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Delayed Administration of WP1066, an STAT3 Inhibitor, Ameliorates Radiation-Induced Lung Injury in Mice

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Abstract
Purpose The present study was designed to investigate the effects of WP1066, a specific inhibitor of STAT3 signaling, on radiation-induced lung injury in mice.
Methods C57BL/6J mice were subjected to a single thoracic irradiation of 15 Gy X-ray and WP1066 was administrated through intraperitoneal injection. The early and delayed treatment groups were treated with WP1066 during the first 2 weeks and the second 2 weeks, respectively. The therapeutic effects of WP1066 were evaluated by survival analysis, histological examination, and measurement of inflammatory parameters and collagen deposition. The activation of STAT3 pathway was also estimated by immunohistochemical staining and Western blotting.
Results Delayed treatment of WP1066, but not early treatment, prolonged survival time and prevented the development of radiation pneumonitis and the subsequent lung fibrosis in mice. WP1066 treatment also significantly suppressed the activation of STAT3 signaling in the irradiated lung tissues.
Conclusions The activation of STAT3 pathway might play an important part in the pathogenesis of radiation-induced lung injury. The protective effects of delayed treatment of WP1066 suggested STAT3 signaling could be a therapeutic target for radiation pneumonitis.

Keywords WP1066 · STAT3 · Radiation pneumonitis · Inflammation · Cytokine

Introduction

Delivery of a high dose of ionizing radiation to tumors in the thoracic region usually causes radiation pneumonitis, which is characterized by an acute phase of interstitial pulmonary inflammation and a subsequent chronic stage of fibrosis. The reported incidence of radiation pneumonitis varies significantly, since the occurrence of radiation pneumonitis is dependent on many factors, such as the total dose delivered, the irradiated lung volume, the fractionation schedule, the individual susceptibility, and the application of chemotherapy [1, 2]. Generally, 5–15 % of patients receiving thoracic radiotherapy would suffer from symptomatic radiation pneumonitis which could be a critical dose-limiting complication and affect the quality of life [3, 4]. Although the underlying mechanism of radiation pneumonitis remains elusive, the radiation-triggered pro-inflammatory cascade and the destruction of vasculature and lung parenchyma may be the critical events in the onset of radiation pneumonitis [5, 6]. Inflammatory mediators and responses are potential therapeutic targets for radiation-induced lung injury, and the clinical management of radiation pneumonitis still relies on the anti-inflammatory action of corticosteroids [7].

Signal transducer and activator of transcription 3 (STAT3) is a multifunctional intracellular signaling pathway and participates in the regulation of cell proliferation, apoptosis, inflammation, differentiation, and transformation [8]. As a member of transcriptional factor family,

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STAT3 could be activated by reactive oxygen species and a number of cytokines and growth factors, many of which have been reported to be upregulated in lung tissue upon radiation exposure [9]. For example, serum levels of interleukin-6 (IL-6) might have predictive value for the risk of radiation pneumonitis [10–12]. Evidences also revealed a critical role of STAT3 in several different kinds of lung inflammatory diseases, such as lipopolysaccharide-induced lung injury and ischemia-reperfusion lung injury [13]. Additionally, activation of STAT3 signaling was involved in idiopathic pulmonary fibrosis and bleomycin-induced pulmonary fibrosis [14–16].

Although targeting STAT3 pathway might represent a therapeutic approach to prevent lung inflammatory diseases and pulmonary fibrosis, its role in the development of radiation pneumonitis is unclear. The aim of the current study is to investigate the effects of WP1066, a specific inhibitor of STAT3 signaling, on a mice model of radiation-induced lung injury. WP1066 was administrated through intraperitoneal injection for 2 weeks [17]. We could not observe any protective effects of WP1066 on radiation pneumonitis if treatment of WP1066 was performed during the first 2 weeks post-irradiation. Nevertheless, delayed treatment of WP1066 in mice, that was initiated 2 weeks after thoracic irradiation, improved survival rate, alleviated inflammatory response, and subsequent pulmonary fibrosis.

Materials and Methods

Animal Experimental Procedures

Pathogen-free 8-week-old C57BL/6J female mice were purchased from Slac Laboratory Animal (Shanghai, China) and maintained under a 12 h light/dark cycle at constant temperature and provided with water and food ad libitum. All animal experimental procedures were in accordance with the guidelines provided by the Animal Ethical Committee of Soochow University.

Mice were anesthetized and a single dose of 15 Gy X-ray (160 kV, 1.15 Gy/min) was delivered to the whole thorax by a biological research irradiator (Rad Source Technologies, Suwanee, GA). Non-irradiated parts of the mice were shielded with 1 cm of lead.

WP1066 (Selleck Chemicals, Houston, TX) was dissolved in DMSO/polyethylene glycol (PEG) 300 (1:4, *v/v*) [17]. Mice were intraperitoneally injected with WP1066 (20 mg/kg) in a total volume of 100 µl on every other day for nine treatments (on Mondays, Wednesdays, and Fridays). Mice of control group were treated with the same volume of DMSO/PEG 300 vehicle.

Histological and Immunohistochemical Studies

Lung tissues were fixed with 10 % formalin and embedded in paraffin. Tissue sections (4 µm) were stained with hematoxylin and eosin (HE) for light microscopy. For immunohistochemistry, lung tissues were fixed with 10 % formalin with phosSTOP phosphatase inhibitor cocktail (Roche Diagnostics, Germany). Then tissue sections were treated with a pressure cooker antigen retrieval procedure in pH 6.0 sodium citrate buffer for 3 min and immunostained with anti-p-STAT3 (Tyr705) antibody (Cell Signaling Technology, Danvers, MA).

Western Blot Analysis

Lung tissues were homogenized in ice-cold RIPA lysis buffer with protease and phosphatase inhibitors. Homogenates containing 30 µg of tissue lysate were separated by 10 % SDS-PAGE and transferred to PVDF membranes. After blocking with 5 % skim milk, the blot was probed with primary antibodies for p-STAT3 and STAT3 (Cell Signaling Technology). Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biochemicals, Santa Cruz, CA) was used as a secondary antibody. Protein levels were visualized by using enhanced chemiluminescence by FluorChem M System (ProteinSimple, San Jose, CA).

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed at 4 week after thoracic irradiation. Mice were anesthetized, and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged three times with 0.5 ml phosphate-buffered saline containing protease inhibitors. The bronchoalveolar lavage fluid (BALF) samples were collected and the number of cells per 100 µl aliquot was determined by using a hemocytometer. The remaining sample was centrifuged, and the protein concentration of supernatant was measured by Bradford method.

RNA Isolation and Real-Time PCR

Total RNA was extracted from lung tissue by using RNAiso Plus (Takara, Japan). RNA was precipitated with isopropanol and dissolved in diethylpyrocarbonate-treated distilled water. First-strand cDNA was generated by using the SuperQuick RT MasterMix (Cwbio, China). Specific primers for each gene were designed as shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control. The real-time PCR reaction mixture consisted of 10 ng reverse transcribed total RNA, 200 nM forward and reverse primers, and

Table 1 Sequences and accession numbers for primers (FOR, forward and REV, reverse) used in real-time RT-PCR

Gene	Primer sequence	Accession no.
IL-1β	Forward: GGTCAAAGGTTTGGAAGCAG	NM_008361
	Reverse: TGTGAAATGCCACCTTTTGA	
IL-6	Forward: ACCAGAGGAAATTTTCAATAGGC	NM_031168
	Reverse: TGATGCACTTGCAGAAAACA	
TNF-α	Forward: AGGGTCTGGGCCATAGAACT	NM_013693
	Reverse: CCACCACGCTCTTCTGTCTAC	
TGF-β	Forward: CAACCCAGGTCCTTCCTAAA	NM_011577
	Reverse: GGAGAGCCCTGGATACCAAC	
GAPDH	Forward: CGTCCCGTAGACAAAATGGT	NM_008084
	Reverse: TTGATGGCAACAATCTCCAC	

2× UltraSYBR Mixture (Cwbio) in a final volume of 20 μl. The PCR reaction was carried out in 96-well plates by using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). All experiments were performed in triplicate.

Serum Sample Assays

Mouse IL-1β, IL-6, tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Norcross, GA) and transform growth factor-β (TGF-β) ELISA kit (eBioscience, San Diego, CA) were used to measure the concentration of cytokine levels in serum samples.

Measurement of Lung Hydroxyproline

Lung collagen deposition was assessed by determining hydroxyproline in lung tissue. Briefly, 20-mg lung tissue was homogenized and hydrolyzed with 6 M HCl at 100 °C for 24 h. Then the hydroxyproline content were measured by using a colorimetric assay kit (Biovision, Milpitas, CA) according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as means ± the standard deviation. Statistical analysis was performed by one-way ANOVA following multiple comparisons by using SPSS 18.0 software. The probability of survival was calculated by Kaplan–Meier product-limited method. The comparison of survival probability of different groups was performed by using log-rank test. Results with a p value less than 0.05 were considered statistically significant.

Results

Prolonged Survival by Delayed Treatment of WP1066 in Thoracic Irradiated Mice

Radiation-induced lung injury was conducted in C57BL/6J mice by thoracic irradiation of 15 Gy X-ray. Then the mice were divided into three groups, the vehicle group, the early treatment group, and the delayed treatment group. It took 2 weeks to finish the nine treatments of WP1066 as described in materials and methods. In the early treatment group, WP1066 injection was performed during the first 2 weeks, whereas in the delayed treatment group, injection started 2 weeks after irradiation. The experimental protocol is shown in Fig. 1a. Delayed treatment of WP1066 significantly increased the life span of mice when compared with early treatment group (Fig. 1b). However, early treatment of WP1066 did not show any beneficial effects on mice. The median survival time was 11.2 and 14.7 weeks in early and delayed treatment group, respectively.

WP1066 Inhibits the Radiation-Induced Expression of p-STAT3 in Lung

Various pro-inflammatory cytokines have been shown to be upregulated in the lung tissue exposed to ionizing radiation [18, 19], some components of them are activators for the STAT3 signaling [13]. Five weeks after thoracic irradiation, the lung tissues of mice were collected and the expression of p-STAT3 was examined by immunohistochemical staining and Western blot analysis. As shown in Fig. 2a–c, 15 Gy X-ray treatment raised the expression levels of STAT3 in the lung. In addition, delayed treatment of WP1066 significantly suppressed the activation of STAT3 induced by irradiation.

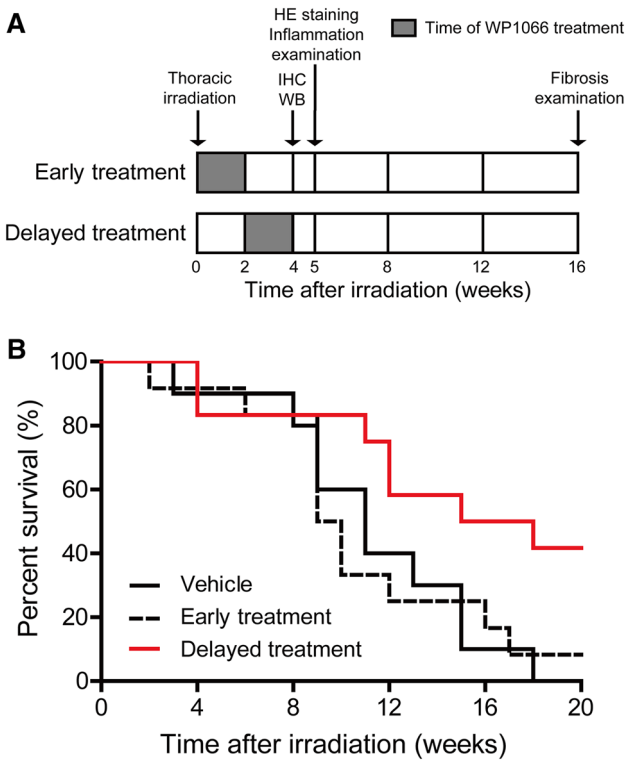

Fig. 1 The schematic diagram of the experimental protocol and Kaplan–Meier survival plots of mice. **a** Mice were irradiated 15 Gy X-ray to the whole thorax. The *gray bars* indicated the period of time of WP1066 treatment for different groups. Lung tissues and blood samples were collected at indicated time points for each experiment. **b** Delayed treatment of WP1066 prolonged the survival time of mice compared to the early treatment group ($p = 0.028$). $n = 10$–12 for each group

Mitigation of Radiation-Induced Lung Inflammation by WP1066

Histological examination revealed the occurrence of radiation pneumonitis in the X-ray-treated mice, including the destruction of the alveolar wall, the thickened alveolar septa, the pronounced inflammatory cell infiltration (Fig. 3a). The cell number and protein levels in the BALF of X-ray-treated mice were markedly increased compared to that of control mice, suggesting an increased permeability of the alveolar–capillary membrane (Fig. 3b). Delayed treatment of WP1066 protected lung against radiation-induced inflammatory injury, therefore retained the intact sac-like structure of alveoli and prevented the leakage of plasma protein and fluid in the alveolar space.

WP1066 Reduces the Cytokine Levels in Thoracic Irradiated Mice

Next, we evaluated the mRNA expressions of cytokines in the lung tissue, as well as the cytokine concentrations in the plasma. As shown in Fig. 4a, mRNA levels of IL-1β, IL-6, and TGF-β, but not TNF-α, were reduced by delayed treatment of WP1066. However, only IL-6 concentration in the plasma were statistically decreased in the mice treated with WP1066, both IL-1β and TGF-β levels in the plasma showed no difference between vehicle and WP1066-treated mice, and the TNF-α level was even higher in WP1066-treated mice than that of vehicle group. Since WP1066 is a potent immune activator [17], administration of WP1066 by intraperitoneal injection might provoke inflammatory response and therefore increase the cytokine levels in the plasma. These data indicated WP1066 could reduce the mRNA expression of some cytokines in the radiation-exposed lung tissue, but not the systemic inflammatory levels.

WP1066 Treatment Attenuates the Radiation-Induced Pulmonary Fibrosis

Pulmonary fibrosis is the late manifestation of radiation-induced lung injury. HE staining was performed 16 weeks post-irradiation to visualize the lung fibrosis. As shown in Fig. 5a, the lung architecture of vehicle-treated mice was extensively distorted, and an attenuation of radiation-induced lung fibrosis was observed in delayed WP1066-treated mice. The hydroxyproline contents of lung homogenates were determined as an indicator of lung fibrosis. In accordance to histological assessment, delayed treatment of WP1066 significantly ameliorated radiation-induced hydroxyproline accumulation in the mouse lung tissues (Fig. 5b).

Discussion

WP1066 is a cell permeable AG490 tyrphostin analog that markedly inhibits the STAT3 phosphorylation and its antitumor effects on multiple types of cancer have been validated both in vitro and vivo [20–23]. Here, we showed the therapeutic potential of WP1066 for radiation-induced lung injury. Delayed treatment of WP1066, but not early treatment, improved survival and prevented the development of radiation pneumonitis and subsequent lung fibrosis in mice. WP1066 treatment also inhibited the expression of p-STAT3 in the irradiated lung tissue. Since radioresistance of lung cancer and esophageal cancer could be overcome by disruption of STAT3 [24, 25], our results suggest that STAT3 signaling might be a reliable target for ameliorating radiation-induced lung injury without reducing the efficacy of thoracic radiotherapy.

In response to cytokines and growth factors, STAT3 is phosphorylated by receptor tyrosine kinases, thus allowing its dimerization and translocation to the nucleus to activate transcription of genes containing STAT3 response elements. As a intracellular hub in inflammation, STAT3

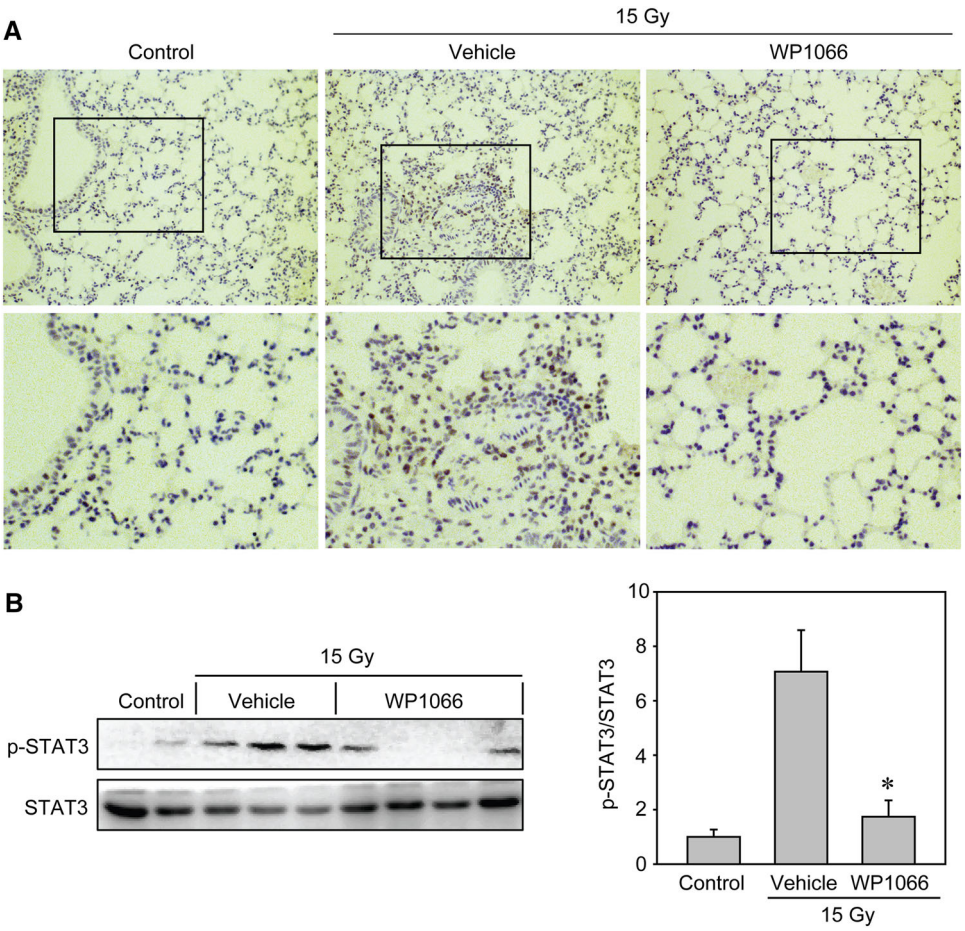

Fig. 2 Inhibition of p-STAT3 expression by WP1066. Five weeks post-irradiation, lung tissues from mice were subjected to immunochemical staining (**a**) and Western blotting analysis (**b**) for p-STAT3 expression. $*p < 0.05$ vs vehicle-treated group

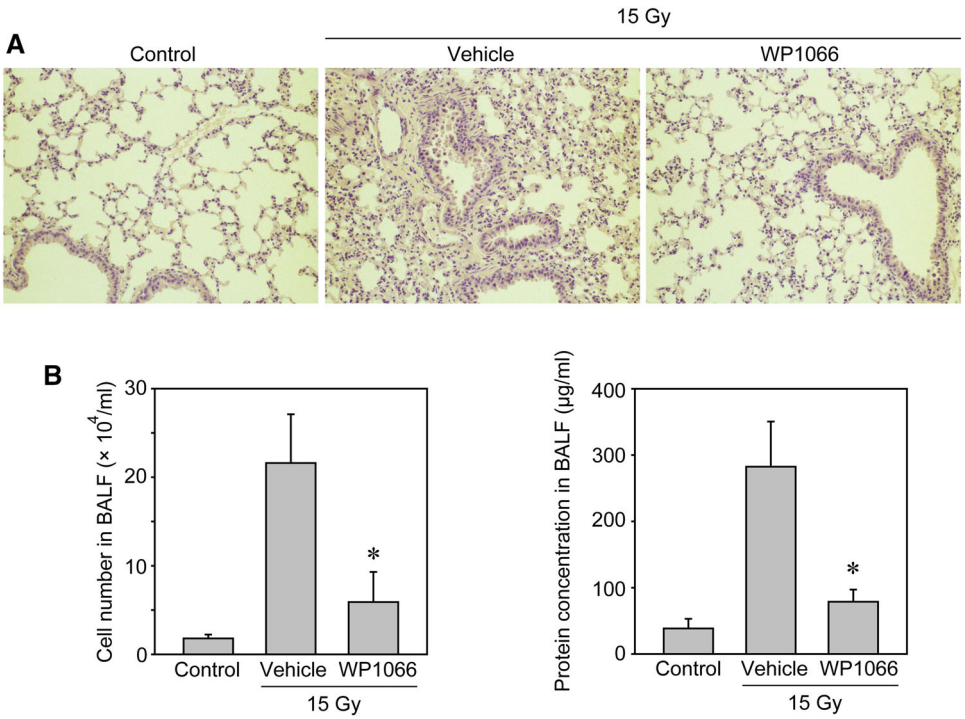

Fig. 3 WP1066 alleviates radiation-induced pulmonary inflammation. **a** HE staining of the irradiated lung tissue. **b** BALF samples were collected and the cell number and protein concentration were measured. $*p < 0.05$ vs vehicle-treated group

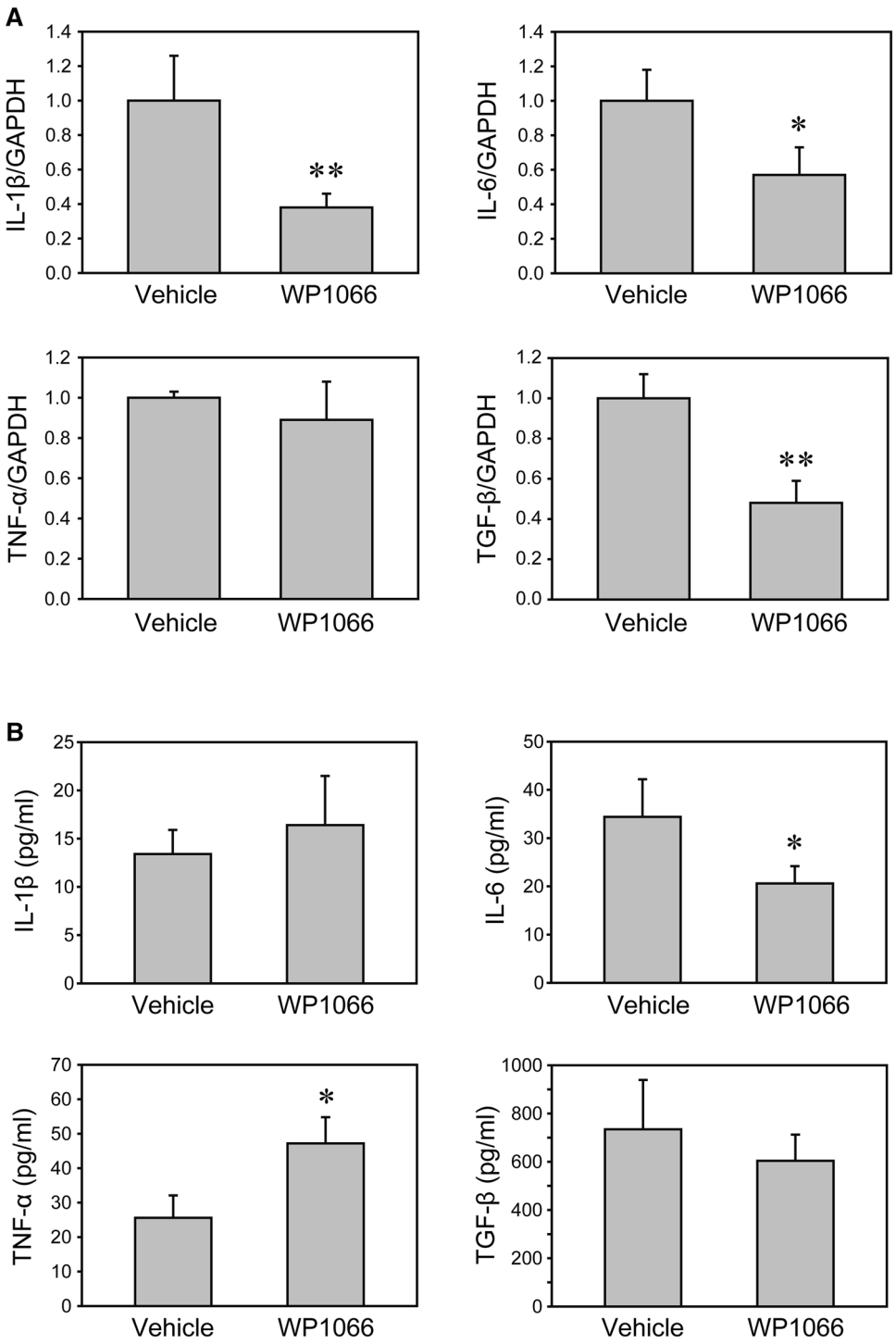

Fig. 4 The effects of WP1066 on the expression levels of cytokines. **a** Real-time PCR was performed to analyze the mRNA expression of cytokines. **b** Serum levels of cytokines were measured by ELISA kit. $*p < 0.05$; $**p < 0.01$ vs vehicle-treated group

signaling plays a crucial role in pulmonary inflammatory and fibrotic diseases [13]. Despite the fact the roles of STAT3 in radiation-induced lung injury remain elusive, IL-6, a well-established upstream regulator of STAT3 signaling, was shown to be increased in C57BL/6 mice exposed to lung irradiation [26], and IL-6 knock-out mice were resistant to C-ion irradiation-induced lung fibrosis [27]. In addition, some prospective studies showed positive correlation between circulating IL-6 levels and the risk of radiation pneumonitis in patients receiving thoracic radiotherapy [10–12]. All these results strongly implicated that STAT3 signaling could be a pharmacological target for the prevention of radiation lung toxicity. However, our initial attempt to inhibit STAT3 activation by WP1066 during the

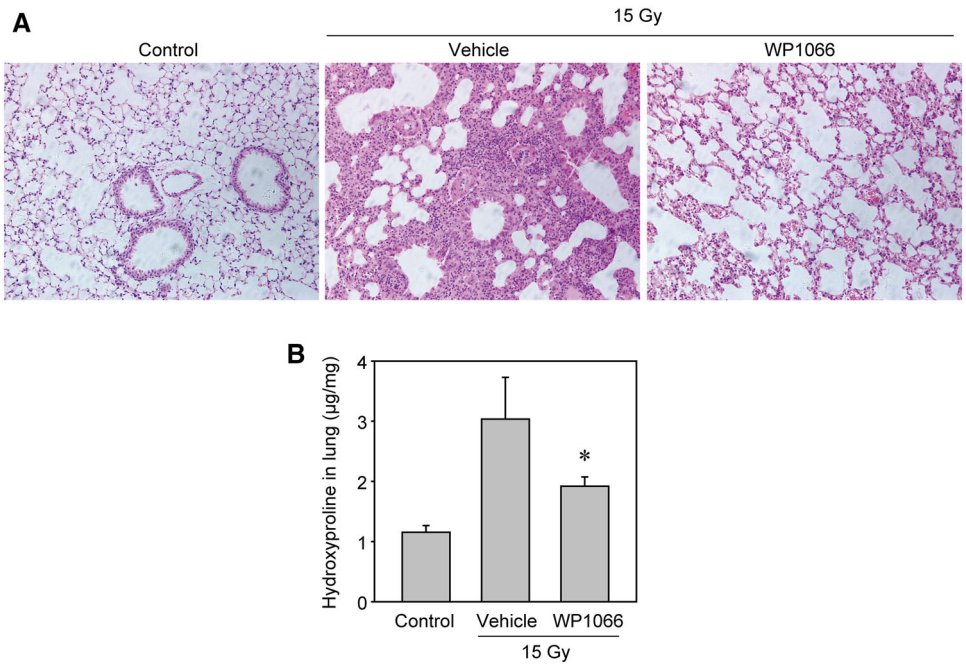

Fig. 5 WP1066 prevents radiation-induced lung fibrosis. **a** Four months post-irradiation, HE staining was performed to visualize lung fibrosis. **b** The hydroxyproline contents were determined as an indicator of lung fibrosis. $*p < 0.05$ vs vehicle-treated group

first 2 weeks post-irradiation did not exert any protective effects against radiation-induced lung injury. In a similar report using a Balb/c mice model of radiation-induced lung injury, early treatment of anti-IL-6 monoclonal receptor antibody failed to increase the survival time [28]. Although the reason for these phenomena is unknown, it seems that early activation of STAT3 is not responsible for the severity of radiation pneumonitis.

Given that the expression of IL-6 has been shown to increase in a biphasic pattern in radiation pneumonitis [26], we next performed a trial to treat the mice with WP1066 2 weeks after the radiation exposure. Intriguingly, the so-called delayed treatment of WP1066 partially, if not completely, prevents the life shortening induced by thoracic irradiation in mice. WP1066 delayed treatment prolonged the mean survival time about 3 weeks, and significantly suppressed the activation of STAT3 signaling in lung tissues. Further examination revealed decreased inflammatory response and less collagen deposition in delayed WP1066-treated mice. It has been shown that the secondary elevation of cytokines in radiation pneumonitis were persistent and coincided with the onset of pathological changes [26]. Together with our results, it was suggested that delayed inhibition of STAT3 activation might have defensive effects against radiation-induced lung injury. It has been reported that sensitization of carcinoma cells to cisplatin by WP1066 is via the inhibition the STAT3/miR-21 axis [23], and miR-21 could modulate both lung inflammatory response and fibrotic process [29, 30]. However, delayed treatment of WP1066 did not alter the expression of miR-21 in the radiation-exposed lung tissue of mice (data not shown).

Notably, the mRNA expression and serum concentration of some cytokines were not accordingly decreased by delayed treatment of WP1066. These data may result from the following reasons. First, the current experimental protocol did not fully prevent the radiation-induced pulmonary toxicity, since suppression of single target may not be sufficient to counteract the radiation-induced inflammatory response. Second, low incidence of acute and chronic toxicity has been reported in multiple WP1066-treated mice through intraperitoneal injection [17]. Third, as a pleiotropic transcriptional factor, STAT3 has both anti-inflammatory and pro-inflammatory functions in different tissue and cellular context [31, 32]. Therefore inhibition of STAT3 by WP1066 in vivo might induce the upregulation of systemic pro-inflammatory cytokines.

In summary, our results demonstrated the beneficial effects of STAT3 inhibition on radiation-induced lung injury in mice. The time window of STAT3 inhibition post-irradiation is important and likely to affect the ultimate outcome of radiation pneumonitis. Targeting STAT3 pathway might be efficacious and feasible in clinical practice to protect patients receiving thoracic radiotherapy, as well as those who suffer from nuclear disaster and radiological terrorism. Further studies should be performed to optimize the dosage and duration regimen of STAT3 inhibition after lung irradiation, to develop highly specific STAT3 inhibitors without undesired side effects and to search for the possible combination of other anti-inflammatory approaches with STAT inhibition.

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Compliance with Ethical Standards

Conflict of interest None.

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