α -PD-L1 mAb enhances the abscopal effect of hypo-fractionated radiation by attenuating PD-L1 expression and inducing CD8⁺ T-cell infiltration

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Aim: We investigated a promising cooperative combination of radiotherapy (RT) and programmed death ligand 1 (PD-L1) monoclonal antibodies (mAb) in both local and abscopal tumors. **Materials & methods:** C57BL/6 mice were randomly grouped and received RT, α -PD-L1 mAb or combination therapy 13 days after implantation of Lewis lung carcinoma cells. Flow cytometry and immunohistochemistry analyses demonstrated CD8⁺ T-cell infiltration and PD-L1 expression in tumor issue. Cytometric bead arrays were used to examine cytokine levels. **Results:** Our studies revealed that administration of 8 Gy $\times$ 3 F with α -PD-L1 mAb promoted both local and distant control. Only local hypofractionated RT enhanced CD8⁺ T-cell infiltration with increased PD-L1 expression at distant foci, which might occur via serum IFN- γ modulation. Addition of α -PD-L1 mAb reduced PD-L1 expression and further increased CD8⁺ T-cell infiltration. **Conclusion:** We identified a novel mechanism through which combination therapy enhanced the abscopal effect.

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Radiotherapy (RT) causes tumor cell death by directly damaging DNA and is one of the main approaches to treat lung cancer. Available data showed that 64.3% of non-small-cell lung cancer (NSCLC) cases required RT [1]. Due to its lymphocytotoxic effect, RT was previously believed to be immunosuppressive [2]. However, many researchers recently demonstrated that radiation-induced T cells impaired tumor growth [3–5]. Extensive preclinical and clinical studies revealed that RT potentially activated the host antitumor immune system and exerted an abscopal effect, in which tumor growth outside the irradiated region was delayed by RT [6,7].

McBride *et al.* stated that RT promoted dendritic cell maturation and activated the immune system through ‘danger’ signals [8]. Further studies revealed that RT activated and recruited antigen-presenting cells, and promoted a tumor antigen-specific T-cell response by excreting damage-associated molecular patterns (DAMPs), including high-mobility group box 1 (HMGB1) and interferon (IFN) type I and II [9–13]. However, researchers also stated that IFN- γ induced the expression of programmed death ligand 1 (PD-L1) on tumor cells as a subsequent immune-escape mechanism [12,14]. The PD-1/PD-L1 axis was reported to inhibit tumor-infiltrating T-cell function, an effect that was defined as T-cell exhaustion [15]. A recent clinical trial with a PD-1-targeting monoclonal antibodies (mAb) showed that immune checkpoint inhibition significantly extended overall and progression-free survival, and exerted fewer adverse events than platinum-based chemotherapy [16]. Moreover, several clinical case reports correlated the abscopal effect with immunoradiotherapy [17,18]. These findings suggested that immune therapy,

particularly checkpoint inhibitors, might augment the abscopal effect. Currently available data indicated that the immune system and inflammatory signaling were key components of the abscopal effect [19–22]. Thus, identification of the optimal combination therapy for the abscopal effect and an exploration of the underlying mechanism are crucial requirements for immunoradiotherapy development.

Materials & methods

Mice & cells

C57BL/6 mice, aged 6–8 weeks, were purchased from the Hubei Research Center of Laboratory Animals. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University. All of the mice were raised under specific pathogen-free conditions. The mice were sacrificed when the tumor burden reached 1500 mm³. Lewis lung carcinoma (LLC) cells were purchased from the Cell Resource Center of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in complete DMEM supplemented with 10% fetal bovine serum and 1% glutamine–penicillin–streptomycin at 37°C in a 5% CO₂ atmosphere.

Tumor inoculation & therapy

LLC cells were implanted subcutaneously (s.c.) into the left upper flank (1×10^6 cells, treatment tumors) and left lower limb (2×10^5 cells, abscopal tumors) [23]. The tumor volume was calculated using the formula $L \times W^2 \times 0.52$, where L is the longer diameter and W the shortest diameter perpendicular to L [24]. The perpendicular tumor diameters were measured using calipers every other day until day 30. On day 13, when the average volumes of the irradiated and nonirradiated LLC tumors were approximately 75 and 30 mm³, respectively, the animals were randomly grouped as indicated (Figure 1A). All of the treatments were primed on day 13, and RT was administered as described previously [20]. Briefly, the mice (including the mice-administered mock radiation) were lightly anesthetized through an intraperitoneal (i.p.) injection of chloral hydrate (35 g/kg). All of the mice were irradiated using the Small Animal Radiation Research Platform (PXi X-RAD 225Cx, CT, USA). The mice received a single dose of 20 Gy on day 13 or three fractions of 8 Gy on consecutive days (days 14, 15 and 16; Figure 1A). PD-L1-blocking mAb (clone 10F.9G2, BioXCell) or vehicle phosphate-buffered saline (PBS) was given intraperitoneally at a dose of 10 mg/kg on days 13, 16, 19 and 22. T-cell depletion was performed four-times every 3 days starting 1 day before treatment. To achieve T-cell depletion, 250 mg of monoclonal α -CD4 (GK1.5 clone, BioXCell #BE003-1) or α -CD8 (2.43 clone, BioXCell #BE0061) antibodies were intraperitoneally administered, and depletion was verified by peripheral blood mononuclear cell staining (Supplementary Figure 3). All of the mice were sacrificed on day 30 to harvest tumors.

Figure 1. Cooperativity of immunoradiation in a metastatic lung cancer model. (A) All of the mice (including mice receiving mock radiation) were lightly anesthetized via the i.p. route. RT was administered locally and exclusively to the irradiated tumor, and the rest of the body was shielded. A single dose or multiple fractions were administered on consecutive days starting on day 13. The PD-L1-blocking mAb 9H10 was given i.p. four-times every 3 days, at days 13, 16, 19 and 22. Tumor volumes were measured every other day until day 30, when the mice were sacrificed. All treatments were primed on day 13. (B–E) Tumor growth curve (left panel) and tumor volume at day 10 after treatment (day 13 after tumor inoculation, right panel). (B) Irradiated tumor. (C) Nonirradiated tumor. (D & E) Depletion of CD4⁺ or CD8⁺ T cells in the 8 Gy $\times$ 3 F + α -PD-L1 group. (D) Irradiated tumor. (E) Nonirradiated tumor. The data are the means $\pm$ SEM of four to six mice per group.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

i.p.: Intraperitoneal; mAb: Monoclonal antibody; PD-L1: Programmed death ligand 1; RT: Radiotherapy; SEM: Standard error of the mean.

Figure 1. Cooperativity of immunoradiation in a metastatic lung cancer model (cont.). (A) All of the mice (including mice receiving mock radiation) were lightly anesthetized via the i.p. route. RT was administered locally and exclusively to the irradiated tumor, and the rest of the body was shielded. A single dose or multiple fractions were administered on consecutive days starting on day 13. The PD-L1-blocking mAb 9H10 was given i.p. four-times every 3 days, at days 13, 16, 19 and 22. Tumor volumes were measured every other day until day 30, when the mice were sacrificed. All treatments were primed on day 13. (B–E) Tumor growth curve (left panel) and tumor volume at day 10 after treatment (day 13 after tumor inoculation, right panel). (B) Irradiated tumor. (C) Nonirradiated tumor. (D & E) Depletion of CD4⁺ or CD8⁺ T cells in the 8 Gy $\times$ 3 F + α -PD-L1 group. (D) Irradiated tumor. (E) Nonirradiated tumor. The data are the means $\pm$ SEM of four to six mice per group.

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Immunohistochemistry

The tumors were fixed in 10% neutral-buffered formalin and embedded in paraffin; 3- μ m-thick sections were antigen-retrieved by citrate buffer (pH 6.0, #S1699, Dako Target Retrieval Solution, Glostrup, Denmark) after deparaffinization with xylene and alcohol gradients. These sections were then treated with 3% H₂O₂, blocked with 10% goat serum (#C-0005, Bioss, Beijing, China), and incubated at 4°C overnight with primary antibodies (Abcam, Ltd, Cambridge, UK): rabbit α -CD11b antibody [EPR1344] (#ab133357), rat α -Ly6c antibody [ER-MP20] (#ab15627) or rat α -Ly6g [RB6-8C5] (#ab25377). Using a Histostain–streptavidin–peroxidase kit (rabbit, #SP-0023, Bioss) and streptavidin–biotin complex (SABC)-POD (rat IgG) kit (#SA1025, Boster, Wuhan, China), the slides were incubated with secondary antibodies, an amplification reagent and either α -fluorescein-HRP or SABC. Finally, DAB chromogen (#C02-04001, Bioss) was added. The sections were counterstained with hematoxylin, dehydrated with alcohol gradients and xylene washes, and mounted with a cover slip. Hematoxylin & eosin (HE) staining was examined using an Autostainer XL (ST5010, Leica, Solms, Germany). To quantify the immunohistochemistry (IHC) staining results, the H-score was calculated under the supervision of an experienced pathologist as follows: H-score = $\sum P_i \times I_i$, where I_i was a score of 0 (no staining), 1 (weak), 2 (moderate) or 3 (strong), and P_i was the percentage of positive cells per field. Ten fields of each section were examined under a light microscope at 400 $\times$ magnification.

Flow cytometry

The harvested tumor tissues were digested with 1 mg/ml collagenase IV (Invitrogen) at 37°C for 40 min, and the resulting single-cell suspensions were stained with fluorescent-labeled antibodies, including CD4 (#553046, BD, CA, USA), CD8 (#553032, BD), CD45 (#561869, BD), PD-L1 (#25-5982-82, eBioscience, CA, USA), CD44 (#553134, BD), CD62L (#561917, BD), PD1 (#562671, BD), CD11b (#101226, Biolegend, CA, USA) and Gr-1 (#108428, Biolegend). The specific Fc γ II/III receptor 2.4G2 purified antibody BD Fc Block™ (#553142, BD) was used to block the FC receptor and thus eliminate nonspecific results caused by binding of fluorescently labeled antibodies to Fc receptors. Isotype controls were used for all antibodies. Gates and quadrants were set based on isotype control staining. The samples were examined with a FACS Aria™ III Cell Sorter (BD), and the data were analyzed using FlowJo software.

Serum cytokine levels

Retroocular artery blood was collected and centrifuged at 5000 rpm for 5 min and then at 3000 rpm for another 5 min. The commercial BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (CBA; Cat. #551287, BD) was used to examine the levels (pg/ml) of IL-2, IL-4, IL-5 and IFN- γ according to the manufacturer's instructions. The samples were analyzed using a flow cytometer (FACS Aria™ III Cell sorter, BD), and the cytokine levels were examined 5, 10 and 17 days after the first day of treatment.

Statistical analyses

All experiments were repeated at least three-times. All of the data were presented as the means $\pm$ SEMs. Two-way analysis of variance was used for assessments in all groups, including tumor growth curves [25]. A p-value less than 0.05 was considered statistically significant.

Results

α -PD-L1 mAb enhances the abscopal effect of hypofractionated radiation

In our animal experiments, RT alone and combination therapy caused a significant delay in tumor growth in the irradiated region (Figure 1B). Analysis of the irradiated tumors showed no significant difference in volume between 8 Gy $\times$ 3 F and 8 Gy $\times$ 3 F + α -PD-L1 (Figure 1B; $p = 0.216$), and these results were similar to those obtained with the 20 Gy treatment and the 20 Gy + α -PD-L1 treatment (Figure 1B; $p > 0.05$). Administration of α -PD-L1 alone slightly inhibited tumor growth in both irradiated and nonirradiated regions (Figure 1B & C; $p < 0.01$ vs the control). The growth of the nonirradiated tumors was significantly inhibited in the mice treated with fractionated RT combined with α -PD-L1 (Figure 1C; $p < 0.01$). All of the treatments except the single 20 Gy treatment decreased tumor growth in the nonirradiated region (Figure 1C). Compared with the control, single high-dose RT did not significantly improve the tumor volume (Figure 1C; $p = 0.756$). The depletion of CD8⁺ cells significantly abrogated the control of irradiated tumor following combined therapy (Figure 1D). Interestingly, our data showed that the abscopal effect appeared only following treatment with fractionated RT combined with

α -PD-L1 (Figure 1C), while depletion of CD8⁺ T cells also significantly abrogated the abscopal effect (Figure 1E). However, mice depleted of CD4⁺ T cells still showed a marked delay in irradiated tumor growth (Figure 1D), and the growth of nonirradiated tumors was restrained during the early period and then accelerated (Figure 1E).

RT combined with immunotherapy improved CD45⁺ T cells in tumors

Given the observations described above, we subsequently explored the mechanisms occurring in the tumor microenvironment 5 and 10 days after beginning RT (at day 13). In the RT region, immunoradiotherapy as well as RT alone increased the infiltration of CD45⁺ cells (Figure 2A; immunoradiotherapy vs control; RT vs control; $p < 0.05$). There was no increase in CD45⁺ cells in the nonirradiated regions, and the proportion of CD45⁺ cells was comparable in the mice receiving combined treatment (Figure 2B; day 5 vs 10; $p > 0.05$). Specifically, the proportion at day 10 was slightly higher than that at day 5 after 8 Gy $\times$ 3 F + α -PD-L1, but this was not significant (Figure 2B). The blockade of PD-L1 improved the percentage of CD45⁺ cells 5 days after the start of

Figure 2. Radiotherapy-induced tumor-infiltrating T cells in the tumor environment showed a combined effect with α -programmed death ligand 1. At days 5 and 10 after RT (day 13 after tumor inoculation), the mice were sacrificed, and the tumor cells were analyzed. The data are representative of at least two independent experiments ($n > 3$). (A & B) CD45⁺ cells among lymphocytes at days 5 and 10 after RT. (A) Irradiated tumor. (B) Nonirradiated tumor (right panel). (C & D) Percentage of CD8⁺ T cells within the CD45⁺ cell population. (C) Irradiated tumor. (D) Nonirradiated tumor. (E & F) Percentage of MDSCs (CD11b⁺ Gr1⁺) in the population of CD45⁺ cells. (E) Irradiated tumor. (F) Nonirradiated tumor.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RT: Radiotherapy; MDSC: Myeloid-derived suppressor cell.

Figure 2. Radiotherapy-induced tumor-infiltrating T cells in the tumor environment showed a combined effect with α -programmed death ligand 1 (cont.). At days 5 and 10 after RT (day 13 after tumor inoculation), the mice were sacrificed, and the tumor cells were analyzed. The data are representative of at least two independent experiments ($n > 3$). (A & B) CD45⁺ cells among lymphocytes at days 5 and 10 after RT. (A) Irradiated tumor. (B) Nonirradiated tumor (right panel). (C & D) Percentage of CD8⁺ T cells within the CD45⁺ cell population. (C) Irradiated tumor. (D) Nonirradiated tumor. (E & F) Percentage of MDSCs (CD11b⁺ Gr1⁺) in the population of CD45⁺ cells. (E) Irradiated tumor. (F) Nonirradiated tumor.

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treatment (at day 13); however, the percentage at day 10 was decreased in both the irradiated and nonirradiated regions (Figure 2A & B).

8 Gy $\times$ 3 F + α -PD-L1 improved the percent of CD8⁺ tumor-infiltrating T cell

Previous studies revealed that CD8⁺ T-cell infiltration in tumors is a pivotal factor for the abscopal effect [20,21]. The degree of local changes in CD8⁺ tumor-infiltrating T cells (TILs) is related to enhanced inhibition of tumor growth and tumor clearance.

In the irradiated tumors 5 days after RT, the 20 Gy + α -PD-L1 treatment led to an increase in CD8⁺ TILs (Figure 2C; $p < 0.001$; 20 Gy + α -PD-L1 vs the control). Interestingly, on day 5, the 8 Gy $\times$ 3 F treatment with or without α -PD-L1 did not show a significant increase compared with the tumors in control mice (Figure 2C). At day 10, RT + α -PD-L1 and RT alone notably enhanced CD8⁺ TIL infiltration in the irradiated tumors (Figure 2C; $p < 0.05$). The data in the nonirradiated region showed that 8 Gy $\times$ 3 F + α -PD-L1 resulted in greater improvement in the proportion of CD8⁺ T cells compared with 8 Gy $\times$ 3 F alone at 5 or 10 days after RT (Figure 2D; $p < 0.01$, respectively). Similarly, we observed the same phenomenon with 20 Gy + α -PD-L1 therapy (Figure 2D; $p < 0.01$). No significant difference was observed between 8 Gy $\times$ 3 F + α -PD-L1 and 20 Gy + α -PD-L1 on day 5 after beginning RT (Figure 2D; $p = 0.121$), while at day 10, the proportion of CD8⁺ T cells after 8 Gy $\times$ 3 F + α -PD-L1 was markedly higher (Figure 2D; $p < 0.01$). Notably, only the mice receiving 20 Gy showed a significant decrease in the percentage of CD8⁺ T cells within 10 days after treatment (Figure 2D; $p < 0.01$).

Analysis of myeloid-derived suppressor cell dynamic changes in tumors

Myeloid-derived suppressor cells (MDSCs) are associated with radioresistance [25,26]. MDSCs in the tumor microenvironment can facilitate tumor relapse. MDSCs were analyzed at days 5, 10 and 17 after RT (at day 13 after tumor inoculation). In the irradiated tumors, immunoradiotherapy reduced the number of MDSCs, but no significant difference was observed (Figure 2E; $p > 0.05$). Interestingly, $8 \text{ Gy} \times 3 \text{ F}$ plus α -PD-L1 did not enhance the reduction in MDSCs compared with $8 \text{ Gy} \times 3 \text{ F}$ alone at day 10 (Figure 2E; $p > 0.05$), whereas the MDSC levels of the both treatments were lower than those of the other treatments. These results of the nonirradiated tumors were similar to those of irradiated tumors (Figure 2F). In particular, in the nonirradiated tumors, $8 \text{ Gy} \times 3 \text{ F} + \alpha$ -PD-L1 did not enhance MDSC reduction compared with $8 \text{ Gy} \times 3 \text{ F}$ alone at day 5 (Figure 2F; $p > 0.05$). Moreover, at day 10, all groups showed marked decreases in MDSCs, except for mice receiving hypofractionated RT combined with α -PD-L1, which showed no difference from the control group (Figure 2F; $p > 0.05$).

The IHC samples harvested at day 17 after RT showed that RT alone or combined with α -PD-L1 decreased the amount of infiltrating MDSCs. The optimal method was the $8 \text{ Gy} \times 3 \text{ F} + \alpha$ -PD-L1 treatment, which inhibited CD11b⁺ cell accumulation in both local and distant tumors (Figure 3A–F). Of note, RT alone caused an increase in MDSCs on day 17. PD-L1 mAb could effectively decrease MDSC levels when combined with $8 \text{ Gy} \times 3 \text{ F}$, while it had no effect when combined with 20 Gy.

Fractionated RT led to a substantial increase in PD-L1 expression in abscopal tumors

Localized RT was reported to regulate tumor clearance in a T-cell-dependent manner [27]. Despite the immune stimulation effects of RT, relapse often occurs, indicating that RT alone does not optimally induce adaptive immunity to regulate complete tumor regression. The negative regulatory signals of the PD-L1/PD-1 axis might participate locally in tumor relapse.

In the irradiated regions, RT induced PD-L1 expression at day 5 after RT, particularly in tumors receiving a single high dose of RT (Figure 4A). Blockade of PD-L1 led to a reduction of detected PD-L1 levels (Figure 4A). The PD-L1-blocking mAb might also contribute to the reduced detection of PD-L1 (Supplementary Figure 4). Similarly, combination of RT and α -PD-L1 treatment significantly decreased the detected levels of PD-L1 at day 5 (Figure 4A; $p < 0.05$ vs RT alone). Ten days after treatment, the PD-L1 levels in all of the groups, except the α -PD-L1 group, were decreased compared with those at day 5 (Figure 4A; $p < 0.05$). Similarly, the levels of PD-L1 at day 10 were also increased in the nonirradiated region (Figure 4B; $p < 0.05$). In the nonirradiated region, no significant difference in PD-L1 detection was observed in the mice that received 20 Gy compared with the controls at day 10 (Figure 4B; $p = 0.166$). In contrast, only fractionated RT led to a substantial increase in PD-L1 levels (Figure 4B & C; $p < 0.01$). The detection of PD-L1 in the α -PD-L1 group increased after treatment, as described above. Interestingly, the levels remained low when combined with fractionated RT (Figure 4B; $p = 0.385$ at day 5 after treatment vs day 10).

$8 \text{ Gy} \times 3 \text{ F} + \alpha$ -PD-L1 reduced the percent of PD-1⁺ CD8⁺ T cells & PD-L1⁺ MDSCs

The examination of PD1 expression on CD8⁺ T cells in irradiated tumors indicated that $8 \text{ Gy} \times 3 \text{ F} + \alpha$ -PD-L1 did not decrease the percentage of PD-1⁺ CD8⁺ T cells (Figure 4D; $p > 0.05$), whereas single high-dose RT increased PD-1⁺ CD8⁺ T expression (Figure 4D). In nonirradiated tumors, this reduction was only observed with combination immunoradiotherapy (Figure 4E). Treatment with 20 Gy increased the number of PD1⁺ CD8⁺ T cells (Figure 4E; $p < 0.05$).

We observed that combination therapy effectively reduced the amount of immune-suppressive PD-L1⁺ MDSCs in both irradiated and nonirradiated tumors at day 5 (Figure 4F & G), whereas α -PD-L1 mAb also showed a notable effect (Figure 4F & G). Similarly, at day 10, RT + α -PD-L1 reduced the numbers of PDL1⁺ MDSCs in both regions with tumors (Figure 4F & G).

These data suggested that RT-induced PD-L1/PD-1 expression inhibited T-cell toxicity in the tumor microenvironment and induced tumor relapse [12]. Furthermore, these data indicated that α -PD-L1 downregulated the PD-L1/PD-1 axis in TILs, which might be one of the mechanisms underlying the cooperativity of immunoradiotherapy.

Fractionated RT altered the peak & level of serum cytokines

To investigate the mechanism through which local RT influences the antitumor immune response, we examined the serum levels of cytokines relevant to inflammation. Levels of IL-2, IL-4, IL-5 and IFN- γ cytokines at days 5, 10

Figure 3. Myeloid-derived suppressor cells in tumors. Mice were treated as described above. (A–C) Tumor samples ($n \geq 3$ per group) were harvested at day 30 after tumor inoculation. The original magnification was 200 $\times$. (A) CD11b⁺; (B) Ly6c⁺; (C) Ly6g⁺. (D–F) H-score obtained after IHC staining; (D) CD11b⁺; (E) Ly6c⁺; (F) Ly6g⁺. At least ten fields were included in the calculation of positive cells per field at 400 $\times$ magnification.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

IHC: Immunohistochemistry.

Figure 3. Myeloid-derived suppressor cells in tumors (cont.). Mice were treated as described above. (A–C) Tumor samples ($n \geq 3$ per group) were harvested at day 30 after tumor inoculation. The original magnification was $200\times$. (A) CD11b⁺; (B) Ly6c⁺; (C) Ly6g⁺. (D–F) H-score obtained after IHC staining; (D) CD11b⁺; (E) Ly6c⁺; (F) Ly6g⁺. At least ten fields were included in the calculation of positive cells per field at $400\times$ magnification.

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and 17 after RT (at day 13) and the dynamic changes in each group are shown in Figure 5A. The changes in the 20 Gy and 8 Gy $\times$ 3 F groups were diverse. The 20 Gy treatment elevated the levels of all of the cytokines, although the relative levels of the four cytokines showed no differences compared with those in the control group (Figure 5A). In addition, the skewing peak was also similar to that in the control, which showed immune-suppression activity in tumors. The combined treatment with PD-L1 did not change the skewing (Figure 5A). The three fractions of 8 Gy not only changed cytokine levels but also shifted the secretion peak earlier to day 5. Additionally, the 8 Gy $\times$ 3 F treatment combined with α -PD-L1 changed the cytokine levels and caused the secretion peak to be on day 10, whereas the other treatments had no effect. These findings might explain why an increased abscopal effect was observed in tumors treated with 8 Gy $\times$ 3 F combined with α -PD-L1.

Previous studies showed that IFN- γ had a tumor cytotoxic effect [13] and enhanced PD-L1 expression [12,24]. Our data showed that fractionated RT increased the serum level of IFN- γ on day 5 (Figure 5B; $p < 0.05$) and that the addition of α -PD-L1 extended the peak to day 10 (Figure 5B; $p > 0.05$ for 8 Gy $\times$ 3F + α -PD-L1 vs the other groups). The 20 Gy treatment, the PD-L1 treatment and the combined treatment induced IFN- γ increased at day 10 (Figure 5B).

RT + α -PD-L1 increases effector memory cells in the spleen

The mouse model gave us the opportunity to quantify the systemic effects of T-memory cell expansion induced by single and combined therapy. For these studies, we defined naive cells as CD62L⁺ CD44⁻, the effector memory phenotype as CD44⁺ CD62L⁻ and central memory cells as CD44⁺ CD62L⁺ [28]. In the mouse model, our data showed that RT alone reduced the relative naive T-cell percentage and augmented the effector memory phenotype (Figure 6A). The addition of α -PD-L1 mAb significantly increased the percentage of effector T-memory cells

(Figure 6A & B; $p < 0.05$; RT + α -PD-L1 vs RT). Effector T-memory cells reached their maximal level with 8 Gy $\times$ 3F + α -PD-L1 therapy (Figure 6B & C). However, PD-L1 blockade alone did not significantly add to this effect at day 5 (Figure 6B; $p > 0.05$; α -PD-L1 vs the control), whereas at day 10, the effect was less than that in the control (Figure 6C; $p < 0.001$, α -PD-L1 vs the control).

Discussion

RT is currently the most popular treatment for lung cancer, and immunotherapy has continued to attract increasing attention due to its specificity and effectiveness. The combination of these two treatments not only causes maximal damage to tumor cells but also destroys the tumor microenvironment. An increasing number of basic and clinical studies have suggested that combination therapy, including immunoradiotherapy and immunochemotherapy, may be the treatment direction with the greatest potential to cure malignant tumors, including lung cancer [6,21,24]. In this study, we demonstrated that fractionated, but not single, local RT combined with PD-L1 blockade inhibited nonirradiated tumors and induced a systemic antitumor immune response. In a lung carcinoma metastasis model, we observed that the addition of α -PD-L1 mAb enhanced the efficacy of RT, similar to the effects observed in various

Figure 4. Radiotherapy-induced programmed death ligand 1 expression in the microenvironment can be downregulated by α -programmed death ligand 1. At days 5 and 10 after the first treatment was administered (at day 13 after tumor inoculation), the mice were sacrificed, and the tumor cells were analyzed. The data are representative of two or three independent experiments ($n > 3$). The control samples were obtained from untreated mice with a time-matched sample. (A–C) PD-L1 expression in tumors: (A) irradiated tumor, (B) nonirradiated tumor and (C) histogram comparing PD-L1 expression in the three groups. (D & E) PD1 expression on intratumoural CD8⁺ T cells: (D) irradiated tumor and (E) nonirradiated tumor. (F & G) PD-L1 expression on intratumoural CD11b⁺ Gr1⁺ cells: (F) irradiated tumor and (G) nonirradiated tumor.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

PD-L1: Programmed death ligand 1.

Figure 4. Radiotherapy-induced programmed death ligand 1 expression in the microenvironment can be downregulated by α -programmed death ligand 1 (cont.). At days 5 and 10 after the first treatment was administered (at day 13 after tumor inoculation), the mice were sacrificed, and the tumor cells were analyzed. The data are representative of two or three independent experiments ($n > 3$). The control samples were obtained from untreated mice with a time-matched sample. (A–C) PD-L1 expression in tumors: (A) irradiated tumor, (B) nonirradiated tumor and (C) histogram comparing PD-L1 expression in the three groups. (D & E) PD1 expression on intratumoural CD8⁺ T cells: (D) irradiated tumor and (E) nonirradiated tumor. (F & G) PD-L1 expression on intratumoural CD11b⁺ Gr1⁺ cells: (F) irradiated tumor and (G) nonirradiated tumor.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

PD-L1: Programmed death ligand 1.

solid tumors [12,23,24]. Our results confirmed that the dose schedule may be a vital factor in checkpoint-blockade therapy because appropriate RT enhanced the abscopal effect and activated systemic antitumor immune responses.

Fractionated RT was superior to single-dose RT when combined with α -PD-L1. Prior clinical trials indicated that only 15–25% of patients responded to PD-L1 or PD-1 mAbs, depending on the tumor type [29]. RT has a complex local effect, inducing the release of DAMPs by tumor cells and IFN secretion for the cross-priming of CD8⁺ T cells [13,27,30]. Moreover, local RT contributes to tumor regression through CD8⁺ T cells and IFN- γ [2,13,27]. Nevertheless, IFNs induce PD-L1 expression and are associated with restricted local immunity and increased tumor relapse. A recent clinical trial reported that high levels of PD-L1 expression (PD-L1-positive expression > 50%) were a biomarker for the administration of a PD-1 antibody as first-line therapy in advanced NSCLC [16,31]. A high level of PD-L1 expression in the tumor microenvironment is an indication for the administration of a checkpoint-blocking antibody, such as α -PD-1/PD-L1 mAb.

Figure 5. Dynamic serum cytokine levels. Serum samples were obtained at days 5, 10 and 17 after the treatments. Three independent experiments were repeated ($n = 4$). **(A)** Four cytokines showed changes in the six groups: 8 Gy $\times$ 3 F + α -PD-L1, 8 Gy $\times$ 3 F, 20 Gy + α -PD-L1, 20 Gy + PBS, α -PD-L1 and PBS. PBS was administered at a dose of 200 μ g through the i.p. route at days 13, 16, 19 and 22. **(B)** IFN- γ was changed in the six groups at three time points.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

i.p.: Intraperitoneal; PBS: Phosphate-buffered saline; PD-L1: Programmed death ligand 1.

This study provides the first demonstration that hypofractionated radiation upregulated PD-L1 expression in both irradiated and nonirradiated tumors postradiation, which indicated that radiation was an optimal combination strategy with α -PD-L1 treatment. RT alone could induce CD8⁺ T-cell infiltration in locally irradiated regions in both preclinical mouse models [27,32] and clinical data [33]. Our data also showed that hypofractionated RT alone could prime CD8⁺ T cells and induce high expression of PD1/PD-L1 in both irradiated and nonirradiated tumors at the same time, while the combination with α -PDL1 mAb reduced both PD-L1 expression and PD1⁺ CD8⁺ T cells.

MDSCs directly enhance tumor cell survival and have indirect effects on local T-cell suppression [20,34]. We first observed a dynamic change in MDSCs with the different treatment strategies, whereas in the irradiated tumors, all treatments effectively reduced the number of MDSCs; hypofractionated RT alone induced MDSCs systemically, and RT combined with α -PD-L1 mAb was not able to reduce the increase of this subgroup. Similarly, in the nonirradiated tumors, hypofractionated RT alone and hypofractionated RT combined with α -PD-L1 treatment caused a peak effect at day 10. This study also explored MDSC dynamics after different doses of RT for the first time. Previous studies have demonstrated that fractionated RT induced intratumoural MDSCs, and the levels of

Figure 6. Spleen T-memory cells. The spleen was harvested at days 5 and 10 after RT (day 13 after tumor inoculation). **(A)** T-memory cells in the spleen at days 5 and 10 after RT. **(B)** Effector T memory at day 5 after RT. **(C)** Effector T-memory cells at day 10 after RT.

* < 0.05; **p < 0.01; ***p < 0.001.

RT: Radiotherapy.

MDSCs reached a peak at day 10 after RT [20]. Moreover, we demonstrated that hypofractionated RT combined with α -PD-L1 also reduced PD-L1 expression on MDSCs (Figure 4F & G) as well as tumor cells. Hypofractionated RT combined with α -PD-L1 treatment, but not RT alone, effectively reduced the PD-L1⁺ MDSC populations. Appropriate RT has also been shown to induce high PD-1/PD-L1 expression and recruited MDSCs to the tumor environment to promote immune escape [12,16]. Therefore, RT alone was incapable of inducing durable antitumor immunity; however, the efficacy of RT was strengthened by the blockade of either PD-1 or PD-L1, indicating that expression of the PD-1/PD-L1 axis might restrict the immune response to RT in a suboptimal manner.

Previous studies on CD4⁺ T cells have been controversial: some researchers have stated that CD4⁺ T cells can exert a cytotoxic function against tumor cells [15,21,35,36], whereas others found that CD4⁺ T cells were dispensable [23]. Nevertheless, in our mouse model, CD4⁺ T cells were not necessary for the efficacy of the therapeutic combination of RT and α -PD-L1 mAb, and CD4 depletion exerted a bidirectional effect by first halting growth of abscopal tumors followed by subsequent acceleration (Figure 1D & F). These data suggested that

Th2 CD4⁺ T cells and Tregs functioned as immune-suppression elements, whereas Th1 cells acted as immune stimulators.

Our research illustrated the dynamic changes in the MDSCs in local and distant foci for the first time. Immunotherapy combined with fractionated RT ultimately resulted in decreases in the MDSC and PD-L1⁺ MDSC populations in both the irradiated and abscopal tumors. In addition, we confirmed the potential of improving systemic antitumor immune responses through immunoradiotherapy, which has advantages over chemotherapy including highly focused tumor targeting and fewer side effects [21,37–39]. For the host immune system, radiation-induced immunogenic tumor cell death and the subsequent generation of danger signals can contribute to an *in situ* vaccine [40–42]; although the generated responses were not therapeutically significant, additional immunotherapeutic interventions might lead to meaningful antitumor immunity.

Our studies are the first to reveal the dynamic changes in spleen memory populations, which indicated changes in the host immune system and revealed that the optimal effects were induced by radiation combined with α -PD-L1 treatment. In addition, the treatments caused changes in serum cytokine levels, and the significant increase in the IFN- γ peak and the comparable duration following fractionated radiation combined with α -PD-L1 mAb clearly showed the advantages of this treatment. We observed that α -PD-L1 exerted few effects on the immune system, including the spleen and cytokines, explaining its low efficacy in attracting TILs into tumor foci. When combined with RT, the skewing was obvious (Figure 6). The effect on T memory cells in the spleen was similar to that found for CD8⁺ T cells in nonirradiated tumors at early time points after fractionated RT with α -PD-L1 (Figures 2D & 6B), suggesting that combination therapy altered the local and systemic immune statuses to an immune elimination status. Although immune therapy is a breakthrough treatment, resistance to α -PD-1/PD-L1 has been reported in several clinical trials [41,43]. In our experiments, we found that this optimized treatment showed a strong abscopal effect; however, we also observed accelerated tumor growth after a certain period. The resistance observed to the combination treatment suggested the possible limitations of immunoradiotherapy. This might result from decreased cytokine levels and a high proportion of MDSCs during the period after treatment. Furthermore, prolonged use of α -PD-L1 led to the upregulation of PD-L1 expression, which might explain the resistance of α -PD-L1. However, further studies on the mechanism of how combination treatment modulates the tumor environment are still required. Together, our data have important implications for the mechanism of combined treatments and for clinical trial design.

There were some limitations in our experiments. The data presented are confined to a single murine model of solid malignancy, the LLC, which is the only common lung cancer cell line capable of neoplasia in mice with normal immune functions, though there some references have studied immunotherapy using a single murine model of solid malignancy [44,45]. The tumor weight was not measured due to the presence of liquid inflammatory necrosis in the tumors. It was difficult to weigh the tumors accurately. Radiation had direct lethal effects on the tumor and immune subsets. Although we excluded the dead cells according to FSC and SSC, dead/live staining reagents were not used to specifically identify dead cells in flow cytometry due to panel limitations. In summary, our experiments provide the first demonstration that therapy with hypofractionated RT and α -PD-L1 mAb upregulated CD8⁺ T-cell infiltration in local foci. Our work revealed a previously unclear mechanism for how PD-L1 blockade increased the efficacy of RT. Our data suggest that fractionated radiation might be appropriate for immunoradiotherapy, as immune therapy is a first-line treatment for advanced NSCLC patients, and the combination of RT and immunotherapy might be a promising therapy regimen for many solid malignancies that are commonly treated with RT.

Conclusion

RT has the advantage of local control, while α -PD-L1 mAb are able to reactivate T cells and the immune system. Thus, the combination of RT and α -PD-L1 mAb is cooperative and shows notable enhancement of the abscopal effect. Our studies suggest that administration of 8 Gy $\times$ 3 F with α -PD-L1 mAb promoted both local and distant control.

Future perspective

Although our study suggested that administration of 8 Gy $\times$ 3 F with α -PD-L1 mAb enhanced the abscopal effect, not all mice were able to respond to the combination therapy. This novel strategy needs further support in clinical trials. We are currently working to identify the relationship between tumor immune microenvironments and combination treatment with RT and immune checkpoint therapy. Deep investigations into the underlying mechanisms will enrich our comprehensive understanding of the tumor microenvironment and immune system.

Summary points

Background

- Radiotherapy (RT) is one of the main approaches to treat lung cancer and its effect on the nonirradiated tumor region is known as abscopal effect. Many studies revealed that radiotherapy potentially activated the antitumor system and exerted an abscopal effect. The immunotherapy, particularly checkpoint inhibitors, can augment the abscopal effect.

Main topics

- Researchers stated that IFN- γ induced the expression of programmed death ligand 1 (PD-L1) that was reported to associate with inhibition of the tumor-infiltrating function of T cells. Our study suggested that α -PD-L1 monoclonal antibodies (mAbs) enhanced the abscopal effect induced by hypofractionated radiation through IFN- γ .

Results

- Our data showed that α -PD-L1 treatment promoted the abscopal effect induced by fractionated RT, whereas depletion of CD8⁺ T cells significantly abrogated the abscopal effect. Moreover, CD4⁺ T cells depletion delayed the irradiated tumor growth.
- In the RT region, not only RT with immunotherapy but also RT alone improved the infiltration of CD45⁺ cells in the nonradiated regions.
- Hypofractionated RT increased CD8⁺ T cell and PD-L1 expression in abscopal tumor. The hypofractionated RT with α -PD-L1 treatment effectively decreased the myeloid-derived suppressor cells both in local and distant tumors.
- Local hypofractionated RT induced the serum levels of IFN- γ and extended its peak, but has no significant effect on other serum cytokines, such as IL-2, IL-4 and IL-5.
- α -PD-L1 mAb combined with RT increased effector memory cells in the spleen, however, PD-L1 blockade alone had no additional effects.

Conclusion

- RT has the advantage of local control, while α -PD-L1 mAb are able to reactivate T cells and the immune system. Thus, the combination of RT and α -PD-L1 mAb is cooperative, and has notable effects on the abscopal effect. Our studies suggested that administration of 8 Gy $\times$ 3 F with α -PD-L1 mAb promoted both local and distant control.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/imt-2018-0049

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