Radiation combined with PD-1 inhibitor induces time-dependent changes in myocardial injury by regulating HMGB1-associated inflammatory microenvironment

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Research Article

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Abstract

Background

PD-1 inhibitors may superimpose the toxicity of radiotherapy while increasing the antitumor effect. However, there are fewer studies on immune myocarditis caused by radiotherapy plus anti-PD-1 and the mechanism is still under exploration.

Methods

40 C57BL/6 mice were randomly assigned to 4 groups. A: Control, B: PD-1 inhibitor, C: cardiac irradiation and D: thoracic irradiation + PD-1 inhibitor, mice were treated with either anti-PD-1 antibody with or without thoracic radiation (15Gy). Each group contained ten mice, five of which were studied for a duration of 1 month and the remaining five for 3 months. Tunel staining was utilized to observe apoptosis of cardiac tissues; histological analysis was performed to analyze the structural and morphological alterations, fibrosis of the heart tissue. The infiltration of CD3+, CD4+, and CD8 + T-cells into the cardiac was analyzed through flow cytometry; Elisa measured the expression levels of TNF-α, IL-1β, and TLR-4 in the cardiac; and immunoprotein blotting and qPCR were used to observe the protein and mRNA expression levels of HMGB1, TLR-4, and NF-κB p65.

Results

Group D exhibited a greater degree of cardiac injury, fibrosis, and apoptosis in comparison to groups A, B, and C. Additionally, there was an increase in injury, AI, and CVF values after three months as opposed to one month (P < 0.05). After one month, there was no statistically significant difference in cardiac damage, AI, or CVF values between groups A and B; however, after three months, there was a significant difference (P < 0.05). Group D also had higher levels of IL-1β, IL-6, TNF-α and T-lymphocyte distribution, HMGB1, TLR4, NF-κB P65 protein, and mRNA expression than the other three groups. However, each group’s index expression declined over the course of three months as opposed to one month, and this difference was statistically significant (P < 0.05).

Conclusion

PD-1 inhibitors exacerbated myocardial injury based on radiation by upregulating the expression of inflammatory factors in the HMGB1 signaling pathway. In the early stages of myocardial damage, inflammatory alterations predominated, while in the later stages, fibrosis.

Background
Radiotherapy is one of the most important treatments for thoracic tumors and numerous studies have confirmed its strong link with cardiac dysfunction [1, 2]. Incidental exposure of the heart during radiotherapy can lead to both acute and chronic cardiovascular consequences, with the latter being more common and having a latency period of years to decades, so the incidence of radiation-induced cardiac dysfunction increases with prolonged patient survival[3–5]. Current studies on the incidence of RIHD have focused on breast cancer and Hodgkin’s lymphoma due to their high 5-year survival rates, whereas other thoracic tumours have shorter follow-up time and poorer survival rates, thus underestimating the true incidence of RIHD [6].

In last decade, immunotherapy has been acknowledged as one of the most important tactics for slowing the progression of malignant tumours. Nevertheless, the majority of patients exhibit primary or acquired resistance during treatment (up to 60–70% in melanoma, with a higher incidence in other cancers), which limits the clinical utility of immune checkpoint inhibitors (ici) [7, 8]. Studies have demonstrated that PD-1 inhibitors not only enhance the local anti-tumour effects of radiotherapy, but also shrink tumours beyond the radiation area, referred to as the "distant effect"[9]. Radiotherapy combined with PD-1 inhibitors has become one of the combination treatments for many cancers.

The use of PD-1 inhibitors is accompanied by a 72% incidence of grade 1–5 irAE, whereas the incidence of ICI-related myocarditis ranges from 0.27–1.14% [10]. Preclinical studies in mice have shown that PD-1 blockade combined with cardiac irradiation increased the risk of myocarditis and mortality by recruiting CD8⁺T lymphocyte infiltration and elevating TNF-α expression in heart, and anti-CD8 treatment reversed the acute mortality, suggesting the CD8⁺T is immmediated in heart toxicly[11]. Nevertheless, the mechanism remains unclear.

High mobility group protein box 1 (HMGB1) acts as an alert signal and functions as an extracellular signalling molecule in a number of processes including tissue regeneration, infection, cell differentiation and tumourigenesis and development[12]. Under pathological conditions such as cardiovascular disease and myocardial ischaemia-reperfusion injury, HMGB1 is released by necrotic cells and participates in the inflammatory response as an inflammatory mediator, and its key receptor TLR4 is also involved in the induction of inflammation. TLR4 regulates apoptosis by stimulating the expression of TNF-α and IL-6 through pathways such as oxygen free radicals, neutrophil aggregation, p38 kinase and NF-κB[13].

Currently, it is unclear how the HMGB1/TLR4 signalling pathway is involved in myocardial injury when PD-1 inhibitors and radiotherapy are combined. We used a mouse model of radiomyocardial injury to assess the effects of PD-1 inhibitors on myocardial inflammation and explore the relationship between the HMGB1/TLR4 pathway and related inflammatory factors and myocardial injury.

**Methods**

**Mice and models**
Male C57BL/6 mice (6–8 weeks old, 20–25 g) were obtained from Beijing Viton River Laboratory Animal Science and Technology Co, Ltd. All mice were housed in a specific pathogen-free (SPF) animal facility with prepared food and water without restriction. All mouse experiments were approved by the Animal Care and Use Committee of the Guizhou Medical University (Certificate No: 1901009) and were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

The mice were randomly assigned to 4 groups with 10 mice in each: control group (injected with PBS, no irradiation); anti-PD-1 group (injected with anti-PD-1, no irradiation); cardiac irradiation group (injected with PBS, cardiac irradiation of 15 Gy); IR + anti-PD-1 group (injected with anti-PD-1, cardiac irradiation of 15 Gy).

**Cardiac irradiation and PD-1 inhibitors treatment**

Mice were anaesthetised and fixed in 1 cm wax models and placed under a linear accelerator. The heart was positioned at the centre of the radiation field by observing the extent of the heart beat on the body surface of the mice. The mice were irradiated with 6 MV x-rays at a dose of 15 Gy, 0° single-field vertical irradiation of the chest; 2.0 × 2.0 cm irradiation area; the radiation to skin was 100 cm., and a dose rate of 600 cGy/min.

The anti-PD-1 group and the IR + anti-PD-1 group were injected intraperitoneally with 200 µg of PD-1 inhibitor 1 week and 30 min before cardiac irradiation, respectively, and 100 µg of PD-1 inhibitor 7, 14, and 21d after irradiation, and the same volume PBS was injected intraperitoneally at the same time in control and IR group.

**Sample collection**

In subsequent studies, 5 mice were anaesthetised and executed 1 and 3 months after cardiac irradiation respectively, and heart tissues were stored in 4% paraformaldehyde or frozen at -80°C.

**HE staining**

HE staining was performed to observe the morphology of cardiomyocytes; according to the manufacturer's instructions, tissue sections were dewaxed, stained with hematoxylin and eosin, then dehydrated with alcohol, treated with xylene for transparency, and sealed with neutral gum (Beijing Solarbio Science & Technology Co. Ltd. Beijing, China).

**Masson staining**

Masson's trichrome staining was used to stain the myocardium and fibrotic tissue red and blue, respectively. Staining was performed according to manufacturer's protocol (Beijing Solarbio Science & Technology Co. Ltd. Beijing, China) Optical microscope was used for observation and photographs were taken. Three areas were randomly selected from each section and semi-quantitative analysis of myocardial collagen volume fraction (CVF) was performed by applying ImageJ software. CVF was equal to the area of collagen in the field of view divided by the total area of the field of view.
**TUNEL staining**

Apoptosis was detected using the TUNEL method, and cell staining was observed under a fluorescence microscope. FITC-stained apoptotic cells emitted green light, and DAPI-stained nuclei of all cells emitted blue light. Cardiomyocyte apoptotic index (AI) was analysed semi-quantitatively using ImagJ software. AI was used as a measure of TUNEL positivity, defined as the number of TUNEL-positive cells divided by the total number of cells.

**Flow cytometry**

30 mg of myocardial tissue was prepared as a single-cell suspension by enzymatic digestion, and cells were labelled with anti-mouse CD3, CD4 and CD8. Staining was performed according to the manufacturer's protocol (Biogem Scarl, Ariano Irpino, Italy). Data were statistically analysed using FlowJo_V10 software.

**Immunoblotting**

Protein solutions were prepared by placing heart tissues in RIPA/PMSF (100:1), sonicated in ice water with interference, and then dissolved for 25 minutes. The protein concentration was determined using a BCA protein assay kit. After electrophoretic transfer of the protein solution, the PVDF membrane was blocked with 5% low-fat skinned milk dissolved in TBST. After diluting the primary antibody overnight at 4°C, the membrane was incubated with hrp-coupled secondary antibody for 1 h at room temperature. PVDF membranes were visualised using enhanced chemiluminescence (ECL) and analysed using ImagJ software.

**Real-time fluorescence quantitative PCR**

Total cardiac tissue RNA was extracted using the Tissue RNA Purification Kit. The purity and concentration of the extracted RNA were detected using UV spectrophotometry. Then cDNA was synthesised according to the instructions of the Rapid All-in-One RT Kit. cDNA was used as a template to detect mRNA expression in the CFX96TM Real-Time PCR system. The cycling parameters were set as follows: pre-denaturation at 95°C for 5 min, cycling once; denaturation at 95°C for 15 s, annealing extension at 60°C for 30 s, annealing extension at 60°C for 30 s, cycling 40 times.

The primer sequences were as follows: GAPDH (forward 5'-GGTTGTTTCTCCTGCGACTTCA-3'; reverse 5'-TGGTCAGGGTTTCTTACTCC-3'), HMGB1 (forward 5'-AGCACAAGAAGAAGCACCCG-3'; reverse 5'-ACGAGCCTTGTACGGCTTGG-3'), TLR4 (forward 5'-GCCATCATTATGAGTGCCAATT-3'; reverse 5' -AGGGATAAAGACGCTGAGAATT-3'); NF-κB p65 (forward 5'-AGACCCAGAGGCTTACAGACC - 3'; reverse 5' -GTACACGGCGAGTTATATGCTTCAG-3').

**ELISA**

IL-1β, IL-6, and TNF-α ELISA was performed according to the manufacturer's instructions (Thermofisher, Shanghai, China). All tests were performed in triplicate.
Statistical analysis

Results were analysed using GraphPad Prism 8.2 software and expressed as mean and standard deviation. Comparisons between groups that conformed to a lognormal distribution were analysed by two-way ANOVA, and further two-by-two comparisons were made by Tukey’s test, and Dunnett’s T3 test was used in case of heteroscedasticity. Data that did not conform to log-normal distribution were compared using non-parametric tests and further Bonferroni tests. Differences were considered statistically significant at \( P < 0.05 \).

Results

PD-1 inhibitors aggravated IR-induced myocardial injury in mice

HE analysis

As shown in Fig. 1a, HE was performed to detect cardiac pathological changes at 1 and 3 months. The results showed that mice treated with PD-1 inhibitors alone did not induce significant myocardial injury, but cytoplasmic lysis and lymphocyte infiltration were observed in some cardiomyocytes in cardiac irradiation alone at 1 and 3 months. However, we found that PD-1 inhibitors resulted in more pronounced cardiomyocytolysis and more lymphocyte infiltration when radiotherapy and PD-1 inhibitors were co-administered compared with PD-1 inhibitors or radiotherapy alone, which further exacerbated the disordered arrangement of myocardial tissues (Fig. 1a).

Masson analysis

As shown in Fig. 1b, Masson was performed to detect the distribution of cardiac collagen fibres at 1 and 3 months. Semi-quantitative analysis showed that collagen fibres were visible in the cardiac tissue at 1 month with a CVF of 5.39 ± 0.77%, which increased to 7.14 ± 0.79% \( (P < 0.05) \) at 3 months in IR group. The CVF was (1.50 ± 0.15%), (2.97 ± 0.57%), (7.14 ± 0.79%) and (10.32 ± 0.86%) at 3 months in the control, anti-PD-1, IR and IR + anti-PD-1 groups respectively. There was an interaction effect of time with the intervening factors as analysed by two-way ANOVA (F3, 16 = 4.490, \( p = 0.0181 \)). Masson staining as described above showed that PD-1 inhibitors exacerbated myocardial fibrosis when co-administered with radiotherapy in mice (Fig. 1b-c).

TUNEL analysis

The results of TUNEL assay were similar to those of HE and Masson assay; AI in the control, anti-PD-1, IR, and IR + anti-PD-1 groups increased gradually at 1 month (1.49 ± 0.41%, 2.19 ± 0.39%, 7.06 ± 0.58%, and 8.38 ± 0.88%, respectively) and at 3 months (1.60 ± 0.32%, 2.68 ± 0.32%, 9.10 ± 0.40%, and 11.73 ± 0.45%, respectively) gradually increased. PD-1 inhibitors combined with radiotherapy increased AI compared
with irradiation alone, and the increase in AI was time-dependent in the IR and IR + anti-PD-1 groups, \( P < 0.05 \) (Figs. 2 and 3).

The PD-1 inhibitors' affect on the intramyocardial immune microenvironment

**Distribution of T Lymphocyte Subsets**

At 1 and 3 months after irradiation (The distribution of cardiac lymphocytes in 1 month was derived from our previously published article[14]), the percentage of CD3\(^+\)T lymphocytes was higher in all groups (\( P < 0.05 \)) than in control group, except for the anti-PD-1 group (3 months). At 1 month after irradiation, the percentage of CD4\(^+\)T lymphocytes was elevated in the IR group and the IR + anti-PD-1 group compared with the control group, and the difference between the remaining groups was not statistically significant, and the difference was not statistically significant when comparing 3 months after irradiation with 1 month after irradiation. At 1 and 3 months after irradiation, there was a trend of gradual increase in the percentage of CD8\(^+\)T lymphocytes among the 4 groups (\( P < 0.05 \)), indicating that CD8\(^+\)T lymphocytes were the main cause of the increase in T lymphocytes. Further comparing the groups with the control group, despite the increase in T lymphocytes (mainly CD8\(^+\)T lymphocytes) at 1 and 3 months after irradiation, there was a decrease in T lymphocytes at 3 months after irradiation compared to 1 month after irradiation (Fig. 4, Fig. 5a-c).

**Expression of Cytokine**

At 1 month after irradiation, the concentrations of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) were higher in the anti-PD-1 and IR groups than in the control group (The data of expression of cytokine in 1 month was derived from our previously published article[14]). At 3 months after irradiation, the expression of IL-1\(\beta\) and TNF-\(\alpha\) decreased in both groups compared with that at 1 month after irradiation, but was still higher than that in the control group, and the difference in IL-6 expression among the 3 groups was not statistically significant. When chest irradiation was combined with PD-1 inhibitors, myocardial IL-1\(\beta\), IL-6, and TNF-\(\alpha\) expression increased at 1 and 3 months after irradiation compared with the other groups, and the trend of expression at 3 months matched that of the other groups, which was slightly lower than that of 1 month (Fig. 5d-f).

It can be seen that PD-1 inhibitors seem to stimulate the secretion of inflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF-\(\alpha\) and the growth of CD8\(^+\)T lymphocytes in myocardium, and the expression gradually decrease over time.

**PD-1 Inhibitors could Enhance Myocardial Injury via HMGB1/NF-\(\kappa\)B Pathway**

**Westernblot Analysis**
Western blotting detected the concentration of HMGB1/TLR4 pathway-related proteins in myocardial tissues, as shown in Fig. 6. The expression trends of HMGB1 and NF-κB p65 were similar to those of lymphocytes (Fig. 7a, b). Their expression at 3 months was lower than that at 1 month, and it was higher in the anti-PD-1 + IR group than in the other three groups at both 1 month and 3 months ($p < 0.0001$). The differences in TLR4 expression between the groups were not statistically significant ($p > 0.05$) at 1 and 3 months after irradiation (Fig. 7c).

**qPCR Analysis**

The results of RT-qPCR assay showed that the mRNA expression trend of HMGB1/TLR4 signalling pathway was consistent with the protein expression trend. Using the control group as a reference, there was no significant difference in TLR4 mRNA expression among the 4 groups at 1 and 3 months ($P > 0.05$) (Fig. 7d), whereas HMGB1 and NF-κB p65 mRNA expression was increased and could be maintained for at least 3 months in the other 3 groups ($P < 0.0001$). After PD-1 inhibitor combined with chest radiotherapy, all groups showed the HMGB1 and NF-κB p65 mRNA expression was highest (Fig. 7e, 7f).

**Discussion**

Combining radiotherapy with PD-1 inhibitors enhances the efficacy of lung cancer, esophageal cancer and other thoracic cancers, which is currently a focus of clinical research, and whether PD-1 blockade aggravate radiation injury is in concern. Accumulating studies mainly reported pneumonia and esophagitis, but less attention paid to cardiac toxicity [15–19].

The inflammatory reaction plays a crucial role in myocardial damage caused by the concurrent thoracic radiotherapy plus PD-1 blocking. HMGB1, an important damage-associated molecular pattern, is rapidly released upon injury or infection, promoting adaptive immune responses and activating APCs. Furthermore, it is also involved in a variety of myocardial injuries as a strong inflammatory stimulant [20]. Radiation induced cardiac damage, ensuing HMGB1 releasing, activation of T lymphocytes and production of inflammatory cytokines, which form an immune microenvironment promoting inflammatory responses and initiating fibrosis [10, 21–23]. Nevertheless, the correlation between HMGB1 and myocardial injury is unclear when radiotherapy is combined with PD-1 inhibitors.

To better understand the correlation of HMGB1 and cardiotoxicity from concurrent thoracic radiotherapy plus anti-PD-1 therapy, we established a model where cardiac radiation plus anti-PD-1 treatment causes cardiomyopathy in mice, it was found that PD-1 inhibitors enhance the infiltration of lymphocytes and elevate the levels of HMGB1, NF-κB p65, IL-1β, IL-6, and TNF-α expression of myocardium on basis of irradiation. PD-1 inhibitors alone do not significantly cause myocardial injury in early stage (1 month), and only slightly injured myocardium and form a little fibrosis in late stage (3 months). In late stage, there was only a minor increase in CVF value compared to the control, which indicated the administration of anti-PD-1 only slightly injured myocardium. In this study, both irradiation alone or plus PD-1 inhibitors resulted in more extensive myocardial injury and fibrosis; and the combination promoted more pronounced injury. The injury manifest early and fibrosis worsened over time, consistent with the gradual
aggravation of the formation process of radiomyocardial fibrosis. Although PD-1 inhibitors alone cause mild myocardial injury, the combination with irradiation can further exacerbate radiomyocardial injury and fibrosis, which should be taken seriously. Du et al. also found that PD-1 inhibitors exacerbated radiomyocardial injury and affected cardiac function in mice, combined with PD-1 inhibitors significantly reduced cardiac output and increased mortality by 30% in mice compared with cardiac irradiation alone[11].

PD-1 inhibitors promote immune-inflammatory responses by activating T lymphocytes. Compared with the IR group, the IR + anti-PD-1 group showed an increase in CD3+T lymphocytes and a significant infiltration of CD8+T lymphocytes; the infiltration of CD4+T lymphocytes was not obvious. Studies have shown that blocking CD8+T lymphocytes combined with radiotherapy can attenuate myocardial injury induced by PD-1 inhibition; however, blocking CD8+T lymphocytes, a key antitumor effector cell, will inevitably reduce the efficacy of anticancer therapy [11, 24]. In-depth exploration of CD8+T lymphocyte subsets and immunomodulatory mechanisms is needed to more accurately combat radiation and PD-1 inhibitor-induced myocardial injury. Myocardial T-lymphocyte infiltration was significantly reduced in the reperfusion group and the reperfusion + anti-PD-1 group 3 months after irradiation compared with 1 month after irradiation. At an early stage, T-lymphocyte infiltration plays a more prominent role in promoting the inflammatory response and exacerbating radiological myocardial injury.

After HMGB1 binds to the cell surface TLR4 receptor, intracellular signalling to the NF-κB complex degrades the IκB protein, releases active NF-κB p65 into the nucleus, and regulates gene transcription of the inflammatory factors IL-1β, IL-6 and TNF-α. A mouse model of autoimmune myocarditis showed improved cardiac function and reduced myocardial fibrosis after injection of HMGB1 antagonist[25]. Yao et al. attenuated doxorubicin-induced myocardial injury by blocking the inflammatory effects induced by hmgb1 [26]. Wang et al. found that HMGB1 in myocardial ischemia promotes inflammatory injury through targeting of TLR4 to promote inflammatory injury, and knockdown of TLR4 attenuated HMGB1-induced injury[27]. In contrast, according to Ma et al.[28], the use of tlr4 blockers amplifies inflammation, inhibits autophagy, and exacerbates adriamycin-induced heart failure and fibrosis. Compared with cardiac irradiation alone, the expression of HMGB1 and P65 was increased in combination with PD-1 inhibitors, whereas there was no significant change in the expression of TLR4. HMGB1-induced inflammatory response may not be a role of the TLR4/NF-κB p65 pathway in myocardial injury caused by radiation combined with anti-PD-1 therapy, and the mechanism deserves further investigation.

When the intrinsic immune system is activated through the classical NF-κB signalling pathway, pro-inflammatory cytokines such as TNF-α and IL-1β are produced, resulting in an inflammatory response, and on the other hand, the cytokines further activate the classical NF-κB pathway, resulting in an inflammatory cascade response[29]. The concentrations of IL-1β, IL-6, and TNF-α in the IR + anti-PD-1 group IL-1β, IL-6 and TNF-α were significantly higher in the IR + anti-PD-1 group than in the IR group, suggesting that these three inflammatory factors play an important role in myocardial injury induced by radiation combined with PD-1 inhibitors. In acute and chronic inflammation, IL-1β acts as a pro-
inflammatory mediator, causing the synthesis and expression of a variety of secondary inflammatory mediators, and is also involved in collagen fibre deposition in atherosclerotic plaques[30, 31]. Furthermore, IL-1β mediated inflammation in the tumor microenvironment plays an important role in cancer invasion, progression and metastasis [32]. Blocking IL-1β reduces the frequency of recurrent myocardial infarction and systemic inflammation and enhances the antitumour activity of anti-PD-1[33, 34]. As a key cytokine of intrinsic immunity, IL-6 is expressed early in the response to infection and tissue injury and is involved in host defence, regulation of immune cells, proliferation and differentiation[35, 36]. By blocking IL-6, myocardial injury induced by PD-1 inhibitor co-irradiation may be reduced[37]. Furthermore, IL-6 promotes tumourigenesis in the tumour microenvironment by regulating cancer hallmarks and multiple signalling pathways, including apoptosis, survival, proliferation, angiogenesis, invasion and metastasis, and most importantly metabolism[38]. Therefore, blockade of IL-1β and IL-6 in combination with PD-1 inhibitors is expected to attenuate myocardial injury without negative anticancer effects in patients undergoing chest radiotherapy. There are currently clinical trials testing drugs targeting IL-6 in patients with haematological malignancies and solid tumours[36]. TNF-α binds to different signalling pathways to form different functional endpoints such as inflammation, survival, apoptosis and necrotic apoptosis, and is therefore referred to as a pleiotropic cytokine. TNF-α increases T-lymphocyte killing capacity, exerts immune and inflammatory functions, and has a homeostatic effect in tumour immunotherapy [39]. Inflammatory cytokines can be reduced when anti-TNF-α therapy is given early in the disease process [40]. High levels of TNF-α reduce myocardial contractility, leading to chamber enlargement and heart failure, and inhibition of TNF-α may be beneficial in patients already experiencing impaired cardiac function. This hypothesis is supported by a short-term clinical study of TNF-α antagonists that showed improved cardiac function in patients with heart failure[41]. However, Andrew et al. found that TNF-α deficient mammals were unable to perform regular immune surveillance activities, leading to tumour immune escape[42]. Therefore, blocking TNF-α in antitumour therapy is not an ideal way to reduce myocardial injury induced by radiotherapy combined with PD-1 inhibitors. In the present study, we found that HMGB1, NF-κB p65, IL-1β, and IL-6 showed a tendency to increase and then decrease during irradiation combined with anti-PD-1 induced myocardial injury, suggesting that early blockade of inflammatory response may be more effective in reducing myocardial injury.

**Conclusions**

PD-1 inhibitors alone are less toxic to the myocardium, and radiation can cause myocardial fibrosis and injury. And when combined, PD-1 inhibitors enhance CD8 + T lymphocyte infiltration, promote HMGB1 and NF-κB expression, and increase the expression of myocardial inflammatory cytokines IL-1β, IL-6 and TNF-α. At the same time, the inflammatory response was intensified, and radiological myocardial injury and fibrosis were aggravated. The inflammatory response was more pronounced in early stage (1 month after irradiation) than in late stage (3 months after irradiation), and early blockade of the inflammatory response may be more effective in reducing myocardial injury.

**Abbreviations**
Declarations

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author upon request.

Ethics approval and consent to participate
Ethics approval and consent to participate were obtained from Guizhou medical university in Henan province.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
Authors' contributions

YL, BBW, YW, JB designed the study. YL, BBW, YW, JB SSZ GW acquired the data. YL, BBW, YW, JB SSZ GW contributed to analysis and interpretation of the data. YL, BBW, YW were involved in drafting the article and revising it for important intellectual content. FSS and BL were involved in funding acquisition. YXH, WWOY, ZNG,JW contributed to irradiation technical guidance. All authors approved the final manuscript.

References


Figure 1

Pathological changes of heart tissue 1 and 3 month after irradiation and/or PD-1 inhibitors treatment in each group. a: HE staining of heart tissue (×400). b: Masson staining of myocardial fibers in red and collagen fibers in blue, 1 and 3 months after heart irradiation (×100). c: Semi-quantitative analysis of cardiac collagen volume fraction (CVF). A: control, B: anti-PD-1, C: IR, D: IR + anti-PD-1. (ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).
Figure 2

TUNEL staining of heart tissue at 1 and 3 months after irradiation. A: control, B: anti-PD-1, C: IR, D: IR + anti-PD-1
Figure 3

Semi-quantitative analysis of cardiomyocyte apoptotic index (AI). ns $P > 0.05$, $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$, $^{****} P < 0.0001$. 
Figure 4

Flow cytometry of cardiac T lymphocytes from various groups of mice after irradiation.
Figure 5

Expression of T-lymphocytes and inflammatory factors in the myocardium of mice after irradiation. The data of 1 month derived from our previously published article[14]. a-c: Percentage of CD3+, CD4+, CD8+ T-lymphocytes in the myocardium of 4 groups of mice at 1 and 3 months after irradiation. d-f: Expression levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α in the myocardium of mice in all groups of
mice at 1 and 3 months after irradiation. (ns P > 0.05, *P < 0.05, ** P < 0.01, ***P < 0.001, **** P < 0.0001)

Figure 6

Protein expression of myocardial HMGB1/TLR4 signalling pathway after irradiation of mice in each group. A: control, B: anti-PD-1, C: IR, D: IR+anti-PD-1.
Figure 7

Western Blot and RT-qPCR were performed to detect the myocardial tissues of irradiated mice. a-c: Relative expression of HMGB1/TLR4 signalling pathway proteins in myocardial tissues. d-f: Quantification of HMGB1, TLR4, NF-κB p65 mRNA. (ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$)