Title: **Nicaraven reduces cancer metastasis to irradiated lungs by decreasing CCL8 and macrophage recruitment**

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Abstract

Radiotherapy for cancer patients damages normal tissues, thereby inducing an inflammatory response and promoting cancer metastasis. We investigated whether nicaraven, a compound with radioprotective and anti-inflammatory properties, could attenuate radiation-induced cancer metastasis to the lungs of mice. Nicaraven and amifostine, another commercial radioprotective agent, had limited effects on both the radiosensitivity of Lewis lung carcinoma cells *in vitro* and radiation-induced tumor growth inhibition *in vivo*. Using experimental and spontaneous metastasis models, we confirmed that thorax irradiation with 5 Gy X-rays dramatically increased the number of tumors in the lungs. Interestingly, the number of tumors in the lungs was significantly reduced by administering nicaraven but not by administering amifostine daily after radiation exposure. Furthermore, nicaraven administration effectively inhibited CCL8 expression and macrophage recruitment in the lungs 1 day after thorax irradiation. Our data suggest that nicaraven attenuates radiation-induced lung metastasis, likely by regulating the inflammatory response after radiation exposure.

1. Introduction

Radiotherapy for cancer patients improves patient survival but is limited by severe side effects, as it injures normal tissue cells [1]. It is also reported that thorax irradiation promotes cancer cell dissemination to the lungs [2, 3]. As metastatic development is the most lethal aspect of human cancer [4, 5], radioprotective agents are urgently needed that can also effectively attenuate cancer metastasis for patients receiving radiotherapy.

Amifostine has been clinically approved as a cytoprotective adjuvant to alleviate the side effects of radiotherapy, but its clinical application is limited by severe side effects [6]. Furthermore, it remains disputable whether amifostine reduces cancer metastasis [7-9]. Nicaraven, a powerful free radical scavenger, can protect normal tissues from ischemia-reperfusion injury [10-12]. We recently found that nicaraven selectively protects normal tissue (stem) cells against radiation-induced injuries [13, 14] as nicaraven has limited radioprotective effects in cancer cells [15]. Interestingly, the radioprotective effects of nicaraven are more likely associated with anti-inflammatory effects [13, 14] rather than free radical scavenging.

Beyond the pro-metastasis effect of primary tumors [16, 17], injuries to normal tissues may induce an inflammatory microenvironment that supports cancer cell metastasis [2, 18]. It has recently been demonstrated that excessive cytokine/chemokine production and increased inflammatory cell infiltration promote cancer metastasis in the lungs [19, 20]. Considering the anti-inflammatory effect of nicaraven, it is possible that nicaraven also attenuates radiation-induced cancer metastasis.

By using experimental and spontaneous metastasis models in mice, we investigated the effects of nicaraven and amifostine on attenuating radiation-induced lung metastasis. Our results showed that nicaraven but not amifostine significantly reduced cancer metastasis to the irradiated lungs, likely by inhibiting CCL8 expression and macrophage recruitment.

2. Materials and methods

2.1 Cells and animals

Mouse Lewis lung carcinoma (LLC) cells used for the experiments were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako, Japan) with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, United States). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

We used 10- to 12-week-old male C57BL/6 mice (CLEA, Japan) for this study. All experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University (No. 1108120943), and animal procedures were performed in accordance with institutional and national guidelines. At the end of the experiments, mice were administered general anesthesia by an intraperitoneal injection of 160 mg/kg pentobarbital and euthanized by severing the aorta.

2.2 In vitro evaluation of cancer cell radiosensitivity

A clonogenic assay was used to evaluate the role of nicaraven and amifostine on cancer cell radiosensitivity *in vitro*. Briefly, LLC cells were seeded into 6-well plates at a density of 100 cells/well. After incubating overnight, cells were exposed to 0, 2 or 5 Gy γ-rays at a dose rate of 1 Gy/min (^{137}Cs source in PS-3100SB γ-ray irradiation system, Pony Industry Co., Ltd., Japan). We treated the cells with 5 mM nicaraven or amifostine for 30 minutes before irradiation, and the medium was replaced approximately 30 minutes after radiation exposure. Colonies with more than 50 cells were counted 7 days after radiation exposure. Plating efficiency was calculated by dividing the number of colonies by the number of plated cells in each well separately.

We also detected DNA damage in the cancer cells by immunofluorescent staining with 53BP1, as previously reported [15]. Briefly, LLC cells were exposed to 0 or 2 Gy γ-rays. At 1 and 8 hours after radiation exposure, cells were fixed and stained with rabbit anti-53BP1 antibody (1:200 dilution, #ab36823, Abcam). Positive staining was examined under a laser confocal scanning microscope (FV10i, Olympus, Japan). The mean number of 53BP1 foci from more than

50 cells was calculated for statistical analysis.

2.3 Evaluation of radiation-induced tumor growth inhibition

To investigate the radiation-induced tumor growth inhibition, mice were subcutaneously injected with 1×10^6 LLC cells in the left flank. When the tumors reached approximately 100 mm^3, they were exposed to 10 Gy X-rays at a dose rate of 1.2 Gy/min (200 kV, 15 mA, 5 mm Al filtration, ISOVOLT TITAN320, General Electric Company, United States). Mice were intraperitoneally injected with nicaraven (100 mg/kg), amifostine (50 mg/kg) or saline, immediately after radiation exposure. Drugs were administered daily for 2 additional days after radiation exposure.

We measured the tumor volumes with calipers every 2-4 days after radiation exposure. The tumor volume in cubic millimeters was determined using the formula, (length × $width^2$)/2. Fourteen days after radiation exposure, the mice were sacrificed and the tumors were excised and weighed.

2.4 Tumor metastasis evaluation in the irradiated lungs

To investigate the influence of radiation exposure on cancer metastasis, two lung metastasis models were established as previously described [21]. For the experimental metastasis model, mice were injected with nicaraven, amifostine or saline immediately after thorax irradiation with 5 Gy X-rays as described above. Twenty-four hours after radiation exposure, we intravenously injected 5×10^5 LLC cells (in 0.5 ml saline) to induce lung metastasis. Drugs were administered daily for another 6 days after radiation exposure. Animals were sacrificed at 4 weeks after cell injection. Lung tissues were excised and weighed. We also counted tumor nodes on the lung surface.

For the spontaneous metastasis model, mice were injected with nicaraven, amifostine or saline immediately after thorax irradiation with 5 Gy X-rays as described above. Twenty-four

hours after radiation exposure, LLC cells (1×10^6 cells in 0.1 ml saline) were subcutaneously injected in the left flank. Tumor nodules were removed 2 weeks after cell injection. Drug were also administered daily for another 6 days after radiation exposure. Animals were sacrificed for evaluation 6 weeks after cell injection. Lung tissues were excised and weighed. We also counted tumor nodes on the lung surface.

2.5 Evaluation of systemic and local inflammatory responses to radiation injury

To evaluate the inflammatory responses to radiation injury, healthy mice were given thorax irradiation with 5 Gy X-rays, then immediately given an intraperitoneal injection with nicaraven, amifostine, or saline as described above. Mice were sacrificed 24 hours after radiation exposure, and plasma and lung tissue samples were collected for subsequent experiments.

We measured the chemokine CCL8 levels in the lung tissue and plasma by using a mouse CCL8 ELISA kit (#DY790, R&D Systems). Briefly, whole lung lysate (100 μg protein) or plasma (0.2 μl) was added to each well and measured per the manufacturer's instructions. The optical density of each well was measured at 450 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

To evaluate inflammatory cell infiltration and CCL8 expression, lung tissues were fixed in 4% paraformaldehyde. Paraffin-embedded lung tissues were cut into 8-μm-thick sections for staining. Briefly, slides were deparaffinized and rehydrated. Immunofluorescence staining for CD206 (#AF2535, R&D Systems), CD11c (#ab11029, Abcam), Ly6g (#ab25377, Abcam), CD4 (#11-0041-81, Thermo Fisher Scientific) and CCL8 (#MAB790, R&D Systems) was performed per the manufacturer's instructions. The positively stained cells were observed under a fluorescence microscope with 100× magnification, and 10 fields per section were randomly selected for cell counts.

We also measured the percentage of $CD206^+$ cells in whole lung suspensions. In brief, lungs were dissected, minced, and digested with Liberase (#05401119001, Roche) and DNase I

(#10104159001, Roche) in HBSS (Hank's Balanced Salt Solution) and passed through a 100 μm cell strainer. For flow cytometry analysis, single-cell suspensions of lung were subjected to red blood cell lysis solution (#00-4333-57, Thermo Fisher Scientific) to remove erythrocytes and washed with PBS (phosphate buffered saline). Immunofluorescence staining for CD206 (#AF2535, R&D Systems) was performed per the manufacturer's instructions. Flow cytometry analysis was performed using a FACSCalibur instrument (Becton Dickinson).

2.6 Statistical analyses

Data are represented as the means ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's test or by the unpaired *t* test between two groups (Dr. SPSS II, Chicago, IL). A p-value less than 0.05 was considered significant.

3. Results

3.1 Nicaraven and amifostine did not change cancer cell radiosensitivity

We evaluated the radiosensitivity of LLC cells by clonogenic assay. Treatment with 5 mM nicaraven or amifostine did not significantly change the ability of LLC cells to form colonies (***Fig. 1A***). Exposing LLC cells to 2 or 5 Gy γ-rays dramatically impaired their colony forming abilities, which was not significantly mitigated by treatment with 5 mM nicaraven or amifostine (***Fig. 1A***).

Radiation-induced DNA damage was evaluated by counting the 53BP1 foci, a sensitive marker of DNA double-strand breaks. Exposure to 2 Gy γ-rays significantly increased the number of 53BP1 foci in the LLC cells 1 hour after radiation exposure (***Fig. 1B***); however, treatment with 5 mM nicaraven or amifostine at different times after radiation exposure did not significantly change the number of 53BP1 (***Fig. 1B***).

3.2 Nicaraven and amifostine had limited effects on radiation-induced tumor growth

inhibition

We established preclinical tumors in C57BL/6 mice by subcutaneously injecting LLC cells into the left flank and determined the potential effect of nicaraven and amifostine on radiation-induced tumor growth inhibition. Exposure to 10 Gy X-rays dramatically inhibited tumor growth (***Fig. 2***). However, radiation-induced tumor growth inhibition was not significantly affected by nicaraven or amifostine administration (***Fig. 2***).

3.3 Nicaraven, but not amifostine, attenuated radiation-induced lung metastasis

We used LLC cells to establish an experimental metastasis model in C57BL/6 mice. Neither nicaraven (p=0.661, *vs.* 0 Gy + placebo) nor amifostine (p=0.998, *vs.* 0 Gy + placebo) administration significantly changed the number of tumors in normal lungs (***Fig. 3***). We further evaluated whether nicaraven or amifostine diminished radiation-induced metastasis. Thorax irradiation with 5 Gy X-rays dramatically increased tumor numbers in the irradiated lungs (p<0.05, *vs.* 0 Gy + placebo) (***Fig. 3***). Interestingly, radiation-induced metastasis was significantly attenuated by nicaraven (p<0.05, *vs.* 5 Gy + placebo), but not by amifostine (p=0.847, *vs.* 5 Gy + placebo) (***Fig. 3***).

Thorax irradiation with 5 Gy X-rays also significantly increased tumor numbers in the irradiated lungs in the spontaneous metastasis model (p<0.05, *vs.* 0 Gy + placebo). Similarly, spontaneous metastasis in the irradiated lungs was significantly reduced by nicaraven (p<0.05, *vs.* 5 Gy + placebo) but slightly decreased by amifostine (p=0.087, *vs.* 5 Gy + placebo) (***Fig. 4***).

3.4 Nicaraven decreased CCL8 and macrophage recruitment in irradiated lungs

To investigate why radiation exposure promotes cancer metastasis to the lungs, we measured CCL8 levels in irradiated lungs. Nicaraven did not affect the baseline CCL8 expression in the lungs (*P=0.507*, *vs.* placebo) (***Supplementary Fig. 1A***). CCL8 significantly increased in the irradiated lungs 1 day after radiation exposure (p<0.05, *vs.* 0 Gy + placebo) (***Fig. 5A***). Compare

with the non-irradiated lung tissues, the expression of CCL8 was mildly increased in the irradiated lung tissues, but was dramatically increased in the bronchial epithelial cells (***Supplementary Fig. 1B***). Radiation-induced CCL8 expression in the lungs was significantly halted by nicaraven ($p<0.05$, *vs.* 5 Gy + placebo) but not by amifostine ($p=0.988$, *vs.* 5 Gy + placebo) (***Fig. 5A***). However, CCL8 levels in the plasma were not significantly different among groups (***Fig. 5B***).

We also investigated inflammatory cell infiltration, which is closely associated with lung metastasis. We found that neutrophil ($Ly6g^+$) and T-cell ($CD4^+$) infiltration in lung tissues did not significantly change 1 day after thorax irradiation (***Supplementary Fig. 2***). However, $CD206^+$ cell infiltration was significantly higher in the irradiated lungs than the non-irradiated lungs ($p<0.01$, *vs.* 0 Gy + placebo) (***Fig. 6A, B and Supplementary Fig. 3***). Interestingly, administering nicaraven ($p<0.01$, *vs.* 5 Gy + placebo), but not amifostine ($p=0.579$, *vs.* 5 Gy + placebo), significantly reduced $CD206^+$ cell numbers in the irradiated lungs (***Fig. 6A, B and Supplementary Fig. 3***). Moreover, the $CD206^+$ cells were highly expressed with CD11c, a well-classified macrophage marker in mouse lungs [22, 23] (***Fig. 6C***).

4. Discussion

Cancer cell metastasis requires an appropriate microenvironment in its destination organs [16, 24]. Radiotherapy, a regular therapy for cancer, is also reported to promote cancer metastasis [2, 3, 25]. As increasing evidence shows the critical role of the inflammatory microenvironment in cancer metastasis [19, 20, 24], it is possible that radiation-induced tissue injuries may result in an inflammatory microenvironment that favors cancer cell metastasis. Therefore, inhibiting the inflammatory response to radiation injury may reduce the cancer metastasis risk after radiotherapy.

We recently found that nicaraven, a small chemical compound, effectively protects normal tissue (stem) cells against radiation-induced injuries by suppressing inflammatory

cytokine/chemokine expression [13, 14]. Here, we investigated whether nicaraven could also attenuate cancer metastasis, especially in radiation-induced injuries. Compared with amifostine, an approved radioprotective agent, neither nicaraven nor amifostine was found to alter cancer cell radiosensitivity *in vitro* or radiation-induced tumor growth inhibition *in vivo*. Our data also showed that neither nicaraven nor amifostine changed the dissemination and metastasis of cancer cells into normal lungs. Consistent with previous reports [2, 3], thorax irradiation with 5 Gy X-rays significantly increased cancer cell dissemination and metastasis to irradiated lungs. As expected, radiation-induced enhancement of cancer metastasis to the lungs was almost completely attenuated by nicaraven in both experimental and spontaneous metastasis mouse models. However, amifostine had little effect on the radiation-induced enhancement of cancer metastasis in these metastasis models. Based on previous reports, amifostine may inhibit spontaneous metastasis [7], promote lung metastases [8], or have little effect on radiation-induced tumor metastasis enhancement [9]; therefore, using amifostine to prevent cancer metastasis remains disputable. As nicaraven protects against radiation injury without serious side effects at protective doses [10, 13, 14, 26], nicaraven is likely an appropriate radioprotective agent for cancer patients after radiotherapy.

It has been demonstrated that radiation injuries to tissue cells may induce cytokine/chemokine release and inflammatory cell infiltration [27], thereby promoting cancer metastasis [9, 11, 22, 28]. Therefore, we investigated whether nicaraven changed the cytokines/chemokines in the lungs by using a cytokine/chemokine protein array (data not shown). We are unable to present the protein array data at this time, owing to a patent application. We found that CCL8, a chemokine known to regulate macrophage infiltration [29], was significantly increased in irradiated lungs. CCL8 was recently demonstrated to promote cancer cell dissemination and metastatic tumor growth [28, 30]. We further investigated whether nicaraven reduced neutrophil, T-cell and macrophage infiltration into the irradiated lungs. Interestingly, radiation exposure only enhanced $CD206^{+}/CD11c^{+}$ cell infiltration into the irradiated lungs [22,

23]. It is accepted that macrophages promote cancer metastasis under various conditions [16, 24]. A previous study also showed that the resident lung macrophages create a pro-metastatic microenvironment for cancer cells [31]. $CD206^{+}$ macrophages are thought to be an anti-inflammatory phenotype of macrophages and have also been classified as pro-tumoral macrophages [32]. Consistent with a previous report [33], administering nicaraven significantly reduced CCL8 and macrophage recruitment in the irradiated lungs. Although further study is required to demonstrate a causal relationship, nicaraven appears to attenuate cancer metastasis by inhibiting inflammatory responses to radiation injury. This study has several limitations. First, we use single cell line in the metastasis models due to a cost problem. Second, we did not confirm our findings by additional experiments, such as the antibody neutralization of CCL8. Otherwise, it is critical to elucidate the critical factors associated with the metastasis of cancer to the irradiated lungs by further experiments.

In conclusion, we demonstrated that nicaraven but not amifostine significantly attenuated the radiation-induced enhancement of lung metastasis, likely by suppressing the inflammatory response to radiation injury. Further study is warranted to determine how nicaraven regulates inflammatory responses and prevents cancer metastasis after irradiation.

Conflicts of interest statement

The authors indicate no potential conflicts of interest.

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Figure Legends

Fig. 1. *Nicaraven and amifostine did not affect cancer cell radiosensitivity.* (A) After treatment with 5 mM nicaraven or amifostine for 30 min, LLC cells were exposed to 0, 2 or 5 Gy γ-rays. The plating efficiency of the LLC cells is shown. (B) LLC cells were exposed to 0 or 2 Gy γ-rays. At 1 and 8 hours after radiation exposure, the cells were fixed and stained with the 53BP1 antibody. The average number of 53BP1 foci in the LLC cells is shown. Data are presented as the means ± SD.

Fig. 2. *Nicaraven and amifostine did not change radiation-induced tumor growth inhibition.* (A) Raw images of the extracted LLC tumors. (B) Changes in tumor volume over time. (C) Tumor weights for each group. Data are presented as the means ± SD, n=5 per group.

Fig. 3. *In the experimental metastasis model, radiation-induced lung metastasis was attenuated by nicaraven, but not by amifostine.* (A) Experimental protocol scheme. (B) Raw images of the lung tissues. (C) Changes in metastasized tumor numbers. (D) Lung weights for each group. Data are presented as the means ± SD, n=7 per group. *: $p<0.05$ *vs.* 0 Gy + placebo, #: $p<0.01$ *vs.* 0 Gy + placebo, †: $p<0.05$ *vs.* 5 Gy + placebo.

Fig. 4. *In the spontaneous metastasis model, radiation-induced lung metastasis was attenuated by nicaraven, but not by amifostine.* (A) Experimental protocol scheme. (B) Raw images of the lung tissues. (C) Changes in metastasized tumor numbers. (D) Lung weights for each group. Data are presented as the means ± SD, n=5 per group. *: $p<0.05$ *vs.* 0 Gy + placebo, #: $p<0.01$ *vs.* 0 Gy + placebo, †: $p<0.05$ *vs.* 5 Gy + placebo, ‡: $p<0.01$ *vs.* 5 Gy + placebo.

Fig. 5. *CCL8 protein levels in mouse lung tissue and plasma.* (A) CCL8 protein levels in whole lung lysate were measured by ELISA. (B) CCL8 protein levels in plasma were measured by

ELISA. Data are presented as the means ± SD, n=3 per group. *: $p<0.05$ *vs.* 0 Gy + placebo, †: $p<0.05$ *vs.* 5 Gy + placebo.

Fig. 6. *Nicaraven reduced macrophage recruitment in irradiated lungs.* (A) CD206 staining in lung tissue. Scale bars, 100 μm. (B) $CD206^+$ cell count averages per mm^2. (C) $CD206^+/CD11c^+$ cell staining in lung tissue. Scale bars, 20 μm. Data are presented as the means ± SD, n=3 per group. #: $p<0.01$ *vs.* 0 Gy + placebo, ‡: $p<0.01$ *vs.* 5 Gy + placebo.

Fig. 1. Nicaraven and amifostine do not affect cancer cells proliferation and radioresistance(*in vitro* and *in vivo*)

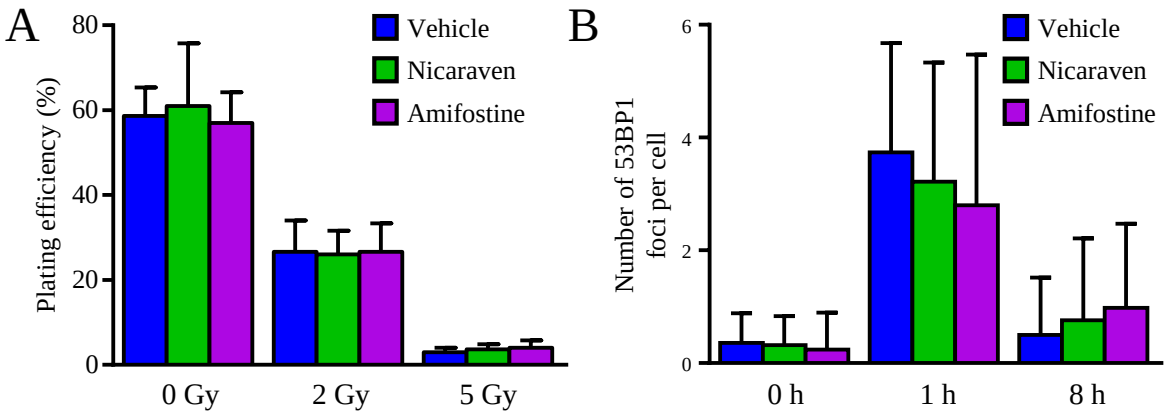


Fig. 2. Nicaraven and amifostine do not affect cancer cells proliferation and radioresistance(*in vitro* and *in vivo*)

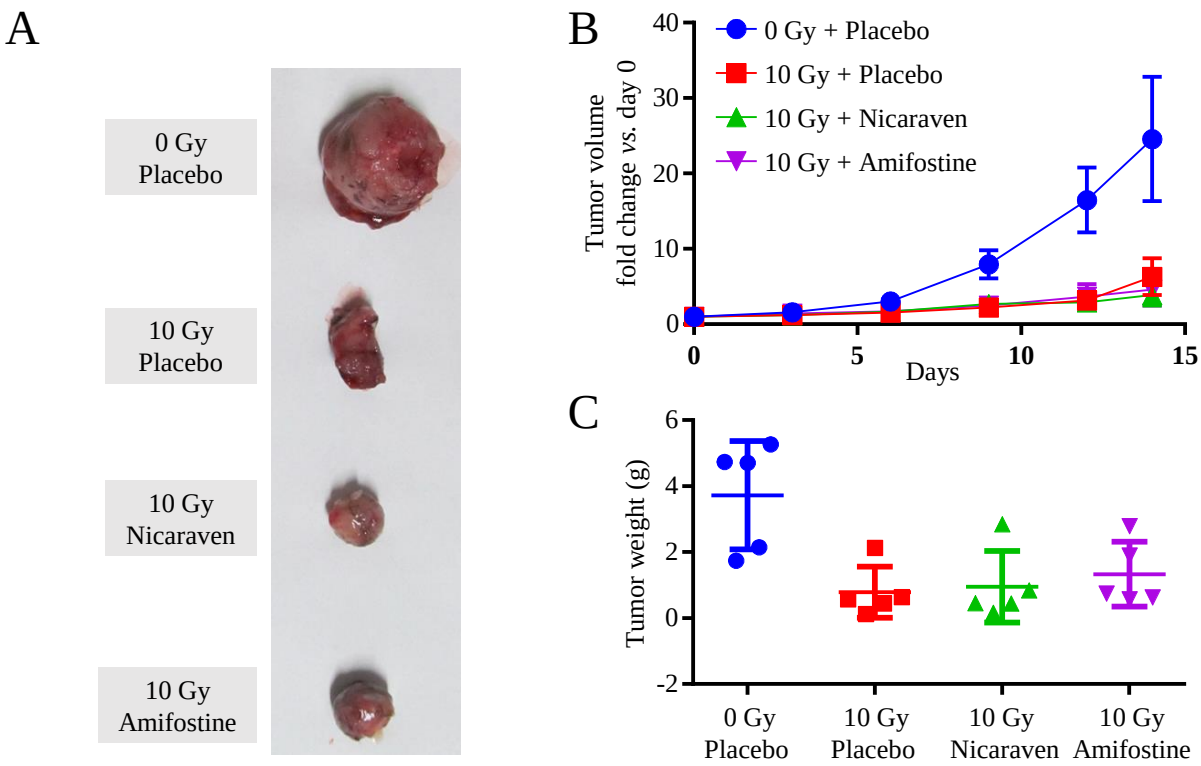


Fig. 3. Nicaraven reduces lung metastasis in mouse experimental metastasis model, but not amifostine

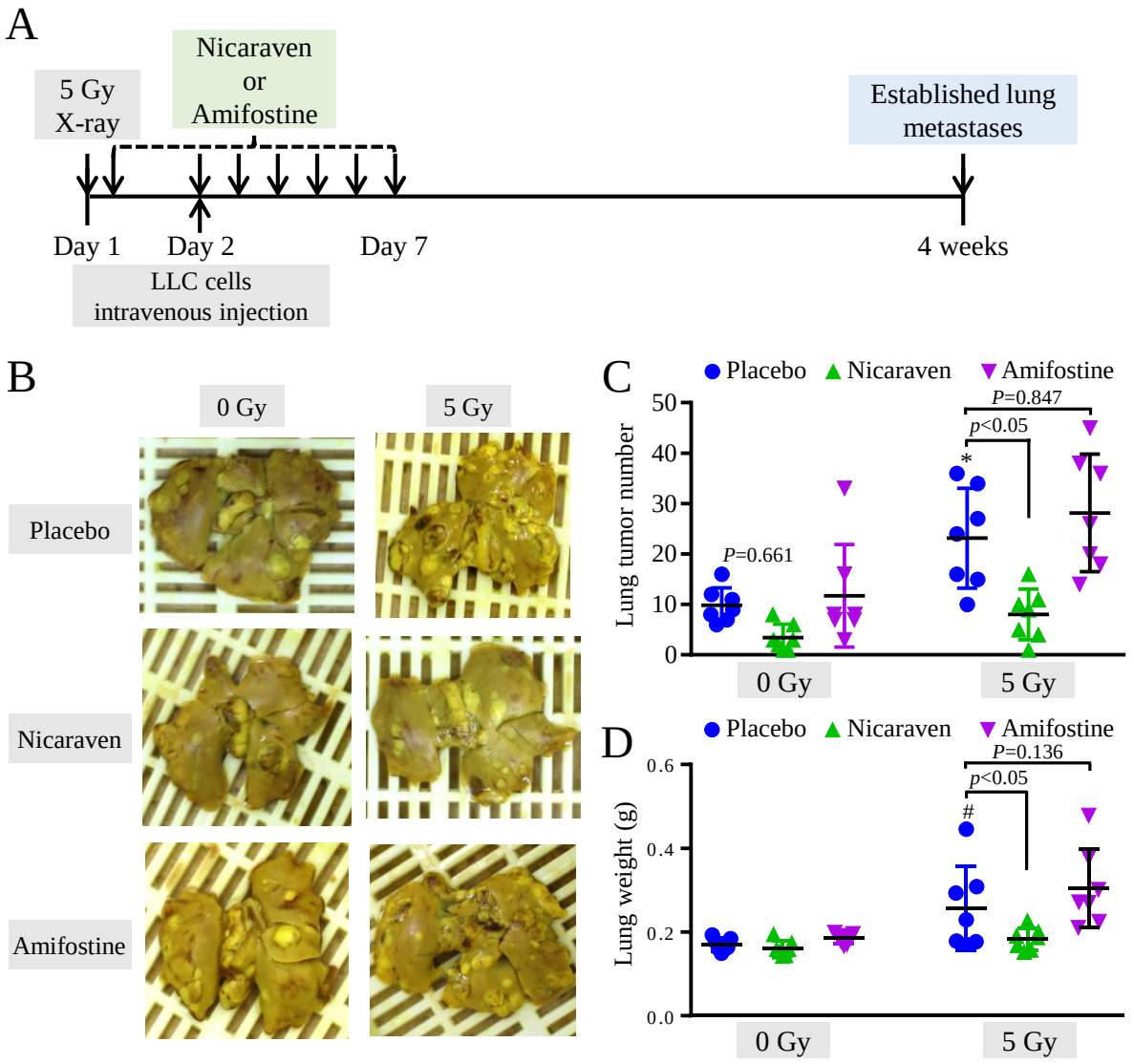


Fig. 4. Nicaraven reduces lung metastasis in mouse spontaneous metastasis model, but not amifostine

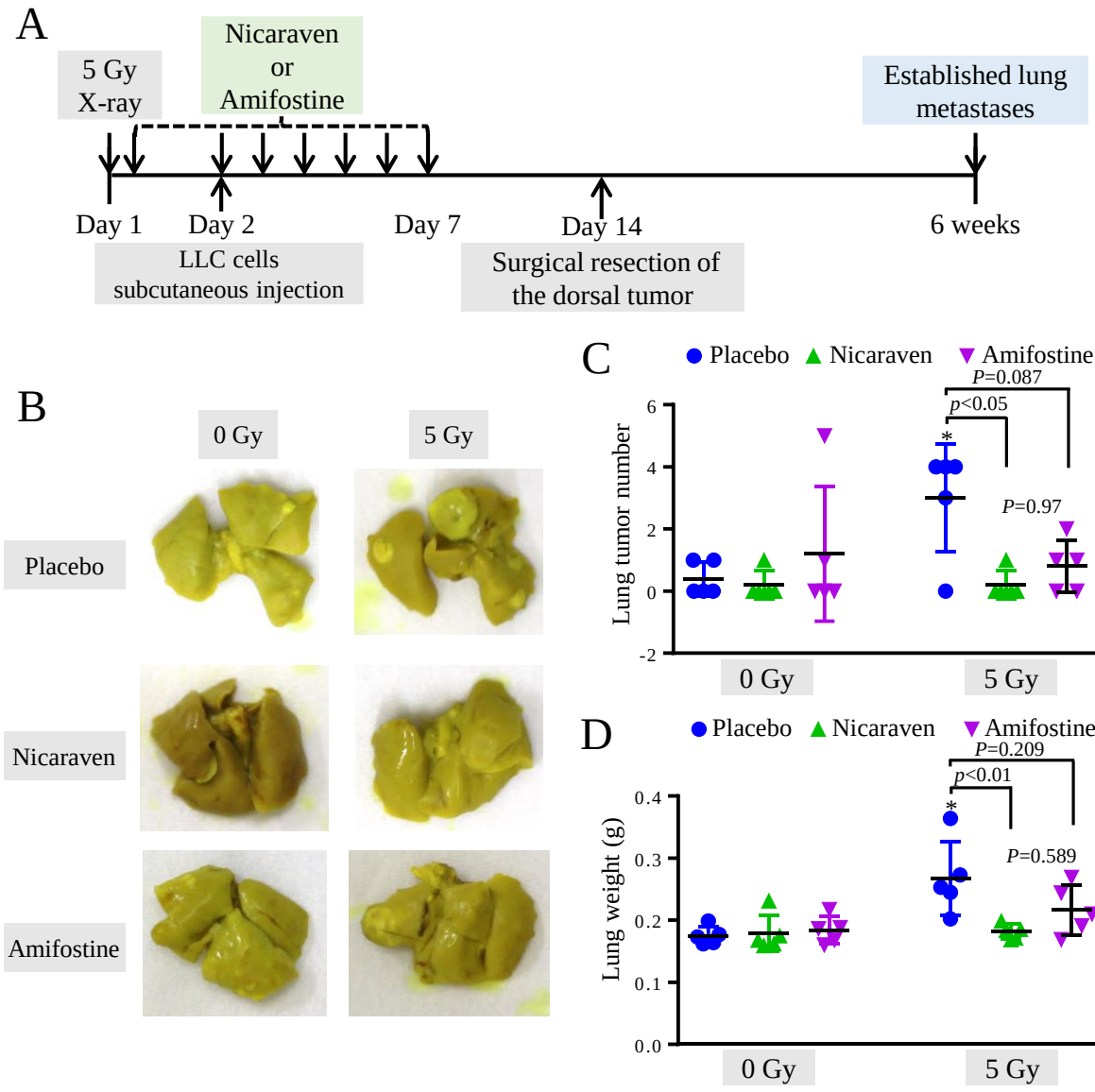


Fig. 5. Nicaraven reduces CCL8 expression

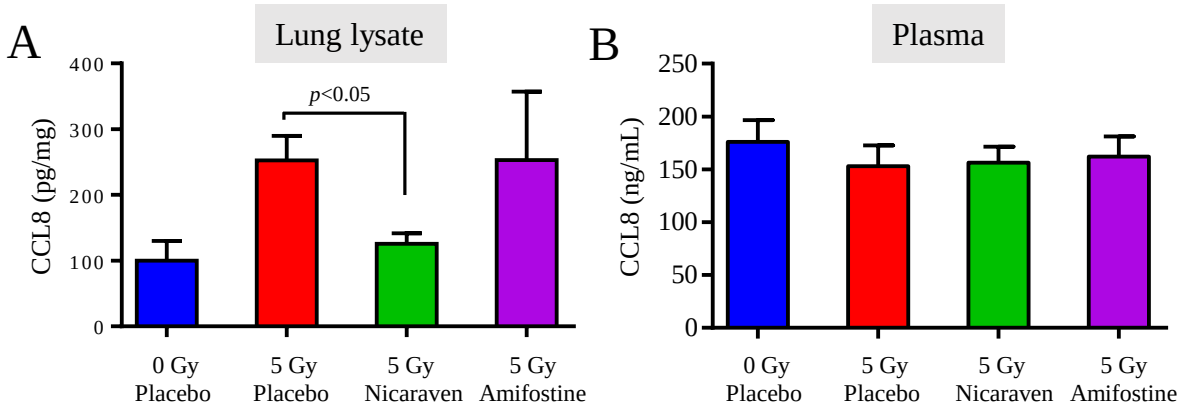
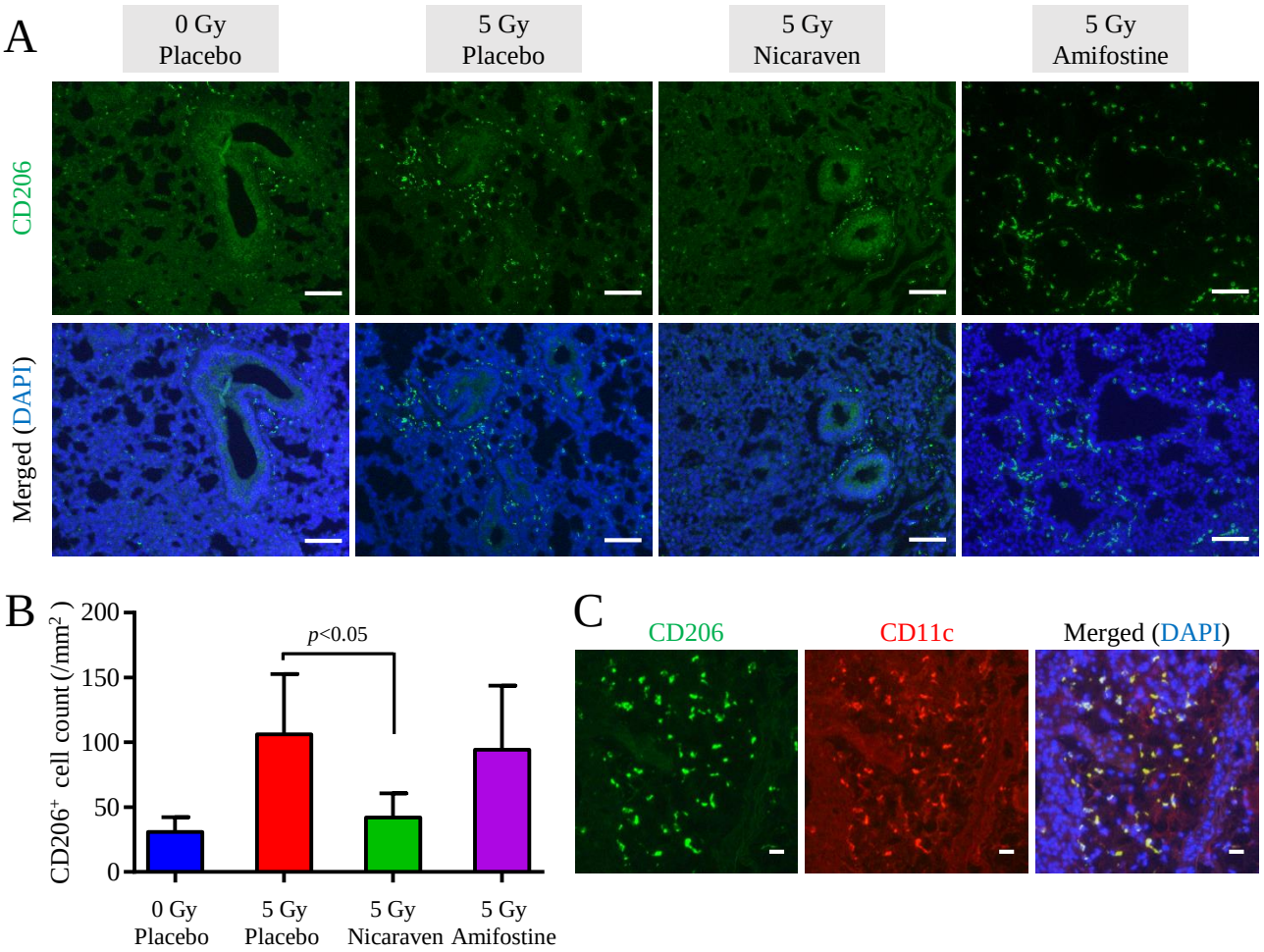
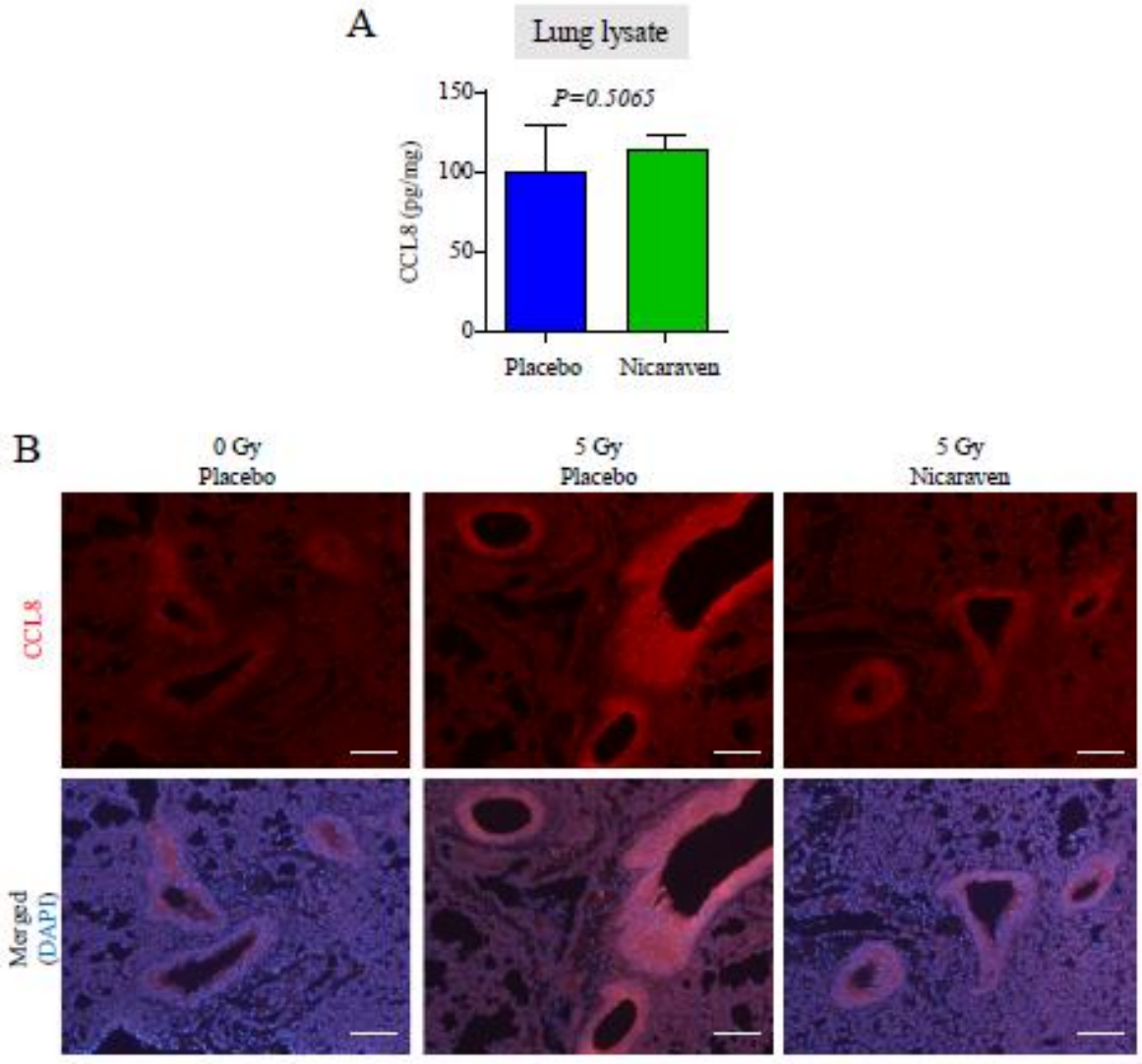
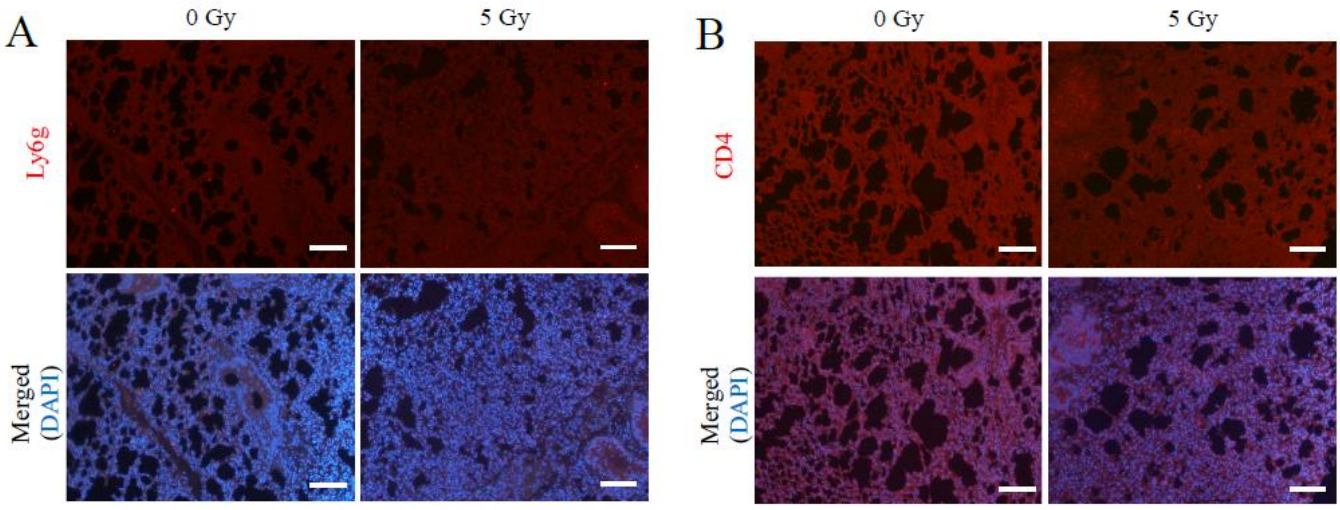


Fig. 6. Nicaraven reduces macrophage cells recruitment

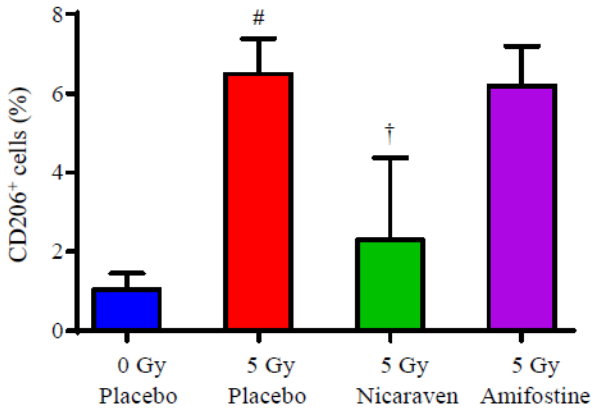




Supplementary figure 1. *The expression of CCL8 in the lungs.* (**A**) CCL8 protein levels in whole lung lysate were measured by ELISA. Data are presented as the means ± SD, n=3 per group. (**B**) The staining of CCL8 protein. Scale bars, 100 μm.



Supplementary figure 2. *The Ly6g$^+$ cells and CD4$^+$ cells in the lungs.* The staining of (A) Ly6g$^+$ cells and (B) CD4$^+$ cells. Scale bars, 100 μm.



Supplementary figure 3. *The CD206$^+$ cells in the lungs.* The percentage of CD206$^+$ cells in whole lung suspensions. Data are presented as the means ± SD, n=3 per group. #: $p<0.01$ *vs.* 0 Gy + placebo, †: $p<0.05$ *vs.* 5 Gy + placebo.