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Low-dose Radiation Improves Tumor Immune Microenvironment, Enhancing the Effects of Anti-CTLA-4 Therapy

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ABSTRACT

Background: Radiotherapy destroys tumor cells primarily through direct DNA damage by high-energy particles or indirect DNA damage by free radicals. High-dose radiotherapy (HDR) destroys tumor cells while also damaging normal cells and may potentially cause immunosuppression. The effect of low-dose radiotherapy (LDR) on the tumor microenvironment (TME) may differ from those of HDR.

Objectives: To determine if combining low-dose radiotherapy with immune checkpoint inhibitors results in synergistic effects.

Methods: We established a mouse model for lung cancer and categorized mice into 4 cohorts: NC (negative control) cohort, LDR cohort, anti-CTLA-4 cohort, and LDR+anti-CTLA-4 cohort. Changes in tumor volume were observed in each group, with particular attention given to the variations in immune cells and cytokines within the mouse tumors following LDR.

Results: The mice in the LDR+anti-CTLA-4 group exhibited the slowest growth in tumor volume, and low-dose radiotherapy tended to inhibit tumor growth. The proportion of infiltrating CD8+T cells increased and the proportion of infiltrating Treg cells decreased in the tumor after LDR. The levels of interferon (IFN) and the chemokines CXCL9, CXCL10 and CXCL11 were increased after low-dose radiotherapy.

Conclusion: LDR has the ability to alter the immune microenvironment of tumors by promoting the production of IFN. Additionally, when combined with anti-CTLA-4, whole-body LDR can effectively suppress tumor growth in mice. The finding is of potential clinical significance and deserves further exploration.

Keywords: Anti-CTLA-4, Interferon, Low-dose radiotherapy, Tumor immune microenvironment

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INTRODUCTION

Radiotherapy plays a crucial role in cancer treatment by destroying tumor cells through direct DNA damage caused by high-energy particles or indirect DNA damage from free radicals. Conventional fractionated radiotherapy is believed to trigger an immune response against tumor cells, stimulating of the immune system to fight against cancer (1). The process includes the liberation of dsDNA when tumor cells die, leading to the generation of interferon (2). The interferon produced reaches the distant tumor tissue and kills the distant tumor, resulting in the so-called "distant effect." (3). However, conventional fractionated or hypofractionated radiation treatment could result in possible immune system suppression and damage to normal cells and tissues (4). The tumor microenvironment (TME) undergo changes as the tumor progresses, establishing a supportive environment for tumor growth by immune system suppression (5). Unfortunately, conventional radiotherapy is not effective in changing this immunosuppressive microenvironment (6, 7). Low-dose radiotherapy has a different effect on the immune system compared to high-dose radiotherapy (HDR) (8-10). Low-dose radiotherapy can improve the immune environment of the tumor (11-13).

Low-dose radiation therapy has the potential to boost immune responses against tumors by inducing tumor cell death and releasing antigens (14, 15). The effects of LDR go beyond just killing cancer cells directly. This includes the stimulation of various immune cell types such as T, B , NK cells, and macrophages and changing their distribution within the tumor microenvironment (16, 17). It also reduces the presence of Treg cells that suppress the immune response, and promote M1 macrophage polarization (15). These mechanisms may be potential reasons for the enhancement of the immune system. Studies have shown that the use of LDR (0.1-0.2 Gy) in clinical trials leads to remission rates and side effects that are comparable, if not superior to, other systemic anticancer therapies, indicating the effectiveness of LDR at a systemic level (10).

By reversing T-cell suppression and boosting the immune response, immune checkpoint inhibitors like CTLA-4 inhibitors can be affected by LDR therapy to enhance the effectiveness of these T cells in altering TME. Research has shown that LDR can increase the expression of immune checkpoint molecules (18), indicating that combining of LDR with immune checkpoint inhibitors may have synergistic effects. Overall, using low-dose radiotherapy alongside immune checkpoint inhibitors introduces a new approach to treating tumors (18, 19). Nevertheless, further research is needed to fully comprehend its effectiveness and mechanisms.

METHODS

Cell Line

The mouse Lewis lung cancer (LLC) cell line, reference number TCM-C742, was obtained from Suzhou Haixing Biological Technology Co. in Suzhou, China. The cells were cultured in DMEM (HyClone, USA SH30243.01) supplemented with 10% FBS (Biological, 04-001-1ACS) and 1% penicillin/streptomycin (biosharp BL505A). They were then incubated at 37°C in an environment containing 5% CO_2.

Modeling Tumors

Female C57BL/6 mice, aged six weeks and weighing 18±2 g, were procured from HFK Bioscience (HFK Bioscience, China). All mouse experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Shandong First Medical University and were approved by them (Ethical approval number: CUTCM /2021/09/113). We strictly follow the animal care and experimental procedures recommended by the committee.

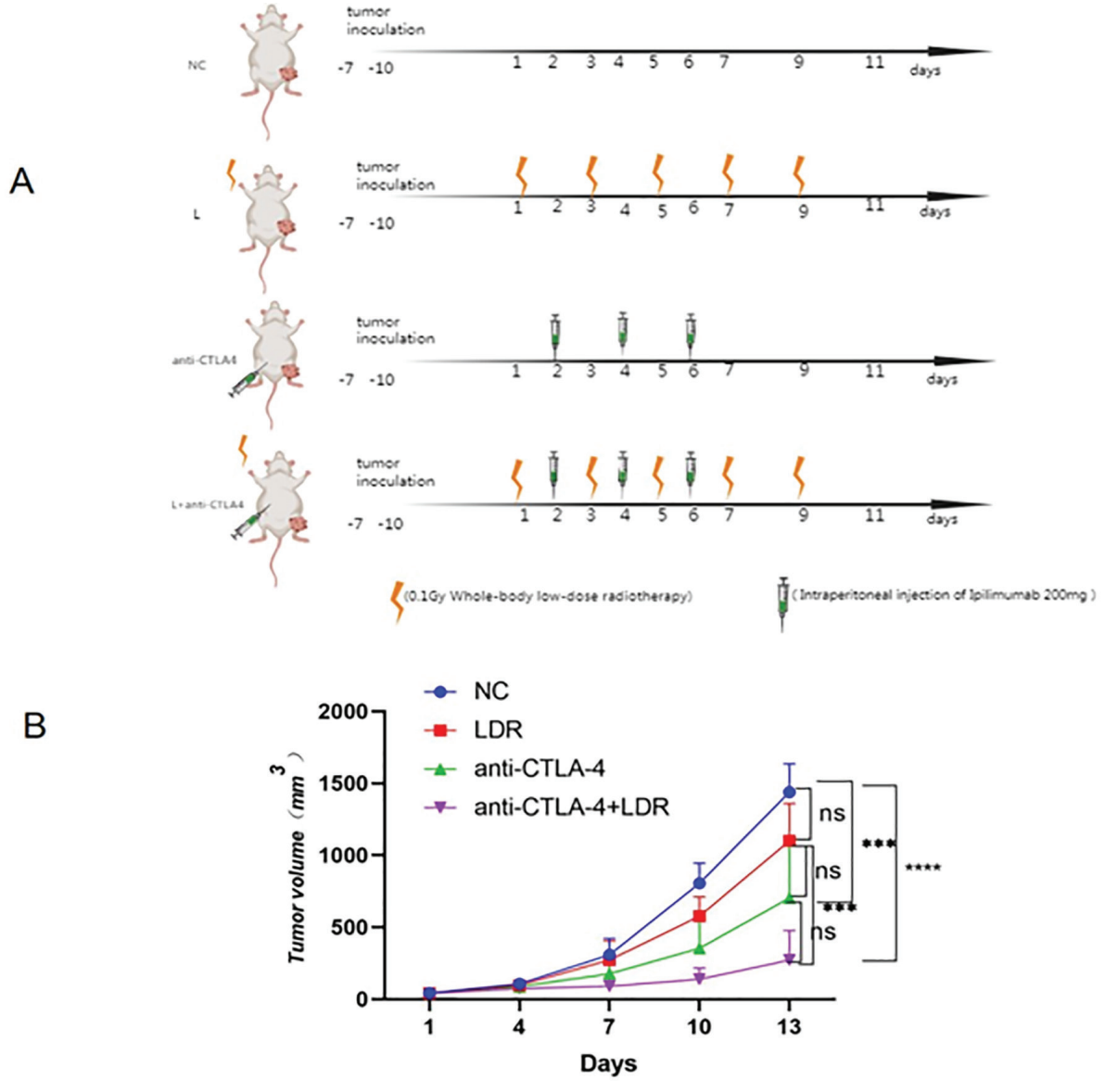

Fig. 1. LDR enhances the antitumor effects of CTLA-4 inhibitors. A. We established a mouse tumor model in LLC-implanted tumor-bearing mice, and divided them into 4 groups (n=6 per group) as shown in A. The NC group served as the negative control, while the LDR group received 0.1Gy whole-body low-dose radiotherapy every other day, totaling 5 occasions. The group that received anti-CTLA-4 was administered 200ug of the treatment via intraperitoneal injection every other day for a total of 3 injections. The LDR+anti-CTLA-4 group followed the same radiotherapy schedule as the LDR group, and also received an intraperitoneal injection of 200ug anti-CTLA-4 one day after LDR, repeated every other day for a total of 3 injections. B. The LDR+anti-CTLA-4 group showed the slowest tumor growth among the four groups. The observed variance was found to be statistically significant when compared to the NC ($P<0.0001$). Our observation showed that mice treated with systemic low-dose radiation (LDR) exhibited a tendency to inhibit tumor growth compared to the control group. Furthermore, the inhibitory impact was more significant in the group receiving combined LDR and anti-CTLA-4 treatment than in those receiving anti-CTLA-4 alone (n=6) (1B).

C57BL/6 mice were subcutaneously injected with 1×10^6 LLC cells in the left hind limb. Once the tumors reached a size of approximately 4 mm in diameter, the mice were randomly divided into four groups (n=6) as depicted in Fig. 1A: the control group (NC), a low-dose radiotherapy (LDR) group receiving 0.1 Gy, an anti-CTLA-4 treatment group, and a combination therapy group receiving LDR+anti-CTLA-4.

Treatment

When the tumor diameter reached approximately 6 mm, all mice were treated accordingly. The NC cohort was used as a negative control. The LDR group received 0.1Gy of LDR, with the treatment given every other day for a total of 5 doses. The group treated with anti-CTLA-4 received 3 intraperitoneal injections of 200ug anti-CTLA-4, administered every other day. The LDR+anti-CTLA-4 group received 0.1Gy LDR every other day, five times in total, along with an intraperitoneal injection of 200 ug anti-CTLA-4 performed one day after LDR. The treatment was given every second day, totaling 3 doses. The mice were euthanized in accordance with ethical guidelines through cervical dislocation once the tumors reached a diameter of 15 mm. No chemicals or substances were used in this process.

Flow Cytometry Analysis

The mice in the study were separated into four distinct categories. The excised tumors were homogenized using a combination of 0.2% collagenase type IV, 0.01% hyaluronidase, and 0.002% DNase I, all sourced from Solarbio Science. The experiment was conducted in a DMEM solution at 37°C for a duration of 40 minutes. The individual cells in the resultant suspension were labeled with fixable viability dye BV510. The cells were labeled with a specific panel of antibodies to analyze T cells that had infiltrated the tumor tissue. Tube 1 utilized CD3+ APC, CD45+ FITC, CD8+ percpcy5.5, and IFN-γ+ PE/APC-Cy7 as the main method for evaluation. Tube 2 assessed Treg cells in tumor tissues using CD4+ FITC, CD25 PE, CD45+ percpcy5.5, and Foxp3 APC following Biolegend's protocols. The cells were cultured in vitro with a cell stimulation cocktail containing protein transport inhibitors from Biolegend for 6 hours to enhance the detection of IFN-γ. After stimulation, the cells were labeled with CD3+ APC, CD45+ FITC, and CD8+ percpcy5.5 antibodies for surface staining. Subsequently, a fixation and permeabilization kit from Biolegend, USA was used for sample processing, followed by staining with IFN-γ antibody at a dilution of 1:1000. Flow cytometry analysis was performed using a BD Fortessa system to examine the stained cells. The FlowJo software, version 10.0, was utilized to process and analyze the data collected from flow cytometry.

ELISA

The tumor samples were thoroughly mixed, and the collected liquids were obtained after being exposed to a lysis buffer containing protease inhibitors provided by Beyotime (P 1045). The levels of chemokines and cytokines, including IFN-α, IFN-γ, TNF-α, CXCL11, CXCL10, and CXCL9 were measured. The experiments were conducted using ELISA kits that rely on specific antibodies, following the suggested procedures provided by Biological J&L (Shanghai, China). The mice were euthanized 24 hours after the final administration of LDR, and their tumor samples were then extracted for examination.

RNA Sequencing Analysis

The tumor tissue samples were rapidly frozen in liquid nitrogen for preservation and total RNA was extracted. Subsequently, libraries were generated using the TruSeq Stranded mRNA Sample Prep Kit (Illumina). Shandong Xiuyue Biotechnology Co. Ltd. in Shandong, China, was responsible for conducting transcriptome sequencing and analyzing the resulting data.

Statistical Analysis

The statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). The results are presented as the mean value with the standard error of the mean (SEM). To assess the impact of tumor proliferation, Two-Way ANOVA was used to analyze different treatments and time intervals. The Unpaired 2-Tailed Student's t-Tests were used to compare two groups, while the One-

Way ANOVA with Bonferroni adjustments was utilized for comparing multiple groups. Statistical significance levels were indicated by asterisks (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). These statistical techniques were carefully selected to comprehensively assess the impact of treatments on tumor progression, immune responses, and other relevant variables in the research.

RESULTS

LDR Enhances the Antitumor Effects of CTLA-4 Inhibitors

Our observation revealed that the LDR+anti-CTLA-4 group exhibited the slowest tumor growth among the four groups, and this difference was statistically significant compared to the NC group (P<0.0001). Although there was no statistical difference between whole-body low-dose radiotherapy and the control group, there was a trend for whole-body low-dose radiotherapy to inhibit tumor growth in mice. Additionally, a trend was observed towards suppressing the tumor growth in the LDR + anti-CTLA-4 group compared to the anti-CTLA-4 alone group (n=6) (Fig. 1B).

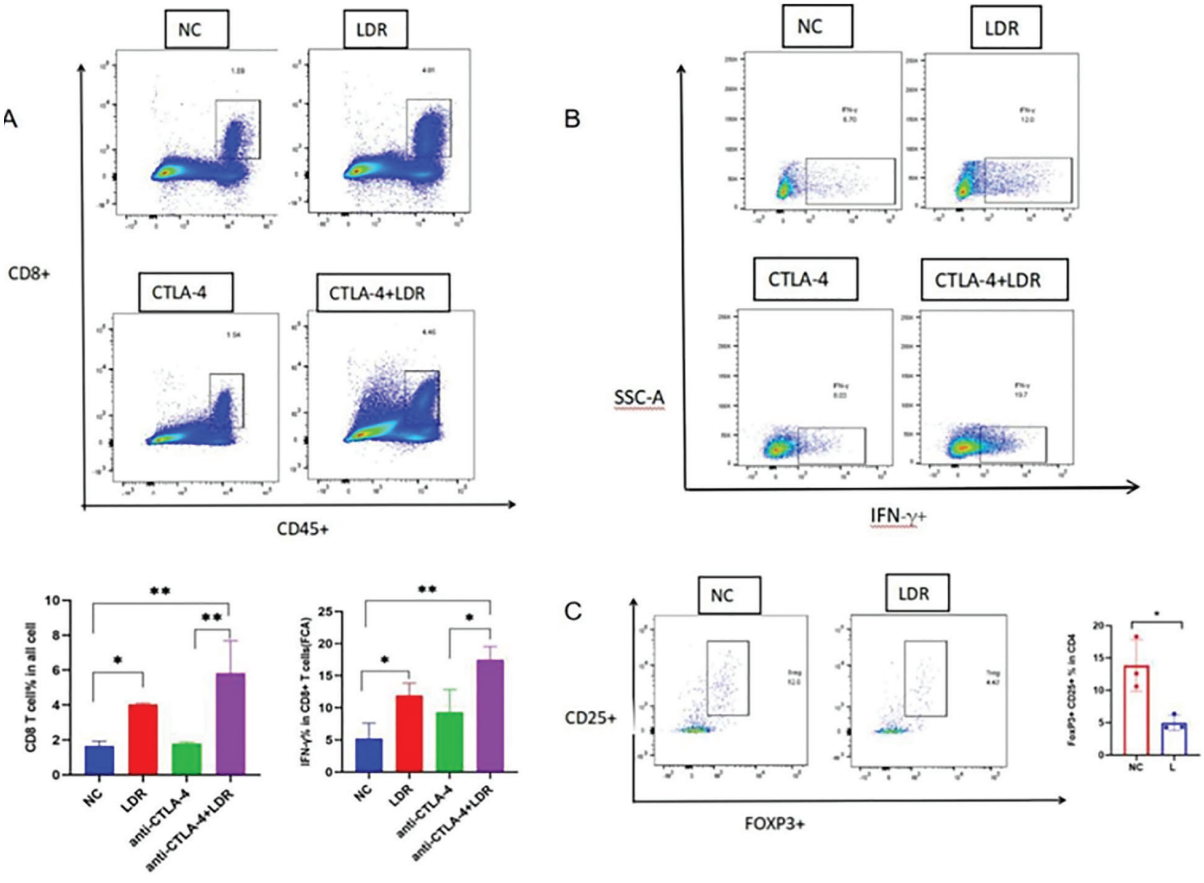

Fig. 2. Low-dose radiotherapy (LDR) enhanced the infiltration of CD8T cells into tumor. A. Following exposure to low-dose radiation, there was a significant increase in the percentage of CD8+ T cells within tumor tissues, rising from 1.65% to 4.14% (P=0.0483, P<0.05). The proportion of CD8+ T cells in tumor tissues was also significantly increased by anti-CTLA4 combined with LDR compared with anti-CTLA4 treatment alone. It increased from 1.777% to 5.9% (P=0.0034, P<0.05) (mean±SEM n=3). B. After exposure to low-dose radiation (LDR), there was a significant increase in the proportion of IFN-γ+ cells among CD8+ T cells, rising from 5.197% to 11.95% (P=0.0478, P <0.05). Additionally, the percentage of IFN-γ+ cells among CD8+ T cells in tumor tissues also significantly increased, going from 9.3% in the group treated with anti-CTLA-4 alone to 17.47% in the combination group (P=0.0188, P<0.05). C. The ratio of Treg cells to CD4+ T cells in tumor tissues significantly decreased from 13.93% before irradiation to 4.92% after irradiation (mean±SEM n=3) (P<0.05).

LDR Can Enhance the Aggregation of CD8+ T cells Within the Tumor and Improve the Immune Environment of the Tumor

The percentage of CD8+ T cells in the tumor showed a significant increase from 1.65% to 4.14% following exposure to low-dose radiation. (P=0.0483, P<0.05) (Fig. 2A). Additionally, the proportion of CD8+ T cells producing IFN-γ also exhibited a significant increase from 5.197% to 11.95% after exposure to low-dose radiation (P=0.0478, P<0.05) (Fig. 2B). Furthermore, the ratio of Treg cells to CD4+ T cells in tumor tissues significantly decreased from 13.93% before irradiation to 4.92% post-irradiation (P<0.05) (Fig. 2C).

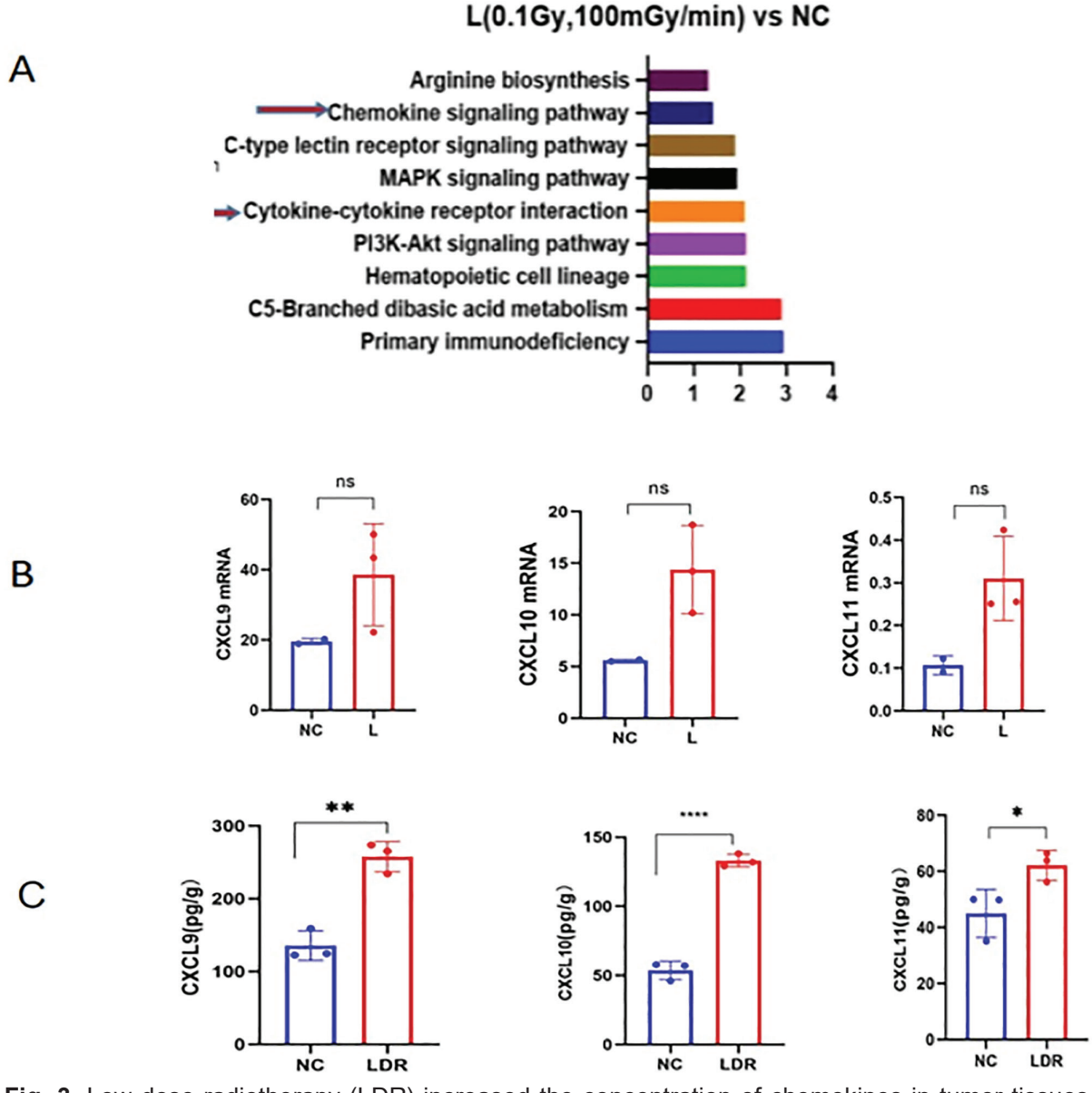

Fig. 3. Low-dose radiotherapy (LDR) increased the concentration of chemokines in tumor tissues. A. Analysis of RNA sequencing (n=3) revealed a significant up-regulation of cytokines and chemokine-related signaling pathways following low-dose irradiation to the entire body. B. RAN sequencing analysis (mean±SEM n=3) indicated a tendency for increased expression of chemokine-related genes Cxcl9, Cxcl11, and Cxcl10. However, the difference observed did not achieve statistical significance. C. The concentration of CXCL9 in tumor tissues showed a significant increase from 135.5 pg/g before irradiation to 257.9 pg/g following irradiation (P=0.0019, P<0.01). The CXCL10 level exhibited a notable increase from 53.68 pg/g before irradiation to 133.1 pg/g after irradiation (P<0.0001). Additionally, the level of CXCL11 increased from 44.99 pg/g before receiving irradiation to 62.16 pg/g after irradiation, with a significant difference (P=0.0419, P<0.05) (mean±SEM n=3).

The co-administration of LDR and anti-CTLA-4 therapy resulted in a higher influx of CD8+ T cells into tumor tissues compared to the group that only received anti-CTLA-4 treatment. The proportion of CD8+ T cells in tumor tissues increased from 1.777% in the group treated with anti-CTLA-4 alone to 5.9%, indicating a statistically significant difference (P=0.0034, P<0.05) (Fig. 2A). Additionally, the proportion of CD8+ T cells producing IFN-γ in tumor tissues increased from 9.3% in the group receiving only anti-CTLA-4 treatment to 17.47% in the combination group, a statistically significant difference as well (P=0.0188, P<0.05) (Fig. 2B).

Receiving LDR Dose Radiotherapy Led to an Increase in the Concentration of Chemokines within the Tumor Tissues

As a result of the impact of chemokines, changes in the composition of immune cells within the tumor microenvironment were observed. We assessed the concentrations of CXCL10, CXCL9, and CXCL11 in the neoplastic tissues (n=3). The concentration of CXCL9 in tumor tissues showed a significant increase from 135.5 pg/g beforirradiation to 257.9 pg/g following irradiation (P=0.0019, P<0.01). The CXCL10 level exhibited a notable rise from 53.68 pg/g before irradiation to 133.1 pg/g after irradiation (P<0.0001), as did the level of CXCL11 which increased from 44.99 pg/g before receiving irradiation to 62.16 pg/g after irradiation (P=0.0419, P<0.05) (Fig. 3C). We conducted RNA sequencing analysis (n=2) on tumor tissues from mice that had received low-dose radiation and observed a significant increase in cytokine-related signaling pathways following whole-body LDR (Fig. 3A). Additionally, there was a trend towards up-regulated expression of chemokine-related genes Cxcl9, Cxcl11, and Cxcl10, although the differences were not statistically significant (Fig. 3B).

LDR Increased the Concentration of IFN in the Tumor Samples

Considering the interactive effect of chemokines and IFN, we investigated the concentration of IFN in the tumor samples

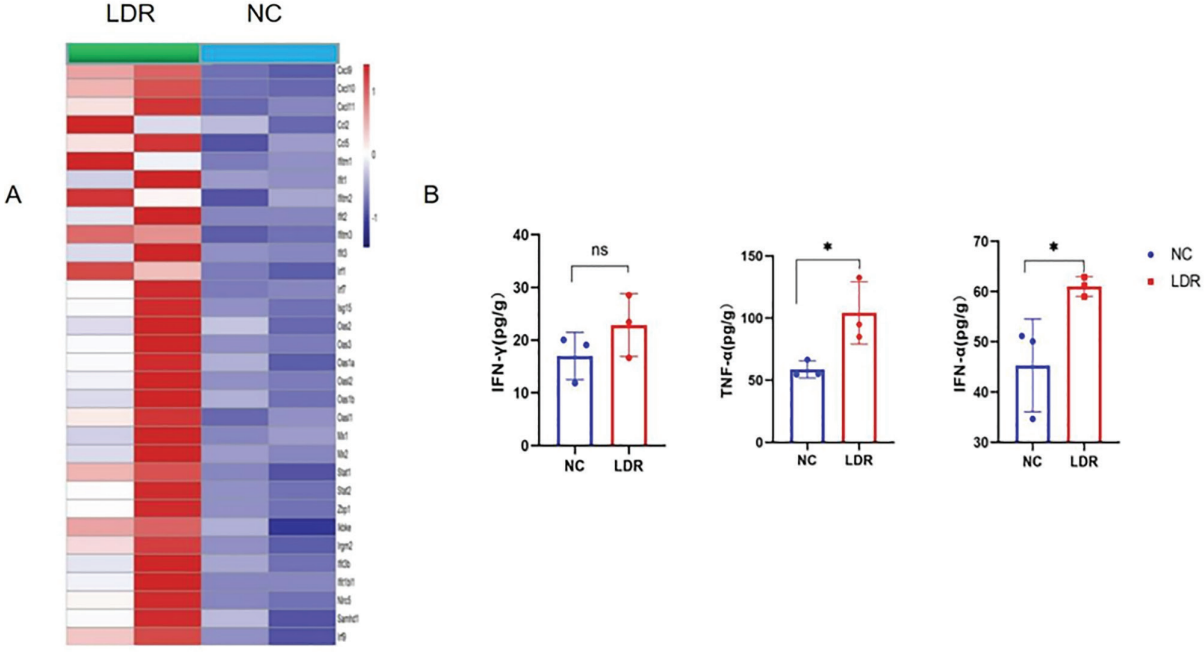

Fig. 4. Low-dose radiotherapy (LDR) increased the concentration of interferon (IFN) in the tumor cells. A. Analysis of tumor tissues from mice treated with radiation (n=2) revealed an up-regulation of IFN-related genes at low doses of irradiation, as determined by RNA sequencing. B. The levels of IFN-γ increased from 17.00 pg/g before irradiation to 22.89 pg/g after irradiation. TNF-α increased from 58.68 pg/g before irradiation to 104.1 pg/g after irradiation (P=0.0390, P<0.05), and IFN-α increased from 45.30 pg/g before irradiation to 61.02 pg/g after irradiation (P=0.0449, P<0.05) (mean±SEM n=3).

(n=3) and observed an increase in IFN-γ from 17.00 pg/g before irradiation to 22.89 pg/g after irradiation, TNF-α from 58.68 pg/g before irradiation to 104.1 pg/g after irradiation (P=0.0390, P<0.05), and IFN-α from 45.30 pg/g before irradiation to 61.02 pg/g after irradiation(P=0.0449, P<0.05) (Fig. 4B). Additionally, RNA sequencing analysis of tumor tissues (n=2) post low-dose irradiation in mice confirmed the up-regulation of IFN-related gene expression following systemic low-dose irradiation (Fig. 4A).

DISCUSSION

The potential influence of LDR as an immunomodulatory instrument extends beyond just treating cancer, offering promising avenues for medical research (20-25). There is evidence to suggest that low dose radiation (LDR) has the potential to regulate immune reactions, which could be particularly crucial in non-cancerous situations such as COVID-19. LDR has shown promise in enhancing the effectiveness of treatments by activating the body's immune response (20-25).

The impact of LDR on crucial immune pathways, such as the CXCR3 ligand axis involving CXCL10, CXCL9, and CXCL11, has been evidenced. These chemokines are essential for attracting immune cells that express the CXCR3 receptor, including Th1 cells, CD8+ T cells, and NK cells, to the tumor microenvironment. These cells are crucial for the immune system's ability to fight against tumors. Recent research has suggested that directly injecting of CXCL9, CXCL11, and CXCL10 into tumors can effectively attract immune cells, leading to decreased tumor growth in various cancer models (26-29). Additionally, the presence of these chemokines in tumor tissues has been linked to improved clinical outcomes for cancer patients, underscoring their significance in combating tumors (26, 28). It has been observed that low-dose radiation may boost the production of these chemokines, potentially aiding the movement of effector T-cells into the tumor environment, enhancing the local immune context and potentially inhibiting tumor growth.

Furthermore, the impact of LDR extends beyond its immediate cytotoxic effects, as it can stimulate immune reactions against cancer involving both innate and adaptive elements. This includes the activation of various types of immune cells such as T cells, B cells, NK cells, and macrophages within the tumor microenvironment, while also decreasing the population of Treg cells that suppress immune responses (30, 31).

Receiving radiation doses between 1 Gy and 2 Gy can boost effector T-cell penetration, promote M1 macrophage polarization,increase NK cell infiltration, and decrease levels of TGF-β (32). Doses between 0.5 Gy and 1 Gy have the potential to impact larger areas of the body and enhance the body's immune responses against tumors (14). Clinical studies using LDR (0.1-0.2 Gy) have demonstrated comprable or improved rates of remission and side effects when compared to other systemic anti-tumor treatments, highlighting the effectiveness of LDR (29-33).

The immune system's response to LDR involves the crucial generation of interferon, a multifunctional cytokine essential for regulating cellular proliferation, innate and adaptive immune responses, and angiogenesis. The use of low levels of radiation has been linked to the stimulation of interferon release, which is influenced by the dosage level and rate of radiation exposure (33). As a result, IFN influences the levels of specific chemokines such as CXCL9 and CXCL10, which play a role in recruiting immune cells, particularly CD8+ T cells, to the tumor site (25-29). This helps to prevent the growth of tumors (34, 35). The important function of IFN in tumor immunology is underscored by the intricate interplay between IFN and the recruitment of immune cells.

Our results suggest that LDR could

potentially help rebalance the immune system and tumor cells in Tumor-associated immune systems (TAIS) by controlling IFN production, which is often disturbed during cancer development (36). By regulating the immune system, especially by increasing the recruitment of immune effector cells through chemokines, LDR may have the ability to revert TAIS back to an "elimination" stage, allowing for the restoration of immune-mediated tumor control.

Additionally, the interesting method of integrating LDRT with additional treatments such as immunotherapy or chemotherapy has demonstrated encouraging results in numerous clinical investigations (37-42). Our research showed that CTLA-4 checkpoint inhibitor combining with low-dose radiation treatment results in a stronger anti-tumor effect in mice. This suggests that combining these treatments has the potential to be more effective than using either one alone.

In summary, LDR's ability to modulate the immune system by inducing IFN and attracting immune cells through chemokines shows potential for improving anti-tumor immune responses. Further research is needed to fully comprehend its impact on the immune system, which may expand LDRT's application beyond cancer. By restoring the balance between tumors and the immune system, LDRT offers a new approach to improving immune-mediated tumor regulation, bringing hope to individuals with various types of cancer and other illnesses.

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AUTHORS' CONTRIBUTION

Jigang Dong: Writing–original draft, Software, Methodology, Investigation, Data curation, Conceptualization. Ying Qi: Writing– review & editing, Funding acquisition. Sha Sha: Soft ware, Data curation.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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