Radiation-induced cardiopulmonary dysfunction
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DECREASING IRRADIATED RAT LUNG VOLUME CHANGES DOSE-LIMITING TOXICITY FROM EARLY TO LATE EFFECTS

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Technological developments in radiotherapy result in smaller irradiated volumes of normal tissue. Since the risk of radiotherapy-induced toxicity generally depends on irradiated volume, changing volume could change the dose-limiting toxicity of a treatment. Recently, in our rat model, we found that early radiation-induced lung dysfunction (RILD) was closely related to irradiated volume dependent vascular remodelling besides inflammation. The exact relation between early and late RILD is still unknown. Therefore, in this preclinical study we investigated the dose-volume relation of late RILD, assessed its dependence on early and late pathologies and studied if decreasing irradiated volume changed the dose-limiting toxicity.

Methods: A volume of 25%, 32%, 50%, 63%, 88%, or 100% of the rat lung was irradiated using protons. Until 26 weeks after irradiation, respiratory rates were measured. Macro-vascular remodelling, pulmonary inflammation and fibrosis were assessed at 26 weeks after irradiation. For all endpoints dose-volume-response curves were made. These results were compared to our previously published early lung effects.

Results: Early vascular remodelling and inflammation correlated significantly with early RILD. Late RILD correlated with inflammation and fibrosis, but not with vascular remodelling. In contrast to the early effects, late vascular remodelling, inflammation and fibrosis showed a primarily dose but not volume dependence. Comparison of respiratory rate increases early and late after irradiation for the different dose-distributions indicated that with decreasing irradiated volumes, the dose-limiting toxicity changed from early to late RILD.

Conclusions: In our rat model, different pathologies underlie early and late RILD with different dose-volume dependencies. Consequently, the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. In patients, early and late RILD are also due to different pathologies. As such, new radiation techniques reducing irradiated volume might change the dose-limiting toxicity of the radiotherapy treatment.
Abstract

Purpose: Technological developments in radiotherapy result in smaller irradiated volumes of normal tissue. Since the risk of radiotherapy-induced toxicity generally depends on irradiated volume, changing volume could change the dose-limiting toxicity of a treatment. Recently, in our rat model, we found that early radiation-induced lung dysfunction (RILD) was closely related to irradiated volume dependent vascular remodelling besides inflammation. The exact relation between early and late RILD is still unknown. Therefore, in this preclinical study we investigated the dose-volume relation of late RILD, assessed its dependence on early and late pathologies and studied if decreasing irradiated volume changed the dose-limiting toxicity.

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Introduction

Radiation therapy plays a pivotal role in the treatment of thoracic cancers. Unfortunately, radiation-induced lung dysfunction (RILD) is a potentially life-threatening and dose limiting side effect of thoracic irradiation and thus the risk should be minimized. Traditionally, RILD is divided into an early inflammatory phase known as “radiation pneumonitis” and a later fibroproductive phase referred to as “lung fibrosis”. Clinically significant symptomatic early RILD occurs in approximately 5-50%, 5-10%, and 1-5% of patients irradiated for cancers of the lung, mediastinal lymphatics, and breast, respectively.

Accurate prediction of the development of RILD is of great importance for treatment optimization. However, controversy exists about which dosimetric parameter(s) optimally predict RILD. Moreover, only models predicting early RILD are described. Since new advances in therapies will lead to a longer life expectancy of cancer patients, the occurrence of late radiation-induced normal tissue toxicity will become more relevant. Besides, technological developments in radiotherapy result in smaller irradiated volumes of normal tissue. Changing the irradiated volume could change the dose-limiting toxicity of a treatment.

In our rat model, early and late RILD occurs as a bi-phasic increase in breathing rates and histopathological changes. Morphologically, distinct types of lung injury can be observed: vascular remodelling, inflammation and fibrosis. Recently, we found that in concert with inflammation, vascular remodelling played a major role in the aetiology of early RILD. It was shown that lung irradiation induced early vascular remodelling resulting in pulmonary hypertension (PH) and right ventricle (RV) hypertrophy eventually leading to cardiopulmonary dysfunction.

The relation between early RILD with its dependence on irradiation dose and volume and late RILD is still unknown. Therefore, in this preclinical study we investigated the dose-volume relation of late RILD, assessed its dependence on early and late pathologies and studied if decreasing irradiated volume could change the dose-limiting toxicity.

Material and Methods

See supplementary data for complete material and methods.

Animals

Adult male albino Wistar rats (n=3-7 per dose-volume group) were used in the experiments. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Irradiation procedure

A volume of 25% (15–28 Gy), 32% (19-28 Gy), 50% (12–20 Gy), 63% (12–19 Gy), 88% (10–14 Gy) or 100% (10–13 Gy) of the rat lung was irradiated with protons. Figure 1 gives an overview of the irradiation ports.
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Breathing rate assay
To assess response of pulmonary function to radiation, the breathing rate (BR) was measured up to week 26, as previously described and shown in Figure 2. The mean increase in BR between 6 and 10 weeks after irradiation was used to assess the level of early RILD. For late RILD, the mean increase in BR between 16 and 26 weeks was measured.

Histologic examinations
Histologic examination was performed 26 weeks after radiation and compared with the histology at week 8 which we previously published. Vascular remodelling was scored by assessing hypertrophy of the macro-vasculature (Fig. 3). Both arterioles and venules were scored since these could not be distinguished in the lung tissue. No affected vessels received a score of 0, hypertrophic vascular walls a score of 1 and heavily affected vessels, meaning smooth-muscle cells of the media layer were thickened and around the arterioles edema or fibrosis a score of 2.

Pulmonary inflammation was scored as the level of inflammatory cells in the lung tissue (Fig. 3). No cells = 0; only a few cells = 1; many non-clustered cells present = 2; and large amounts of clustered cells present and total affected area volume of 50% or more of the total tissue cross-section = 3.

Late fibrosis was scored 0-3 (Fig. 3). No fibrosis = 0; small foci present = 1; medium foci present = 2; large foci present and total affected area ≥ 50% of the total tissue cross-section = 3.

Statistical analysis
Pearson’s linear correlation coefficient r was calculated to test for associations between respiratory changes after irradiation with vascular remodelling and inflammation. To evaluate dose- or volume-dependencies, a multivariate logistic regression analysis was performed in Matlab using glmfit. The values of p<0.05 were considered significant.
Results

Relation of vascular remodelling and inflammation with respiratory rate
To investigate early and late lung function changes we assessed respiratory rate up to 26 weeks after lung irradiation (Fig. 2). In general we see a dose dependent biphasic increase in respiratory rate as reported before 5,6.

![Figure 2: Respiratory rate of lung irradiated rats.](image)

Next we assessed the level of known radiation-induced lung pathologies - vascular remodelling, pulmonary inflammation and fibrosis (3, 6, 7, 9, 13-18) - by scoring the histology of lung slides (Fig. 3). Finally, to investigate the role of these different pathologies in the development of late RILD, we correlated the level of early and late lung effects with respiratory rate changes.
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Resuls

To investigate early and late lung function changes we assessed respiratory rate up to 26 weeks after lung irradiation (Fig. 2). In general we see a dose dependent biphasic increase in respiratory rate as reported before 5,6.

**Figure 2:** Respiratory rate of rats irradiated to a lung volume of 25% (A), 32% (B), 50% (C), 63% (D), 88% (E) and 100% (F) with doses ranging from 10 -28 Gy. The grey dotted line in all panels indicates the breathing rate of unirradiated animals. N=3 -7 per dose-volume group. The error bars indicate the standard error of the mean.

Next we assessed the level of known radiation-induced lung pathologies - vascular remodelling, pulmonary inflammation and fibrosis (3, 6, 7, 9, 13 -18) - by scoring the histology of lung slides (Fig. 3). Finally, to investigate the role of these different pathologies in the development of late RILD, we correlated the level of early and late lung effects with respiratory rate changes.

**Figure 3:** Quantification of rat lung morphology early and late after irradiation. A score of 0 for macro-vascular tumour indicates thin vascular walls, a score of 1 hypertrophic vascular walls and a score of 2 extreme hypertrophic vascular walls. A score of 0 for pulmonary inflammation represents normal lung tissue with sporadic inflammatory cells. A score of 1 a moderate increase of inflammatory cells, a score of 2 a lot of nonclustered inflammatory cells and 3 large foci of inflammatory cells. A score of 0 for fibrosis shows normal lung tissue without fibrosis, a score of 1 small foci of fibrosis. A score of 2 medium foci of fibrosis and a score of 3 large foci of fibrosis.

In contrast to early vascular remodelling, which is closely related to respiratory rate (Fig. 4A) as reported earlier 7, no clear correlation was found for late vascular remodelling with respiratory rate (Fig. 4B) (r=0.79 (0.53-0.91 95% C.I.) resp. 0.53 (0.07-0.81 95% C.I.)). Consequently, the role of vascular remodelling in late RILD seems less important than in early RILD.

Next, we assessed the relation between early and late pulmonary inflammation and respiratory rate. Typically, fibrosis is used as a late marker of RILD 11. However, since in this study relatively low radiation doses were used not leading to early fibrosis, inflammation was assessed to compare pathologies underlying early and late RILD. This parameter is present in both phases. Contrary to early RILD, where vascular remodelling and inflammation correlated strongly with respiratory rate (Fig. 4A and C, resp. r=0.79 (0.53-0.91 95% C.I.) and r=0.81 (0.57-0.92 95% C.I.)), late after irradiation a similarly strong correlation was observed only for inflammation (Fig. 4D, r=0.78 (0.48-0.92 95% C.I.)). To assess the possible influence of early vascular remodelling and early inflammation on late
RILD, we correlated early effects with late respiratory rate increases. Figure 4E shows that there is no correlation between early vascular remodelling and late respiratory rate increase.

**Figure 4: Correlation of respiratory rate changes with vascular tumour and pulmonary inflammation.** Correlation between macro-vascular tumour and respiratory rate early (A) and late (B) after irradiation. Correlation of inflammation and respiratory rate early (C) and late (D) after irradiation. Correlation between early macro-vascular tumour and late respiratory rate (E) and of early inflammation and late respiratory rate (F). The Pearson’s linear coefficients $r$ and the 95% confidence intervals are depicted in the graphs.
increase (r=0.11 (-0.52-0.67 95% C.I.)). No correlation was found between early inflammation and late respiratory increase either (r=0.59 (-0.02-0.88 95% C.I.)) (Fig. 4F). Therefore, vascular remodelling and inflammation play a role in the development of early RILD whereas the role of vascular remodelling seems to be reduced in late RILD. Furthermore, neither early vascular remodelling nor early inflammation seems to influence late respiratory difficulties.

**Dose-volume dependency of vascular remodelling, inflammation and fibrosis**

Next, we investigated the dose-volume dependency of late RILD and compared it with early effects. Figure 5A shows the dose-response curve of the score of vascular remodelling after irradiation of 50% as an example. Consistent with early RILD, at 26 weeks after irradiation vascular remodelling was already observed at low doses (12 Gy) (Fig. 5A), albeit at a low level. Similar to early vascular remodelling, the out-of-field effects were virtually as severe as the in-field effects (Fig. 5A). However, in contrast to early vascular remodelling, which was both dose- (Fig. 5G) and volume-dependent (Fig. 5G), late vascular remodelling was associated only with dose- (Fig. 5A and G) and not with volume (Fig. 5B and G).

Next, we investigated the dose-volume dependency of late inflammation and fibrosis. Figure 5C shows the dose-response curve of the score of inflammation after irradiation of 50% as an example. Consistent with early inflammation, a dose-dependent increase in the in- and out-of-field number of inflammatory cells was observed at 26 weeks (Fig. 5G). This phenomenon was observed for all irradiated volumes, but with increasing volumes, the number of out-of-field inflammatory cells became more similar to the in-field score (Fig. 5D). Early after irradiation, the number of inflammatory cells increased with irradiated volume at a fixed dose level (Fig. 5G). This volume-dependency was not observed at 26 weeks (Fig. 5D and G). The dependence on dose and irradiated volume of late inflammation and fibrosis are similar (see Fig. 5C-F). Comparable to inflammation, no volume-dependency was shown for the level of fibrosis late after irradiation (Fig. 5F and G). Thus, the development of late RILD in our model is mainly dependent on radiation dose whereas early RILD is dependent on irradiated dose as well as volume.

**Irradiated volume determines which toxicity is dose-limiting**

So far we showed that early RILD is associated with vascular remodelling and inflammation in a dose- as well as a volume-dependent manner. Late RILD on the other hand was mainly associated with inflammation and fibrosis in a dose-dependent manner. As such, this model shows that different prediction models may be required for early and late RILD.

To investigate if dose-limiting toxicity varies with irradiated volume, we assessed early and late respiratory rate after irradiation for various volumes (Fig. 6A-B). These early and late respiratory changes were compared in Figure 6C to assess which toxicity would be more severe and therefore dose-limiting. With decreasing irradiated volumes the dose-limiting toxicity changed from early RILD towards late. At higher irradiated volumes (100%, 88%) early function loss was dose-limiting due to its dependence on irradiated volume-dependent vascular remodelling (Fig. 5G, ref 7 (Fig. 3D)), while no significant late RILD was observed. With decreasing irradiated volume, the tolerance dose for early RILD increased, while dose-dependent late inflammation and fibrosis increased (Fig. 5C-G) leading to late
RILD (e.g. 63%, 50%). At an irradiated volume of 32% the tolerance dose for early RILD even exceeded that for late RILD. Therefore, respecting dose-volume limits for early RILD might not always prevent late RILD.

![Graphs showing dose-volume dependencies for various pathologies](image)

**Figure 5:** Quantification of the level of late vascular tumour, pulmonary inflammation and fibrosis after irradiation of the rat lung. Relationship between: A) the level of macro-vascular tumour and dose after 50% lung irradiation. B) The level of macro-vascular tumour and irradiated volume at fixed dose levels. C) The number of inflammatory cells and dose after 50% lung irradiation. D) The number of inflammatory cells and irradiated volume at fixed dose levels. E) The dose and level of fibrosis after 50% lung irradiation. F) The level of fibrosis and irradiated volume at fixed dose levels. G) Table showing the β-coefficients and p-values of multiple linear regression analysis predicting dose- and volume-dependencies of vascular tumour and inflammation and fibrosis early and late after irradiation.

The solid lines in panels (B), (D) and (F) indicate the in-field levels of lung damage and the dotted lines the out-of-field damage. N=3-7 per dose-volume group.

<table>
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<tr>
<th>Time</th>
<th>Variable</th>
<th>Dose</th>
<th>Volume</th>
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<td>p-Value</td>
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<tr>
<td>LATE - Week 26</td>
<td>Late Fibrosis</td>
<td>0.08</td>
<td>&lt; 0.00001</td>
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* Increase in histopathology score per Gy
* Increase in histopathology score per % irradiated volume
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**Figure 6: Variations in the increase in respiratory rate as a function of dose for various irradiated lung volumes.** Respiratory rate increases early (A) and late (B) after irradiation of different dose-distributions. C) Comparison of the in panel A and B depicted respiratory rate increases early and late after irradiation. The arrow indicates that with decreasing irradiated volumes the dose-limiting toxicity changes from early RILD towards late. The grey dotted lines in all 3 panels indicate respiratory rate increase of unirradiated animals (early phase: 19 bpm, late phase: 11 bpm). Error bars indicate the standard error of the mean. N=3-7 per dose-volume group.

**Discussion**

In our rat model, we show that depending on time after lung irradiation, different pathologies determine functional outcome. In addition, we observed that these pathologies differ in their dependence on irradiated dose and volume. Late RILD was associated with inflammation and fibrosis, mainly depending on dose. In contrast, early RILD was associated with vascular remodelling besides inflammation and mainly depended on irradiated volume. These observations were described before by us and others \cite{3,5,8,12,14}. 

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**Table:**

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<td>Late</td>
<td>Inflammation</td>
<td>0.06 &lt; 0.0001</td>
<td>0.16 0.48</td>
</tr>
</tbody>
</table>

**Dose-volume dependencies assessed by multivariate logistic regression analysis:**

- Effect at fixed volume
  - Vascular remodeling 0.04 < 0.01 0.03 0.90
- Effect at variable volumes
  - Vascular remodeling 0.03 0.01 0.76 < 0.001
- Effect at fixed dose
  - Early Fibrosis 0.02 < 0.0001 < 0.001 0.57
  - Late Fibrosis 0.08 < 0.00001 < 0.001 0.74
  - Pulmonary inflammation 0.06 < 0.00001 0.55 < 0.01

**Figure:**

A) Early RILD  
- Lung 25% right-base  
- Lung 32% lateral  
- Lung 50% lateral  
- Lung 63%  
- Lung 88%  
- Lung 100%

B) Late RILD  
- Lung 25% right-base  
- Lung 32% lateral  
- Lung 50% lateral  
- Lung 63%  
- Lung 88%  
- Lung 100%

C) Comparison of the in panel A and B depicted respiratory rate increases early and late after irradiation. The arrow indicates that with decreasing irradiated volumes the dose-limiting toxicity changes from early RILD towards late. The grey dotted lines in all 3 panels indicate respiratory rate increase of unirradiated animals (early phase: 19 bpm, late phase: 11 bpm). Error bars indicate the standard error of the mean. N=3-7 per dose-volume group.
Interestingly, we observed a new phenomenon. We found that the dose-limiting toxicity can change depending on irradiated volume. With decreasing irradiated volume the dose-limiting toxicity changed from early to late RILD. This finding may be very relevant in an era of technical developments in radiotherapy leading to smaller irradiated volumes of normal tissue.

The radiation-induced lung pathologies — vascular damage, inflammation and fibrosis — assessed in this study have been recognized before in animals as well as in patients. However, the impact on RILD and the exact dose-volume relations of these different pathologies have not been investigated before. Besides, so far, studies aimed at investigating the dose-volume relation of the development of “radiation pneumonitis” and not “late fibrosis”. As reported in our previous studies, we show that besides early inflammation and early fibrosis, vascular remodelling may play an important role in the development of early RILD. Late RILD on the other hand was associated with inflammation and fibrosis whereas the role of vascular remodelling seemed to be reduced. This was supported by the finding that the pulmonary pressure and right ventricle hypertrophy did not further increase and even decreased at 26 weeks after irradiation (Suppl. Fig. S1). This might indicate that vascular remodelling in our model stabilizes, is repaired or compensated for over time, while inflammation and fibrosis remain or even further progress. As such, early and late RILD have different dose-volume dependencies due to the different underlying pathologies. Therefore, different prediction models may be required.

Interestingly, we found that late RILD is not always preceded by early dysfunction. Irradiating small rat lung volumes (25%) up to a single dose of at least 28 Gy does not lead to functional changes either early or late after irradiation. However, irradiating a somewhat larger volume, 32%, leads to severe late RILD with, in contrast, a minor loss of function early after irradiation. Larger irradiated volumes (50%-63%) lead to both early and late RILD with more severe dysfunction in the early than in the late phase. Irradiated volumes of 88% and 100% showed early RILD, but no late RILD. However, the doses of these irradiated lung volumes could not exceed 14 Gy, whereas the threshold dose of inflammation/fibrosis may be somewhere in the range of 14-15 Gy. As such, early and late RILD is limited by different dose-volume constraints. Consistent with our findings Fröhlich et al. reported the presentation of symptomatic fibrosis without preceding pneumonitis in patients. Thus, late symptomatic RILD is not always a consequence of the clinical occurrence of early RILD. As such, prediction models of early RILD might not necessarily predict late RILD. In patients also different pathologies underlie early and late RILD. Therefore, different prediction models might also be required for early and late RILD in patients. Clinically, however, this is only relevant if respecting the dose-volume limitation of early RILD does not also prevent late RILD. Since this study shows that late RILD is not always proceeded by early RILD, late prediction models may be relevant. Unlike our rat model, in patients e.g. genetic differences and pre-existing pulmonary disease exist which may influence the development of the different pathologies and consequently the development of RILD. As such, to establish these models clinical studies should be performed including the aforementioned factors.

The development of new models predicting early as well as late RILD may be of importance since new advances in therapies, like stereotactic radiation technique, irradiation with protons or specific molecular targeted therapies lead to a longer life expectancy of
cancer patients. Moreover, new radiation techniques lead to a reduced irradiated volume of normal tissue. This preclinical study showed that the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. This could be explained by different pathologies underlying early and late RILD with different dose-volume dependencies. In patients, also different pathologies underlie early and late RILD. As such, to optimize radiotherapy treatment, models predicting both early and late toxicity may have to be used. To establish these models clinical modelling studies should be performed.

Conclusions

In contrast to early RILD in rats, late RILD predominantly depends on dose and is associated with inflammation and fibrosis, rather than irradiated volume and vascular remodelling. Consequently, the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. In patients, early and late RILD are also due to different pathologies. As such, new radiation techniques reducing irradiated volume might change the dose-limiting toxicity of the radiotherapy treatment.
References

Supplementary Material and Methods

Animals
Adult male albino Wistar rats (n=3-7 per dose-volume group, 270gr-320gr) of the Hsd/Cpb:WU strain, bread in a specific pathogen free colony (Harlan-CPB, Rijswijk, The Netherlands) were used in the experiments. They were housed five to a cage under a 12 h light - 12 h dark cycle and fed rodent chow (RMH-B, Hope Farms, Woerden, The Netherlands) and water ad libitum. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Irradiation procedure
The rats were anesthetized with an i.p. injection of xylazine (Rompun; Bayer, Leverkusen, Germany) plus S-ketamine (Ketalar; Pfizer, Capelle aan de IJssel, The Netherlands) and placed in a holder hanging on a positioning rod by their upper incisors (1) for CT scanning or irradiation. The use of 150-MeV protons in a fixed beam line facilitated the irradiation of subvolumes of the lung with sharply demarcated (20–80% penumbra of approximately 1 mm) radiation fields (2, 3). Radiation portals were designed using planning CT images of 5 age-matched rats, as described previously (1, 4). Using protons, 100% (10–13 Gy), 88% (10–14 Gy), 63% (12-19 Gy), 50% (12–20 Gy), 32% (19-28 Gy) or 25% (15–28 Gy) of the lung was irradiated with a single fraction. The dose distributions were limited to prevent mortality of the animals and were chosen such that the heart was either spared or irradiated only to a dose well below the threshold dose of 18 Gy where it starts influencing loss of pulmonary function early after irradiation (5). This ensured that the observed irradiation-induced injuries could be attributed to dose to the lung only. Control animals were anaesthetized and sham irradiated. Figure 1 gives an overview of irradiated volumes, the shape of the openings of the collimators used to achieve this, and the dose range used for each volume.

Breathing rate assay
To assess response of pulmonary function to radiation, breathing rate (BR) was measured before and every two weeks after the irradiations up to week 26, as previously described (2, 4) and shown in Figure 2. After two training sessions, a breathing rate (BR) at rest was recorded for each rat which took place less than a week before irradiation. As described earlier (6, 7), an unrestrained animal was placed in a 1500 ml airtight but transparent tube of a whole-body plethysmograph connected to a pressure transducer. The frequency of pressure changes inside the tube was recorded and displayed on a calibrated chart as breaths per minute (bpm). A mean BR of an animal was then calculated from minimum of 4 steady regions of the recording lasting ≥ 15 seconds. If the measurement required more than 5 minutes to obtain, the animal was let out of the tube and rested to prevent anxiety as well as drop of oxygen inside the tube. A mean BR of a dose group (bpm) with its standard error (SEM) was calculated from the means of individual animals at each time point. The increase of BR, relative to the mean BR in weeks 0–2 after irradiation, was used as an indicator of early and
late loss of pulmonary function. The mean BR increase between 6 and 10 weeks after irradiation was used to assess the level of early lung dysfunction. For late dysfunction, the mean BR increase between 16 and 26 weeks was assessed.

Histologic examinations
Histologic examination was performed 26 weeks after radiation and compared with the histology at week 8 which we published previously (8). Lung tissue samples were taken from both inside and outside the radiation field, with sufficient margins to ensure that the tissue was either irradiated (“in-field”; i.e., tissue receives more than 97% of the prescribed dose) or shielded (“out-of-field”; tissue receives less than 7% of the prescribed dose) (2, 3). The tissue was collected as published previously (4, 9). Briefly, the animals were heparinized while the heart was still beating. After opening the thoracic cavity both pulmonary and systemic circulation were perfused in situ via the right ventricle and liver incision with PBS (pH 7.3). Subsequently the lungs were removed and inflated by intratracheal infusion of 4% formaldehyde in PBS (pH 7.3) under a hydrostatic pressure of 20 cm H2O. The trachea was tied and the entire specimen was immersed in 4% buffered formaldehyde for overnight fixation. A clear margin between the irradiated and shielded tissues was visible macroscopically, conforming the shape of the irradiation portal. Lung tissue samples were taken from both inside and outside the radiation field, with sufficient margins to ensure that the tissue was either irradiated (“in-field”; i.e., tissue receives more than 97% of the prescribed dose) or shielded (“out-of-field”; tissue receives less than 7% of the prescribed dose) (2, 3), and embedded in paraffin. Sections of 3 μm containing standardized samples of irradiated or non-irradiated lung tissue were stained with Hematoxylin & Eosin and Masson’s trichrome and examined by light microscopy. In the entire tissue cross-section on each slide, blinded scoring of vascular remodelling and pulmonary inflammation/fibrosis was carried out separately using three semi quantitative scoring scales independently by two observers.

Vascular remodelling
Vascular remodelling was scored by assessing macro-vascular remodelling: hypertrophy of the macro-vasculature (>100 μm). Both arterioles and venules were scored since these could not be distinguished in the lung tissue. Macro-vascular hypertrophy, meaning that the smooth-muscle cells from the media layer are thickened, was scored as 0–2. An observation of no affected vessels received a score of 0 (Fig. 3), hypertrophic vascular walls (200x magnification field) a score of 1 (Fig. 3), and heavily affected vessels, meaning smooth-muscle cells of the media layer are thickened and around the arterioles edema or fibrosis can be seen a score of 2 (Fig. 3).

Pulmonary inflammation/fibrosis
Pulmonary inflammation was scored as the level of inflammatory cells in the lung tissue on each slide. No distinction was made between the different cell types. No inflammatory cells = Score 0 (Fig. 3); only a few inflammatory cells in the lung = Score 1 (Fig. 3); many non-clustered inflammatory cells present (200x magnification) = Score 2 (Fig. 3); and large amounts of clustered inflammatory cells present (100x magnification) and total affected area volume of 50% or more of the total tissue cross-section = Score 3 (Fig. 3).
Late fibrosis was scored 0-3. No fibrosis = Score 0 (Fig. 3); small foci present (focus < 1/2 of 100x magnification field) = Score 1 (Fig. 3); medium foci present (focus ≤ 100x field) = Score 2 (Fig. 3); large foci present (focus ≤ 40x field) and total affected area ≥ 50% of the total tissue cross-section = Score 3 (Fig. 3).

Statistical analysis
Pearson’s linear correlation coefficient r was calculated to test for associations between respiratory changes early and late after irradiation with vascular remodelling and pulmonary inflammation. To evaluate dose- or volume-dependency of vascular remodelling and inflammation/fibrosis early and late after irradiation, a multivariate logistic regression analysis was performed in Matlab using glmfit. The values of p<0.05 were considered significant.

Supplementary References
Supplementary Figure S1: Early radiation-induced pulmonary hypertension and right ventricle hypertrophy decreases in time. Pulmonary artery pressures and right ventricle hypertrophy at week 8 (A-B) and week 26 (C-D) after rat lung irradiation with different dose distributions, resp. 25% lung with a dose of 28 Gy, 33% lung with a dose of 22 Gy and 75% with a dose of 17 Gy. N=3. Error bars indicate standard error of the mean. Early data was adapted from Ghobadi et al. (1).

Reference
Supplemental material

Figure S1: Early data was adapted from Ghobadi et al. (1)
