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Optimal radiation dose to induce an abscopal effect by combining carbon-ion radiotherapy and anti-CTLA4 antibody

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ABSTRACT

Background and purpose: Although carbon-ion radiotherapy (CIRT) has led to good outcomes, controlling metastasis is still crucial for improving overall survival. This study aimed to evaluate the effectiveness of by two combinations, one of CIRT and anti-CTLA4 antibody, the other of CIRT and anti-PD-1 antibody, applied at different radiation doses for distal tumour and metastasis suppression.

Materials and methods: Murine cancer cells (colon carcinoma Colon-26 cells for experiments and osteosarcoma LM8 cells for verification) were grafted into both sides of the hind legs of syngeneic mice. Right-side tumours were irradiated with 3 Gy or 10 Gy CIRT while the left-side tumours were not irradiated, followed by the administration of the anti-CTLA4 antibody or anti-PD-1 antibody. The diameter of the tumours in both legs was measured 3 times per week after irradiation. The number of pulmonary metastases was evaluated within 3 weeks after irradiation.

Results: Compared with the control group, the high-dose group showed promising anti-cancer benefits in terms of both irradiated tumours and lung metastasis, but neither 10 Gy CIRT combined with the anti-CTLA4 antibody nor 10 Gy CIRT combined with the anti-PD-1 antibody suppressed the growth of distant unirradiated tumours. In the low-dose group, the effect on primary tumour control was slightly weaker than that in the high-dose treatment group, but significant suppressive effects on both distant unirradiated tumours and metastases were observed following 3 Gy CIRT combined with anti-CTLA4 antibody treatment. Specifically, the volume of distant unirradiated tumours decreased by 40 % compared with that of the control group, and no lung metastasis was observed.

Conclusion: Our findings suggest that there is an optimal dose range for the abscopal effect generated with the CIRT combined with anti-CTLA4 antibody, and it highlights a new opportunity for increased induction efficiency of the abscopal effect of combination therapy.

Introduction

Radiotherapy (RT) is a noninvasive treatment for cancer patients. One of the RT modalities, carbon-ion radiotherapy (CIRT) is considered the most advanced technology because of its excellent biological properties and dose distribution, especially in radioresistant tumours, which are difficult to cure via conventional photon RT [1–3]. Over the past several decades, many clinical trials completed in Japan and Germany have shown that CIRT can improve outcomes with extremely low toxicity at various tumour sites [4–7]; however, the control of distant metastases remains a difficult therapeutic challenge for CIRT [8]. Immunotherapy shows potential anti-metastatic potency because of its ability to enhance systemic anti-tumour immunity. However, its efficacy against solid tumours is unstable, and its curative rates are usually low [9]. CIRT combined with immunotherapy was therefore developed as a strategy that could have good therapeutic effects on both local tumour

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control and metastasis suppression.

Cancer immunotherapy, especially immune checkpoint blockade, exhibits systemic anti-tumour activity against primary local tumours and even distant tumours [10]. Clinical data show that immune checkpoint therapy (ICT) has good effects on melanoma, renal cell carcinoma, prostate cancer, urothelial carcinoma, non-small cell lung cancer, ovarian cancer and bladder cancer [11]. Cancer cells escape from cytotoxic T lymphocytes through the functional inactivation of immunocytes resulting from immune checkpoint molecule expression [12, 13]. Checkpoint blockade on the T-cell surface by anti-PD-1 and anti-CTLA4 antibodies has been shown to induce the proliferation of tumour-infiltrated T cells, thus launching an immune attack against the cancer [14]. Nevertheless, the efficacy of this treatment against solid tumours is not comparable to that of CIRT, and the systemic anti-tumour effect varies widely among individuals.

RT combined with immunotherapy, as a novel anti-metastatic strategy, is progressing rapidly [15]. Several reports have revealed that RT combined with virus-mediated vaccination or immune checkpoint blockade immunotherapies can be used to manage local tumours [16–18]. Interestingly, some studies reported a very rare abscopal effect in which single-photon RT may also regress metastatic tumours outside the radiation field in addition to its contribution to irradiated tumours [19–23]. Further studies revealed that the abscopal effect suppressed metastatic tumours of some types of cancer in combination with conventional photon RT and immune checkpoint blockade or granulocyte-macrophage colony-stimulating factor (GM-CSF) [24–26]. Recently, it has been reported that proton or heavy-ion beam monotherapy can also induce similar phenomena with low probability [27, 28]. This finding has led to great interest in exploring potential abscopal effects by combining heavy-ion RT and immunotherapy, which may further improve the efficiency of metastasis suppression [29]. However, related studies thus far have been very limited, and the abscopal effect of CIRT combined with immune checkpoint blockade has been little understood.

Controlling distant metastasis is an urgent issue to be solved to improve CIRT outcomes, especially overall survival. Induction of the abscopal effect is a suitable solution; however, the induction efficiency of the abscopal effect is not sufficient for use in treatment with confidence. The aim of this study was to investigate the impact of different irradiation doses on the abscopal effect and determine an appropriate radiation dose of CIRT combined with immune checkpoint blockade immunotherapy. In this study, two doses, 10 Gy and 3 Gy, were selected to evaluate the abscopal effect of combining CIRT with immunotherapy.

Materials and methods

Mice and cancer cell lines

Six-week-old female BALB/c and C3H/He mice were obtained from Japan SLC Co., Ltd. (Shizuoka, Japan). All experiments were conducted in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the National Institutes for Quantum Science and Technology Institutional Animal Care and Use Committee (protocol 11-2021 and 16-2013), and the Animal Care and Experimentation Committee of Gunma University (16-030). Colon-26 murine colon carcinoma and LM8 murine osteosarcoma cell lines were purchased from Riken BioResource Center (Tsukuba, Japan). Colon-26 cells were cultured in RPMI 1640 (HyClone, Logan, UT, USA). LM8 cells were grown in high-glucose DMEM (HyClone). All media contained antibiotics and 10 % fetal bovine serum (HyClone). All cells were maintained at 37°C in an incubator containing 5 % CO_2.

Irradiation characteristics

Carbon-ion (C-ion) irradiation was conducted at National Institutes for Quantum Science and Technology (Chiba, Japan) and Gunma University Heavy Ion Medical Center (GHMC) (Gunma, Japan). An irradiation collimator (5 cm thick brass with a 28 mm × 100 mm irradiation field) was used to limit and collimate the particle beam to ensure the accuracy of the irradiation area. The parts other than the irradiated lower limbs (the entire torso, including the anus and the opposite lower limbs) were placed outside the irradiation field. Tumour allografts were locally irradiated with 3 or 10 Gy of C-ion beams (290 MeV/n) at the center of the spread-out Bragg peak (SOBP, 6 cm). Cultured cells were irradiated with 3 or 10 Gy C-ion (290 MeV/n, mono-energetic, LET approximately 70 keV/μm).

Gene expression analysis

Colon-26 and LM8 cells were seeded separately on dishes for subsequent gene expression analysis. One day after seeding, the cells were irradiated with C-ions. At 3 days after C-ion irradiation, total RNA was isolated from irradiated or nonirradiated cancer cells using the Reliaprep™ RNA Miniprep System (Promega, Tokyo, Japan). The isolated total RNA was reverse-transcribed using the PrimeScript RT−PCR Kit with the random primers N6 (Takara Bio, Shiga, Japan). Quantitative reverse transcription-polymerase chain reaction (qRT−PCR) was performed using an CFX Connect Real-Time PCR system (Bio Rad, Hercules, CA, USA). The housekeeping gene HPRT1 was selected as an internal standard. The comparative $2^{-\Delta\Delta CT}$ method was used to analyze the relative gene expression levels. The gene-specific primers used for qRT−PCR are listed in Table S1. Each reaction was performed in triplicate.

Evaluation of the anti-tumour and anti-metastatic abilities of CIRT and ICT

A suspension of 5×10^5 cancer cells in 0.02 mL Hank's medium was used for subcutaneous injection into both hind legs of the mice. One week after the subcutaneous injection into the mice, only the right-sided subcutaneously transplanted Colon-26 or LM8 tumours were irradiated with C-ions. Anti-CTLA4 (Clone: 9H10) or anti-PD-1 (Clone: RMP1-14) antibodies (BioLegend, San Diego, CA, USA; 1 mg/mL, in 0.2 mL per mouse) were administered intraperitoneally every 3 days for a total of 4 injections (Colon-26 tumour-bearing BALB/c mice) or 2 injections (LM8 tumour-bearing C3H/He mice) [24] starting the day after CIRT (Figs. 2A, 3A, S3A). We measured the distal unirradiated tumour diameter to clarify whether an abscopal effect could be produced by CIRT combined with an anti-PD-1 antibody (PD-1-Ab) or an anti-CTLA4 antibody (CTLA4-Ab). The tumour diameter was measured three times per week with callipers. The tumour volume was calculated using the following formula: $(a \times b \times c \times \pi)/6$, where a, b, and c represent the three orthogonal diameters of the tumour. At 17–20 days after RT, the pulmonary metastatic nodules on the surfaces of all the pulmonary lobes were macroscopically counted. The sizes of the metastases were measured using ImageJ software (National Institutes of Health, ver.1.53a; Bethesda, MD, USA). A total of six groups were used for the evaluation, including the control (no treatment; saline), groups treated solely with the PD-1-Ab (200 μg/mouse for single treatment) or CTLA4-Ab (200 μg/mouse for single treatment), another group that received only CIRT (either 3 Gy or 10 Gy), and combination groups in which CIRT was administered alongside either the PD-1-Ab or CTLA4-Ab. (n = 5 mice per group).

ELISA analysis

On the 18th day after irradiation, the mice were sacrificed, and their serum was collected. Interferon-gamma (IFN-γ) levels in the serum were detected using a two-step sandwich IFN-γ kit (BD OptEIA Mouse IFN-γ ELISA Kit II, BD Biosciences, NJ, USA) according to the manufacturer's instructions (n = 5 serum samples per group).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Prism version 8.4.3 for Mac OS X; GraphPad Software, San Diego, CA, USA; www.graphpad.com). Data normality was pre-examined before analysis using the Shapiro–Wilk and Kolmogorov–Smirnov tests. Parametric or nonparametric tests were selected according to the normality results. The statistical significance of differences was tested using one-way ANOVA (parametric) followed by Sidak's or Tukey's multiple comparison tests and Kruskal–Wallis (nonparametric) tests. Differences between the means were considered statistically significant if $P < 0.05$.

Results

Characterization of murine cancer cells used in the CIRT combined with ICT models

The characteristics of Colon-26 colorectal cancer cells treated with CIRT were analyzed. The expression of immune checkpoint molecules (such as PD-L1) in cancer cells after CIRT is acknowledged as a notable indicator of CIRT efficacy [30]. Furthermore, blocking immune checkpoint molecules to treat PD-L1-high tumours after RT treatment can prolong the delay in tumour growth, thereby improving the therapeutic effect [31]. Therefore, to determine whether the Colon-26 model is suitable for CIRT combined with anti-PD-1 antibody or anti-CTLA4 antibody therapy, changes in the expression of key genes in the immune checkpoint-associated pathway were detected by PCR (Fig. 1). Notably, we found that both a low dose of 3 Gy and a high dose of 10 Gy C-ion irradiation increased in PD-L1, CD80, LAG-3, IRF1 and CCL2 gene expression, but no significant difference was observed between the two radiation doses. Based on these results of enhanced immune checkpoint molecule expression, Colon-26 cells are considered to be capable of generating an immune response for ICT after CIRT.

Induction of potential abscopal effects by CIRT combined with ICT

High irradiation dose group

In the case of Colon-26 tumour-bearing BALB/c mice, the anti-PD-1 antibody or anti-CTLA4 antibody alone had a limited therapeutic effect on the two transplanted tumours compared with the control group (Figs. 2B, 2C, S1); however, the irradiated tumour shrank significantly when 10 Gy of CIRT was applied in the irradiation group. Although no significant improvement was observed with anti-PD-1 or anti-CTLA4 antibody administration in the combined group, a positive trend was observed between curves (Figs. 2B, S1A). Interestingly, the volume of distant unirradiated tumours tended to increase with CIRT treatment alone (Figs. 2C, S1B). However, this effect was diminished in this study by the combined application of the anti-CTLA4 antibody (Figs. 2C, S1B). Compared with the single CIRT or CIRT combined with anti-PD-1 antibody group, the 10 Gy CIRT combined with anti-CTLA4 antibody group presented clear smaller tumour at distant unirradiated sites ($P < 0.05$), but no clear difference was observed compared with the control group ($P = 0.39$). Compared with the control, CIRT alone almost completely

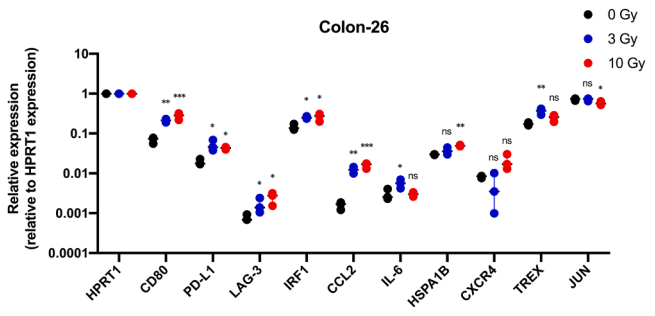

Fig. 1. Gene expression (relative to HPRT1 expression) in Colon-26 cells after C-ion irradiation. Colon-26 cells were subjected to 0 Gy (black filled circles), 3 Gy (blue filled circles) or 10 Gy (red filled circles) C-ion irradiation with a linear energy transfer value of approximately 70 keV/μm by using a mono-energetic beam. The expression of immune checkpoint-associated genes was detected on day 3 after irradiation. Bars indicate the medians with 95 % CIs. Statistical significance was determined using the One-way ANOVA followed by Sidak's multiple comparisons test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, no significant difference vs. 0 Gy.

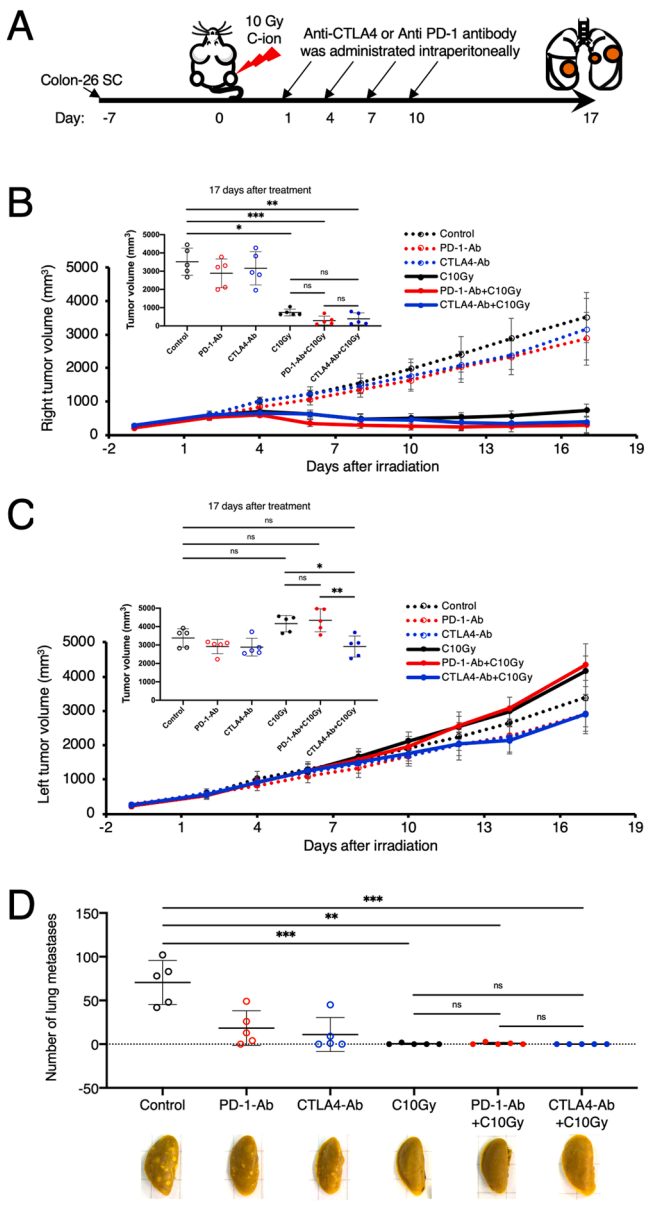

Fig. 2. Anti-cancer effects of 10 Gy CIRT and/or ICT. (**A**) Experimental schedule for treatment with 10 Gy CIRT (C10Gy) combined with anti-PD-1 (PD-1-Ab) or anti-CTLA4 (CTLA4-Ab) antibodies. Right-sided irradiated tumour growth (**B**), left-sided distal unirradiated tumour growth (**C**) and the number of lung metastases (**D**) in Colon-26 tumour-bearing mice are shown (n = 5 mice per group). Symbols: Control (black dotted line, black circles), PD-1-Ab (red dotted line, red circles), CTLA4-Ab (blue dotted line, blue circles), C10Gy (black line, black filled circles), PD-1-Ab + C10Gy (red line, red filled circles), and CTLA4-Ab + C10Gy (blue line, blue filled circles). The bars indicate the standard errors calculated using data from three independent trials. Statistical significance was determined using the Kruskal–Wallis test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, no significant difference.

eliminated lung metastasis, even though a difference in unirradiated tumour cell proliferation was not observed (Fig. 2C, 2D). CIRT combined with the anti-PD-1 antibody or anti-CTLA4 antibody also strongly inhibited metastasis. Notably, no metastatic nodules were observed in the lungs of any of the mice in the CIRT combined with anti-CTLA4 antibody group. Although combination therapy had significant anti-cancer benefits on irradiated tumours and lung metastases, especially in the 10 Gy CIRT combined with anti-CTLA4 antibody group, this effect was caused by the high irradiation dose. A single 10 Gy dose of CIRT inhibited most lung metastases, indicating that ICT had a limited contribution to these effects (Fig. 2D). However, all the treatments had little effect on unirradiated tumour cell proliferation compared with the control, indicating that the abscopal effect was not sufficient.

Low irradiation dose group

The impact of different doses of CIRT on the abscopal effect on the Colon-26 tumour-bearing mouse model was investigated using 3 Gy CIRT (Fig. 3A). Like 10 Gy CIRT, 3 Gy CIRT treatment alone significantly inhibited irradiated tumour growth compared with the control, and no significant changes were observed between CIRT and the combination therapies (Figs. 3B, S2A). However, compared with the other groups, the 3 Gy CIRT combined with anti-CTLA4 antibody group presented a significant anti-tumour effect on unirradiated tumours ($P < 0.05$), revealing the greater ability of low-dose CIRT combined with the anti-CTLA4 antibody to induce an abscopal effect than high-dose CIRT alone (Figs. 3C, S2B). Notably, unlike those in the 10 Gy CIRT group, more mice with lung metastases were observed in the 3 Gy CIRT group because of the lower dose of CIRT (Figs. 2D, 3D). However, no lung metastases were observed in the 3 Gy CIRT combined with anti-CTLA4 antibody group, which indicates that combination effects occurred and led to greater suppression of lung metastasis by the combination of low-dose irradiation and ICT therapy (Fig. 3D).

We repeated the experiments using an LM8 tumour-bearing C3H/He mouse model to verify the abscopal effect (Fig. S3A). Note that, LM8 cells were previously used to investigate the therapeutic effect of combination therapy with CIRT and dual ICTs (both anti-PD-1 and anti-CTLA4 antibodies) [32,33]. The combination therapy showed a strong ability to induce an abscopal effect. In this study, the efficacy of CIRT followed by anti-CTLA4 antibody alone was evaluated. C-ion irradiation also induced an increase in immune checkpoint-associated gene expression in LM8 cancer cells (Fig. S3B). Compared with CIRT alone, neither 10 Gy nor 3 Gy CIRT combined with an anti-CTLA4 antibody significantly improved the anti-tumour efficacy against irradiated site tumours (Fig. S3C). Similar to the Colon-26 tumour-bearing BALB/c mouse model, both the 10 Gy CIRT group and the 10 Gy CIRT combined with anti-CTLA4 antibody group showed no significant suppression of distant unirradiated tumours compared with the control group (Fig. S3D). Importantly, 3 Gy CIRT combined with anti-CTLA4 antibody treatment had the greatest effect on the suppression of unirradiated tumours compared with that of the control group (Fig. S3D). Therefore, low-dose CIRT combined with an anti-CTLA4 antibody treatment seems to be a better option to control distant unirradiated tumours by inducing a stronger abscopal effect than the high-dose protocol.

Classification of metastatic foci after low-dose CIRT and/or ICT

The size of the lung metastatic foci was not uniform, which is related to the time at which the metastasis process occurred. Compared with those in the control group, metastatic foci larger than 0.3 mm^2 were not observed after anti-PD-1 antibody and/or 3 Gy CIRT treatment, whereas metastatic foci larger than 1 mm^2 were still detected after anti-CTLA4 antibody treatment (Fig. S4). The value of the third quartile in the control group was 0.084 mm^2. In accordance with the size of the lung metastasis nodules in Colon-26 tumour-bearing mice, the metastases were divided into the following two groups: large ($\geq$ 0.1 mm^2, larger than 80 % of metastatic foci in the control group) and small ($<$ 0.1 mm^2)

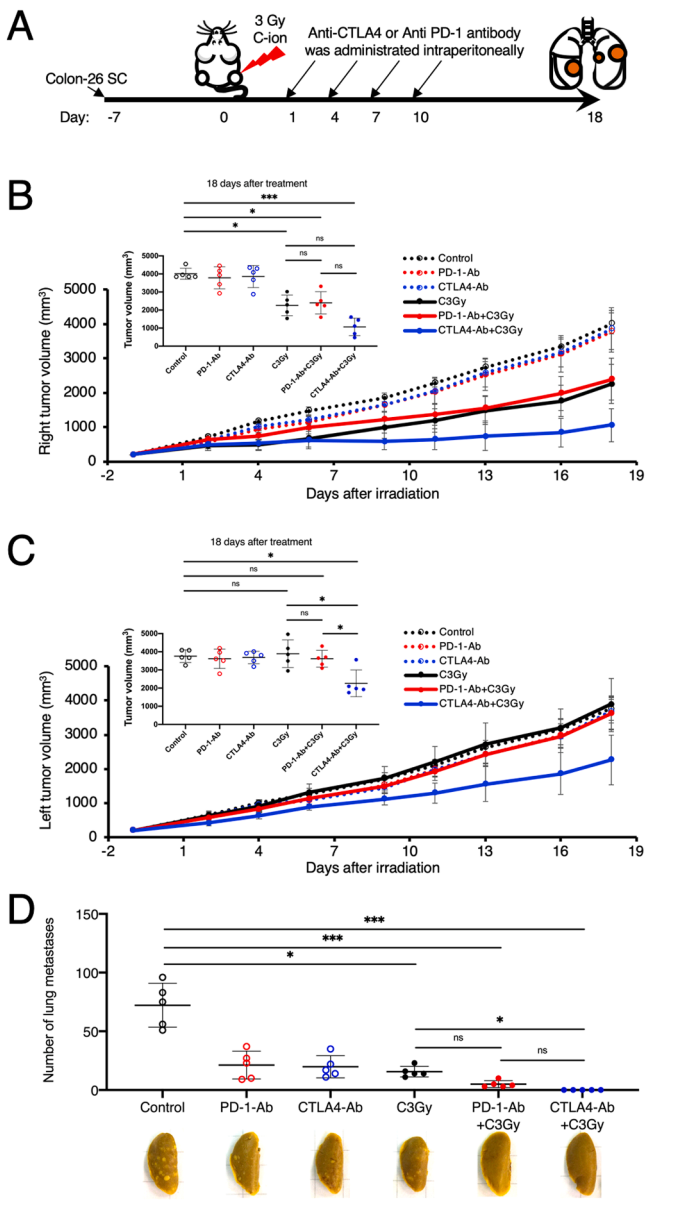

Fig. 3. Anti-cancer effects of 3 Gy CIRT and/or ICT. (**A**) Experimental schedule for treatment with 3 Gy CIRT (C3Gy) combined with anti-PD-1 (PD-1-Ab) or anti-CTLA4 (CTLA4-Ab) antibodies. Right-sided irradiated tumour growth (**B**), left-sided distal unirradiated tumour growth (**C**) and the number of lung metastases (**D**) in Colon-26 tumour-bearing mice are shown (n = 5 mice per group). Symbols: Control (black dotted line, black circles), PD-1-Ab (red dotted line, red circles), CTLA4-Ab (blue dotted line, blue circles), C3Gy (black line, black filled circles), PD-1-Ab + C3Gy (red line, red filled circles), and CTLA4-Ab + C3Gy (blue line, blue filled circles). The bars indicate the standard errors calculated using data from three independent trials. Statistical significance was determined using the Kruskal–Wallis test. ***, $P < 0.001$; *, $P < 0.05$; ns, no significant difference.

(Fig. 4A, 4B). The numbers of large metastases in the control, anti-PD-1 antibody, anti-CTLA4 antibody, 3 Gy CIRT, 3 Gy CIRT combined with anti-PD-1 antibody, and 3 Gy CIRT combined with anti-CTLA4 antibody groups were 73, 30, 55, 16, 6 and 0, respectively. The number of large metastases decreased after anti-PD-1 antibody and/or 3 Gy CIRT treatment but not after anti-CTLA4 antibody administration (Fig. 4A). On the other hand, the numbers of small metastases in the above experimental groups were 288, 76, 44, 62, 19 and 0. Notably, anti-CTLA4 antibody treatment significantly inhibited small metastases (Fig. 4B). Our data indicated that anti-PD-1 antibody therapy and 3 Gy CIRT have anti-

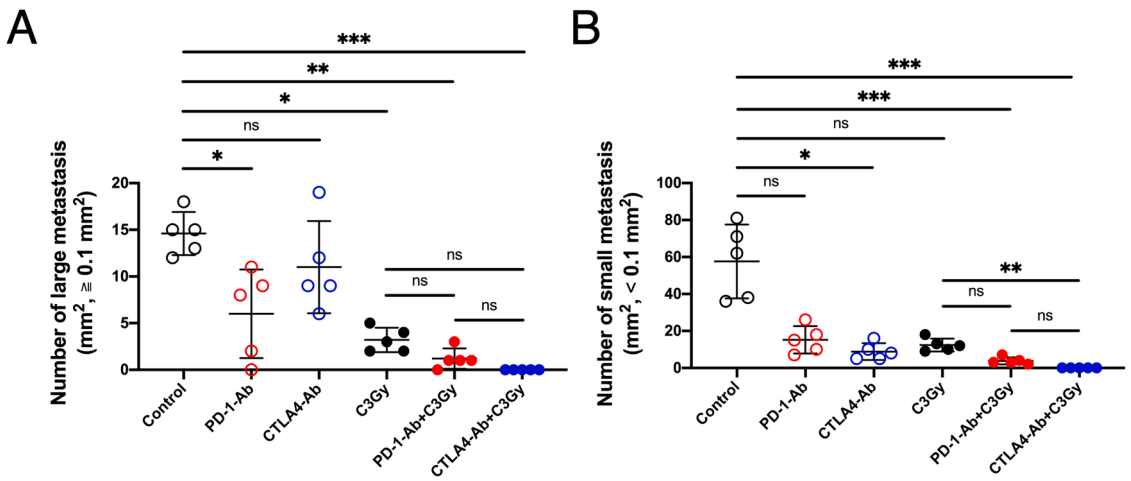

Fig. 4. Classification of metastatic foci after CIRT and/or ICT. Large metastases (**A**) and small metastases (**B**) in Colon-26 tumour-bearing mice are shown (n = 5 mice per group). Symbols: Control (black circles), PD-1-Ab (red circles), CTLA4-Ab (blue circles), C3Gy (black filled circles), PD-1-Ab + C3Gy (red filled circles), CTLA4-Ab +C3Gy (blue filled circles). The bars indicate the standard errors calculated using data from three independent trials. Statistical significance was determined using the Kruskal–Wallis test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, no significant difference.

metastatic effects on the formation of large metastatic nodules; in contrast, anti-CTLA4 antibody therapy reduces the formation of small metastatic nodules. However, metastasis was completely inhibited in the CIRT combined with anti-CTLA4 antibody group, which indicates that a single treatment is not adequate for eliminating distant cancer metastasis.

Release of IFN-γ after low-dose CIRT and/or ICT

We measured the release of IFN-γ to determine the anti-tumour immunity mechanism underlying CIRT combined with the anti-CTLA4 antibody in a Colon-26 tumour-bearing mouse model. The serum level of IFN-γ in the control mice (untreated) was 9.4 ng/mL (Fig. 5). No changes in the serum IFN-γ concentration were observed after single immune checkpoint inhibitor administration or single CIRT treatment. Notably, 3 Gy CIRT combined with the anti-CTLA4 antibody significantly increased the concentration of IFN-γ to 13.1 ng/mL. Therefore, anti-CTLA4 antibody administration after CIRT enhances the IFN-γ-based anti-tumour immune response.

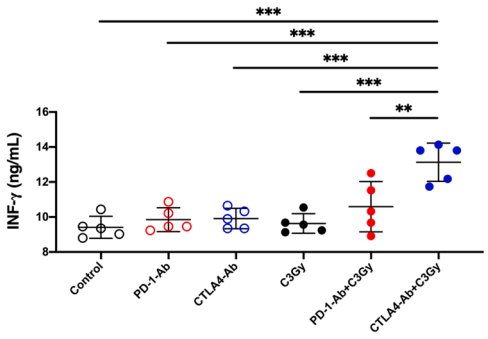

Fig. 5. IFN-γ release in response to 3 Gy CIRT and/or ICT. The concentrations of IFN-γ in the control, PD-1-Ab, CTLA4-Ab, C3Gy, PD-1-Ab + C3Gy and CTLA4-Ab + C3Gy groups are shown (n = 5 mice per group). Symbols: Control (black circles), PD-1-Ab (red circles), CTLA4-Ab (blue circles), C3Gy (black filled circles), PD-1-Ab + C3Gy (red filled circles), CTLA4-Ab + C3Gy (blue filled circles). The bars indicate the standard errors calculated using data from three independent trials. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison tests. ***, $P < 0.001$; **, $P < 0.01$.

Discussion

Since invasion into adjacent tissues and distant metastasis are known to account for 90 % of cancer-related deaths [34], a feasible and effective method to cure metastasis is needed to improve patient outcomes. Combining CIRT with immunotherapies, such as dendritic cell (DC) vaccines and immune checkpoint inhibitors, is advantageous for preventing the spread of metastasis via the activation of systemic immunity [15,35]. Recent studies have shown that CIRT combined with DC immunotherapy inhibits distant lung metastases in tumour-bearing immunocompetent mouse models [36–38]. Compared with DC immunotherapy, immune checkpoint inhibitors have the advantages of wider application, greater therapeutic effectiveness, and lower cost. Furthermore, recent research has indicated that immune checkpoint inhibitors combined with conventional radiotherapy can further improve therapeutic effectiveness [24]. In this study, we evaluated immunotherapy with an anti-PD-1 antibody or anti-CTLA4 antibody in combination with CIRT. When CIRT was administered at a high dose (10 Gy), both types of combination therapy produced significant anti-cancer benefits on irradiated tumours, but did not induce an abscopal effect on distal unirradiated tumours. On the other hand, at a low dose (3 Gy), CIRT combined with an anti-CTLA4 antibody widely suppressed tumours, not only in irradiated primary tumours, but also induced the abscopal effect on distal unirradiated tumours and metastatic tumours. These findings emphasize that relatively low-dose CIRT combined with an anti-CTLA4 antibody has an efficient abscopal effect on tumour-bearing immunocompetent mouse models and may be helpful for optimizing treatment in the clinic.

Since immunotherapy uses the body's immune system to fight cancer cells, the completion of the immune system is a prerequisite for fully inducing anti-tumour effects in this experimental animal model. For both BALB/c and C3H/He mice, since both are inbred strains, the genetic background of each strain of mouse remains consistent, and both have a complete immune system. Moreover, both Colon-26 cells and LM8 cells result in distant organ metastasis, and the expression of immune checkpoint-associated genes increases after C-ion irradiation (Figs. 1, S3B). The immune-related genetic variation of mouse strains can be divided into Th1- or Th2-type strains based on the style of helper T (Th) cell cytokine production [39]. Our previous preclinical animal study indicated that the therapeutic effect of CIRT combined with dendritic cell immunotherapy depended on the Th differentiation bias of the mice [38]. In this study, to clarify whether there are differences in the therapeutic effects of Th1- and Th2-type mouse strains in combination

therapy with CIRT and ICT, Th1-type C3H/He and Th2-type BALB/c mice were investigated. The abscopal effect was induced in both the tumour-bearing C3H/He model and the tumour-bearing BALB/c model, suggesting that the therapeutic effects of combining CIRT with anti-CTLA4 antibody is independent of the host's Th type.

Several studies have documented that CIRT combined with dual immune checkpoint inhibitors (anti-CTLA-4 and anti-PD-L1) can effectively inhibit distal unirradiated tumours; in other words, an abscopal effect occurs [32,33]. In this study, we evaluated the abscopal effect of CIRT combined with a single immune checkpoint inhibitor. Although we did not observe any beneficial effects of anti-PD-1 antibody administration after CIRT on distal unirradiated tumours, the distal tumour volume decreased after treatment with 3 Gy CIRT combined with the anti-CTLA4 antibody in the Colon-26 tumour-bearing and LM8 tumour-bearing mouse models (Figs. 3, S3). Similarly, C Twyman-Saint Victor *et al.* reported that X-ray radiation administered concurrently with an anti-CTLA4 antibody blocks cell proliferation in distal unirradiated tumour [24]. RT treatment is able to induce the immunogenicity of tumour cells via damage-associated molecular patterns (DAMPs) expression and DC activation [37,40]. Moreover, anti-CTLA4 antibody administration mainly inhibits regulatory T cells, thereby increasing the activated T-cell population [41]. Therefore, both RT and anti-CTLA4 antibody administration affect the systemic immune response. IFN-γ is a cytokine that plays a key role in host anti-tumour immunity [42]. Our subsequent mechanistic study revealed that 3 Gy of CIRT combined with an anti-CTLA4 antibody can increase the serum IFN-γ level (Fig. 5). The whole-body activation of immune cells and the increased release of anti-tumour cytokines by immune cells may be important reasons for the inhibition of distal tumour growth and metastasis formation. These results demonstrated that CIRT combined with anti-CTLA4 antibodies has potential benefits in producing abscopal effects, possibly through enhanced whole-body anti-tumour immunity.

The induction of the abscopal effect, which is mediated by the systemic anti-tumour immune response, is considered influenced by physical radiation parameters, such as the radiation dose, dose rate and dose fractionation [15]. An appropriate dose threshold exists for the induction of a strong anti-cancer immune reaction. The administration of conventional radiotherapy at extremely low doses may not produce a sufficient immune reaction, which is triggered by "eat me" and "danger" signals from dying cancer cells [37]. High-dose radiotherapy may not only kill cancer cells but also damage blood vessels and further promote the apoptosis of immune cells [43]. This property can induce excellent effects on local tumour, but the anti-tumour immune response at the distal tumour and metastasis sites may be limited. The results of this study revealed that the expression of many immune-related genes tended to increase in a dose-dependent manner, whereas the expression of the CXCR4 gene tended to decrease after 3 Gy C-ion treatment (Fig. 1). CXCR4 plays an important role in colorectal cancer development, and high expression of CXCR4 is significantly associated with metastasis in colorectal cancer patients [44]. We suggest that the genes exhibiting different patterns of expression after treatment within a certain dose range are related to our findings. However, the changes in gene expression vary among different cell lines, and more extensive data are needed in the future. Not only radiation dose, but also dose rate was reported to be associated with distant unirradiated tumour control using the same mouse model used in this study [45]. Whether the irradiation dose rate is one of the key factors affecting the induction of the abscopal effect needs to be further proved by subsequent experiments in the future.

Recently, conventional radiotherapy combined with ICT using an optimal radiation dose was shown to potentially cause an abscopal effect [46]. Therefore, exploring the ideal dose of CIRT in combination therapy may enhance the effective recognition of tumour-specific antigens by immune cells and their activation, thereby maximizing the effective anti-tumour immune response. Both CIRT and conventional RT can change the immune characteristics of the tumour microenvironment, but their effects differ [47]. Recent studies have shown that even low-dose CIRT can induce higher levels of DAMPs than photons can, which gives CIRT natural advantages in inducing the anti-tumour immune response [37]. In this study, the abscopal effect was stably induced by 3 Gy CIRT combined with the anti-CTLA4 antibody but not by 10 Gy CIRT combined with the anti-CTLA4 antibody (Figs. 2, 3, S3). Although the effect on primary tumour control was slightly worse than that of the high-dose treatment, a good balance between primary tumour control and distant metastasis suppression occurred with the 3 Gy CIRT combined with anti-CTLA4 antibody treatment. Considering that severe metastatic lesions can be life-threatening, the abscopal effect is important for suppressing metastasis under this balance. Hence, overall survival may be further improved.

Interestingly, anti-PD-1 antibody administration and CIRT had anti-metastatic effects on larger lung metastatic nodules, and anti-CTLA4 antibody administration suppressed smaller metastatic nodules (Fig. 4). This phenomenon has not been reported before. A larger size of metastasis indicates the possibility of circulating cancer cells adhering to the lung tissue earlier, and a smaller size of metastasis is considered to indicate later adhesion. These data suggest that the effective therapeutic period for anti-PD-1 antibody administration or CIRT may be early after treatment and that of anti-CTLA4 antibody administration may be later after treatment. This difference in the duration of metastasis inhibition may be due to the direct effect on cancer cells treated with the anti-PD-1 antibody or CIRT; meanwhile, anti-CTLA4 antibody therapy has a time delay, as it acts indirectly on cancer cells after dendritic cell-mediated T-cell activation. Therefore, CIRT combined with anti-CTLA4 antibody treatment has the advantage of different durations of metastasis inhibition for both CIRT and anti-CTLA4 antibody therapy, providing sequential long-term systemic anti-metastasis effects during both the early and late stages of treatment. Further research is needed to confirm this hypothesis.

The unsatisfactory anti-metastasis effect of the combination of CIRT and dendritic cells in BALB/c mice is generally believed to be due to the lack of efficient activation of dendritic cells in BALB/c mice by irradiated cancer cells [38]. Ando *et al.* reported that B7 costimulatory molecules (CD80 and CD86) are more sensitive to stimulants from tumours than radiation factors [37]. The high expression of B7 molecules represents a double-edged sword for anti-tumour immune regulation. Binding of the B7 molecule (CD80 and CD86) to CD28 expressed on T cells is necessary for T-cell activation, whereas binding to CTLA4 prevents T-cell activation [48]. Anti-CTLA4 antibody therapy induces T-cell activation by blocking CTLA4 expressed on T cells, thereby promoting the binding of CD28 and B7 molecules and ultimately enhancing anti-tumour immunity [14]. In this study, a preclinical experiment in BALB/c mice revealed that an abscopal effect can also be induced in this mouse strain. Therefore, whether the abscopal effect can be triggered does not depend on whether the dendritic cells are efficiently activated but rather on the activation level of T cells, which largely depends on the binding of CD28 and B7 molecules.

In summary, we showed that relatively low-dose CIRT combined with anti-CTLA4 antibody treatment induces a strong abscopal effect on tumour-bearing immunocompetent mouse models, which may be caused by an enhanced anti-tumour immune response. At the optimal dose, CIRT combined with anti-CTLA4 antibody therapy represents an effective combination therapy model in which CIRT-induced activation of anti-tumour immunity may be a key factor in inducing the abscopal effect, and ICT further amplifies this immune response and enhances the systemic anti-cancer effect to cure distant tumours/metastases. This finding suggests an opportunity to increase the induction efficiency of the abscopal effect for combination therapy and highlights a new strategy for optimizing the radiation dose for cancer metastasis treatment.

CRediT authorship contribution statement

Liqiu Ma: Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Yang Li:** Writing – review & editing, Resources. **Yoshimitsu Sakamoto:** Resources. **Lin Xie:** Resources. **Saaya Suzuki:** Resources. **Yukari Yoshida:** Resources. **Li Sui:** Resources. **Gang Guo:** Resources. **Jialing Wen:** Resources. **Wangcai Ren:** Resources. **Kazuhiro Kakimi:** Writing – review & editing, Resources. **Kensuke Osada:** Investigation, Resources, Writing – review & editing. **Akihisa Takahashi:** Writing – review & editing, Resources, Project administration. **Takashi Shimokawa:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement for this work

Research data are not available at this time.

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Supplementary materials

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