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Preclinical Evaluation of Dose-Volume Effects and Lung Toxicity Occurring in- and out-of-field

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Summary

Small animal irradiators enable the interrogation of translationally relevant irradiation protocols and beam geometries. This study reports on the characterisation of early and late pulmonary toxicities occurring both in- and out-of-field, and the corresponding clinically-relevant dosimetric parameters. Significant correlations were observed between the mean lung dose (MLD) and V10 for late pulmonary toxicity in-field whereas toxicities outside the targeted volume showed no dependence on MLD and V10, suggesting non-targeted effects may have a role in driving toxicities outside the treatment field.
Abstract

Purpose

The aim of this study was to define the dose and dose-volume relationship of radiation induced pulmonary toxicities occurring in and out-of-field in mouse models of early inflammatory and late fibrotic response.

Materials and methods

Early radiation induced inflammation and fibrosis were investigated in C3H/NeJ and C57BL/6J mice respectively. Animals were irradiated with 20 Gy delivered to the upper region of the right lung as a single fraction or as three consecutive fractions using the Small Animal Radiation Research Platform (SARRP, Xstrahl Inc., Camberley, UK). Cone Beam Computed Tomography (CBCT) was performed for image guidance prior to irradiation and to monitor late toxicity. Histological sections were examined for neutrophil and macrophage infiltration as markers of early inflammatory response, type I collagen staining as a marker of late occurring fibrosis. Correlation was evaluated with the Dose Volume Histogram (DVH) parameters calculated for individual mice and changes in the observed CBCT values.

Results

Mean Lung Dose (MLD) and the volume receiving over 10 Gy (V10) showed significant correlation with late responses for single and fractionated exposures in directly targeted volumes. Responses observed outside the target volume were attributed to non-targeted effects and showed no dependence on either MLD or V10.

Conclusions

Quantitative assessment of normal tissue response closely correlates early and late pulmonary response with clinical parameters demonstrating this approach as a potential tool to facilitate clinical translation of preclinical studies. Out-of-field effects were observed but did not
correlate with dosimetric parameters suggesting non-targeted effects may have a role in driving toxicities outside the treatment field.
Introduction

Radiation induced lung toxicity (RILT) is the most common dose limiting adverse sequelae in patients receiving thoracic irradiation (1, 2). Despite technological advances in conformal delivery techniques, dose escalation has failed to demonstrate significant overall survival (OS) benefits compared to the previously established lower dose regimen, as demonstrated by the Radiation Therapy Oncology Group (trial RTOG-0671) (4). Furthermore, as both the number and age of cancer survivors increases, addressing critical gaps in understanding normal tissue responses is of significant importance towards improving quality of life following radiotherapy (5, 6).

RILT describes multiple pulmonary pathologies that can develop weeks to years after radiotherapy and significantly compromise quality-of-life (7). It represents a spectrum of biological events evolving from the initial stages of early inflammation, symptomatic radiation pneumonitis (RP) through to late stages of radiation induced fibrosis (RIF). Clinical scoring systems for RILT use a combination of radiologic, functional and symptomatic criteria towards a global toxicity score (8), yet prediction of patients most likely to develop RILT remains challenging in the clinic and may be an important factor towards optimising personalised radiotherapy.

Over the past two decades, progress has been made in defining normal tissue tolerances (9, 10). By establishing the dose-volume relationship between the delivered dose and resulting normal tissue injury, dose escalation can be achieved with minimum impact on normal tissue complication (8). Several dose thresholds used in clinical practice have been investigated with strong correlation observed between different dosimetric parameters (V5 and V20) within individual institutions (10). However, these parameters are dependent on radiotherapy techniques specific to each institution, making it difficult to establish general dose-volume constraints based on retrospective clinical data (8, 10, 12).
Preclinical studies of pulmonary response have largely investigated dose-response using whole thorax irradiation (11, 12). Previous studies have used partial thorax irradiation to explore the differential sensitivity of different lung structures and the relationship of radiation dose and volume to damage and morbidity in the mouse lung. These studies identified a heterogeneous response of the lung to partial volume irradiation, and suggested this is due to critical target cells located in the base in and apex of the mouse lung; moreover, proton irradiation studies indicated a change in dose-limiting toxicity with the change of the irradiated volume (13–15). Whilst these studies have provided vital information on the pulmonary radiation response of different rodent strains and defined critical structures and dose thresholds for lung toxicities, the implementation of small animal irradiators has enabled major refinements in the precision and accuracy of dose delivery, allowing for more clinically relevant dose distributions and fractionation schedules to be achieved experimentally (16–18).

This study aimed to characterise early and late pulmonary responses of partially-irradiated lung volumes using small animal image guided radiotherapy. Biological responses occurring in- and out-of-field were quantified and compared with dosimetric parameters such as MLD and V10 to determine their dose-volume dependence. This study supports the development of refined preclinical models of RILT incorporating clinical dosimetric parameters and the role of non-targeted effects occurring outside the treatment field.

**Materials and Methods**

**Preclinical Study Design**

RILT manifested as early inflammation (RP) and late toxicity (RIF) was investigated within and outside of the irradiated target volume in C3H/NeJ and C57BL/6J mice respectively. All
animals were obtained from Charles River Laboratories (Oxford, UK) and irradiated at 8-10 weeks old under UK Department of Health Approved procedures. A minimum of 5 mice per experimental group were sacrificed at each of the time points investigated, along with time matched sham irradiated control animals who received only CBCTs. A schematic overview of the study design is presented in Figure 1a, with further details given in the Supplementary Material.

All mice were irradiated with 220 kVp X-rays under CBCT image guidance using a SARRP calibrated using the IPEMB code of practice (19). Lung injury was induced by delivering a total dose of 20 Gy as a single fraction or as three daily fractions of 6.67 Gy/day. Using an α/β value of 2.5 for mouse lung (20), the Biologically Effective Doses (BEDs) for single fraction and fractionated deliveries were calculated as 180 and 73.4 Gy respectively. A parallel opposed beam geometry with a 5 x 5 mm collimator (dose rate 2.70 ± 0.16 Gy/min) was used to target an isocentre in the upper right lung chosen to minimise displacement during breathing and to spare organs at risk.

**CBCT imaging, planning and analysis**

CBCT scans were performed prior to irradiation and at 4, 12 and 26 weeks post irradiation. Lungs were outlined and DVHs were calculated retrospectively for each mouse using Muriplan (Xstrahl Inc., Suwannee, GA). Dosimetric parameters including MLD for the irradiated lung and V10 were extracted from the individual DVHs using Matlab R2017a. The percentage of the irradiated lung receiving at least 50% of the dose was 50.7 % ± 19% for single fraction exposures and 47.2 % ± 24% for fractionated arm of the study. Significant variations in the DVHs were observed as shown in Figure 1b due to anatomical variations for individual mice at the time of the imaging which allowed investigation of dose-volume relationships.
Histological assessment of RILT

Lung tissues were fixed by intra-tracheal infusion with 4% formaldehyde and processed for histological evaluation as detailed in the Supplementary Material. Five different areas of 0.25 mm$^2$ were analysed for both in- and out-of-field responses in the regions delineated in Figure 1c. In-field regions were defined as volumes irradiated with at least 80% of the prescribed dose, whilst out-of-field regions were defined as volumes irradiated with less than 2% of the prescribed dose (equivalent of scatter). This was considered to avoid any regions where respiratory motion may contribute towards penumbra and to reduce uncertainty.

Early inflammatory response was quantified as macrophage and neutrophil infiltration at 72 h, 1 and 4 weeks after irradiation. Late occurring fibrosis was quantified from type I collagen deposition detected by the trichrome blue positive staining in each image at 4, 12 and 26 weeks post irradiation. Representative histological images of early and late responses are presented in Supplementary Figures 1 and 2. A Matlab 2017a code was used to cluster the histological images and then extract the blue fraction from each image.

Data and statistical analysis

Statistical differences between populations were calculated using unpaired two-tailed Student t-tests, or Mann Whitney tests where appropriate, with a significance threshold of $p < 0.05$. Data is presented either as an average for the entire experimental arm ± standard error, or per individual mouse ± standard deviation. Linear regression lines were fitted to the data using Prism GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.) with the correlation $R^2$ values for MLD and V10 reported in supplementary table 1. V20 and V5 correlation $R^2$ parameters are shown in Supplementary Table 2. Statistical significance of slope parameters
was calculated using an F-test comparing the linear regression model to a model with a slope of 0.

Results

In-field effects

Early inflammatory responses were determined as the level of neutrophil and macrophage infiltration in the target volume after irradiation as shown in Figures 2a and b. In CBCT only control animals, cell counts of 115 ± 6.27 cells/mm$^2$ were observed for neutrophils, and 97 ± 5.05 cells/mm$^2$ and macrophages. At 72 hours after irradiation with 20 Gy, the numbers of inflammatory cells increased to 312.3 ± 36.79 for neutrophils and to 220.6 ± 12.31 cells/mm$^2$ for macrophages (p <0.0002), equivalent to increases of approximately 2.7 and 2.3 fold respectively. The numbers of inflammatory cells proceeded to decline with time, but remained significantly elevated in-field even after 4 weeks (p=0.012). The observed early increase returned to control levels within 1 week which may be due to the neutrophil response preceding other immune cells types or inflammatory cytokines in a chronological manner.

Late responses determined by CBCT tissue density analysis shown in Figures 2c and d, indicated significant increases which gradually reached a maximum 26 weeks after irradiation.

CBCT numbers increased from 0.73 ± 0.13 % to 6.31 ± 0.34 % for the single fraction exposures, and up to 5.41 ± 0.51 % for fractionated arm, with no significant differences due to fractionation (p=0.67). Furthermore, collagen deposition was also found to correlate with the observed changes in the CBCT numbers, as shown in figure 3 (p<0.001). This indicates that the changes can be non-invasively resolved using imaging. CT numbers showed no variation outside the targeted volume (data not shown).
Out-of-field effects

Out-of-field effects were quantified in the lower region of the irradiated lung for both early and late responses as in Figure 4. The mean total dose delivered to this volume was 0.2 ± 0.04 Gy for the single fraction exposures, and 0.15 ± 0.03 Gy for fractionated exposures. Outside the target area, neutrophil and macrophage infiltration (shown in Figure 4a and b) was significantly lower than inside the irradiated area (p<0.0001). However, temporal variation was still observed where at the 72 hour time point, both markers (135.9 ± 6.42 neutrophils/mm$^2$ and 123.9 ± 6.66 macrophages/mm$^2$) were significantly higher than those for the CBCT only animals (p<0.01). The level of inflammatory cells returned to baseline levels at later time-points. In addition, out-of-field effects show no significant difference between single and fractionated exposures.

Late responses showed significant increases at 26 weeks post irradiation from 0.38 ± 0.09 % trichrome in the CBCT only group to an average of 2.88 ± 0.29 % and 2.27 ± 0.21 % for acute and fractionated exposures respectively, despite the out-of-field tissues seeing less than 2% of the prescribed dose. The lower trichrome levels in different locations before irradiation are the result of differences in lung anatomy. Interestingly, fractionated exposures showed significantly lower out-of-field effects compared to single exposures, unlike in-field effects (p=0.012).

In- and out-of-field effects vs. MLD

MLD was calculated for the whole volume of irradiated lung to analyse RILT in the context of clinical dosimetric parameters. Correlation with early inflammatory responses is presented in Figure 5 and shows that although the total numbers of macrophages and neutrophils increased following irradiation, no statistically significant trend was seen with MLD in- or out-of-field (p>0.29).
For late responses, after 26 weeks a strong correlation between MLD and in-field fibrosis was observed which reached maxima of 7.48% and 6.60% for single and fractionated exposures respectively (Figures 5c and e). Out-of-field fibrosis effects were shown to be weaker with maxima of 3.43% and 3.0% for single and fractionated doses respectively, and were also shown to be independent of the individual MLD (Figure 5d and f).

**In- and out-of-field effects vs. V10**

The dependence of RILT on V10 was also investigated. A statistically significant positive correlation was found with in-field neutrophil infiltration shown in Figure 6a, in contrast to the observed levels of macrophage infiltration which did not reach significance (p=0.12). As with MLD, no significant variation with dose was seen out-of-field for either of the inflammatory cell types. Supporting early inflammation observations, increases in collagen staining in-field strongly correlated with V10 for both radiation exposure schedules (Figure 6c and e), although the strength of this correlation was slightly reduced. Similarly, there was no significant trend in the out-of-field effects with V10, suggesting these effects are not purely dose-dependent (Figure 6d and f).

**Discussion**

This study aimed firstly, to demonstrate correlations between early and late pulmonary toxicities with clinical dose and volume parameters following partial lung irradiation and secondly, to characterise these effects occurring within the target volume and out-of-field. Many clinical studies have focussed on analysing specific DVH parameters to assess the risk of RILT development (5, 8, 21, 22). These studies report that the percentage of the total lung volume exceeding 20 Gy (V20), and the MLD are independent predictors of symptomatic RP.
Early immune infiltration precedes RP and was used in this study as an early effect of RILT (23), whilst collagen deposition was examined longitudinally representing late occurring fibrosis (24). MLD and V10 were found to be relevant parameters in preclinical studies, correlating these biological endpoints of normal tissue toxicity. The precise delivery and individual analysis presented in this study enables the calculation of dosimetric parameters specific for each individual mouse. This enables this study to explore effects of inter-mouse variations in anatomy, in contrast the large-scale differences in irradiation geometry as used in previous partial thorax irradiation studies (13, 25).

Our data show that whilst irradiation clearly increases immune cell infiltration at 72 h post irradiation, there was limited evidence of a dependence on MLD across individual mice. Fitting of early responses occurring in-field suggested immune cell gradients up to 50 cells/mm²/Gy which were not statistically significant whilst out-of-field responses were negligible (<2 cells/mm²/Gy). In contrast, for late responses, clear trends were seen in trichrome accumulation occurring in-field for both single and fractionated doses (1.06 and 2.75 % trichrome/Gy), while out-of-field responses appeared to be independent of MLD. Similarly, late occurring in-field responses show clear dependencies on V10, with similar rates of trichrome accumulation between single and fractionated exposures (with gradients of 0.30 and 0.22 % trichrome/ %V10 respectively). Importantly, these data suggest that differences in dose distribution, rather than inter-fraction recovery, may drive the observed differences compared to fractionated exposures in this model system.

Whilst exploring other relevant dosimetric parameters, V5 and V20 were investigated and showed inferior correlation with observed pathological changes (Supplementary Table 2). Taken together, these data strongly support the use of MLD and V10 as optimal preclinical parameters relating to the measured early and late RILT. Furthermore, tissue density measured by CBCT showed strong correlation with the collagen staining measured within the
irradiated volume of the lung in agreement with previous studies (26). CBCT values showed no variation for the out-of-field volume in this study (data not shown), suggesting a sensitivity threshold for CBCT, or differences in the progression of fibrosis out-of-field.

Although out-of-field effects have been reported both in vitro and in vivo, their clinical significance remains controversial, with a recent analysis reporting on only 46 cases detected between 1969 and 2014, despite millions of patients being treated worldwide (27). The data presented in this study explores the relationship between in vivo cellular response and DVH parameters. With early and late responses occurring out-of-field reaching a significantly higher level than predicted for the scattered dose delivered, these results support previous in vitro work from our laboratory (22). The observed poor correlation of these effects with dosimetric parameters further strengthens previous observations that have indicated a threshold dose dependence and that they cannot be predicted solely from target doses (30, 31), which may also contribute to the observations that there is no dose-volume threshold below which there is no risk of RILT (8).

C3H/NeJ and C57BL/6J mice were selected to model early and late responses respectively. As there are known strain differences in the intermediate and late phases of the radiation response of mouse lung (25, 32), it is important to note that whilst the general trends of MLD and V10 dependence are conserved between strains, the magnitude may quantitatively differ. Further exploration of these effects in different strains may provide additional insight into the mechanisms underlying these effects and further advance our understanding of RILT in murine models.

The present study assessed the predictive power of clinical parameters in the context of early and late pulmonary responses routinely investigated in preclinical studies. While clear correlations are observed in-field, this study was not powered to fully and definitively
characterise the dose-volume dependency of normal lung tissue. However, it can be used as a model to further inform larger scale studies to fully explore the effects of dose, volume and fractionation on normal tissue toxicity markers, particularly in out-of-field regions. This may further improve the translational potential of preclinical studies applied to multiple lines of investigation including drug radiotherapy combinations with systemic agents that may exacerbate RILT, trials of potential mitigation or prevention strategies, and exploration of dose constraints in preclinical scenarios for novel treatment protocols.

Conclusions

Specific cellular responses in preclinical models can be quantitatively assessed in the context of clinically relevant parameters. Significant correlations were observed between the mean lung dose (MLD) and V10 for late pulmonary toxicity in-field. Toxicities were observed outside the targeted volume, but showed no dependence on MLD and V10, suggesting non-targeted radiation effects may have a role in driving toxicities outside the treatment field.

References


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**Figure 1.** a) Schematic diagram of experimental design detailing the time points and fractionation schedule for each experimental arm; b) DVHs for irradiated lungs seeing lowest and highest doses in study cohort; c) Schematic diagram of analysed targeted and non-targeted volumes of the irradiated lung; percentages are of the prescribed dose.

**Figure 2.** In field effects after targeted irradiation of normal lung, assessed as early effects in C3H mice of a) neutrophil and b) macrophage infiltration following single fraction 20 Gy exposure. Late effects were assessed in C57BL/6J mice and quantified as trichrome stained % after irradiation with 20 Gy delivered as c) single fraction and d) 3 equal consecutive fractions. Data is presented as an average for 5-7 mice per treatment group ± SEM. Significance values were classified as *** (p < 0.001), ** (p < 0.005), * (p < 0.05).

**Figure 3.** Correlation between imaging cone-beam CT numbers in the irradiated volume and trichrome % as a measure of late radiation induced toxicity in the lung after 20 Gy delivered as a) single fraction and b) 3 consecutive fractions of 6.67 Gy. Each data point represents the CT number and corresponding trichrome % for one C57BL/6J mouse; x error bars represent the collagen variation within the irradiated volume. Blue symbols: 4 week; red symbols: 12 week; black symbols: 26 week time points.

**Figure 4.** Out of field effects after targeted irradiation of normal lung, assessed as early effects of a) neutrophil and b) macrophage infiltration following single fraction 20 Gy
exposure of C3H mice. Late effects were quantified as trichrome stained % after irradiation with 20 Gy delivered as c) single fraction and d) 3 equal consecutive fractions in C57BL/6J. Data is presented as an average for 5-7 mice per treatment group ± SEM. Significance values were classified as *** (p < 0.001), ** (p < 0.005), * (p < 0.05).

**Figure 5.** Quantified in- and out-of-field effects represented as a function of MLD calculated for individual C3H mice for early effects a) in field and b) out-of-field effects (closed symbols - neutrophils; open symbols – macrophage; red symbols: 72 hours, blue symbols: 1 week, black symbols: 4 week time points). For early effects, correlation is assessed at 72h post irradiation. Late effects were quantified after 20 Gy single dose irradiation of C57BL/6J c) in field and d) out-of-field; and 20 Gy delivered as 3 consecutive fractions of 6.67Gy e) in field and f) out-of-field. Blue symbols: 4 week; red symbols: 12 week; black symbols: 26 week time points. Correlation is analysed for 26 weeks post irradiation.

**Figure 6.** Quantified in- and out-of-field effects represented as a function of the V10 for individual C3H mice for early a) in field and b) out of field (closed symbols - neutrophils; open symbols – macrophage; red symbols: 72 hours, blue symbols: 1 week, black symbols: 4 weeks’ time points). For early effects, correlation is assessed at 72h post irradiation. Late effects were assessed after 20 Gy single dose irradiation of C57BL/6J mice c) in field and d) out of field and 20 Gy delivered as 3 consecutive fractions of 6.67 Gy e) in-field and f) out-of-field. Blue symbols: 4 week, red symbols: 12 week, black symbols: 26 weeks’ time points. Correlation is analysed for 26 weeks post irradiation.
Figure 1.

a) Diagram showing the process of radiation-induced inflammation (C3H) over 72 hours, 1 week, and 4 weeks, followed by late toxicity (RiF) in C57B/L6 mice over weeks 4, 12, and 26.

b) Graphs showing the volume change over dose ranges.

c) Illustration of non-targeted volume with total dose < 2% and targeted volume with PoP geometry, 5x5 mm collimator, and total dose > 80%.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
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