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Research Article

Low-Dose Radiation Improves the Tumor Immune Microenvironment through IFN Production: An *In Vivo* Study Using Murine Lung Cancer Model

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ABSTRACT

Background: Exposure to radiation prompts apoptosis within cancer cells, initiating 'consume me' signals including phosphatidylserine and calreticulin. This activation of dendritic cells paves the way for immune responses mediated by T and NKT cells, as well as the engulfment of cells by macrophages.

Objectives: In our study, we focused on the effects of low-dose radiation on Interferon (IFN) production, thereby enhancing immunogenic cell death in tumor cells.

Methods: In a study utilizing a murine lung cancer model, Lewis Lung Carcinoma (LLC) cells were administered to C57BL/6 mice. Following tumor development, these mice were segregated into cohorts. One cohort received a regimen of low-dose radiation therapy, while another was treated with CTLA-4 antibody injections.

Results: We found that low-dose radiation of 0.1 Gy promoted the production of IFN, and the contents of *CXCL9*, *CXCL10* and *CXCL11* in the tumor tissues were likewise significantly elevated after low-dose irradiation, which promoted the infiltration of CD8T cells in the tumor tissues, and ultimately inhibited the growth of the tumors in mice.

Conclusion: Low-dose radiation enhances immunogenic cell death in tumor cells by stimulating IFN production. These findings highlight the potential therapeutic significance of LDR in remodeling the tumor immune microenvironment, which warrants further exploration of its clinical applications in cancer therapy.

Keywords: Immune enhancement; Microenvironment; Interferon low-dose radiotherapy

INTRODUCTION

Radiotherapy is a pivotal treatment for cancer, primarily functioning through the destruction of tumor cells by high-energy particles that directly harm DNA or free radicals that indirectly damage it. Conventional fractionated or hypo-fractionated radiation is believed to induce "immunogenic" tumor cell death, which activates the immune system to combat cancer [1-3]. This process involves the release of double-stranded DNA (dsDNA) during tumor cell death, which can activate the cGAS-STING signaling pathway, ultimately leading to the creation of IFN-I [4]. This mechanism is also associated with the regression of distant tumor lesions, known as the abscopal effect [3].

However, traditional fractionated or hypo-fractionated radiation may potentially causes immunosuppression and can harm healthy cells and tissues [5]. The tumor microenvironment can evolve during tumor progression, fostering an immunosuppressive milieu that supports tumor growth [6]. Unfortunately, conventional radiation does not effectively alter this immunosuppressive microenvironment.

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Recent studies have suggested that Low-Dose Radiotherapy (LDR) can positively impact the immunosuppressive microenvironment in tumor tissues [7-9]. Defining LDR can be somewhat variable, but in radiation oncology, a fraction of less than 2 Gy is considered low dose, as a conventional single fraction typically delivers 2 Gy [9-11]. However, in the fields of radiobiology and radiation protection, "Low dosage" means a dose of 0.1 Gy or lower. Notably, LDR influences the immune system differently than High-Dose Radiation (HDR) [12-14]. Low-dose radiation therapy has emerged as a promising approach to cancer treatment by triggering immunogenic cell death, which signals the immune cell to recognize the existence of cancer. This process enhances T-cells enter the tumor microenvironment, reduces immunosuppressive elements, and stimulates the release of immune-stimulating molecules. By doing so, low-dose radiation addresses challenges such as tumor immune evasion and resistance to immunotherapies, potentially improving the overall effectiveness of cancer treatment, especially when used in combination with other therapies [13-15].

LDR is believed to modify the tumor microenvironment through various mechanisms, such as promoting M1 macrophage polarization, increasing CD8T cells, and reducing immunosuppressive Treg cells [15-17]. LDR also stimulates DNA repair and induces selective apoptosis or senescence in abnormal cells, further amplifying immune system function [18]. These mechanisms are some of the potential explanations for this immune enhancement.

LDR has garnered interest not only in the context of cancer but also for its potential applications in non-cancer conditions [14,19]. It is recognized for its biological effects and the hormetic response it triggers, particularly in relation to the immune system, with implications beyond cancer [18]. Some studies even explore the potential of LDR in non-cancer disease management, such as the treatment of COVID-19 pneumonia, underscoring its potential for managing viral infections [20,21]. A systematic review by Mortazavi et al. in 2021 further reinforces the potential use of LDR for COVID-19, expanding its scope to non-cancer diseases [22].

Consequently, it is proposed that low-dose radiation can stimulate IFN production due to its capacity to induce immunogenic cell death in tumor cells, providing another potential mechanism for the heightened sensitivity to low-dose radiation [23]. This implies that low-dose radiation may stimulate IFN production and contribute to immunogenic cell death in tumor cells. Therefore, studies exploring the effects of low-dose radiation on immunogenic cell death and its potential in cancer therapy are a promising way to reveal an approach that can enhance anti-tumor immune responses and improve therapeutic outcomes.

By harnessing the ability of low-dose radiation to stimulate immunogenic cell death, we anticipate a shift in the tumor microenvironment towards a more immune-activated state. This may lead to increased immune cell infiltration, particularly cytotoxic T cells, and reduced immunosuppressive elements like regulatory T cells. Ultimately, such modifications are expected to result in more effective cancer treatment, potentially offering a novel strategy to address the challenges in current cancer therapies, including immunotherapy resistance and limited treatment efficacy.

MATERIALS AND METHODS

Ethics approval and animal care

The Institutional Animal Care and Use Committee of Shandong First Medical University authorized all the procedures on animals used in experiments, ensuring adherence to the established ethical standards and guidelines. (Ethical approval no. CUTCM/2021/9/113).

Animal model and tumor induction

In this study, we designed an experiment where female C57BL/6 mice, aged six weeks, were divided into four distinct groups (N=7): NC group (N=7): A control set, L group (N=7): Subjected to low-dose radiation (0.1 Gy radiation every other day for five times, CTLA-4 group (N=7): Treated solely with anti-CTLA-4 (injections of 200 μg of anti-CTLA-4 once every other day for three times, L+CTLA-4 group (N=7): Combination of low-dose radiation and anti-CTLA-4 treatment as shown in Figure 1.

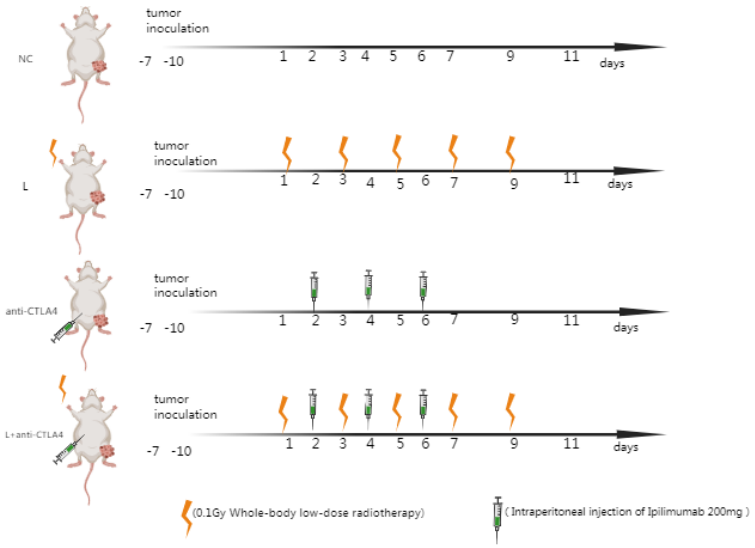

Figure 1: Development of an animal model. **Note:** (): 0.1 Gy whole-body low-dose radiotherapy; (): Intraperitoneal injection of ipilimumab 200 mg

Irradiation geometry

In the conducted experiments, irradiation was precisely delivered at a rate of 10 cGy/min, encompassing a total of five cycles. The irradiation was performed using a Linear Accelerator (LINAC) as the radiation source. The LINAC emitted X-rays with a mean energy of 6 MV (Megavolts), ensuring consistent and controlled irradiation conditions throughout the experimental cycles.

Cell lines

Dr. Xiaoyang Yi kindly provided the LLC cell line utilized in our research. We cultivated this cell line in DMEM with high glucose content (sourced from Gibco, USA), enriched with 10% fetal bovine serum and a 1% mixture of penicillin-streptomycin (acquired from Biosharp, China). These cells were consistently incubated at a stable temperature of 37°C, in an atmosphere containing 5% CO_2, and under conditions of elevated humidity. Upon reaching near 80% confluence, the cells were transferred to new Corning 100 mm culture dishes (originating from Corning, USA) for further growth and passaging.

Tumor models

We acquired six-week-old female C57BL/6 mice, each weighing around 18 ± 2 grams, from HFK Bioscience in Beijing, China, ensuring they were kept in an environment free from specific pathogens. The Shandong First Medical University's Institutional Animal Care and Use Committee granted approval for the mouse studies. We administered a subcutaneous injection of 1×10^6 LLC cells into the left posterior limb of the mice. Subsequent to the tumors reaching an estimated volume of 100 mm^3, we allocated the mice into four distinct experimental groups, which are elaborated upon in the following section.

Flow cytometry analysis

In this study, the mice were allocated into four separate cohorts. The excised tumors were subsequently subjected to homogenization using a mixture containing 0.2% collagenase type IV, 0.01% hyaluronidase, and 0.002% DNase I, all sourced from Solarbio Science in Beijing, China. This process was performed in a DMEM medium and maintained at a temperature of 37°C for a duration of 40 minutes. The resulting single-cell suspension was stained using fixable viability dye BV510. The cells obtained were then marked with a set of antibodies for analysis: (Tube 1) contained antibodies such as $CD45^+$ FITC, $CD3^+$ APC, $CD8^+$ percpcy5.5, and IFN- γ^+ PE/APC-Cy7 to primarily assess the T cells infiltrating the tumor tissue; (Tube 2) was used with $CD45^+$ percpcy5.5, $CD4^+$ FITC, CD25 PE, and foxp3 APC to primarily evaluate Treg cells within the tumor tissues, following the guidelines provided by Biolegend, USA. For the detection of INF-γ, cells underwent *in vitro* stimulation using a cell stimulation cocktail, which included protein transport inhibitors from Biolegend, USA, for a period of 6 hours. Post-stimulation, cells were labeled on the surface with antibodies $CD45^+$ FITC, $CD3^+$ APC, $CD8^+$ percpcy5.5, followed by processing with a fixation and permeabilization kit from Biolegend, USA, and subsequent staining with IFN-γ antibody at a dilution of 1:1000. Flow cytometry analysis was performed on the stained cells using a BD LSDFortessa system. The data from the flow cytometry were processed and analyzed using FlowJo software, version 10.0.

Immunohistochemistry

The experimental mice were evenly distributed into four distinct groups. Following this, the tumor specimens were preserved in 10% neutral-buffered formalin, then embedded within paraffin blocks. From these blocks, sections with a thickness of 4 μm were prepared for subsequent Immunohistochemical analysis (IHC). These sections underwent staining using specific antibodies: CD8, FOXP3, GZMB with the procedure aligned with the guidelines provided by abcam, China. Image capturing was conducted with the aid of an optical microscope provided by Olympus, Tokyo, Japan. For the quantification of immunoreactivity, in each tumor specimen, cells that exhibited positive staining for CD8, FOXP3 and GZMB were tallied across five fields selected at random under a magnification of 200x. From these counts, the proportion of cells showing positive staining was determined.

ELISA measurements

The tumor specimens were homogenized, and the resulting supernatants were collected after being treated with a lysis buffer that included protease inhibitors provided by beyotime (P1045). The concentrations of cytokines and chemokines, specifically IFN-γ, IFN-α, IFN-β, *CXCL9*, *CXCL10*, and *CXCL11*, were quantified using the ELISA technique. These measurements were performed with specific antibody-based ELISA kits, following the protocols recommended by J and L Biological, based in Shanghai, China. More specifically, the mice were euthanized 24 hours subsequent to the final session of low-dose radiation, and their tumor tissues were excised for analysis.

Western blot

LLC cell proteins and those from related samples were isolated using RIPA buffer supplied by bioss (C5029-100 ml) with the addition of a cocktail that inhibits proteases from beyotime (P1045). The BCA technique, utilizing beyotime's kit (P0010S), was employed to ascertain the protein levels. Each sample contributed 50 μ g of protein, which was then separated on a 10% SDS-PAGE gel under a steady 120 V. Proteins were then transferred to a pre-activated PVDF membrane (Millipore, Cat #IPVH00010) at a current of 220 mA and temperature of 4°C throughout the night. The membranes were incubated with primary antibodies targeting Interferon alpha 2 (ab193055 at a dilution of 1:1,000), and GAPDH (Proteintech 10494-1-AP, also at a dilution of 1:1,000) in cold conditions overnight. Subsequent to triple washes, the blots were exposed to a secondary antibody specific to rabbit IgG (Abcam ab150077; at a dilution of 1:5,000) at ambient temperature for half an hour. Protein bands were visualized using a chemiluminescence method (Millipore WBKLS0100 ECL), and images were captured with an Azure c600 imager from Azure Biosystems. Bands corresponding to molecular weights of 25, 37, and 55 kDa were pinpointed and annotated on the film prior to gel scanning. The unaltered scans of the western blots are presented in the supplementary materials the ratio of Interferon alpha 2 to GAPDH for each specimen was calculated for relative quantification using the imageJ software.

RNA sequencing analysis

Specimens of tumor tissue were immediately preserved by freezing in liquid nitrogen, and from these specimens, total RNA was isolated. Following this, the construction of libraries was carried out with the aid of the Truseq Stranded mRNA LT Sample Prep Kit (Illumina, based in San Diego, CA, USA). Shandong Xiuyue Biotechnology Co., Ltd., located in Shandong, China, was responsible for conducting the transcriptome sequencing as well as the subsequent data analysis.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, based in La Jolla, CA, USA) was utilized for all statistical evaluations. The results are depicted as the mean ± SEM (standard error of the mean). The analysis in this research included the use of Two-Way ANOVA for analyzing the influence of varying treatments and time intervals on the proliferation of tumors. Comparisons between two sets were conducted using unpaired 2-tailed student's t-tests, and for comparing multiple groups, One-Way ANOVA followed by Bonferroni adjustments was applied. The levels of statistical significance were indicated by asterisks (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). These statistical methods were carefully chosen to provide a thorough assessment of the effects of treatments on tumor progression, immune reactions, and other pertinent factors within the research.

RESULTS

Low-dose radiotherapy promotes IFN-α production in lung cancer cell lines

We chose LLC cell lines that were exposed to doses of 0.1 Gy, 0.5 Gy, 1 Gy, and 5 Gy respectively. Twenty-four hours after exposure, we performed PCR and western blot to identify changes in the IFN-α gene as shown in Figure 2A and IFN-α protein as shown in Figure 2B. In our study, we found that the low-dose radiation of 0.1 Gy promoted the production of IFN.

Low-dose radiotherapy increases the level of IFN in tumor tissues

Based on RNA sequencing of tumor tissues from radio-treated mice, we discovered that IFN-related genes could be upregulated when exposed to a low dose of 0.1 Gy as shown in Figure 3A. This finding is consistent with our observation at the cellular level and further proves that IFN production was correlated with the dose rate of irradiation. By using an ELISA to detect the amount of IFN in tumor tissues, we also discovered that IFN-γ, IFN-α, and TNF-α levels were increased in tumor tissues following low-dose irradiation, with TNF showing the most significant statistical difference (p<0.01) as shown in Figure 3B.

Low-dose radiotherapy increases the level of chemokines in tumor tissues

By using RAN sequencing analysis, we discovered that chemokine-related genes were significantly elevated following whole-body low-dose irradiation, with *CXCL11* and *CXCL10* being the most dramatically increased as shown in Figures 4A,4B and 4C. The levels of *CXCL9*, *CXCL10*, and *CXCL11* in tumor tissues were likewise found to be significantly higher following low-dose irradiation, and the difference showed statistical significance (p<0.01) in our subsequent ELISA measurements as shown in Figure 4D.

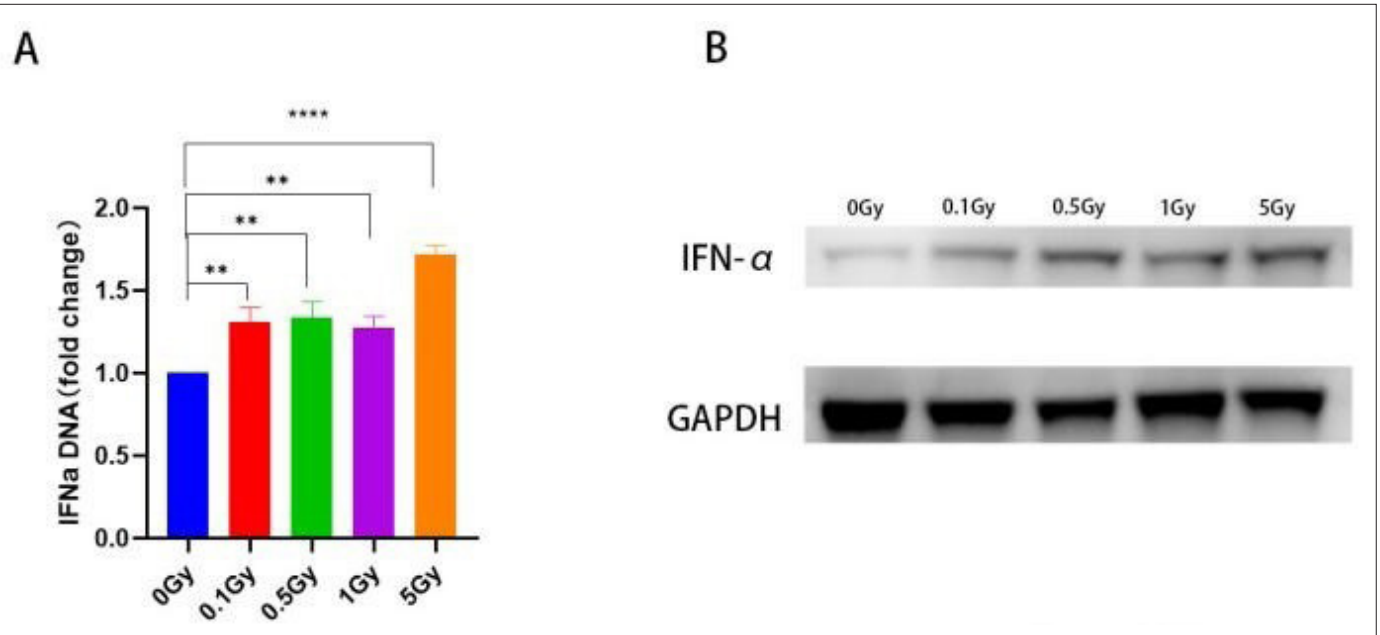

Figure 2: Low-dose radiotherapy promotes IFN-α production in lung cancer cell lines. (A): After LLC cells were exposed to 0.1 Gy, 0.5 Gy, 1 Gy and 5 Gy doses for 24 hours, we detected IFN-α gene expression by PCR and found that IFN-α gene was upregulated by 0.1 Gy of low-dose radiation; (B): After LLC cells were exposed to 0.1 Gy, 0.5 Gy, 1 Gy and 5 Gy doses for 24 hours, we detected IFN-α protein by Western blot and found that the low dose radiation of 0.1 Gy promoted IFN-α protein expression.

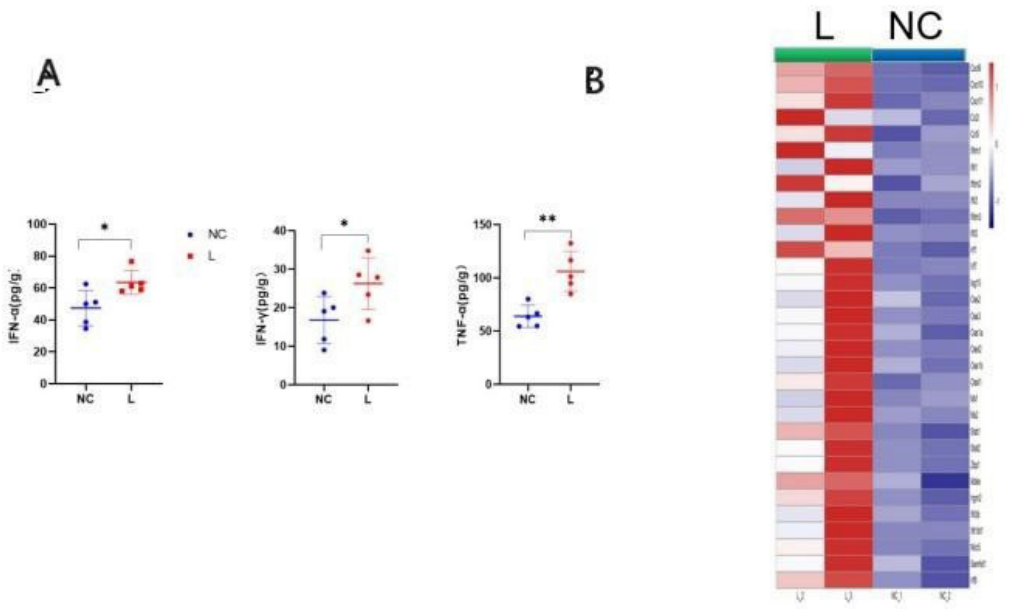

Figure 3: Low-dose radiotherapy increases the level of IFN in tumor tissues. (A): Based on RNA sequencing of tumor tissues from radio-treated mice, we discovered that IFN-related genes could be upregulated when exposed to a low dose of 0.1 Gy. (B): By using an ELISA to detect the amount of IFN in tumor tissues, we also discovered that IFN-γ, IFN-α, and TNF-α levels were increased in tumor tissues following low-dose irradiation, with TNF showing the most significant statistical difference ($p<0.01$).

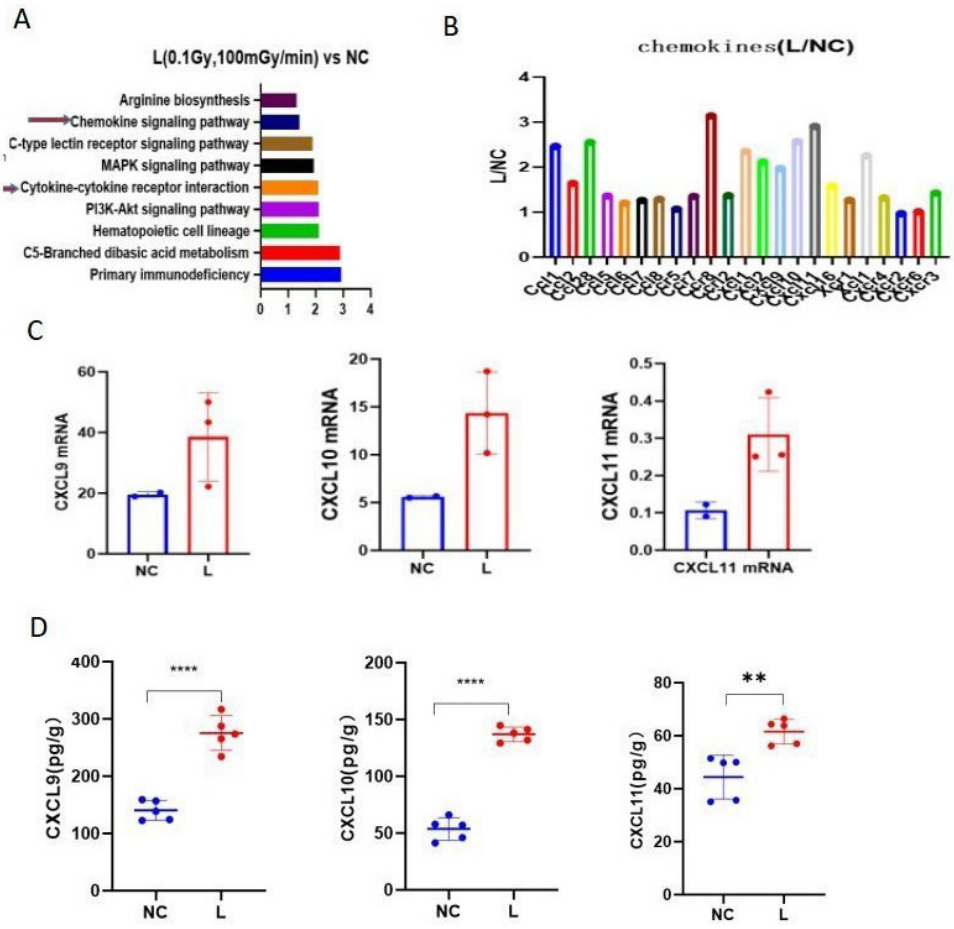

Figure 4: Low-dose radiotherapy increases the level of chemokines in tumor tissues. (A-C): By using RAN sequencing analysis, we discovered that chemokine-related genes were significantly elevated following whole-body low-dose irradiation, with *CXCL11* and *CXCL10* being the most dramatically increased; (D): The levels of *CXCL9*, *CXCL10* and *CXCL11* in tumor tissues were likewise found to be significantly higher following low-dose irradiation, and the difference showed statistical significance ($p<0.01$) in our subsequent ELISA measurements.

Low-dose radiation boosted CD8T cell infiltration in tumor tissue

Additionally, we discovered that systemic low-dose radiation boosted CD8T cell infiltration in tumor tissue. We used multicolor flow cytometry analysis to collect immune cells from tumor tissue, and we discovered an increase in CD8T cells as shown in Figure 5A, particularly IFN-γ^+ CD8T cells as shown in Figure 5B, in tumor tissues after low-dose radiotherapy. The rise in CD8T cells in the tumor tissue following low-dose radiation was further confirmed by immunohistochemistry as shown in Figure 5D, and the GZMB$^+$ cells that infiltrate tumors were also dramatically enhanced as shown in Figure 5E. On the other hand, immunosuppressive Treg cells are decreased in the tumor tissue by systemic low-dose radiation as shown in Figure 5C. By using multicolor flow cytometry and immunohistochemistry as shown in Figures 5C,5D,5E,and 5F, we discovered a drop in Treg in tumor tissue following low-dose radiotherapy. We also discovered a decrease in FOXP3$^+$Treg cells in tumor tissue following low-dose radiotherapy as shown in Figure 5F. Low-dose radiation administered throughout the body enhances the tumor microenvironment. We used CTLA-4 to lower the threshold of T-cell activation to improve immunity as shown in Figure 5A LDR combined with CTLA-4 application promotes tumor tissue IFN-γ^+ T cell infiltration ($p<0.01$) as shown in Figure 5B. The same structure was obtained in experiments performed by multicolor flow cytometry ($p<0.01$) as shown in Figure 5E.

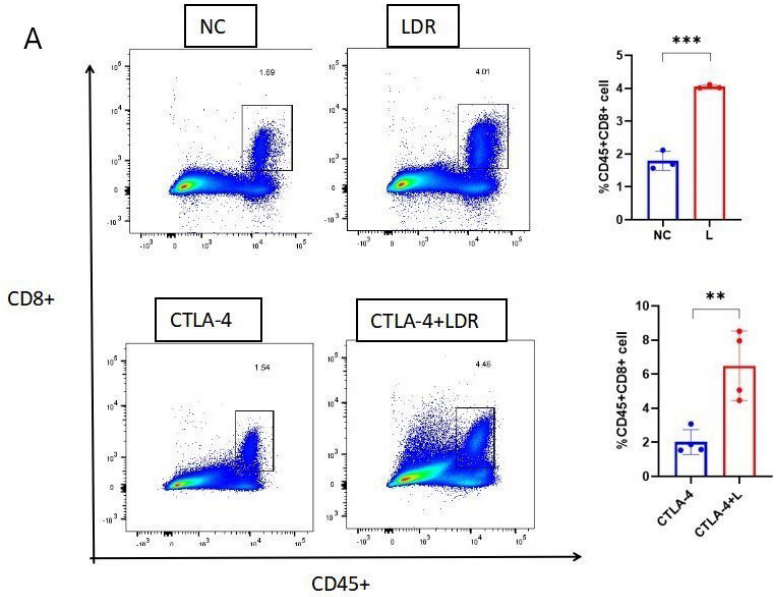

Figure 5A: We used multicolor flow cytometry to analyze the immune cells in the collected tumor tissues and found an increase in CD8T cells in the tumor tissues after low-dose radiotherapy ($p<0.01$).

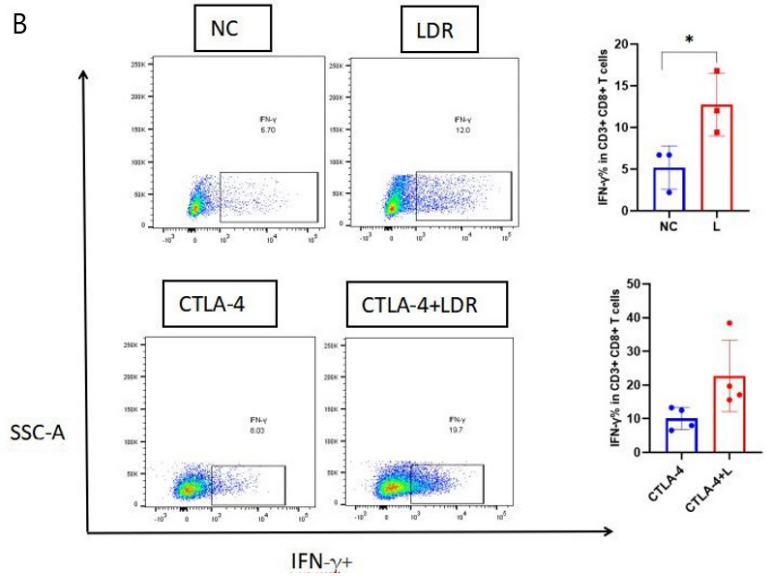

Figure 5B: We used multicolor flow cytometry to analyze the immune cells in the collected tumor tissues and found that the proportion of IFN-γ^+ CD8T cells as a percentage of CD8T cells increased ($p<0.05$).

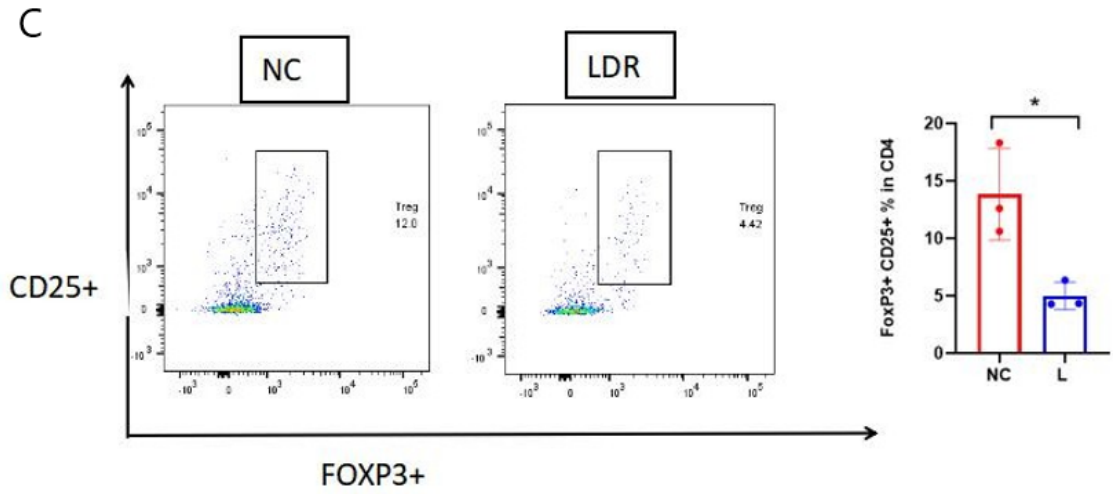

Figure 5C: Systemic low-dose radiation was found to result in a decrease of immunosuppressive Treg cells in tumor tissues by multicolor flow cytometry ($p<0.05$).

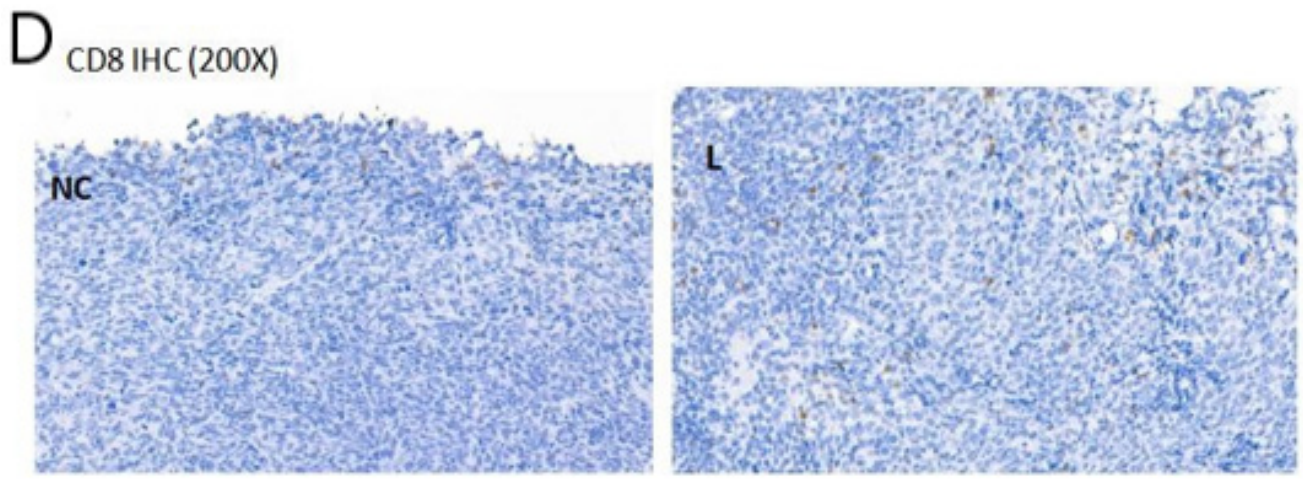

Figure 5D: Immunohistochemistry further confirmed the increase of CD8T cells in tumor tissues after low-dose radiotherapy ($p<0.01$).

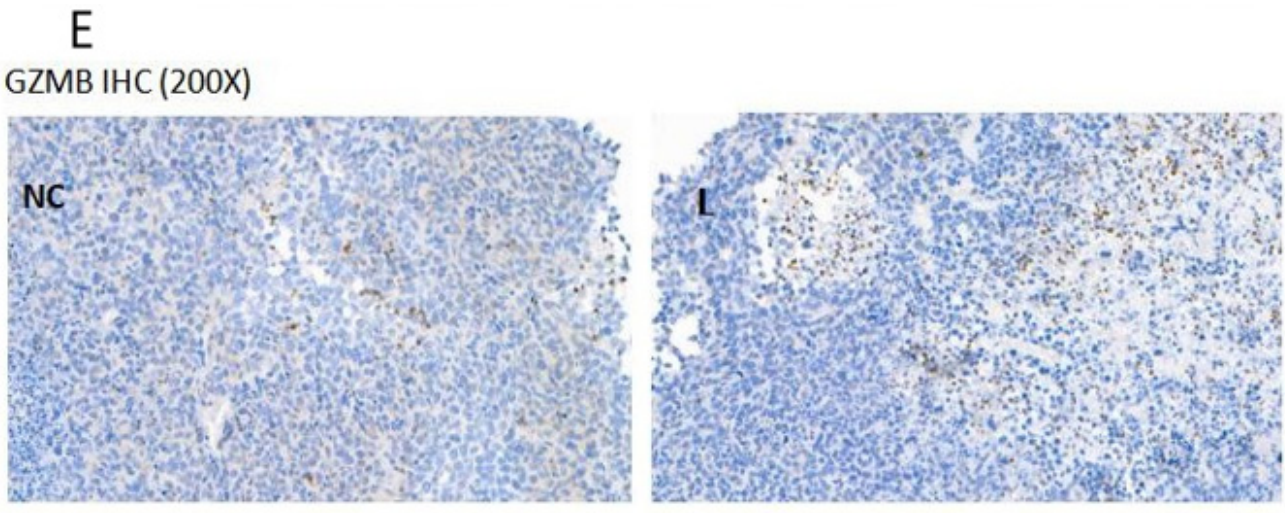

Figure 5E: Immunohistochemistry further confirmed a significant increase in tumor-infiltrating $GZMB^+$ cells in tumor tissues after low-dose radiotherapy as well ($p<0.01$).

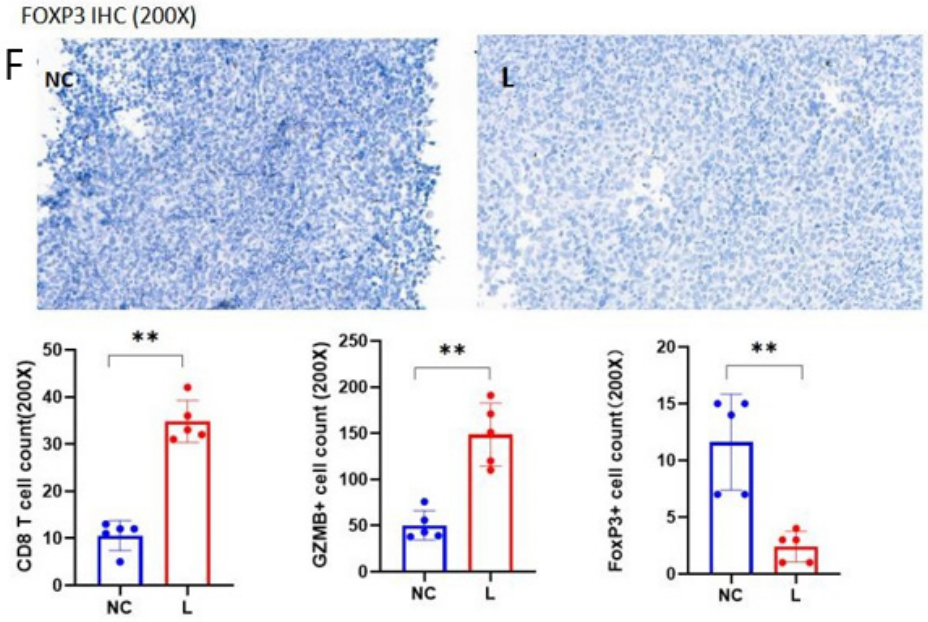

Figure 5F: A decrease in FOXP3$^+$ cells was found in tumor tissues after low-dose radiotherapy by immunohistochemistry ($p<0.01$).

DISCUSSION

The introduction of Low-Dose Radiation Therapy (LDRT) as an immunomodulatory tool with the potential to impact a broad spectrum of diseases, not limited to cancer, presents a promising avenue for medical research [14,18-23]. Beyond its known role in cancer treatment, there's growing evidence that LDRT can modulate immune responses, which may be particularly relevant in non-oncological conditions like COVID-19. By stimulating the immune system, LDRT has shown potential in enhancing therapeutic outcomes [14,18-23].

LDR has been shown to affect critical immune pathways, notably the *CXCR3* ligand axis, which encompasses *CXCL9*, *CXCL10* and *CXCL11*. These chemokines are vital for drawing immune cells that express the *CXCR3* receptor, such as Th1 cells, CD8$^+$ T cells, and NK cells, into the tumor microenvironment. These cells are essential components of the immune system's tumor-fighting response. Research has indicated that administering *CXCL9*, *CXCL10* and *CXCL11* directly into tumors can successfully recruit these immune cells, leading to a reduction in tumor growth across several cancer models [24-26]. Furthermore, a correlation has been observed between the heightened presence of these chemokines in tumor tissues and improved clinical outcomes for cancer patients, underscoring their significance in combating tumors [24,26]. It has also been discovered that LDR can amplify the expression of these chemokines, potentially enhancing the infiltration of effector T-cells into the tumor environment, which improves the local immune context and may result in the diminution of tumor development [27].

Moreover, the impact of low-dose whole-body irradiation extends beyond its direct killing effect on tumor cells. It possesses an extraordinary capacity to initiate anti-cancer responses from both the innate and adaptive branches of the immune system. This encompasses the stimulation of various immune cells, including T cells, B cells, NK cells, and macrophages, and concurrently diminishes the number of Treg cells that suppress immune responses, all within the tumor's microenvironment [28,29].

Studies on low-dose radiotherapy have highlighted the significance of dosage and dose rate. The selection of radiation dosage in the range of 1 Gy to 2 Gy, primarily targeting metastatic sites, has been shown to increase effector T-cell infiltration, facilitate M1 macrophage polarization, promote NK cell infiltration, and reduce TGF-β levels [30]. Doses of 0.5 Gy to 1 Gy can irradiate larger areas of the body, enhancing anti-tumor immune responses. Clinical trials using ultra-low levels of ionizing radiation (0.1-0.2 Gy) have demonstrated remission rates and side effect profiles comparable to or better than other systemic anti-tumor modalities, underlining the effectiveness of whole-body low-dose radiation [27-31].

A crucial element in the immune system's response to low-dose irradiation is Interferon (IFN) production. Interferon (IFN), a versatile cytokine, is pivotal in the control of a myriad of biological processes, such as cellular growth, both branches of the immune system-innate and adaptive-and the formation of new blood vessels. The release of IFN has been connected to the application of low-dose radiation, with its generation being dependent on the level and pace of the radiation dose [31]. Subsequently, IFN affects the levels of certain chemokines, including *CXCL9*,*CXCL10* [23-27], which are responsible for attracting immune cells, notably CD8$^+$ T cells cells, to the tumor site, contributing to the suppression of tumor growth [32,33]. The intricate feedback loop between IFN and immune cell recruitment underscores the critical role of IFN in tumor immunology.

Our findings suggest that LDRT, through its modulation of IFN production, can restore the equilibrium between the immune system and tumor cells within the TAIS, a balance often disrupted during cancer development [34]. By enhancing the immune milieu, particularly by increasing the recruitment of immune effector cells through chemokines, LDRT has the potential to

shift the tais back into an "elimination" phase, where immune-mediated tumor control can be restored.

Furthermore, combining LDRT with other therapies, such as immunotherapy or chemotherapy, offers an intriguing approach. Several clinical studies have shown promising outcomes when LDRT is combined with other treatments [35-40]. In our study, the combination of systemic low-dose radiation with a CTLA-4 checkpoint inhibitor had a synergistic anti-tumor effect in mouse model. This suggests that combination therapies have potential.

CONCLUSION

LDRT's immunomodulatory effects through IFN induction, coupled with the recruitment of immune effector cells *via* chemokines, hold promise for enhancing anti-tumor immune responses and potentially extending LDRT's applications beyond cancer, but this requires further studies to fully elucidate the effects of LDRT on immune dynamics.

This study provides evidence to support the potential of LDR as an immunomodulatory tool in the treatment of a variety of diseases, not limited to cancer. By restoring immune-tumor equilibrium, LDRT offers a novel approach to reinvigorate immune-mediated tumor control, providing renewed hope for patients battling various malignancies and other diseases.

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