPD-1 alone recapitulated our previously reported therapeutic responses and demonstrated significant inhibition or slowed tumor enlargement compared to naive tumors, respectively (8, 12). The antitumor efficacy of RT was significantly better than PD-1 blockade in Kras/Lkb1 tumors. However, application of dual therapy was not synergistic (Figure 5A). To exclude the possibility that this lack of tumor shrinkage may be masked by influx of recruited immune cells leading to a “pseudo-progression,” we correlated the absolute numbers of tumor versus immune cells per milligram tumor among the treatment cohorts and observed similar increases of immune cell numbers (Supplemental Figure 4A). A small number of Kras/Lkb1 mice treated with the combination therapy survived beyond 4 weeks of study, and we were able to document a massive tumor volume increase accompanied by pleural effusion (Supplemental Figure 4B).

To understand the poor efficacy in Kras/Lkb1 tumors, we performed immune panel analysis on these tumors to identify the pharmacodynamic changes upon treatment. Although we found that the highest degree of usually considered favorable antitumor changes within the T cell compartment was detected upon combinatorial treatment (Figure 5B), these changes did not reach significance. Predominantly, the counts of CD8+ and CD4+ T cells switched, leading to a more beneficial CD8+/Treg ratio (Figure 5C). Although the expression of T cell inhibitory markers on CD8+ T cells was not significantly altered among the treatment groups, our data showed an increase in Lag3, Tim3, and CTLA-4 on CD4+ T cells when comparing untreated and dual-treated Kras/Lkb1 tumors (Figure 5D). Moreover, we were surprised to find a marked influx of tumor-homing eosinophils in Kras/Lkb1 tumors treated with αPD-1 (Figure 2B). This was consistent with a recent analysis by Carretero et al., showing that tumor-associated eosinophilia contributes to tumor rejection through directed recruitment of tumor-specific CD8+ T cells, macrophage polarization, and normalization of the tumor vasculature (44). Whereas the latter were not part of our investigation, CD8+ T cell recruitment to the tumor was not changed in comparison to naive tumors.

Our findings suggest that combined therapy of RT and PD-1 blockade may recruit cytotoxic CD8+ T cells to the microenvironment of an Lkb1-deficient tumor; however, the expression of the T cell inhibitory markers Lag3 and Tim3 on these CD8+ T cells was significantly increased compared with Kras tumors, potentially indicating reduced cytotoxic activity (Supplemental Figure 4D).

Discussion

The purpose of this study was to examine the feasibility and efficacy of combined RT and immune checkpoint blockade in a highly predictive animal-based translational cancer research platform utilizing Kras-driven NSCLC-specific GEMMs. Despite a growing body of literature describing in vivo studies of com-
bined RT and immunotherapy, the majority of these experiments were performed in syngeneic mouse models with ectopic implantation of cancerous cells and potential lack of altered tumor vasculature and concomitantly developed tumor microenvironment (19, 45, 46). However, the relevance of tissue location in terms of RT-induced immune phenotype changes was previously demonstrated in the ALTS1C1 glioma model (47). RT-mediated clustering of TAM was only seen in intracranial tumors, whereas tumors grown intramuscularly did not show these effects. However, besides our preclinical trial, only one other study employed an autochthonous model (prostate cancer) to examine synergistic effects of RT and immunotherapy, specifically granulocyte/macrophage colony-stimulating factor-secreting cellular immunotherapy (48).

Here, we provide evidence that clinical evaluation of combined RT and immunomodulatory therapy with αPD-1 appears promising for patients with KRAS-driven NSCLC, as our pronounced and durable tumor responses in a highly predictive GEMM are striking. We speculate that the observed tumor responses could potentially be extended beyond 12–17 weeks in the majority of treated mice, omitting the limitations of our tumor model. As mentioned above, 6 responding mice had to be discontinued owing to contralateral tumor growth, although the dual-treated tumor within the left lung remained small. Although our experimental setting was not designed to analyze the abscopal effect in Kras-driven mNSCLC, we were surprised by this finding, which suggests that using RT on a local tumor nodule to boost systemic immune response together with checkpoint blockade may not work. Given the importance of this observation in regards to clinical translation, careful evaluation of our tumor model is warranted. In contrast to humans, the incidence of metastasis in this tumor model is very low (49). We therefore hypothesize that immediate recovery of the mouse after intrathoracic injection of Adeno-Cre into the left lung lobe causes small amounts of virus to spill into the right lung lobes, which promote tumor growth with an extended latency (8). Furthermore, although these contralateral tumors grow over a longer time period, we believe that these tumors do not harbor mutations other than the KrasG12D-driver mutation, as these tumor models are considered genetically stable (50). Whether the emergence of these delayed contralateral lesions indicates that the concept of an abscopal effect is either not valid in Kras-driven GEMMs or generally in GEMMs needs much further in-depth evaluation with appropriate experimental design and additional tumor models of other tumor types. Of note, until recently, only case reports, but no large clinical studies, have been published on the systemic immune-related effects of RT (51). Thus, it is possible that this phenomenon is also a rather rare event in humans. As commonly observed in the clinical setting, we also monitored tumor relapse in 2 of 10 mice, despite several weeks of response (Figure 1C and Supplemental Figure 1B). Whether this disease progression could have been influenced by reirradiation should be addressed in future analyses.

The translation of treatment doses and fractionation schemes used in humans to mice is difficult and not very well studied (8, 18). Whereas in the majority of in vivo RT studies a single dose is prescribed, Dewan and colleagues specifically analyzed the efficacy of different dose-fractionation regimens with CTLA-4 blockade in two syngeneic mouse models (breast and colorectal cancer) and showed superiority of the multi-fraction versus single-fraction approach (19). We therefore chose to apply one of our previously tested fractionated regimens in these models: tumors were irradiated to a dose of 17 Gy (8.5 Gy × 2) (8).

The timing for best harnessing the combinatorial effects of RT and immune-stimulating agents is essential, and likely dependent on various factors, such as tumor type, tumor-associated immune microenvironment, and the immunotherapeutic agent itself. In melanoma, αCTLA-4 antibody application prior to RT was shown to enhance antitumor immunity by removal of Tregs (52). However, immunogenic cell death mediated by RT occurs within 1 to 3 days after treatment (19, 53). We reasoned that RT-induced immunogenic cell death could be improved by blocking the PD-1/PD-L1 axis shortly after RT administration, as similar therapeutic effects have been observed in a syngeneic colon cancer model using either αPD-L1 or αPD-1 antibodies in combination with RT of 10 Gy in 5 fractions (46). This study further demonstrated increased expression of tumor cell PD-L1 up to 7 days after RT. Additionally, the authors provide evidence that combination of RT with αPD-1 was superior to combination with αPD-L1 (curative in 80% vs. 66%).

The recent review by Vatner and Formenti summarizes the effects of RT on tumor myeloid cells (54). Consistent with their classification, we also observed long-lived recruitment of TAM after RT in some tumors (Figure 3B). However, contrary to prior published preclinical data, we observed major removal of TAM within the first week after RT (Figure 2B) (45). This discrepancy might originate from the different models used in the distinct studies. Furthermore, the definition of TAM likely differs in both studies due to the particular situation in the lung compartment and, therefore, precludes formal comparison. Besides several other subgroups, AMs, which are considered tissue-resident macrophages, represent the predomi-
niant group and remain viable for prolonged periods (55). Although caution is required when comparing immune cell behavior observed in healthy lung tissue or acute lung injury to phenomena in the cancer context, others have noted that bleomycin-induced injury led to similar marked shrinkage of the AM pool as our RT regimen (56). Importantly, we expected to monitor an increment of neutrophil influx due to the RT-induced inflammation within the tumor microenvironment, which did not occur. An explanation could be that this inflammation-mediated neutrophil recruitment is potentially masked by continuous neutrophil death within 24 to 48 hours after activation in the tumor (57). Alternatively, AMs have been described to prevent neutrophil influx (58). Whether this is a realistic scenario regarding the drastic diminution of TAM remains elusive.

Special emphasis on treatment scheduling, with regard to the efficacy of RT and PD-1/PD-L1 blockade, is warranted in patients with NSCLC to limit adverse reactions. These patients are likely to have poor lung reserve function due to smoking, chronic obstructive pulmonary disease, advanced disease, and prior cancer therapy, and cumulative toxicity of pneumonitis would be of particular concern. RT of lung malignancies almost always inevitably involves normal lung parenchyma, which could potentially lead to variable extents of pulmonary inflammation and radiation pneumonitis (59). Although clinical pneumonitis rates for the αPD-L1 antibody nivolumab are similar to or lower than those of other drugs commonly used in NSCLC (docetaxel: 4.6%, ref. 60; gefitinib: 3.5%, ref. 61), two patient deaths associated with pneumonitis were reported (62). Whether αPD-L1 antibodies have a lower risk of pneumonitis is currently under clinical investigation. Despite the specific predispositions for cumulative toxicity in patients with NSCLC, our preclinical data do not suggest that treatment of RT-refractory KRAS-driven tumors with αPD-L1 or αPD-1 antibodies would be beneficial in the clinic. It remains to be determined how αPD-1 treatment induces increased expression of Lag3, Tim3, and CTLA-4 in RT-relapsed Kras tumors and if concomitant application of neutralizing antibodies against these inhibitory T cell markers might reverse the therapeutic responsiveness. Given our observations, we hypothesize that acute effects of RT play an essential role in provoking the synergistic treatment outcome. Currently, a few clinical trials assessing the combinatorial effects of RT and immunomodulatory agents in patients with NSCLC have been initiated: RT with ipilimumab (NCT02221739) and RT with granulocyte/macrophage colony-stimulating factor (NCT02623595).

Although we observed durable and prolonged synergistic treatment efficacy of RT and PD-1 blockade in Kras-driven mNSCLC, this effect was limited to 60% to 80% of recipient mice and not potent enough to induce tumor regression in Kras/Lkb1 tumors. Our immune analysis indicates that the combinatorial treatment predominantly induces changes within the T cell compartment resulting in a more antitumor environment. However, increased expression of Lag3 and Tim3 potentially prevent exertion of synergistic therapeutic effects to its fullest extent. Furthermore, the increased accumulation of TAN in Kras/Lkb1 versus Kras tumors (Supplemental Figure 4C) and subsequent secretion of tumor-promoting cytokines might play a critical role in lack of therapeutic efficacy (12). Hence, this finding suggests that addition of further immunomodulatory agents to increase response rates might be valuable, despite the potential risk of side effects. Following the idea of combining RT with multiple checkpoint blockades, Twyman-Saint Victor et al. provided evidence of feasibility and efficacy in patients with metastatic melanoma and verified their observations in mice (63). Briefly, taking advantage of the inhibitory effects of αCTLA-4 antibodies on Tregs resulting in an augmented CD8⁺/Treg ratio and increased diversity of the T cell receptor repertoire of intratumoral T cells upon RT, the authors demonstrated that further application of αPD-L1 inverted T cell exhaustion and led to improved T cell expansion. This concept of targeting tumor-infiltrating Tregs was previously shown to have pronounced preclinical effects if applied transiently and in combination with RT (64). Furthermore, the relative radioresistance of Tregs in contrast to other lymphocytes argues strongly for the rationale of combined RT and Treg ablation (65).

Although our pharmacodynamic studies of RT and PD-1 blockade in Kras/Lkb1 tumors showed favorable antitumor changes in regards to improved CD8⁺/Treg ratio, this marker alone may not be sufficient to predict therapeutic response. Potential explanations might be that the observed changes compared with the other treatment groups are only modest or that the expression of T cell inhibitory markers on CD8⁺ T cells was significantly altered compared with Kras tumors (Supplemental Figure 4D). It remains uncertain what underlying mechanisms exert compensatory signaling such that the milieu remains suppressive of CD8⁺ T cells. Inclusion of additional immunomodulatory drugs could be a logical next step towards enhancing the treatment option for Kras/Lkb1 tumors.

Albeit our current data allow the assumption that transient Treg depletion and/or multiple checkpoint
blockade against PD-1, Lag3, and Tim3 in addition to RT are pertinent to improve therapeutic outcome of Kras/Lkb1 tumors, one should keep in mind that low-dose RT (single dose ≤1.0 Gy) may also be utilized to attenuate inflammatory processes (66). Furthermore, Klug et al. demonstrated that low-dose γ irradiation led to differentiation of inducible NOS+ M1 macrophages, subsequent recruitment of cytotoxic CD8+ T cells, and tumor cell killing in a spontaneous insulinoma tumor model (67). Whether these phenomena are operative in the case of an inflammatory tumor microenvironment, as observed in Kras/Lkb1 tumors, remains to be explored in further studies.

Additional genomic alterations may possibly also contribute to immune resistance. This is of particular importance in the case of RT-refractory tumors. However, the molecular determinants of immune resistance are currently poorly understood. Two recent studies provide initial evidence that oncogenic pathway signaling may support resistance to antitumor immune response (68, 69). Spranger and colleagues found that melanoma cell–intrinsic activation of the WNT/β-catenin signaling pathway correlated with lack of intratumoral T cell infiltration (69). Similar decreased T cell trafficking into melanomas mediated by expression of immunosuppressive cytokines was observed upon loss of phosphatase and tensin homolog (PTEN) (68). Therapeutic efficacy of αPD-1 and αCTLA-4 antibodies could be improved by additional treatment with a selective PI3Kβ inhibitor in a mouse model. Together, these findings demonstrate that further examination of underlying molecularly driven mechanisms of immune resistance is warranted in the future.

In summary, we provide evidence that clinical evaluation of combined RT and immunomodulatory therapy with αPD-1 appears promising for patients with KRAS-driven NSCLC. We demonstrated that the therapeutic outcome strongly depends on the timing of PD-1 blockade, resulting in continuous tumor growth in RT-refractory Kras tumors upon αPD-1 application. We showed that addition of αPD-1 in these tumors led to enhanced expression of T cell inhibitory markers on tumor-infiltrating T cells. Finally, loss of the tumor suppressor Stk11/Lkb1 attenuates the synergistic efficacy. Due to the distinct immune phenotype of Kras/Lkb1 tumors, we suggest alternative immunomodulatory concepts to optimally harness the therapeutic efficacy of RT and the adaptive immune response.

**Methods**

**Mice.** Mouse strains harboring a conditional activating mutation (G12D) at the endogenous Kras locus and conditional Lkb1 knockout were described previously (41). Male and female mice of KrasG12D/wt and KrasG12D/lox/Lkb1lox/lox genotype were used for all experiments. All experimental mice were maintained on a mixed genetic background (C57BL/6, BALB/c, and S129). Single nodule murine lung cancer was induced by intrathoracic injection of Adeno-Cre virus (Gene Transfer Vector Core Facility at the University of Iowa) at the age of 6–8 weeks as described previously (8). Animals were randomly assigned to various treatment groups once tumors had formed as indicated. Irradiation experiments were performed using the small animal radiation research platform at the Dana-Farber Cancer Institute. Irradiation parameters and settings were previously described (8). Radiotherapy was given at a fractionation scheme of 8.5 Gy × 2 on 2 consecutive days. Data regarding unirradiated Kras, RT-treated Kras, and some RT-treated Kras/Lkb1 mice was previously published and are therefore historical (8). However, RT-treated Kras mice used to produce RT-refractory tumors (Figure 3) were treated simultaneously as the other experimental arms (αPD-1 and RT+αPD-1 in Figure 1) and showed similar tumor growth curves as our previous data (Supplemental Figure 3). Animals that received a single treatment with PD-1 blocking antibodies still underwent the stress of placement on the irradiation platform and also underwent cone beam computed tomography imaging as often as the irradiated mice. Rat anti–PD-1 monoclonal blocking antibodies (clone 29F.1A12, generated in-house) (70) or vehicle (PBS) was given i.p. at a dose of 200 µg/mouse (8–10 mg/kg) 3 times per week starting 6 hours after the second radiation dose. Tumor growth was evaluated every 2 weeks using MRI imaging until the tumor burden met euthanasia criteria. Tumor volume quantification was performed as described previously (8).

Currently, no standard response criteria exist to evaluate the effect of drug treatment on murine lung tumors. Contrary to human studies, which rely on unidimensional or bidimensional tumor measurements, we used tumor volume measurements to determine tumor treatment response as described previously (71). Briefly, we applied the following criteria: (a) complete response (CR) = target lesion disappeared completely; (b) partial response (PR) = at least 30% volume reduction of target lesion (compared with the baseline tumor volume); (c) progressive disease (PD) = at least 20% volume increase of target lesion (compared
with the baseline tumor volume); and (d) stable disease (SD) = neither sufficient reduction or increase of tumor volume to qualify for PR or PD, respectively (compared with the baseline tumor volume). These are in keeping with RECIST response criteria used in human clinical studies.

**Immune cell isolation and analysis.** Tumor and tumor-associated immune cells were isolated, identified, and counted as previously described (9), with some modifications. Briefly, after blood and bronchoalveolar lavage fluid collection, lungs were perfused with cold PBS containing 5 mmol/l EDTA from the right ventricle. Tumors from the left lung lobe were resected and used for histologic, flow cytometry, and immune cell gene expression analysis, respectively. The majority of the tumor sample was used for flow cytometry analysis. Total cell count was divided by tumor weight utilized for each assay. AccuCheck Counting Beads (Invitrogen) were used according to the manufacturer's protocol to count absolute numbers of immune cell populations. After dissociation of the tumor tissue, the cells were treated with rbc lysis (BioLegend) and used directly for staining. Prior to surface marker staining, isolated cells were stained using the LIVE/DEAD fixable dead cell stain kit (Invitrogen). A complete list of antibodies for flow cytometry analysis can be found in Supplemental Table 1. Detection of anti–PD-1 antibody binding was performed using anti–rat IgG2a (r2a-21B2: eBioscience) without prior Fc blocking (BioLegend), which was performed for all other stainings. All antibodies were used at 1:50 dilution. Intracellular staining for CTLA-4 and Foxp3 was performed according to the manufacturer's protocol (eBioscience). Cell analysis was examined with the use of a BD FACS Canto II cytometer equipped with Diva software (version 8.0) and FlowJo (version X, Tree Star). The gating strategy for immune cell population analysis was previously described (12). Briefly, after gating live, single CD45+ cells from total tumors, cells were differentiated in 3 distinct populations: (a) the lymphocyte population (CD11c−CD11b−), which is mainly composed of CD4+ T cells, CD8+ T cells, B cells, and NK cells; (b) a population of CD11c+CD11b− cells, which include CD103− alveolar macrophages (AMs) and CD103+CD11c+ DCs; and (c) a population of CD11b+ cells, which includes Ly-6G+ neutrophils (CXCR2+) and a Ly-6G− population. The CD11b+Ly-6G− population contains subpopulations, such as SiglecF−CCR3+ eosinophils and SiglecF+Ly-6C+ monocytes (Supplemental Figure 5).

**NanoString experiments.** Total cellular RNA was extracted from homogenized tumor tissues using TRIzol reagent (Invitrogen). It was then DNase treated using an Ambion Kit (Ambron) and column purified using a RNaseasy Mini kit (QIAGEN). Gene expression profiling was performed on 50–75 ng of RNA using the NanoString Immune Profiling Kit (NanoString Technologies). Capture and Reporter Code sets were added to the samples following the manufacturer's protocol and allowed to hybridize at 65°C for 16 hours. Samples were washed and loaded onto a cartridge using the nCounter Analysis System Prep Station per the manufacturer's protocol. The cartridge was scanned using the nCounter Digital Analyzer at the maximum resolution of 1,150 fields of view. Data Analysis was completed using nSolver software (NanoString Technologies).

**Statistics.** Statistical analysis was performed with GraphPad Prism (version 6.0d). Differences between the groups were evaluated by 2-tailed unpaired Student's t test, 1-way ANOVA with Tukey's multiple comparisons test, and log-rank test corrected for multiple comparisons with Bonferroni threshold where appropriate. Correlation was evaluated using Pearson's correlation coefficient. Data are presented as mean ± SEM, and P values of less than 0.05 were considered statistically significant.

**Study approval.** All in vivo experiments performed in this study were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute and the Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

**Author contributions**

GSHS, SK, PSH, ACK, and KKW conceived and designed the experiments. GSHS, SK, HK, JH, KAB, JD, AKG, SU, KR, JDC, NPN, and CLC performed the experiments. GSHS, SK, YYL, CLC, and JMH collected and analyzed data. GSHS, SK, PSH, ACK, and KKW wrote the manuscript. GMM, FSH, GJF, and GD gave technical support and provided reagents and conceptual advice. All authors contributed to the final manuscript.

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Address correspondence to: Alec C. Kimmelman, NYU Langone Medical Center, 560 First Avenue, 2nd FL, Radiation Oncology Department, New York, New York, 10016, USA. Phone: 646.501.8940; E-mail: alec.kimmelman@nyumc.org. Or to: Kwok-Kin Wong, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, Massachusetts, 02215-5450, USA. Phone: 617.632.6084; E-mail: kwong1@partners.org.

Shohei Koyama’s present address is: Division of Respiratory Medicine, Department of Respiratory Medicine, Allergy, and Rheumatic Disease, Osaka University Graduate School of Medicine, Osaka, Japan.

Glenn Dranoff’s present address is: Novartis Institutes for BioMedical Research, Cambridge, Massachusetts, USA.

Alec C. Kimmelman’s present address is: Department of Radiation Oncology, Perlmutter Cancer Center, New York University, School of Medicine, New York, New York, USA.

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