Acquired Resistance to Fractionated Radiotherapy Can Be Overcome by Concurrent PD-L1 Blockade


Abstract

Radiotherapy is a major part in the treatment of most common cancers, but many patients experience local recurrence with metastatic disease. In evaluating response biomarkers, we found that low doses of fractionated radiotherapy led to PD-L1 upregulation on tumor cells in a variety of syngeneic mouse models of cancer. Notably, fractionated radiotherapy delivered in combination with αPD-1 or αPD-L1 mAbs generated efficacious CD8⁺ T-cell responses that improved local tumor control, long-term survival, and protection against tumor rechallenge. These favorable outcomes were associated with induction of a tumor antigen–specific memory immune response. Mechanistic investigations showed that IFNγ produced by CD8⁺ T cells was responsible for mediating PD-L1 upregulation on tumor cells after delivery of fractionated radiotherapy. Scheduling of anti–PD-L1 mAb was important for therapeutic outcome, with concomitant but not sequential administration with fractionated radiotherapy required to improve survival. Taken together, our results reveal the mechanistic basis for an adaptive response by tumor cells that mediates resistance to fractionated radiotherapy and its treatment failure. With attention to scheduling, combination immunoradiotherapy with radiotherapy and PD-1/PD-L1 signaling blockade may offer an immediate strategy for clinical evaluation to improve treatment outcomes.

Introduction

Radiotherapy is the most important nonsurgical treatment in the management of solid malignancies with around 50% to 60% of all patients with cancer receiving this treatment. The inclusion of radiotherapy in treatment regimens reduces disease recurrence and improves overall survival in most common cancers (1–3). In addition to the direct cytoreductive effect of radiotherapy, emerging evidence suggests that the generation of antitumor immune responses may play an important role in the effectiveness of this treatment (4, 5). Radiotherapy can lead to expression of ecto-calreticulin on tumor cells as well as the release of several damage-associated molecular patterns (DAMP), including High Mobility Group Box 1 (HMGB1) and ATP, which can lead to recruitment and activation of antigen-presenting cells and priming of tumor antigen–specific T-cell responses (6–10). Despite this, immune-escape frequently occurs with tumor recurrence remaining the leading cause of mortality in patients receiving radiotherapy (11). The identification and inhibition of key drivers of immunosuppression may augment antitumor immune responses with the potential to improve patient outcome.

The PD-1/PD-L1 axis is involved in the maintenance of peripheral tolerance and modulation of acute inflammatory responses through inhibition of T-cell function such as loss of T-cell receptor signal transduction or through apoptosis of activated T cells (12, 13). In addition to binding PD-1, PD-L1 can also suppress T-cell function through interaction with CD80 (14). Expression of PD-L1 is inducible and thought to respond to local inflammatory milieu, particularly type I and II IFN (12, 15, 16). Although barely detectable in most normal tissues, expression of PD-L1 has been described in multiple malignancies (reviewed in ref. 17). Importantly, recent clinical studies with either PD-1 or PD-L1 targeting mAbs have demonstrated encouraging responses in patients with advanced disease (18–21).

In this study, we demonstrate that radiotherapy leads to an adaptive upregulation of tumor cell PD-L1 expression that is dependent on CD8⁺ T-cell production of IFNγ and may attenuate the efficacy of the anticancer immune response. Using syngeneic models of melanoma, colorectal and triple-negative breast cancer, we show that the efficacy of low doses of fractionated radiotherapy can be enhanced when delivered in
combination with mAb targeted against PD-1 and PD-L1. Importantly, we show that dose scheduling is critical to the synergistic effect of combination therapy with concurrent but not sequential administration (αPD-L1 mAb administered 7 days after completion of radiotherapy) generating effective antitumor immunity and long-term tumor control. The results of this study provide evidence for the potential mechanisms that may underlie treatment failure with low-dose fractionated radiotherapy and have important implications for clinical translation demonstrating concurrent but not sequential blockade of the PD-1/PD-L1 axis with radiotherapy is required for long-term tumor control.

Materials and Methods

Mice and cell lines
BALB/c and C57Bl/6 mice were obtained from Harlan. Animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. CT26 murine colon carcinoma cells (ATCC) and 4434 cells isolated from BrafV600E p16−/− mice (Richard Marias, Cancer Research UK Manchester Institute, Manchester, United Kingdom) were maintained in DMEM, and 4T1 cells and performed under a United Kingdom Home Office license. CT26 murine colon carcinoma cells (ATCC) and 4434 cells isolated from BrafV600E p16−/− mice (Richard Marias, Cancer Research UK Manchester Institute, Manchester, United Kingdom) were maintained in DMEM, and 4T1 triple-negative breast cancers (ATCC) maintained in RPMI-1640, supplemented with 10% FCS, 1% l-glutamine (Invitrogen). All cell lines were cultured to limited passage before absence of Mycoplasma contamination.

Tumor therapy
Mice were inoculated subcutaneously with either 5×10⁵ CT26, 1×10⁵ 4T1, or 5×10⁵ 4434 cells. Irradiations were performed 7 to 10 days after inoculation (when tumors were at least 100 mm³) as described previously (22). Administration of αPD-1, αPD-L1, or isotype control mAb commenced on day 1 of the fractionated radiotherapy cycle (unless otherwise stated) and was administered intraperitoneally (i.p.) 3qw for up to 3 weeks at a dose of 10 mg/kg in a dose volume of 100 µL/10 g in PBS. Tumor volume (1,000 mm³ for CT26 and 4434 models and 500 mm³ for 4T1) was the primary endpoint for efficacy studies. For cellular and cytokine depletion experiments, mice received either αCD8 mAb, clone YTS169 (a gift from M. Glennie, Southampton University, Southampton, United Kingdom); αCD4 mAb, clone GK1.5 (Biolegend); αAsialo-GM1 (Wako Chemicals); or αIFNγ, clone XMG1.2 (BioXcell). Peripheral blood was sampled during therapy to confirm cellular depletion. For tumor rechallenge experiments, long-term surviving (LTS) mice were implanted contralaterally with tumor cells a minimum of 100 days after previous tumor implantation. Experimental groups contained at least 5 mice/group and are representative of at least two independent experiments.

Measurement of cytokine production by CD8+ T cells isolated from long-term surviving mice
Splenocytes were isolated from either LTS or control mice and cocultured with either irradiated tumor cells (50 Gy) or 1 µmol/mL of the H2-Ld restricted peptides SP3VYHQQ (AH1)/TPEHARIGL (β-galactosidase; Anaspec) as described previously (22). Experimental groups contained 3 to 5 mice and are representative of two independent experiments.

Tumor and immune cell phenotyping by flow cytometry
To obtain single cell suspensions, tumors were processed using a gentleMacs dissociator and a murine tumor dissociation kit (Miltenyi Biotec). For analysis, nonspecific binding was blocked as described above and expression of CD4, CD8 (BD Biosciences), CD45, CD11b, GR1, NKp46, PD-L1, and PD-L1 examined by multiparameter flow cytometry (all Biosoience unless otherwise stated). For analysis, live cells were gated (by vital dye exclusion, Invitrogen) and populations phenotyped (as described above). An example of the gating strategy used for selection of either CD45− or CD45+ cells is provided in Supplementary Fig. S1.

In vitro cocultures
Tumor cells were cultured in the presence of 20 ng/mL IFNγ and/or TNFα for 24 hours before evaluation of PD-L1 expression by flow cytometry as described above. For coculture assay either resting or activated splenocytes (treated with PBS or phorbol 12-Myristate 13-Acetate and Ionomycin cell stimulation cocktail respectively, eBioscience) were cocultured at a 1:1 ratio with tumor cells and tumor cell expression of PD-L1 determined as described above. Silencing of IFNγR1 expression was achieved by lentiviral transduction of cells with shRNA (cells were also transduced with nontargeting shRNA as controls; Thermo Scientific). Measurement of splenocyte cytokine production (IFNγ and TNFα) was measured by intracellular flow cytometry as described above.

Results
Blockade of PD-1 or PD-L1 enhances the therapeutic efficacy of radiotherapy
Radiotherapy has been shown to modulate the immunogenicity of tumor cells but is rarely able to generate durable therapeutic responses that lead to systemic antitumor immunity alone. We show that low doses of local fractionated-dose radiotherapy delivered as 10 Gy in 5 fractions lead to increased tumor cell expression of PD-L1 with elevated expression evident 1, 3, and 5 days after the last dose of radiotherapy when compared with time-matched, nontreated (NT) mice (Fig. 1A and B). This radiotherapy-mediated increase in tumor cell PD-L1 expression peaks at 72 hours after the last dose of radiotherapy and although it declines significantly by 7 days after radiotherapy (when compared with expression at days 1 and 3; P < 0.05 and P < 0.01, respectively; Mann–Whitney test) remains elevated when compared with NT mice (P < 0.05, Mann–Whitney test). A similar pattern of expression was also found on CD11b+ GR1hi but not on CD11b+ GR1lo cells (Supplementary Fig. S2A and S2B).

Given these observations, we hypothesized that the immune response generated following radiotherapy may be limited through the PD-1/PD-L1 axis. Local radiotherapy delivered as 10 Gy in 5 daily fractions was found to significantly improve survival in mice bearing established CT26 tumors when compared with NT controls (Fig. 1C and D; P < 0.05 log-rank; Mantel–Cox test). Our data demonstrate that this radiotherapy-mediated local tumor control can be substantially improved through combination with either αPD-L1 mAb or αPD-1 mAb (Fig. 1C and D; P < 0.001 log-rank; Mantel–Cox test). Combined therapy was curative in 66% and 80% of mice that received either
radiotherapy and αPD-L1 mAb or αPD-1 mAb, respectively. No additional benefit was observed when mice received radiotherapy in combination with both αPD-1 and αPD-L1 mAb versus either mAb alone (Supplementary Fig. S3). In contrast with combination with radiotherapy, monotherapy with either αPD-L1 mAb or αPD-1 mAb did not significantly improve survival (Fig. 1C and D; \( P > 0.05 \) log-rank; Mantel-Cox test).

Blockade of PD-L1 also improved response to radiotherapy in mice bearing established 4T1 tumors where combined therapy significantly reduced tumor burden by 38%, when compared with radiotherapy alone (10 days after start of therapy; 184.3 ± 13.5 mm² vs. 292.8 ± 14.3 mm², respectively; \( P < 0.001 \) Mann-Whitney test) and significantly improved survival (\( P = 0.001 \) log-rank; Mantel-Cox test; data not shown). Similar results were also...
observed in mice bearing established 4434 melanomas (Fig. 1E). Combination therapy with local radiotherapy and mAb targeting either PD-1 or PD-L1 was well tolerated in both BALB/c and C57Bl/6 mice (Supplementary Fig. 5A and 5B). These data clearly demonstrate the potential to improve outcome following low-dose fractionated radiotherapy of established solid tumors by blockade of the PD-1/PD-L1 axis.

**NK cells contribute to local tumor control following combination therapy, but long-term survival is dependent on CD8<sup>+</sup> T cells**

We next investigated the mechanisms underlying this long-term tumor control observed following combination radiotherapy and αPD-L1 mAb therapy. Initially colony forming assays were used to confirm that αPD-1 and αPD-L1 mAb were not acting as radiation sensitizers through direct interaction with the tumor cells (Supplementary Fig. S5A–S5C). Using depleting antibodies, we next explored the roles for effector T cells and NK cells in mediating antitumor efficacy following radiotherapy/αPD-L1 mAb combination therapy. Our data demonstrate that as early as 7 days after the start of a 5-day fractionated radiotherapy cycle (with PD-L1 mAb therapy commencing on day 1 of the radiotherapy cycle), reduced tumor burden is evident in mice following combination therapy when compared with NT mice (207.5 ± 29.2 mm<sup>2</sup> vs. 409.4 ± 86.88 mm<sup>2</sup>, respectively; P = 0.067, Mann–Whitney U test; Fig. 2A). However, this statistical trend to reduced volume was lost following depletion of either CD8<sup>+</sup> T cells or NK cells.

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**Figure 2. Therapeutic efficacy of fractionated radiotherapy and αPD-L1 mAb combination is dependent on the activity of CD8<sup>+</sup> T lymphocytes.**

A, tumor volume on day of therapy, 7 and 11 days after combination therapy with 5 fractions of 2 Gy and αPD-L1 mAb. Immune cell subsets (either CD8, CD4, or NK cell) were depleted 1 day before therapy with depletion maintained for 2 weeks. *** P < 0.001; ** P < 0.01; * P < 0.05, Mann–Whitney test. B, survival curve. *** P < 0.001, log-rank (Mantel–Cox) test. Data representative of 10 mice per cohort. C, representative density plots of peripheral blood, confirming depletion of immune cell subsets. RT, radiotherapy.
where tumor volumes were not significantly different from those in NT cohorts ($P = 0.52$ and $P = 0.70$, respectively, Mann–Whitney U test) but significantly larger than mice that received combination therapy without immune cell depletion ($P < 0.01$; combined therapy vs. CD8 depletion, and $P < 0.05$; combined therapy vs. NK cell depletion, Mann–Whitney U test). By day 11 after treatment, combination therapy significantly reduced tumor burden when compared with NT controls ($P < 0.001$, Mann–Whitney U test). Although the depletion of either CD8$^+$ T cells or NK cells at this time point reduced the efficacy of combination therapy ($P < 0.001$ and $P < 0.05$, respectively, Mann–Whitney U test), the relative contribution of CD8$^+$ T cells and NK cells became more evident with significantly reduced tumor control in CD8 versus NK cell-depleted mice ($P < 0.05$, Mann–Whitney U test). Our data also reveal that the depletion of CD4$^+$ T cells improved local tumor control following combination therapy (153.2 ± 27.0 mm$^3$ vs. 72.7 ± 17.3 mm$^3$, respectively; $P < 0.05$, Mann–Whitney U test).

In contrast with early tumor control following combination radiotherapy/αPD-L1 mAb therapy, LTS was not affected by the depletion of NK cells (70% LTS mice following treatment with radiotherapy/αPD-L1 vs. 77.8% following combination therapy with NK cell depletion; Fig. 2B). The depletion of CD4$^+$ T lymphocytes increased the frequency of LTS mice from 70% to 87.5% (combination vs. combination + CD4 depletion) but this did not achieve significance ($P > 0.05$ log-rank; Mantel–Cox test). We show that the depletion of CD8$^+$ T cells completely abrogated the therapeutic efficacy of combination radiotherapy/αPD-L1 mAb therapy ($P < 0.001$ log-rank Mantel–Cox test). Depletion of immune cell populations was confirmed by flow cytometry on peripheral blood samples (Fig. 2C). These data suggest that although NK cells may exert some local tumor control, this does not affect overall survival and although CD4$^+$ T cells are dispensable or even capable of suppressing responses, CD8$^+$ T cells are critical for mediating effective long-term tumor control following treatment with radiotherapy and αPD-L1 mAb.

**T-cell responses**

We next investigated whether immunologic memory was generated following treatment with radiotherapy and αPD-L1 mAb. We show that LTS mice originally treated with radiotherapy and αPD-L1 mAb were able to completely reject tumors following contralateral rechallenge (Fig. 3A). To quantify this memory response, splenocytes were harvested from LTS mice that had more than 100 days of disease-free survival and the capacity of CD8$^+$ T cells to produce IFNγ following coculture with either a CT26 tumor-associated antigen (AH1: SPSYVYHQF), control peptide (β-galactosidase: TPHARIGL), or with irradiated CT26 cells was assessed (Fig. 3B and C). Our data

![Figure 3](image-url)

**Figure 3.** Fractionated radiotherapy and αPD-L1 mAb combination generates protective immunologic memory. A, survival curve of LTS mice following contralateral rechallenge with 5 × 10$^5$ CT26 cells. $P < 0.05$ compared with control mice (log-rank: Mantel–Cox test). Experimental groups contained at least four mice and are representative of at least two independent experiments. RT, radiotherapy. B, representative dot blot of IFNγ production by CD8$^+$ T cells isolated from either tumor naïve or LTS mice previously treated with radiotherapy and αPD-L1 mAb. C, frequency of IFNγ$^+$ CD8$^+$ T cells isolated from either tumor naïve or LTS mice originally treated with radiotherapy and αPD-L1 mAb following coculture with either H2-Ld restricted peptides (AH1 (SPSYVYHQF); a defined CT26 tumor-associated antigen or β-galactosidase (TPHARIGL); control peptide of prokaryotic origin) or 50 Gy irradiated CT26 cells for 5 days, followed by priming with 50 Gy irradiated CT26 cells. $P < 0.05$ (Mann–Whitney test). Data representative of two independent experiments.
reveal that LTS mice were endowed with a significantly greater frequency of IFNγ-producing CD8+ T lymphocytes following coculture with AH1 peptide than naïve mice (6.6% ± 0.8 vs. 2.3% ± 0.2, respectively; \( P < 0.05 \), Mann–Whitney test). A similar response was observed following coculture of splenocytes with CT26 cells. Comparison of peptide and tumor cell cocultures, however, revealed that the frequency of memory CD8+ T cells was approximately 3-fold lower in LTS mice following coculture with tumor cells than with AH1 peptide and may reflect tumor cell-mediated suppression of T-cell activation. Taken together, these data demonstrate that radiotherapy when combined with blockade of the PD-1/PD-L1 axis can generate protective immunologic memory in long-term survivors.

**Fractionated radiotherapy leads to CD8+ T-cell-dependent adaptive upregulation of tumor cell PD-L1 expression**

Initially we confirmed that treatment of tumor cells with a range of radiotherapy doses *in vitro* did not have any direct impact on expression of PD-L1 (Fig. 4A). To identify which cellular populations within the tumor microenvironment were responsible for modulating tumor cell expression of PD-L1, mice received a fractionated radiotherapy cycle in combination with CD8, CD4, and NK cell depleting antibodies. Our data reveal that the depletion of CD8+ T cells but not NK cells completely abrogates the radiotherapy-mediated upregulation of PD-L1 on tumor cells (Fig. 4B and C). Interestingly, the
depletion of CD4+ T cells was found to further augment radiotherapy-mediated upregulation of PD-L1 on tumor cells (~2-fold compared with treatment with radiotherapy alone).

**Adaptive upregulation of PD-L1 by tumor cells following fractionated radiotherapy is IFNγ dependent**

Given the clinical correlation between IFNγ in the tumor microenvironment and PD-L1 expression (16) and the impact of TNFα on this response (23), we evaluated the impact of these cytokines on PD-L1 in our cell lines. Coculture of tumor cells with recombinant IFNγ leads to a significant 20-fold increase in cell surface expression of PD-L1 in vitro (Fig. 5A). Furthermore, although addition of recombinant TNFα alone has no impact on tumor cell expression of PD-L1, a cocktail of both IFNγ and TNFα can further augment tumor cell PD-L1 expression (~2-fold) when compared with expression with IFNγ alone (Fig. 5A). We show that silencing of IFNγ-receptor 1 (IFNγR1) on CT26 tumor cells using shRNA completely abrogates the upregulation of PD-L1 following coculture with either recombinant IFNγ or both IFNγ and TNFα (Fig. 5A).

To establish whether activated immune cells could lead to an adaptive change in tumor cell expression of PD-L1 through the production of IFNγ and TNFα, WT CT26 cells and those transduced with either nontargeting shRNA (NTC shRNA) and
IFNyR1 shRNA were cocultured with isolated resting (PBS treated) and activated [phorbol 12-myristate 13-acetate (PMA) and ionomycin-treated] splenocytes (Fig. 5B and C). Importantly, these experiments demonstrate that activated immune cells can elevate tumor cell PD-L1 expression at physiologically relevant concentrations of IFNy and TNFα, in an IFNyR1-dependent manner.

To confirm the role of IFNy on tumor cell expression of PD-L1 in response to radiotherapy in vivo, mice received radiotherapy in combination with αIFNy-blocking antibody (or isotype control). IFNy blockade reduced tumor cell expression of PD-L1 by 2.6-fold in NT mice to the level observed on CT26 cells cultured in vitro, suggesting that adaptive upregulation of PD-L1 occurs following implantation of tumor (Fig. 5D and E). However, the significant upregulation of PD-L1 observed following radiotherapy (2.4-fold compared with NT control) was completely abrogated by IFNy blockade confirming this as the driver of adaptive tumor cell expression of PD-L1.

Concomitant scheduling of αPD-L1 mAb and radiotherapy is critical for the generation of an effective antitumor immune response

The optimal schedule for combining radiotherapy and immune checkpoint inhibitors is currently unclear and may significantly affect the generation of a durable therapeutic antitumor immune response. We evaluated three distinct combination schedules where mice bearing established CT26 tumors received a fractionated radiotherapy cycle of 10 Gy in 5 fractions with administration of αPD-L1 mAb commencing either on day 1 of the fractionated radiotherapy cycle (schedule A), day 5 of the cycle (schedule B), or 7 days after completion of radiotherapy (schedule C; Fig. 6A). No significant difference in overall survival was found between schedule A and B with LTS of 60% and 57%, respectively (P >0.05 log-rank: Mantel–Cox test; Fig. 6B and Supplementary Fig. S6A). In contrast, sequential treatment with radiotherapy followed 7 days later by αPD-L1 mAb (schedule C) was completely ineffective at enhancing overall survival when compared with radiotherapy alone (median survival of 30 vs. 35 days, respectively; P >0.05 log-rank: Mantel–Cox test) despite similar tumor burden across groups (Supplementary Fig. S6B and S6C).

Analysis of tumor-infiltrating CD4⁺ and CD8⁺ T cells 24 hours after the last dose of radiotherapy revealed increased expression of PD-L1 when compared with time-matched NT controls (P < 0.05, Mann–Whitney test; Fig. 6C). In contrast, by 7 days after radiotherapy, no change in PD-1 expression was evident on CD4⁺ T cells and was found to be significantly decreased on CD8⁺ T cells when compared with time-matched NT controls (P < 0.05, Mann–Whitney test; Fig. 6D). Expression of PD-1 was consistently found to be higher on tumor-infiltrating CD8⁺ than CD4⁺ T-cells (Fig. 6C and D). We next compared efficacy with an acute versus extended αPD-L1 mAb dosing protocol where mice received either three doses of mAb concurrent with radiotherapy or the same with dosing extended (3qw) for an additional 2 weeks. No additional benefit was observed with extended αPD-L1 mAb administration (Supplementary Fig. S7).

Taken together these data demonstrate that treatment with low-dose fractionated radiotherapy leads to an acute increase in PD-1 expression on T cells and that sequential therapy where blockade of the PD-1/PD-L1 signaling axis is delayed until completion of an radiotherapy cycle may be ineffective potentially due to deletion or anergy of tumor-reactive CD8⁺ T cells.

Discussion

In this manuscript, we show that treatment with low doses of fractionated radiotherapy leads to upregulation of PD-L1 expression on tumor cells secondary to CD8⁺ T-cell production of IFNy. In models of melanoma, colorectal, and breast cancer, we demonstrate that the efficacy of radiotherapy can be enhanced through combination with αPD-L1 mAb, leading to the generation of memory immunity in LTS mice capable of protecting against tumor recurrence. Furthermore, our data reveal that dose scheduling may be critical for outcome with concurrent but not sequential therapy effective at improving local tumor control and survival.

Recent clinical trials have begun to evaluate blockade of the PD-1/PD-L1 axis with encouraging responses observed in multiple disease settings with mAb targeted against both PD-1 and PD-L1 (18–20, 24, 25). Despite this, combination approaches may be required to improve response rates and to generate durable antitumor immunity. We and others have demonstrated the potential to improve systemic antitumor immune responses through combination of immunotherapy with local radiotherapy, which has distinct advantages over chemotherapy in terms of highly focused tumor targeting and reduced systemic impact on the immune system (5, 22, 26, 27). Radiotherapy can lead to tumor cell DAMP release, DC recruitment, and type I IFN-dependent cross-priming of tumor-specific CD8⁺ T-cell responses (4, 6, 28). This is the first preclinical study to demonstrate that fractionated radiotherapy leads to increased tumor cell expression of PD-L1 through CD8⁺ T-cell production of IFNy. Here, tumor cell expression of PD-L1 may act as a biomarker of local antitumor response, suggesting that local radiotherapy may be sufficient to prime CD8⁺ T-cell responses. However, although treatment with radiotherapy alone was unable to generate durable anticancer immunity, efficacy was found to be enhanced through blockade of either PD-1 or PD-L1, suggesting that signaling through this axis may limit the immune response to radiotherapy suboptimally. We saw no significant differences in overall survival between radiotherapy delivered in combination with either αPD-1 or αPD-L1, or a combination of both mAb. Given this observation, it seems likely that the efficacy of these mAb when delivered in combination with radiotherapy is through blockade of PD-1/PD-L1 signaling and not mediated through the PD-L1/CD80 or PD-1/PD-L2 axes although further experiments are needed to confirm this.

In addition to generating adaptive immunity, radiotherapy has been shown to enhance recognition and antitumor activity of NK cells through increased tumor cell expression of NKG2D (29). Our data reveal that the depletion of NK cells reduced local tumor control at early time points (up to 11 days after completion of radiotherapy) but did not affect overall survival. In addition, the depletion of NK cells did not affect tumor cell expression of PD-L1 following radiotherapy, suggesting their limited contribution to local IFNy production. In contrast, although depletion of
CD4\(^+\) T cells did not affect survival following combination therapy, substantial increases in tumor cell expression of PD-L1 after radiotherapy was observed. The depletion of CD4\(^+\) T cells affects both helper T-cell and Treg populations. Although further studies are needed to delineate the relative contributions of these subpopulations of CD4\(^+\) T cells, we can speculate that CD4\(^+\) helper T cells may be dispensable for the generation of effective CD8\(^+\) T-cell responses after radiotherapy/PD-L1 mAb therapy. In addition, it is possible that tumor-infiltrating Tregs may be actively suppressing IFN\(\gamma\) production in the local tumor.

Figure 6. Dosing schedule is critical to outcome with radiotherapy potentiation only observed with concurrent but not sequential \(\alpha\)PD-L1 mAb therapy. A, schema for dose-scheduling studies. Mice received fractionated-dose radiotherapy (as 10 Gy in 5 daily fractions of 2 Gy) alone or in combination with \(\alpha\)PD-L1 mAb starting either on day 1 of radiotherapy cycle (schedule A), day 5 of radiotherapy cycle (schedule B) or 7 days after the last dose of radiotherapy (schedule C). B, survival curves of therapy. "++" \(P < 0.01\) compared with monotherapy (log-rank; Mantel–Cox test). C and D, expression of PD-1 on CD4\(^+\) (C) and CD8\(^+\) (D) T cells 24 hours and 7 days after the last dose of radiotherapy. "*" \(P < 0.05\) (Mann–Whitney test). Experimental groups contained at least five mice and are representative of at least two independent experiments. RT, radiotherapy.
milieu; however, further studies to assess the impact of Tregs on tumor cell PD-L1 expression following radiotherapy are required.

PD-L1 expression can be modulated by a number of cytokines, including type I and II IFNs, TNFα, and TGFβ (16, 23, 30–32). We show that tumor cell expression of PD-L1 can be enhanced following coculture with IFNγ and that addition of TNFα can further augment this response. However, blockade of IFNγR1 or in vivo depletion of IFNγ demonstrates dependence on IFNγ-mediated signaling for upregulation of PD-L1, with TNFα alone incapable of modulating tumor cell PD-L1 expression. Interestingly, in vivo depletion of IFNγ reduces PD-L1 expression on syngeneic tumor, matching the expression profile of tumor cells in vitro. This suggests that the local tumor microenvironment in the absence of therapeutic intervention may foster immunologically driven tumor adaptation, which may support tumor development. Similarly, in human melanocytic lesions, expression of PD-L1 has been shown to colocalize with areas of CD8+ T-cell infiltration and is postulated to represent an adaptive mechanism of immune escape (16). However, in our preclinical models, monotherapy with mAb targeting PD-1/PD-L1 only provides modest efficacy, which suggests that targeting this axis alone may be unlikely to bring about durable antitumor immune responses and underscores the requirement for a combinatorial approach.

A recent study in the TUBO breast cancer model found that treatment with a single dose of 12 Gy radiotherapy could also increase both tumor cell and monocyte-derived suppressor cell expression of PD-L1 (30). In contrast with the use of a single 12-Gy dose, our results demonstrate that upregulation of PD-L1 occurs at much lower biologic effective doses involving delivery of fractionated radiotherapy using 10 Gy in 5 daily fractions. This is an important finding given that this dose per fraction is commonly used in routine clinical practice. Several studies have demonstrated the superiority of either single ablative doses or fractionated radiotherapy on the generation of antitumor immune responses (4, 5, 22, 26, 33, 34). However, the varying intrinsic radiosensitivity of the cell lines used, their inherent immunogenicity, and the disparate phenotype of the tumor microenvironments (which may be influenced both by the tumor cells and the background of the mouse) may contribute to the lack of concordance across these published studies. What is clear from these studies is that for a given model, radiotherapy dose fractionation does influence the phenotype and antitumor activity of the immune response. Additional studies where the immunogenicity of different radiotherapy dose fractionation regimens is directly compared are clearly required.

To date, no studies have addressed the impact of sequencing on the efficacy of radiotherapy and αPD-L1 mAb therapy. This is especially pertinent given the predisposition to adopting a sequenced combination regimen in the clinic in an effort to limit adverse reactions. We show that only blockade of PD-L1 at the time of radiotherapy delivery can enhance therapeutic response with sequential therapy no better than treatment with radiotherapy alone. In addition, our data reveal that PD-L1 expression is elevated by 24 hours after completion of a fractionated radiotherapy cycle and remains elevated for at least 7 days after completion. Moreover, we found elevated PD-L1 expression on tumor-infiltrating CD4+ and CD8+ T cells 24 hours after radiotherapy when compared with control cohorts. Expression of PD-L1 was found to be consistently higher on CD8+ versus CD4+ T cells. Taken together, these data suggest that local fractionated radiotherapy can prime antitumor CD8+ T-cell responses but these may be attenuated by signaling through the PD-1/PD-L1 axis and early inhibition of this axis may be critical for the generation of durable effective antitumor responses. Furthermore, the requirement for chronic versus acute blockade of the PD-1/PD-L1 axis when combined with radiotherapy, is unclear. We observed no difference in survival when αPD-L1 mAb was administered 3qw over 1 week concurrent with radiotherapy delivery versus an extended schedule of up to 3 weeks. These data suggest that combination approaches with radiotherapy may permit a reduction in the duration of αPD-1/PD-L1 mAb therapy required to achieve therapeutic antitumor immune responses. Together these data have important implications for clinical trial design.

In summary, this study demonstrates that treatment with fractionated radiotherapy leads to upregulation of tumor cell expression of PD-L1 and that blockade of the PD-1/PD-L1 axis can enhance the immune response to fractionated radiotherapy in multiple syngeneic models. Our data suggest that dose scheduling of αPD-L1 mAb with radiotherapy may be critical to the generation of therapeutic immune responses with the capacity to reduce tumor burden and improve survival. This therapeutic combination may be a promising approach for the treatment of many solid malignancies where radiotherapy is commonly used and translation to early-phase clinical trial is clearly warranted.

Disclosure of Potential Conflicts of Interest
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