Peer Review File

Article Information: https://dx.doi.org/10.21037/tlcr-24--649

Reviewer A

The study delves into examining the involvement of the stimulator of interferon genes (STING) in radiation-induced lung injury (RILI) and the potential therapeutic benefits of the STING inhibitor H-151. The research employs a mouse model subjected to 20 Gy whole-thorax irradiation and subsequent treatment with H-151. The findings indicate that inhibiting STING significantly diminishes inflammatory cell infiltration, the release of pro-inflammatory cytokines, and the development of fibrosis in lung tissue. Furthermore, the study investigates the pathway through which STING facilitates the transition of fibroblasts into myofibroblasts via the PERK-eIF2α pathway, hinting at a promising avenue for managing RILI.

This investigation sheds light on the significance of the cGAS-STING pathway in RILI, demonstrating the potential of STING inhibition in alleviating the condition in mice. By offering fresh insights into the molecular origins of radiation injury, the study proposes that targeting the STING-PERK-eIF2α pathway could inform future pharmacological interventions for RILI.

Nevertheless, certain aspects warrant careful consideration before acceptance for publication in Translation Lung Cancer Research.

Comment 1. Discrepancies Between Mice and Human Cell Lines:

Exploring the impact of STING inhibition on human fibroblasts and comparing it with its effects on murine counterparts could enhance the study's relevance. A comparative analysis through western blotting to showcase the expression of the STING-PERK-eIF2α pathway might prove insightful.

Reply 1: We are grateful for your insightful comment. We totally agree that exploring the effect of STING inhibition on human cell lines is necessary to enhance that STING inhibitor significantly relieves fibrosis. As mentioned, our current study embarked on exploring the mechanisms underlying STING inhibition in RILI using mice and subsequent experiments with mouse epithelial and fibroblast cells. Indeed, we have demonstrated the therapeutic potential of STING inhibition in mitigating RILI both *in vivo* and *in vitro*. In line with your recommendation, we have already conducted a parallel line of research exploring the regulatory roles of novel STING modulators in RILI, with a primary focus on human fibroblasts. Although this aspect has not been directly addressed in the current manuscript, we are excited about the progress made so far and its potential impact on advancing the field. Therefore, we have added the following statements to the Discussion section (see page 21, line 408-412).

Changes in the text: Firstly, while our current results strongly support the therapeutic promise of STING inhibition in RILI using mouse models and cellular studies, we recognize the importance of translating these findings into clinical settings. Therefore, it is highly necessary to conduct research with human cell lines, especially human fibroblasts, to further enhance the underlying mechanisms of STING inhibitors in RILI.

Comment 2. Impact of STING Inhibition in Oncology Radiotherapy:

The implications of inhibiting the STING-PERK-eIF2α pathway as a therapeutic approach should be approached cautiously. Activation of the cGAS-STING pathway is pivotal in eliciting an anti-tumor immune response. Inappropriate administration of a STING inhibitor like H-151 during oncological radiotherapy could potentially dampen the therapeutic effects of ionizing radiation (IR). The authors need to elucidate how the study addresses this concern after disseminating its findings to translational research. Detailed information on the concentration of H-151 used in in vivo and in vitro studies is essential. Additionally, it is crucial to ensure that this concentration does not interfere with the therapeutic mechanisms of IR. This could be added in the conclusion as part of future research directions.

Reply 2: Thank you for your insightful comment. We know that the events that lead to radiation-induced lung injury are diverse. For example, for healthy people at risk of radiation exposure, the primary concern would be the therapeutic efficacy of STING inhibitors themselves, with less emphasis on tumor-specific immune responses. However, from a clinical point of view, the consideration of RILI becomes intricate in the context of cancer patients, particularly those undergoing radiotherapy for lung cancer, where the focus naturally shifts towards balancing tumor response with mitigation of adverse effects. The utilization of STING inhibitors demands rigorous planning and experimental verification regarding the optimal timing, duration, dosage, and administration route. Our top priority is to guarantee that the employment of STING inhibitors does not interfere with the therapeutic mechanisms of IR or undermine the immune response against the tumor, while simultaneously providing therapeutic advantages against RILI. Of course, it still needs to be confirmed by a series of clinical trials. As you suggested, we have expanded our discussion to address this issue in the revised manuscript (see page 21, line 412-417).

Changes in the text: Secondly, activation of the cGAS-STING pathway is pivotal in inducing an anti-tumor immune response. It is necessary to ensure that STING inhibitors can effectively treat RILI without interfering with the radiotherapy effect and the anti-tumor immune response. Specifically, we have elaborated on the potential clinical applications of STING inhibitors in the context of RILI in lung cancer patients, highlighting the need for rigorous preclinical and clinical investigations to optimize their use and ensure their safety and efficacy.

Comment 3. STING Role in Lung Injury with Old and Modern Radiotherapy:

In contemporary radiotherapy modalities like Stereotactic Body Radiation Therapy (SBRT), high radiation doses are precisely delivered to bolster the anti-tumor efficacy of IR while minimizing collateral damage. Nevertheless, some normal cells surrounding tumor cells are still affected by the radiation. The impact on normal cells between traditional and modern radiotherapies could vary, influencing STING stimulation or inhibition. This comparison could be a valuable addition to the conclusion as part of future research directions.

Reply 3: Thank you for your guidance and advice. Indeed, contemporary radiotherapy methods such as SBRT have been demonstrated remarkable efficacy in reducing the amount of radiation exposure to surrounding normal tissues, thereby mitigating adverse effects to a considerable extent, which is different from traditional radiotherapy. It is worth noting that traditional and modern radiotherapies may have different effects on normal cells. Whether the treatment strategy of using STING inhibitors as a potential direction for RILI requires adjustment or more precise administration deserves further investigation. As you suggested, we have added these contents in the discussion section to provide a more valuable reference for the clinic (see page 22, line 417-421).

Changes in the text: Finally, traditional radiotherapy and modern radiotherapy such as stereotactic body radiation therapy (SBRT) may have different effects on normal cells due to different modes of action.

Therefore, it is worth further investigation whether the STING inhibitor, as a potential therapeutic strategy for RILI, should be adjusted or implemented more accurately based on different radiotherapy modes.

Reviewer B

The present study investigates the use of a STING inhibitor on mice to reduce the secondary inflammatory effects following lung irradiation which lead to the development of RILI. Overall - new targets and medications for this condition are desperately needed and this is a pilot work which suggests that the STING pathway could be a potential target.

Some questions the authors might address are:

1.) Given as they state the inhibitor might only delay the onset of fibrosis/inflammation - was it considered to follow the mice for a longer time point to see if following the subacute pneumonitis if the sting inhibitor changed the long-term impact of the thoracic radiation

Reply 1: Thank you for your comment. The confirmation of the long-term efficacy of STING inhibitors in RILI is indeed crucial for assessing their potential clinical significance. This study, which focused on the assessment of STING inhibitor treatment at 1, 2, 4, 12 and 24 weeks post-irradiation, has reported promising therapeutic effects, particularly at the 24-week time point, where remarkable attenuation of radiation-induced lung fibrosis was observed. As you suggested, extending the follow-up period to one year or beyond would be invaluable in elucidating the potentially enhanced effectiveness of STING inhibitors in managing RILI. This would strengthen the argument for their potential clinical application. In a follow-up study, we intend to observe the mice for at least one year post-irradiation to comprehensively evaluate the long-term benefits of STING inhibitor treatment.

2.) Does the inhibitor need to be given daily injections for 4 weeks? This may be troublesome to convert into a clinical investigation. Was a shorter duration or just before treatment considered? Why was daily for 4 weeks picked?

Reply 2: Thank you for your insightful query about the drug administration in our animal model. Firstly, we would like to emphasize that the primary objective of our animal study was to investigate whether early application of the STING inhibitor could exert therapeutic effects on both the early inflammatory phase and the later fibrotic stage of lung injury. Given this goal, we designed a 4-week treatment regimen as an early intervention strategy. The choice of daily injections was based on the consideration that longer follow-up times would require a sustained and consistent therapeutic effect to accurately assess the impact on disease progression. Moreover, daily injections maintain a steady drug concentration in the animals, maximizing the potential therapeutic benefits. During our experiments, we observed that the mice tolerated the daily injections well, without significant adverse effects. Secondly, the study referenced (Cell Death Dis. 2019 Dec 20;10(12):957.) serves as an important precedent and offers valuable insights for our experimental design. However, we recognized that while preclinical studies like ours offer promising results, the translation of these findings into clinical practice requires rigorous optimization of dosing schedules, concentrations, and routes of administration in the future.

3.) In the background and discussion more should be said about how RP is treated with steroids - which can decrease severity of symptoms but prolong the inflammatory process and will not offset possibility of fibrosis and how using a STING inhibitor here the goal is prevention of RP development in the first place.

Reply 3: Thank you for your valuable feedback. We are sorry for neglecting the current treatment landscape of RILI, which indeed deserves reflection in the text. We fully agree that this information is crucial for providing a comprehensive understanding of the field and motivating the need for further research. As advised, we have modified our text in the revised manuscript (see page 6, line 72-77).

Changes in the text: Currently, RILI is typically treated with high doses of corticosteroids to temporarily relieve symptoms. However, these treatments come with significant side effects, such as edema, sleep disturbances and weight gain, which may make corticosteroids inappropriate for the prevention of RILI or long-term use. Therefore, it is urgent to further explore its mechanism, identify more effective intervention targets, and develop new drugs to prevent RILI.

Reviewer C

The research presented in the manuscript titled "STING facilitates the development of radiation-induced lung injury via regulating the PERK/eIF2α pathway" is an important topic. I congratulate the authors for taking interest in radiation-induced injury to lungs. The manuscript would benefit greatly with some revision for the benefit of the readers.

Below are the suggested edits/ comments –

Comment 1

Line118: there were 30 mice / group. How many biological variables were taken out every timepoint? Total time points discussed in the manuscript ranged from 12 h to 24 weeks.

Reply 1: Thank you for your insightful comment. As you pointed out, we have now clarified the two distinct phases of our animal experiments. In the first phase, mice were randomly divided into four groups: the control, 12 hours post-irradiation, 24 hours post-irradiation, and 48 hours post-irradiation (n=6 per group). This setup allowed us to observe the initial temporal dynamics of radiation-induced lung injury, which our preliminary findings suggest to be closely linked with the cGAS-STING pathway. Proceeding to the second phase, mice were randomly assigned to three groups prior to irradiation: the control group, the irradiation (IR) group, and the irradiation plus drug administration group (n=30 per group). Samples were collected at predetermined time points of 1 week, 2 weeks, 4 weeks, 12 weeks, and 24 weeks, enabling us to comprehensively evaluate the long-term effects of radiation and potential therapeutic interventions. We have revised the manuscript to more clearly detail the experimental design (see page 9, line 141-151).

Changes in the text: The study includes two parts of animal experiments. Mice in the first stage were randomly divided into four groups: the control group, 12 hours after irradiation, 24 hours after irradiation, and 48 hours after irradiation (n=6 per group). In the second part of animal experiments, mice were randomly divided into three groups before irradiation, including the control group, the irradiation group (IR), and the irradiation plus administration group (n=30 per group), and the time points of sample collection were 1 week, 2 weeks, 4 weeks, 12 weeks and 24 weeks, respectively. For

irradiation, the mice were anesthetized by intraperitoneal injection of Pentobarbital Sodium (50 mg/kg) and then placed in a special box that fully exposed their chests and kept their chests at the same level. Thick lead plates were carefully positioned to cover all areas of the mouse except for the chest. A single dose of 20 Gy whole-thorax X-ray radiation was performed on mice with the RS2000 Biological Irradiator (irradiation dose: 1.325 Gy/min).

Comment 2

Line 120: More details regarding the irradiation procedure needs to be added. How was the dosimetry carried out?

Reply 2: Thank you for your feedback. First, our instrumentation undergoes rigorous and periodic testing and calibration by a team of dedicated professionals. This ensures that the measurements, including the dose rate, are precise. Moreover, in light of the fixed single radiation dose we have determined for our study, we have adjusted the exposure time to ensure that the delivered dose remains accurate and consistent with our experimental protocol. This approach has been carefully implemented to maintain the integrity of our results.

Comment 3

How was it ensured that each animal was positioned correctly? Discoloration of fur is not sufficient evidence.

Reply 3: Thank you for your thoughtful comment. To ensure the accurate positioning of each mouse, we have implemented a meticulous protocol. Prior to irradiation, the mice are anesthetized to minimize movement, and then they are carefully placed and fixed within a specialized radiation box. We ensured that the chest region of each mouse was fully exposed and maintained at the same level. Furthermore, we used thick lead plates to prevent unwanted radiation exposure. These plates were carefully positioned to cover all areas of the mouse except for the chest, which was left exposed within a precisely defined field of approximately 2 cm in width.

Comment 4

How was the rest of the body not exposed to x-rays? Lead shielding?

Reply 4: Thank you for your comment. As mentioned in Reply 3, we did use thick lead shielding plates to shield any non-target regions and prevent unwanted exposure to X-rays. We have supplemented the irradiation method in the revised manuscript (see page 9, line 147-151).

Changes in the text: For irradiation, the mice were anesthetized by intraperitoneal injection of Pentobarbital Sodium (50 mg/kg) and then placed in a special box that fully exposed their chests and kept their chests at the same level. Thick lead plates were carefully positioned to cover all areas of the mouse except for the chest. A single dose of 20 Gy whole-thorax X-ray radiation was performed on mice with the RS2000 Biological Irradiator (irradiation dose: 1.325 Gy/min).

Comment 5

RS2000 has an output energy of 160 kVp. Have the authors considered the biological effects due to orthovoltage x-rays? (Reference: Radiation Research 193, 506–511 (2020)).

Reply 5: Thank you for your feedback. We used X-ray radiation in this study, as the use and management of radioactive isotopes are subject to stringent regulations. And through literature review, we found that X rays have now surpassed ^{60}Co and ^{137}Cs gamma rays to become the most common type

of radiation used in mouse studies. Regarding the biological effects you mentioned, we are well aware of the nuances and have conducted further investigations to ensure our understanding is comprehensive. Transitioning from high-energy gamma-ray irradiators to orthovoltage X-ray irradiators may indeed lead to notable differences in biological outcomes, particularly in terms of their impact on bone tissue and adjacent hematopoietic cells in the bone marrow. However, for the majority of our experiments involving localized irradiation of soft tissues in experimental animals, X-rays covering a range of beam energies exhibit a relatively close degree of biological equivalency. This translates to a smaller range of potential consequences, making X-rays a suitable choice for our study.

Comment 6

What was the lethality at 20 Gy thoracic irradiation?

Reply 6: Thank you for your feedback. In terms of this experiment, we found that none of the mice suffered from short-term mortality directly under a single chest X-ray irradiation of 20 Gy, and by the end of the 30-week period, we observed a mortality rate of approximately 10%.

Comment 7

Line121: Please represent the dose of H151 in terms of mg/kg body weight of the mice as conventionally done.

Reply 7: Thank you for your suggestion. We have converted 750 nM to 10.5 mg/kg according to the conventional dose formulation, and corrected the statement in the revised manuscript (see page 8, line 112; page 10, line 152; page 14, line 258).

Comment 8

Line127: There is no mention of sample collection prior to 1 week whereas there is data on dsDNA presented for the shorter time points. No mention of tissue collection. How were the lung tissue collected for histopathology and molecular work?

Reply 8: We are sorry for this missing information. For the sample collection time points prior to 1 week, we have now stated the collection times for bronchoalveolar lavage fluid and serum at 12, 24 and 48 hours post-irradiation. The revised manuscript clearly describes the methods for collecting these samples, as detailed in the relevant paragraph. Furthermore, we have added a description of lung tissue collection, specifying that the tissues were divided into portions for different analytical purposes. Some tissues were stored at -80 °C for molecular experiments such as western blot and RT-qPCR, while others were preserved in paraformaldehyde for subsequent histopathological analyses, including H&E staining, Masson's trichrome staining, and immunohistochemistry. These details have been incorporated into the Methods section (see page 10, line 158-168).

Changes in the text: Mice were euthanized at corresponding time points after irradiation, and subsequently blood was collected and left at room temperature for 1 hour. After centrifugation at 3000 rpm for 15 min, serum from the upper layer of the centrifuge tube was collected and stored at -80 °C. Bronchoalveolar lavage fluid (BALF) was collected from mice at time points 12, 24 and 48 hours after irradiation. The trachea was exposed after mice were sacrificed, and a puncture needle was inserted into the trachea. Then, BALF was collected by lavage three times with 0.8 mL PBS, and the supernatant was collected after centrifugation and stored at -80 °C. Collected serum and BALF were used for Enzyme-linked immunosorbent assay (ELISA) and determination of dsDNA content. The lung tissues were separated into several parts. Some of them were stored at -80 °C and then used for molecular

experiments such as western blot. Other tissues were temporarily preserved in paraformaldehyde for subsequent histopathological analysis.

Comment 9

Lines 160 and 167: As there is no mention of cells prior to this, it would benefit to present the cell culture method prior to these sections.

Reply 9: We are grateful for your feedback. We have put cell culture method before the western blot and RT-qPCR part in the revised manuscript (See page 11, line 193-199).

Comment 10

Line188: What is the rationale to use 10 Gy while the mice were irradiated at 20 Gy?

Reply 10: Thank you for your feedback. We conducted a large amount of literature research prior to initiating our study, and found that for the establishment of the model of RILI in mice, a single whole-thorax irradiation was often selected, and the dose was mostly 18/20 Gy. Our preliminary experiments found that the mice were well tolerated under the dose of 20 Gy, which made us chose the condition of 20 Gy for animal experiments. For cell experiments, on the one hand, the tolerance of cells to high-dose irradiation is relatively limited; on the other hand, in animal experiments with lung injury, irradiation has a certain degree of penetration, and the radiation response of lung cells in the body is also relatively limited. A single irradiation dose of 8/10 Gy was often selected for cell experiments in a large number of RILI-related literature, and 10Gy irradiation was finally selected through the preliminary experiments (Reference: 1. Respir Res. 2022 Apr 28;23(1):104. 2. Clin Transl Med. 2024 May;14(5):e1690. 3. Respir Res. 2024 Aug 7;25(1):299. 4. Cell Death Dis. 2019 Dec 20;10(12):957.).

Comment 11

Results section: Please stick to describing the results of the studies done in this manuscript.

Lines 220-221: Reported results with speculations should be in the discussion.

Reply 11: We appreciate your constructive feedback. We have revised our discussion to more appropriately position the speculative aspects of our findings. Specifically, we have moved the results with speculations into the Discussion section, as outlined below (see page 20, line 378-382).

Changes in the text: To explore the relationship between dsDNA and lung inflammation, our study showed that irradiation could cause an abnormal elevation of dsDNA in serum and alveolar lavage fluid in mice. dsDNA has been reported as an upstream stimulus of the cGAS-STING pathway. Further, the expression levels of cGAS and STING proteins in lung tissue were significantly increased, which suggests that radiation-induced lung injury may be closely related to the cGAS-STING pathway.

Comment 12

Line241: Suggesting use of H&E in place of HE in the rest of the manuscript.

Reply 12: Thank you for your correction. We have modified our text in the revised manuscript (see Page 11, line 178; Page 15, line 275; Page 28, line 543).

Comment 13

Line 304: Authors probably meant ‘vital component’ instead of ‘virtual component’

Reply 13: We apologize for this mistake. The "virtual component" should be changed to "vital component". We have corrected this sentence in the revised manuscript (see Page 18, line 338).

Comment 14

Line391: No funding information is unusual. How was the work even carried out?

Reply 14: Thank you for your comment. Regarding the absence of funding information in our manuscript, we would like to provide some clarification. While our research is indeed part of a larger project that is partially funded by a confidential funding source, our specific study and the content presented in this manuscript have been deemed non-confidential by our institution. However, due to the sensitive nature of the funding body and its policies regarding public disclosure, our organization has advised us not to include any explicit funding acknowledgments that could potentially reveal their identity or the nature of their support. We understand that transparency in funding sources is an important aspect of scientific publications, and we have taken this decision with utmost caution and respect for the confidentiality agreements we are bound by. Rest assured that the research conducted and reported in our manuscript adheres to the highest standards of scientific rigor and integrity, regardless of the funding source. We hope that this explanation addresses your concern.

Reviewer D

This is an interesting study of the effect of a STING inhibitor; namely, H-151 on radiation lung injury in C57BL/6J mice exposed to 20 Gy thoracic irradiation. The drug was delivered intraperitoneally on the day of irradiation for four weeks. The results are very interesting, but they are preliminary. The studies are based on the effect of the drug, which is supposed to inhibit the STING pathway, but the validation of the drug, the demonstration of the pathway described, which is PERK/eiF2a is not well delineated. The authors should carry out more extensive studies. They need radiation dose response curves. They need to separate acute lung injury from radiation fibrosis, which occurs in C57BL/6J mice at around 120 days.

Major Comments:

Comment 1: The results are preliminary and need to be substantiated with another model system in which a drug, which inhibits STING is used to complement the studies with the current drug. A knockout mouse strain would also be very helpful.

Reply 1: Thank you for your suggestions. Our study has confirmed that H-151 can significantly alleviate radiation-induced lung injury in mice. The choice of H-151 as the primary drug was strategic, grounded on its established efficacy as a highly potent and selective small-molecule antagonist of STING that has noteworthy inhibitory activity both in human/mouse cells and *in vivo* (Nature. 2018 Jul;559(7713):269-273). Therefore, H-151 has more advantages than other STING inhibitors, rendering it an ideal candidate for our study. We fully agree with your suggestion regarding the potential reinforcement of our conclusions through the use of gene knockout mice. However, we were constrained by two considerations. Firstly, our study timeline was limited by the lengthy breeding cycle associated with gene knockout mice; Secondly, we aimed to explore the therapeutic potential of early intervention with H-151, rather than a comprehensive analysis of STING deficiency. Therefore, we adopted the

intervention model of H-151. However, we acknowledge the merit of using gene knockout mice. We will take note of this suggestion and plan to include gene knockout mice in our follow-up studies.

Comment 2: The most important thing is the failure of the authors to differentiate acute radiation pneumonitis from radiation fibrosis.

Reply 2: Thank you for your comment. We fully agree with the distinction between the early phase of RILI, which encompasses RP and the later phase characterized by RIPF. While these two stages have a temporal sequence, they indeed represent a continuous pathological process that cannot be neatly severed. We acknowledge that within the entire lung tissue, multi-site lesions may demonstrate asynchronous progression, highlighting the significance of presenting images at various time points to demonstrate the trend of pathological changes. Through our previous literature research, several studies on RILI choose to present pathological outcomes from different stages collectively (References: 1. Cell Death Dis. 2019 Dec 20;10(12):957. 2. J Transl Med. 2022 Dec 14;20(1):597). However, in the case of fibrosis, we have consistently emphasized our primary attention on the pathological outcomes at 24 weeks post-radiation, a crucial juncture in the evolution of RIPF, which we have highlighted in the article (see page 16, line 289-292).

Changes in the text: Masson staining showed that with the extension of time after irradiation, the lung pathology of mice in the irradiation group showed obvious collagen deposition, while the content of collagen in the treatment group significantly decreased, especially after 24 weeks.

Specific Comments:

Comment 3:The results are based on STING inhibitor, H-151, and a PERK activator.

Drug studies are very important, but need to be confirmed with mouse strains that have genetic deletion in pathways that are supposed to be involved.

Reply 3: Thank you for your suggestion. We totally agree with your emphasis on the importance of multidimensional validation in our research, which helps to strengthen the conclusions. In our current study, we attempted a drug intervention strategy, specifically focusing on the therapeutic potential of STING inhibitors in alleviating RILI. There is a multitude of approaches available for establishing animal intervention models. Therefore, we intend to conduct follow-up studies using gene knockout mouse models or by delivering adeno-associated viruses (AAVs) to further validate our findings of the mechanisms underlying the protective effects of STING inhibitors against RILI.

Comment 4: Irradiation: The dose rate of radiation is not described. The radiation dose used is 20 Gy whole thoracic irradiation, but the technique is not described. What was the dose to the abdomen and head and neck region? The radiation dose rate is 1.325 Gy/min, however, it is not clear what this RS2000 biological irradiation device is? Is this releasing gamma irradiation? Is this orthovoltage x-ray? How is the irradiation calibrated? Were thermoluminescent dosimeters used? Several radiation doses need to be studied. The drug is given once a day for four consecutive weeks. The mice are then studied for their irradiation effects. The results are confined to the figures.

Reply 4: Thank you for your comments. As mentioned in the Method section of the manuscrript, the dose rate of radiation is 1.325 Gy/min. And we have described the method of exposure in more detail: For irradiation, the mice were anesthetized by intraperitoneal injection of Pentobarbital Sodium (50 mg/kg) and then placed in a special box that fully exposed their chests and kept their chests at the same level. Thick lead plates were carefully positioned to cover all areas of the mouse except for the chest. A single dose of 20 Gy whole-thorax X-ray radiation was performed on mice with the RS2000

Biological Irradiator (irradiation dose: 1.325 Gy/min). The irradiation device RS2000 belongs to orthovoltage x-rays, which is also supplemented in the text. Besides, our instrumentation undergoes rigorous and periodic testing and calibration by a team of dedicated professionals. This ensures that the measurements, including the dose rate, are precise. Moreover, in light of the fixed single radiation dose we have determined for our study, we have adjusted the exposure time to ensure that the delivered dose remains accurate and consistent with our experimental protocol. We administered the drug to mice once a day for four weeks, then collected samples, and finally validated the results through a variety of pathological experiments and a series of molecular biology experiments. We have modified the relevant contents in the revised manuscript (see page 9, line 147-151).

Changes in the text: For irradiation, the mice were anesthetized by intraperitoneal injection of Pentobarbital Sodium (50 mg/kg) and then placed in a special box that fully exposed their chests and kept their chests at the same level. Thick lead plates were carefully positioned to cover all areas of the mouse except for the chest. A single dose of 20 Gy whole-thorax X-ray radiation was performed on mice with the RS2000 Biological Irradiator (irradiation dose: 1.325 Gy/min).

Comment 5: Figure 1 shows that H-151 was delivered, and the results are quantitated by looking at DS DNA in bronchoalveolar lavage specimens at different time points up to 48 hours, which is very early. The data was also in lung coefficient levels of something that is not well delineated after 20 Gy irradiation. A western blot, which is purported to show activation of C Gas STING is not clear. The T-TBK1 panel on the top shows decrease in the IR+H-151, but there appears to be no difference between NC and irradiation. The blots are not very well quantitated at all.

Reply 5: Thank you for your comments. Early determination of dsDNA in alveolar lavage fluid and plasma, as well as increased protein expression of GAS and STING, suggest that the cGAS-STING pathway may be closely related to RILI. Indeed, this is a preliminary hypothesis that deserves further validation, and as you suggested, we have carried out subsequent animal experiments. With regard to the lung coefficient mentioned in your review, we appreciate your attention to this detail. As stated in our manuscript (see page 15, line 263-264), the lung coefficient primarily serves as an indicator of the extent of pulmonary edema or fibrosis. Furthermore, concerning the western blot results, we confirm that our data show significant upregulation of cGAS, STING, and p-TBK1 proteins after radiation exposure. And we also observed a notable downregulation of these proteins upon drug intervention, suggesting the potential role of the cGAS-STING pathway in mediating RILI and highlighting the therapeutic potential of targeting this pathway.

Changes in the text: In addition, we calculated the lung coefficient (lung weight/body weight) of the mice, which served as an indicator of the extent of pulmonary edema or fibrosis.

Comment 6: Figure 2 shows early application of STING inhibitor H-151 decreasing radiation pneumonitis. Radiation pneumonitis is not detected in C57BL/6J mice at quantitated values. The authors should look at C3H/HeN or BALB/C mice. C57BL/6J mice develop radiation fibrosis at around 110 – 120 days, and, perhaps, the authors wish to study this late pathology.

Reply 6: Thank you for your comments. As you mentioned, Figure 2 primarily demonstrated the impact of H-151 on early radiation pneumonia. It encompassed both H&E staining for histological assessment and quantification of various inflammatory markers in serum. We acknowledge that the quantitative analysis presented focuses on the efficacy of the drug intervention. Given that the histological scores and levels of inflammatory factors were significantly elevated in the irradiation group compared to the controls at various time points post-exposure, our goal is to evaluate the effects of H-151 on this radiation-induced inflammation. Hence, the statistical comparisons in the manuscript primarily feature

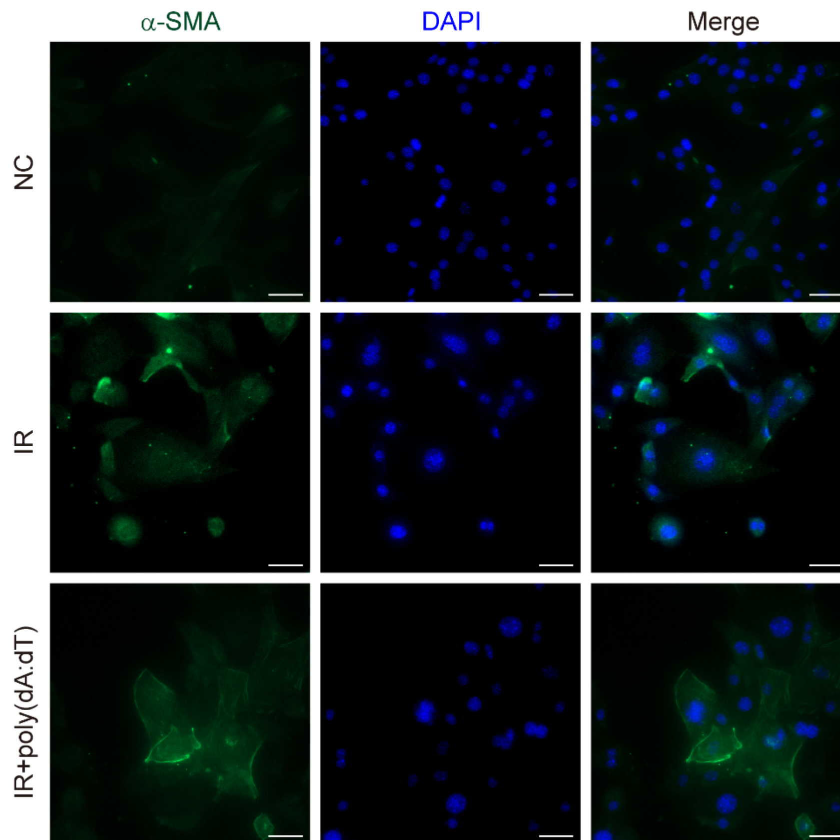
the drug administration group versus the irradiation group. We apologize for the confusion arising from the lack of comparison between the irradiation and the control group, which may suggest an erroneous assumption of no quantitative difference between these two groups. Indeed, the purpose of our study was not to disprove the fact of radiation-induced tissue damage but rather to evaluate the potential therapeutic benefit of H-151 in relieving these effects. Moreover, while we are also interested in the late pathology, Figure 2 was designed to emphasize the early manifestations of RP, where drug intervention is most likely to be effective. Nevertheless, we recognize the importance of comprehensively assessing the disease course and will extend our analyses to include the late pathology in future studies.

Comment 7: Figure 3 shows pulmonary fibrosis. This is quite interesting, particularly, looking at alpha-SMA. The data at 24 weeks is quite impressive, and this would be more consistent with radiation fibrosis. The IR staining specimens at 4, 12 weeks do not show an increase at 24 weeks. In contrast, IR-H-151 shows more intense, deposition at 24 weeks, than does the irradiation alone. This is in panel A. Panel B is even more confusing. The quality of the staining is inconsistent between the data points making it very difficult to detect differences.

Reply 7: Thank you for your valuable feedback. Regarding your concern on the results presented in Figure 3, we have performed Masson staining in Figure 3A, which indeed displayed a notable increase in the blue staining of collagen fibers at 4 weeks and 12 weeks post-irradiation compared to the control group. This finding reflects the progression of radiation-induced fibrosis over time. Notably, at 24 weeks post-irradiation, we observed a marked reduction in the blue collagen fibers following drug administration compared to irradiation alone. While acknowledging the slight variations in staining efficacy, this observation strongly suggested that the administration of the inhibitor effectively relieved the extent of fibrosis. To further corroborate our findings, we have conducted various experiments, including immunohistochemical staining, Western blot and qPCR assays. These complementary approaches consistently demonstrated the efficacy of the inhibitor in alleviating radiation-induced lung fibrosis.

Comment 8: Figure 4 shows the dsDNA from pulmonary epithelial cells. The panel H is nearly completely black and no color is visible.

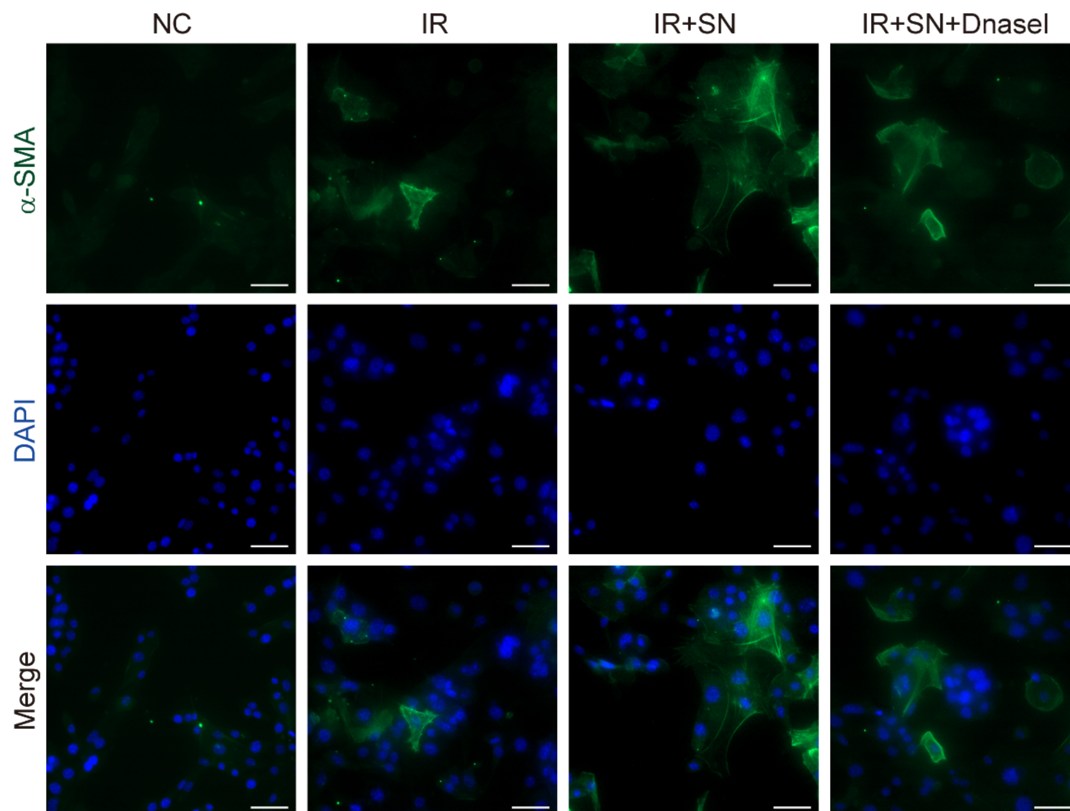
Reply 8: We appreciate your constructive feedback regarding the figures. It is possible that the resolution settings of our confocal microscope during image acquisition and subsequent processing for output cause the observed darkness in the images. We re-performing the immunofluorescence experiments under optimized conditions, ensuring that the microscope settings are adjusted to maximize image brightness, and replaced the images (Figure 4H).



Comment 9: Figure 5 shows DNASE1 effectively interfering with transformation of fibroblasts. It is not clear what transformation means. Panel E is completely black, and I can see no colors to evaluate.

Reply 9: Thank you for your comments. As you pointed out, our primary aim is to demonstrate that DNase I effectively interferes with the transformation of fibroblasts into myofibroblasts. We fully recognize that fibrosis is characterized by the accumulation of myofibroblasts, which are primarily derived from the differentiation of fibroblasts. These myofibroblasts play a pivotal role in producing excessive amounts of ECM adhesion and structural proteins, marked by the expression of α-SMA. As you suggested, we have revised the relevant section (see page 29, line 566-567). Furthermore, we have conducted additional immunofluorescence experiments under optimized conditions and replaced the images in Figure 5E.

Changes in the text: Fig. 5 Dnase I effectively interfered with the transformation of fibroblasts into myofibroblasts caused by supernatant.



Comment 10: Figure 6 shows STING mediated activation of fibroblasts to myofibroblasts. This is completely unclear, and the blot in Panel I is not interpretable as the spots appear to be very dense.

Reply 10: Thank you for your comment. As highlighted in the figure, our aim was to demonstrate that STING mediates the transformation of fibroblasts into myofibroblasts through the PERK-eIF2α pathway. Our results showed that the irradiated supernatant significantly enhanced the protein expression of both α-SMA and collagen I, markers commonly associated with myofibroblast activation. Concurrently, we observed increased phosphorylation of PERK, indicating activation of the PERK-eIF2α pathway. Importantly, these effects were reversed after STING knockdown, suggesting that STING is essential for activating this pathway and subsequently driving the transition. We then treated STING-knockdown fibroblasts with a PERK activator, and observed the expression of α-SMA and collagen I was increased again, confirming that STING mediates the activation of fibroblasts to myofibroblasts through the PERK-eIF2α pathway.

Comment 11: Figure 7 can be eliminated, as this is stated in the text.

Reply 11: Thank you for your suggestion. Figure 7 is our model diagram, and we hope to show the research content of the article more intuitively through the mechanism diagram.

Comment 12: The supplementary figures are not helpful given the problems with the presentations in the regular figures.

Reply 12: Thank you for your advice. Supplementary figure is intended to further support our findings, but is not necessary. We have removed the supplementary figure according to your suggestion.

Comment 13: It is suggested that the authors remove the information on acute effects since radiation pneumonitis is not quantitated in C57BL/6J mice and get better photographs of the histochemistry for the radiation fibrosis model. Most importantly, simply giving drugs does not prove the STING pathway is activated. The downstream effects of STING need to be measured including interferon, and other downstream products.

Reply 13: Thank you for your guidance and advice. As explained in Reply 2, we found that mice had a certain degree of inflammatory damage in the early stage by H&E and ELISA. Although our mechanistic research focuses on late fibrosis, we wanted to demonstrate the effect of early application of inhibitor on RILI from early to late stages. The STING-PERK-eIF2α pathway has been reported to play an important role in organ fibrosis. The study embarked on exploring the mechanism of fibroblasts and confirmed that the pathway is important for fibrosis in RILI disease. As you suggested, in-depth studies of innate immune effects of STING and classical pathways would be invaluable in elucidating the potential enhancing effectiveness of STING inhibitors in the treatment of RILI. This will undoubtedly strengthen the argument for its potential clinical application. In follow-up studies, we intend to conduct in-depth studies of the downstream effects of STING in different effector cells to fully assess the long-term benefits of STING inhibitor therapy.