ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2023; 22(2):163-171.
DOI: 10.18502/ijaai.v22i2.12677

Evaluation of Molecular-level Changes of Programmed Cell Death Ligand-1 after Radiation Therapy in a BALB/c CT26 Colorectal Mouse Tumor Model

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Received: 2 May 2022; Received in revised form: 29 January 2023; Accepted: 15 February 2023

ABSTRACT

The effects of radiation therapy (RT) for cancer can be systemic and partially mediated by the immune system. However, radiation alone is unlikely to transform an immunosuppressive environment into an immunostimulatory one. Therefore, an effective combination of RT and immunotherapy may provide a new, more efficient treatment approach. Here, we investigated how the expression of programmed cell death-ligand 1 (PD-L1) in the tumor microenvironment varied in different RT regimens with the same biologically effective dose.

In this study, female BALB/c mice inoculated with CT26 tumor cells were irradiated with 3 different RT regimens using the same BED of 40 gray (Gy). These included ablative RT (1*15 Gy), hypo-fractionated RT (2*10 Gy), and conventional (Hyper-fractionated) RT (10*3 Gy). PD-L1 expression was analyzed with immunohistochemical staining on days 2 and 20 and when the size of tumors had reached 2 cm^2 after RT.

All treated groups expressed PD-L1, but the group receiving single ablative high-dose RT showed higher expression compared to the other groups. No significant differences in PD-L1 expression were observed at different times in the same group.

These findings showed that different regimens of RT have different effects on the TME, so a combination of RT and immune checkpoint blockade could be clinically used in cancer patients.

Keywords: Immunohistochemistry; Programmed cell death 1 ligand 1 protein; Radio-frequency therapy; Tumor microenvironments

INTRODUCTION

Radiation therapy (RT) is one of the major therapeutic modalities used in the treatment of locally advanced solid tumors and is used to treat about 50% of all cancer patients. Also, 40% of patients who show improvements continue to use it to manage their disease.

For some time, research into improving the results of RT focused on the cancer cell itself, regardless of the complex interaction between the cancer cell and the tumor microenvironment (TME). Over the last two decades, radiation therapists have moved towards considering the effects of TME. RT works in various ways, including direct destruction of tumor cells, modulation of the TME, and stimulation of the host immune response against the tumor cells.[1–3] RT can exert a wide range of cytotoxic effects against tumors,

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which at the molecular level cause unrepairable DNA strand breaks that ultimately lead to cellular senescence and apoptosis. Moreover, RT causes a specific type of cell death called immunogenic cell death (ICD), which is characterized by the release of danger signals, also known as damage-associated molecular patterns (DAMPs). DAMPs that occur due to ICD after RT include cell surface exposure of calreticulin (CRT), the extracellular release of ATP, and high mobility group box 1 (HMGB1).[4]

Since RT can have both stimulatory and inhibitory effects on the immune system, it should be combined with immunotherapy (IT) to activate the immune system and overcome any immunosuppressive effects it may have. Some preclinical and clinical studies have been performed using this combination, and some good results have been obtained.[5] We know that TME components are involved in both tumor progression and treatment response;[6] as such, a better understanding of the changes in TME following RT will help us to design improved therapeutic combinations.

Programmed death-ligand 1 (PD-L1) is one of the molecules that inhibit the activity of immune cells within the TME. Upon interaction with the programmed death 1 (PD-1) receptor expressed on cytotoxic T cells, it plays an essential role in decreasing their attack against the tumor cells. Therefore, PD-L1 seems to be a good target for combining IT with other cancer treatments, such as RT.[7,8] Mimura et al, showed that PD-L1 expression was positively correlated with the presence of cytotoxic lymphocytes (CTLs) in the stroma and IFN-γ expression in the tumor, and that expression of PD-L1 was a key limiting factor for CTL activity.[9] Accordingly, in radiobiology, the concept of biologically effective dose (BED) based on a linear quadratic (LQ) model indicates the biological effect of RT treatment according to changes in the dose per fraction. Therefore, the BED describes the cell response to RT and is used to compare different treatment regimens.[10]

In our previous study,[11] when an anti-PD-L1 antibody was combined with high single-dose RT, we observed an increase in IFN-γ and tumor-infiltrating CTLs in the tumor, resulting in increased antitumor activity. As a result, the combination therapy resulted in effective tumor control and increased survival, and consequently, no recurrence of the tumor was observed. Dovedi et al, showed that tumor cell expression of PD-L1 was increased after hyper-fractionated RT.[12] Studies have shown that combining RT with immune checkpoint inhibitors promotes localized tumor control and the release of tumor antigens that activate the immune system.[13] In addition, to improve the treatment results, the optimal radiation dose in each session should be considered.[14,15]

Therefore, as a continuation of our previous study and using the same samples, and considering that PD-L1 expression is dependent on IFN-γ production, in this study, we examined the expression of PD-L1 and IFN-γ after RT with various doses and fractionation in a BALB/c mouse CT26 tumor model. We euthanized the mice at day 2, day 20, and when the size of the tumors reached $2cm^2$ after RT. The difference in PD-L1 expression after RT regimens with the same BED allowed the optimal therapeutic dose to be determined.

MATERIALS AND METHODS

Cell Line

We used the CT26 cell line, which is a mouse epithelial colon adenocarcinoma line. To culture CT26 cells, RPMI-1640 medium was used with 100 U/mL penicillin, 100 μg/mL streptomycin, 20% heat-inactivated fetal bovine serum (FBS), and 1% L-glutamine. All cells were maintained at 37°C in 5% CO_2. These cells were also free from mycoplasma infection.

Mouse Model

BALB/c mice, 5-6 weeks old, were purchased from Pasteur Institute of Iran, Tehran, Iran, and kept in standard conditions with a 12-hour light/dark cycle and free access to food and water.

All experiments on animals, as well as their care, were performed with the approval of the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Tumor Induction

For induction, 1×10^6 CT26 tumor cells were injected subcutaneously into the right flank of the anesthetized mice 43.[16]

Radiation Treatment Program

The BED equivalent of 40 Gy was delivered with 3 different radiation therapy regimens, including ablative (15 Gy×1), hypo-fractionated (10 Gy×2), and hyper-fractionated (3 Gy×10); the dose in each regimen was

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calculated based on LQ models. The BED of the 3 radiation regimens included the single dose receiving 15 Gy, 2 fractions receiving 10 Gy per fraction, and 10 fractions receiving 3 Gy per fraction (approximately 40 Gy). The control group did not receive RT.

Fourteen days after tumor cell injection, the mice were randomly divided into 3 treatment groups (each group consisting of at least 12 mice) plus 1 group as control. Eighteen days after the initial inoculation, when the size of the tumors reached at least 300 to 400 mm^3, all groups except the control group started the irradiation. [11,17] The ablative RT regimen was a single fraction of 15 Gy on day 18, the hypo-fractionated RT regimen was 2 fractions of 10 Gy delivered on days 18 and 28, and the conventional (hyper-fractionated) RT regimen was 10 fractions of 3 Gy delivered daily from day 18 to day 31 as shown in Figure 1.

Mice were anesthetized by intraperitoneal injection with ketamine (100 mg/kg) and xylazine (12.5 mg/kg). They were then treated with radiation using a linear clinical accelerator (6 MV photons, Elekta Synergy linear accelerator, Stockholm, SE), with ethical considerations taken to minimize their suffering. The mice were placed in a modified 50 mL plastic tube so that only the tumor area would be irradiated. Other body parts were protected by a 9-cm–thick lead plate. RT was delivered to a 3×3 cm^2 field with 5-mm margins at 350 Gy/min with 6 MV X-rays using tangential beam delivery. A layer of 1.5-cm Superflab Bolus Material was placed on the tumor, and the radiation source was adjusted to a distance of 100 cm from the skin.

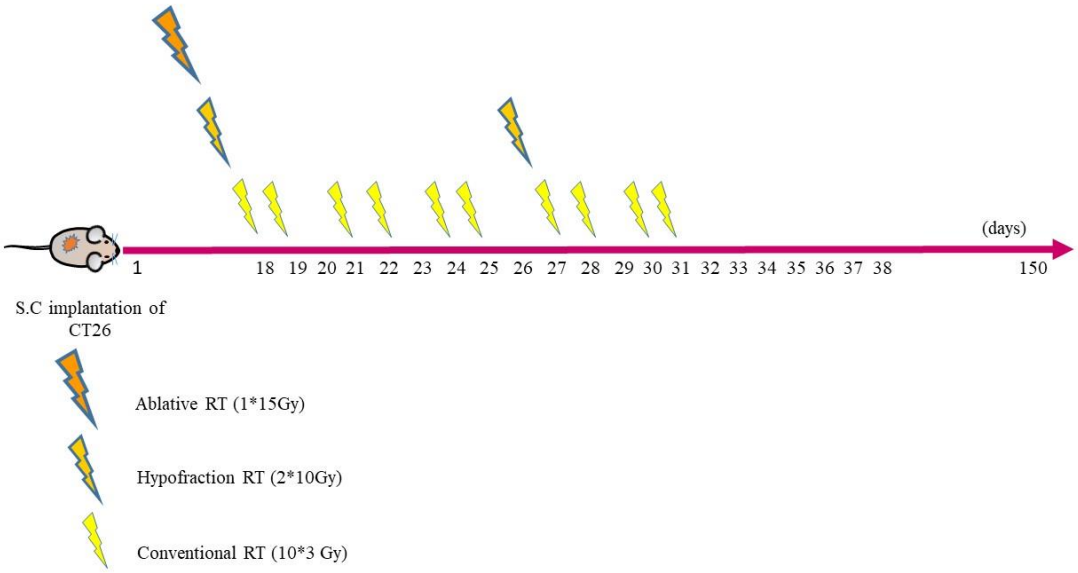

Figure 1. The experimental schedule of radiation therapy in mice.

The tumor tissues were isolated from the mice after they were euthanized (by cervical dislocation) at the end of the treatment period. The euthanasia was performed at different time points: 2 days, 20 days, and when the tumor size reached 2 cm^2. The tissue was divided into 2 parts: one part was prepared for flow cytometry analysis, while the remaining part was immediately fixed in formalin. After the fixation process, the tumor tissues were embedded into paraffin wax blocks.

Flow Cytometry Analysis

Isolated tumor tissues were digested with type I collagenase (Sigma Aldrich C0130, Massachusetts US) in Roswell Park Memorial Institute (RPMI-1640) (1:1 ratio) and incubated at 37°C for 2 hours. The cell mixture was filtered through a 70-µm cell strainer and centrifuged at 1,500 rpm for 10 min. These cells were stained by specific antibodies. Cells were suspended in a flow cytometry staining buffer containing PBS + 5% FBS.

They were analyzed using flow cytometry (BD FACS Calibur flow cytometer, Becton Dickinson, USA) using fluorochrome-labeled antibodies against programmed cell death- ligand 1 (PD-L1) (Biolegend, 155404), IFN-γ (clone XMG1.2), CD8 (clone 53–6.7) and an IgG1 isotype control (clone RTK2071) (Biolegend, San Diego, California). As CD8 is a surface antigen, a fluorescent antibody was added to the cells, followed by 30 minutes of incubation at 4°C, and they were washed twice with a staining buffer. IFN-γ is an intracellular marker, so cells were fixed and permeablized by True-Nuclear Transcription Factor Buffer Set (Biolegend, San Diego, California) kit before adding the fluorescent antibody. After reading the samples by flow cytometry, the results were analyzed with FlowJo software version 7.6.1.[18]

PD-L1 Analysis

Tumor sections (4 μm thick) were cut from formalin-fixed, paraffin-embedded tumor blocks and mounted on positively charged glass slides. For the deparaffinization step, tumor slides were placed twice in xylene for 10 minutes at room temperature. Subsequently, sections were successively immersed in absolute ethanol, 90% ethanol, 70% ethanol, and 50% ethanol, followed by one rinse with water. For antigen retrieval, sections were incubated with Tris-EDTA buffer (pH 9.0) for 30 minutes at 95°C and then cooled down for 40 minutes. Endogenous peroxidase and proteins were blocked. Slides were incubated with a primary antibody against PD-L1 (ab233482 anti-mouse IgG1, Cambridge, UK) for 1 hour at a concentration of 10 μg/mL according to a standard protocol.

After washing in tris-buffered saline (TBS), the secondary antibody (envision Dako, K4006, California, US) that was conjugated with HRP was applied for 30 minutes at room temperature. After each incubation step, the slides were washed with TBS, incubated in substrate solution for 5 minutes, and washed with distilled water. They were then counterstained with hematoxylin for 1 minute. Finally, they were dehydrated in graded ethanol, cleared in xylene, mounted on a coverslip, and examined with a light microscope.

The staining results were interpreted by experienced pathologists who were blinded to the groups. Cancer cells that showed membranous and cytoplasmic staining for PD-L1 were considered positive cells.

The samples were classified based on the percentage of tumor cells stained with any intensity in each section containing approximately 100 tumor cells. The classification was 1+ for 1–5%, 2+ for 5–10%, and 3+ for 10–20%.

Tonsil and placenta tissues were stained as negative and positive controls with and without primary antibodies.

Statistical Analysis

Descriptive statistics were analyzed using the independent t-test and the post hoc test for one-way analysis of variance (ANOVA) carried out by GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Evaluation of PD-L1 Expression in CT26 Tumor Cells in Control Group

In the first step, the tumor tissues of the control group of mice that did not receive radiation therapy were assessed for PD-L1 expression both by fluorescence-activated cell sorting (FACS) and immunohistochemistry (IHC). After tumor induction in mice, the tumors were isolated and examined for PD-L1 expression, which was confirmed by the median fluorescence intensity (MFI) value in the FACS histogram (Figure 2A) and also by IHC staining of sections (Figure 2B).

Interferon-gamma (IFN-γ) Expression in the Ablative Radiation Therapy Group Showed the Highest MFI of IFN-γ Compared to the other RT Groups

The histogram of IFN-γ expression showed an increased MFI in the ablative radiation therapy group, with a 4.43-fold increase in IFN-γ expression in immune cells within the tumor tissue compared to the unstained control. In the other two regimens, there was a relative decrease in IFN-γ expression between regimens with the same BED, but it was not statistically significant (Figure 3).

$CD8^+IFN\text{-}\gamma^+$ Cells Were Higher in the Ablative Treatment Group than in the Other Groups

Because of the increase in interferon-gamma (IFN-γ) after treatment, $CD8^+IFN\text{-}\gamma^+$ cells in the tumor were examined. The ablative RT regimen increased the infiltration of $CD8^+$ cells expressing IFN-γ more than that of the hypo-fractionated and conventional regimen groups (Figure 4).

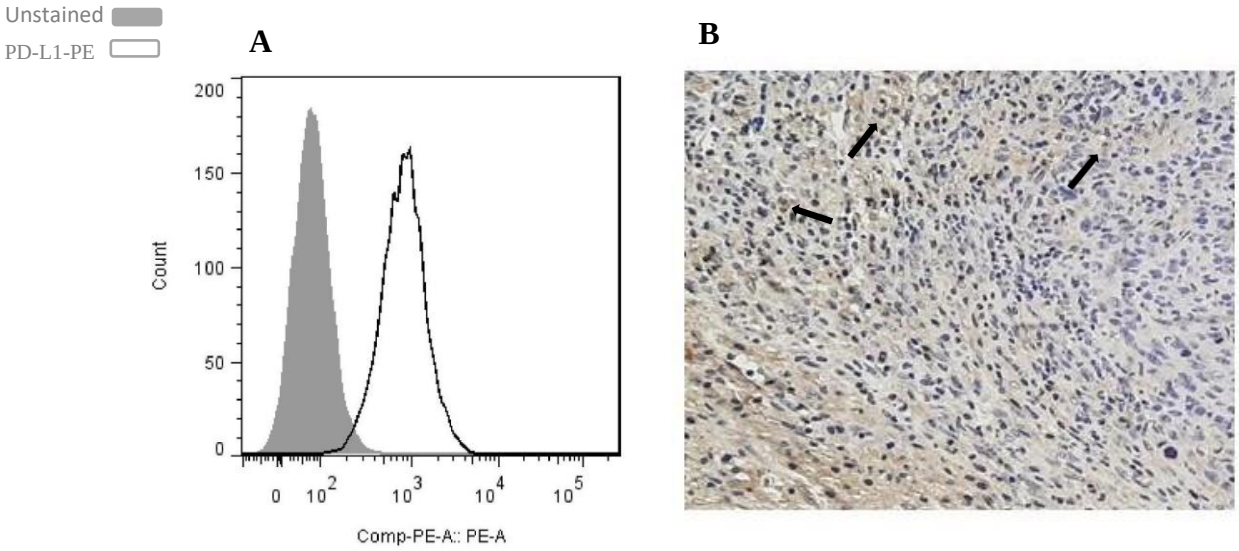

Figure 2. Analysis of programmed cell death- ligand 1 (PD-L1) expression at baseline. A: MFI in the FACS histogram of CT26 tumor tissues showing PD-L1 expression on CT26 cells. B: CT26 tumor tissues stained with immunohistochemistry to detect PD-L1.

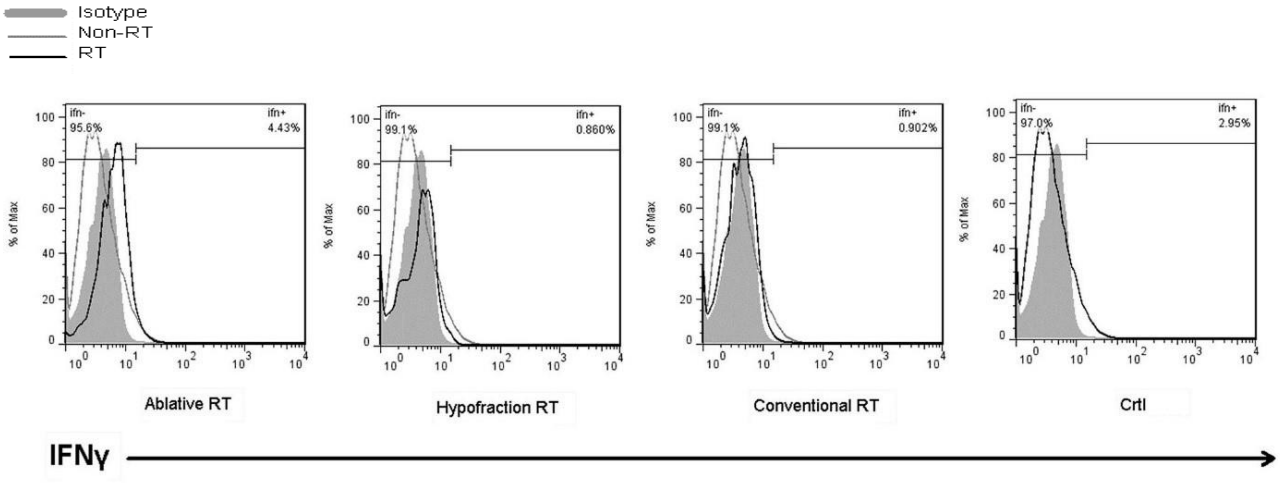

Figure 3. Altered interferon-gamma (IFN-γ) expression following treatment with different radiation therapy regimens. The histogram of IFN-γ expression shows a shift to the right on the x-axis, indicating an increase in IFN-γ expression in immune cells within the tumor tissue in the ablative group that received a single dose (15 Gy), the hypo-fractionated group that received two fractions (10 Gy per fraction), and the hyperfraction group that received 10 fractions (3 Gy per fraction). The control group did not receive RT.

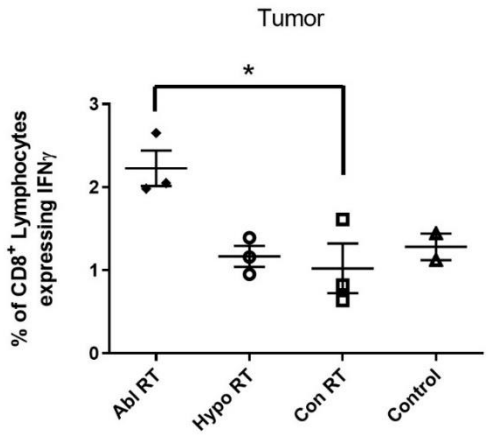

Figure 4. Percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells infiltrated in tumor tissues. The ablative radiation therapy regimen increased the infiltration of $CD8^{+}$ cells expressing IFN-γ more than that of the hypo-fractionated and conventional regimen groups ($p<0.05$). IFN-γ, interferon-gamma.

Ablative RT Showed Higher PD-L1 Expression Compared to Other RT Regimens with the Same BED

After confirming the expression of PD-L1 on the surface of CT26 tumor cells, radiation therapy-treated tumor tissues were examined for this marker. The results of the analysis showed that the CT26 tumor tissues of mice who received the ablative RT regimen had 3+ expression, with 10 to 20% of cells showing cytoplasmic and membranous staining, while in the hypo-fractionated group, 5 to 10% of tumor cells showed membranous and cytoplasmic staining with a score of 2+. In the conventional RT group, only 1% to 5% of tumor cells showed staining with a score of 1+ (Figure 5).

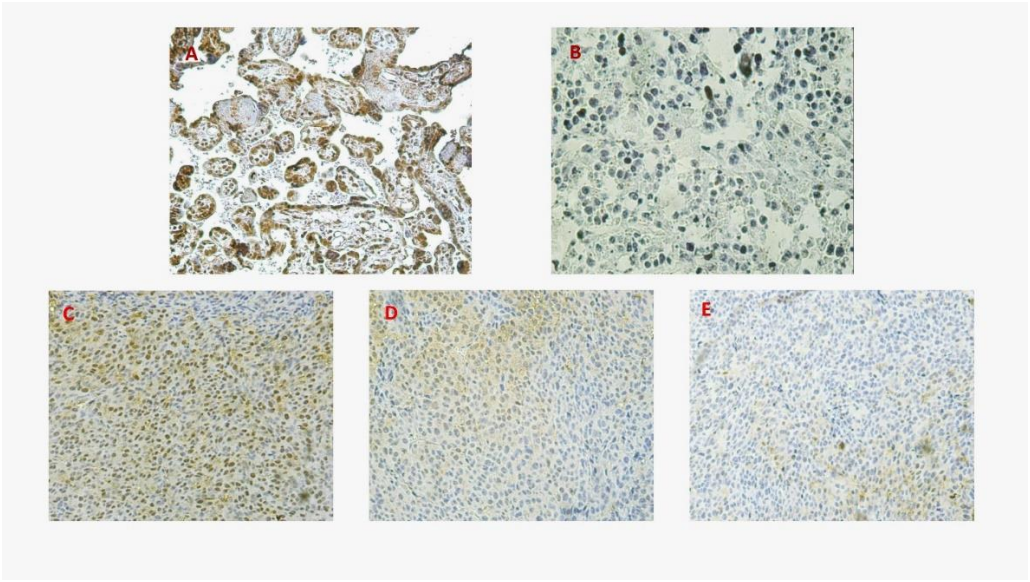

Figure 5. programmed cell death-ligand 1 (PD-L1) immunohistochemical staining in CT26 tumor tissues of BALB/c mice after radiation therapy showed different levels of PD-L1 expression. A: placenta tissue as a positive control. B: Negative controls consisted of tonsil tissue stained without primary antibodies. C: Tumor tissues treated with ablative RT showed PD-L1 3+ expression. D: Tumor tissues from mice treated with hypo-fractionated RT showed PD-L1 2+ expression. E: Tumor tissue treated with conventional RT showed PD-L1 1+ expression.

Moreover, we analyzed and compared the expression of PD-L1 in tumor tissues sampled at different times: after 2 days, 20 days, and when the size of the tumors had reached 2 cm^2. We observed that there were no significant differences between the time points.

Overall, we showed that the high expression of PD-L1 after ablative RT treatment could be associated with a high expression of IFN-γ in this group. The results of our study showed that the expression of IFN-γ in the ablative group has the highest MFI. At the same time, in the IHC analysis, we observed that the expression of PD-L1 in the ablative group also had the highest expression.

DISCUSSION

Considering that no study has examined programmed cell death-ligand 1 (PD-L1) expression after different radiation therapy regimens so far, in this study, we measured the expression of PD-L1 after 3 different RT regimens with the same BED so that we could make a rational decision on appropriate combination therapy. Like other studies in this field, our study confirmed that RT can affect the immune system and alter the tumor microenvironment.

Also, we showed that each type of treatment regimen induces different effects, although the BED of these 3 treatment regimens was the same. Our findings showed significantly greater expression of PD-L1 after ablative RT than hypo-fractionated or conventional RT. However, there was no significant difference between the expression of PD-L1 at different times, which could be confirmed by increasing the number of samples. These findings are consistent with previous studies found that PD-L1 expression is correlated with IFN-γ production and is important for IT based on blockade of the PD-1/PD-L1 axis.[19,20]

Dovedi et al, showed that the treatment of tumor cells with a range of RT doses in vitro did not have a direct effect on PD-L1 expression. Furthermore, they suggested that $CD8^+$ cells produce IFN-γ and are responsible for upregulating PD-L1 expression in tumor cells following RT.[12]

On the other hand, several studies have shown that RT can induce the activation of anti-inflammatory pathways. For instance, the secretion of the immunosuppressive cytokine transforming growth factor-beta (TGF-β) was increased by localized RT with a single dose of 5 or 10 Gy.[21,22] These are just a few examples of the effects of RT on the immune system and the TME, each of which affects the progression and response of tumors to treatment. This poses many questions relevant to tumor biology and cancer treatment.

Since PD-L1 expression is not limited to tumor cells alone and is also expressed by other cells within the tumor, including dendritic cells, natural killer (NK) cells, and tumor-associated neutrophils (TANs), it is necessary to examine PD-L1 in other cells. It is clear that PD-L1 is usually expressed in tumor cells and inhibits the function of PD-1–expressing T cells. As a result, the tumor has the opportunity and ability to escape from the attack by the immune system.[23]

Deng et al, showed that PD-L1 expression was increased in dendritic cells and tumor cells after a single 12 Gy dose of RT.[24] Dong et al, found that NK cells could be divided into 2 groups based on the presence or absence of PD-L1. They suggested that PD-L1 positive NK cells had significant cytotoxicity and higher IFN-γ production compared to PD-L1 negative NK cells. They showed that an anti-PD-L1 antibody directly potentiated the anticancer effect of NK cells, independently of the expression of PD-L1 on the surface of the tumor cells. In this regard, the anti-PD-L1 monoclonal antibody can act directly on NK cells expressing PD-L1 to enhance the attack on the tumor, which may explain why anti-PD-L1 has the same therapeutic effect on tumors without any PD-L1 expression on the tumor cells.[25]

Furthermore, Wang et al, suggested that TANs present in gastric cancer tumors could directly suppress the T cell immune response by the increased PD-L1 expression induced by Granulocyte Monocyte-colony Stimulating Factor (GM-CSF).[26] In addition to PD-L1, RT can also affect Cytotoxic T-Lymphocyte- Associated protein 4 (CTLA-4) expression. Katalin Balázs et al, suggested that RT (median of 57.6 Gy delivered over a median of 44 days) induced a strong increase in CTLA-4 levels on $CD4^+$ T cells measured in head and neck carcinoma patients.[27] Therefore, in future studies, the effects of various RT regimens on the expression levels of CTLA-4 should be considered as another important inhibitory checkpoint in the TME.

Our previous study showed that after the ablative treatment regimen, the amount of IFN-γ expressing $CD8^+$ cells increased significantly compared to other treatment regimens, including hypofraction and hyperfraction, and finally, the combination of anti-PD-

L1 therapy with ablative RT has had the best results in inhibiting tumor growth.[11] On the other hand, several previous studies in animal models, as well as the result of our present study, have shown a link between increased PD-L1 expression and the increased production of IFN-γ.

However, to date, there has been only limited information on humans. The question of how exactly PD-L1 expression is increased at the tumor cell surface and in the TME after RT has not been clearly answered. On the other hand, it is known that IFN-γ can have a dual effect on the antitumor immune response because it can induce the expression of both PD-L1 and HLA class I simultaneously. Therefore, the antitumor activity of CTL could depend on the balance between the inhibitory effect of PD-L1 and the stimulatory effects of HLA-I, both induced by IFN-γ.[9] As mentioned at the beginning of the discussion, no study has been done to investigate the expression of PD-L1 after 3 different RT regimens with the same BED. This study aimed to introduce the best treatment regimen in combination with anti-PD-L1 IT according to the expression level of PD-L1. Finally, our study's results could help select a suitable IT intervention (such as anti-PD-L1) to be combined with a single high dose of RT to achieve the best therapeutic outcome. However, more extensive studies are needed to examine the effects of RT on other cells and molecules involved in the antitumor immune response in the TME.

STATEMENT OF ETHICS

All experiments on animals, as well as their care, were performed with the approval of the Ethics Committee of Shahid Beheshti University of Medical Sciences (approval number: IR.SBMU.MSP.REC.1397.616.)

FUNDING

This work has been supported by Shahid Beheshti University of Medical Sciences research grant number: 18162.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

This article has been extracted from the thesis written by Ms. Fateme Khani Chamani, School of Medicine, Shahid Beheshti University of Medical Sciences.

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