DNA damage at respiratory distress, but not acute time points, correlates with tissue fibrosis following thoracic radiation exposure in mice

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Running title: DNA damage correlates with lung fibrosis

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Key words: Inbred mouse strains, double strand breaks, thoracic irradiation, , fibrosis

Abstract

Purpose: Radiation exposure can result in DNA damage but whether extent of DNA damage correlates with the radiation-induced tissue injury in the lung is not known. We aimed to determine whether numbers of γH2AX foci, representing histone H2AX phosphorylation a marker of DNA damage, measured within days of radiation exposure, correlate with known later lung injury responses in 8 inbred mouse strains.

Materials and Methods: Mice received 18 Gy pulmonary irradiation and numbers of γH2AX positive nuclei in the lung were immunohistochemically determined.

Results: Numbers of γH2AX foci, assessed up to seven days post irradiation did not correlate with pulmonary fibrosis. γH2AX counts from mice in respiratory distress, however, significantly correlated with fibrosis and lungs from mice treated with a fibrosis-reducing antagonist had fewer γH2AX foci.

Conclusions: Acute response measures of pulmonary DNA damage did not predict for pathology, but levels of this marker in distressed mice were correlative of fibrosis.

Introduction

Thoracic radiation therapy is one of the most important therapeutic modalities for lung cancer (Ding et al., 2011). However, it is often associated with serious dose limiting side effects such as the pathologies of excessive inflammation (pneumonitis) or deposition of extracellular matrix in the normal lung tissue interstitium (fibrosis) which is necessarily also exposed. If excessive, this damage can result in impaired lung function (Kong et al., 2005; Carver et al., 2007). Clinically, there is a latent period between the radiation exposure and the presentation of lung diseases, and this time may vary from months to years (Kong et al., 2005). Assays which could predict, early on, the individuals likely to develop the side effects of fibrosis and pneumonitis would therefore be useful in monitoring response to therapy (Barnett et al., 2009).

Several types of assays (such as single nucleotide polymorphisms, levels of chromosomal aberration, lymphocyte apoptosis, or cytokine production) of radiation-induced primary responses have been investigated for their possible correlations with the long term health effects (Wurm et al., 1994; Borgmann et al., 2002; Ozsahin et al., 2005; Fleckenstein et al., 2007; Barnett et al., 2010; Jérôme et al., 2013). Although limited success has been reported, no assay yet robustly correlates with tissue injury (Chua et al., 2013). Recently, DNA double strand breaks (DSB) have gained renewed attention as a predictive cellular marker of radiosensitivity in cells/tissues and in vivo model systems given that DNA is the prime sub-cellular target of radiation (Rube et al., 2008a; Bhogal et al., 2009; Ivashkevich et al., 2012; Pouliliou and Koukourakis, 2014). Histone H2AX phosphorylation is a recognized marker of DNA damage (Ivashkevich et al., 2012, Sharma et al., 2012) and immunofluorescence/immunochemistry based methodologies to detect phosphorylated γH2AX in tissues have been established (Rube et al.,

2008a; Bhogal et al., 2009; Brunner et al., 2011; Toyoda et al., 2013; Pouliliou and Koukourakis, 2014).

The radiation response of residual DNA damage, indicated by γH2AX foci, has been investigated in mice and shown to be both dose responsive and inbred strain dependent. For example, Bhogal et al. (2010) reported the numbers of foci in skin at 7 days following total body irradiation exposure to be highest in BALB/cJ mice, followed by levels in the C3H/HeJ and C57BL/6J strains. Rube et al. (2008b) also determined the numbers of radiation-induced γH2AX foci to depend on strain as levels in blood lymphocytes of BALB and C57BL/6J mice differed, and further, this strain difference in DNA damage was also evident in different tissues including the intestine and lung suggesting there may be strain variability in DNA damage level due to the inherent differences among inbred strains.

Inbred strains of mice are also known to vary in their susceptibility to develop pneumonitis or fibrosis in response to whole thorax irradiation (Sharplin and Franko 1989a and 1989b; O'Brien et al., 2005; Thomas et al., 2010) and to present these injuries over a time course similar to that evident clinically. We have recently reported the pulmonary injury response of a panel of 27 strains (Paun and Haston, 2012), and in this work all mice developed lethal pneumonitis following thoracic irradiation and some strains additionally presented significant pulmonary fibrosis. Specifically the inbred strains C57BL/6, 129S1/SvImJ, KK/HIJ and NZW/LacJ developed significant fibrosis while the strains C3H/HeJ, A/J, AKR/J and CBA/J succumbed to radiation-induced respiratory distress with pneumonitis only. In addition to histologically evident lung disease, time to distress was identified to vary significantly among the strains. For example, mice of the KK/HIJ, C3H/HeJ, NZW/LacJ and AKR/J strains presented distress at 10-14 weeks after radiation treatment while this trait was evident in C57BL/6J,

129S1/SvImJ, CBA/J and A/J mice at 21-26 weeks after 18 Gy. As it has previously been shown that survival time correlated with the inflammatory response of pneumonitis (Haston et al., 2007), we have included the parameter of asymptomatic survival time in the current investigation.

In present study we quantified the residual DNA double strand breaks in the lung tissue of mouse strains differing in their susceptibilities to radiation induced lung diseases with the aim to evaluate the predictive potential of the levels of these radiation-induced DNA double strand breaks for the eventual lung disease phenotypes.

Materials and methods

Mice: Mice of inbred strains (C3H/HeJ, C57BL/6, A/J, AKR/J, 129S1/SvImJ, KK/HIJ, CBA/J and NZW/LacJ) were purchased from the Jackson Laboratory (Bar Harbor, ME,USA) and housed in the animal facility of the Meakins-Christie Laboratories. All mice were handled according to national guidelines and regulations, under a protocol approved by the Animal Care Committee of McGill University. The strains were selected to present a range in the parameter of onset of distress and in extent of pulmonary fibrosis occurring as a consequence of radiation exposure. The radiation-induced tissue injury phenotype values of pulmonary fibrosis score and time to respiratory distress, for these eight strains, were taken from a previous report (Paun and Haston, 2012).

Radiation treatment: Eight week old female mice were anesthetised with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The mice were then partially shielded with 3 cm of lead and lung damage was elicited by whole thorax radiation exposure (18 Gy; dose rate 0.54 Gy/minute) using a 160 kV Faxitron X ray machine (Tucsun AZ, USA). This dose of radiation had been established previously to produce lung disease in these strains of mice (O'Brien et al., 2005; Haston et al., 2007; Thomas et al., 2010; Paun and Haston, 2012), thus to evaluate the correlation between radiation-induced DNA damage and radiation-induced lung disease, the radiation dose was kept constant. After irradiation, the animals were housed under normal laboratory conditions and specific groups of 3-6 mice sacrificed at 6h, 24h, 7 days and 8 weeks post irradiation. The control mice of each strain were not treated and were euthanized at the 7 day time point.

Lung tissue preparation: At necropsy, each lung was inflated with 1 mL of 10% neutral buffered formalin before being rapidly removed and washed with cold PBS. The left lobe from each mouse was submitted for standard histological processing. Additionally, left lung tissue sections from mice of the same eight inbred strains, harvested upon presentation of respiratory distress as presented in (Paun and Haston, 2012), and from DF2156A (an inhibitor of IL-8 receptors CXCR1 and CXCR2) -treated KK/HIJ mice (Fox and Haston, 2013) were studied. The radiation-induced lung disease phenotype of average fibrosis score of DF2156A-treated mice was taken from the prior report (Fox and Haston, 2013). In each of these studies (Paun and Haston, 2012; Fox and Haston, 2013) respiratory distress in mice was defined as the loss of >20% of body weight, and the exhibition of signs of distress including ruffled fur, visibly accelerated breathing and a hunched posture. The same indications of distress were used to evaluate mice of all strains. All mice euthanized due to the presentation of distress symptoms had significant lung disease by histological examination.

Immunohistochemistry of p-γH2AX: The left lung tissue sections (5 μm thickness), after dewaxing in xylene and rehydration in graded alcohols, were boiled in antigen unmasking solution (Vector Laboratories, Burlington ON, Canada) for 50 min to expose antigenic determinants and blocked with 4 % goat serum. Afterwards sections were incubated with anti-*p(ser139)*-γH2AX antibody (Upstate Biotechnology, Lake Placid, NY, USA), followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlington ON, Canada). Sections were labeled using avidin-biotin-alkaline phosphatase and red alkaline phosphatase substrate kits (Vector Laboratories, Burlington ON, Canada) as per the manufacturer's

instruction. Finally sections were counterstained with methylene blue, mounted with non-aqueous media and viewed at 200X under binocular microscope by a user blinded to mouse strain and treatment. The cells with red stained nuclei were counted as γH2AX foci positive nuclei. The number of γH2AX foci positive nuclei for each section was calculated as average of the γH2AX foci positive nuclei counted from five different microscopic fields. One section from each of 3-6 different mice per strain was analysed and results are presented as mean ± SEM.

Statistical analysis: The statistical significance of the variability among the means of the treatment groups, the strain dependence of the phenotype, was determined by one way ANOVA. Pearson's rank correlation test was used to assess an association between numbers of γH2AX foci positive nuclei with fibrosis score or asymptomatic survival time (days).

Results

Strain dependent levels of γH2AX foci positive nuclei in the lung post thoracic radiation exposure

To determine whether the extent of residual DNA DSB varied among inbred strains following whole thorax radiation exposure, numbers of γH2AX foci positive nuclei in the lungs of mice of eight strains were enumerated at various timepoints after radiation treatment. As shown in Figure 1, control tissue sections were almost all negative for γH2AX foci, whereas thoracic irradiation resulted in a significant increase in the numbers of pulmonary γH2AX foci positive nuclei in all strains. The γH2AX positive nuclei were observed throughout the tissue section

irrespective of the cell type. Within each strain, the average numbers of pulmonary γH2AX foci positive nuclei varied significantly ($p<0.01$) over the time course post irradiation which resulted in strain dependent variability in this trait ($p<0.0005$; by ANOVA of counts for each strain at one time point) when measured at each of 6h, 24h, 7d and 8w post irradiation and in animals presenting respiratory distress (Fig. 2).

Correlation of pulmonary γH2AX foci positive nuclei and the lung disease phenotypes

Correlation analyses were completed to test whether the strain-dependent residual DNA DSB accumulated during the post irradiation period were associated with the late lung tissue responses of fibrosis or asymptomatic survival time. As shown in Figure 3A, a negative correlation (Pearson coefficient = -0.67, $p = 0.063$) between average number of γH2AX foci positive nuclei, at 8w post irradiation, and asymptomatic survival time was suggested. Additionally, the average numbers of γH2AX foci positive nuclei, evident at the point of respiratory distress, were positively correlated with extent of pulmonary fibrosis (Pearson coefficient = 0.71, $p = 0.044$), as illustrated in Figure 3B. No other significant correlations were evident for average numbers of γH2AX foci positive nuclei and the phenotypes of asymptomatic survival time or fibrosis at any of the remaining assay times ($p>0.13$, data not shown). Further, analyses completed within the γH2AX foci dataset revealed the numbers of pulmonary foci measured at early time points from 6 hours to 7 days post irradiation did not correlate with the numbers of foci evident in the lungs from mice in distress ($p>0.17$, data not shown).

γH2AX foci positive nuclei in experimentally reduced pulmonary fibrosis

We had previously demonstrated that DF2156A treatment reduces radiation-induced pulmonary fibrosis in KK/HIJ mice, without affecting their survival time i.e. the time post irradiation at which respiratory distress was evident (Fox and Haston 2013). This prior study was completed with whole thorax irradiation of 14 Gy. Therefore, to investigate the correlation of γH2AX foci count with fibrosis, in experimentally manipulated fibrotic lung disease, we assayed the lungs of KK/HIJ mice treated with DF2156A throughout the post-irradiation period. As shown in Figure 4, the average number of γH2AX foci positive nuclei in the lungs of KK/HIJ mice treated with DF2156A was significantly lower ($p < 0.001$) than the levels evident in irradiated, but untreated, KK/HIJ mice, each euthanized at the common respiratory distress point of approximately 60 days post irradiation.

Discussion

In the present investigation, by quantifying the level of residual DNA DSB as γH2AX foci positive nuclei in the lung tissue of eight strains of mice, which differ in their susceptibilities to thoracic radiation-induced lung disease, we showed the acute DNA damage response post irradiation did not correlate with fibrosis or asymptomatic survival time, rather numbers of γH2AX foci positive nuclei, procured from mice in distress, correlated with extent of pulmonary fibrosis in these mice.

By assaying the tissue response within hours to days of radiation exposure we detected significant variability in the kinetics of the accumulation of γH2AX foci positive nuclei in the lungs of this panel of inbred mice. The strain dependence of the numbers of γH2AX foci at these times after normal tissue irradiation is in agreement with prior reports in mice (Rube et al., 2008b; Bhogal et al., 2010). As numbers of γH2AX foci positive nuclei recorded at these times did not, however, correlate with later lung disease we have no evidence that this early radiation response would be useful as an assay predictive of tissue injury. Related to this finding, Langan et al. (2006) showed that administration of reactive oxygen species scavenger, Eukarion-189, to rats within 3 days of irradiation significantly decreased the DNA damage level, measured by micronucleus assay, but did not reduce the incidence of the later response of pneumonitis. The potential association between the levels of γH2AX foci positive nuclei at 8w post irradiation and the lung disease phenotype of the presentation of respiratory distress may suggest the existence of a useful time for monitoring residual DNA DSB occurring after 7d and up to 8 weeks post irradiation. It is also possible that DNA damage assayed at a point in time before 6 hours post irradiation may be demonstrated to correlate with the later injury response. In addition, in this work, as in others (Rube et al., 2008a; Bhogal et al., 2010) we did not identify the specific cell

types harbouring the γH2AX foci positive nuclei, and thus the potential of a correlative response to tissue injury, based on cell type specific DNA damage, exists.

While the increase in the level of residual DNA DSB measured at times up to 24h post irradiation could be attributed to primary effects of radiation exposure (Olive et al., 1990; Rube et al., 2008a, 2008b and 2010), the significant increase in this marker at respiratory distress was likely due to the presence of pro fibrotic oxidizing environment (Poli and Parola, 1997; Sprung et al., 2013). Indeed the observation that the majority of the strains had greater numbers of γH2AX foci positive nuclei in their lungs when they were in distress, compared to levels at 8 weeks post irradiation, indicates a change in the lung environment affected the DNA DSB level, as no second radiation dose was given. In support of an altered lung environment effecting DNA damage, Khan et al. (1998, 2003) reported there to be both DNA damage in the shielded portions of the lungs of rats exposed to thoracic irradiation and for this DNA damage to be decreased in animals treated with superoxide dismutase. Further to the link of oxidative stress and DNA damage are reports of the elevated levels of the ROS-induced DNA damage marker 8OHdG in the fibrotic response of arthroplasty patients (Freeman et al., 2009) and the presence of increased numbers of γH2AX foci in lung tissue from COPD patients (Pastukh et al., 2011).

We investigated the finding that the fibrotic environment could indeed lead to the elevated DNA damage levels in the lungs of thoracic irradiated mice, by quantifying the number of γH2AX foci positive nuclei in the lungs of thoracic irradiated mice treated with a CXCR2 antagonist DF2156A. This experimental model was chosen based on our previous study wherein treatment of mice with DF2156A during post irradiation period resulted in an attenuation of the fibrosis response, without affecting the time to onset of distress (Fox and Haston, 2013). The significantly reduced number of γH2AX foci positive nuclei in the lung under experimentally

(DF2156A treatment) reduced fibrosis condition supports the finding that the lung fibrotic environment is correlated to the DNA damage response. The immune environment in the lung, specifically, may be a key component of the putative fibrotic environment as macrophages and neutrophils are known sources of reactive oxygen species (Dupré-Crochet et al. 2013). Thus differences in macrophage number or phenotype, or neutrophil number, between the strains manifesting pulmonary fibrosis in response to thoracic irradiation and those manifesting pneumonitis only may exist and may have produced the correlation of DNA damage with fibrosis. As an example of a strain difference, we (O'Brien et al., 2005) have previously reported there to be more apoptotic macrophages in the lungs of fibrosis prone C57BL/6J mice compared to levels in pneumonitis responding C3H mice, after pulmonary irradiation. Apart from an inflammatory response, the grouping of inbred strains according to the numbers of γH2AX foci evident in the lungs at respiratory distress, depicted in Figure 3, may indicate a shared underlying biology of response to radiation injury in these strains.

In summary, the present study demonstrated potential correlations between the extent of residual DNA DSB accumulated at later times during post irradiation period (at 8 weeks and at respiratory distress), measured through γH2AX assay, and lung disease phenotypes of asymptomatic survival and fibrosis. In this panel of strains the marker of DNA damage, assessed within hours to days post irradiation, did not predict for eventual lung disease.

Declaration of interest: The authors have no conflicts of interest to disclose. Funded by a grant from the Canadian Institutes of Health Research (to CKH).

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Figure legends

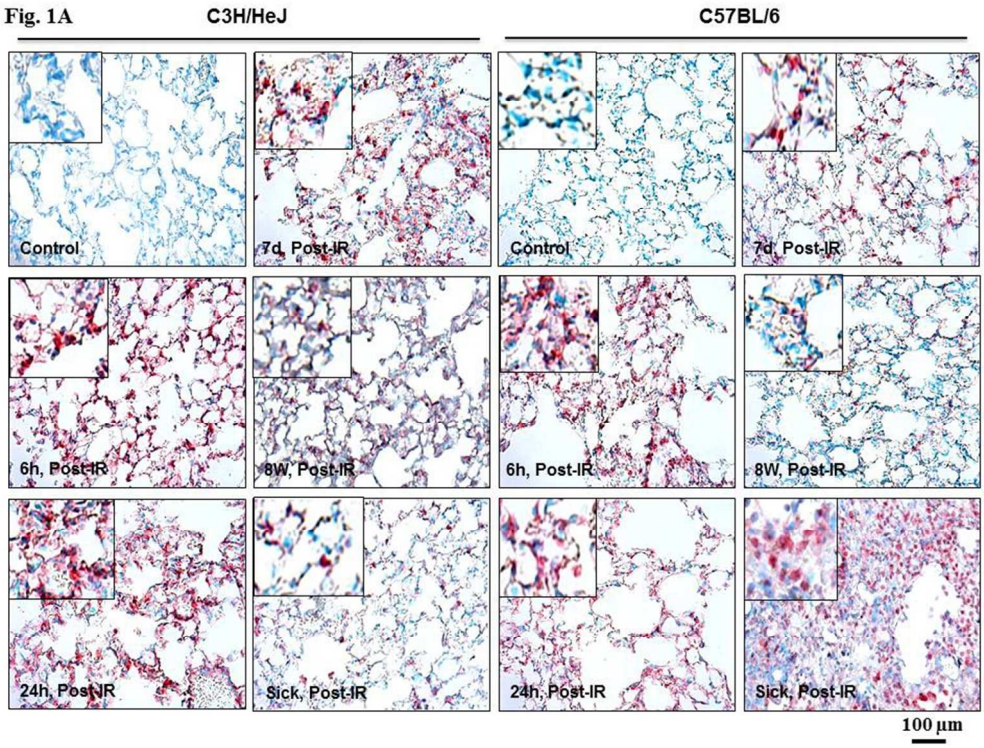
Figure 1. Representative sections showing the γH2AX foci positive nuclei in lungs of (A) C3H/HeJ and C57BL/6 (B) KK/HIJ and AKR/J (C) 129 S1/SvlmJ and A/J and (D) CBA/J and NZW/LacJ inbred strains, following whole thorax radiation exposure. Lung tissue was immuno-stained with anti-γH2AX antibody from tissue procured at 6h, 24h, 7d, 8w and upon presentation of respiratory distress ("sick") of mice following exposure to 18 Gy; 200X magnification. Inset shows γH2AX foci positive nuclei in the representative lung sections at magnification of 400X.

Figure 2. Radiation-induced numbers of γH2AX foci in the lungs is strain dependent. Lung sections as in figure 1. Counts of γH2AX foci positive nuclei from five microscopic fields/section/mouse scored at 200X magnification, normalized to area of lung; mean ± S.E.M (n = 3-6). "Distress" indicates samples procured from mice in respiratory distress. Line above the groups indicates significance level by one way ANOVA.

Figure 3. Correlation of pulmonary γH2AX positive nuclei with lung disease phenotypes. (A) Scatter plot showing the regression of asymptomatic survival time on the numbers of γH2AX foci positive nuclei in lung at 8w post irradiation. (B) Scatter plot showing the regression of fibrosis score on the numbers of γH2AX foci positive nuclei in lung at the point of respiratory distress post irradiation. r is Pearson correlation coefficient, P value indicates significance of

correlation by Pearson's rank correlation test. Data are expressed as the average for a strain ± std error of the mean.

Figure 4. Effect of the post irradiation treatment with DF2156A on the numbers of γH2AX foci positive nuclei in lungs of KK/HIJ mice. (A) Representative lung tissue sections from KK/HIJ mice exposed to radiation only and irradiated and DF2156A treated; each euthanized due to respiratory distress, and sections immunostained with anti-γH2AX antibody; 200X magnification. (B) Counts of γH2AX foci positive nuclei from five microscopic fields/section/mouse scored at 200X magnification; normalized to area of lung; mean ± S.E.M (n = 6). Line above the groups indicates significance level by two-tailed Student's t-test.



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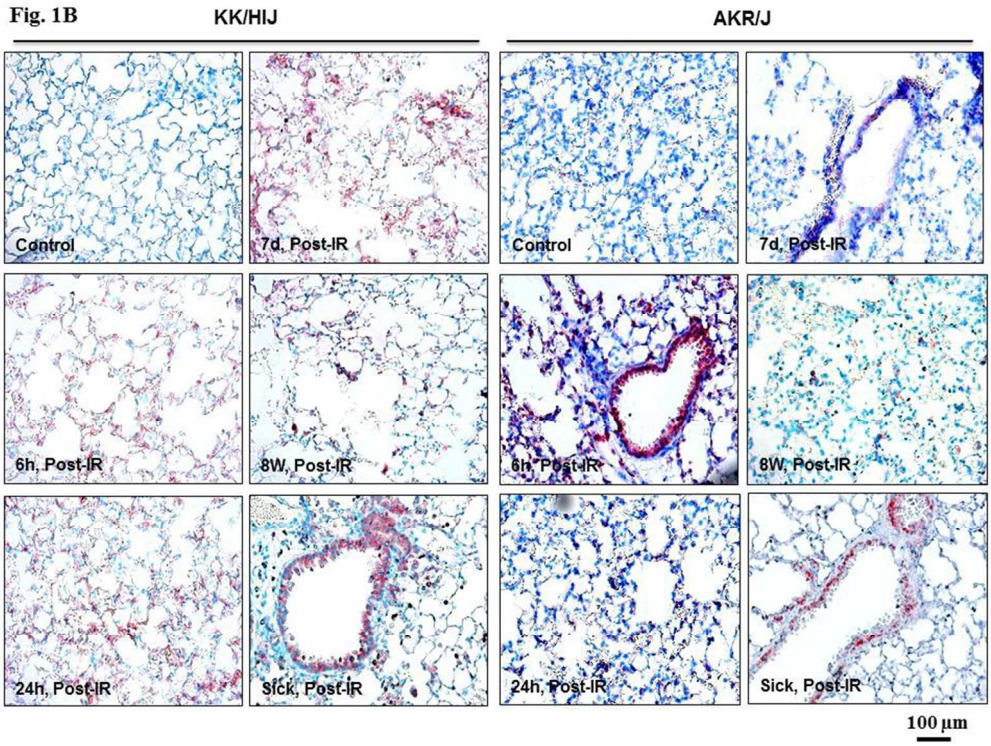

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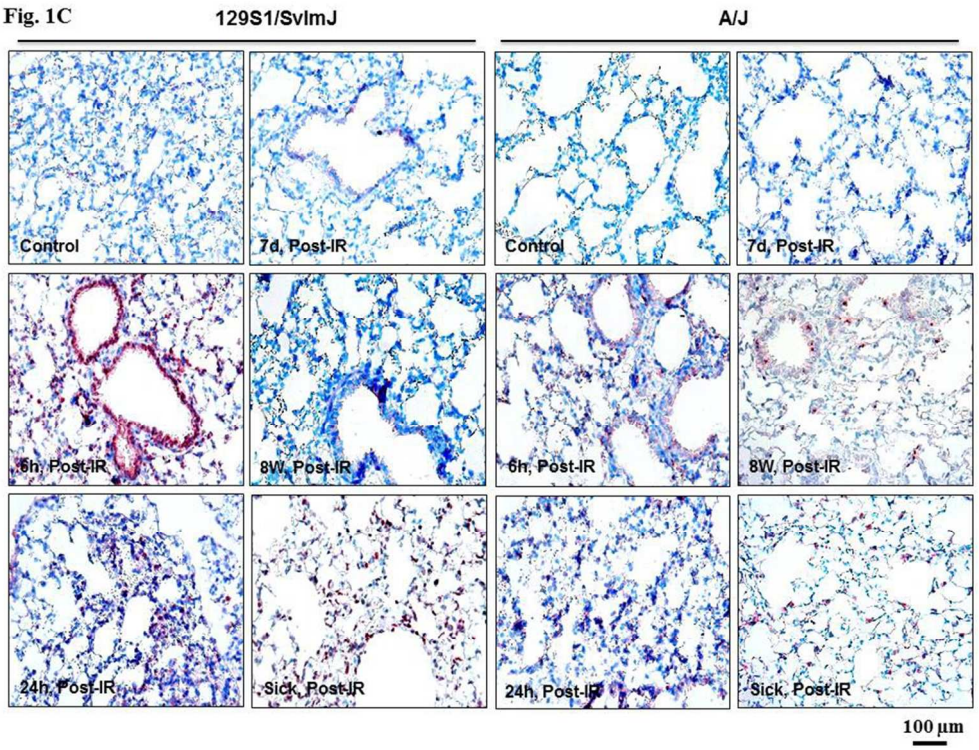

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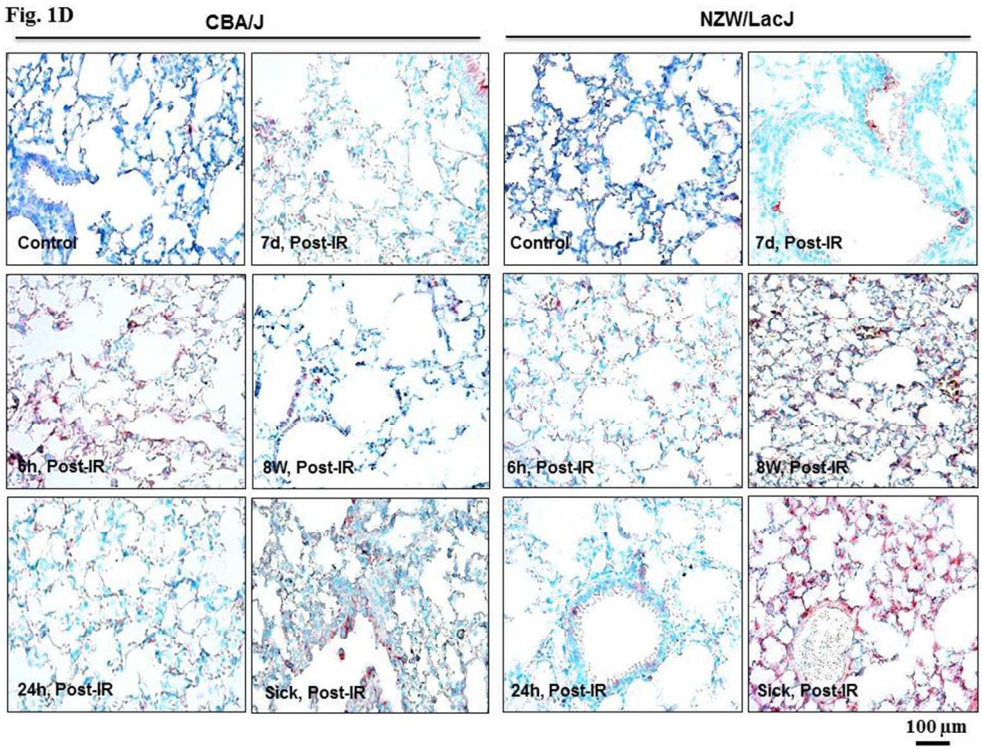

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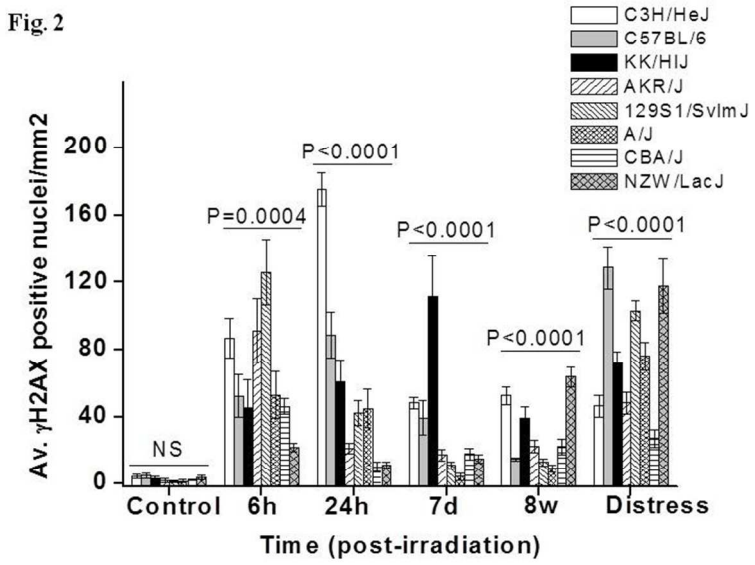

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Fig. 3

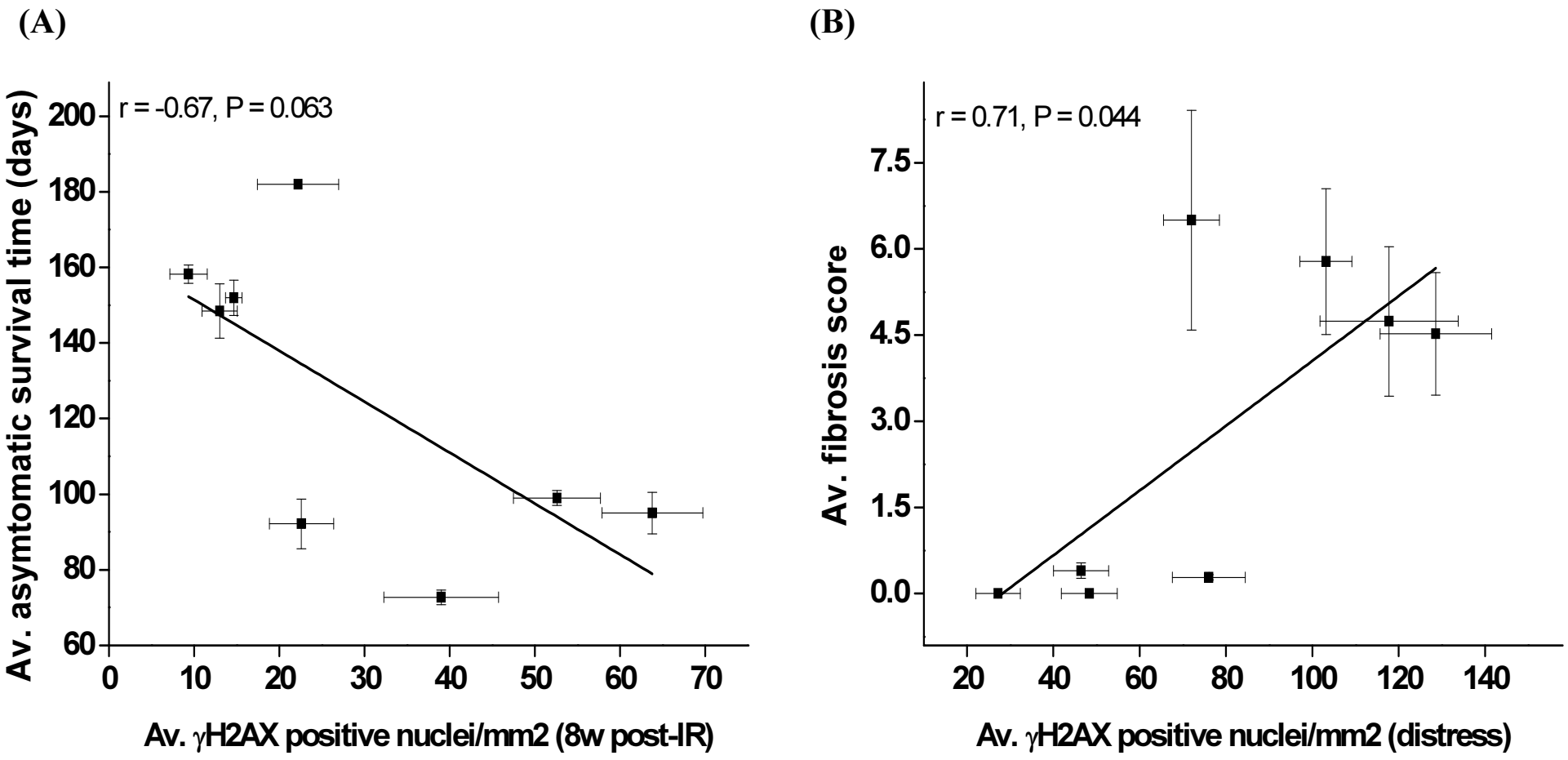
(A)
r = -0.67, P = 0.063
Av. asymtomatic survival time (days)
200
180
160
140
120
100
80
60
0
10
20
30
40
50
60
70
Av. γH2AX positive nuclei/mm2 (8w post-IR)
(B)
r = 0.71, P = 0.044
Av. fibrosis score
7.5
6.0
4.5
3.0
1.5
0.0
20
40
60
80
100
120
140
Av. γH2AX positive nuclei/mm2 (distress)

Fig. 4

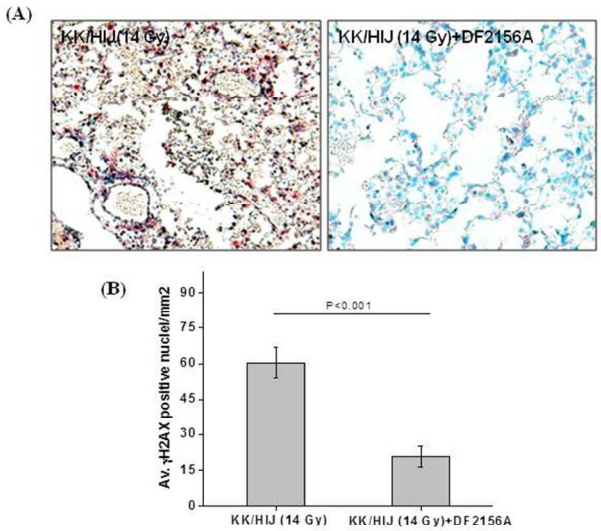

Figure 4. Effect of the post irradiation treatment with DF2156A on the numbers of γH2AX foci positive nuclei in lungs of KK/HIJ mice. (A) Representative lung tissue sections from KK/HIJ mice exposed to radiation only and irradiated and DF2156A treated; each euthanized due to respiratory distress, and sections immunostained with anti-γH2AX antibody; 200X magnification. (B) Counts of γH2AX foci positive nuclei from five microscopic fields/section/mouse scored at 200X magnification; normalized to area of lung; mean ± S.E.M (n = 6). Line above the groups indicates significance level by two-tailed Student's t-test.
254x190mm (96 x 96 DPI)