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Radiation Triggered Phenotypic Changes of Regulatory T Cells

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Abstract: Ionizing radiation disrupts the immune balance easily. Regulatory T (Treg) cells directly affect the immune balance after radiation exposure. Meanwhile, the present study is to investigate the survival ability and phenotypic changes of Treg cells induced by ^{60}Co γ-rays to expand the understanding of how Treg cells are modulated by radiation. Mice lymphocytes were isolated from the thymus and spleen at 1, 4, and 10 days after 2 Gy ^{60}Co γ-rays irradiation. The proliferation and phenotype of Treg cells were analyzed by flow cytometry. Helio$^+$ Treg cells were calculated and ATP luminescence was measured by ELISA. CD39 from the thymus and spleen was measured by flow cytometry. Moreover, miR-31 expression in the thymus and spleen post-irradiation was determined by RT-PCR. The apoptosis rate of Treg cells was increased to 13.1% in the thymus and 44.2% in the spleen after 2Gy irradiation. The proliferation of Treg cells in the thymus decreased from 31.6 to 14.2% post-irradiation ($t = 3.56, 5.72, P<0.05$). The percentage of Helio$^+$ Treg cells increased and the mean fluorescence intensity of Helio in Treg cells increased from 5,677.7 to 6,529 in the thymus and increased from 3,968.7 to 4,558.7 in the spleen. The ratio of CD39$^+$ Treg in the thymus was significantly increasing from 43.2 to 83.8 after 10 days of radiation ($t = -18.29, P<0.05$). ATP released by Treg cells was down-regulated after radiation (0.15 μmol/L, $t = 31.98, p<0.05$). Furthermore, miR-31 expression in CD4$^+$T cells significantly decreased after 2 Gy radiation in the thymus. This study indicates the relationship between radiation-induced Treg cell apoptosis and phenotype changes in vivo and provided a theoretical basis for the prevention and treatment of radiation-induced immune imbalance. Our study offered a new idea for radiotherapy combined with immunotherapy by target-regulating CD39 and miR-31.

Keywords: Regulatory T Cell, Ionizing Radiation, microRNA, miR-31, Immune Imbalance

Introduction

Inflammation function depends on the dynamic change between lymphocyte subsets. Whole-body irradiation dramatically disturbs the lymphocyte pool. Regulatory T cells (Treg) play an important role in immune balance (McCulloch *et al*., 2017; Sakaguchi, 2006). Ionizing radiation is an important factor that induces acute and chronic inflammation. A recent study showed the critical role of Treg cells in radiation-induced lung injury and indicated radiotherapy combined with Treg-targeting immunotherapy offers beneficial outcomes in lung tissue protection (Guo *et al*., 2020). Moreover, the characteristics of Treg cells can be used to assess the risk of the cancer patient who is receiving ionizing radiation (Gong *et al*., 2020).

Different subsets of lymphocytes have different sensitivities to irradiation (Park *et al*., 2000; Qu *et al*., 2010). The proportion of CD4$^+$CD25$^+$ Treg is about 5–10% of peripheral CD4$^+$ T cells in humans or mice. Moreover, the CD4$^+$CD25$^+$ Treg were also enriched in various cancers which reduced the killing effect of radiation (Oweida *et al*., 2019; Hou *et al*., 2020). Reports show that the radiation sensitivity of CD4$^+$CD25$^+$ Treg cells differed from that of CD4$^+$CD25$^-$ T effector cells in vivo (Qu *et al*., 2010; Liu *et al*., 2010). Also, it has been reported that the phenotype and proportion of Treg cells changes after local tumor irradiation or whole-body irradiation (Cao *et al*., 2007).

However, the change in the proportion of CD4$^+$CD25$^+$ Treg cells owing to the effect of ionizing irradiation on peripheral T cells or thymus function remains unclear.

Significant cell damage and ATP release outside of the cell are observed after irradiation. It is speculated that ATP is an important inflammatory response factor that

mediates radiation-induced Treg cell apoptosis. Moreover, research reports showed ATP induces a pro-inflammatory response (Mariathasan and Monack, 2007). A high ATP concentration has toxic side effects that were reported on T cells in mice (Borsellino *et al*., 2007). Also, mouse Treg cells are particularly sensitive to ATP. Meanwhile, Treg cells highly express CD39, which helps in coping with the harm caused by increased ATP concentration. CD39 expression and ATP concentration were up-regulated in Treg cells after radiation exposure.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally repress the expression of target genes (Borsellino *et al*., 2007). Many findings showed that different microRNAs control the phenotype and function of Treg cells in different microenvironments (Shukla *et al*., 2011; Liston *et al*., 2008; Lu *et al*., 2010; McCulloch *et al*., 2017; Park *et al*., 2000; Qu *et al*., 2010a). The microRNA-31 (miR-31) was recently identified as a pleiotropically acting tumor suppressor miRNA (Creighton *et al*., 2010; McCulloch *et al*., 2017; Qu *et al*., 2010b; Oweida *et al*., 2019). CD39 was reported as the potential target of miR-31. Thus, it is important to investigate whether the phenotype change of Treg cells is induced directly by radiation or by the large amount of ATP released owing to radiation exposure.

Besides, the conclusions of the studies are inconsistent in vivo and in vitro. In this study, we investigate the radiation response characteristics of Treg cells, and the viability and proliferation of lymphocytes and Treg cell subsets in different immune organs post-radiation exposure.

Materials and Methods

Animals

BALB/c male mice which were aged 4-6 weeks (body weight 19-21 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Animals were housed in a Specific-Pathogen-Free (SPF) environment at room temperature (20-22°C) and 50-60% relative humidity. The schematic diagram of the experiment design was showed in supplemental Fig. 1.

Irradiation Conditions

Mice were subjected to whole-body irradiation with a standard IAEA/WHO ^{60}Co γ-ray source (dose rate, 0.27 Gy/min; distance 0.85 m). The doses on the body surface were 0 Gy and 2 Gy in both the control and irradiation groups. There are 5 mice in each group. Animal testing has been approved by the Animal Ethics Committee and the approval number is C2019002.

Isolation of Mouse lymphocytes

Mice irradiated with different doses were sacrificed at 1, 4, and 10 days after exposure (5 mice in each group). The thymus and spleen were isolated and homogenized in a 1 × Phosphate-Buffered Saline (PBS). The supernatant was removed by filtration through a 300-mesh filter and centrifugation. The precipitate was resuspended in 1 × PBS containing 10% Fetal Bovine Serum (FBS) to prepare a single-cell suspension. Fluorescein Isothiocyanate (FITC) - labeled hamster anti-mouse Helios antibody (Bioscience, USA) was used to measure Helios-Treg cells. PE-Cy-labeled hamster anti-mouse Helios antibody (Bioscience, USA) was used to measure CD39-Treg cells.

Treg Cell Sorting

The single-cell suspension samples were individually placed in flow tubes (5×10^6 each sample). FITC-labeled rat anti-mouse CD4 (BD Bioscience, USA), PE-labeled rat anti-mouse CD8 (BD Bioscience, USA), and APC-labeled rat anti-mouse CD25 (BD Bioscience, USA) antibodies were added to thymus and spleen samples. The samples were incubated at 20-22°C for 30 min and then samples were loaded into the flow cytometer for sorting. Treg cells and $CD4^+CD25^-$ T (Tcon) cells were sorted separately (5×10^5 each).

Cell Proliferation Analysis

The single-cell suspension (200 μL) was placed in a flow tube. 7-AAD chemical dye or Ki-67 antibody was added to each sample and incubated at room temperature (20-22°C) for 30 min. Unbound antibodies were removed by washing with PBS at 4°C. The samples were filtered with a 400-mesh filter before loading on the flow cytometer.

Determination of Intracellular ATP Concentration

Sorted Treg cells were centrifuged at 2000 rpm for 5 min and the supernatant was discarded. ATP luminescence detection kit (Vigorous Biotechnology Beijing Co., Ltd.) was used to measure intracellular ATP concentration. Standard ATP was diluted with ddH_2O to 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 50 nmol/L, and 100 nmol/L. The cell lysate was added to 96 well plates (10 μL/well) and a 50 μL assay reagent from the kit was added to each well. The luminescence was recorded in relative luminescence units of firefly luciferase. ATP concentration was calculated based on a standard curve. Each sample was used in triplicate.

MicroRNA Extraction and Specific MicroRNA Expression Analysis (RT-PCR)

MicroRNA was extracted from $CD4^+$T cells. (1) TRIzol RNA Isolation Reagents (0.75 mL) (Thermo Fisher) were added to $CD4^+$T cells isolated from the thymus and spleen and then transferred to a 1.5 mL tube. (2) Chloroform (0.2 mL) (Thermo Fisher) was added to the sample and mixed well and the mixture was incubated at room temperature for 3 min. (3) The mixture was centrifuged at 12,000 × *g* for 15 min at 4°C. (4) The upper aqueous phase was removed and one-third of the volume of the organic phase was added to the tube and mixed well. (5) The mixture was transferred to another tube and centrifuged at 10,000 rpm for 15 s. (6) Then, 700 μL miRNA

wash solution (QIAGEN´s RNA isolation kit) was added and centrifuged at 10,000 rpm for 10 s. (7) Next, 100 µL of pre-heated (95°C) Elution Solution was added to the filter and centrifuged at 10 000 rpm for 30 s. Total microRNA concentration was determined based on the $OD_{260\ nm}$ value measured using the ultraviolet spectrophotometer. MicroRNA purity was determined based on the ratio of $A_{260\ nm}/A_{280\ nm}$ (1.8 and 2.1). MiroRNA gene expression was calculated by Comparative Ct ($2^{-\Delta\Delta C}$t) method.

Statistical Analysis

Five mice (n = 5) were included in each group. The experiments were performed in triplicate for each group. Statistical analysis was conducted using SPSS 19.0 software to perform generalized factor variance analysis on more than three sets of data. Bonferroni method was used for pairwise comparison between groups. An Independent sample of *t*-test was used to analyze two sets of data. The results are expressed as mean ± standard deviation (x ± s) and $P<0.05$ was considered statistically significant.

Results

Treg Cells Viability in Thymus After 2 Gy Exposure

To analyze Treg cell viability, thymocytes were separated followed by 2 Gy radiation immediately. The survival rate of Treg cells after 9 h of irradiation was analyzed by detecting the level of 7-AAD, which bind double-stranded DNA and thus can be used to identify viable cells. The apoptosis rate of $CD4^+CD25^+$ Treg cells and Tcon cells in the thymus was significantly higher in the experimental group (after 2 Gy exposure; 13.8 and 6.9%, respectively) than in the control group (13.1 and 4.3%, respectively; t = -4.18; $P<0.05$; Fig. 1A and supplementary Fig. 2). The apoptosis rate of $CD4^+CD25^+$ Treg cells and Tcon cells in the spleen was significantly higher in the experimental group (44.2 and 40.6%, respectively) than in the control group (43.4 and 18.7%, respectively; t = -4.18; $P<0.05$; Fig. 1B).

Treg Cell Proliferation after Exposure in Vivo

To analyze the Treg cell proliferation induced by radiation, the proportion of Ki-67^+ Treg cells in the thymus was analyzed. On 1 day after radiation exposure, the proportion of Ki-67^+ Treg cells in the thymus was significantly lower in the experimental group (8.6%) than in the control group (13.8%; Fig. 2A) and the proportion of Ki-67^+ Tcon cells in the thymus was significantly lower in the experimental group (14.2%) than in the control group (31.6%; t = 3.56, 5.72; $P<0.05$; Fig. 2B). However, at 4 days post-radiation exposure, the proportion of both Ki-67^+ Treg cells and Ki-67^+ Tcon cells was significantly higher in the experimental group (24.3 and 38.0%, respectively) than in the control group (13.8 and 31.6%, respectively; t = -10.96, -2.78; $P<0.05$; Fig. 2A, B).

On 1 day of radiation exposure, the proportion of Ki-67^+ Treg cells in the spleen was significantly higher in the experimental group (11.5%) than in the control group (9.5%; t = -3.41; $P<0.05$), whereas the proportion of Ki-67^+ Tcon cells in the spleen was not significantly different between the two groups (Fig. 2C, D). At 4 days post-radiation exposure, the proportion of both Ki-67^+ Treg cells and Ki-67^+ Tcon cells in the spleen was higher in the experimental group than in the control group, but the difference was not statistically significant (Fig. 2C, D). These results indicate that Treg cell proliferation initially decreased post-irradiation. However, Treg cells start to proliferate after 4 days of irradiation in the spleen.

Change in the Proportion of Thymus-Derived Treg Cells Post Irradiation

Natural Treg (nTreg) cells mainly differentiate and mature in the thymus and then reach the peripheral lymphoid tissues and non-lymphoid tissues. However, the nTreg cells produced in the thymus are not the only source of Treg cells in the peripheral tissues as induced (iTreg) cells may exist under certain conditions. $Helios^+$Treg cells were used as a marker of thymus-derived Treg (Thornton *et al*., 2010; Gottschalk *et al*., 2012). To clarify the stability of nTreg cells in the thymus and the proportion changes in the spleen. The proportion of $Helios^+$ Treg cells in the thymus and spleen is relatively stable after 2Gy irradiation. At 10 days post-radiation exposure, the Mean Fluorescence Intensity (MFI) of Helios in a single Treg cell increased both in the thymus and spleen. The MFI of Helios in a single Treg cell in the thymus increased from 5677.7 in the control group to 6529.5 in the experimental group (Fig. 3A) and that in the spleen increased from 3968.7 in the control group to 4558.7 in the experimental group (t = -3.76, -7.09; $P<0.05$; Fig. 3A). However the proportion of $Helios^+$ Treg cells in the thymus increased 4 days after radiation exposure, and it decreased again 10 days after exposure (Fig. 3B). And, there was no statistical difference between control and experimental groups 4 and 10 days post-exposure. The proportion of $Helios^+$ Treg cells in the spleen was significantly higher in the experimental group (74.3%) than in the control group (58.9%; t = -3.15; $P<0.05$; Fig. 3B). Compared with the control group, the ratio of $Helios^+$ Treg cells was decreased in both thymus and spleen 10 days after exposure, from 1.4 to 1.0 (Fig. 3C).

The Expression of CD39 in Treg

Our result found the proportion of CD39+Treg cells increased in both thymus and spleen after 2 Gy exposure. The ratio of CD39+Treg in the thymus was a significant increase from 43.2 to 83.8 compared with control after 10 days of radiation (t = -18.29, $P<0.05$; Fig. 4A). Meanwhile, the ratio of CD39+Treg cells between the thymus and spleen is up-

regulated after exposure (Fig. 4B). The MFI value of CD39 of Treg cells in the thymus was up-regulated and increased from 2720.8 to 4659.3 (t = -4.51, P<0.05) after 10 days of exposure (Fig. 4C). While the MFI value of CD39 of Treg cells in spleen decreased at 4 days after exposure (t = 2.61, P<0.05) and up-regulated at 10 days (t = -5.12, P<0.05; Fig. 4C).

Change in ATP Concentration and mir-31 Expression in Thymic Treg Cells Post Irradiation

The ATP concentration of Treg cells decreased 1 d after exposure to 2 Gy radiation (0.15 μmol/L, t = 31.98; P<0.05). Moreover, 4 days after 2 Gy exposure, the ATP concentration in the experimental group (0.17 μmol/L) remained significantly lower than that in the control group (0.2 μm) (t = 6.14; P<0.05; Fig. 5A). The results of real-time PCR showed that $CD4^+$ T cells in the thymus showed increased miR-31 expression 1 and 4 days after 2 Gy irradiation (Fig. 5B) but decreased miR-31 expression 10 days after 2 Gy irradiation. However, miR-31 expression in the $CD4^+$ T cells in the spleen was significantly decreased at 1, 4, and 10 d post 2 Gy radiation (Fig. 5C).

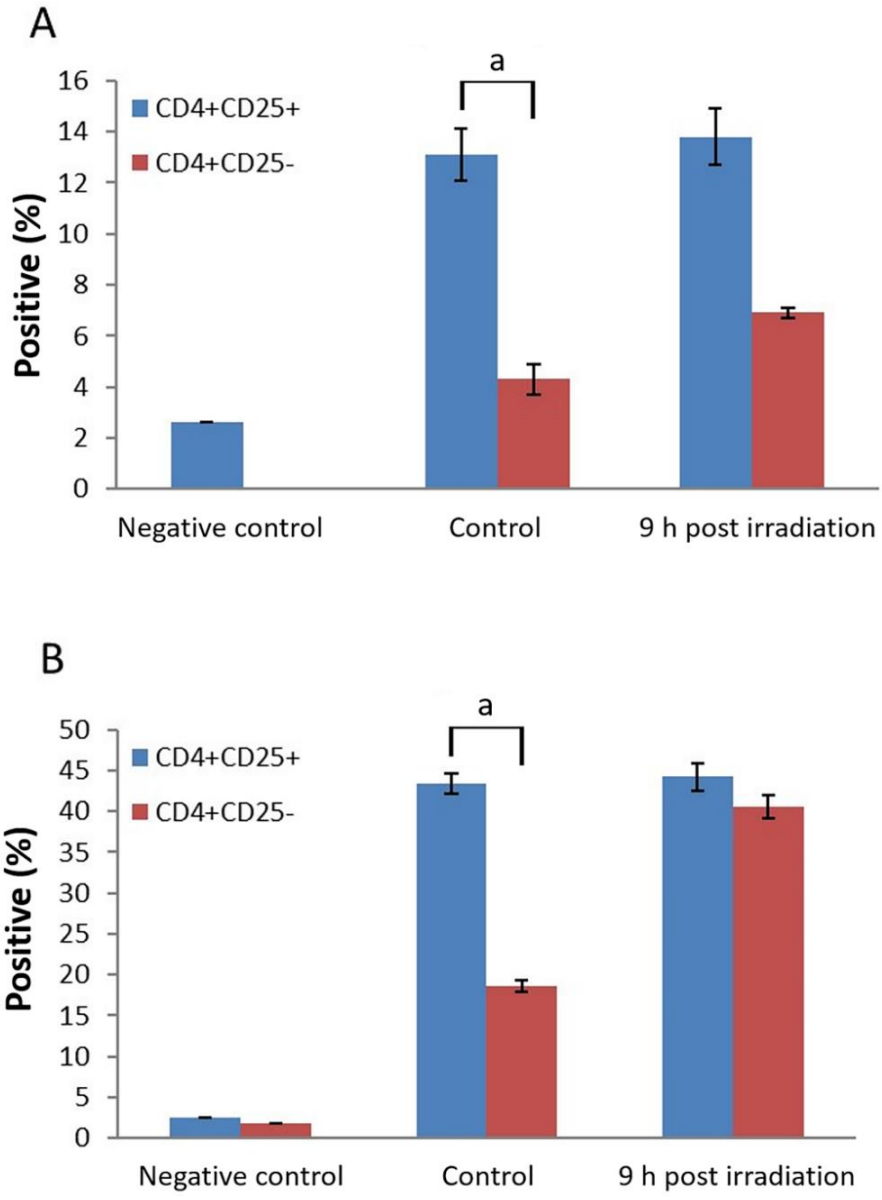


Fig. 1: Viability analysis of Regulatory T (Treg) cells in the thymus. Isolated thymocytes and spleen cells were incubated with CD4 antibodies. The cell suspension was filtered before loading on the flow cytometer. A. Analysis of cell viability from isolated thymocytes 9 h post-irradiation. a, vs. Control, t = 4.18; p<0.05; B. Analysis of cell viability from isolated spleen cells 9 h post-irradiation. a, vs. Control, t = 14.03; p<0.05

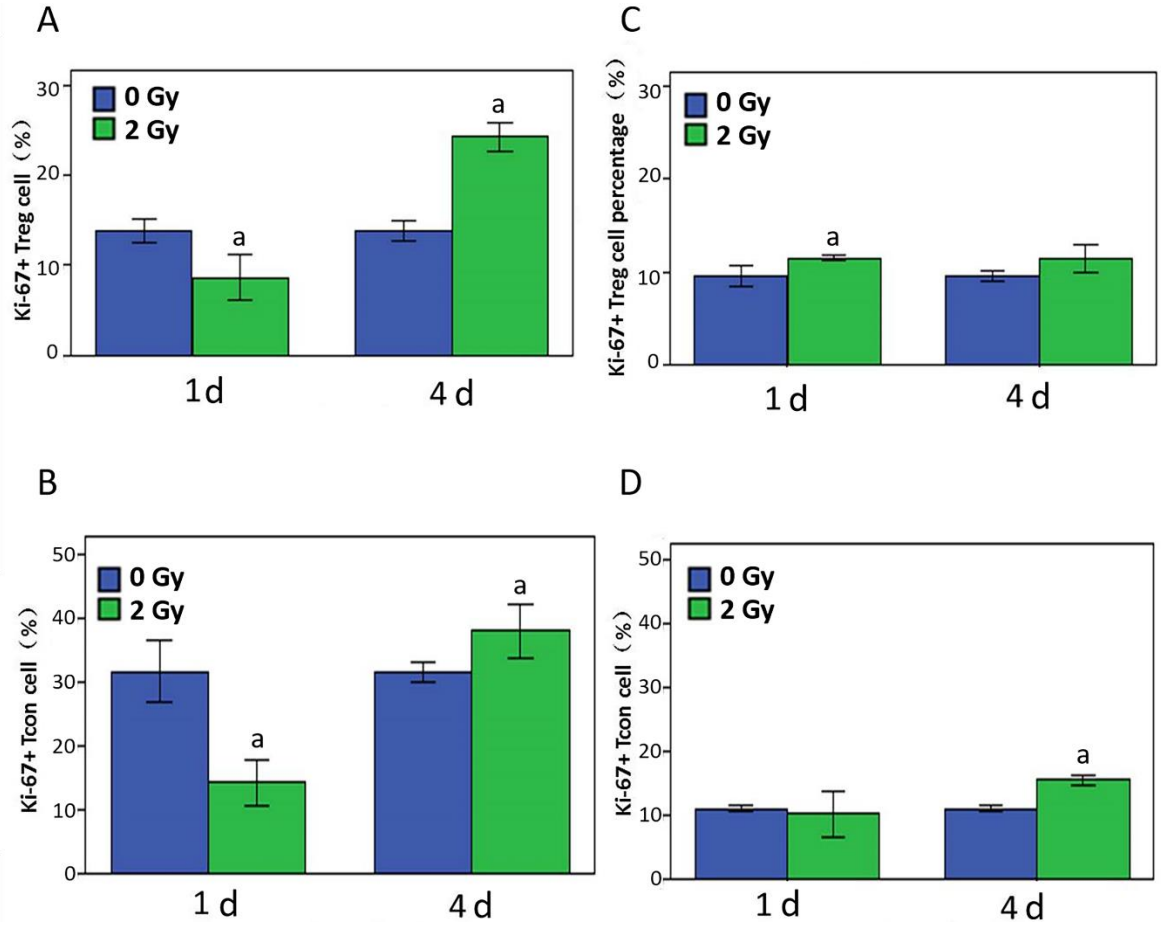
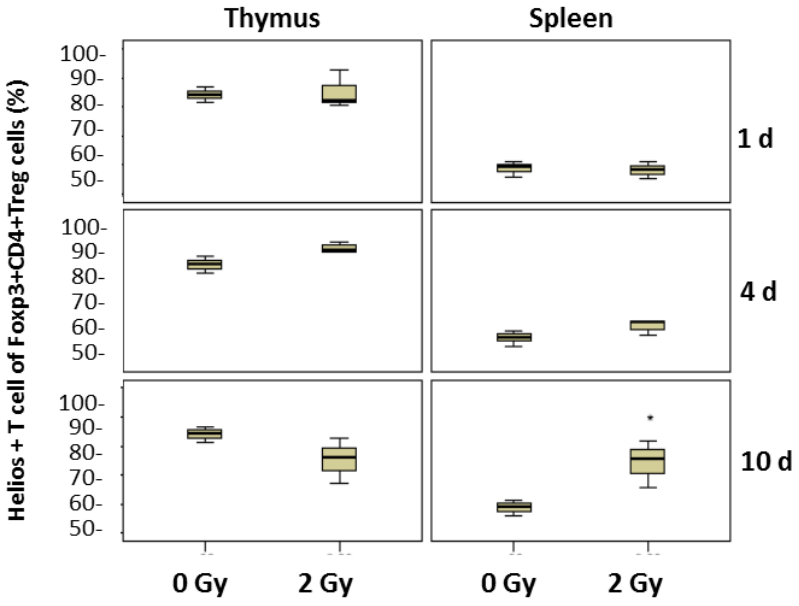


Fig. 2: Analysis of Treg cell proliferation. Treg cells were isolated 1 day and 4 days post 2 Gy irradiation and incubated with a Ki-67 antibody. A, B The proportion of Ki-67$^+$ Treg cells in the thymus post-irradiation. a, vs. Control, t = 3.56, -3.41, -10.96; p<0.05; C, D The proportion of Ki-67$^+$ Tcon cells in the spleen post-irradiation. a, vs. Control, t = 5.72, -2.78, -10.36; p<0.05



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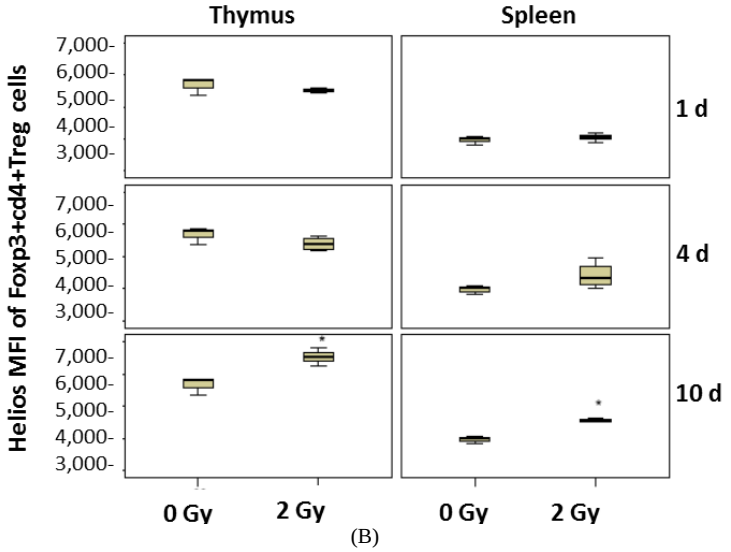

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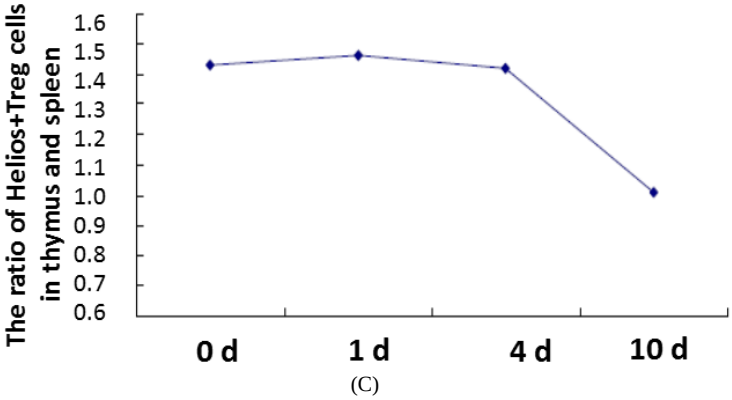
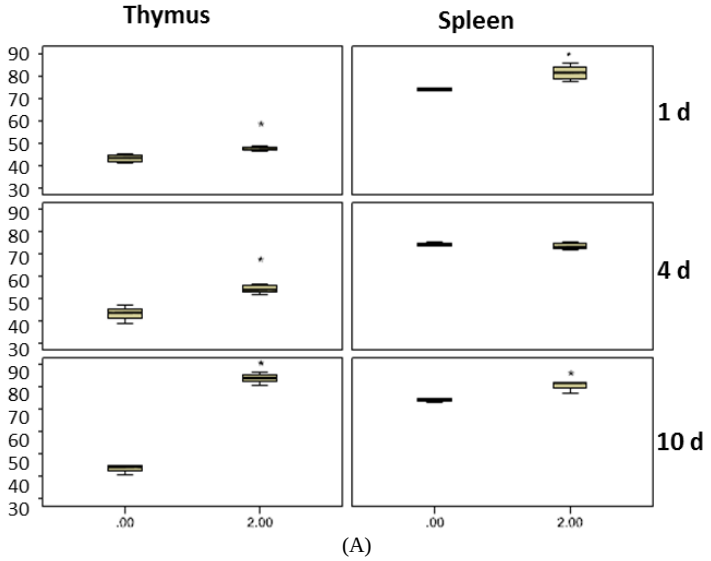


(C)

Fig. 3: Radiation-induced changes in the proportion of thymus-derived Treg cells. The Mean Fluorescence Intensity (MFI) was measured by flow cytometry. A. The MFI of Helios in Treg cells post 2 Gy irradiation. * vs. Control, t = 3.76, -7.09; $p<0.05$; B. Proportion of Helios$^+$ Treg cells post 2 Gy irradiation. *vs. Control, -3.15; $p<0.05$; C. Proportion of Helios$^+$ Treg cells in the thymus and spleen post 2 Gy irradiation



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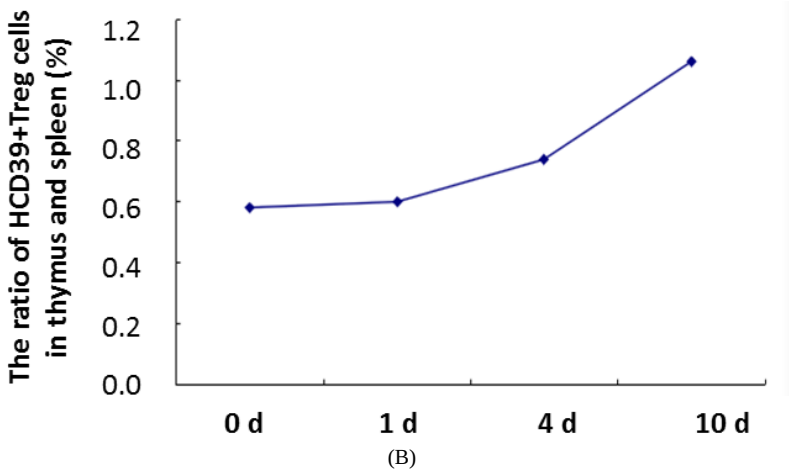

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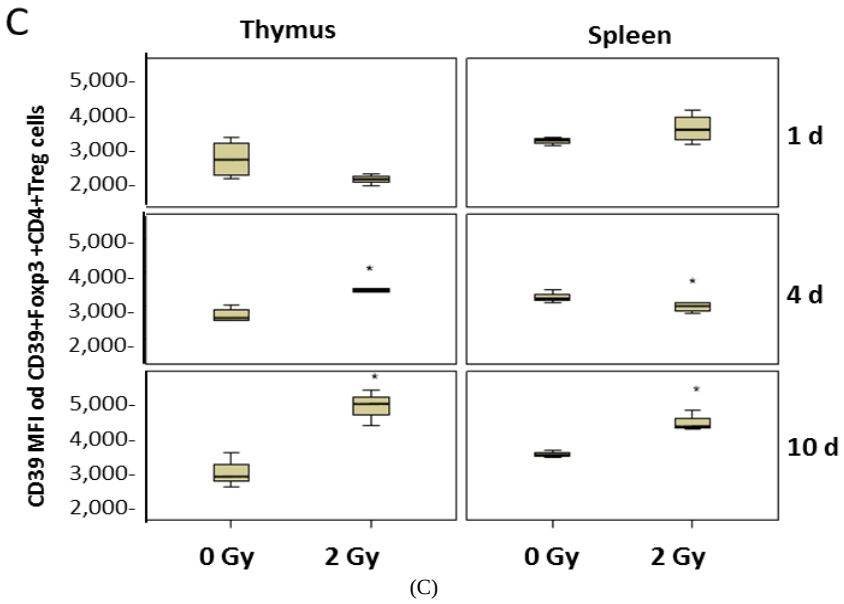


(C)

Fig. 4: The expression of CD39 in Treg cells. (a) Proportion of CD39+Treg cells after 2Gy irradiation in thymus and spleen. *vs. Control, t = -3.74, -5.63, -18.29,-4.35,-3.55, p<0.05; (b) Ratio of CD39+Treg cells in thymus and spleen after 2Gy irradiation. (c) MFI changes of CD39 in Treg cells after 2Gy irradiation. *vs. Control, t = -6.63, -4.51, 2.61, -5.12, p<0.05

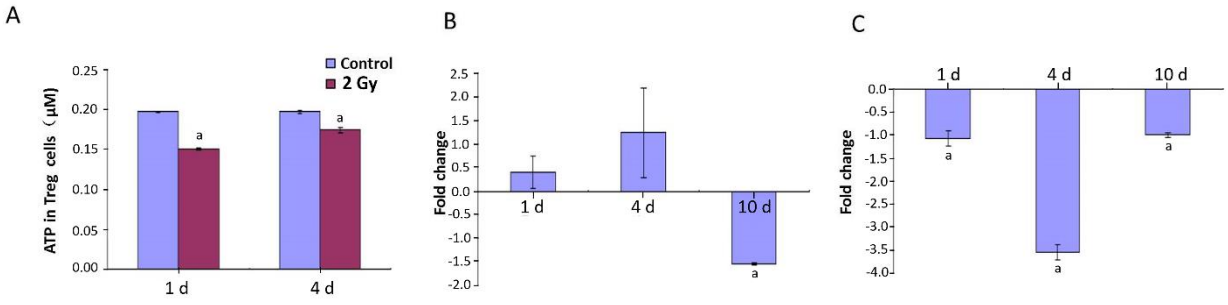
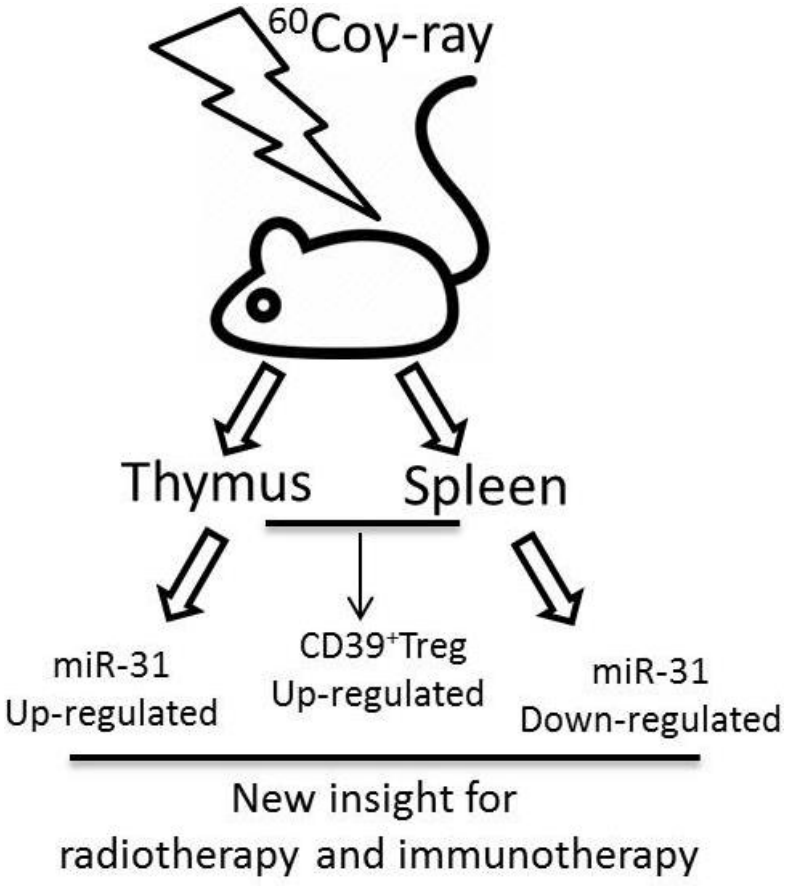
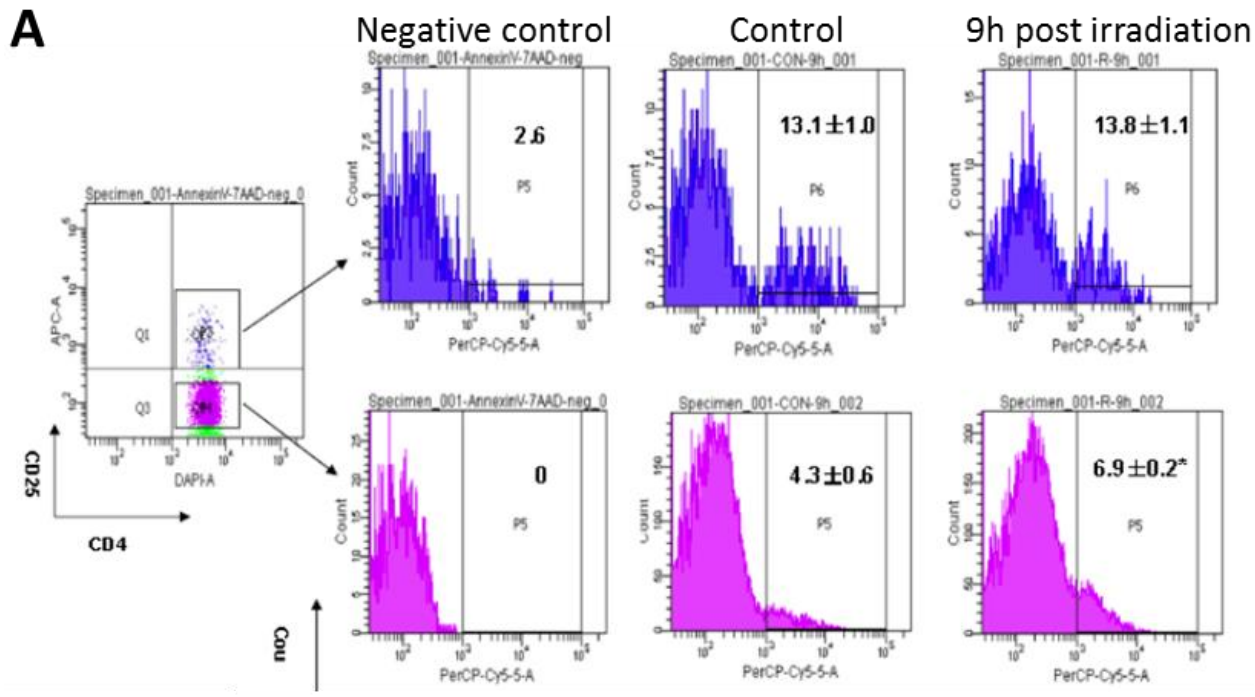
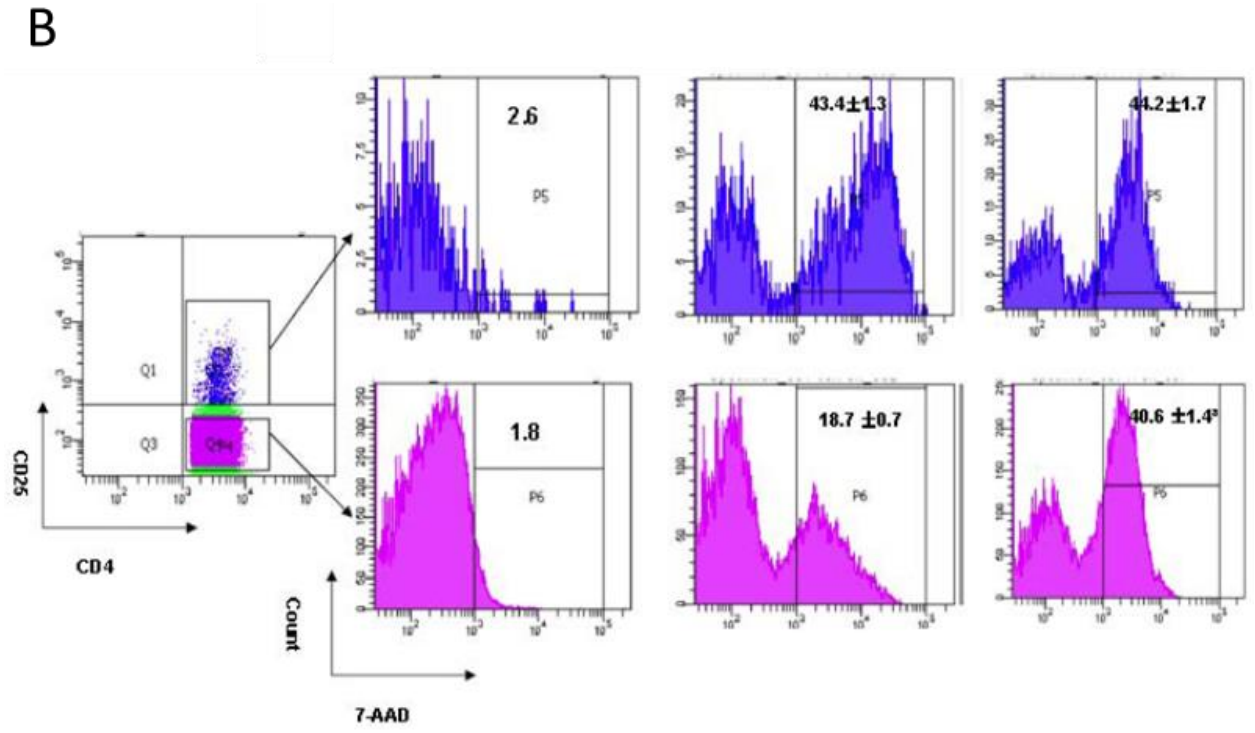


Fig. 5: Changes in ATP concentration and miR-31 expression in thymic Treg cells post 2 Gy irradiation A. ATP concentration of thymus Treg cells decreased post irradiation. a, vs. Control, t = 31.98, 6.14; p<0.05; B. miR-31 gene expression in the thymus post irradiation. a, vs. Control, t = 3.61; p<0.05; C. miR-31 gene expression in the spleen post irradiation. a, vs Control, t = 2.43,12.32,2.64, p<0.05



Supplemental Fig. 1: A schematic diagram of experimental design





Supplemental Fig. 2: Viability analysis of Regulatory T (Treg) cells by flow cytometer. A. Analysis of 7-AAD from isolated thymocytes 9 h post-irradiation. B. Analysis of 7-AAD from isolated spleen cells 9 h post-irradiation

Discussion

Treg cells promote tumor development by inhibiting the immunity system (Dannull *et al*., 2005; Gottschalk *et al*., 2012). Treg cell treatment in pre-clinical animal models showed the enormous therapeutic potential of these cells in immune-mediated diseases and placed the foundations for their applications in therapy in humans (Giganti *et al*., 2021). The stability of Treg cells is changed in radiation injury and radiation oncology. Studies have shown that lymphatic cells are damaged post-irradiation. Tumor irradiation mainly changes the phenotype and proportion of Treg cells. The sensitivities of using Treg to predict radiotherapeutic responses were 61.5% and the specificity was 84.2% (Song *et al*., 2021). Therefore, many researchers have tried to modify the proportion of Treg cells to alter the autoimmune and to achieve the purpose of adjuvant treatment of tumors and autoimmune diseases (Wang and Huang, 2020). Recently, research reported ionizing radiation modulates the phenotype and function of human CD4+ induced regulatory T cells (Beauford *et al*., 2020). Our study found that the proportion of Treg cells in the thymus and spleen increased after 2Gy γ-ray irradiation. This finding indicated that Treg cells were radiation-resistant and their survival ability was different at each time point after irradiation. The result is consistent with the result of a previous study which also showed radiation-resistant of Treg cells (Liu *et al*., 2013).

Usually, apoptotic cells are quickly engulfed by macrophages. Moreover, there are interactions between Treg cells in the organ and other stimulating factors which affect the apoptosis rate of single cells. Therefore, thymocytes were isolated immediately post-irradiation in this study. Our study also found a radiation-induced significant increase in the apoptotic rate of Treg cells in the thymus than in the spleen. This result indicated that microenvironment changes significantly affect the viability of Treg cells and, the expression of Helios in Treg cells shows the survival Tregs are still mainly natural ones in the spleen post-irradiation. Our study revealed that radiation induces apoptosis accompanied by the proliferation of surviving cells. There is a different proliferation decreasing rate between Treg cells and Tcon cells post-irradiation indicating the direct effect of radiation on cells varies greatly among different cell proportions. Moreover, radiation-induced changes in Treg cell proliferation vary with time post-exposure and between organs.

Many factors affect the apoptosis rate of Treg cells including the ATP released from cells. The up-regulation of CD39 in thymic Treg cells can accelerate the decomposition of extracellular ATP to maintain the low level of ATP (Maj *et al*., 2017). Therefore, it is reasonable to consider that radiation-induced high expression of CD39 in Treg cells is closely related to the resistance of radiation-induced Treg cells apoptosis. The expression of CD39 in Treg cells has a similar changing pattern as

the apoptosis rate of Treg cells indicating a similar stimulation mechanism. Therefore, it is speculated that the up-regulation of the extracellular ATP level is one of the possible mechanisms. "This finding showed that the miR-31 expression was different between CD4+ T cells in thymus and spleen with temporal correlation and its expression pattern is similar to CD39 expression which was responsible for converting ATP into ADP (Badimon *et al*., 2020). This result indicated that miR-31 may play a regulatory role in CD39 expression induced by radiation. And, miR-31 expression 10 days after exposure may be related to the developmental stage of Treg cells in the thymus (Kachikwu *et al*., 2011).

In summary, our study found that Treg cells are resistant to radiation exposure. Although radiation induced a significant increase in the apoptosis rate of Treg cells in vivo, cell proliferation still increased 4 days post-irradiation. Moreover, up-regulation of extracellular ATP levels was associated with a miR-31 expression which target-regulating CD39. Even though our findings improve the understanding of the mechanism of radiation-induced changes in Treg cell phenotype and its role in immune regulation, research studies on signal transduction pathways still need to be carried out in the future.

Authors Contributions

Lantao Liu: Analysis and interpretation of data and drafted the manual.

Yan Peng: Analysis of data and performed part of the experiment.

Deqin Zhang: Performed part of the experiment.

Dongsheng Niu: Designed the project.

Ethics

The study was approved by the institutional research ethics committee of the Beijing Institute for Occupational Disease Prevention and Treatment.

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