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ORIGINAL RESEARCH

The Effect of PD-1 Inhibitor Combined with Irradiation on HMGB1-Associated Inflammatory Cytokines and Myocardial Injury

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Purpose: To explore the effect of PD-1 inhibitors combined with irradiation on myocardial injury and the changes of HMGB1-associated inflammatory markers.

Methods: Four groups of five mice were used, each groupformed by randomly dividing 20 mice (group A control; group B PD-1 inhibitors; group C Irradiation; group D PD-1 inhibitors+irradiation; n = 5 for each). The mice were treated with either PD-1 inhibitors or a 15 Gy dose of single heart irradiation, or both. Hematoxylin-eosin staining assessed the morphology and pathology of heart tissue; Masson staining assessed heart fibrosis; Tunel staining evaluated heart apoptosis; flow cytometry detected CD3+, CD4+, and CD8+ T lymphocytes in heart tissues; enzyme linked immunosorbent assay evaluated IL-1β, IL-6, and TNF-α of heart tissue; Western blot and quantitative real-time PCR (qPCR) detected the expression of protein and mRNA of HMGB1, TLR-4, and NF-κB p65 respectively.

Results: The degree of heart injury, collagen volume fraction (CVF) and apoptotic index (AI) in groups B, C, and D were higher than group A, but the differences between the CVF and AI of group A and group B were not statistical significance ($P>0.05$). Similarly, the absolute counts and relative percentage of CD3+ and CD8+ T lymphocytes and the concentrations of IL-1β, IL-6, and TNF-α in heart tissue with group D were significantly higher than the other groups ($P<0.05$). In addition, compared with group A, the expression of protein and mRNA of HMGB1 and NF-κB p65 in other groups were higher, and the differences between each group were statistically significant while TLR4 was not. In addition, interaction by PD-1 inhibitors and irradiation was found in inflammatory indicators, especially in the expression of the HMGB1 and CD8+ T lymphocytes.

Conclusion: PD-1 inhibitors can increase the expression of HMGB1-associated inflammatory cytokines and aggravate radiation-induced myocardial injury.

Keywords: HMGB1, PD-1 inhibitors, radiotherapy, heart injury

Introduction

The number of cancer patients is increasing worldwide, and thoracic malignancies, including lung cancer, breast cancer, and esophageal cancer, account for more than 26% of malignant tumors.[1,2] Radiotherapy plays an essential role in the treatment of chest oncology, as a radical, adjuvant, or palliative method.[3,4] Immune checkpoint inhibitors have recently entered the immunotherapy mainstream with the widespread use of programmed death 1 (PD-1) inhibitors.[5] PD-1 inhibitors function by blocking PD-1/PD-L1 binding in order to restore T cell immunity and have a significant antitumor potential.[6] However, it is estimated that approximately 20% of tumor patients respond to PD-1

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axis blockers alone in clinical treatment, which suggests that immunotherapy alone is not sufficient to activate the immune system to perform anti-neoplastic effects.[7] Studies have confirmed that the abscopal effect, a evidence of synergistic effect of radiotherapy and anti-PD-1 therapy, shows that the combination of PD-1 inhibitors and radiotherapy can increase efficacy.[8,9] Considering the superimposed toxic side effects, they may affect patients' quality of life and even endanger lives, which is one of the major foci of attention, although patients may benefit from the combination therapy modality.

Radiation-induced myocardial injury (RIMI) is one of the leading causes of noncancer-related death.[10] The process of RIMI mainly refers to the changes of immune microenvironment included inflammatory cell activation and secretion of numerous cytokines.[11] A fatality rate of immune myocarditis led by anti-PD-1 treatment in cancer immunotherapy is up to 50%, though its incidence is low.[12,13] Researchers found that PD-1 inhibitors make the T cell more effective in fighting cancer but can also incur the fatal immune myocarditis which has a large number of CD8+ T cells in T cell infiltration foci of myocardial tissue under pathological examination, and the same phenomenon is observed in the animal models.[14,15] The study shows that PD-1 inhibitors increase the infiltration of CD8+ T lymphocyte in myocardium, and blocking CD8+ T lymphocyte can reduce myocardial under combined therapy.[16] However, the blocking is also likely to have a negative impact on tumor treatment effect, because PD-1 inhibitors and irradiation need CD8+ T lymphocytes to exert anti-tumor effects. Therefore, it is of great significance to explore the pivotal cytokines that regulate the myocardial injury induced by irradiation combined with PD-1 inhibitors for further accurate prevention of myocardial injury without reducing the anti-tumor effect.

High mobility group box-1 protein (HMGB1), a common damage-associated molecular pattern and intense pro-inflammatory factors, was heralded as the nuclear weapon in the immune arsenal.[17] HMGB1 can regulate the systemic immune response to boost the immune response or immunologic tolerance by acting on inflammatory cells. New inflammatory microenvironment mediated by HMGB1 plays an important role in inflammatory and traumatic diseases.[18,19] Moreover, it can produce different biological effects in myocardial injury caused by different etiologies.[20] Our previous study and foreign research have discovered that the combination group produced more severe cardiac injury by comparison with the radiotherapy group,[16] which demonstrated that PD-1 inhibitors aggravate inflammatory damage of RIMI. At present, whether HMGB1 participates in the process of the aggravation of RIMI by PD-1 inhibitors has not been reported until now. Owing to the difficulty with obtaining heart tissue from patients, we performed studies in mouse models and tried to find whether HMGB and its inflammatory cytokines participate in the process.

Materials and Methods

Animals

Animal experiment protocols were all approved by our institute's ethics committee. Twenty specific-pathogen-free C57BL/6 male mice, (aged 56 days and weighing 20–22 g), were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Animal care was in compliance with the Laboratory Animals-Guidelines for ethical review of animal welfare of China (GB/T 35892–2018), and animal experiments were approved by the Animal Experimental Ethics Committee of Guizhou Medical University (No. 1901009).

Major Instruments, Equipment, and Reagents

The Precise medical linear accelerator was from Elekta, Co. Ltd. (Stockholm, Sweden); the Navios flow cytometer was from Guangzhou Flow Biotechnology Co. Ltd. (Guangzhou, China); the multifunctional microplate reader was from BioTek Co. Ltd. (Winooski, VT, USA); the electrophoresis and blotting devices were from Beijing Orient Ruili Technology Co. Ltd. (Beijing, China); the chemiluminescence imaging instrument was from Shanghai Qinxiang Scientific Instrument Co. Ltd. (Shanghai, China); and the CFX96TM Real-Time PCR system was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The anti-mouse PD1 monoclonal antibody (catalogue number BE0146) was from BioXcell Co. Ltd. (West Lebanon, NH, USA); the HE staining and Masson staining kits were from Beijing Solarbio Science & Technology Co. Ltd. (Beijing, China); the apoptosis detection kit was from Shanghai Yisheng Bio-Technology

Co. Ltd. (Shanghai, China); the antibodies for flow cytometry were from Biogem Scarl (Ariano Irpino, Italy); and the ELISA kits were from Thermofisher (Shanghai, China).

Irradiation and PD-1 Inhibitors Treatment

Four groups of five mice were formed by randomly dividing 20 mice. Mice in group A (group control) were not given any treatment; mice in group B (group PD-1 inhibitors) were injected PD-1 inhibitors and given mock single heart irradiation; mice in group C (group irradiation) were given single heart irradiation of 15 Gy and injected with saline; mice in group D (group combination) were treated with PD-1 inhibitors plus a single heart irradiation of 15 Gy. Mice in groups C and D were fixed on the accelerator and surrounded by a 1-cm wax mold after anesthesia. The thoraxes of mice underwent a vertical 0-degree single-field irradiation of the chest with 6 MV X-rays, at a dose of 15 Gy. The irradiation field was 2.0×2.5 cm; the source to skin distance was 100 cm, and the dose rate was 600 cGy/min. A spontaneous recovery process was allowed for the mice. PD-1 antibody of 200 μg was given intraperitoneally to the mice 7 days and half an hour before irradiation and 100 μg was given 7, 14, and 21 days after irradiation to mice in groups B and D. The same volume of saline was administered intraperitoneally to mice ingroups A and C.

Pathological Staining

Each group of mice were humanely killed 28 days after irradiation and the one third of the left atrium and ventricle were used for tissue embedding, which were sliced into 5 μm sickness for HE staining, Masson staining and TUNEL staining respectively.

HE Staining

HE staining was used to observe the morphology of cardiomyocytes in the excised heart. According to routine protocols, after deparaffinization and rehydration, tissue sections were stained with hematoxylin solution and eosin solution respectively. Then the sections were dehydrated by graded alcohol and cleared in xylene.

Masson Staining

Masson staining was performed following standard protocols. A red stain was used to stain myocardial fibers, while a blue stain was used to stain collagen fibers. Under an optical microscope, each section selected five random fields and five sections were analyzed in total. With ImageJ software,[21] a semi-quantitative analysis was conducted to calculate collagen volume fractions (CVF) by dividing collagen area by the total area of each specimen.

TUNEL Staining

Apoptosis detection kit instructions were followed when performing the TUNEL assay and the stained cells were observed by fluorescent microscopy. The DNA of all cells was stained with DAPI to emit blue light, and apoptotic cells were stained with FITC to emit green light. Under a fluorescence microscope, each section selected five random fields and five sections were analyzed in total. Apoptotic index (AI), the TUNEL-positive rate, which was equal to the number of TUNEL-positive cells divided by the total cell number × 100%. The numbers of cells were calculated by ImageJ-5.0 software.

Flow Cytometry

The percentages of CD3, CD4 and CD8 lymphocytes were calculated using flow cytometry. 30 mg of myocardial tissue was prepared into a single-cell suspension by enzymatic digestion, lysed with erythrocyte lysate, washed and resuspended in PBS, and incubated in the dark with anti-mouse CD3 antibody conjugated to APC, anti-mouse CD4 antibody conjugated to FITC, anti-mouse CD8 antibody conjugated to PE. Flow cytometry was performed after 30 minutes. Data were analyzed with FlowJo_V10 software.

ELISA

Tissue lysates were assayed for mouse IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) as directed by the manufacturer. Microplate readers were used to measure OD value at 450 nm and sample concentrations were calculated in accordance with the standard curve equation.

Western Blot Analysis

25 mg of myocardial tissue was lyzed in ice-cold RIPA buffer, supplemented with a Protease inhibitor cocktail, sonicated for 30 seconds and collected the supernatant after centrifugation. The concentration of lysate protein was measured by BCA protein assay. After being denatured at 100 °C for 10 minutes in loading buffer, protein samples were electrophoresed. Wet-blotting was used to transfer proteins after electrophoresis, followed by blocking in 5% low-fat milk in TBS-T buffer. Membranes were incubated with primary antibodies diluted in at 4 °C overnight. HRP-conjugated secondary antibodies were diluted in 5% low-fat milk in TBS-T buffer at room temperature for 60 min and exposed with a Clinx using the ECL method.

qPCR Analysis

mRNA expression was analyzed by the Real-time Quantitative PCR Detecting System (qPCR). A CFX96TM Real-Time PCR system was used for qPCR amplification of reverse-transcribed cDNA from total RNA. Normalizing mRNA levels to the GAPDH expression, relative gene expression levels were calculated using 2-ΔΔCt. Cycling conditions were set as follows: pre-incubation, 1 cycle of 95 °C for 5 min; amplification, 40 cycles of 95 °C for 10s, 60 °C for 10s and 65 °C for 30s. The following primers were used: GAPDH (forward 5'-GGTTGTTCTCCTGCGACTTCA-3'; reverse 5'-TGGTCCAGGGTTTCTTACTCC -3'), HMGB1 (forward 5'-AGCACAAGAAGAAGCACCCG-3', reverse 5'-ACGAGCCTTGTCAGCCTTTG-3'), TLR4 (forward primer 5'-GCCATCATTATGAGTGCCAATT-3', reverse 5'-AGGGATAAGAACGCTGAGAATT-3'); NF-κB p65 (forward 5'-AGACCCAGGAGTGTTCACAGACC-3', reverse 5'-GTCACCAGGCGAGTTATAGCTTCAG-3').

Statistical Analysis

Mean and standard deviation (SD) were used to show results. Using two-way factorial ANOVA analyzed the interaction of PD-1 inhibitors and irradiation, followed by post-hoc Bonferroni tests. With $P<0.05$ considered statistically significant. Statistical analyses was done using IBM SPSS Statistics, Version s3.0 and Graphpad Prism Windows, Version 8.2 was used to make the graphs.

Results

Pathological Characteristics of Heart Tissue

HE Staining

The samples from group A mice presented the orderly arrangement and clear structure of myocardial cells, where no abnormalities were observed in the cardiomyocytes. In group B, the heart tissue was in mild disorder in the partial structure, but their arrangements were relatively well ordered. In group C, a perturbed cell structure was seen, with cytoplasmic vacuolation. In group D, the normal structure was significantly destroyed and the arrangement was more disorderly than the other groups. Besides that, rupture of myocardial fibers and inflammatory cell infiltration were discovered (Figure 1).

Masson Staining

Masson staining of group A and group B showed that the heart interstitial tissue did not exhibit significant collagen fiber deposition. And there was a small amount of collagen fiber in group C. By comparison with other groups, collagen fibers in the heart interstitium and around the vessel were more evident in group D (Figure 2).

Semi-quantitative analysis of collagen in heart tissue presented that the CVF of groups A, B, C, and D were 1.89 ±0.56, 2.68±0.22, 5.50±0.51, and 6.91±0.43%, respectively. Two-factor ANOVA presented that mice treated with combination modality did not see significant interaction in CVF ($F1,16 = 1.889$, $P = 0.188$). $P = 0.150$ for comparison of CVF between groups A and B suggested that they was no statistical significance, while group D was significantly higher by comparison with groups A, B and C ($P<0.01$) (Figure 3).

TUNEL Analysis

AI in the heart of mice in groups A, B, C, and D were 1.76±0.43, 2.58±0.36, 7.56±0.71, and 8.81±0.52%, respectively. Two-factor ANOVA presented that mice treated with combination modality did not see significant interaction in AI

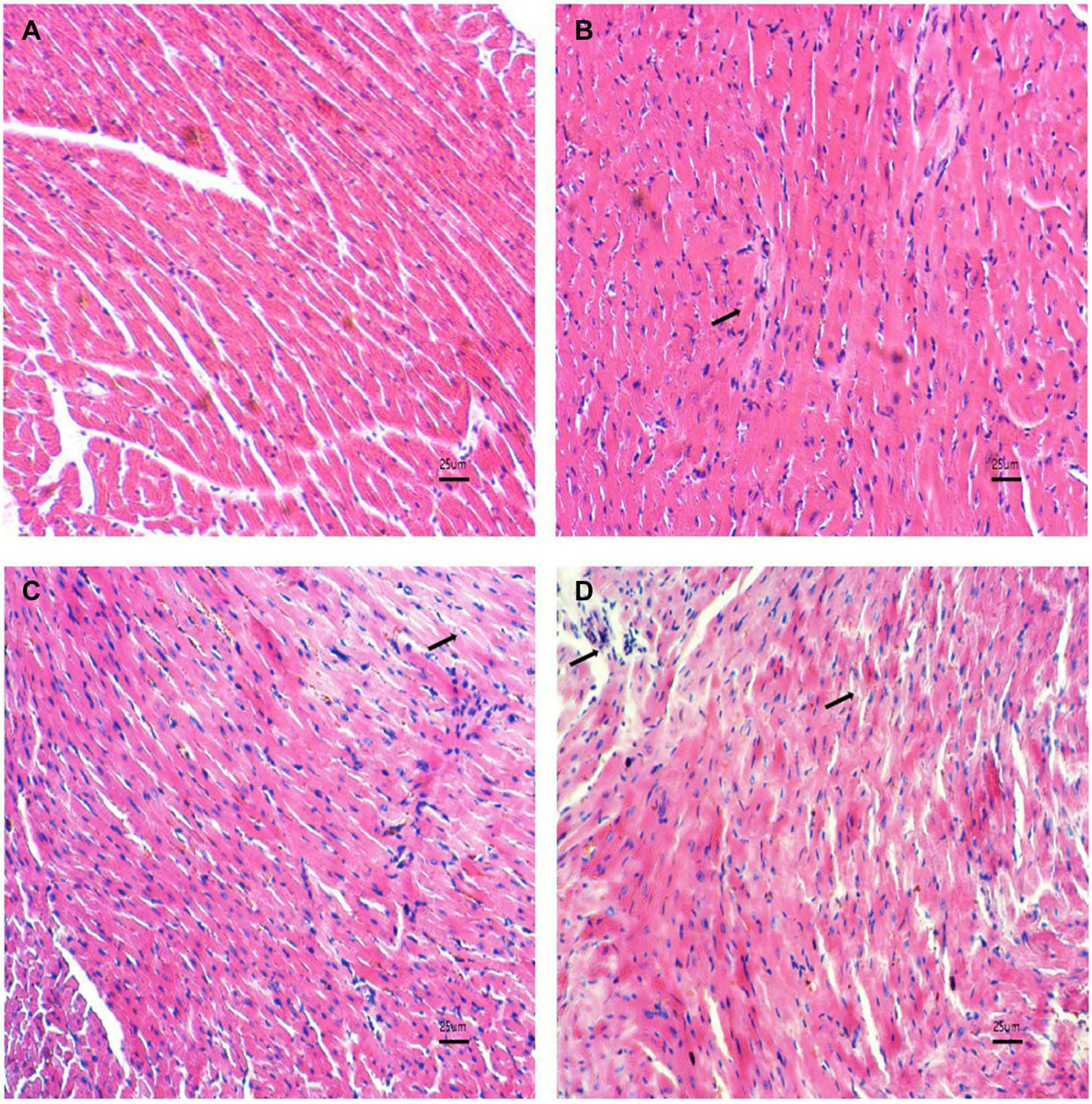

Figure 1 HE staining of heart tissue 1 month after irradiation and/or PD-1 inhibitors treatment in each group. "↑" shows cardiac structural impairments and/or lymphocyte aggregation. (**A**) Control; (**B**) PD-1 inhibitors; (**C**) Irradiation; (**D**) PD-1 inhibitors + irradiation.

($F_{1,16} = 0.674$, $P = 0.424$). Figures 4 and 5 showed that, compared with group A, apoptotic cells in other groups were higher and the differences of groups C and D were statistically significant ($P<0.0001$), however, there was no statistical significance with the differences between groups A and B ($P = 0.248$). In addition, post-hoc analysis presented that group combination was significantly higher by comparison with group irradiation and PD-1 inhibitors ($P<0.05$).

Distribution of T Lymphocyte Subsets

Relative percentages of CD3+, CD4+, and CD8+ T lymphocytes are present in the heart tissue of the four groups in Figures 6 and 7 and absolute count of CD3+, CD4+, and CD8+ T lymphocytes in the four groups were recorded (Table 1). No matter in relative percentage or absolute count of T lymphocyte subsets, two-factor ANOVA presented that mice treated with combination modality

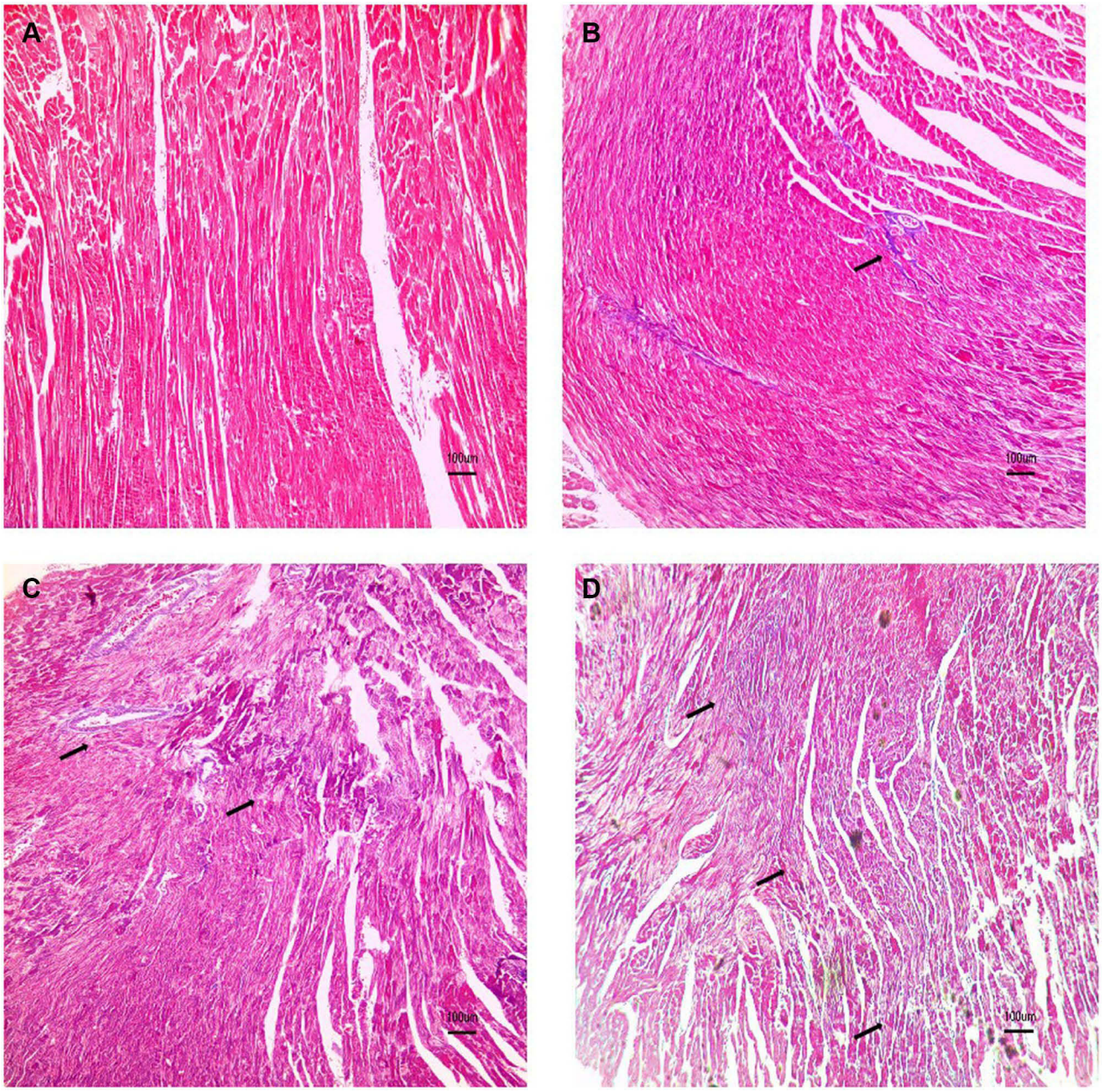

Figure 2 Masson staining of heart tissue 1 month after irradiation and/or PD-1 inhibitors treatment in each group. "↑" shows collagen fibers. (**A**) Control; (**B**) PD-1 inhibitors; (**C**) Irradiation; (**D**) PD-1 inhibitors + irradiation.

saw significant interaction with CD3+, CD8+ T lymphocytes (CD3+: relative percentage: $F_{1,8} = 48.796$, $P<0.001$, absolute count: $F_{1,8} = 156.260$, $P<0.0001$; CD8+: relative percentage: $F_{1,8} = 7.664$, $P<0.05$; absolute count: $F_{1,8} = 7.898$, $P<0.05$), while they did not in CD4 (relative percentage: $F_{1,8} = 0.357$, $P = 0.567$; absolute count: $F_{1,8} = 0.047$, $P=0.835$); post-hoc analysis in CD3+, CD8+ T lymphocytes presented that group D was significantly higher by comparison with other groups (CD3+: relative percentage: $P<0.001$, absolute count: $P<0.001$; CD8+: relative percentage: $P<0.001$, absolute count: $P<0.0001$).

Expression of Downstream Cytokine

IL-1β Expression

IL-1β concentrations in the heart of mice of groups A, B, C, and D were 145.24±8.46, 559.34±26.94, 635.13±25.18, and 864.96±22.20 pg/mL, respectively (Figure 8A). Two-factor ANOVA presented that mice treated with combination

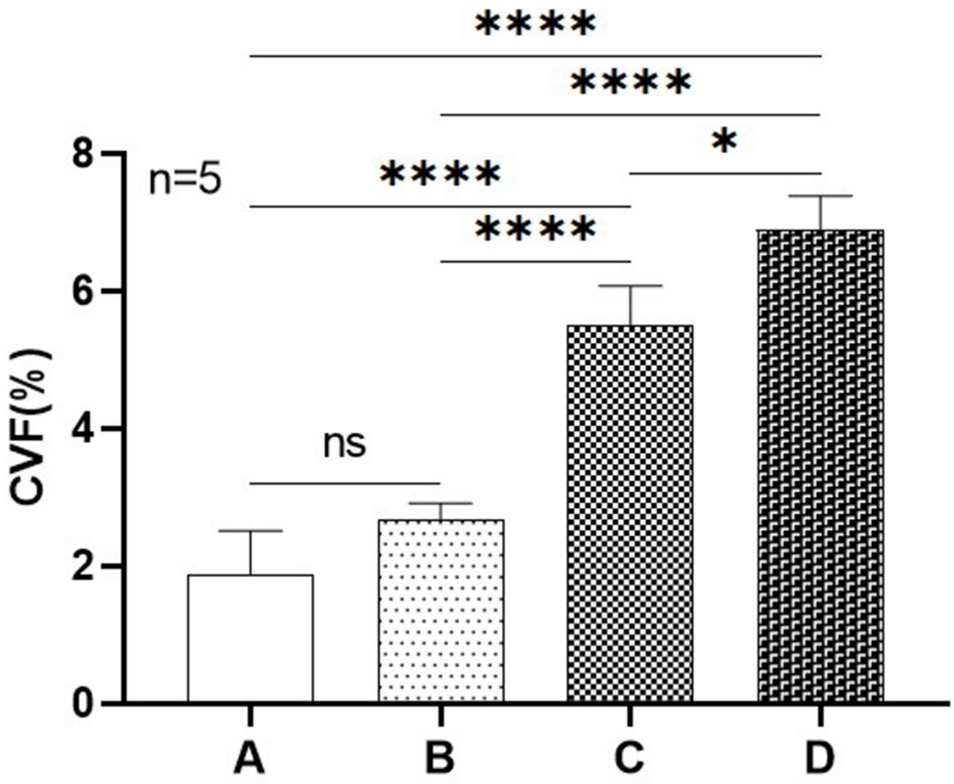

Figure 3 Collagen volume fraction in heart tissue in the four groups of mice (n = 5). (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, ***P<0.0001.

modality were recorded significant interaction (F1,8 = 26.470, P<0.001) in the IL-1β expressions. Post-hoc analysis presented that IL-1β expression in group D was significantly higher by comparison with group A, B and C. (P<0.001).

IL-6 Expression

The concentrations of IL-6 in the heart of mice in groups A, B, C, and D respective;y, were 123.18±7.80, 220.67±14.87, 276.95±11.36, and 340.29±15.27 pg/mL (Figure 8B). Two-factor ANOVA showed that the IL-6 expression of mice treated with PD-1 inhibitors (F1,8 = 60.226, P<0.0001) and irradiation (F1,8 = 174.029, P<0.0001) were significantly increased, but there was no significant interaction in combination modality (F1,8 = 2.715, P = 0.138). Post-hoc analysis presented that group D was significantly higher in IL-6 expression than the other three groups (P<0.05) and statistically significant differences could be found in the comparison between groups ($p < 0.001$).

TNF-α Expression

TNF-α concentrations in the heart of mice in groups A, B, C, and D, respectively, were 313.03±8.38, 617.37±26.55, 803.88±22.42, and 1003.1±15.47 pg/mL (Figure 8C). Two-factor ANOVA presented that mice treated with combination modality witnessed significant interaction (F1,8 = 10.926, P<0.05) in the TNF-α expression. Post-hoc analysis showed that TNF-α expression in group D was significantly higher than the other three groups (P<0.001) and statistically significant differences could be found in the comparison between groups ($p < 0.001$).

Western Blot Analysis

HMGB1, TLR4, and NF-κB p65 protein in the heart of mice in groups A, B, C, and D by Western blot analysis are shown in Figure 9A and the expression is shown in Figure 9B–D, with 1, 1.48±0.07, 1.67±0.07, and 2.33±0.05 in HMGB1; 1, 1.11±0.06, 1.47±0.07, and 1.15±0.07 in TLR4; and 1, 1.27±0.04, 1.49±0.04, and 2.06±0.08 in NF-κB p65 recorded. Two-factor ANOVA presented that mice treated with a combination modality witnessed significant interactions in the proteins HMGB1 (F1,8 = 9.896, P<0.05) and NF-κB p65 (F1,8 = 12.198, P<0.001). Post-hoc analysis

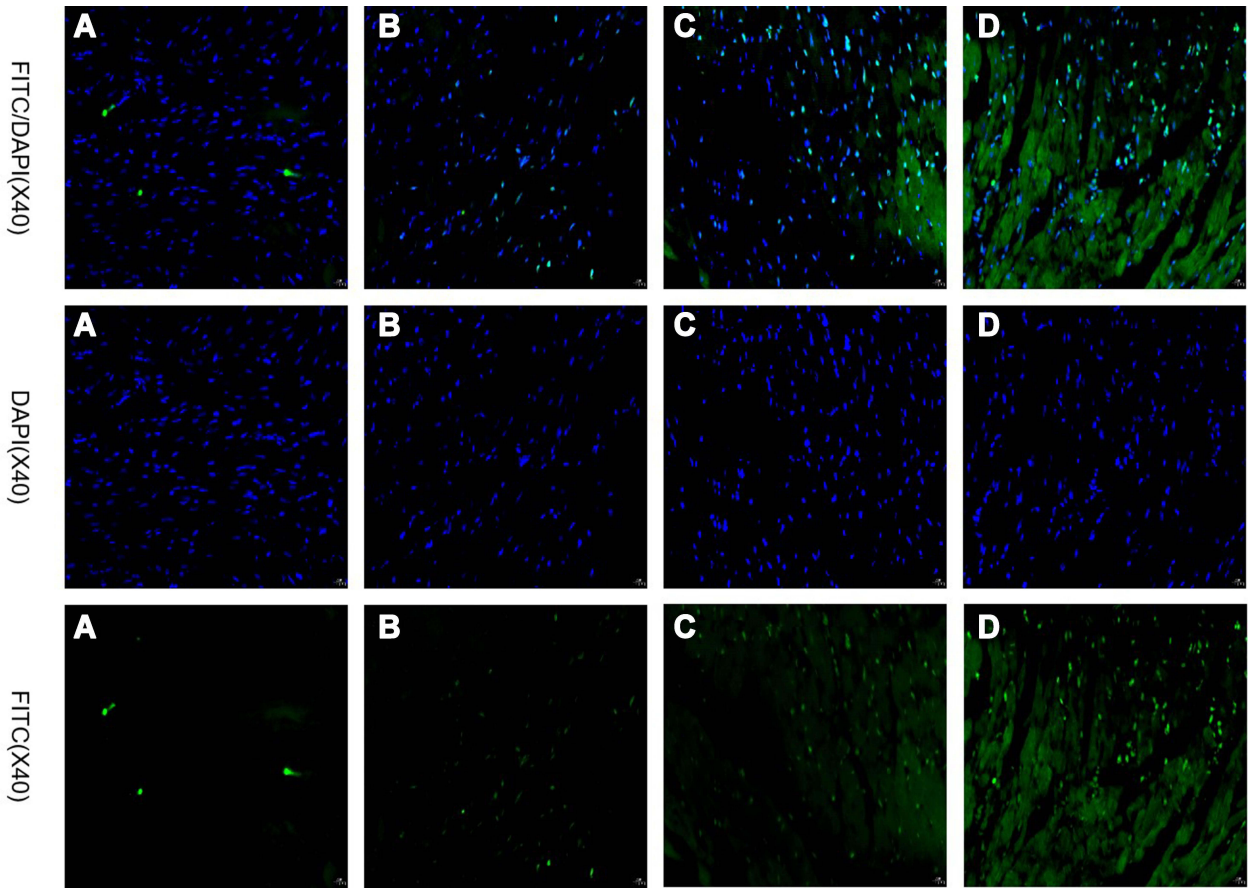

Figure 4 TUNEL staining of heart tissue 1 month after irradiation and/or PD-1 inhibitors treatment in each group. (**A**) Control; (**B**) PD-1 inhibitors; (**C**) Irradiation; (**D**) PD-1 inhibitors + irradiation.

showed that HMGB1 (P<0.0001) and NF-κB p65 (P<0.0001) protein in group combination therapy were significantly higher than other groups. Besides that, the significant differences of HMGB1 and NF-κB p65 protein expressions in each group could be found from the experimental data (P<0.05), however, there was no significant differences of TLR4 protein in each group (P>0.05).

qPCR Analysis

mRNA HMGB1, TLR4, and NF-κB p65 expressions in the heart of mice in groups A, B, C, and D are shown in Figure 10, with 1, 148±0.03, 1.64±0.05, and 2.47±0.04 in HMGB1; 1, 1.10±0.03, 1.15±0.06, and 1.18±0.04 in TLR4; 1, 1.25±0.02, 1.47±0.04, and 1.95±0.09 in NF-κB p65, recorded. Two-factor ANOVA presented that mice treated with a combination modality witnessed significant interaction in the mRNA of HMGB1 ($F_{1,8} = 5.664$, P<0.05) and NF-κB p65 ($F_{1,8} = 12.198$, P<0.01). Post-hoc analysis showed that HMGB1 (P<0.0001) and NF-κB p65 (P < 0.001) mRNA in group combination therapy were significantly higher than other groups. In addition to this, statistically significant differences existed in the four groups for HMGB1 and NF-κB p65 mRNA expression (P<0.05), while no statistically significant difference of TLR4 mRNA was discovered in the four groups (P>0.05).

Discussion

At present, the combined-modality treatment of radiotherapy with immunotherapy is increasingly being explored, but less attention to cardiac toxicity is reported in studies.[22–27] As a significant proinflammatory factor, HMGB1 and its signal pathway plays a crucial role in aggravating or repairing damage of heart diseases.[28] After ionizing radiation (IR) damage to cardiomyocytes, HMGB1 can be released and promotes a new immune microenvironment consisting of

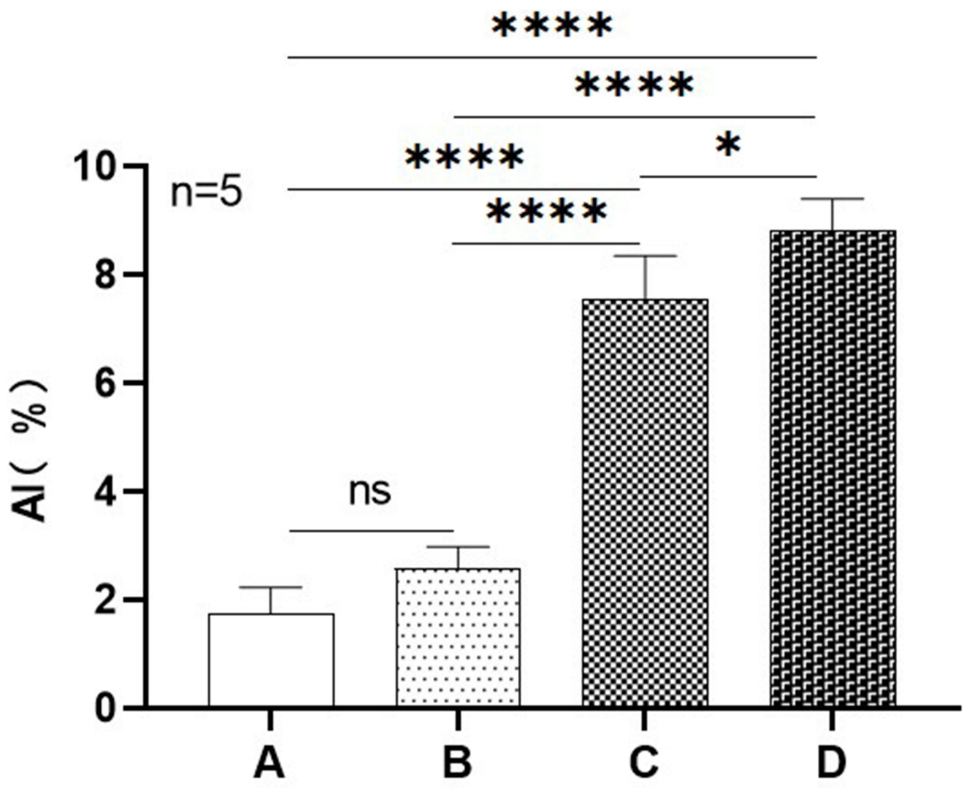

Figure 5 Apoptotic index in heart tissue in the four groups of mice (n = 5). (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, ***P<0.0001.

T lymphocytes and inflammatory cytokines, and PD-1 inhibitors which may aggravate RIMI through acting on them.[29,30] Therefore, we speculate that the HMGB1-associated inflammatory markers play an important role in the aggravation of RIMI by PD-1 inhibitors.

At the pathological level, either the results of HE, Masson or TUNEL staining, the morphological damage, CVF and AI conducted by treatment with PD-1 inhibitors alone are not much different in comparison to the control, while treatment with irradiation or/and PD-1 inhibitors present more severe damage and apoptosis in the results, suggesting that although PD-1 inhibitors can act on lymphocytes to start an immune response, it does not cause excessive inflammation in unhurt experimental models. HMGB1 will be passively released in the microenvironment to initiate an inflammatory response from necrotic cardiomyocytes damaged by radiation.[17] Apart from passive release, in the group with combination treatment modality, activated immune cells by PD-1 inhibitors synergistically induce HMGB1 in a positive way to aggravate the inflammatory response and myocardial damage.[17] In addition to inflammatory injury, HMGB1 has also been demonstrated that there are relative associations between HMGB1 with myocardial fibrosis and apoptosis. In the termed experimental autoimmune myocarditis animal models, injecting HMGB1 inhibitors can effectively reduce myocardial fibrosis and improve cardiac function.[31] Inhibiting the expression of HMGB1 can discover the myocardial apoptosis decrease in the doxorubicin-induced myocardial apoptosis model.[32]

According to the flow cytometry, when irradiation and PD-1 inhibitors are combined, CD8+ T lymphocytes are more likely to be infiltrated in heart injury than CD4+ T lymphocytes, which witness the same phenomenon as the other research.[16] Although the research has found that anti-CD8 antibody can reduce myocardial injury in combined treatment,[16] it may also decrease the effect of anti-tumors, which is not a win-win method. It is necessary to explore a crucial factor that can prevent myocardial damage without cutting down the anti-neoplasm in this therapy modality. We discover that the expression of HMGB1 and its relative inflammatory markers increase in the heart tissue treated with PD-1 inhibitors and irradiation. Meanwhile, PD-1 inhibitors and irradiation have significant interaction in the expression of HMGB1. In addition, Several studies have shown that intervention or blocking of HMGB1 and its receptors can reduce the production of CD8+

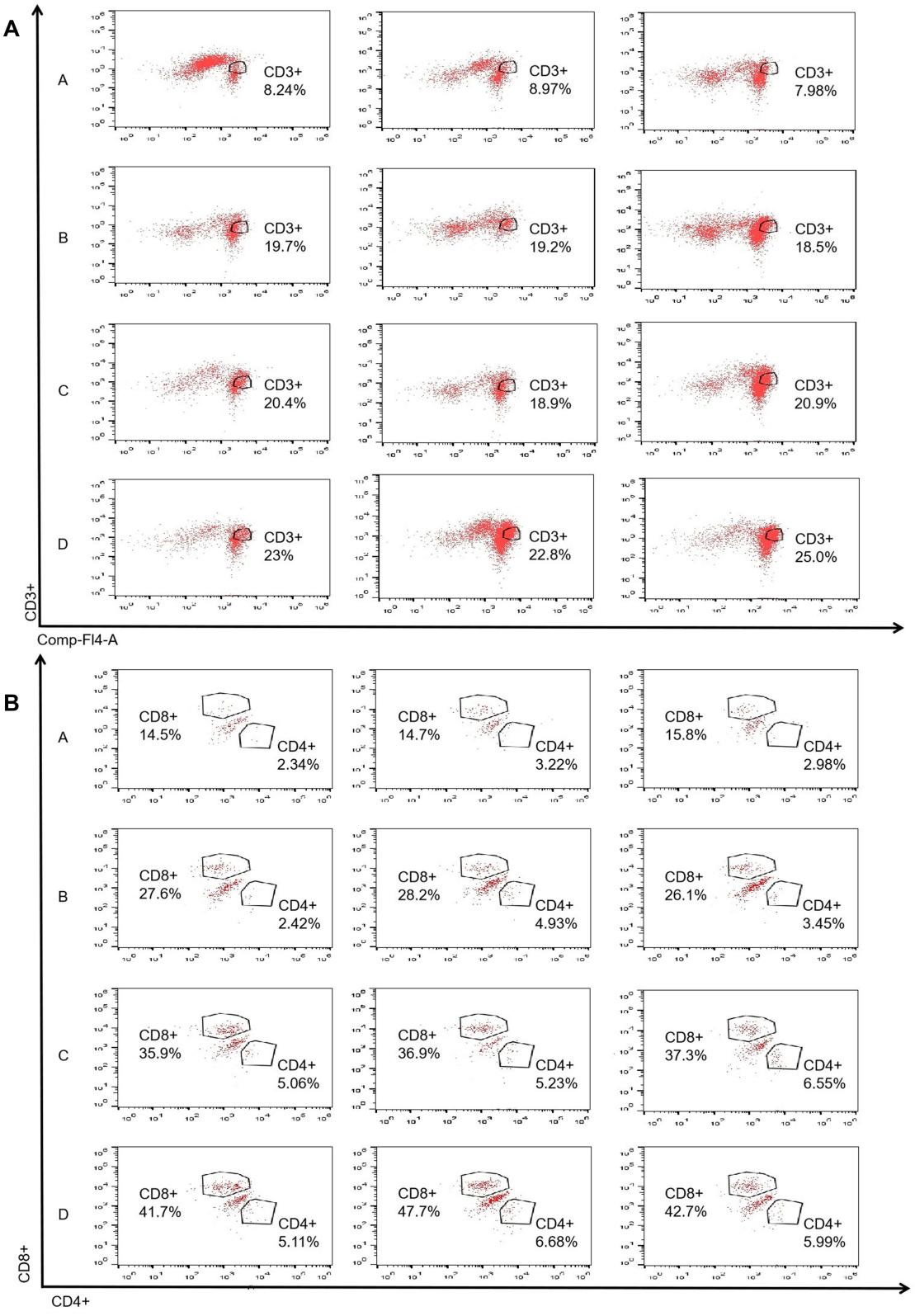

Figure 6 Distribution of T lymphocytes subsets in heart tissue in the four groups of mice. (**A**) Distribution of CD3+ T lymphocytes; (**B**) Distribution of CD4+, CD8+ T lymphocytes. (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation.

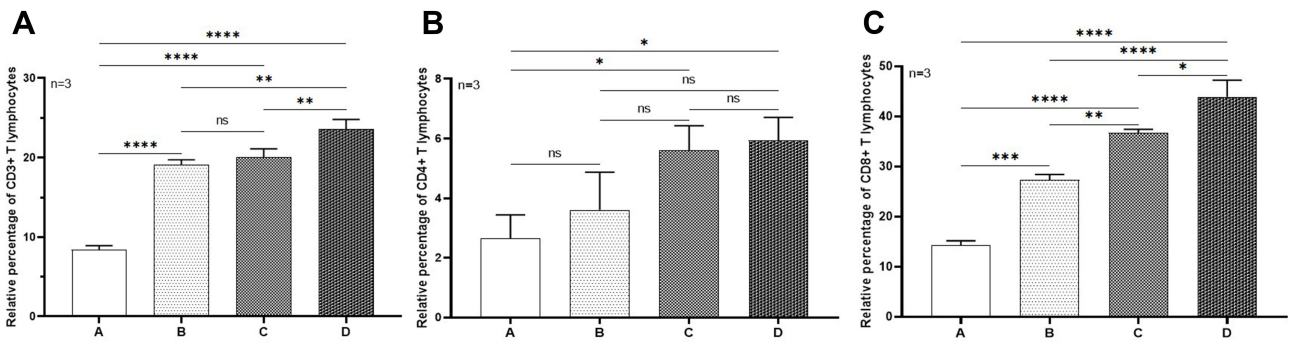

Figure 7 Relative percentage of T lymphocytes subsets in heart tissue in the four groups of mice (n = 3). (**A**) Relative percentage of CD3+ lymphocytes; (**B**) Relative percentage of CD4+ lymphocytes; (**C**) Relative percentage of CD8+ T lymphocytes. (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

T lymphocytes, inhibit tumor growth and metastasis, and improve the anti-tumor effect of PD-1 inhibitors.[33–37] Therefore, HMGB1 may be the ideal regulatory factor. However, the assessment of the mechanisms of myocardial injury induced by combined therapy is insufficient. There is a need for further research on HMGB1 and its mediated pathway after upregulation or downregulation in order to clarify the role in myocardial injury led by PD-1 inhibitors combined with irradiation.

As a significant transcription factor downstream of HMGB1, NF-κB p65 is closely related to the promotion of immune system development and immune cell function.[38] NF-κB p65 often exists in the cytoplasm in the form of a NF-κB complex (containing p50/p65 heterodimer and inhibitory IκB proteins), which is inactive in the absence of signaling. In the present study, HMGB1 was produced by irradiation and/or PD-1 inhibitors, combining with TLR4 and initiating the downstream signaling pathways, degrading IκB protein and releasing active NF-κB p65, which enters the nucleus to affect gene transcription involved in the inflammatory response.[39] In accordance with the expression of protein and mRNA, there is a positive correlation between the release of HMGB1 and the activation of NF-κB p65, supporting an intimate connection between the inflammation caused by irradiation and/or PD-1 inhibitors and this pathway. Interestingly, different interventions do not alter the expression of TLR4. This happens possibly because TLR4 does not change in the early stage of inflammation caused by radiation,[40] or it may only act as a transduction receptor in this experiment and does not expand.

After translocation into the nucleus, NF-κB p65 induces IL-1β, IL-6, and TNF-α to further promote inflammatory response. By identifying what cytokines are associated with RIMI plus PD-1 inhibitors, researchers may be able to find how to prevent and treat RIMI without compromising their antitumor properties. IL-1β activates T cells and other immune cells to produce inflammatory cytokines and chemokines, and facilitates a positive feedback loop of HMGB1 activation, which exacerbates inflammatory responses.[41–43] After IR for heart injury, IL-1β participates in the inflammatory response of arterial injuries led by radiation.[44] Blocking binding of IL-1β to its receptor may help to alleviate cardiac systolic dysfunction, counterbalance the longstanding deleterious effects of radiation on the arterial wall, and enhance the anti-tumor effect of PD-1 inhibitors.[45,46]

Table 1 Absolute Count of CD3+, CD4+ and CD8+ T-Lymphocytes in Each Group of Mice (Cells/μL, Mean±SD)

Groups	CD3+	CD4+	CD8+
Control	153±10.01	6±0.47	22±2.49
PD-1 inhibitors	429±7.41	12±1.63	117±5.73
Irradiation	465±16.11	26±2.16	171±8.96
PD-1 inhibitors+irradiation	534±11.43	32±3.40	234±12.28

Abbreviations: HMGB1, high mobility group box 1; qPCR, quantitative real-time PCR; CVF, collagen volume fraction; AI, apoptotic index; RIMI, radiation-induced myocardial injury; IR, ionizing radiation.

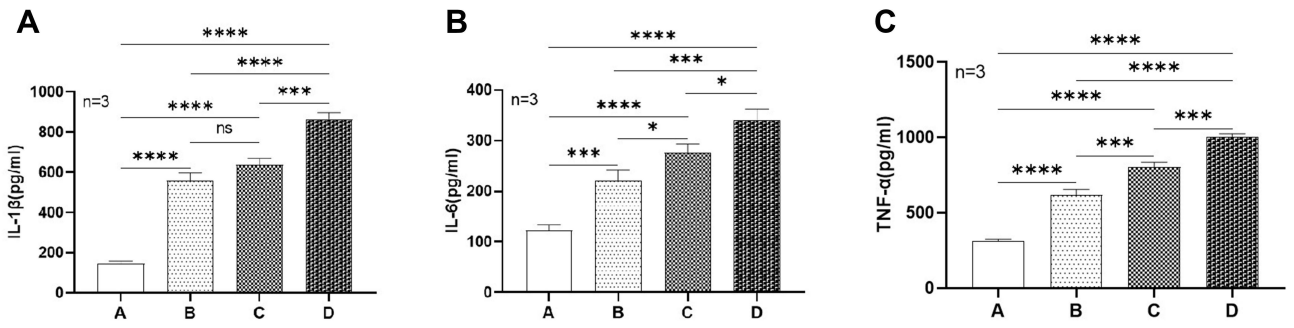

Figure 8 Concentrations of cytokine in heart tissue in te four groups of mice (n = 3). (**A**) IL-1β concentration; (**B**) IL-6 concentration; (**C**) TNF-α concentration. (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, ***P<0.001, ****P<0.0001.

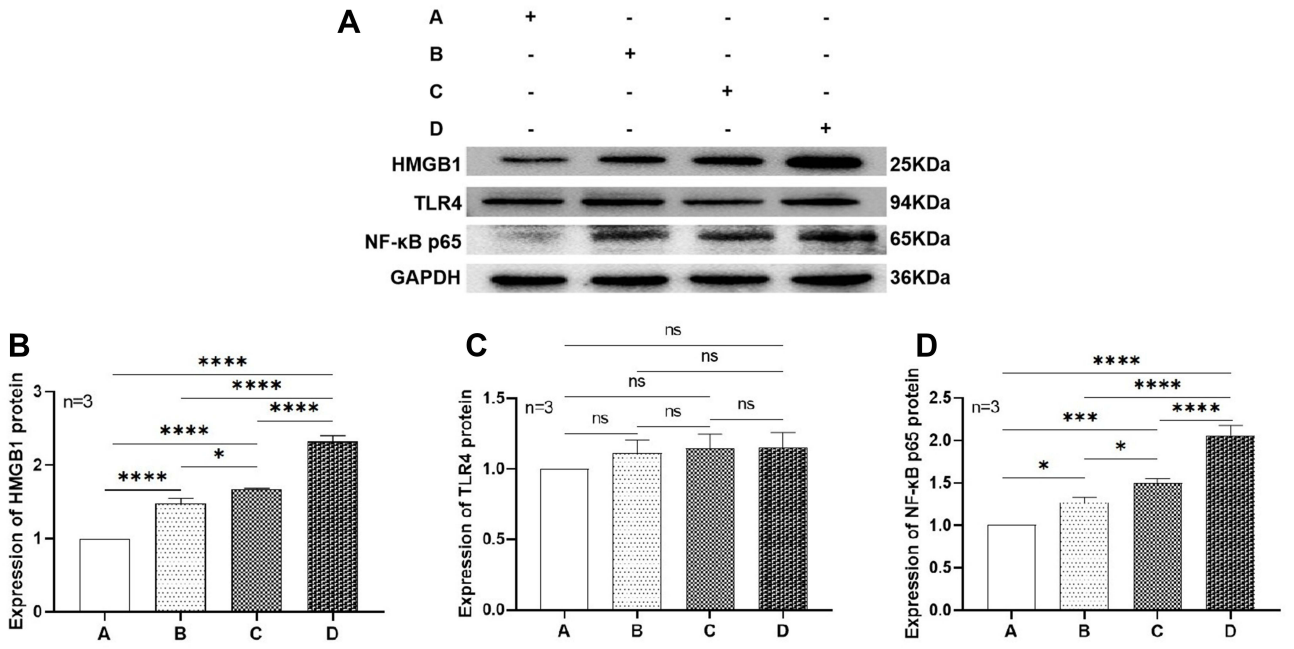

Figure 9 Blot and expression of relative protein in heart tissue in the four groups of mice (n = 3). (**A**) HMGB1, TLR4, NF-κB p65 protein; (**B**) expression of HMGB1 protein; (**C**) expression of TLR4 protein; (d) expression of NF-κB p65 protein. (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, ***P<0.001, ****P<0.0001.

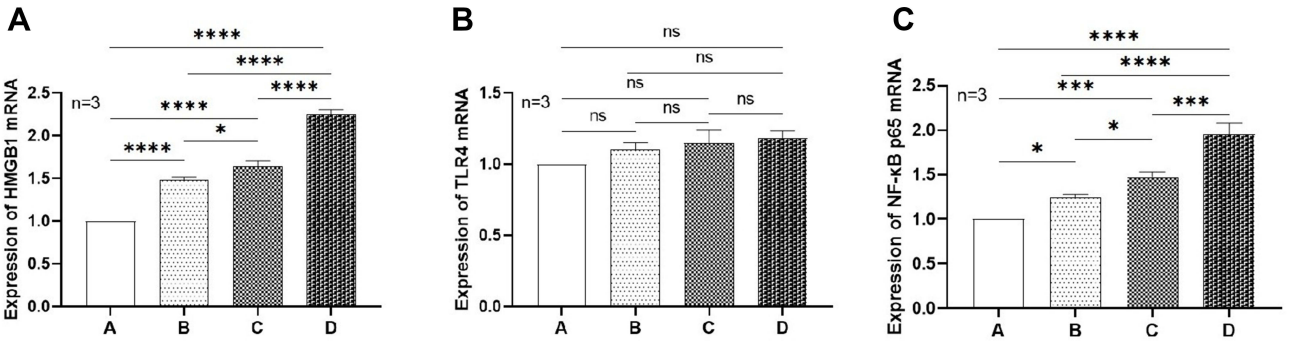

Figure 10 Expression of relative mRNA in heart tissue in the four groups of mice (n = 3). (**A**) Expression of HMGB1 mRNA; (**B**) Expression of TLR4 mRNA; (**C**) Expression of NF-κB p65 mRNA. (A) Control; (B) PD-1 inhibitors; (C) Irradiation; (D) PD-1 inhibitors + irradiation. nsP>0.05, *P<0.05, ***P<0.001, ****P<0.0001.

IL-6 is a typical pro-inflammatory factor in autoimmune and inflammatory diseases. For lethal myocarditis patients caused by immunotherapy, IL-6 can be detected in the plasma at an early stage of treatment.[47] The study shows that myocardial IL-6 levels increased significantly in the group of PD-1 inhibitors in the aggravation of RIMI. Specific antibodies against IL-6 receptors may offer hope for reducing myocardial injury from radiation combined with PD-1 inhibitors.[48] Moreover, IL-1β and IL-6 contribute to the occurrence, development, and metastasis of cancer.[49,50] Therefore, more patients with thoracic radiotherapy and immunotherapy may benefit from the combined use of anti-IL -1β and anti-IL-6 during treatment.

TNF-α plays multiple roles in tumor therapy. TNF-α is considered as a cardiotoxic factor, inhibiting cardiac contractility and inducing ventricular hypertrophy after heart injury led by radiotherapy.[51] Intervention in the expression of TNF-α can alleviate cardiomyocyte apoptosis and inhibit myocardial fibrosis.[52,53] However, TNF-α can enhance the tumor-killing effect of T cells in tumor immunotherapy. In mammals with a knockout of TNF-α, T cells cannot perform normal immune surveillance functions, resulting in the immune evasion of tumor cells.[54]

Conclusion

PD-1 inhibitors may increase the infiltration of CD8+ T lymphocytes in myocardial tissue and the expression of HMGB1, NF-κB P65, IL-1β, IL-6, and TNF-α through affecting the cardiac immune microenvironment. HMGB1 signaling axis may play the crucial role in the myocardial injury of a combination of PD-1 inhibitors and irradiation. The future direction of our research is to understand if the HMGB1 signaling axis changes are the primary mechanism contributing to the changes in the immune microenvironment.

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Disclosure

The authors report no conflicts of interest in this work.

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