Erb-(IL10)2 Induces Abscopal Antitumor Effects of Radiotherapy through the Activation and Recruitment of Lymph node CD8+ T Cells

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Abstract

**Background:** Although radiotherapy (RT) has been widely used in cancer treatment, it provides limited benefits in patients with metastatic cancers due to rare abscopal antitumor effects. The recent progression in cancer immunotherapy provides a potential new strategy to boost abscopal antitumor effects of RT.

**Methods:** We fused Interleukin 10 (IL10) dimer onto an anti-epidermal growth factor receptor antibody Cetuximab (Erbitux) to form a new bispecific protein Erb-(IL10)₂. The antitumor effect and biological activity of Erb-(IL10)₂ was measured in B16-EGFR-OVA tumor model. *In vivo* cell depletion and flow cytometry analysis were used to access the mechanism of antitumor effects.

**Results:** Erb-(IL10)₂ treatment alone showed modest tumor growth inhibition, while local single dose RT (10 Gy) retarded irradiated tumor growth without affecting on the growth of nonirradiated tumors. Notably, the combination therapy of RT and Erb-(IL10)₂ not only additively inhibited irradiated tumor growth, but also induced abscopal antitumor responses. *In vivo* depletion of CD8⁺ T cells abrogated the combinational antitumor effects, while blockade of lymphocyte trafficking by FTY720 treatment abolished the abscopal antitumor responses without affecting the antitumor effects on the irradiated tumor sites.

**Conclusion:** This study provides evidences for the radio-sensitivity role of Erb-(IL10)₂ in B16-EGFR-OVA tumor model. Our findings suggest a novel strategy to elicit abscopal antitumor effects of RT through combining tumor-targeted therapy of IL10.

Introduction

Surgery, radiotherapy (RT) and chemotherapy (CT) are the main cancer treatment modalities for decades. RT is used by 50% of cancer patients in the course of their disease and received by 40% of those cured cancer patients(1). The biological responses of tumors to RT include the induction of DNA damage, the generation of inflammatory signals and the modulation of signal transduction. RT could be immunosuppressive since RT also kills immune cells and induces bone marrow suppression, while many studies suggest RT can also stimulate antitumor immunity by releasing tumor-associated antigens (TAAs) and induce tumor regression in a T cell dependent manner(2–5). Despite the immune stimulating effects of irradiation, as well as the technical improvements, such as three-dimensional conformal RT (3D-CRT), intensity-modulated RT (IMRT) and image-guided RT (IGRT), many patients still suffer from localized disease recurrence after RT, and advanced cancer patients could not achieve long-term survival benefit due to distant metastasis. The main reason is that RT alone is insufficient to activate antitumor immunity to completely eliminate tumor cells, especially the unirradiated tumor lesions(6–8). Therefore, the combination of a complementary approach with radiotherapy to potentiate adaptive immunity and to induce abscopal antitumor activities would be critical to eliminate tumor cells body-wide and to achieve long-term survival benefits. RT kills localized tumor cells and releases more tumor antigens, and the
complementary approaches enhance antitumor immunity and mobilize systemic immunity to clear residual tumor cells.

Immunotherapy has achieved considerable progress in controlling cancer in recent years, which is the fourth main approach for cancer treatments. Cancer immunotherapy stimulates the body’s own immune system to identify and eliminate tumor cells. Ipilimumab, which blocks cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) that is a negative regulator of T-cell activation(9), has shown exciting antitumor activity in malignant melanoma and other cancers(10) and has been approved by the US Food and Drug Administration (FDA) in 2011. Anti-programmed death 1 (PD-1) antibodies, such as Pembrolizumab and Nivolumab, have been approved by FDA in multiple cancer types, including metastatic melanoma, non-small-cell lung cancer (NSCLC), and colorectal cancer(11). Interleukin-10 (IL-10), expressed by most T cells, macrophages, antigen presenting cells (APC) as well as epithelial cells(12), is considered as an immune suppressive cytokine by inhibiting the secretion of the proinflammatory cytokines IFNγ, TNFα, and the expression of major histocompatibility complex (MHC) molecules(12, 13). At the same time, the administration of IL-10 has also been shown to elicit antitumor immunity in mice(13–15). High local concentrations of IL-10 led to tumor rejections and inhibited tumor metastasis in mice(16). Polyethylene glycol-IL-10 (PEG-IL-10) which prolongs IL-10 circulation time and enhances IL10 effectivity(17), showed its capability to enhance T cells activation and increase intratumoral cytotoxic CD8+ T cells(18). A clinical phase I study showed that PEG-IL-10 therapy was well-tolerated and stimulated systemic antitumor immune responses in advanced solid tumors such as melanoma, renal cell cancer (RCC), NSCLC, colorectal cancer, ovarian cancer, and pancreatic cancer, castrate-resistant prostate cancer(19).

The combination of radiation therapy and immunotherapy has exhibited successful antitumor effects in multiple preclinical studies(20–26). RT combined with immunocytokine IL-2 resulted in long-lasting antitumor effects by stimulating antitumor immune response of T cells and macrophages(27, 28). IL-10 could also be a good candidate to facilitate RT. Owing to the low plasma half-life of IL-10(29), we used Erb-(IL10)2 (a gift from DingFu Biotarget Co. Ltd., Suzhou, China) in this study. The epidermal growth factor receptor (EGFR/ErbB1) is part of the ErbB family of receptor tyrosine kinases and overexpressed in many types of malignancies(30). Erb (Erbitux), also named as Cetuximab, targets extracellular regions of human tumor EGFR with higher affinity to block access of the ligands to EGFR domain III and induce EGFR internalisation and degradation, finally leading to cell death(31, 32). While, Erb in Erb-(IL10)2 just exerts the binding targeted effect of EGFR without causing downstream signal transduction in the model. In this study, we developed a method to combine Erb-(IL10)2 and RT and showed additive antitumor effects. Moreover, Erb-(IL10)2 successfully induces abscopal antitumor effects of RT. Furthermore, we demonstrated that the combination therapy suppressed tumor growth in a CD8+ T cell dependent manner and elicited abscopal antitumor effects via the mobilization and activation of lymphocytes from tumor DLNs.

Materials And Methods
Animals and cell culture

Female C57BL/6 mice were obtained from the Experimental Animal Centre of Chinese Academy of Science (Shanghai, China) at 6- to 8-week-old and maintained under specific pathogen-free conditions. All animals were used in accordance with the local ethics committee. All of the experimental procedures were performed in compliance with the Animal Care and Use Regulations of China. The B16-EGFR-OVA melanoma cell line expressing a chimeric EGFR (cEGFR) and a K\textsuperscript{b}-binding peptide antigen SIINFEKL peptide (OVA\textsubscript{257-264}) was obtained from DingFu Biotarget Co. Ltd., Suzhou, China. The cEGFR is the full-length mouse EGFR with the mutation of six amino acids that are required for Cetuximab binding (Li et al., 2005). B16-EGFR-OVA melanoma cells were grown in DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco Invitrogen).

Tumor growth and treatments

B16-EGFR-OVA melanoma cells (5×10\textsuperscript{5}) were inoculated subcutaneously (s.c.) into the flanks of mice and allowed to grow for about 10 days. The two perpendicular diameters of a tumor were measured. Tumor volumes were calculated by $V = \frac{ab^2}{2}$, where $a$ and $b$ are the longest and the shortest diameter, respectively. Mice were randomly assigned into groups according to their tumor sizes. Before radiotherapy, C57BL/6 mice were anesthetized by intraperitoneally (i.p.) injecting with 150µl/20g 1% (w/v) pentobarbital sodium. Each anesthetized mouse was positioned lateral on the flat horizontal surface of the block protected with a lead shield with a 10-mm ×10-mm hole. Local irradiation was then carried out with a single dose (Electron beam irradiation, 3 Gy/min; Medical Linear Accelerators, SIEMENS Primus, Germany) through the hole at the Department of Radiotherapy, the First Affiliated Hospital of Soochow University. Tumor volumes were measured twice weekly. Erb-(IL10)\textsubscript{2} or isotype control mAb was administrated i.p. on days 3 after RT, every 3-4 days for a total of 3 times at a dose of 1mg/kg (unless otherwise stated). FTY720 (SIGMA, Cat # SML0700-5MG), a lipophilic immunomodulatory sphingosine-1-phosphate analog, induces severe peripheral blood lymphopenia and sustains lymphopenia in mice by preventing lymphocytes egress from lymph organs through agonist-induced receptor internalization(33-35). FTY720 (1 mg/kg body weight dissolved in sterile saline) was injected i.p. once a day for 3 days from days 13, and then turned to on alternate days and maintained on drinking water (2 µg/ml) for the duration of the treatment.

ELISPOT assay

The enzyme-linked immunospot (ELISPOT) assay was used to quantify cells secreting interferon gamma (IFN\textgamma) as spots on 96-well plates. ELISPOT assays were performed by using an ELISPOT kit (BD Biosciences Cat# 551083) according to the manufacturer's instructions. Tumor-draining lymph nodes (DLNs) were removed to obtain single-cell suspensions as described previously(2). A 96-well ELISPOT plate was precoated with 5 µg/ml purified anti-mouse IFN\textgamma (BD Biosciences Cat # 51-2525kc) overnight.
at 4°C. 5×10^5 lymph node cells per well was cultured in the presence of 5 mg/mL SIINFEKL peptide (OVA257-264) (InvivoGen Cat # vac-sin) or SIYRYYGL (SIY) peptide (SL-9 GL Biochem Cat # 057787) which was used as a negative control. After 72 hrs of incubation, cells were removed, 2μg/ml biotinylated anti-mouse IFNγ (BD Biosciences Cat # 51-1818kz) was added, and the plate was incubated for 2 hrs at room temperature. Spots were visualized using Streptavidin-HRP (BD Biosciences Cat # 557630) and AEC substrate (BD Biosciences Cat # 551951), followed by image analysis and spot enumeration. Each condition was plated in triplicate.

**In vivo cell depletion and flow cytometry analysis**

For immune cell depletion experiments, anti-mouse CD4 mAb (clone TIB207), anti-mouse CD8 mAb (clone TIB210) or anti-mouse NK1.1 mAb (clone PK136) was delivered once a week by i.p. injection of 200 μg antibody per mouse for up to 2 weeks. Starting on days 10 post B16-EGFR-OVA melanoma cell implantation, mice in the radiation therapy plus Erb-(IL10)_2 group received their first injection. Three days after the first administration of depletion antibodies or FTY720, lymphocyte populations in their peripheral blood were analyzed by labeling with APC-anti-mCD4 (clone GK1.5, BioLegend)FITC-anti-mCD8a (clone53-6.7, BioLegend) or FITC-anti-mCD3 (clone 17A2, BioLegend)APC-anti-mNK1.1 (clone PK136, BioLegend). Samples were collected and analyzed on a Life Attune Flow Cytometer (Life).

**Statistical analysis**

Data were analyzed by using GraphPad Prism version 5.0 software (San Diego, CA). Data are presented as means ± standard error of the mean (SEM). Two group comparisons were performed by using unpaired 2-tailed Student's t-test. More than two group comparisons were assessed by one-way analysis of variance (ANOVA). The statistical significance level was set as *p<0.05, **p<0.01, and ***p<0.001. And a P value of less than 0.05 was considered as statistically significant.

**Results**

**Radiation therapy at 10 Gy enhances the efficacy of Erb-(IL10)_2 treatments**

Although RT therapy often shows potent antitumor effects to a single tumor, the efficacy of RT monotherapy remains limited in many conditions, such as multiple tumors or tumor with special locations. Therefore, the combination of RT with other therapies, especially cancer immunotherapy, is necessary to achieve better outcomes. The combination of tumor local RT with immunotherapy can arouse antitumor immunity better than either stand-alone mode(27, 36). Previous study showed that IL10 targeted therapy can elicit strong antitumor immunity. To explore the strategy to combine RT and IL10
targeted therapy, we developed a IL10 target fusion protein Erb-(IL10)$_2$ by linking IL10 with an anti-EGFR antibody, as well as a B16-EGFR-OVA melanoma cell line expressing a chimeric EGFR (cEGFR).

Firstly, we tested the effects of a single dose of RT on the growth of B16-EGFR-OVA tumors. By days 21 after treatment, a local single-dose of RT from 5 to 30 Gy (Supplementary Figure S1) inhibited B16-EGFR-OVA tumor growth compared to control group. Higher dose of RT showed better tumor growth inhibition. 30 Gy almost eliminated the tumors while 5 Gy had minor antitumor effect. These results suggest that a single dose of RT exhibits dose-dependent antitumor effect in the B16-EGFR-OVA tumor model.

To determine whether the time to administer Erb-(IL10)$_2$ relative to radiotherapy could play a role in its ability to induce antitumor effect, Erb-(IL10)$_2$ treatment was started on different days. We started to inject i.p. Erb-(IL10)$_2$ on the same day of RT (day 10), three days after RT (day 13) or six days later (day 16) (Supplementary Figure S2). Although there was no significant difference in tumor growth for any of the treatment regimens, the administration of Erb-(IL10)$_2$ and RT simultaneously or delaying administration of it until day 16 reduced the therapeutic effect. These data indicate that the administration of Erb-(IL10)$_2$ three days after RT show the best trend to inhibit tumor growth.

In Supplementary Figure S1, we showed that tumor control increased with the dosage of radiation. Schaue's studies suggest that only a single dose above 7.5 Gy were immunostimulatory(37), while Demaria showed that RT 2 Gy in combination with Flt3-Ligand could trigger antitumor T cells response(38). Therefore, we chose RT 5 Gy and 10 Gy to evaluate the impacts of RT dose on Erb-(IL10)$_2$ treatments. C57BL/6 mice were inoculated s.c. with B16-EGFR-OVA cells on day 0, irradiated locally on day 10 and subsequently injected i.p. with 200 μl of Erb-(IL10)$_2$ (1 mg/kg) or isotype control on day 13, every 3-4 days for a total of 3 treatments. RT 5 Gy had a slight impact on tumor growth compared with isotype control group, whereas RT 10 Gy or Erb-(IL10)$_2$ monotherapy slowed tumor growth (Figures 1A, B). The combination of RT 10 Gy and Erb-(IL10)$_2$ inhibited tumor growth compared with monotherapy (Figure 1B), while, RT 5 Gy plus Erb-(IL10)$_2$ did not show better antitumor effect than Erb-(IL10)$_2$ alone (Figure 1A). Tumors treated with RT 10 Gy and Erb-(IL10)$_2$, 5/5 did not reach 2000 mm$^3$ volume at day 40 post RT, as compared with 1/5 of the isotype control group (Figure 1C). Together, these data indicate that RT at 10 Gy improves the efficacy of Erb-(IL10)$_2$ treatments and prolongs the survival in B16-EGFR-OVA tumors.

The combination of Erb-(IL10)$_2$ and radiation therapy inhibits tumor growth in a CD8$^+$ T cell dependent manner

We then investigated the mechanisms underlying tumor control following the combination of RT and Erb-(IL10)$_2$ therapy. We collected tumor tissues and analyzed tumor-infiltrating immune cells. Although Erb-(IL10)$_2$ treatments or RT alone did not change tumor-infiltrating CD8$^+$ T cells, the combination of Erb-
(IL10)$_2$ treatments and RT significantly increased tumor-infiltrating CD8$^+$ T cells, compared to control tumors (Figure 2). Erb-(IL10)$_2$ treatments, RT or their combination therapy neither altered tumor-infiltrating CD4$^+$ T cells (Figure 2), nor changed tumor-infiltrating myeloid cells, including neutrophils, monocytes and TAMs (Supplementary Figure S3). These results indicate that CD8$^+$ T cells may mediate the antitumor effects of the combination of Erb-(IL10)$_2$ treatments and RT. To determine the causal roles of major immune effectors in the combination therapy, we performed in vivo depletion of CD4$^+$ T cells, CD8$^+$ T cells or NK cells. In B16-EGFR-OVA tumor-bearing mice treated with RT and Erb-(IL10)$_2$, we also i.p. injected anti-CD4 mAb (clone TIB207), anti-CD8a mAb (clone TIB210), or anti-NK1.1 mAb (clone PK136) (200 μg/mouse) twice a week starting on day 10 for two weeks. The depletion of CD8$^+$ T cells significantly reduced the efficacy of the combination treatments (Figure 3B), while the depletion of CD4$^+$ T cells or NK cells did not affect tumor growth in the combination treatment group (Figures 3A, C). These results demonstrate that CD8$^+$ T cells are essential for the antitumor effect of the combination therapy.

**Erb-(IL10)$_2$ treatments induce the abscopal effects of radiation therapy**

The induction of abscopal effects, the effect of RT on the tumors outside of the field of RT, is crucial to realize long-term survival benefits of a RT combination therapy. To determine whether Erb-(IL10)$_2$ could elicit abscopal effects of RT, we inoculated mice with B16-EGFR-OVA cells at both flanks: the primary tumor on the right flank was irradiated to determine the direct effect of RT, whereas the secondary tumor on the left flank was not irradiated and served to measure the potential indirect, abscopal effects. Consistent with previous data (Figure 1B), the combination treatments led to a significant tumor growth delay in the primary tumors (Figure 4A). The effects of Erb-(IL10)$_2$ treatments alone on either primary or secondary tumors were modest and similar (Figure 2). RT 10 Gy as single modality significantly inhibited the growth of the primary tumor, but had no effect on secondary tumors (Figure 4). In contrast, the combination of Erb-(IL10)$_2$ with RT on the primary tumors significantly inhibited the growth of secondary nonirradiated tumors compared with the administration of Erb-(IL10)$_2$ alone (Figure 2B). Taken together, these results show that a single dose of local radiation (10 Gy) is unlikely to trigger an abscopal effect, and Erb-(IL10)$_2$ induces the abscopal antitumor effect of RT.

**Erb-(IL10)$_2$ treatments enhance the local and abscopal effect of radiation therapy via distinct mechanisms**

Next, we investigate the mechanisms of abscopal effects induced by Erb-(IL10)$_2$ therapy. Erb-(IL10)$_2$ treatments could induce abscopal effects through the recruitment of T cells from the tumor microenvironment or the tumor-draining lymph nodes (tumor-DLNs). To answer this question, we used FTY720, which blocks T cells egress from lymph organs, to distinguish T cell activation in the tumor
microenvironment from occurring in the tumor-DLNs(33-35). The addition of FTY720 treatments to Erb-(IL10)\textsubscript{2} 1 mg/kg did not decrease its antitumor effect (Figure 5). The additional FTY720 treatments to the combination of Erb-(IL10)\textsubscript{2} and RT therapy did not reversed their antitumor effects in the irradiated tumors (Figure 5A). Interestingly, the additional FTY720 treatments to the combination therapy of Erb-(IL10)\textsubscript{2} and RT abrogated the abscopal antitumor effects on the secondary nonirradiated tumors (Figure 5B). The results suggest that Erb-(IL10)\textsubscript{2} treatments induce the abscopal antitumor effects of RT and enhance RT efficacy via distinct mechanisms.

We then investigate whether the combination of RT and Erb-(IL10)\textsubscript{2} treatment could elicit tumor antigen-specific T cell immune responses in the tumor-DLNs. We used OVA peptides to stimulate OVA-reactive CD8\textsuperscript{+} T cells. Lymphocytes from tumor-DLNs were isolated from the mice inoculated with B16-EGFR-OVA tumor cells on days 19, three days after the second administration of the Erb-(IL10)\textsubscript{2}, which has been shown to induce the strongest anti-tumor immune responses according to previous data. Lymphocytes from the tumor-DLNs in each group of mice were separated and then activated \textit{in vitro} with OVA or SIY peptides in ELISPOT assays. The frequency of IFN-\gamma -secreting T cells was then determined (Figure 6). The frequency of OVA-specific IFN-\gamma -producing CD8\textsuperscript{+} T cells in the DLNs of the mice that received combination treatment of RT and Erb-(IL10)\textsubscript{2} was significantly increased compared with those that underwent RT or Erb-(IL10)\textsubscript{2} treatment alone (P= 0.0026, isotype control vs. RT + Erb-(IL10)\textsubscript{2}; P= 0.0034, Erb-(IL10)\textsubscript{2} vs. RT + Erb-(IL10)\textsubscript{2}; P= 0.0064, RT vs. RT + Erb-(IL10)\textsubscript{2}). The results suggest that the combination of RT and Erb-(IL10)\textsubscript{2} therapy induces tumor antigen specific T cell stimulation in tumor-DLNs.

According to the results in Figures 5 and 6, Erb-(IL10)\textsubscript{2} combined with RT likely activates tumor-infiltrated CD8\textsuperscript{+} T lymphocytes to eliminate irradiated tumor cells, while the abscopal antitumor effects is likely mediated by the recruitment and activation of circulating CD8\textsuperscript{+} T lymphocytes from tumor-DLNs.

\textbf{Discussion}

Radiation therapy is a palliative treatment modality for many cancer patients, especially early-to-intermediate stages. RT exhibits curative potential for localized cancers, but it is rarely demonstrated long-term survival benefits in advanced cancer patients due to limited systemic antitumor effects. In this study, we developed a combination of RT with tumor-targeted Erb-(IL10)\textsubscript{2} therapy. Notably, the addition of Erb-(IL10)\textsubscript{2} to RT not only improved the tumor growth inhibition in the irradiated tumors, but also induced the abscopal antitumor effect of RT. Mechanically, the combination of Erb-(IL10)\textsubscript{2} and RT activated intratumoral CD8\textsuperscript{+} T cells to kill tumor cells, while Erb-(IL10)\textsubscript{2} therapy elicited abscopal tumor suppression via the recruitment and activation of CD8\textsuperscript{+} T cells from tumor-DLNs. Thus, our findings suggest a novel approach to generate potent systemic tumor control via the integration of tumor-targeted IL-10 therapy with RT.
IL-10 has long been considered as an immunosuppressive cytokine. Macrophages and Tregs usually produce high levels of IL-10. IL-10 inhibits the secretion of pro-inflammatory cytokines from T-Helper-1 (Th1) cells, such as IL-12, and suppresses antigen-presenting function of macrophages and DCs. However, IL-10 knockout mice develop colon cancer which is associated with the activation of macrophages and Th1 CD4^+ T cells. Subsequent studies demonstrated that IL-10 can inhibit tumor growth through promoting the infiltration of cytotoxic CD8^+ T cells and eliciting the production of IFN-γ and granzymes(13). These opposing effects may be due to the dosage, the administration approach, and function status of IL-10. In patients with Crohn's disease, IL-10 at intermediate doses shows better efficacy than higher doses. High doses of IL-10 increase the production of IFN-γ, which was contrary to the treatment of inflammatory conditions. In this study, Erb-(IL10)_2 binds to cEGFR overexpressing in tumor cells, resulting in the enrichment of IL-10 in the tumor microenvironment. The local abundance of IL-10 facilitates CD8^+ T cell activation and inhibits tumor growth systemically.

The combination therapy of RT 10 Gy and Erb-(IL10)_2 1 mg/kg displayed advance treatment benefit compared with monotherapy. Some studies suggested that higher than conventional 2 Gy doses were immunostimulatory(2, 38). In this study, we showed that the combination of RT 10 Gy, but not RT 5 Gy, improved the efficacy of Erb-(IL10)_2. This result is consistent with another study showing that a single doses above 7.5 Gy improved immunity to eliminate tumor cells(37). One of the mechanisms for RT to activate immunity is the release of antigen from dying tumor cells(3-5). The immunostimulatory effect of RT 10 Gy over to RT 5 Gy might be due to its stronger ability to kill tumor cells. This immunostimulatory ability of RT 10 Gy was confirmed by the ELISPOT assay, but RT 10 Gy alone can't elicit an abscopal effect on distant tumors. It suggests that local antitumor immunity strengthened by RT 10 Gy is not sufficient to elicit systemic antitumor immune responses. Therefore, it is necessary to combine RT with other therapies to achieve systemic tumor suppression. Indeed, the combination of Erb-(IL10)_2 with RT induced stronger abscopal antitumor effects.

The timing is another critical factor which affects the outcomes of the combination of RT with immunotherapy. The administration of anti-CTLA-4 mAbs 2 days or anti-PD-L1 mAbs 7 days after the completion of RT reduce the therapeutic efficacy even ineffective(20, 23). In this model, it seems that the administration of Erb-(IL10)_2 3 days after RT showed better therapeutic potential. RT could enhance immunotherapy via several mechanisms. For example, tumor antigens released from death cells by RT, or tumor microenvironment remodeling by RT permits more activated T cells access(5-8). RT 10 Gy has been shown to increase the expression of MHC I in the human melanoma MelJuSo cells and it peaked at 3 days post RT(6). MHC I is used to present endogenous peptides to cytotoxic T lymphocytes (CTLs). This result is consistent with our findings. The supplying of Erb-(IL10)_2 concurrently or 6 days after RT showed relatively weak antitumor effects compared with affording it at 3 days post RT.

In general, the therapeutic effects of high-dose RT on local tumor require CD8^+ T cells and the abscopal effect induced by RT also depends on T cells(2, 38). The antitumor effects of IL-10 treatment have been showed to be mediated by either CD8^+ T cells and NK cells(16). In B16-EGFR-OVA tumor model, the
combination of Erb-(IL10)$_2$ and RT displayed additive antitumor effects compared to either therapy alone. The *in vivo* depletion of CD4$^+$ T cells or NK cells did not reverse the therapeutic improvement, while the *in vivo* depletion of CD8$^+$ T cells abrogated the enhanced efficacy and reversed to the level comparable to that of RT alone, suggesting that Erb-(IL10)$_2$ therapy improves RT efficacy through CD8$^+$ T cells. In order to determine what kinds of CD8$^+$ T cells, for example intratumoral CD8$^+$ T cells and recruiting CD8$^+$ T cells, play critical roles in tumor growth control under this combination therapy, we used FTY720 to induce lymphocytopenia by preventing T cells egress from lymph organs. Interestingly, preventing T cell tumor infiltration did not influence the tumor growth inhibition in irradiated tumors, nor affected the tumor suppression by Erb-(IL10)$_2$ therapy alone, however, the induction of abscopal antitumor effects was compromised to the levels which is comparable to that of Erb-(IL10)$_2$ therapy alone. These results indicate that the combinational antitumor effects in the irradiated tumors depend on intratumoral T cells, while the abscopal antitumor effects rely on recruiting T cells. This consequence is in agreement with the observations of RT as a single modality and IL-10 maintains antitumor CD8$^+$ T-cell effector function in situ(2, 15). A recent study shows that tumor target-delivery and retention of IL-10 acts as a balance stimulation of antitumor immunity. The local accumulation of IL-10 activates CD8$^+$ T cells to increase the secretion of IFN-γ and to promote DC priming. Meantime, IL-10 interacts with IL-10 receptor in DCs to reduce their IL-12 production, preventing the over-production of IFN-γ, and thus suppress antigen-specific CD8$^+$ T cell apoptosis mediated by DCs. Therefore, the addition of IL-10 to RT may not only facilitate local CD8$^+$ T cell activation in irradiated tumors, but also enhance tumor antigen priming and presentation to elicit abscopal antitumor effects.

In conclusion, our study shows that the combination therapy of RT with Erb-(IL10)$_2$ provides additive antitumor effects via the activation of intratumoral CD8$^+$ T cells. The addition of Erb-(IL10)$_2$ induces the abscopal antitumor effects of RT in a manner of stimulating and recruiting CD8$^+$ T cells from tumor-draining lymph nodes. Our work provides new insight into the design of novel combination of RT with local enrichment of IL-10 to achieve systemic tumor eradication.

**Conclusions**

Together, these results suggest that Erb-(IL10)$_2$ improves the efficacy of RT through the stimulation of intratumoral CD8$^+$ T cells and promotes abscopal antitumor responses via the recruitment and activation of tumor-draining lymph node T cells. Thus, our findings suggest a novel strategy to elicit abscopal antitumor effects of RT through combining tumor-targeted therapy of IL10.

**Abbreviations**

RT: Radiotherapy; IL10: Interleukin 10; CT: Chemotherapy; TAAs: Tumor-associated antigens; 3D-CRT: Three-dimensional conformal RT; IMRT: Intensity-modulated RT; IGRT: Image-guided RT; CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4; FDA: Food and Drug Administration; PD-1: Programmed...
Declarations

Author contributions

S.Q. conceived and supervised the study. J.X., Z.Q., Y.Y., Q.Z., Z.Z., J.Z., Z.L., and H.X. performed the experiments and analyzed the data. S.F. and L.Z. interpreted the data and provided intellectual input. Y.H and S.Q. designed the experiments, analyzed the data, and interpreted the results. S.Q. wrote and revised the manuscript with J.X., Y.Y., Q.Z., Z.Z., Y.H. and with inputs from other authors.

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Availability of data and materials

The raw data supporting the conclusions of this article were all included in figures and supplementary figures.

Ethics approval and consent to participate

All of the experimental procedures were performed in compliance with the Animal Care and Use Regulations of China and approved by the Institutional Laboratory Animal Care and Use Committee of Soochow University.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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**Figures**
Erb-(IL10)2 treatments potentiate the antitumor effects of RT. (A) C57BL/6 mice were inoculated s.c. on day 0 with 5×10⁵ B16-EGFR-SIY cells on the right flank. Tumor-bearing animals were treated with isotype control, RT (10 Gy, day 10), Erb-(IL10)2 (i.p. injections of 1 mg/kg Erb-(IL10)2 on day 13, every 3-4 days for a total of 3 times), and RT combination with Erb-(IL10)2 (RT 10 Gy on day 10 followed by Erb-(IL10)2 on day 13, every 3-4 days for a total of 3 times). (B) Survival curves of the model, showing the fraction of
tumor volume not reaching 2000 mm$^3$. (C) Tumor-bearing animals were treated with isotype control, RT (5 Gy, day 10), Erb-(IL10)$^2$ (i.p. injections of 1 mg/kg Erb-(IL10)$^2$ on day 13, every 3-4 days for a total of 3 times), and RT combination with Erb-(IL10)$^2$ (RT 5 Gy on day 10 followed by Erb-(IL10)$^2$ on day 13, every 3-4 days for a total of 3 times). Representative data are shown from three independent experiments conducted with 5 mice per group.

Fig. 2

The combination of RT and Erb-(IL10)$^2$ treatments increase tumor infiltration of CD8$^+$ T cells in the B16-EGFR-SIY tumor model. C57BL/6 mice injected s.c. on day 0 with 5×10$^5$ B16-EGFR-SIY cells on the right flank of mice. The tumors were treated locally precisely with RT 10 Gy on day 10 and 1 mg/kg Erb-(IL10)$^2$ was administered i.p. on day 13 every 3-4 days for three times as indicated. The tumor tissues were
harvested and tumor-infiltrated T cells were analyzed by flow cytometry. Representative flow cytometry figures are shown from three independent experiments conducted with 5 mice per group.

**Fig. 3**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**Figure 3**

Erb-(IL10)2 treatments enhance RT efficacy in a CD8+ T cell dependent manner. C57BL/6 mice were inoculated s.c. on day 0 with 5×10^5 B16-EGFR-SIY cells on the right flank. Tumor-bearing animals locally received RT 10 Gy on day 10, and/or 1 mg/kg Erb-(IL10)2 or isotype control i.p. on day 13, every 3-4 days...
for a total of 3 times. Mice received an i.p. injection of 200 μg per mouse anti-CD4 mAb (clone TIB207) (A), anti-CD8 mAb (clone TIB210) (B), or anti-NK mAb (clone PK136) (C) twice a week from day 10. The additive antitumor effects of the combination therapy were abolished when CD8+ T cells were depleted (B). Representative data are shown from three independent experiments conducted with 5 mice per group.

**Fig. 4**

**A**

The primary tumors with RT

| Tumor volume (mm$^3$) |
|-----------------------|
| Control              |
| RT 10 Gy             |
| Erb-(IL10)$_2$ 1 mg/kg |
| RT 10 Gy + Erb-(IL10)$_2$ 1 mg/kg |

**Days after tumor inoculation**

**B**

The secondary tumors without RT

| Tumor volume (mm$^3$) |
|-----------------------|
| Control              |
| RT 10 Gy             |
| Erb-(IL10)$_2$ 1 mg/kg |
| RT 10 Gy + Erb-(IL10)$_2$ 1 mg/kg |

**Days after tumor inoculation**

**Figure 4**
Erb-(IL10)2 treatments induce abscopal antitumor effects of RT 10 Gy in the B16-EGFR-SIY tumor model. C57BL/6 mice injected s.c. on day 0 with 5×10^5 B16-EGFR-SIY cells on the right flank (primary tumor) and with equivalent number of cells on the left flank (secondary tumor). The primary tumors were treated locally precisely with RT 10 Gy on day 10 and 1 mg/kg Erb-(IL10)2 was administered i.p. on day 13, every 3-4 days for three times as indicated. Primary (A) and secondary (B) tumor volumes were measured. Representative tumor growth curves are shown from three independent experiments conducted with 5 mice per group.

**Fig. 5**

![Graph A](image)

**The primary tumors with RT**

- Control
- Erb-(IL10)2 1 mg/kg
- RT 10 Gy+
- Erb-(IL10)2 1 mg/kg+
- FTY720
- RT 10 Gy+
- Erb-(IL10)2 1 mg/kg+
- FTY720

![Graph B](image)

**The secondary tumors without RT**

- Control
- Erb-(IL10)2 1 mg/kg
- RT 10 Gy+
- Erb-(IL10)2 1 mg/kg+
- FTY720
- RT 10 Gy+
- Erb-(IL10)2 1 mg/kg+
- FTY720
Figure 5

Blockade the trafficking of lymphocytes into tumor tissues abrogates the abscopal antitumor effects without affecting on the combinational antitumor effects. C57BL/6 mice injected s.c. on day 0 with $5 \times 10^5$ B16-EGFR-SIY cells on the right flank (primary tumor, A) and with equivalent number of cells on the left flank (secondary tumor, B). The primary tumors were treated locally precisely with RT 10 Gy on day 10 and 1 mg/kg Erb-(IL10)2 was administered i.p. on day 13, every 3-4 days for three times as indicated. FTY720 (1 mg/kg) treatment was initiated once a day from days 13 to 15, then turned to on alternate days and maintained on drinking water (2 µg/ml) until the end of the experiment. Primary and secondary tumor volumes were measured and their tumor growth curves were presented.
The combination of irradiation (10 Gy) and Erb-(IL10)2 treatments elevates IFNg production in tumor DLN in the B16-EGFR-SIY tumor model. C57BL/6 mice were injected with 5x10^5 B16-EGFR-SIY cells and treatments were conducted as described in Fig. 2. After mice received RT 10 Gy and 1 mg/kg Erb-(IL10)2 treatments, tumor-DLN were removed to acquire single cell suspensions and IFNy producing immune cells were determined by ELISPOT assays.
Supplementary Files

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