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ATP protects anti-PD-1/radiation-induced cardiac dysfunction by inhibiting anti-PD-1 exacerbated cardiomyocyte apoptosis, and improving autophagic flux

Jing Wang [a,b], Jing Zhao [a], Zhijun Meng [a], Rui Guo [a], Ruihong Yang [b], Caihong Liu [a], Jia Gao [a], Yaoli Xie [a], Xiangying Jiao [a], Heping Fang [a], Jianli Zhao [c], Yajing Wang [c,*], Jimin Cao [a,**]

[a] Department of Physiology, Shanxi Medical University, Taiyuan, Shanxi 030001, China
[b] Department of Thoracic Radiotherapy, Shanxi Provincial Cancer Hospital, Taiyuan, Shanxi 030013, China
[c] Department of Biomedical Engineering, University of Alabama at Birmingham, AL 35294, USA

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ABSTRACT

The synergy between radiotherapy and immunotherapy in treating thoracic cancers presents a potent therapeutic advantage, yet it also carries potential risks. The extent and nature of cumulative cardiac toxicity remain uncertain, prompting the need to discern its mechanisms and devise effective mitigation strategies. **Radiation alone or in combination with an anti- Programmed cell death protein1 (PD-1) antibody significantly reduced cardiac function in C57BL/6J mice, and this pathologic effect was aggravated by anti-PD-1 (anti-PD-1 + radiation). To examine the cellular** mechanism that causes the detrimental effect of anti-PD-1 upon cardiac function after radiation, AC16 human cardiomyocytes were used to study cardiac apoptosis and cardiac autophagy. Radiation-induced cardiomyocyte apoptosis was significantly promoted by anti-PD-1 treatment, while anti-PD-1 combined radiation administration blocked the cardiac autophagic flux. Adenosine 5′-triphosphate (ATP) (a molecule that promotes lysosomal acidification) not only improved autophagic flux in AC16 human cardiomyocytes, but also attenuated apoptosis induced by radiation and anti-PD-1 treatment. Finally, ATP administration *in vivo* significantly reduced radiation-induced and anti-PD-1-exacerbated cardiac dysfunction. We demonstrated for the first time that anti-PD-1 can aggravate radiation-induced cardiac dysfunction via promoting cardiomyocyte apoptosis without affecting radiation-arrested autophagic flux. ATP enhanced cardiomyocyte autophagic flux and inhibited apoptosis, improving cardiac function in anti-PD-1/radiation combination-treated animals.

1. Introduction

Radiation therapy (RT), as one of the main treatments for cancer, plays an irreplaceable role in improving the therapeutic effect [1]. Unprecedented advances have been made in cancer treatment with the use of immune checkpoint blockade (ICB) [2,3]. Preclinical and

* Corresponding author.
** Corresponding author
E-mail addresses: yajingwang@uab.edu (Y. Wang), caojimin@126.com (J. Cao).

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clinical studies demonstrated that radiotherapy could synergize anti-programmed cell death protein 1 (PD-1) therapy in multiple manners during treated patients with cancer [4]. However, whether the combination may produce greater cardiological response is unclear. Radiation-induced cardiotoxicity (RICT) occurs following radiation to the thorax [5]. The heart can be adversely affected by irradiation treatment for patients with cancers of the breast, lungs, esophagus, and lymphomas [6]. Modern radiotherapy techniques may not have decreased cardiac toxicity even though they have reduced the exposure of the heart to radiation [7]. Research indicates that RICT remains the leading cause of non-malignant tumor-related mortality among cancer survivors [8]. Although immune checkpoint modulating antibodies have revolutionized clinical immunotherapy, cardiac injury has been implicated as a severe outcome in a subset of patients, and particularly myocarditis, can be associated with significant morbidity and mortality [9,10]. PD-1 knockout animal models have shown dilated cardiomyopathy [11], which indicates that PD-1 is associated with cardiomyopathy and cardiac dysfunction. Thus, it is essential to know whether and how PD-1 inhibition combined with radiation aggravates cardiotoxicity in order to determine the best therapeutic strategy for the patients.

The autophagic process maintains metabolic balance and cell homeostasis in cells [12], whilst the disturbance of this process may lead to an inappropriate apoptosis, and ultimately to cardiovascular dysfunction. Although radiotherapy can promote apoptosis to assist cancer therapy, the role of autophagy and apoptosis in radiotherapy combined immunotherapy on cardiomyopathy is still unclear and controversial [13]. Hence, it remains unclear whether apoptosis and autophagy are associated with myocarditis and cardiotoxicity caused by radiation or radiation combined with anti-PD-1 therapy.

Thus, the aim of this study is to examine the impact of combining PD-1 inhibition with radiotherapy on cardiac injury and to elucidate the underlying cellular mechanisms, particularly apoptosis and autophagy, within this combined therapeutic context. This investigation will establish an empirical foundation for devising efficacious treatment approaches aimed at mitigating cardiotoxicity resulting from the combination of PD-1 inhibition and radiation.

2. Material and methods

2.1. Animals model and in vivo treatment

The experiments of this study were performed in adherence to the guidelines for the Care and Use of Experimental Animals issued by the National Institutes of Health and approved by the Ethics Committee of Experimental Animals of Shanxi Medical University Committee on Animal Care. C57BL/6J (8–10weeks) mice were purchased and randomly assigned to various experimental groups. According to the experimental procedures described previously for animal radiation [14,15], the mouse was immobilized, and the heart was irradiated with 6 MeV-electron beam from the Varian Clinac iX linear accelerator (Varian, USA). Single local heart doses of 20 Gy (Gy) were applied, and the dose rate was 2 Gy/min [16]. To determine the degree of cardiac injury caused by irradiation, the radiation field was constrained within 11x11mm^2. Animals that were used as controls were exposed to zero Gy. Mice in the anti-PD-1 + radiation group were intraperitoneally (IP) injected with 200 μg of anti-PD-1 (Bio X Cell, NH, USA) [17] in 100 μl of phosphate buffer saline (PBS) at 2, 4 days before irradiation. A booster injection of 100 μg of antibody was then administered to the animals at the third and seven days following irradiation [18]. Mice in radiation + Adenosine 5′-triphosphate (ATP) group or anti-PD-1 + radiation + ATP group received intramuscular injections of ATP (6 mg/kg, KOAISEI Co., Japan) after radiation for 14 days [19,20]. The autophagic flux was assessed with or without the presence of Bafilomycin A1 (BafA1) (1.5 mg/kg, IP) (MedChemExpress, USA) [21].

2.2. Echocardiography assessment of cardiac function

Transthoracic echocardiography was performed using the GE Vivid 7 Pro System equipped with a 13-MHz probe (General Electric Company, USA) as previously described [22]. After the mice were anesthetized, acoustic coupling gel was applied to the chest after hair removal. Gains were adjusted to eliminate background noise, and five to 10 cycles were recorded to ensure accurate readings. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were obtained by an ultrasonic instrument analysis system.

2.3. Cell culture and treatment

AC16 human cardiomyocyte cell line(ATCC, USA) (4–8 passages) received radiation as previously described with some modifications [23]. To adjust the X-Ray to suit cell challenges, the angle of the gantry of the Varian Clinac iX linear accelerator was rotated 180°, then 6 MV-XRays were applied externally to the cells. The dose of radiation was 8 Gy, and the dose rate was 2 Gy/min. The control cells were exposed to 0 Gy at a dose rate of 0 Gy/min. At the end of radiation, cells were received different treatment in different groups: anti-PD-1(1 μg/ml) + radiation group; radiation + ATP (50 μmol/L) group; anti-PD-1 + radiation + ATP group. Apoptosis was determined by flow cytometry and Caspase 3 activation. Bafilomycin A1 (BafA1) is an inhibitor of autophagosome-lysosome fusion [24]. To evaluate whether autophagy is involved in the process of anti-PD-1 and radiation induced injury, autophagic flux was determined with or without BafA1 (100 nmol/L) administration 4 h prior to radiation, and then followed by the same treatment to determine the autophagy flux state [25,26].

2.4. Western blot analysis

Western blot assay was conducted as described in the previous study [27]. Briefly, 50 μg total proteins per sample were separated

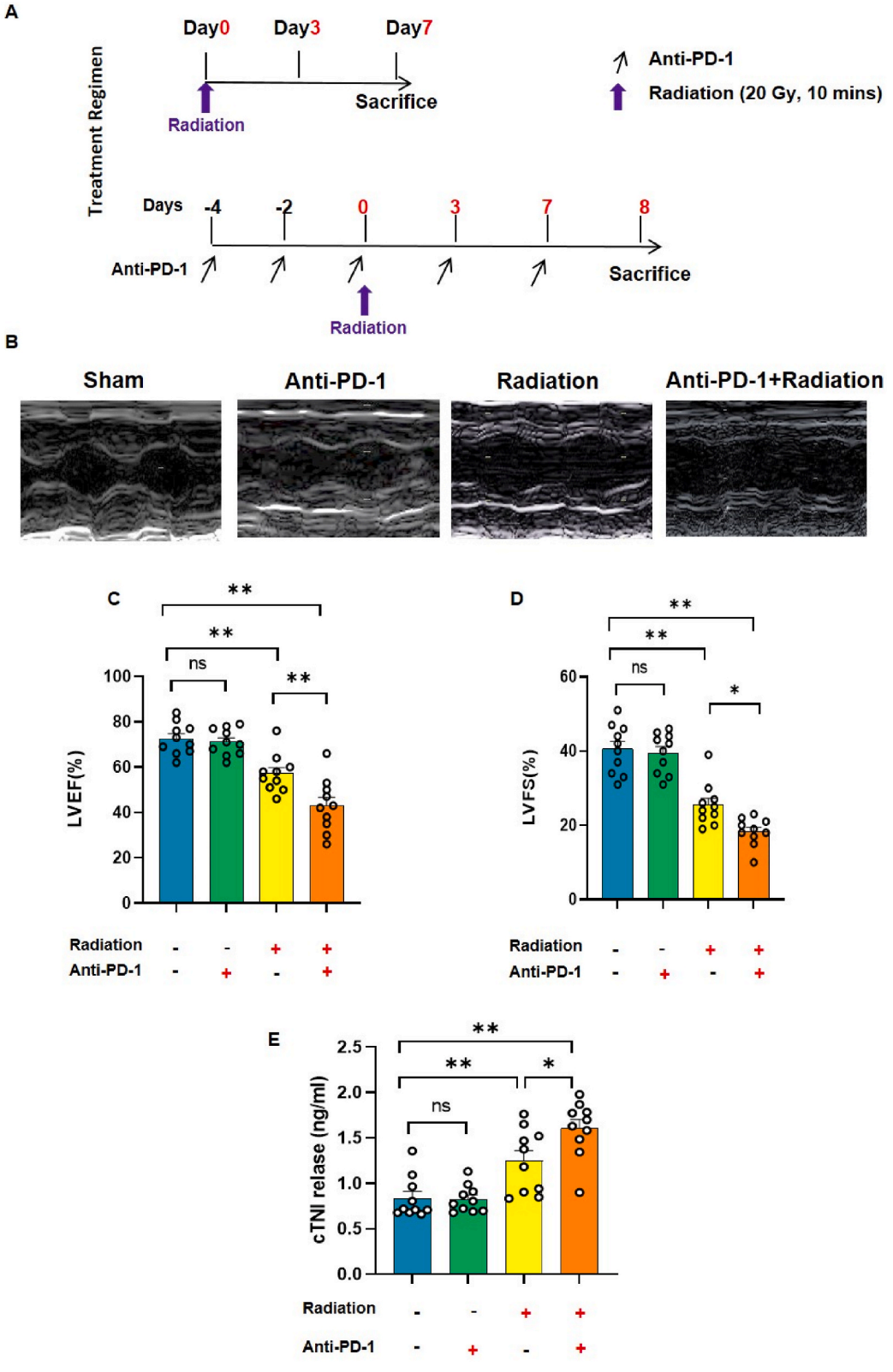

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Fig. 1. Anti-PD-1 combined radiotherapy deteriorates cardiac function. **A** Treatment Regimen in mice model. Mice received anti-PD-1 antibody (black arrow) and 20 Gy of radiation (red arrow) exposure. **B** Representative echocardiogram recording image after radiation and radiation with anti-PD-1. **C** Bar graph analysis of left ventricle ejection fraction. **D** Bar graph analysis of Left ventricle of fraction shortening. n = 10 mice/group. **E.** Plasma cTnI in different experimental groups. $^{*}P < 0.05$. $^{**}P < 0.01$. LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene difluoride (PVDF) member and incubated with primary antibodies against microtubule-associated protein 1 light chain 3 (LC3) (1:1000, Cell Signaling Technology, USA), sequestosome1 (SQSTM1/p62) (1:1000, Cell Signaling Technology, USA), lysosome-associated membrane protein 2 (LAMP2) (1:1000, Cell Signaling Technology, USA), cathepsin D (CTSD) (1:1000, Abcam, USA) which is used to measure the autophagic flux; cysteine aspartic acid specific protease-3 (Caspase-3) (1:1000, Cell Signaling Technology, USA), Cleaved Caspase-3 (1:1000, Cell Signaling Technology, USA), which is used to determine apoptosis; B-cell lymphoma-2 (Bcl-2) (1:1000, Abcam, USA), BCL2-associated X (Bax) (1:1000, Cell Signaling Technology, USA), which used to evaluate the mitochondrial apoptosis pathway; and the internal loading control reference, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam, USA) overnight at 4 °C, followed by secondary HRP-conjugated antibody. The images were captured on the ChemiDoc (Bio-Rad) and quantified by densitometry (Image Lab).

2.5. Detection of lysosome pH

LysoSensor Green DND-189 (Molecular Probes Invitrogen Detection Technologies, Eugene, OR, USA) was used to determine the pH of acidic lysosomes according to the manufacturer's instructions [28]. Briefly, LysoSensor probes were mixed with medium to obtain the final working solution. Prewarmed (37 °C) LysoSensor probe-containing medium was added to the cells. After incubation for 1h at 37 °C, the cells were examined by fluorescence microscopy (Leica). The images were analyzed using Image J (NIH).

2.6. Flow cytometry

For apoptosis detection, cells were washed twice with PBS and stained with Annexin V-FITC and propodium iodide (KeyGEN, Jiangsu, China). Cells were incubated in the dark for 15 min at room temperature. The apoptotic rate was measured by flow cytometry [29]. For cell membrane protein detection, cells were digested and collected. After washing with PBS, cells were incubated with Programmed cell death- Ligand 1 (PD-L1) (Sino, Beijing, China) and PD-1 antibodies or IgG negative control (Elabscience, Wuhan, China) in the dark for 30 min at room temperature. Data (10000 events) were acquired on a BD flow cytometer (BD Biosciences, San Diego, CA) and analyzed with Flowjo software (FlowJo LLC, Ashland, OR).

2.7. Cell Counting Kit-8 cell (CCK8) viability assay

After exposure to radiation for 24 h, AC16 were seeded into 96-well plates at a density of 1×10^3 cells/well. The conditioned medium was aspirated, and 100 μl fresh CCK8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) with serum-free Roswell Park Memorial Institute (RPMI)-1640 medium was carefully added to each well. According to the manufacturer's protocol, the plates were then incubated at 37 °C for 0.5–4 h in the dark. The absorbance was periodically measured at 450 nm using a microplate reader (Bio-Tek) [30].

Mouse cardiac troponin I (cTnI) enzyme-linked immunosorbent assay.

Serum myocardial injury marker, plasma cTnI was detected with ELISA (Bio-Swamp, China) by following the manufacturer's instruction.[31]

2.8. Immunohistochemistry

Paraffin-embedded sections were deparaffinized, and antigen retrieval was carried out using Sodium Citrate Antigen Retrieval Solution. After blocking with 5% bovine serum albumin (BSA), the slices were incubated overnight at 4 °C in a wet box with LC3 (1:500, Cell Signaling Technology, USA), p62 (1:250, Cell Signaling Technology, USA) and cleaved Caspase 3 (1:1000, Cell Signaling Technology, USA) antibodies. The corresponding secondary antibody was incubated. The images were acquired via a microscope (Leica, Buffalo Grove, IL).

2.9. Statistical analysis

Data were expressed as mean ± SEM. Statistical differences between groups were determined using one-way ANOVA followed by Tukey's post hoc multiple groups comparisons. Student's t-test was performed to make a comparison between two groups. All data were analyzed via GraphPad Prism 9.0 software. A P value < 0.05 was considered to be of statistical significance.

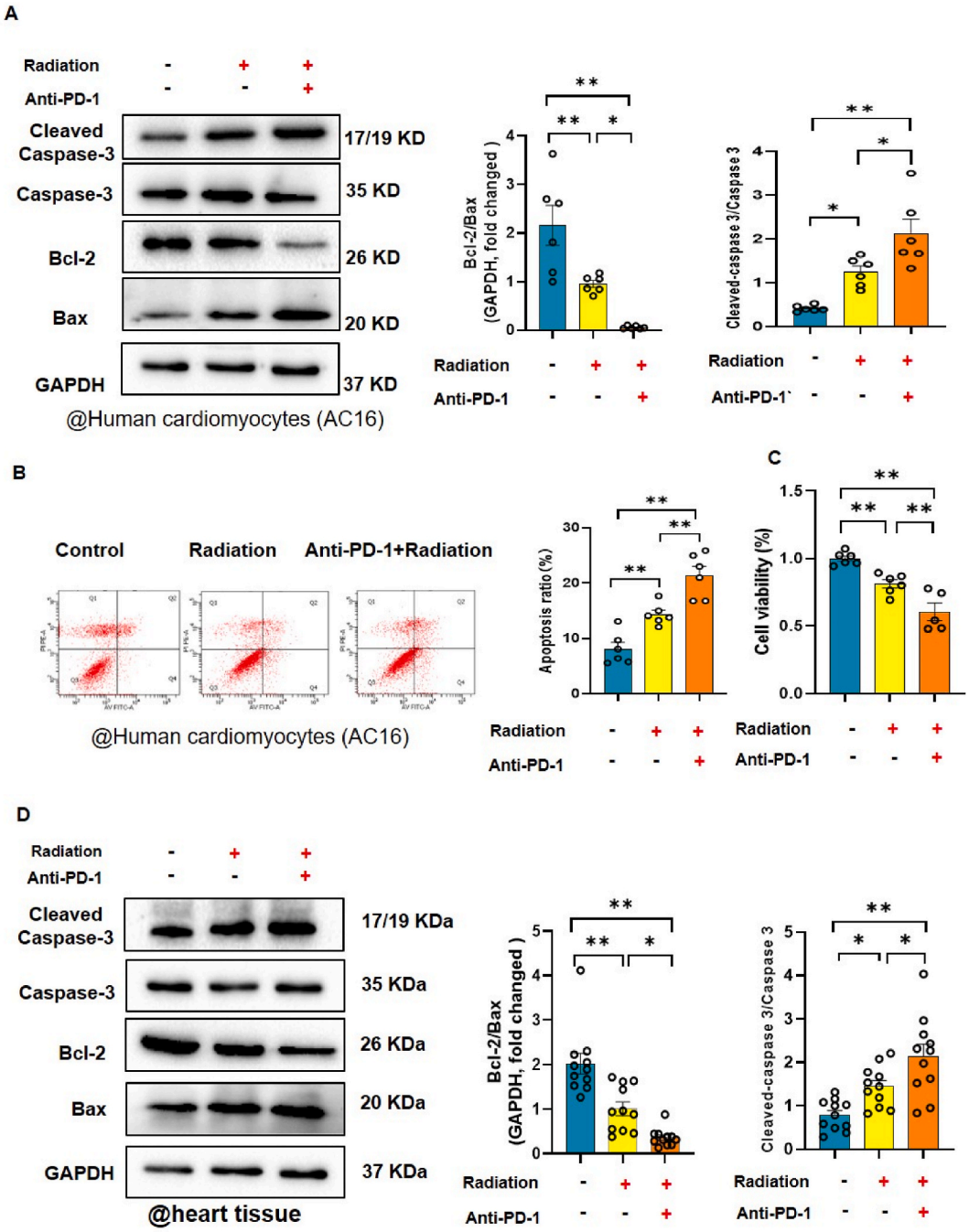

Fig. 2. Combined challenges of Anti-PD-1 antibody and radiation aggravates cardiomyocytes apoptosis. **A** Representative Western blots and density analysis for Bcl-2, Bax, Cleaved-caspase3 and Caspase3 in AC16 human cardiomyocytes after challenges of radiation or radiation combined with anti-PD-1. **B** Apoptotic rate analyzed by flow cytometry with Annexin V-FITC/PI staining. **C** Quantitative analysis of AC16 human cardiomyocytes cell viability. **D** Representative Western blots and density analysis for Bcl-2, Bax, Cleaved-caspase3, and Caspase3 from C57BL/6 mice's heart tissue treated with radiation and radiation combined with anti-PD-1. $^{*}P < 0.05$; $^{**}P < 0.01$; ns: Negative significance. GAPDH: glyceraldehydes 3-phosphate dehydrogenase.

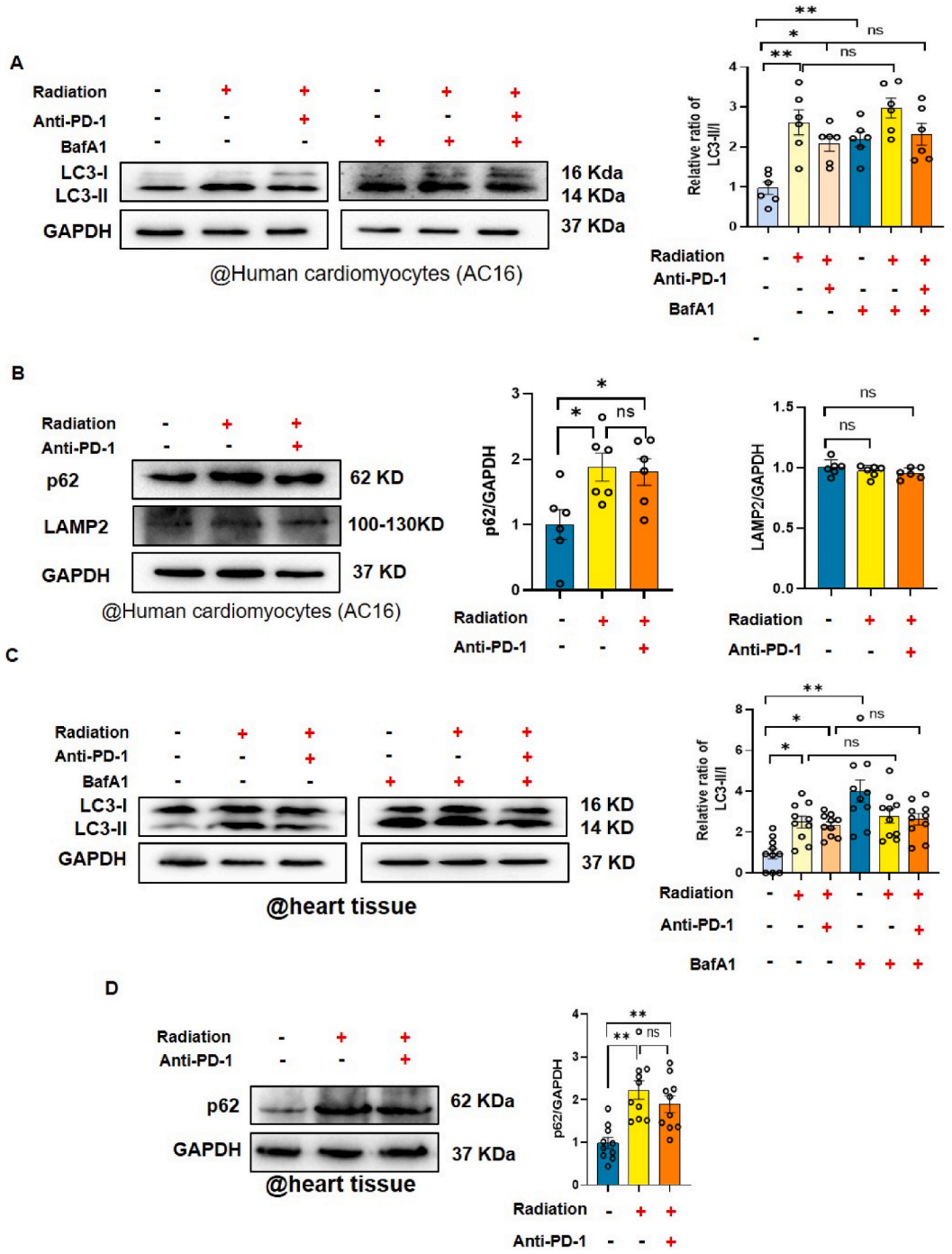

Fig. 3. Anti-PD-1 combined with radiation-induced degradation stage inhibition of autophagic flux in mouse cardiomyocytes. **A** Representative Western blots and density analysis of autophagy-related proteins for LC3-II before and after an intervention with BafA1 in AC16 human cardiomyocytes treated with radiation and radiation combined with anti-PD-1. n = 6. **B** Representative Western blots and density analysis of autophagy-related proteins for p62 and LAMP2 in AC16 human cardiomyocytes exposed radiation and radiation combined with anti-PD-1. n = 6. **C** Representative Western blots and density analysis of autophagy-related proteins for LC3-II before and after an intervention with BafA1 on heart tissue after radiation and radiation combined with anti-PD-1. n = 10 mice/group. **D** Representative Western blots and density analysis of p62 on heart tissue after radiation and anti-PD-1 combined with radiation. n = 10 mice/group. *$P < 0.05$; **$P < 0.01$; ns: Negative significance. LC3: Microtubule-associated protein 1 light chain 3; LAMP2: lysosomal-associated membrane protein 2. BafA1: Bafilomycin A1.

3. Results

3.1. Anti-PD-1 administration followed by radiation challenge aggravated cardiac dysfunction

To clarify whether anti-PD-1 administration followed by exposure of radiation (anti-PD-1 + radiation) is harmful to heart, echocardiography was used to evaluate the cardiac function after challenges. The experimental exposure scheme is displayed in Fig. 1A. The results demonstrated that anti-PD-1 challenge alone did not cause significant cardiac dysfunction. However, radiation was harmful to cardiac function manifested by decreased left ventricle ejection fraction (LVEF) and left ventricle fraction shortening (LVFS) compared to sham control. Most importantly, LVEF and LVFS of the anti-PD-1 + radiation group were significantly further decreased compared with the radiation alone group (Fig. 1B, C, 1D). Consistent with cardiac functional assessment, anti-PD-1 treatment alone had no significant effect on plasma cTnI levels. However, anti-PD-1 treatment on top of radiation significantly exacerbated radiation-induced plasma cTnI elevation (Fig. 1E). These results suggest that radiation exposure-induced cardiac dysfunction and cardiac injury were aggravated by anti-PD-1.

3.2. Anti-PD-1+ radiation increases apoptosis of cardiomyocytes

To determine how cardiomyocytes are injured by anti-PD-1 + radiation, serial experiments were performed. It is important to note that apoptosis is a common death form for cardiomyocytes, defined as a caspase-dependent, genetically controlled form of cell death [31]. To evaluate changes in apoptosis, we quantified markers of apoptosis in control, radiation group and anti-PD-1 + radiation group. Bcl-2 is an anti-apoptotic protein, Bax is a pro-apoptotic protein, Low Bcl-2/Bax ratio promotes apoptosis [32], while Caspase3 is the ultimate executor of apoptosis [33]. As the protein expression of GAPDH in the cell or tissue is generally relatively stable, we used it as a standardized internal loading control. First, we detected the apoptosis rate in the AC16 human cardiomyocytes. Compared with the control group, the ratio of Bcl-2/Bax in the radiation group and anti-PD-1+radiation group were significantly decreased (Fig. 2A). In addition, the Caspase 3 activation was increased significantly in the radiation group. It was worth noting that the Caspase 3 activation in the anti-PD-1 + radiation group was further elevated compared with the radiation alone group (Fig. 2A). Second, Annexin V-FITC/PI staining was employed to detect early-stage apoptosis. The result showed that the apoptosis was aggravated in the anti-PD-1 + radiation group compared with the radiation alone group (Fig. 2B). Third, in order to obtain more evidence that anti-PD-1 + radiation exacerbated cardiomyocyte injury, CCK-8 assay indicated that radiation markedly reduced cell viability when co-treated with anti-PD-1 (Fig. 2C). Fourth, we determined the effects of anti-PD-1+radiation upon cellular apoptosis in the mouse heart tissues. Consistent with *in vitro* results, the ratio of Bcl-2/Bax in the anti-PD-1+radiation group and radiation group were significantly decreased compared with the sham group, whereas Caspase 3 activation was significantly increased in the anti-PD-1+radiation group and radiation group. It is worth noting that the apoptosis in the anti-PD-1 + radiation group was exacerbated compared with the radiation alone group (Fig. 2D). Collectively, these results indicate that radiation induces apoptosis in cardiomyocytes both *in vitro* and *in vivo*, a pathological alteration exacerbated by anti-PD-1.

3.3. Radiation reduced lysosomal acidification and inhibited autophagic flux, a pathological alteration not aggravated by anti-PD-1

Autophagy is an evolutionarily conserved mechanism by which cytoplasmic elements are degraded intracellularly. Autophagy has also emerged as a major regulator of cardiac homeostasis and function [34]. To identify whether autophagy is implicated in cardiac injury induced by radiation and more importantly, whether anti-PD-1 may impact radiation-induced autophagy alteration, several experiments were performed. LC3 is classified into type I and type II. LC3-I conjugates to phosphatidylethanolamine on the surface of the autophagosome to form LC3-II. LC3-II is a sign of the presence of autophagosomes [35–37]. First, we detected the level of LC3-II after radiation and anti-PD-1 + radiation challenge in AC16 human cardiomyocytes. Compared with a control group, the level of LC3-II was increased in both the radiation and anti-PD-1 + radiation groups (Fig. 3A). Bafilomycin A1 (BafA1) inhibits lysosomal acidification and thus blocks late-stage autophagy. Therefore, BafA1 was employed to test which stages of autophagy (autophagic formation or degradation) was affected by radiation and anti-PD-1 + radiation in AC16 human cardiomyocytes. As shown in Fig. 3A, the protein level of LC3-II increased significantly after BafA1 treatment for 4 h, reflecting the basic state of autophagy. Furthermore, no significant difference of LC3-II expression was detected after radiation and anti-PD-1 + radiation challenges in the presence or absence of BafA1, indicating that the autophagy degradation was inhibited after radiation and anti-PD-1 + radiation treatment in AC16 human cardiomyocytes. Second, we further explored the alteration of LAMP2 and p62, two molecules responsible for lysosome/autophagosome merge. As illustrated in Fig. 3B, the abundance of p62 was increased with the challenge of radiation, while anti-PD-1+ radiation administration did not further increase p62 expression. The abundance of LAMP2 did not change following anti-PD-1 + radiation compared to radiation challenge alone, suggesting the challenges of anti-PD-1 + radiation did not further alter the merge of autophagosome and lysosome. Third, to further explore the effects of radiation and anti-PD-1 + radiation *in vivo*, we challenged animals with radiation and anti-PD-1 + radiation and detected the expression of LC3-II and p62 in mouse heart tissue. The results showed that LC3-II and p62 were increased in both the radiation and anti-PD-1+radiation groups compared with the sham group. However, no significant difference of LC3-II and p62 was detected between radiation and the anti-PD-1 + radiation group (Fig. 3C and D). Meanwhile, similar to the *in vitro* results, the protein expression of LC3-II was not further increased after co-administration of anti-PD-1 + radiation and BafA1 (Fig. 3C). These results indicate that radiation blocked the degradation of autophagy in mouse heart tissue, but it did not affect the formation of the autophagosome. Additionally, anti-PD-1 had no significant effect upon radiation-suppressed autophagic flux.

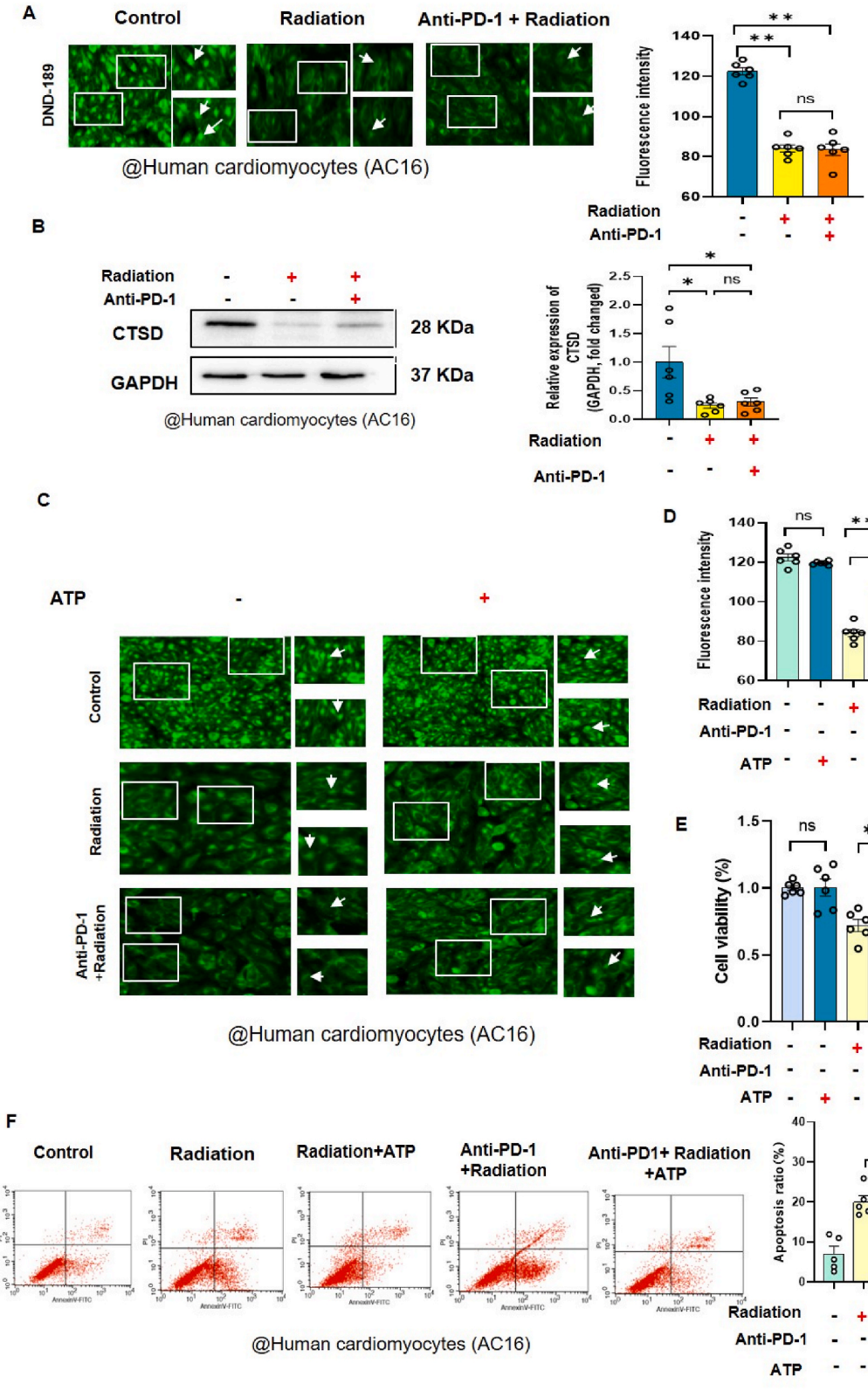

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Fig. 4. ATP presents a protective effect on radiation-induced and radiation combined with anti-PD-1-induced cardiomyocytes injury. **A** Right panel, the pH of lysosome in AC16 human cardiomyocytes was detected by Lysosensor Green DND-189 probe before and after ATP intervention; Left panel, fluorescence intensity was qualitatively analyzed before and after ATP intervention. **B** Representative Western blots and density analysis for CTSD in AC16 human cardiomyocytes. n = 6–10. Bars: 200 μm. **C** The pH of the lysosome was detected by the Lysosensor Green DND-189 probe. D **F**luorescence intensity was qualitatively analyzed and presented with a bar graph. **E** The cell viability assay AC16 human cardiomyocytes after exposure to radiation and radiation combined with anti-PD-1with or without ATP treatment. **F**. Effect of ATP on cardiomyocyte apoptosis determined by Flow cytometry. n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$; ns: Negative significance. CTSD: cathepsin D. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Maintenance of the acid environment of the lysosome is the key to promoting autophagic degradation, preserving autophagy flux [38,39]. Hence, we evaluated lysosomal pH using a lysosome green DND-189 probe. As illustrated in Fig. 4A**,** lysosomal acidification was reduced in the radiation group compared with the control group. However, no further reduction was observed in the anti-PD-1 + radiation group compared with the radiation alone group. This result indicates that the disturbed lysosomal acidification with subsequent inhibition of autophagosome degradation is responsible for radiation-suppressed autophagy flux. As a hydrolase specifically activated by the acid environment of the lysosome, CTSD relies on the acid environment to form mature enzymes with proteolytic function in the lysosome [40]. In order to confirm the inhibitory effect of radiation on lysosomal acidification, we detected the content of mature CTSD in AC16 human cardiomyocytes. The expression of mature CTSD was similarly reduced in the radiation group and anti-PD-1 + radiation group compared with the control group (Fig. 4B). These results indicate that radiation inhibits autophagosome degradation due to reduced lysosomal acidification. However, anti-PD-1 treatment did not aggravate radiation suppressed autophagic flux.

3.4. ATP attenuated radiation suppression of autophagy flux, as well as radiation-induced, anti-PD-1 exacerbated cardiomyocyte apoptosis

The activity of lysosomal enzymes is strictly controlled by pH [41], and the regulation of the lysosomal acid environment mainly depends on the activity of vacuole V-ATPase [41]. V-ATPase is an ATP-dependent enzyme, which can significantly increase the activity of V-ATPase after ATP intervention, thus promoting lysosomal acidification. The results presented in Fig. 3 indicated that radiation blocked autophagy degradation due to an abnormal lysosomal acid environment. Therefore, we first detected the pH of lysosomes in AC16 human cardiomyocytes after ATP intervention. As shown in Fig. 4C and D, the acid environment of AC16 human cardiomyocytes was improved after ATP treatment. In addition, the cell viability of AC16 human cardiomyocytes was detected by CCK-8, and the results showed that cell viability of AC16 was significantly improved after ATP treatment compared with the counterpart group (Fig. 4E). These results indicate that ATP treatment may improve lysosomal acidification and cell viability.

We determined whether ATP could attenuate radiation-induced and anti-PD-1-exacerbated cardiomyocyte apoptosis through flow cytometry. As illustrated and summarized in Fig. 4F, ATP treatment significantly attenuated cardiomyocyte apoptosis in both radiation as well as in the anti-PD-1 + radiation group.

3.5. ATP treatment significantly reduced apoptosis and improved cardioprotection after radiation and anti-PD-1+radiation

Our *in vitro* experimental results presented in Fig. 4 demonstrated that ATP improved not only radiation-suppressed autophagy flux but also attenuated radiation-induced, anti-PD-1 exacerbated cardiomyocyte apoptosis. To evaluate whether ATP treatment alleviate cardiac injury induced by radiation and anti-PD-1+radiation *in vivo*, echocardiography was employed to measure cardiac function after ATP supplement (Fig. 5A). A significant improvement in cardiac function was observed after ATP intervention, as evidenced by increased LVEF and LVFS (Fig. 5B–C).

Next, *in vivo* effect of ATP on autophagy and apoptosis were determined. The ratio of LC3-II/I was decreased after ATP treatment in both the radiation and anti-PD-1 + radiation groups compared with the control groups (Fig. 5D). These results indicate that ATP accelerates autophagic flux *in vivo*. In addition, the protein level of cleaved caspase 3 in mouse heart tissues was detected by immunohistochemistry (Fig. 5E). After radiation, the level of cleaved caspase 3 increased, and this was further exacerbated after treatment with anti-PD-1 plus radiation. Importantly, the level of cleaved caspase 3 was significantly decreased after ATP treatment both in the radiation group and the anti-PD-1 + radiation group (Fig. 5E).

4. Discussion

We provide preclinical evidence to support the conclusion that anti-PD-1 exacerbates irradiation-induced cardiac dysfunction by promoting cardiomyocyte apoptosis, while not altering irradiation-suppressed autophagy. Importantly, all cellular injuries (including irradiation-induced and anti-PD-1 promoted cardiomyocyte apoptosis and irradiation-induced autophagic arrest) were significantly attenuated by ATP treatment *in vitro*, which results in significant cardiac functional improvement when ATP was administrated *in vivo* (Fig. 6).

Immune checkpoint inhibitors (ICIs) have shown promising clinical effects in patients with advanced stages of cancer as one of the most successful immunotherapies [42–44]. The combination of RT with PD-1/PD-L1 inhibitors has been proven to enhance the priming and effect phases of antitumor T-cell response, making it an attractive therapy [45]. The results of our study, however, were consistent with those reported by Du et al. [46] but suggest that anti-PD-1 exacerbates radiation-induced cardiac dysfunction. A sufficient amount of anti-PD-1 antibody was administered before radiation to mask PD-1 level changes; however, anti-PD-1 antibody

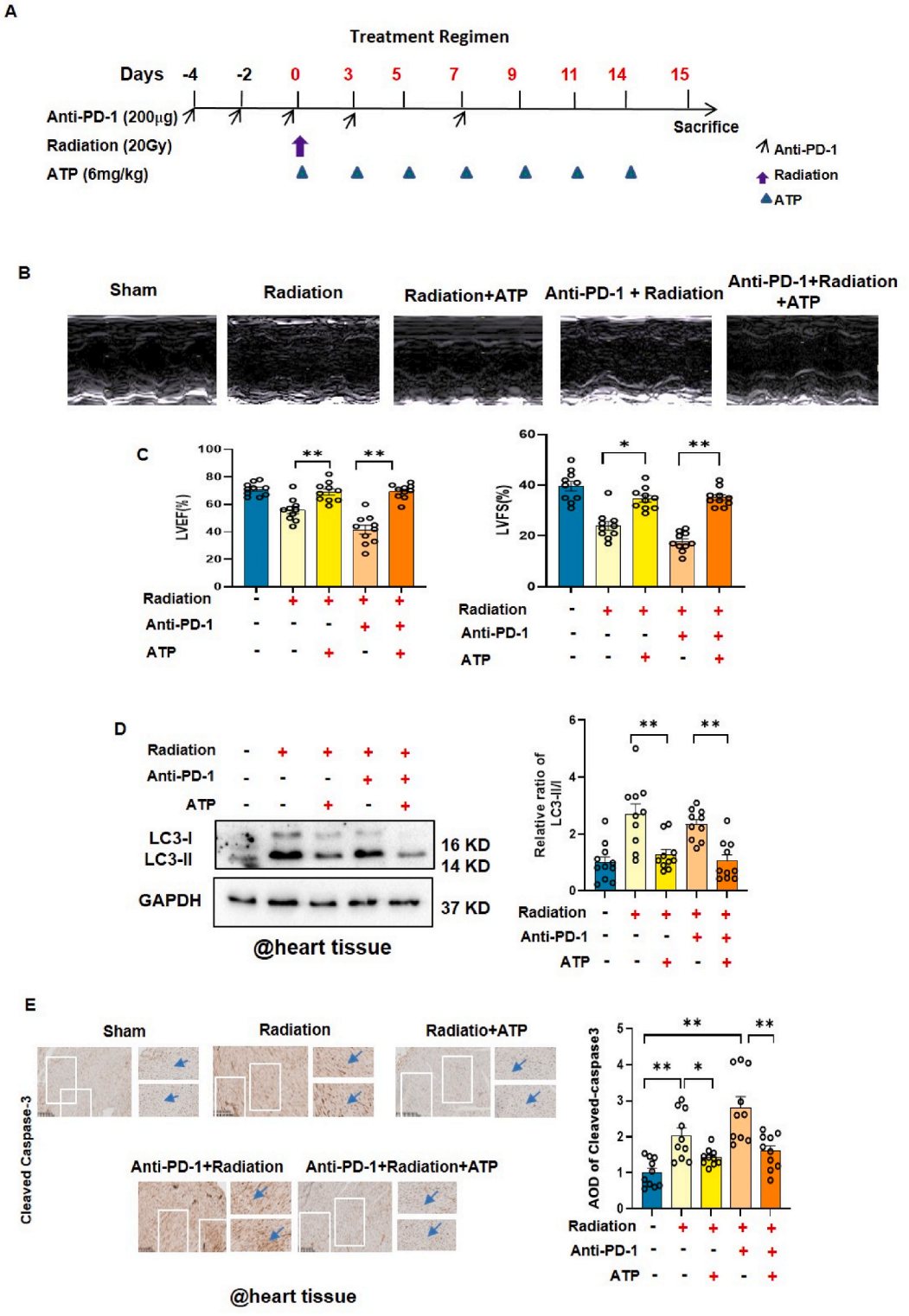

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Fig. 5. ATP administration improved cardiac function after mice were exposed to radiation or PD-1 inhibitor with radiation. **A** Treatment regimen. Mice were challenged with radiation and anti-PD-1+radiation group with or without ATP administration. **B** Representative echocardiogram images before and after radiation and anti-PD-1 challenges and ATP intervention. **C** LVEF and LVFS. **D** Representative Western blots and density analysis for LC3-II before and after ATP intervention in heart tissue. n = 6–10 mice/group. **E** Representative immunochemical images for cleaved-caspase 3 from heart tissue after radiation or anti-PD-1 combined with radiation with or without ATP intervention. Bars: 200 μm. n = 6–10 animal/group. $^{*}P < 0.05$; $^{**}P < 0.01$. LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening.

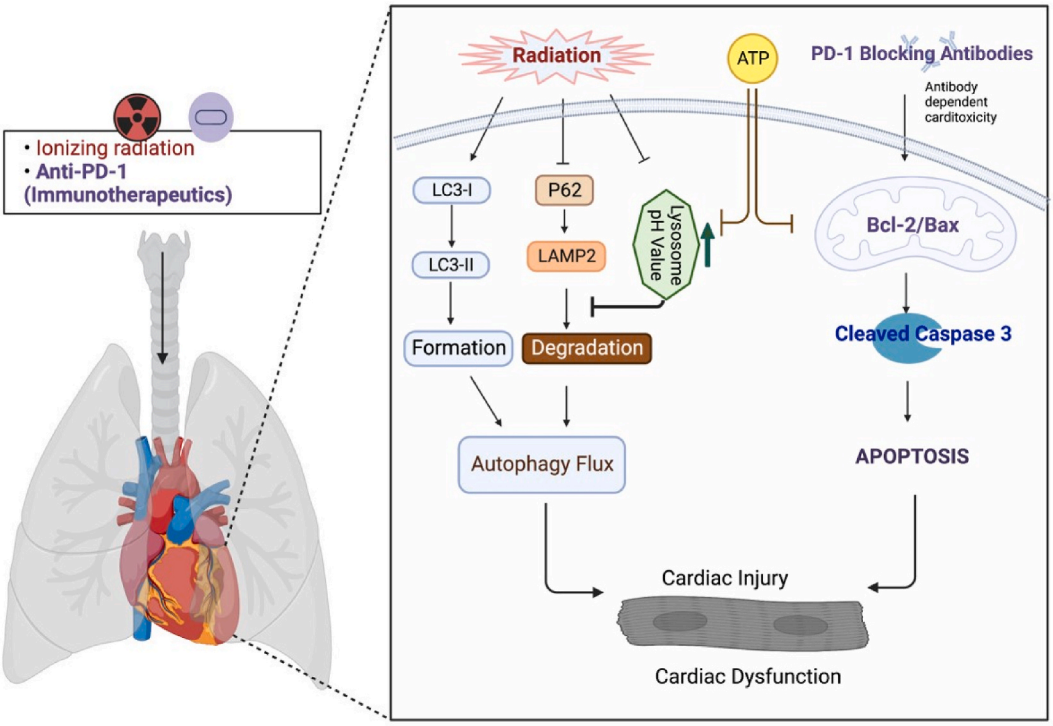

Fig. 6. ATP protects cardiac function from radiation and anti-PD-1 combined with radiation-induced cardiotoxicity by reducing myocardial apoptosis and rescuing the blocked autophagic flux.

delivery alone did not produce the significant cardiac dysfunction. We clarified that cardiomyocytes apoptosis was further increased after anti-PD-1+radiation treatment *in vitro* and *in vivo* compared to radiation alone or anti-PD-1 administrated alone. Our experimental results agreed with the reports that radiation exposure induced direct cardiac damage typically manifested by cardiomyocyte dilatation but we provided new evidence to support that anti-PD-1 treatment significantly enhanced radiation-induced cardiomyocyte apoptosis [46]. These new findings warrant a careful reevaluation of treatment strategies involving anti-PD-1 immunotherapy and radiation therapy. It is of paramount importance that clinicians remain vigilant in monitoring the cardiac health of patients subjected to this combined regimen. Regular assessments, including cardiac imaging modalities and biomarker measurements, may be necessary to detect early signs of cardiac dysfunction. Moreover, close collaboration between oncologists, radiation specialists, and cardiologists becomes crucial in devising comprehensive treatment plans that balance the therapeutic benefits of anti-PD-1 immunotherapy with the potential risks of cardiac complications [47]. Closely monitoring cardiac function with echocardiography, electrocardiogram and cardiac injury biomarkers is advised during the combined immuno and radiotherapy [5,48]. Meanwhile, the enhanced understanding of the interplay between anti-PD-1 treatment, radiation therapy, and cardiac function presented by our study underscores the necessity for personalized and multidisciplinary approaches in clinical decision-making. As anti-PD-1 immunotherapy continues to play a pivotal role in cancer treatment, optimizing its combination with radiation therapy demands a holistic perspective that prioritizes both cancer control and the preservation of cardiac integrity. By elucidating these intricate interactions, this research has raised awareness of the adverse effects to the heart when combining these treatments, ultimately ensuring better outcomes and quality of life for patients facing the challenges of cancer and its treatment.

Given that autophagy is a significant player in the pathophysiological progression of cardiovascular function [49], we found that radiation treatment resulted in an increase of p62 and LC3-II, indicating that autophagy flux was impeded. Distinct from cardiomyocyte apoptosis, anti-PD-1 failed to exacerbate radiation suppression of autophagic flux. We further revealed that radiation suppressed acidification of lysosomes in the radiation group when compared to the control group. According to a recent study that chemotherapy drug can impair lysosome acidification and lysosomal function in cardiomyocytes, which inhibits autophagic flux [21]. Despite the fact that acidification of lysosomes was reduced in the radiation group in this study, anti-PD-1 did not have any additional

additive effect on autophagy degradation. Therefore, anti-PD-1 exacerbates radiation cardiac toxicity by enhancing apoptosis as the primary cellular mechanism.

The exciting part is that after exploring several treatment strategies, ATP treatment increased lysosome pH in AC16, which resulted in autophagy activation (decreased expression of p62 and LC3-II) and alleviated cardiac injury caused by radiation or anti-PD-1 + radiation. In addition to being an intracellular energy source, ATP is an important signaling molecule outside the cell as well [50,51]. As a result of improving the inhibitory state of the lysosomal acid environment, we found that ATP significantly reduced cardiomyocyte apoptosis as well as accelerated autophagic flux. ATP treatment significantly alleviates the heart injury caused by radiation or radiation combined with anti-PD-1.

Although our experimental animal model has limitations to reflect the human heart receiving radiation, our data raise concerns for cardiotoxicity when patients are inevitably exposed to high radiation doses while undergoing anti-PD-1 immunotherapy. Future studies need to address several important questions. First, combination treatment with anti-PD-1 and radiation accelerates cardiotoxicity is related to cytotoxic $CD8^+$ T cell-mediated myocarditis and fibrosis. What the molecular mediators are in between needs to further be explored. Hormone-centered therapy has been shown to reduce cardiotoxicity caused by ICIs [52–54]. However, more comprehensive therapeutic strategies are still needed. A second concern is that although ATP-reduced apoptosis exists in other type of cells as well [55], future studies must investigate the molecular mechanism by which ATP decreases cardiomyocyte apoptosis under anti-PD-1 + radiation. Furthermore, extracellular ATP is rapidly degraded to adenosine monophosphate (AMP) and adenosine by ectoenzymes [56,57]. The adenosine pathway has been identified as a significant barrier to the effectiveness of immune therapies and has become an essential therapeutic target for cancer [12]. In an attempt to address the compromised autophagy flux, we conducted experiments aimed at restoring its functionality through the use of adenosine triphosphate (ATP), with the intention of elevating lysosomal pH in cardiomyocytes. Remarkably, our findings demonstrated the potential of this approach to rescue radiation-induced autophagy impairment. However, notably absent was any discernible impact on mitigating the adverse effects of the combined anti-PD-1 and radiation treatment on cardiac function. It is therefore important to clarify the balance of AMP and adenosine in radiation or anti-PD-1 + radiation treatment in future studies.

5. Conclusion

We reported that combining anti-PD-1 with radiation increased myocardial dysfunction and subsequent worsen cardiac injury by elevating myocardial apoptosis. As part of a combined treatment scheme for cancer patients, oncologists should consider cardiac complications. For cardiac injury caused by radiation or a combination of radiation and anti-PD-1, ATP, a drug that promotes lysosomal acidification, offers a new therapeutic option. It is expected to play a guiding role in clinical practice to achieve effective prevention and to reduce cardiac complications.

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CRediT authorship contribution statement

Jing Wang: Investigation, Methodology, Project administration, Resources, Writing – original draftWriting – original draft. **Jing Zhao:** Formal analysis, Investigation, Methodology, Project administration, Validation. **Zhijun Meng:** Investigation, Methodology, Resources, Software, Validation. **Rui Guo:** Investigation, Methodology, Resources, Software, Validation. **Ruihong Yang:** Investigation, Methodology, Project administration, Resources, Software, Validation. **Caihong Liu:** Investigation, Methodology. **Jia Gao:** Investigation, Methodology, Project administration, Resources, Software. **Yaoli Xie:** Investigation, Methodology, Project administration, Resources, Validation. **Xiangying Jiao:** Funding acquisition, Methodology, Resources, Software, Validation. **Heping Fang:** Methodology, Project administration, Software. **Jianli Zhao:** Methodology, Project administration, Resources, Software, Supervision, Validation. **Yajing Wang:** Visualization, Writing – review & editingWriting – review & editing. **Jimin Cao:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editingWriting – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jimin Cao reports equipment, drugs, or supplies was provided by Shanxi Medical University. Jimin cao reports a relationship with Shanxi Medical University that includes: employment. No has patent na pending to na. NA.

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Abbreviations

PD-1 Programmed cell death protein 1
ATP Adenosine 5′-triphosphate
RT Radiation therapy
ICB Immune checkpoint blockade
RICT Radiation-induced cardiotoxicity
PBS Phosphate buffer saline
LVEF Left ventricular ejection fraction
LVFS Left ventricular fractional shortening
BafA1 Bafilomycin A1
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PVDF Polyvinylidene difluoride
LC3 Microtubule-associated protein 1 light chain 3
p62 Sequestosome-1
LAMP2 Lysosome-associated membrane protein 2
Caspase-3 Cysteine aspartic acid specific protease-3
Bcl-2 B-cell lymphoma-2
Bax BCL2-associated X
CTSD Cathepsin D
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
PD-L1 Programmed cell death- Ligand 1
CCK8 Cell Counting Kit-8 cell
cTnI cardiac troponin I
BSA Bovine serum albumin
ICIs Immune checkpoint inhibitors
AMP Adenosine monophosphate

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