# Azithromycin attenuates acute radiation-induced lung injury in mice

FEI TANG[1,2], RUI LI[1], JIANXIN XUE[1], JIE LAN[1], HE XU[1], YONGMEI LIU[1], LIN ZHOU[1] and YOU LU[1]

[1]Department of Thoracic Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041; [2]Department of Medical Oncology, Guizhou Province People's Hospital, Guiyang, Guizhou 550002, P.R. China

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**Abstract.** Radiation-induced lung injury (RILI) is a common and major obstacle in thoracic cancer radiotherapy, resulting in considerable morbidity and limiting the dose of radiation. However, an effective treatment option remains to be established. Therefore, the present study aimed to investigate the effects of azithromycin (AZM) in acute RILI with a mouse model. In the present study, C57BL/6 mice were given a single thoracic irradiation of 16 Gy and administered orally with AZM. The lung histopathological findings, the levels of malondialdehyde (MDA; an indicator of oxidative damage) and the concentration of pro-inflammatory and pro-fibrotic cytokines in plasma were assessed on 28 day following irradiation. In addition, the total cell counts in bronchoalveolar lavage fluid (BALF), the pro-inflammatory and pro-fibrotic cytokine gene expression in lung tissue were evaluated on day 7, 14 and 28 following irradiation. Administration with AZM markedly alleviated acute RILI as indicated by hematoxylin and eosin and Masson staining. The levels of MDA and total cell counts in BALF significantly reduced in AZM treated mice. AZM also down-regulated the concentration and mRNA expression of interleukin (IL)-1β, IL-6, tumor necrosis factor-α and transforming growth factor-β1. In addition, AZM attenuated the irradiation-induced increases in the mRNA expression of fibrotic markers (α-smooth muscle actin and α-1 type I collagen). AZM treatment mitigated the radiation-induced acute lung injury possibly by its anti-inflammatory and anti-fibrotic effects.

*Correspondence to:* Mr. You Lu, Department of Thoracic Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, 37 Guoxue Lane, Chengdu, Sichuan 610041, P.R. China
E-mail: radyoulu@hotmail.com

*Key words:* azithromycin, radiation-induced lung injury, inflammation, fibrosis, cytokine

## Introduction

Radiation therapy (RT) is an essential therapeutic modality for treating thoracic malignancies, including lung cancer and breast cancer (1). Unfortunately, radiation-induced cell death is not confined to tumors. Normal lung tissue is damaged due to the generation of reactive oxygen species and subsequent inflammation and fibrosis (2). Radiation-induced lung injury (RILI) is a common and major obstacle in thoracic cancer radiotherapy, which results in considerable morbidity and limits the dose of radiation (3). The clinical incidence of radiation-induced pneumonitis ranges from 5 to 30% (4) and is an increasingly prevalent cause of morbidity and mortality (5). Therefore, alleviating RILI is critical for improving tumor control and patient quality of life. However at present, there is no known effective therapeutic strategy to prevent, mitigate, and treat RILI.

The exact pathophysiology of RILI is not completely understood, but the evidence suggests that inflammation has a central role in the initiation and establishment of RILI, especially acute RILI (6). It is generally hypothesized that this process is regulated by the release and activation of various pro-inflammatory and pro-fibrotic cytokines by damaged and activated cells, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) (2).

Azithromycin (AZM) is a second-generation macrolide antibiotic with broad-spectrum efficacy against gram-positive, gram-negative and atypical pathogens. In addition to its antibiotic activity, several studies have established that AZM possesses anti-inflammatory and immunomodulatory properties, and reaches very high and stable lung concentrations (7). AZM treatment has been demonstrated to decrease pulmonary exacerbations and improve lung function in patients with cystic fibrosis (CF) (8,9), chronic obstructive pulmonary disease and non-CF bronchiectasis (10,11). AZM has also been shown to be beneficial in lung transplantation for the prevention and treatment of chronic allograft rejection (12). The mechanisms of action involved in the anti-inflammatory and immunomodulatory properties of AZM are being investigated, but remain unclear and are independent of its traditional antimicrobial activity (13,14). AZM was also previously reported to inhibit mRNA and protein expression of pro-inflammatory cytokines

(Tumor necrosis factor-α and interleukin-1β) in cultured human corneal epithelial cells stimulated by Toll-like receptor agonists (15).

The therapeutic potential and usefulness of AZM in RILI treatment has not been studied. In the present study, the authors investigated the effects of AZM on acute RILI in a C57BL/6 mouse model.

## Materials and methods

*Animals and irradiation*. Female C57BL/6 mice (n=65; weight, 18-20 g, 8 weeks old) were purchased from the Experimental Animal Center, Chinese Academy of Medical Sciences (Beijing, China) and kept under conventional pathogen-free conditions. The mice were maintained at 23±2˚C, with a relative humidity of 50±5%, artificial lighting from 08:00-20:00 and 13-18 air changes/h. The mice were given a standard diet for laboratory rats or mice and water *ad libitum*. The investigation was performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (16), and approved by the Animal Care and Use Committee of Sichuan University (Chengdu, China).

The mice were anesthetized and subjected to 16 Gy whole thoracic irradiation using 6 MV X-rays (Varian Clinac 600C X-ray; Varian Medical Systems, Palo Alto, CA, USA). The head, abdomen, and extremities were shielded with lead strips. Non-irradiated mice were treated in the same manner but without radiation.

*Treatment protocol*. AZM (Dalian Meilun Biology Technology Co., Ltd, Liaoning, China) was dissolved in vehicle (3.5% ethyl alcohol absolute and 96.5% corn oil). The recommended dose in humans is 500 mg, which is ~10 mg/kg in mice. Furthermore, a higher dosage of AZM is required in mice than in humans due to rapid liver metabolism in mice, resulting in an elimination half-life of 2.3 h compared with 68 h in humans (17,18). In order to select an appropriate dose, 10 and 100 mg/kg/day of AZM were used in the pilot study.

In the preliminary study, the mice were randomly divided into four groups: Control (non-irradiated control + vehicle, n=5), RT (irradiation + vehicle, n=5), AZM-10 (irradiation + 10 mg/kg/day AZM, n=5) and AZM-100 groups (irradiation + 100 mg/kg/day AZM, n=5). In the formal study, the mice were randomly divided into three groups: Control (non-irradiated control + vehicle, n=15), RT (irradiation + vehicle, n=15) and AZM groups (irradiation + AZM, n=15).

One day prior to irradiation, the mice received AZM (10 mg/kg or 100 mg/kg) or the same volume of vehicle alone (5 ml/kg) by gavage. Following irradiation, the animals were treated with the original protocol, as described in the preliminary study, once daily until sacrificed under anesthesia.

*Liquichip assay*. At anesthesia, peripheral blood was collected following enucleation of eyeball, and the blood was permitted to clot at 4˚C for 24 h and centrifuged at 1,500 x g for 15 min. The plasma was collected and stored at -80˚C until analysis. The concentration of cytokine in the plasma was assayed using a Mouse Cytokine/Chemokine liquichip kit (EMD Millipore, Billerica, MA, USA). As TGF-β1 was not included in this kit, a separate TGF-β1 liquichip kit was used (EMD Millipore).

*Bronchoalveolar lavage fluid (BALF) analysis*. On day 7, 14 and 28 following irradiation, the mice were sacrificed and the thorax was dissected. The lung tissues were exposed, and the right lobe of lung was ligated with a 6-0 suture, while the left lung was not. An open tracheotomy was performed, and a small plastic tube was inserted into the trachea. To obtain BALF, ice-cold PBS (0.35 ml) was infused into the lung and withdrawn via tracheal cannulation three times (total volume, 1.05 ml). BALF was centrifuged (400 x g, 15 min, 4˚C), and the cell pellet was suspended in 1 ml modified Hank's balanced salt solution. The total number of nucleated cells was counted under a light microscope (Imager A2; Zeiss AG, Oberkochen, Germany). Differential cell count in BALF was performed in a double-blind manner by two independent observers.

*Histopathology*. The lung tissues were fixed with 10% formalin and embedded in paraffin. The tissue sections (thickness, 4 $\mu$m) were stained with Mayer's hematoxylin (H) for 30 sec and eosin (E) for 20 sec at room temperature and Masson's trichrome (ponceau red acid magenta dye for 10 min and aniline blue for 5-10 min) at room temperature. Images of the slides were obtained using a digital camera mounted on a light microscope (Imager A2; Zeiss AG). Each H&E tissue section was given a score between 0-4 based on the area affected by interstitial inflammation, alveolar wall thickening, peribronchial inflammation and interstitial edema as follows: Score 0, ≤10%; 1, ≤30%; 2 ≤50%; 3, ≤70% and 4, ≥70%. A mean inflammation score was determined for each group of mice (19). The grade of fibrosis of each section stained with Masson's trichrome was evaluated with a modified scale of 0-8, as previously reported (20). Briefly, on a scale of 0-8, grade 0 represents normal lung and grade 8 represents total fibrous obliteration of the field. This evaluation was performed by two blind independent observers (Department of Thoracic Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China).

*Malondialdehyde (MDA) activity assay*. The concentration of MDA was determined in plasma and lysates of radiated lung tissue by using the MDA assay kit (Nanjing Jiancheng Bio-engineering Institute, Jiangsu, China), according to the manufacturer's instructions. The MDA levels were expressed as nmol/ml for plasma samples and nmol/mg of tissue for lung tissue homogenate.

*Quantitative reverse transcription polymerase chain reaction (RT-qPCR)*. A total of 100 mg irradiated lung tissue was freshly isolated from each sample. Total RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 1 mg total RNA from each sample was used for first-strand complementary DNA synthesis (37˚C for 15 min; 85˚C for 5 sec) with a RT-PCR kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR (95˚C for 1 min; 95˚C for 10 sec; 58˚C for 10 sec; 72˚C for 10 sec; all for 40 cycles) was performed with the SYBR RT-PCR kit (Takara Bio, Inc.) on the Chromo4 Real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The level

Table I. Primer sequences for quantitative reverse transcription polymerase chain reaction.

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| IL-1β | TTCTTGGGACTGATGCTG | CTCATTTCCACGATTTCCC |
| IL-6 | CAGGCTCCGAGATGAACAA | CAGACTCCACTTTGCTCTTGAC |
| TNF-α | CTGTGAAGGGAATGGGTGTT | CAGGGAAGAATCTGGAAAGGTC |
| TGF-β1 | ATGGTGGACCGCAACAAC | AGCCACTCAGGCGTATCAG |
| α-SMA | TGCTGGACTCTGGAGATGGT | ATCTCACGCTCGGCAGTAGT |
| COL1A1 | ACGCCATCAAGGTCTACTGC | CGGGAATCCATCGGTCAT |
| GAPDH | GGTGAAGGTCGGTGTGAACG | CTCGCTCCTGGAAGATGGTG |

COL1A1, α-1 type I collagen; TGF, transforming growth factor; IL, interleukin; α-SMA, smooth muscle actin.

of GAPDH mRNA in each sample was used as an internal control. All reactions were performed in duplicate, and the results were analyzed by the $2^{-\Delta\Delta Cq}$ method (21). The primer sequences are stated in Table I.

*Western blot analysis*. The lung tissues were homogenized in ice-cold RIPA lysis buffer with protease and phosphatase inhibitors (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Homogenates containing 30 $\mu$g tissue lysate were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore). The buffer used for blocking was 5% skimmed milk for 1 h at room temperature. The membranes were incubated with rabbit monoclonal TGF-β1 antibodies (1:500; sc-146; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and antibodies against β-actin, which were used as a loading control (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), overnight at 4˚C. Immunoreactivity was detected using horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin G antibody (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) in blocking solution for 1 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence kit (EMD Millipore). The western blots were imaged and analyzed by The ChemiDoc MP Imaging System of BIO-RAD and the software used was Image Lab 5.2.1 (Bio-Rad Laboratories, Inc.).

*Statistical analysis*. Data are presented as the mean ± standard error. The data from different groups during various time points were compared using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were carried out using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

*AZM treatment attenuates RILI histopathology*. The experimental protocol is shown in Fig. 1A. In the preliminary study, the authors evaluated RILI-associated histological changes on day 28 using H&E and Masson stained lung sections. Compared with the control group, lung tissue in the RT group showed markedly thickened alveolar walls, collapsed alveoli and marked inflammatory pathological changes, including local inflammatory cell infiltration and inflammatory exudation (Fig. 1B). By contrast, treatment with AZM decreased the thickness of alveolar walls and alleviated interstitial edema (Fig. 1B). Masson staining showed radiation-induced collagen deposition in parts of the lung tissues, and AZM treatment attenuated this deposition (Fig. 1C). Similarly, when lung tissue inflammation and grade of fibrosis were evaluated, the increased inflammation score and grade of fibrosis caused by irradiation were significantly decreased following 100 mg/kg/day AZM treatment (both P<0.01; Fig. 1D and E). Unfortunately, compared with the RT group, the score and grade in the AZM-10 group were lower. However, the differences in score and grade in the AZM-10 group were not statistically significant (both P>0.05; Fig. 1D and E).

*AZM treatment reduces the level of lipid peroxidation*. The present authors measured the levels of MDA in plasma and lung tissue homogenates in order to investigate the effects of AZM on radiation-induced lipid peroxidation. Irradiation treatment increased the levels of MDA. However, the levels of MDA in the AZM-100 group significantly decreased in plasma and lung tissue (both P<0.01 vs. RT group; Fig. 2A and B). These results indicated that 100 mg/kg/day AZM was effective in reducing the level of lipid peroxidation. Compared with the RT group, the levels of MDA in the AZM-10 group were lower in the plasma and lung tissue. However, no significant differences were observed in plasma and lung tissue. Therefore, 100 mg/kg/day was selected as the high AZM dose in subsequent experiments.

*AZM administration decreases total cell counts in BALF*. In the formal study, the mice were sacrificed under anesthesia on day 7, 14 and 28 post-irradiation. The authors evaluated the effect of AZM on total cell counts in BALF following irradiation (Fig. 3). In the RT group, the counts decreased on day 7 compared with the control, and the levels in the RT group decreased further on day 14 (day 7 and 14 vs. control, P<0.01; Fig. 3). By day 28, a marked increase was observed compared with the control (day 28 vs. control). However, in the AZM-100 group, total cell counts were not significantly different to those in the control group on day 7 (P>0.05 vs. control. The total cell counts in the AZM-100 group were increased on day 14 compared with the RT group (P<0.05 vs. RT group; Fig. 3), while the influx of total cells

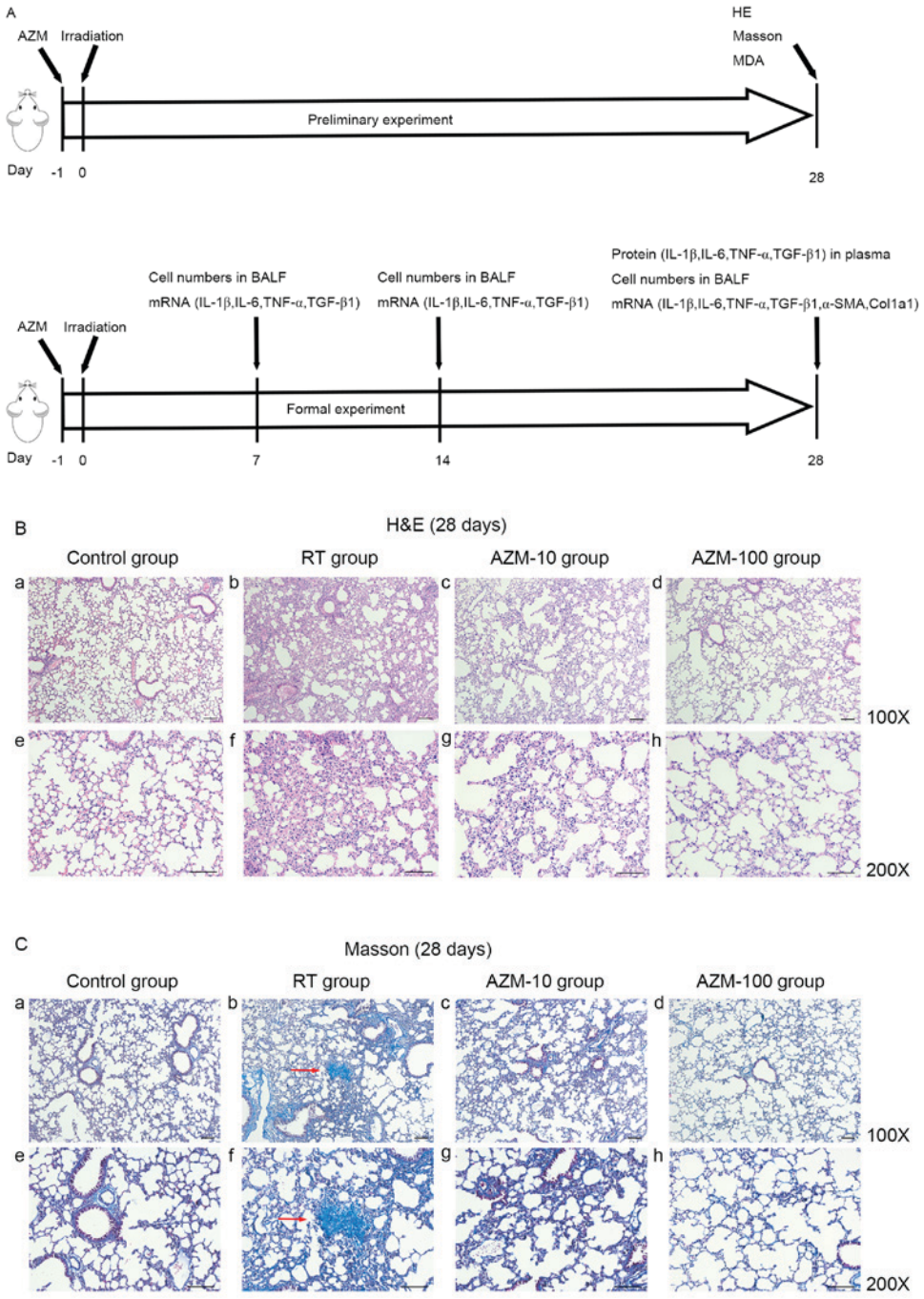


Figure 1. Schematic diagram of the experimental protocol and the effect of AZM on histological changes in the control, RT, AZM-10 and AZM-100 groups on day 28 following irradiation. (A) The mouse thorax was irradiated with 16 Gy X-ray. One day prior to radiation, the mice received AZM or vehicle alone and were administered with the original protocol, as described in the preliminary study, once daily until the mice were sacrificed under anesthesia. Lung tissues, plasma and bronchoalveolar lavage fluid were collected at indicated time points for each experiment. (B) Representative photomicrographs of hematoxylin and eosin stained lung sections. Characteristic morphology of each group is shown at magnifications (a-d) 100x and (e-h) 200x. Scale bar, 100 $\mu$m. (C) Representative photomicrographs of Masson stained lung sections. Characteristic morphology of each group is shown at (a-d) 100x and (e-h) 200x. Examples of collagen deposition lesions (red arrows) are marked. Scale bar, 100 $\mu$m. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. AZM, azithromycin; BALF, bronchoalveolar lavage fluid; COL1A1, α-1 type I collagen; H&E, hematoxylin and eosin; MDA, malondialdehyde; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin; α-SMA, smooth muscle actin.

in the AZM-100 group was significantly decreased on day 28 compared with the RT group (P<0.05; Fig. 3).

*AZM treatment reduces the levels of pro-inflammatory cytokine expression in plasma*. The concentrations of pro-inflammatory cytokines in plasma, including IL-1β, IL-6 and TNF-α, were measured by liquichip on day 28 following irradiation. Thorax irradiation resulted in the abundant production of IL-1β, IL-6 and TNF-α, and the plasma levels of these cytokines significantly increased in RILI mice on day 28 (all P<0.05 vs. control group). By contrast, AZM treatment significantly decreased the irradiation-induced protein

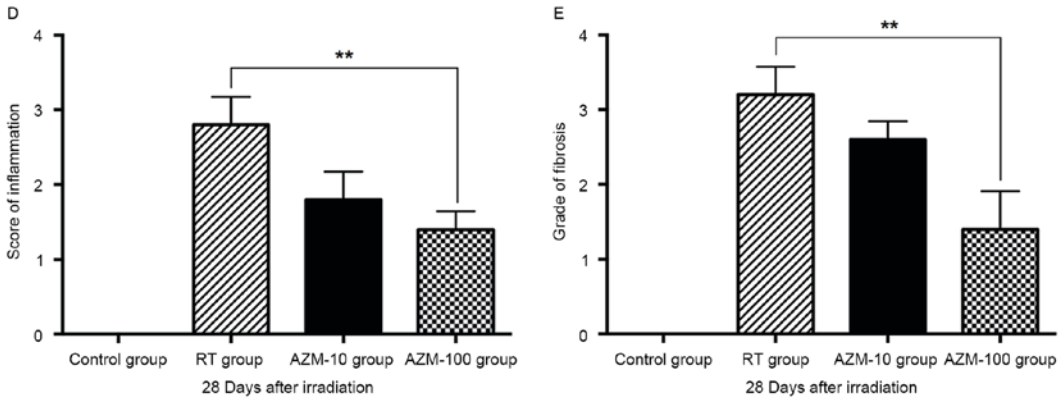


Figure 1. Continued. (D) Scoring of lung tissue inflammation as assessed by one-way ANOVA. Data are represented as the mean ± standard error. **P<0.01, RT group vs. AZM-100 group; n=5 per group. (E) Grading of lung tissue fibrosis as assessed by one-way ANOVA. Data are represented as the mean ± standard error. **P<0.01, RT group vs. AZM-100 group; n=5 per group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. ANOVA, one-way analysis of variance; AZM, azithromycin.

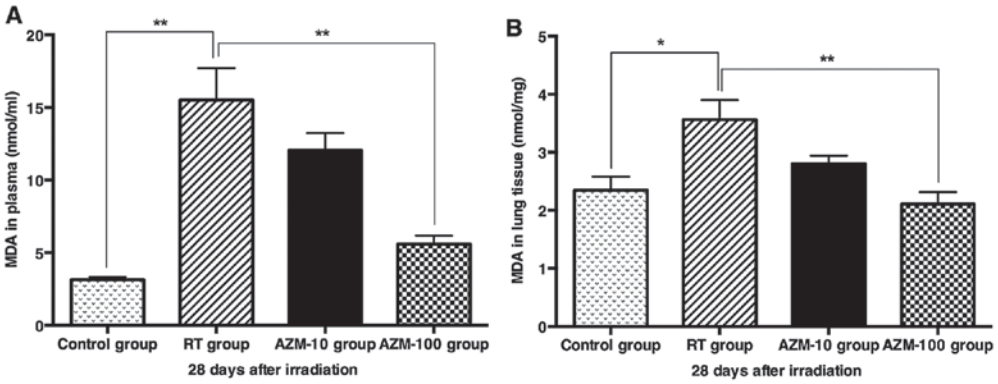


Figure 2. Effect of AZM on the levels of MDA. Changes in (A) plasma and (B) lung tissues in the control group, RT, AZM-10 and AZM-100 groups on 28 day following irradiation. Data are represented as the mean ± standard error. In (A) both Control vs. RT group and RT group vs. AZM-100 group: **P<0.01. In (B) control vs. RT group: *P<0.05, RT group vs. AZM-100 group: **P<0.01; 5 samples/group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. AZM, azithromycin; MDA, malondialdehyde.

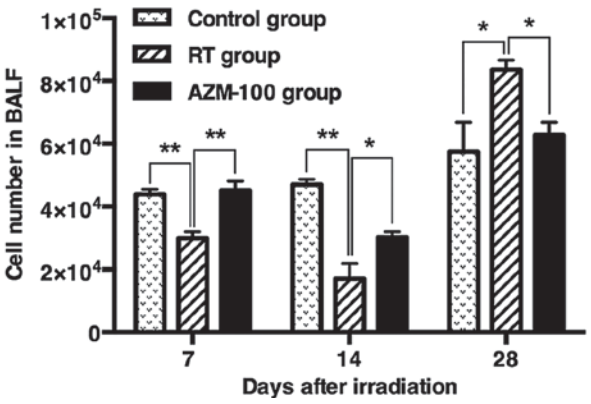


Figure 3. Effect of AZM on changes in total cell counts in BALF on day 7, 14 and 28 following irradiation. Control group vs. RT group and AZM-100 group at the same time points, by one-way analysis of variance. Data are represented as the mean ± standard error. Day 7: Control vs. RT group and RT group vs. AZM-100 group, **P<0.01; day 14: Control vs. RT group, **P<0.01 and RT group vs. AZM-100 group, *P<0.05; day 28: Control vs. RT group and RT group vs. AZM-100 group, *P<0.05; 5 samples/group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. AZM, azithromycin; BALF, bronchoalveolar lavage fluid.

release of IL-1β, IL-6 and TNF-α in plasma compared with the RT group (Fig. 4A-C).

*AZM treatment reduces pro-inflammatory cytokine gene expression in lung tissue*. To gain further insight into the effect of AZM on RILI, lung mRNA samples from these mice were measured on day 7, 14 and 28 following irradiation using RT-qPCR. As shown in Fig. 5A and B, irradiation resulted in a slight increase in the levels of IL-1β and IL-6 on day 7, 14 and 28 compared with the control group (Fig. 5A and B). By contrast, AZM treatment significantly inhibited this increase on day 28 (Fig. 5A and B). On day 14 following irradiation, there was an increase in the levels of TNF-α compared with the control group, and this increase was significantly reduced in AZM-treated mice (Fig. 5C).

*AZM treatment reduces pro-fibrotic factor expression*. The present authors also examined TGF-β1 expression in plasma and lung tissue using liquichip or western blotting on day 28 following irradiation. As expected, AZM treatment significantly decreased irradiation-induced TGF-β1

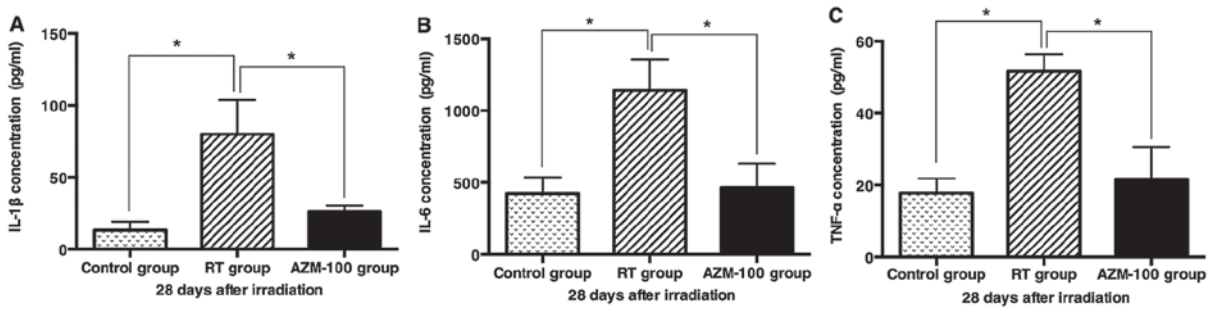


Figure 4. Analysis of concentration of pro-inflammatory cytokines in plasma by Liquichip on 28 day following irradiation. Analysis of (A) IL-1β, (B) IL-6 and (C) TNF-α concentration. Data are represented as the mean ± standard error. Control vs. RT group and RT group vs. AZM-100 group, *P<0.05; 5 samples/group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. AZM, azithromycin; TNF, tumor necrosis factor; IL, interleukin.

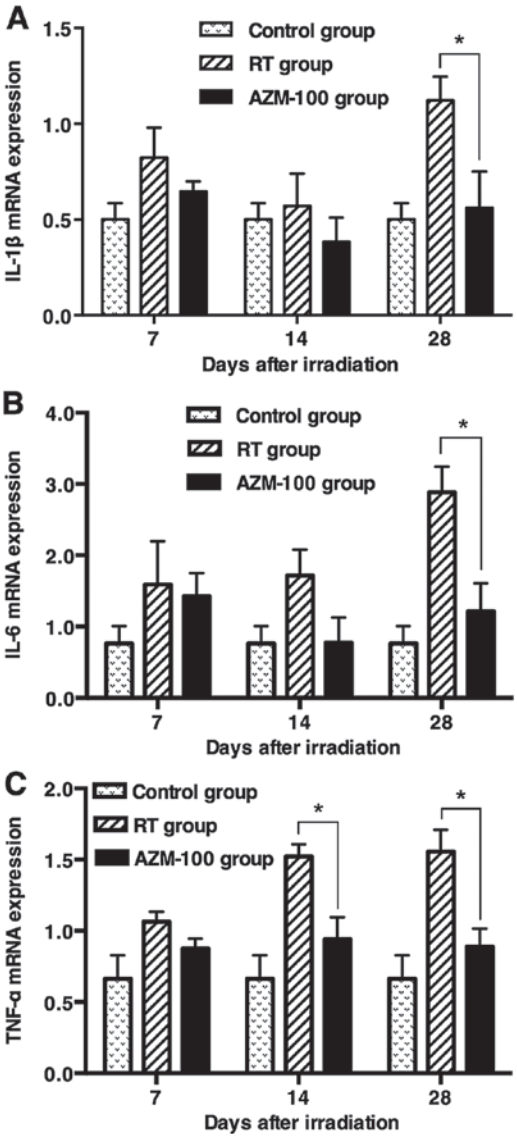


Figure 5. Analysis of changes in pro-inflammatory cytokine gene expression in lung tissues by quantitative reverse transcription polymerase chain reaction on day 7, 14 and 28 following irradiation. (A) IL-1β expression. (B) IL-6 expression. (C) TNF-α expression. Data are represented as the mean ± standard error. In (C) on day 14, RT group vs. AZM-100 group, *P<0.05; in (A-C) on day 28, RT group vs. AZM-100 group, *P<0.05; 5 samples/group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. TNF, tumor necrosis factor; IL, interleukin.

expression in plasma and lung tissues compared with the RT group (Fig. 6A-C). In order to further determine the changes in TGF-β1, the authors measured TGF-β1 mRNA expression in injured lungs using RT-qPCR on day 7, 14 and 28 following irradiation. The irradiation-induced increase in TGF-β1 mRNA expression was significantly decreased in the AZM-100 group on day 7, 14 and 28 (Fig. 6D).

The mRNA expression of α-smooth muscle actin (α-SMA) and α-1 type 1 collagen (COL1A1) was examined in lung tissue on day 28 following irradiation. The irradiation-induced increase in α-SMA and COL1A1 mRNA expression in injured lung tissue significantly decreased in the AZM-100 group (Fig. 6E and F).

## Discussion

In the present study, a murine model was used to investigate the effect of AZM, as a new biological strategy, to ameliorate acute RILI. The results showed that AZM decreased radiation-induced early lung injury. Oxidative stress, inflammatory cell infiltration, cytokine production, and associated gene expression have pivotal roles in the pathogenesis of RILI (22). Although, the exact mechanisms by which RILI is mitigated by AZM are not well described, anti-inflammatory and anti-fibrotic effects may be involved.

Lipid peroxidation is one of the oxidative conversions of polyunsaturated fatty acids to products such as MDA, which is an important indicator of oxidative damage (23,24). In the present study, marked increases in MDA content were observed between week 1 and 24 following whole-lung irradiation, which demonstrated oxidative stress due to radiation-induced pneumonitis and lung fibrosis. Oxidative stress has been previously shown to ameliorate RILI (25). A previous study indicated that AZM decreased the levels of MDA in a pig model of otitis media (26). These findings were confirmed by the results of the present study. In addition, radiation is known to induce the expression of NO synthase (NOS) and results in nitrotyrosine formation in the lungs (27). Evidence to support the harmful role of NOS in RILI includes a study in which partial attenuation of RILI was observed following treatment with L-nitro-arginine methyl ester, a relatively nonspecific NOS inhibitor (28). Notably, AZM also significantly reduced NOS activity in a rat model of colonic damage (29).

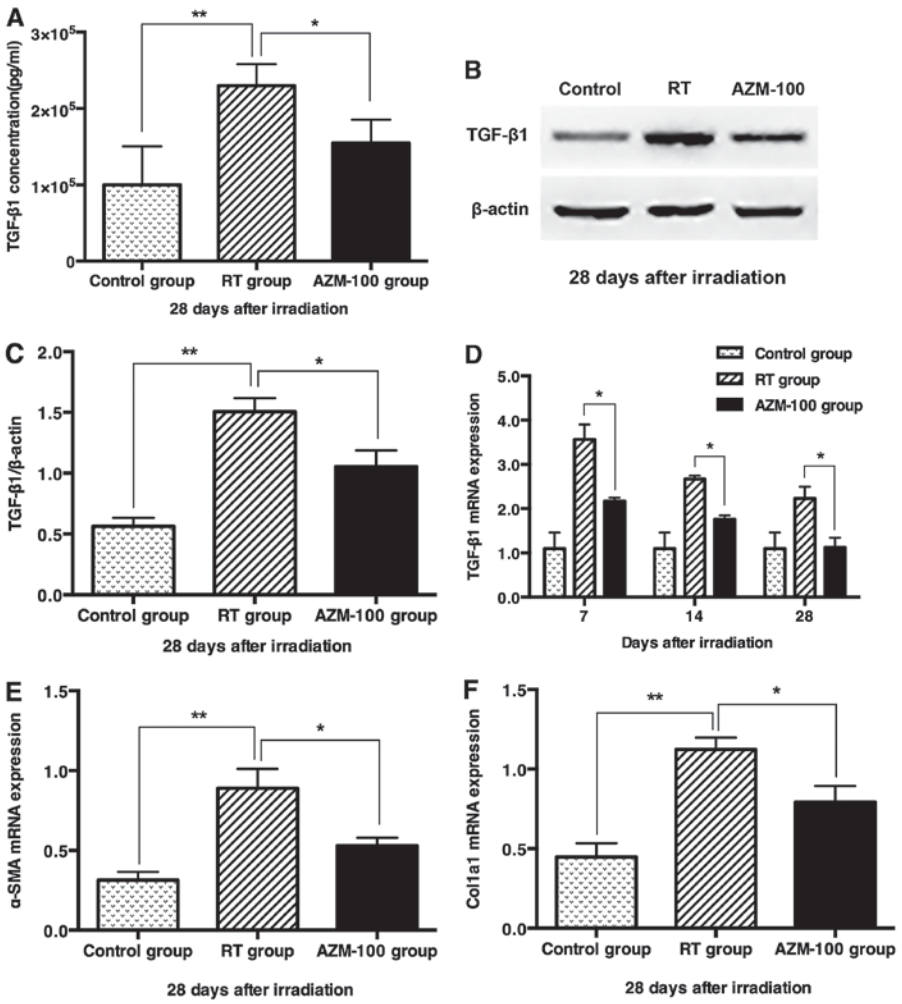


Figure 6. Effect of AZM on changes in the level of pro-fibrotic factor in plasma and lung tissue following irradiation. (A) Changes in TGF-β1 concentration in plasma on day 28 following irradiation. (B) Western blot analysis of TGF-β1 in lung tissue on day 28 following irradiation. (C) Relative TGF-β1 protein expression in lung tissue. Densitometry values were normalized to β-actin. (D) Changes in TGF-β1 mRNA expression in lung tissue on day 7, 14 and 28 following irradiation. (E) α-SMA mRNA expression in lung tissue on 28 day following irradiation. (F) COL1A1 mRNA expression in lung tissue on 28 day following irradiation. Data are represented as the mean ± standard error. Control vs. RT group, $^{**}P<0.01$; RT group vs. AZM-100 group, $^{*}P<0.05$; 5 samples/group. Control, non-irradiated control + vehicle; RT group, irradiation + vehicle; AZM-10 group, irradiation + 10 mg/kg/day AZM; AZM-100 group; irradiation + 100 mg/kg/day AZM. AZM, azithromycin; COL1A1, α-1 type I collagen; TGF, transforming growth factor; IL, interleukin; α-SMA, α-smooth muscle actin.

Evidence suggests a central role for the inflammatory response in the initiation and establishment of RILI. Local recruitment of inflammatory cells and the production of inflammatory cytokines exert an important role in mediating, amplifying and maintaining the RILI process. Intensive anti-inflammatory treatment mitigates the signs and symptoms of RILI (30,31).

Many studies have investigated radiation-induced lung damage using BALF, as it is thought to reflect the lung inflammatory response (32,33). Previous studies have indicated that most patients did not show clinical symptoms of radiation pneumonitis, and lymphocytosis was not very pronounced. However in symptomatic patients, changes in BALF, including total cellularity and lymphocytosis [which appeared to be mainly activated cluster of differentiation (CD)$4^+$ cells], were significantly greater compared with asymptomatic patients (34). In the present study, the total number of cells obtained by BALF decreased on day 7 following irradiation and was very low on day 14. However on day 28, a marked increase was observed. These dynamic changes are in agreement with previous studies (35,36). A previous study indicated that the late increase in BALF cell number was associated with the development of radiation-induced pulmonary lethality (35,36). As shown in the present study, administration of AZM inhibited these dynamic changes. In particular, the number of cells on day 28 was reduced in the AZM-treated group compared with the control. These results are supported by previous data which showed that AZM treatment decreased total cell counts in BALF in a mouse model of ventilator-associated pneumonia and bleomycin-induced acute lung injury (37,38). In addition, inflammatory cell infiltration or exudation into lung parenchyma appears to have to an important role in the development of RILI. Agents that decrease inflammatory cell exudation have the potential to alleviate RILI (39). The present study demonstrated that AZM markedly reduced inflammatory infiltration in the alveolar septa, therefore alleviating the extent of RILI (as determined by H&E staining).

Many studies have indicated that AZM reduces the inflammatory response by decreasing the levels of pro-inflammatory cytokine in lipopolysaccharide- and bleomycin-induced acute lung injury models. These findings are consistent with those in the present study (40,41). Alveolar macrophages have an important role in alveolar physiology. Activated macrophages are the main source of pro-inflammatory cytokines in the early stages of radiation-induced lung disease (42). A number of studies have confirmed that the anti-inflammatory properties of AZM can be attributed, at least partly, to its action on macrophages (41,43). For example, AZM prevented the production of pro-inflammatory cytokines by macrophages in the lipopolysaccharide-induced lung neutrophilia mouse model and inhibited inflammatory cytokine production by J774A.1 macrophage cell lines (41,43). Therefore, the authors hypothesize that AZM regulates inflammatory cytokine production by targeting macrophages following irradiation.

TGF-β, which is a potent stimulator of collagen protein synthesis, exerts a critical role in the pathogenesis of radiation-induced lung fibrosis (44-46). Gene expression of TGF-β has been demonstrated to increase markedly 1-14 days following irradiation, in parallel with changes in fibroblast gene expression of collagen I and fibronectin. The administration of anti-TGF-β1 antibody 1D11 or the TGF-β receptor inhibitor LY210976 is now an option for ameliorating RILI (46-48). In a rat model of bleomycin-induced pulmonary fibrosis, the levels of TGF-β protein and mRNA were reduced following treatment with AZM in the early stage of pulmonary fibrosis (49). In the present study, it was observed that AZM treatment significantly decreased expression of TGF-β1, α-SMA and COL1A1, and grade of fibrosis in Masson stained lung sections, indicating that AZM may contribute to the anti-fibrotic effects of post-irradiation.

Thorax irradiation not only affects macrophages in lung tissue, but also triggers the recruitment of various immune cells into the lung, including $CD4^+$ T-lymphocytes, which exert a critical role in the pathogenesis of radiation-induced pneumonitis preceding lung fibrosis (50). A pronounced increase in $CD4^+$ lymphocytes was demonstrated 4 weeks following irradiation. Co-culture of isolated $CD4^+$ T cells from irradiated lungs with fibroblasts resulted in increased collagen production (51). Furthermore, depletion of $CD4^+$ T cells by specific antibodies prior to partial lung irradiation decreased the degree of radiation-induced lung fibrosis (50). As mentioned previously, AZM has also been shown to be beneficial in lung transplantation for the prevention and treatment of chronic allograft rejection due to its immunomodulatory properties (52). AZM treatment significantly decreased $CD4^+$ T cells in BALF and lung collagen deposition in a murine model of noninfectious lung injury (53). The authors of the present study hypothesize that the anti-fibrotic effects of AZM may be attributed to its immunomodulatory properties.

The authors observed an improvement in radiation-induced lung tissue morphology with high-dose (100 mg/kg/day) and low-dose (10 mg/kg/day) AZM in the preliminary study. As shown in Fig. 1D and E, compared with the RT group, the inflammation score and the grade of fibrosis in the AZM-10 group were lower, but not significantly different. In addition, a lower but not a statistically significant difference in MDA content was observed in plasma and lung tissue in the AZM-10 group compared with the RT group. These results indicate that 100 mg/kg/day is a more appropriate dose for AZM in acute RILI. Future studies are required to determine the optimal dose of AZM with the highest efficacy and minimal dose-limiting toxicities in acute RILI.

RILI refers to a continuous process, which is triggered following lung RT (3). Additionally, acute pneumonitis is associated with radiation fibrosis (39). A limitation in the present study is the lack of a long-term investigation on the survival rate of RILI mice treated with AZM. A future study by the present authors will focus on whether AZM has a similar capacity to ameliorate late RILI and improve survival in mice.

In conclusion, AZM has therapeutic potential in RILI management. The present study demonstrated the beneficial role of AZM in treating RILI, including its anti-inflammatory and anti-fibrotic effects.

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