PD-1 restrains IL-17A production from γδ T cells to modulate acute radiation-induced lung injury

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Background: Combining radiotherapy (RT) with programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) inhibitors has been shown to enhance anti-tumor effects in the treatment of non-small cell lung carcinoma (NSCLC). Pulmonary toxicity is a major adverse effect of thoracic RT in NSCLC patients, whether it is administered alone or in combination with PD-1/PD-L1 inhibitors. This study aimed to evaluate the potential pulmonary toxicity of RT combined with concurrent PD-1 inhibitor and to clarify the underlying mechanisms.

Methods: Radiation-induced lung injury (RILI) was induced in C57BL/6 mice by given 24 Gy in three fractions on consecutive days, with or without concurrent injection of anti-PD-1 antibody. On days 3, 7, 14, and 28 after the first exposure to irradiation, lung tissue and peripheral blood samples were collected from the mice. Histological injury was analyzed, and inflammatory cell infiltration and interleukin (IL)-17A production in the lung tissues were quantified.

Results: Mice that received irradiation with concurrent administration of anti-PD-1 antibody had the highest histological score for RILI. In the murine lung tissues, the levels of PD-1 and IL-17A expression were increased in γδ T cells but not in the other CD3^{+} T cells after irradiation. Concurrent blockade of PD-1 enhanced IL-17A production from γδ T cells in the lung tissues after irradiation. In the mice with acute RILI, concurrent administration of anti-PD-1 antibody exaggerated pulmonary inflammation, with significantly increased levels of neutrophilic infiltration and IL-17A detected in both the lung and blood.

Conclusions: PD-1 could restrain IL-17A production from γδ T cells to modulate acute RILI. The concurrent administration of anti-PD-1 antibody aggravates the severity of acute RILI. More attention should be paid to pulmonary toxicity in patients undergoing thoracic RT with concurrent anti-PD-1 immunotherapy.

Keywords: Radiotherapy (RT); radiation-induced lung injury (RILI); programmed cell death 1 (PD-1); γδ T cells; interleukin-17A (IL-17A)

Introduction

Radiotherapy (RT) is a mainstay of treatment for thoracic neoplasms (1). Radiation-induced lung injury (RILI) is the most common dose-limiting complication associated with thoracic RT. Patients with RILI may suffer from cough, panting, dyspnea, or even respiratory failure, which can compromise the effect of this therapy (2,3).

Immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) have revolutionized the clinical management of non-small cell lung carcinoma (NSCLC) (4). The synergistic efficacy of RT with PD-1/PD-L1 blockade has tremendously improved overall survival (OS) and progression-free survival (PFS) rates among patients with NSCLC (5,6). However, the toxicity profile of RT with concurrent anti-PD-1 therapy in regard to non-malignant lung tissue has not been well defined.

PD-1 is an inducible inhibitory receptor expressed on activated effector T cells (7). T-cell function is significantly inhibited when PD-1 is engaged with PD-L1 or PD-L2, both of which are expressed on tumor cells or immune cells within tumors (8). Anti-PD-1/PD-L1 monoclonal antibodies effectively interdict the immune inhibitory signals, thereby recovering the immune response against the tumor (9). Blockade of PD-1/PD-L1 can also cause collateral damage in non-malignant tissues, which are referred to as immune-related adverse events (10,11). Immune-mediated pneumonitis is usually manageable. However, in a small number of cases, it results in the withdrawal of treatment, and occurrence of fulminant or lethal events (12,13). Considering the potential overlapping pulmonary toxicity of RT and anti-PD-1 therapy, we hypothesized that concurrent PD-1 blockade would exacerbate RILI.

The mechanisms underlying the potential synergistic adverse effects of thoracic RT and anti-PD-1 therapy involve several cytokines (14-16). Interleukin (IL)-17A, which is mainly produced by T helper 17 (Th17) cells and γδ T cells, is a newly discovered cytokine involved in lung immunity and mediates pro-inflammatory responses (17). Several studies have reported that irradiation induces an increase of Th17 cells (18,19). In various inflammatory conditions, the expression of PD-1 on γδ T cells is upregulated (20,21). Genetic deficiency of PD-1 in γδ T cells in the skin exacerbates psoriasiform dermatitis (20), while recombinant PD-L1 protein alleviates psoriatic inflammation by inhibiting γδ T cells (21). As γδ T cells are abundant in lung tissue (22), the role of IL-17A-producing γδ T cells in RILI needs to be addressed.

In this study, we established a mouse model to mimic lung irradiation and concurrent administration of anti-PD-1 antibody. We found that PD-1 expression on γδ T cells could restrain the production of IL-17A to modulate acute RILI. The concurrent administration of anti-PD-1 antibody aggravated the severity of acute RILI. Therefore, when RILI occurs, the administration of anti-PD-1 antibody should be carefully considered.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-838).

Methods

Animals

As the C57BL/6 model is suited for the analysis of RILI, we purchased 6 to 8-week-old male C57BL/6 mice from HFK Bioscience (Beijing, China). The mice were maintained in a specific pathogen-free (SPF) facility under a 12-hour light-dark cycle at 22–25 ℃ and acclimated for three days prior to the experiments. Experiments were performed under a project license (No. NCC2018A144) granted by the Institutional Animal Care and Use Committee at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (NCC/CH, CAMS, Beijing, China), in compliance with institutional guidelines for the care and use of animals.

Radiation schedule

Mice were anesthetized with 2% isoflurane inhalation, and immobilized in a dedicated mouse holder with a lead shield that allowed for selective thoracic irradiation. Based on the published literatures (16,23), we administrated the thoracic irradiation using a linear accelerator (Varian Medical

Systems, Palo Alto, CA, USA) on the mice at a dose rate of 3 Gy/min with the regimen of 8 Gy ×3 on days 0, 1 and 2. The source-surface distance was 100 cm. The irradiation field was 4 cm × 2 cm. Mice were randomly assigned to different groups with the numbers of 5–12 for each time-point per group. Control mice underwent the same procedure and exposed to 0 Gy.

Antibody treatment

Mice were intraperitoneally injected with 10 mg/kg of either rat immunoglobulin G (IgG) isotype or rat anti-PD-1 antibody (Bio X Cell, West Lebanon, NH, USA) one day before irradiation and twice a week for three consecutive weeks thereafter, in order to maintain circulating levels in previous studies (24,25).

Histological analysis

Mice were euthanized by carbon dioxide (CO_2) asphyxiation on days 3, 7, 14, and 28 after the first exposure to irradiation. The fixed left lungs were embedded in paraffin and sectioned for hematoxylin and eosin (HE) staining. Based on the reports (26,27), the pathological grades of injury were evaluated blindly by two independent investigators, using the following criteria: 0= no lung abnormality; 1= presence of inflammation involving <25% of the lung parenchyma; 2= lesions involving 25–50% of the lung; 3= lesions involving >50% of the lung. The mean of the pathological grade for two sections was individually determined for each mouse.

After removal of the lungs, the cardiac vasculature was perfused with cold heparinized phosphate buffer saline (PBS) and the hearts were collected for histological analysis as described (28).

Cell preparation from various organs

Murine blood samples were drawn via cardiac puncture with heparin solution as an anticoagulant on the day of sacrifice. The right lungs of the mice were cut into small pieces and digested in 1 mg/mL collagenase D (Roche, Basel, Switzerland) and 40 U/mL DNase I (Roche, Basel, Switzerland) for 30 minutes at 37 ℃ while shaking, as previously reported (29), with minor modifications. After digestion, 10 mM EDTA was added to stop the enzymatic process. The lung and spleen tissues were triturated and filtered through 70 μm cell strainers. Erythrocytes were lysed in red blood cell lysing buffer (Sigma-Aldrich, St Louis, MO, USA).

Flow cytometry

Single cell suspensions were labeled with Fixable Viability Dye and surface staining was subsequently performed. For analysis of neutrophils, cell suspensions were incubated with antibodies against CD45, Ly6G, and CD11b (30). To detect the expression of PD-1 on γδ T cells, cell suspensions were incubated with antibodies against CD45, CD3, γδ TCR, and PD-1 (29). For intracellular staining, cells suspensions were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin in the presence of 5 μg/mL brefeldin A for 5 hours. After stimulation, the harvested cells were firstly stained with CD45, CD3, and γδ TCR (29). Then the cells were fixed, permeabilized and stained with IL-17A. Stained cells were detected using a LSR-II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the results were analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA). The antibodies and reagents used for flow cytometry were purchased from eBioscience (San Diego, CA, USA) or BioLegend (San Diego, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-17A in plasma from the mice or cell culture supernatant was measured by a commercialized mouse IL-17A (eBioscience, San Diego, CA, USA) ELISA kit according to the manufacturer's instructions.

IL-17A neutralization and recruitment of neutrophils

Lung interstitial liquid was prepared as reported previously (31) with modification. Briefly, each 100 mg lung tissue was cut into small pieces in 400 μL ice cold normal saline and incubated on ice for 15 minutes. Lung interstitial liquid was collected after centrifugation at 500 g for 10 minutes. To neutralize the activities of IL-17A, the lung interstitial liquid collected from one mouse was treated with 1 μg rat anti-IL-17A neutralizing antibodies (eBioscience, San Diego, CA, USA) at 37 ℃ for 2 hours. As a control, the lung interstitial liquid was treated with 1 μg rat IgG isotype in the same way. The treated lung interstitial liquid was then injected intraperitoneally into untreated mice. All peritoneal lavage cells were harvested 4 hours later, and the numbers of neutrophils were determined by flow cytometry.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was extracted from the lung tissues using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The cDNA was synthesized using PrimeScript Reverse Transcription Reagent (Takara Bio, Shiga, Japan). The primers used for IL-17A quantification were as follows: forward 5'-TCCCTCTGTGATCTGGGAAG-3', reverse 5'-AGCATCTTCTCGACCCTGAA-3'. Quantification was performed using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) on an Applied Biosystems 7500 qPCR system (Life Technologies, Carlsbad, CA, USA). Each sample was determined in duplicates. The relative mRNA levels were calculated based on GAPDH according to the report (31).

Magnetic activated cell sorting (MACS) and in vitro treatment

Standard laboratory protocols were used to prepare the splenocytes and isolate the γδ T cells by MACS using a TCRγδ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instruction. We diluted the anti-PD-1 antibody (eBioscience, San Diego, CA, USA) into 10 and 20 µg/mL, the PD-L1-Fc fusion protein (PD-L1-Fc, R&D Systems, Minneapolis, MN, USA) into 5 and 10 µg/mL with PBS respectively. According to the report (21), The anti-PD-1 antibody and PD-L1-Fc were coated in 96-well plates overnight at 4 ℃. In each well, 2×10^5 MACS-purified γδ T cells (purity was greater than 90.0%) were added, and cultured in RPMI 1640 medium containing 10% FBS with 5 µg/mL anti-CD3 antibody for 48 hours. In control group, the cells were cultured in the plate precoated with rat IgG. IL-17A-producing γδ T cells were examined by flow cytometry and the level of IL-17A in culture supernatant was determined by ELISA.

Statistical analysis

All data are presented as means ± SD. Two-tailed Student's t-tests were used to compare differences between two groups. Multiple group comparisons were analyzed by performing one-way analysis of variance (ANOVA), followed by Tukey's tests. GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 22.0 (IBM SPSS Inc., Chicago, IL, USA) were used to perform all these analyses. A two-sided P<0.05 was considered to be statistically significant.

Results

Concurrent administration of anti-PD-1 antibody aggravated acute RILI

While receiving 8 Gy ×3 of thoracic irradiation, the mice were concurrently administered rat anti-PD-1 antibody or IgG isotype (*Figure 1A*). We maintained the irradiated mice for 28 days and no death occurred during this period. Histopathological assessment of lung tissue injury following irradiation was carried out. It revealed that tissue injury was aggravated in a time-dependent manner. On day 3 post-irradiation, no tissue injury was apparent, while on day 7, alveoli collapse, lung capillary hyperemia, hemorrhage, and inflammatory exudate were observed (*Figure 1B*). We calculated the percentage of the section occupied by pulmonary inflammatory lesions and scored it according to published histological scoring criteria for RILI (26). On day 7 post-irradiation, the mice that received irradiation plus anti-PD-1 antibody demonstrated the most severe pulmonary injury, with lesions involving more than 50% of the lung. The mice that received irradiation plus anti-PD-1antibody had a higher histological score for RILI than those treated with irradiation plus isotype (*Figure 1C*). On days 14 and 28 post-irradiation, in the irradiated mice, inflammatory cell infiltration was increased and thickened alveolar septum was observed, which worsened when anti-PD-1 antibody was synchronously administrated (*Figure 1B*). On days 7 and 14 post-irradiation, no obvious heart injury was observed in the mice that received irradiation plus IgG or in combination with anti-PD-1 antibody (Figure S1). These results suggest that concurrent administration of anti-PD-1 antibody aggravates acute RILI.

Irradiation enhanced PD-1 expression on γδ T cells in lung tissues

We harvested the lung tissues from the irradiated mice for flow cytometry analysis on day 7 post-irradiation. Among the CD3+ T cells, PD-1 expression was increased on γδ T cells
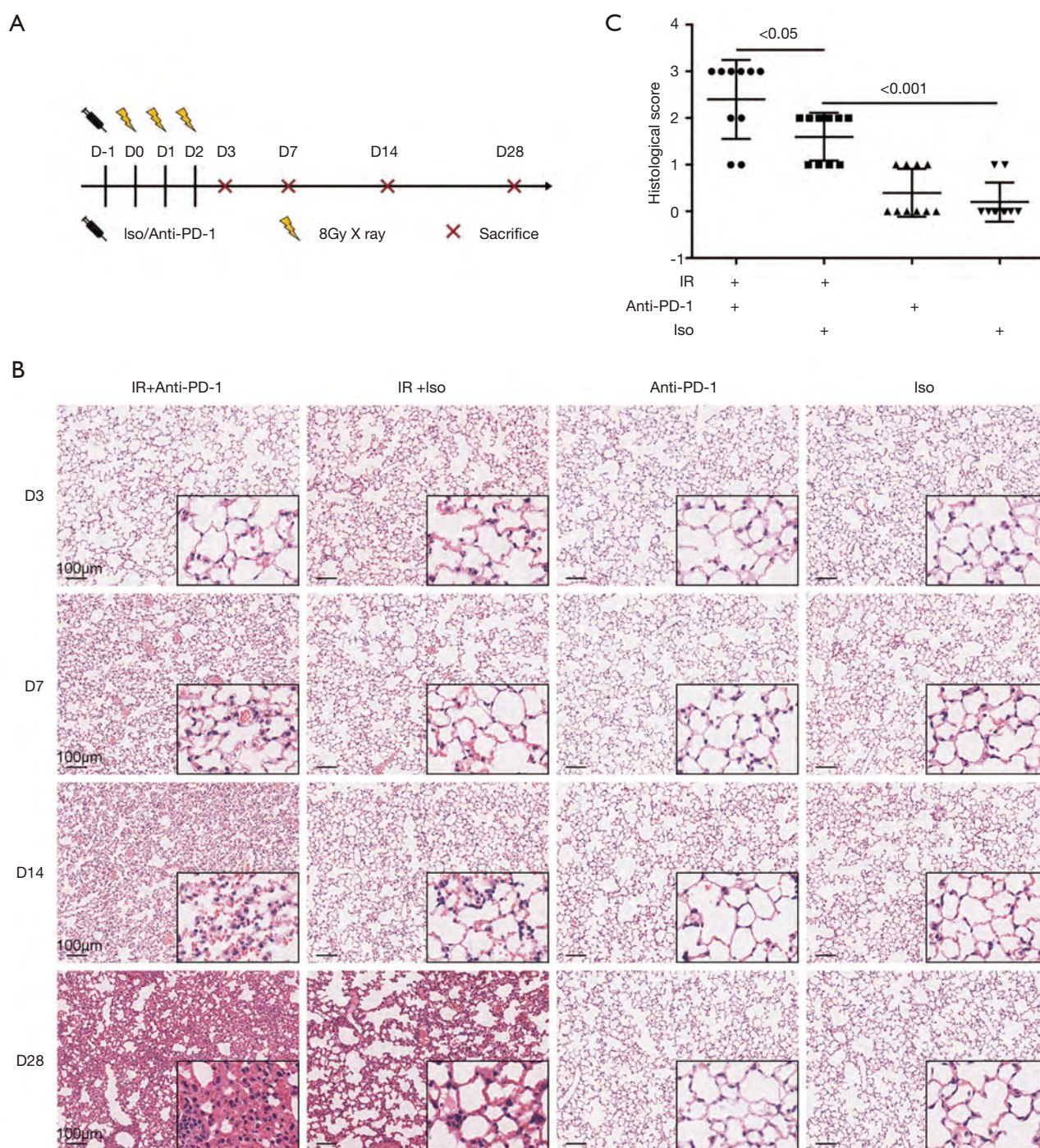
Figure 1 Concurrent administration of anti-PD-1 antibody aggravated acute RILI. (A) The experimental schedule. Rat anti-PD-1 antibody or rat IgG isotype was intraperitoneally administrated one day before irradiation and twice a week for three consecutive weeks thereafter. (B) Representative images of HE-stained lung tissues from different groups on days 3, 7, 14 and 28 after the first exposure to irradiation. Original magnification, 100×; inset magnification, 400×. (C) Histology scores of HE staining on day 7 post-irradiation. Each dot represents one mouse. D, day; Iso, IgG isotype; PD-1, programmed death 1; IR, irradiation; RILI, radiation-induced lung injury.

but not on the other T cells after irradiation (*Figure 2A,B*). The potent pro-inflammatory cytokine IL-17A was mainly detected in γδ T cells at a steady state, and the production increased after irradiation (*Figure 2C*). Irradiation resulted in increased IL-17A production by γδ T cells, but no increased IL-17A production was detected in the other CD3+ T cells (*Figure 2D*). Compared with the irradiation plus isotype group, irradiation with concurrent administration of anti-PD-1 significantly enhanced IL-17A production in lung γδ T cells (*Figure 2E,F*).

Concurrent administration of anti-PD-1 antibody exacerbated lung inflammation and increased IL-17A levels in the blood and lungs after irradiation

We then examined the recruitment of inflammatory cells into the irradiated lung tissues. In the non-irradiated mice, the total numbers of CD45+ leukocytes in the lung tissues were comparable between the anti-PD-1 antibody and isotype treated groups. In the irradiated mice, concurrent administration of anti-PD-1 antibody significantly increased leukocyte infiltration on days 14 and 28 post-irradiation (*Figure 3A*). The levels of IL-17A in the blood showed no alteration in the mice that received isotype alone or anti-PD-1 antibody alone. However, from day 7, IL-17A was gradually upregulated, reaching the peak on day 14, and remained higher on day 28 in the irradiated mice given anti-PD-1 antibody (*Figure 3B*). In the combined treatment group, the mRNA expression of IL-17A was increased in lung tissues on day 7 post-irradiation (*Figure 3C* and Figure S2). Flow cytometry analysis showed that anti-PD-1 therapy alone did not significantly alter the neutrophil (CD45+ Ly6G+ CD11b+) counts in the lung tissues compared to treatment with the isotype. In the irradiated mice, the total numbers of neutrophils in the lung tissues increased significantly when the mice received concurrent administration of anti-PD-1 antibody than those received isotype on days 14 and 28 post-irradiation (*Figure 3D,E*, Figure S3). On day 7 post-irradiation, lung interstitial liquids were collected from mice that received irradiation with concurrent anti-PD-1 therapy and were pretreated with IL-17A neutralizing antibodies. Compared with no treatment, neutralizing IL-17A significantly reduced the recruitment of neutrophils in peritoneal lavage fluid (*Figure 3F*). These data suggest that the combination of irradiation with anti-PD-1 therapy elevates the production of IL-17A and exacerbates the pulmonary inflammation caused by IL-17A.

The numbers of IL-17A-producing γδ T cells were elevated in the lungs of mice administered irradiation and concurrent anti-PD-1 antibody

γδ T cells in lung tissues were further analyzed at different time-points after treatment. In the non-irradiated mice, flow cytometry analysis indicated no significant difference in the total numbers of γδ T cells (CD45+ CD3+ TCRγδ+) between the anti-PD-1 antibody and isotype-treated groups. In the irradiated mice, concurrent anti-PD-1 therapy resulted in significantly up-regulated numbers of lung γδ T cells on days 14 and 28 post-irradiation (*Figure 4A,B*, Figure S4). Among the CD3+ T cells, no significant change was observed in the frequency or absolute numbers of IL-17A-producing γδ T cells between the anti-PD-1 and isotype-treated mice. However, among the irradiated mice, the concurrent anti-PD-1 therapy mice were observed to have more IL-17A-producing γδ T cells in their lung tissues on days 14 and 28 post-irradiation (*Figure 4C,D*, Figure S5). Increased numbers of IL-17A-producing γδ T cells were also found in the peripheral blood and spleens of the combination group (Figures S6,S7).

Anti-PD-1 antibody promotes anti-CD3-induced IL-17A production from γδ T cells

MACS-purified γδ T cells were stimulated with anti-CD3 antibody in the presence of anti-PD-1 antibody or PD-L1-Fc. After 48 hours of culture, the activation of γδ T cells was measured. Flow cytometry analysis showed that anti-PD-1 antibody significantly up-regulated the percentage of IL-17A-producing γδ T cells after stimulation with anti-CD3 antibody. ELISA measurement in the culture supernatant confirmed that anti-PD-1 antibody resulted in a higher level of IL-17A from γδ T cells. Conversely, a higher dose of PD-L1-Fc (10 μg/mL) exhibited the opposite effect (*Figure 5*). These results further demonstrated that the expression of PD-1 on γδ T cells suppresses the production of IL-17A.

Discussion

In the present study, we examined the safety of concurrent RT and anti-PD-1 immunotherapy by establishing a mouse model of acute RILI. The results indicated that concurrent administration of anti-PD-1 antibody with irradiation aggravated the severity of acute RILI. Further analysis indicated that an increased expression of PD-1 on γδ T cells in lung tissues might restrain their over-activation after

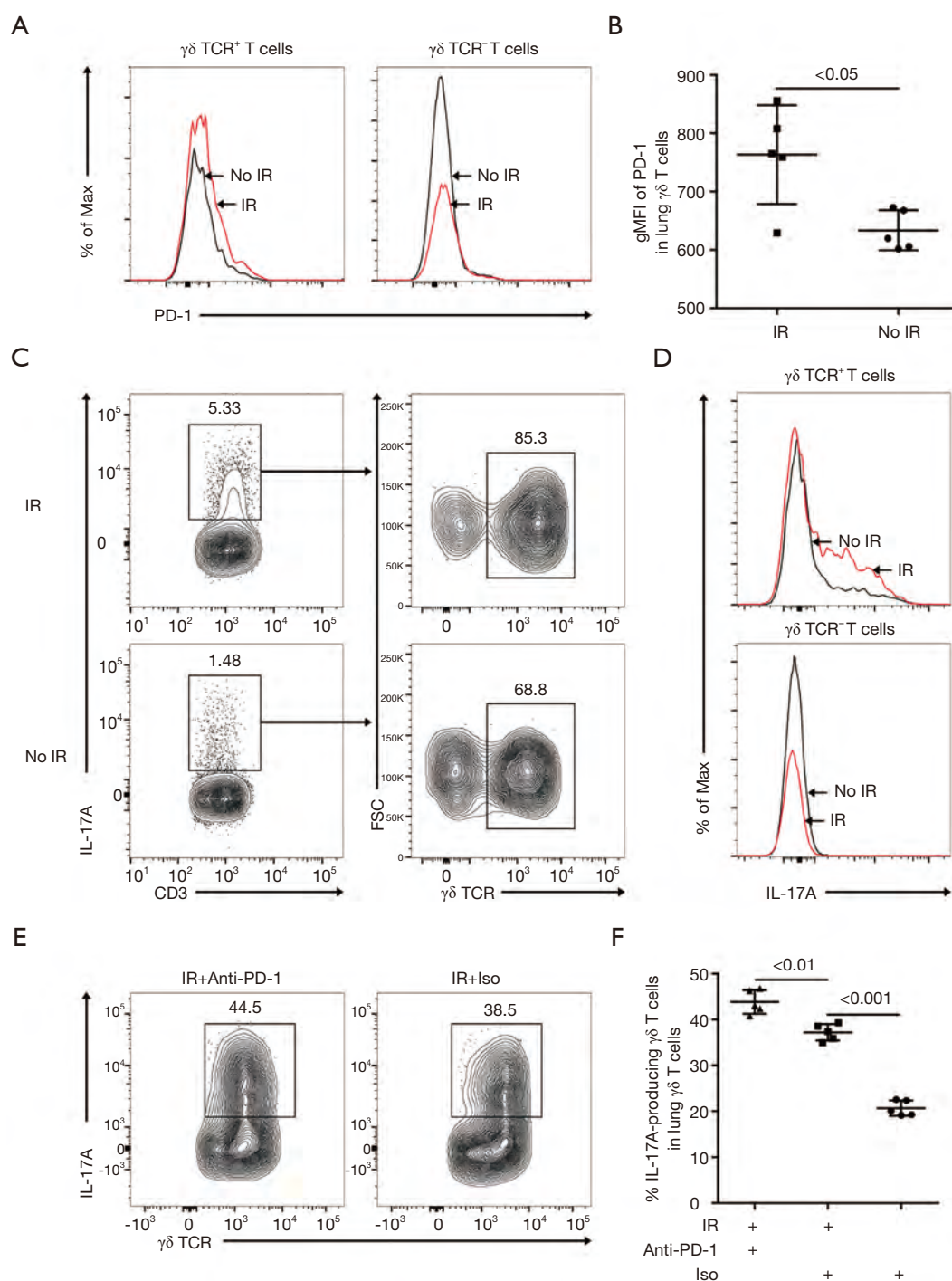**Figure 2** Irradiation enhanced the expression of PD-1 on γδ T cells in murine lung tissues. (A) Representative histograms of PD-1 expression on CD3$^+$ γδ TCR$^+$ T cells or CD3$^+$γδ TCR$^-$ T cells in lung tissues on day 7 post-irradiation. (B) gMFI of PD-1 were analyzed in lung γδ T cells on day 7 post-irradiation (n=5). (C) CD3$^+$ IL-17A$^+$ T cells were further divided into T cell subsets: CD3$^+$ γδ TCR$^+$ T cells and CD3$^+$ γδ TCR$^-$ T cells. (D) Representative histograms displaying intracellular IL-17A staining of CD3$^+$ γδ TCR$^+$ T cells or CD3$^+$ γδ TCR- T cells in lung tissues on day 7 post-irradiation. (E and F) The relative frequency of IL-17A-producing γδ T cells in lung γδ T cells on day 7 post-irradiation (n=5). PD-1, programmed death 1; gMFI, geometric mean fluorescence intensity; IL-17A, interleukin-17A.

Figure 3 Concurrent administration of anti-PD-1 antibody increased lung inflammation in the acute RILI. (A) Total numbers of CD45^{+} leukocytes in the lung on days 3, 7, 14, and 28 post-irradiation. Empty bars indicate the mice received rat anti-PD-1 antibody. Filled bars indicate the mice received same amount of Isotype (n=5). (B) IL-17A concentration in the plasma at different time points determined by ELISA (n=5). (C) qRT-PCR analysis of IL-17A in lung tissues on day 7 post-irradiation (n =12 in IR + Anti-PD-1 group, n=6 in IR + Iso group). (D) Representative dot plots in flow cytometric analysis of CD45^{+}Ly6G^{+}CD11b^{+} neutrophils in the lung on day 14 post-irradiation. The percentage of neutrophils is based on the gating of total CD45^{+} leukocytes. (E) Graph showing percentages (left) and total numbers (right) of neutrophils in the lung on days 3, 7, 14, and 28 post-irradiation (n=5). (F) The percentages (upper) and total numbers (lower) of neutrophils in peritoneal lavage cells examined after IL-17A neutralization (n=3). PD-1, programmed death 1; RILI, radiation-induced lung injury; IL-17A, interleukin-17A; ELISA, enzyme-linked immunosorbent assay.
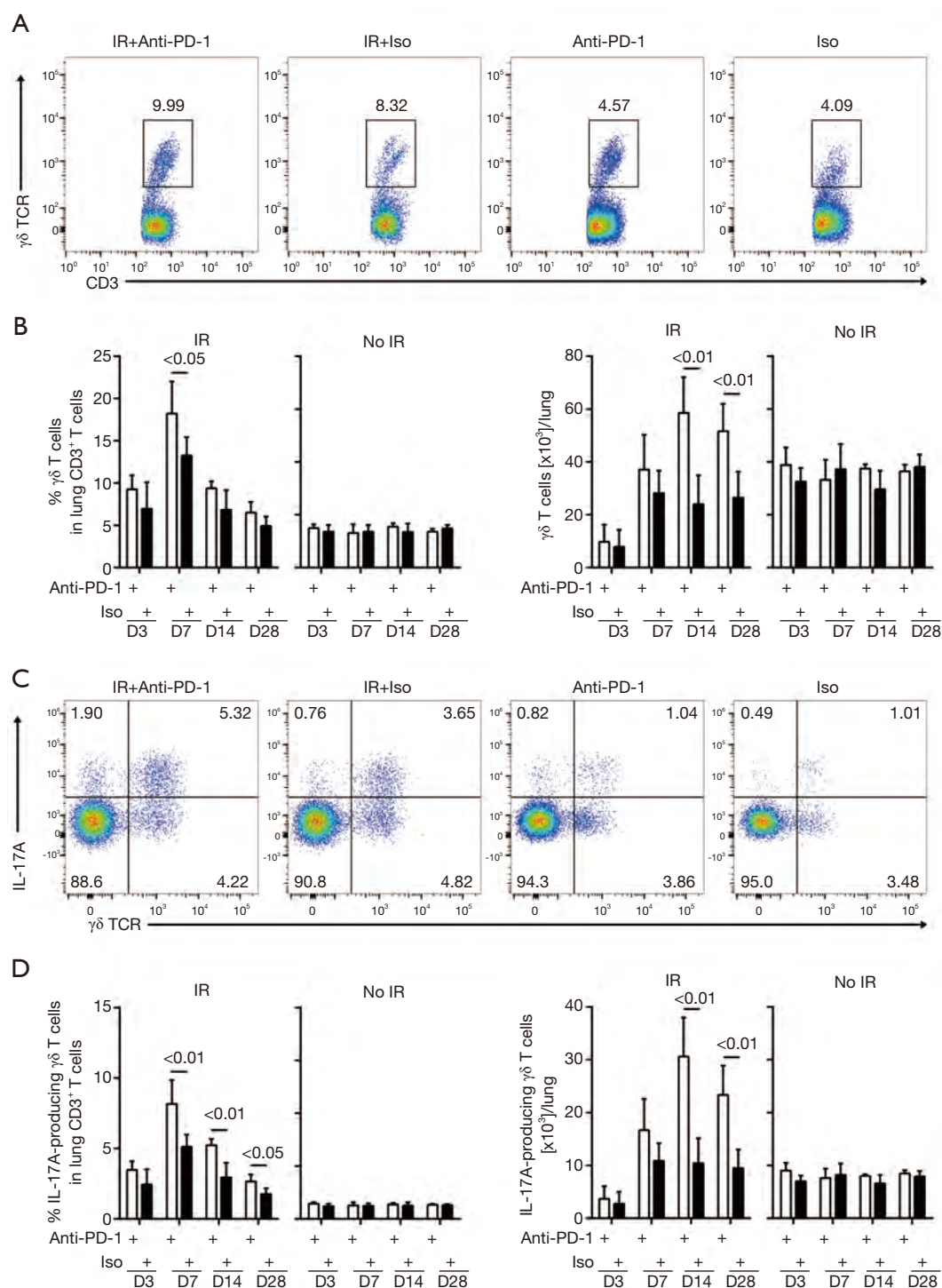
Figure 4 The numbers of IL-17A-producing γδ T cells increased with concurrent administration of anti-PD-1 antibody after irradiation. (A,C) Flow cytometry plots of lung CD45⁺ CD3⁺ γδ TCR⁺ T cells (A) and CD45⁺ CD3⁺ γδ TCR⁺ IL-17A⁺ T cells (C) on day 14 post-irradiation. The numbers were the percentage of objective cell among total CD3⁺ T cells. (B,D) The percentages (left) or numbers (right) of γδ T cells (B) and IL-17A-producing γδ T cells (D) were respectively enumerated by flow cytometry in lungs on days 3, 7, 14, and 28 post-irradiation (n=5). PD-1, programmed death 1.

Figure 5 *In vitro* effects of anti-PD-1 antibody or PD-L1-Fc on γδ T cells. (A) IL-17A production was analyzed in γδ T cells. Representative dot plots of flow cytometry analysis are presented. The numbers represented the percentage of IL-17A-producing γδ T cells among total γδ T cells. (B) The relative frequency of IL-17A-producing γδ T cells in different culture systems (n=6). (C) IL-17A in the supernatant was determined by ELISA (n=6). PD-1, programmed death 1; IL-17A, interleukin-17A; ELISA, enzyme-linked immunosorbent assay.

irradiation. When anti-PD-1 antibody was concurrently administered in combination with RT, the production of IL-17A from γδ T cells was significantly enhanced, which mediated the aggravated severity of acute RILI.

RT is one of the major treatment approaches for patients with lung cancer (32). This conventional therapy is generally unable to subvert the immune tolerance of the tumor (33). Therapies targeting PD-1 and PD-L1, such as pembrolizumab, nivolumab and atezolizumab, release the brakes on activated T cells and renew their anti-cancer function. Immune checkpoint inhibitors have been approved for lung cancer treatment (34-36). As only about 20% of patients are able to benefit from the use

of inhibitors, multimodal combinations, including the integration of RT, have been investigated to augment anti-tumor effects (37,38). Most of the available data indicate that the combination of RT and immune checkpoint blockade is generally safe, whereas others have reported significantly higher rates of pulmonary toxicity (39-42). We explored the effects of combined thoracic irradiation and anti-PD-1 therapy to better understand how non-malignant lung tissue may be affected during this combination therapy.

RILI involves the destruction of the lung structure and a sequence of inflammatory events, which is characterized by the recruitment of diverse immune cells and a perpetual cascade of cytokines/chemokines (3,43). Consistent with

previous findings, we observed that irradiation triggered the infiltration of time-dependent leukocytes into the lung, most likely due to acute radiation responses, including DNA damage, reactive oxygen species (ROS) induction, and apoptosis (3,43). Concurrent administration of anti-PD-1 antibody increased the infiltration of radiation-induced inflammatory cells in the lung tissues, which conformed with the aggravated histopathological changes.

γδ T cells preferentially localize to mucosal surfaces, such as the skin, respiratory and digestive tracts (22). Furthermore, γδ T cells are largely pre-programmed in the thymus and are able to respond faster than Th17 cells. Once triggered, γδ T cells rapidly produce pro-inflammatory cytokines, thus they predominate in the early stages of inflammatory response (44). In this study, among the IL-17A-producing CD3+ T cells recruited upon irradiation in lung tissues, only γδ T cells significantly expressed IL-17A. Concurrent anti-PD-1 antibody application promoted the infiltration of IL-17A-producing γδ T cells in the irradiated lungs on days 14 and 28 post-irradiation. In line with the trend of increased numbers of IL-17A-producing γδ T cells, the level of IL-17A in the combined group increased gradually, peaking on day 14 post-irradiation and remained higher on day 28 post-irradiation. One hallmark function of IL-17A is attracting myeloid cells such as neutrophils to the injured tissue (17). Concurrent anti-PD-1 therapy significantly up-regulated the numbers of neutrophils in the lung tissues on days 14 and 28 post-irradiation. Lung interstitial liquid from the combined treatment group was intraperitoneally injected into untreated mice. In these mice, IL-17A was observed to recruit neutrophils into the peritoneal cavity and the chemotaxis was inhibited after IL-17A neutralization. All these data suggest that IL-17A production from γδ T cells plays an important role in the pathological manifestations of aggravated inflammation.

The coinhibitory receptor PD-1 is induced in activated T cells and increases after continuous or repetitive stimulation of T cells by antigen (7). Irradiation is well known to promote antigen liberation (45). Among the CD3+ T cells in this study, upregulated PD-1 expression was observed on γδ T cells in the lung tissues following thoracic irradiation. The results indicate that γδ T cells can be activated after irradiation, which may be attributed to the stimulation of radiation-induced antigens. Hoverer, it remains unclear which antigens activate γδ T cells via their T cell receptors in RILI. *In vivo* synchronous administration of anti-PD-1 antibody resulted in increased IL-17A production from γδ T cells after irradiation. In an *in vitro* experiment, we applied

anti-CD3 antibodies to mimic the effect of radiation-induced antigens on naïve γδ T cells. Consistently, blockade of the PD-1/PD-Ls pathway further enhanced the secretion of IL-17A from γδ T cells after CD3 stimulation, whereas PD-L1-Fc showed the opposite effect. Taken together, these findings suggest that PD-1 expressed on activated γδ T cells after irradiation probably functions as a brake for RILI, and those cells may be further unleashed by anti-PD-1 therapy, thereby producing higher levels of IL-17A.

Reassuringly, available data, such as those from the PACIFIC trial, demonstrate that the rate of pulmonary toxicity in patients receiving RT and immunotherapy is not very high (6). However, participants in the PACIFIC clinical trial received radiochemotherapy before they received anti-PD-L1 treatment, and patients who suffered grade 2 or higher pneumonitis as a result of previous radiochemotherapy were routinely excluded from the clinical trial. In contrast, a multicenter analysis reported a relatively higher incidence of grade 3 pneumonitis in the concurrent RT and immune checkpoint inhibitor cohort (46). Whether the combination of RT and concurrent immune checkpoint inhibitors increases the risk of pulmonary toxicity currently remains to be answered. Our study provides evidence that the concurrent application of thoracic irradiation and anti-PD-1 antibody could intensify pulmonary toxicity. In another study, concurrent PD-1 inhibition and cardiac irradiation increased radiation-induced cardiac toxicity (RICT) (25). RICT is a recognized late sequela of thoracic RT (47). In the current study, we tried to address the acute toxicities associated with thoracic RT, such as acute pulmonary toxicity. Chronic toxicities including RICT and pulmonary fibrosis need to be explored in the future. Our experimental model may not truly reflect the management of lung cancer in clinical practice, and pulmonary toxicity should be monitored during treatment with thoracic RT and concurrent PD-1 inhibitors. Further verification of the potential effects of this combination is needed in a clinical setting.

Conclusions

In summary, we have provided evidence that PD-1 restrains IL-17A production from γδ T cells in the lung tissues to modulate acute RILI. The concurrent application of anti-PD-1 antibody aggravates acute RILI. We recommend that patients receiving a combination of thoracic RT and concurrent PD-1/PD-L1 blockade are monitored closely for pulmonary toxicity.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/tlcr-20-838

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tlcr-20-838

Peer Review File: Available at http://dx.doi.org/10.21037/tlcr-20-838

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr-20-838). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. NCC2018A144) granted by the Institutional Animal Care and Use Committee at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (NCC/CH, CAMS, Beijing, China), in compliance with institutional guidelines for the care and use of animals.

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698

Sheng et al. PD-1 restrains IL-17A production from γδ T cells in RILI

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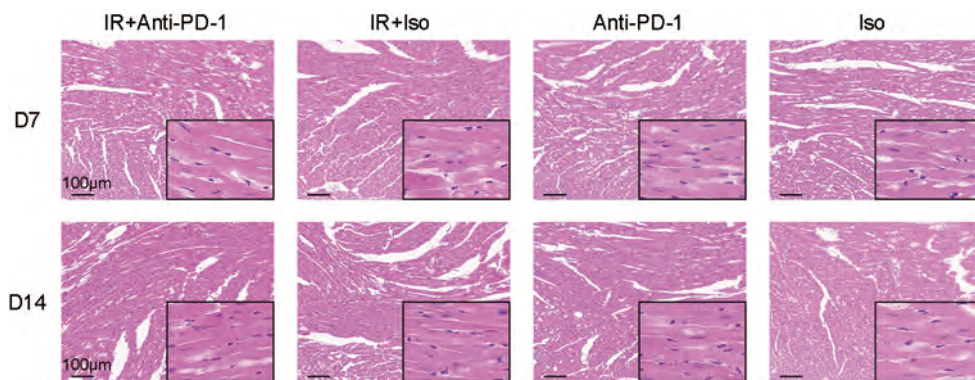
Figure S1 Irradiation with concurrent anti-PD-1 antibody exerted no influence on murine hearts. Representative images of HE-stained heart tissues from different groups on days 7 and 14 after the first exposure to irradiation. Original magnification, 100×; inset magnification, 400×. D, day; HE, hematoxylin and eosin; Iso, IgG isotype; PD-1, programmed death 1; IR, irradiation
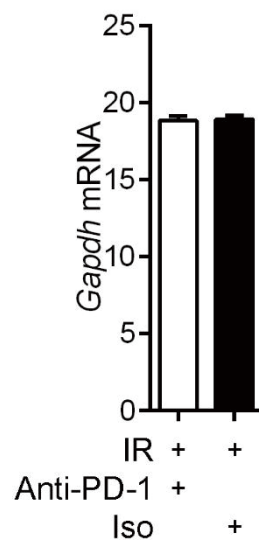

Figure S2 Expression levels of GAPDH were unchanged in the lung tissues of mice. qRT-PCR analysis of Gapdh in the lung tissues on day 7 post-irradiation. Empty bars indicate the mice received rat anti-PD-1 antibody. Filled bars indicate the mice received same amount of Isotype (n =12 in IR + Anti-PD-1 group, n = 6 in IR + Iso group).
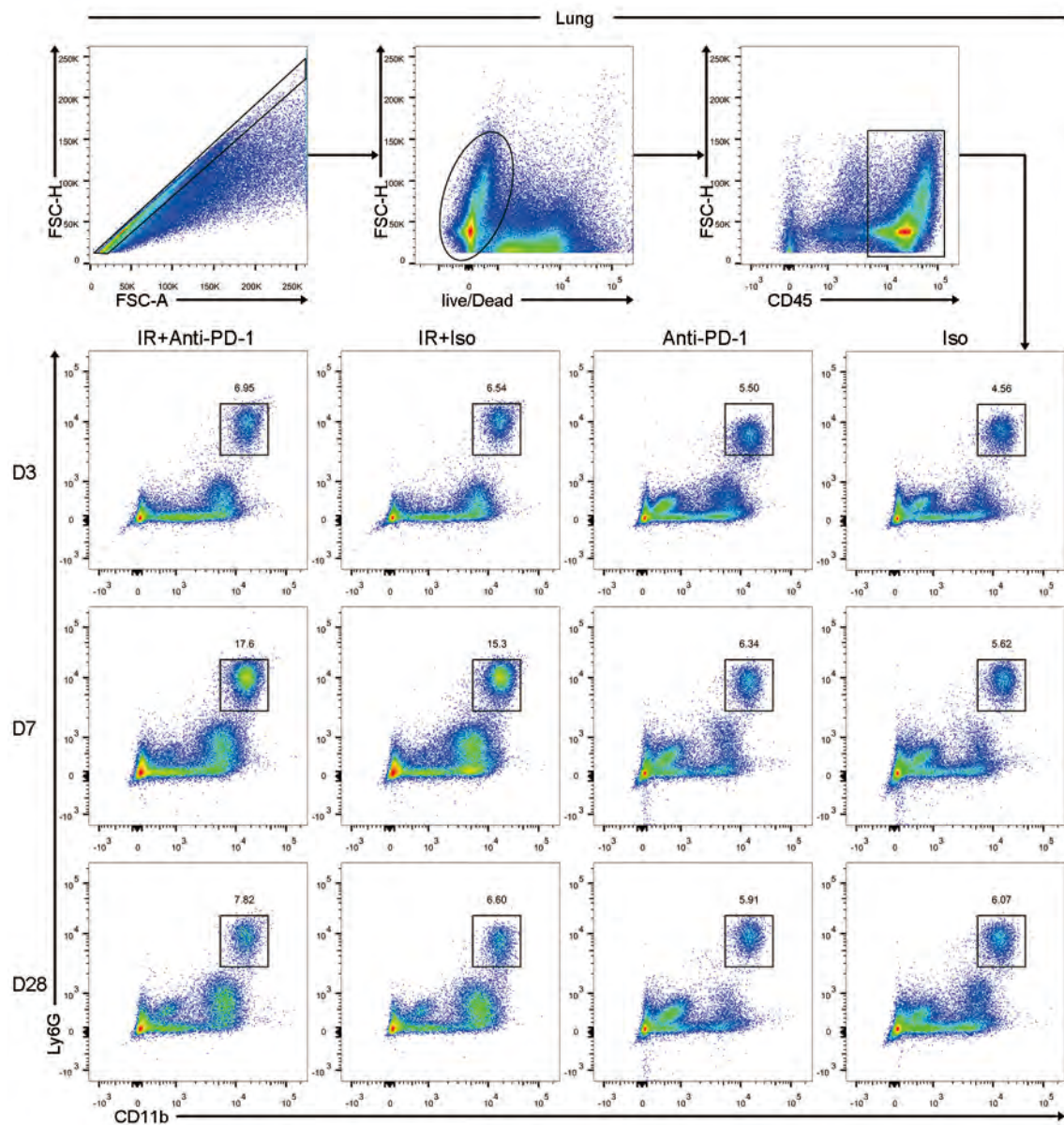
Figure S3 Concurrent administration of anti-PD-1 antibody significantly upregulated the numbers of lung neutrophils in mice with radiation-induced lung injury (RILI). Living cells were gated to determine the presence of CD45$^+$ Ly6G$^+$ CD11b$^+$ neutrophils in the lung tissues on days 3, 7, and 28 post-irradiation. The numbers were the percentage of neutrophils cells among total CD45$^+$ leukocyte cells.
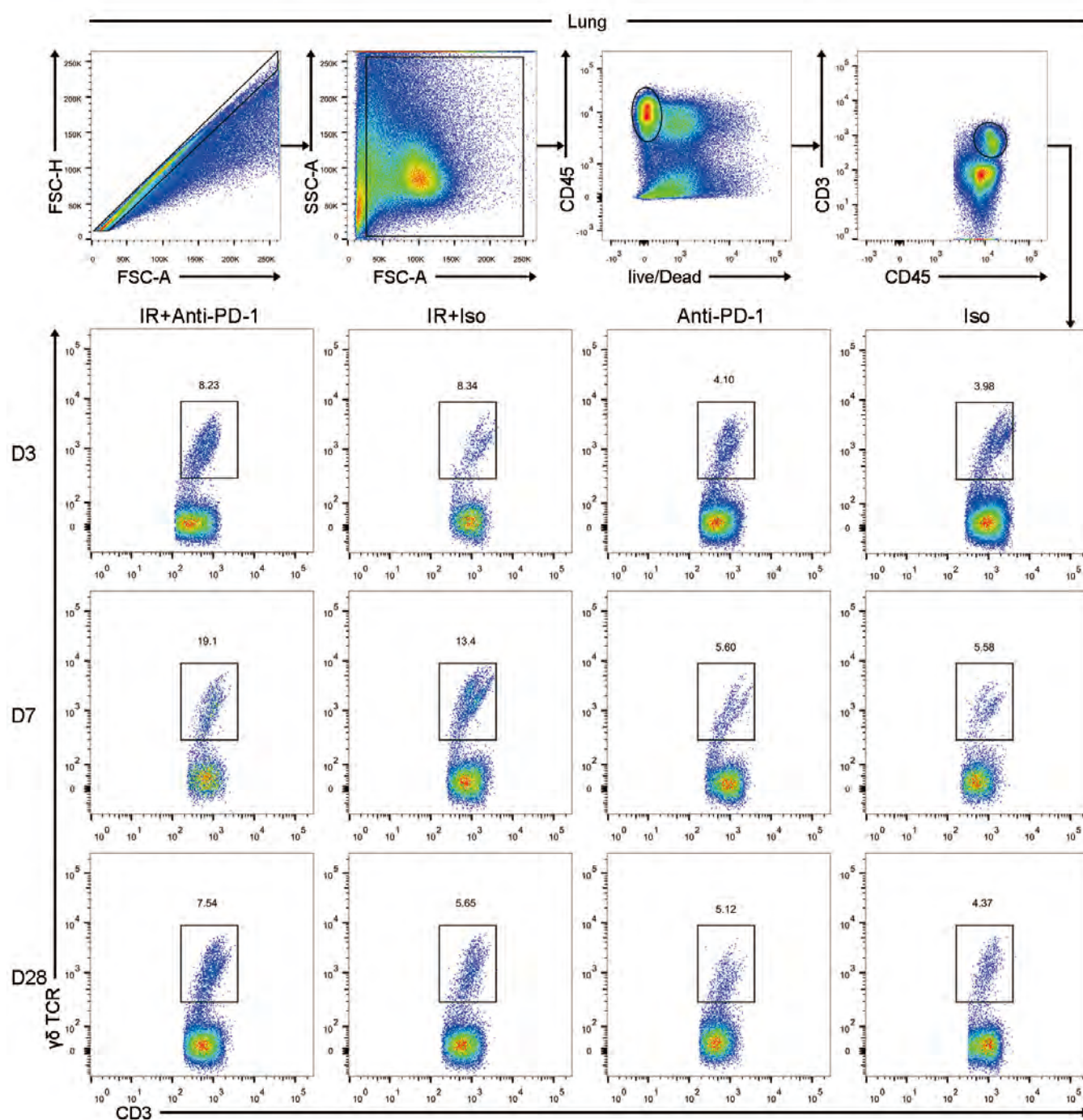
Figure S4 Concurrent administration of anti-PD-1 antibody resulted in a significant increase in γδ T cells in the lung tissues of mice with RILI. Identification of CD45$^+$ CD3$^+$ γδ TCR$^+$ T cells in the lung tissues on Days 3, 7, and 28 post-irradiation. The numbers were the percentage of γδ T cells among total CD3$^+$ T cells.

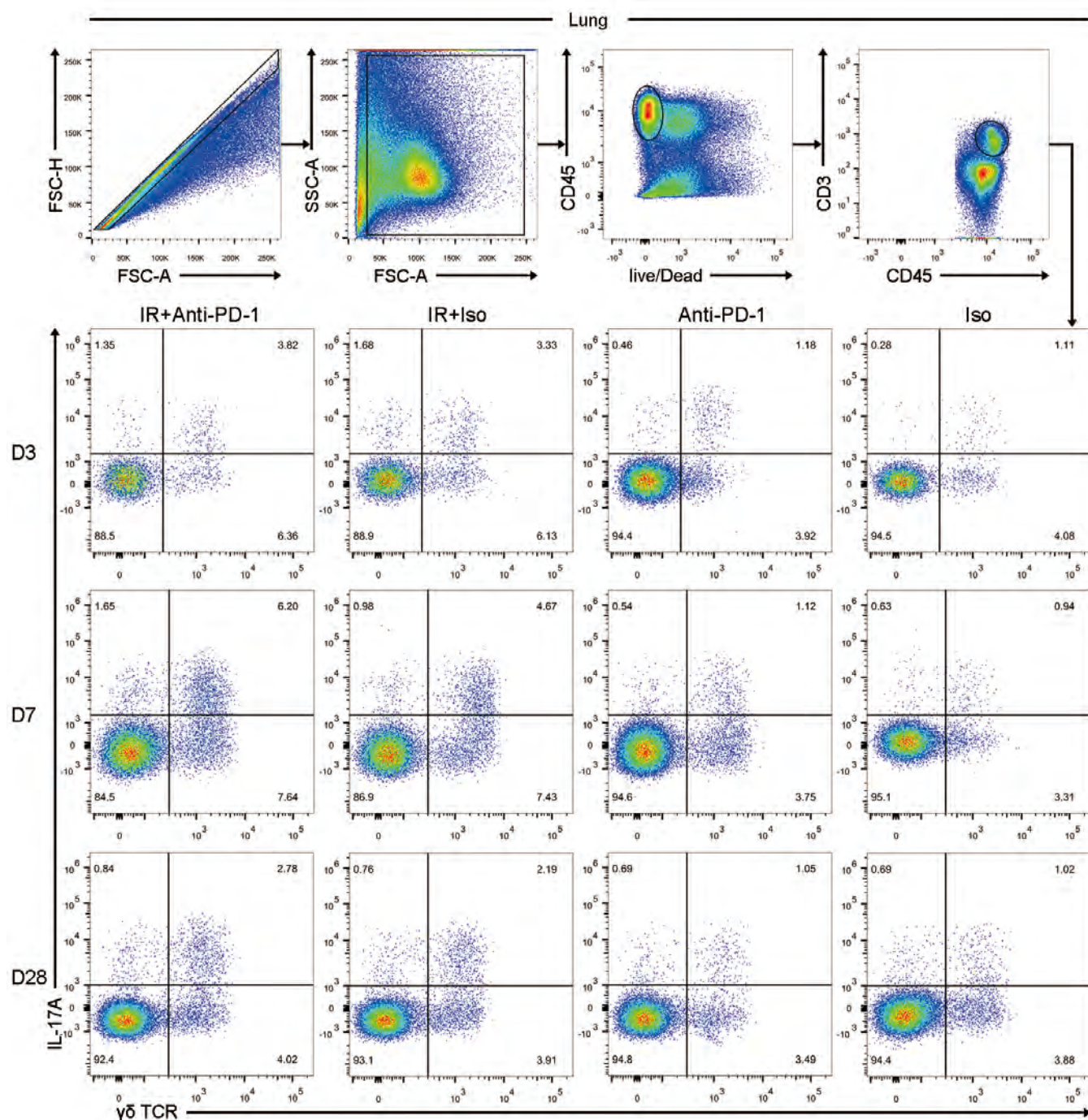**Figure S5** The numbers of IL-17A-producing γδ T cells were elevated in the irradiated lungs with concurrent anti-PD-1 immunotherapy. Flow cytometry plots of lung CD45⁺ CD3⁺ γδ TCR⁺ IL-17A⁺ T cells on Days 3, 7, and 28 post-irradiation. The numbers were the percentage of IL-17A-producing γδ T cells among total CD3⁺ T cells.
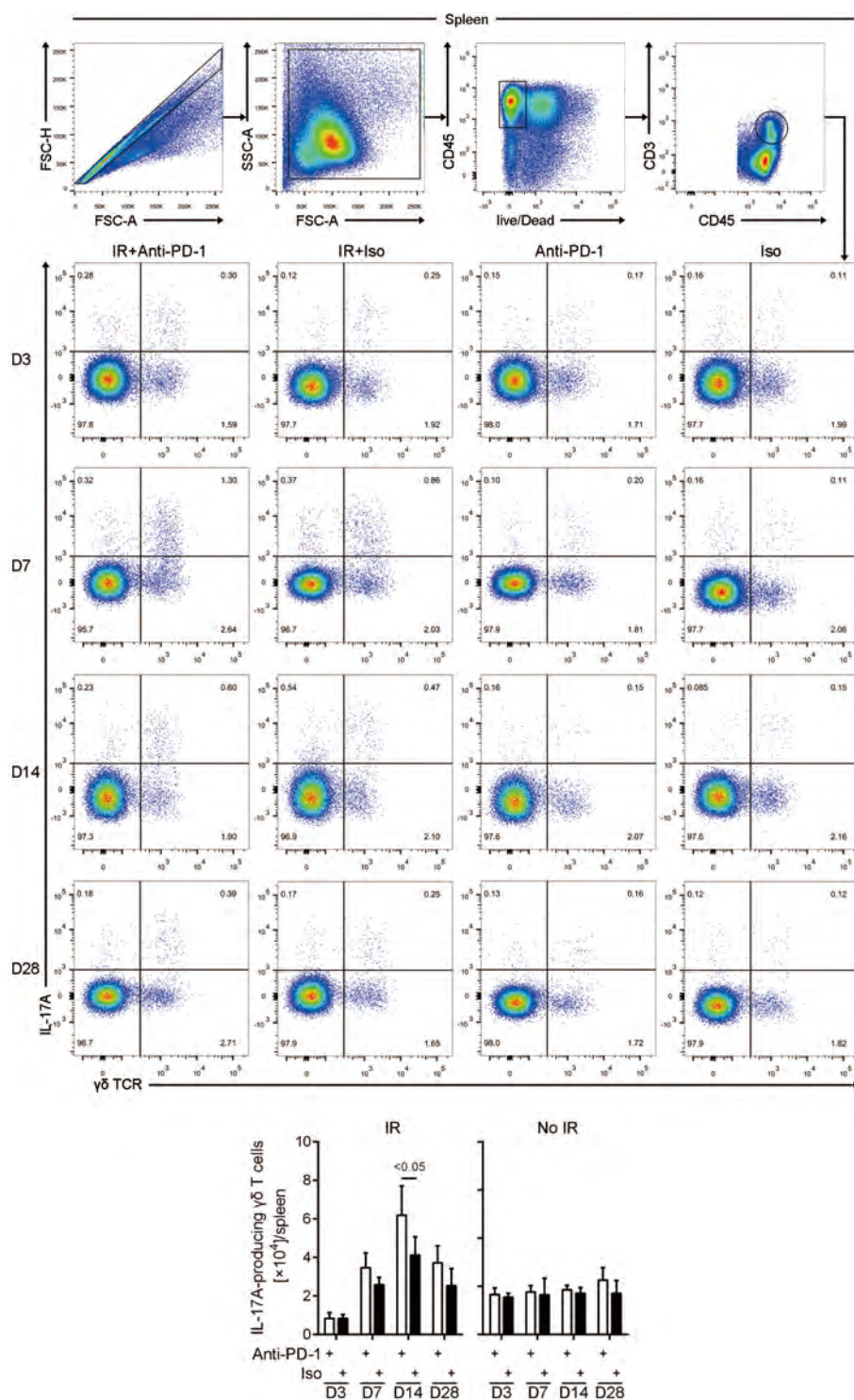
Figure S6 Concurrent administration of anti-PD-1 antibody significantly increased the numbers of splenic IL-17A-producing γδ T cells in RILI CD45⁺ CD3⁺ γδ TCR⁺ IL-17A⁺ T cells were progressively gated (upper panel) and counted (lower panel) in the spleen on days 3, 7, 14 and 28 post-irradiation. Empty bars indicate the mice received rat anti-PD-1 antibody. Filled bars indicate the mice received same amount of isotype. The numbers were the percentage of IL-17A-producing γδ T cells among total CD3⁺ T cells (n = 5).

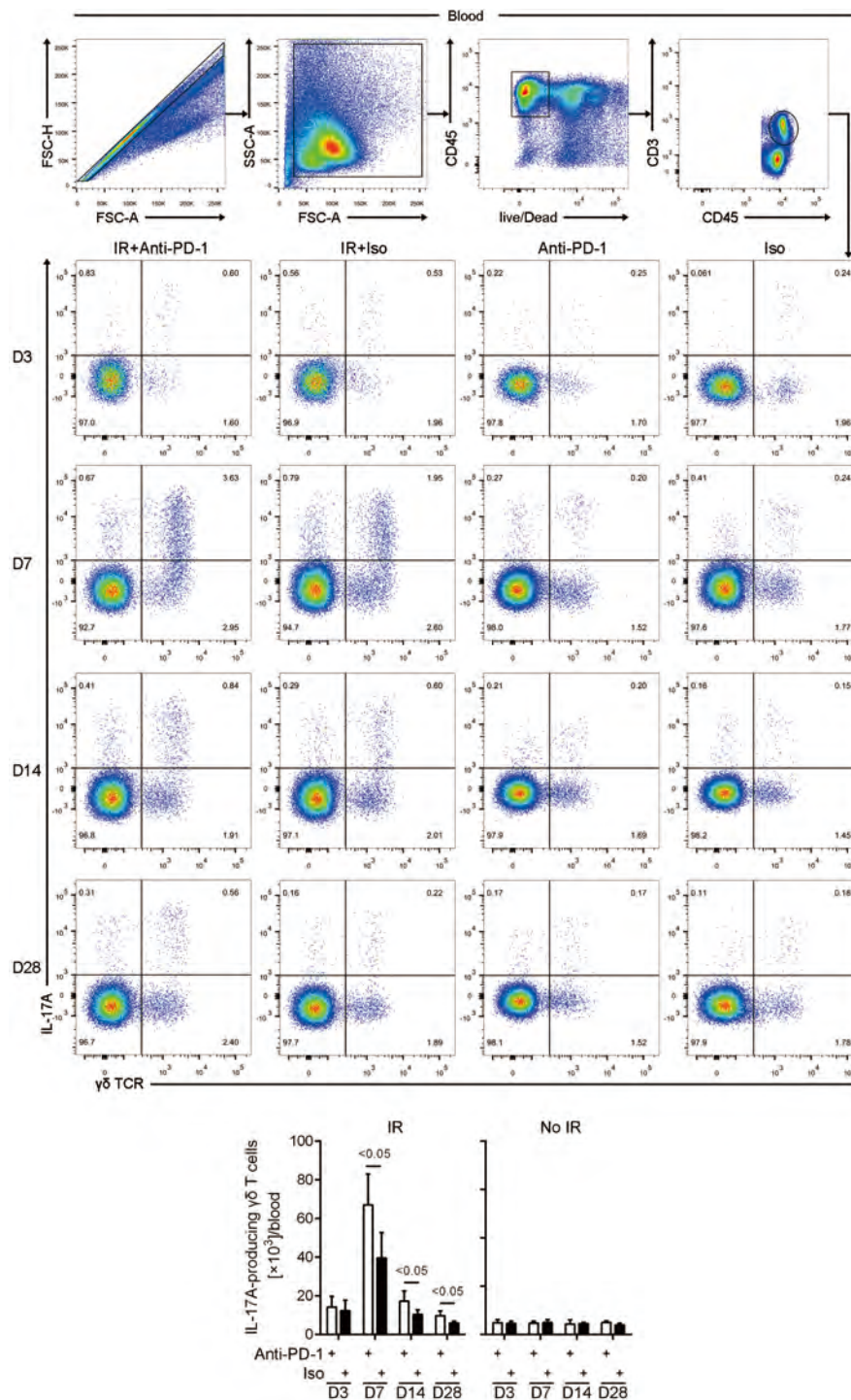**Figure S7** Concurrent administration of anti-PD-1 antibody significantly increased IL-17A-producing γδ T cell counts in the peripheral blood of RILI Flow cytometry gating strategies for CD45$^+$ CD3$^+$ γδ TCR$^+$ IL-17A$^+$ T cells in the peripheral blood on Days 3, 7, 14 and 28 post-irradiation. Empty bars indicate the mice received rat anti-PD-1 antibody. Filled bars indicate the mice received same amount of Isotype. The numbers represented the percentage of IL-17A-producing γδ T cells among total CD3$^+$ T cells. Quantified data was shown in the lower portion of the panel (n = 5).