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NKT Deficient Mice are not Spared Lung Disease after Exposure to Thoracic Radiotherapy

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The specific pathways through which radiation produces the lung injuries of pneumonitis (alveolitis) and fibrosis are unknown but may involve an altered immune response. In this study, we investigated the hypothesis that the radiation-induced lung phenotype of *Ja18^{-/-}* mice [which lack invariant natural killer T (iNKT) cells] is altered relative to that of C57BL/6J genetic background strain. After 18 Gy whole-thorax irradiation male C57BL/6J mice succumbed to respiratory distress at 28–30 weeks postirradiation and although confirmed by flow cytometric analysis to be deficient in iNKT cells, the postirradiation survival of *Ja18^{-/-}* mice was not significantly different from that of C57BL/6J mice ($P = 0.87$). Histologically, the lungs of both C57BL/6J and *Ja18^{-/-}* mice developed fibrosing alveolitis over a similar time course with the same severity ($P = 0.15$). Analysis of the bronchoalveolar lavage revealed that the C57BL/6J mice and female *Ja18^{-/-}* mice succumbed to respiratory distress with neutrophil numbers exceeding those of the *Ja18^{-/-}* male mice and untreated control mice. In conclusion, the radiation-induced lung disease of *Ja18^{-/-}* mice did not significantly differ from that of C57BL/6J mice. © 2014 by Radiation Research Society

INTRODUCTION

Thoracic radiotherapy, a common treatment modality for thoracic cancers, has pulmonary side effects of either excessive inflammation (alveolitis) or pulmonary fibrosis, which can impair treatment effectiveness. Fibrosis consists of an unresolved inflammatory response to the initial radiation insult followed by excessive collagen deposition, leading to impaired pulmonary function, however the interactions between the various cellular and molecular players in this response are not completely understood. The

tissue reacts to the initial radiation insult by producing pro-inflammatory cytokines (1–3), which can perpetuate the infiltration and activation of innate and adaptive immune cells (4) whose presence may contribute to the radiation injury. Several reports of increased lymphocyte numbers in the bronchoalveolar lavage (BAL) of patients after thoracic radiotherapy (5–7) and of the protective effect of thymectomy prior to whole-body irradiation (8) emphasize the relevance of lymphocyte infiltration, specifically to the radiation response.

Natural killer T (NKT) cells are a lymphocyte subset with both T and natural killer (NK) cell markers, capable of rapidly producing Th1-, Th2- and Th17-type cytokines upon stimulation (9, 10). Based on their T cell receptor variability and antigen recognition, NKT cells are divided into three subtypes. Type I (invariant) NKT cells have an invariant T cell receptor – V α 14J α 18 in mice and recognize glycolipid antigens (α -GalCer) presented by the MHC-like molecule, CD1d. Type II NKT cells have a variable T cell receptor and are Cd1d-restricted, whereas type III NKT cells are both Cd1d-independent and possess diverse T cell receptors (11). Whether the specific subtypes of NKT cells exhibit distinct functions is not yet known (12).

NKT cells, through their cytokine secretions, have been established to effect the development of different pulmonary pathologies. For example, in animal models NKT cells were shown to contribute to allergen-induced airway hyperreactivity (13, 14) but to protect against bronchopneumonia following influenza challenge (15). In a model of pulmonary fibrosis induced by bleomycin, NKT cells were identified as protective (16) and treatment with the NKT antigen, α -GalCer, was found to attenuate the extent of fibrosis (17).

An NKT contribution to radiation-induced lung disease has not been reported, but Morris *et al.* have shown that total-body irradiation, used to condition mice for bone marrow transplant, resulted in NKT cell activation and subsequent complications of graft versus host disease in a murine model (18). Herein we investigated the response of *Ja18^{-/-}* mice (which exclusively lack type I NKT cells (19)) to thoracic radiation therapy. We compared the response of these mice to that of the background strain, C57BL/6J an inbred strain which we (20–22) and others (23, 24) have

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shown succumb to fibrosing alveolitis after thoracic irradiation. Although C57BL/6J mice are commonly used in pulmonary response studies, one group (25) suggests the response of this strain may have limitations.

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the Meakins-Christie Laboratories. Mice of the *Ja18*^{-/-} strain [C57BL/6J background (19)] were obtained through a material transfer agreement with Dr. Taniguchi of RIKEN Research Japan, and experimental mice were bred from these in-house. All mice were handled according to guidelines and regulations of the Canadian Council on Animal Care.

Thoracic Irradiation and Experimental Groups

At the age of 8–10 weeks, mice were exposed to a single dose of 18 Gy to the thorax using a Gamma Cell Cesium-137 source as previously described (22, 26). Mice were weighed weekly beginning 8 weeks after irradiation. Mice were euthanized when they showed signs of distress (ruffled fur, accelerated breathing, hunched posture, weight loss >15% of body weight). For these studies, groups of C57BL/6J and *Ja18*^{-/-} mice were euthanized not only when they showed signs of distress but also at specific time points (16, 20, 26 and 35 weeks) postirradiation. Control mice were not irradiated and were euthanized at matching time points.

Lung Histopathology and Bronchoalveolar Lavage Fluid Analysis

Bronchoalveolar lavage collection was performed by cannulating the trachea, and then injecting and removing 1 mL phosphate-buffered saline from the lungs one time. The lungs were then removed and the single left lobe was perfused with 10% buffered formalin and processed histologically. Lung sections of 5 μ m were cut and stained with Masson's trichrome and the fibrosis score was calculated as the lung surface covered by fibrosis relative to the total lung surface using Image-Pro Plus software (Media Cybernetics, Rockville, MD) (20, 21). To determine the degree of alveolitis, lung sections were stained with hematoxylin and eosin and evaluated semi-quantitatively through subjective scoring by an investigator blinded to strain and treatment. Alveolitis was indicated by extent of airspace with cellular influx and thickening of alveolar walls. A score of 0–6 was given, 0 being no alveolitis and 6 being severe alveolitis, as in previous studies (21, 22), based on the amount of lung tissue involved, without consideration to the extent of fibrosis. The BAL fluid was centrifuged (300g for 10 min at 4°C) and the cellular pellet was re-suspended in 125 μ L PBS. Inflammatory cell counts were performed at 400 $\times$ magnification on centrifuged cells (214.2g for 3 min) after staining with hematoxylin and eosin (Hema-3 Stain Set) and are reported as percentage of 500 counted cells.

Lymphocyte Profiling

At necropsy all lobes of left and right lungs were cut into small pieces and placed in PBS containing 1 mg/mL collagenase (Roche, Indianapolis, IN) and 1 mg/mL DNase (Roche) at 37°C for 45 min. The tissue was further disrupted using a Cell Dissociation Kit (Sigma-Aldrich, St. Louis, MO) and the total number of cells retrieved was determined using a Hemacytometer. Following a blockade of Fc receptors with Fc block (BD Biosciences, San Jose, CA), iNKT cells were identified by staining with CD3 and either PBS57-loaded Cd1d tetramer or NK1.1 antibodies and corresponding isotype controls. Cells were acquired using the FACSCalibur cytometer and the lymphocyte population was identified based on size and granularity on

a forward scatter/side scatter plot. The analysis of cell counts was completed using FlowJo software (<http://www.flowjo.com/>).

Data Analysis

Differences in survival between *Ja18*^{-/-} and inbred C57BL/6J mice were assessed with the log-rank test using GraphPad Prism software (<http://www.graphpad.com/>). Phenotypic differences between groups were evaluated using unpaired *t* tests ($\alpha = 0.05$), which were performed using R (<http://cran.r-project.org/>) or Microsoft Excel software.

RESULTS

Survival and Pulmonary iNKT Levels of *Ja18*^{-/-} Mice

To investigate whether a deficiency in invariant NKT cells affects the development of radiation-induced lung disease, *Ja18*^{-/-} mice and C57BL/6J mice were irradiated with 18 Gy and their survival to the onset of respiratory distress or to 35 weeks postirradiation, was recorded. As shown in Fig. 1, the majority of female *Ja18*^{-/-} and C57BL/6J mice succumbed to disease at 22–26 weeks postirradiation which is similar to our published results of survival of identically treated female C57BL/6J mice (20–22, 27). Male *Ja18*^{-/-} and C57BL/6J mice developed respiratory distress past the 26 week time point, and 10% of the male animals of each strain survived to the experimental end point of 35 weeks. Postirradiation survival did not depend on genotype in male ($P = 0.87$) or female ($P = 0.79$) mice.

To determine whether irradiation affected iNKT cell counts, we measured the numbers of NK1.1+CD3+ and PBS57-loaded CD1d tetramer+CD3+ cells in lungs from eight C57BL/6J and eight *Ja18*^{-/-} mice euthanized due to respiratory distress. Shown in Fig. 2 are scatterplots of NKT cells among 15,000 lymphocytes in lung tissue from *Ja18*^{-/-} and C57BL/6J mice. The wild-type strain had more NK1.1+CD3+ (98.6 ± 10.2 cells representing $0.66 \pm 0.07\%$ of total lymphocytes) and PBS57-loaded CD1d tetramer+CD3+ (169.6 ± 15.1 cells representing $1.13 \pm 0.10\%$ of total lymphocytes) cells on average compared to *Ja18*^{-/-} mice (7.0 ± 1.3 total NK1.1+CD3+, representing $0.047 \pm 0.009\%$ of lymphocytes; $P = 0.00001$ and 12.3 ± 3.0 total PBS57-loaded CD1d tetramer+CD3+ cells, representing $0.082 \pm 0.02\%$ of lymphocytes, $P = 10^{-5}$), confirming that the latter are indeed deficient in iNKT cells.

Lung Disease Phenotypes of *Ja18*^{-/-} Mice

All mice presenting respiratory distress developed histological evidence of alveolitis and pulmonary fibrosis (Fig. 3), and the extent of these phenotypes did not differ among C57BL/6J and *Ja18*^{-/-} male and female animals (ANOVA $P = 0.67$ for alveolitis and 0.15 for fibrosis).

To monitor the development of lung disease, *Ja18*^{-/-} and C57BL/6J mice were exposed to 18 Gy whole-thorax irradiation and their lung responses assayed in mice surviving to 16, 20, 26 and 35 weeks post-treatment. As

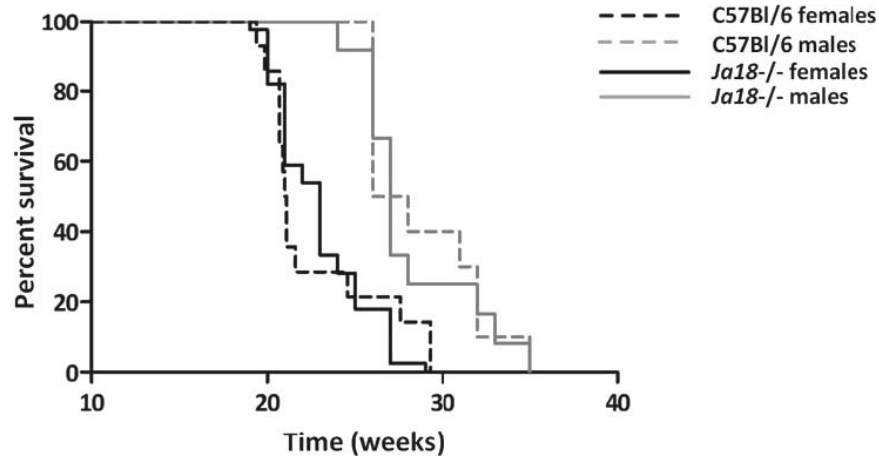


FIG. 1. Post-thoracic irradiation survival of *Ja18^{-/-}* and C57BL/6J mice. After a single dose of 18 Gy radiation to the thorax, C57BL/6J WT and *Ja18^{-/-}* mice were euthanized when in respiratory distress or at 35 weeks postirradiation which was the end of experiment. $n = 10\text{--}12$ mice per strain.

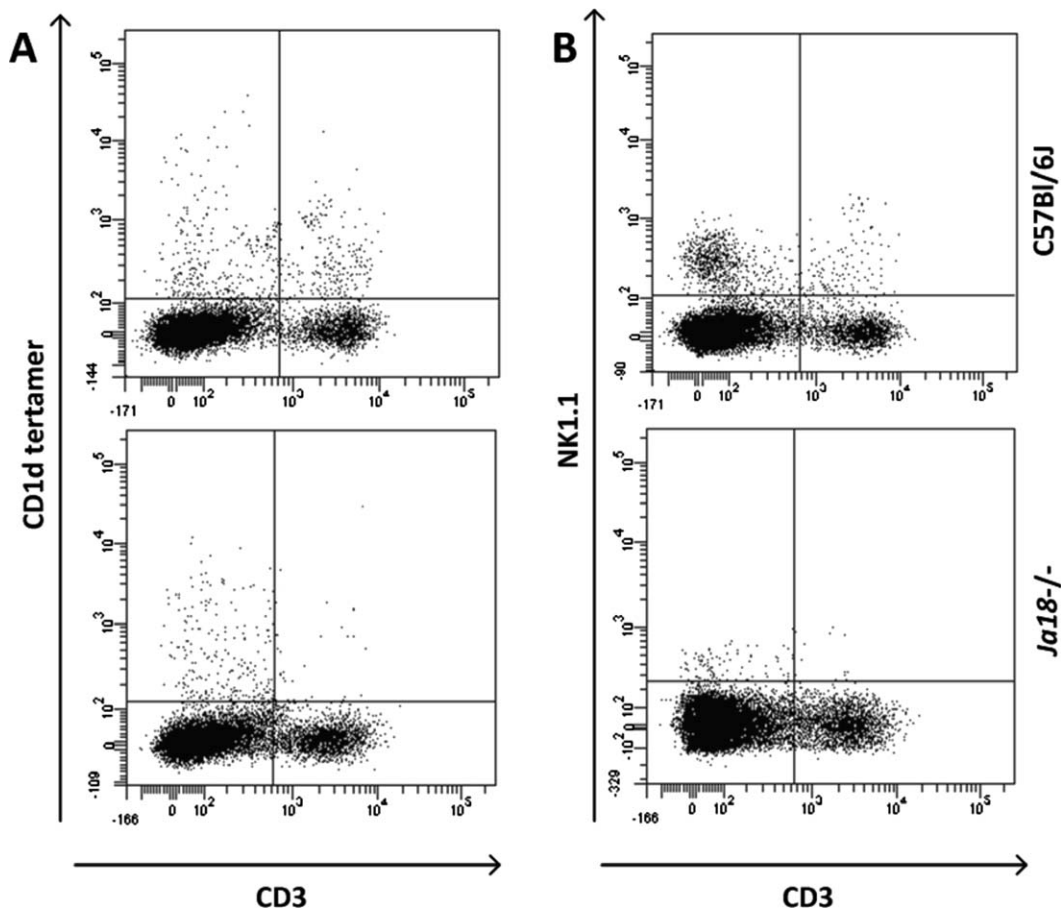


FIG. 2. Representative scatter plots of pulmonary tissue from *Ja18^{-/-}* and C57BL/6J mice showing invariant NKT deficiency. Lung tissue was procured from irradiated *Ja18^{-/-}* and C57BL/6J mice and flow cytometry was performed on isolated cells stained with the following combinations of antibodies: CD3 and Cd1d tetramer loaded with PBS-57 (panel A); and CD3 and NK1.1 (panel B). Scatter plots are representative of 15,000 lymphocytes in *Ja18^{-/-}* and C57BL/6J mice.

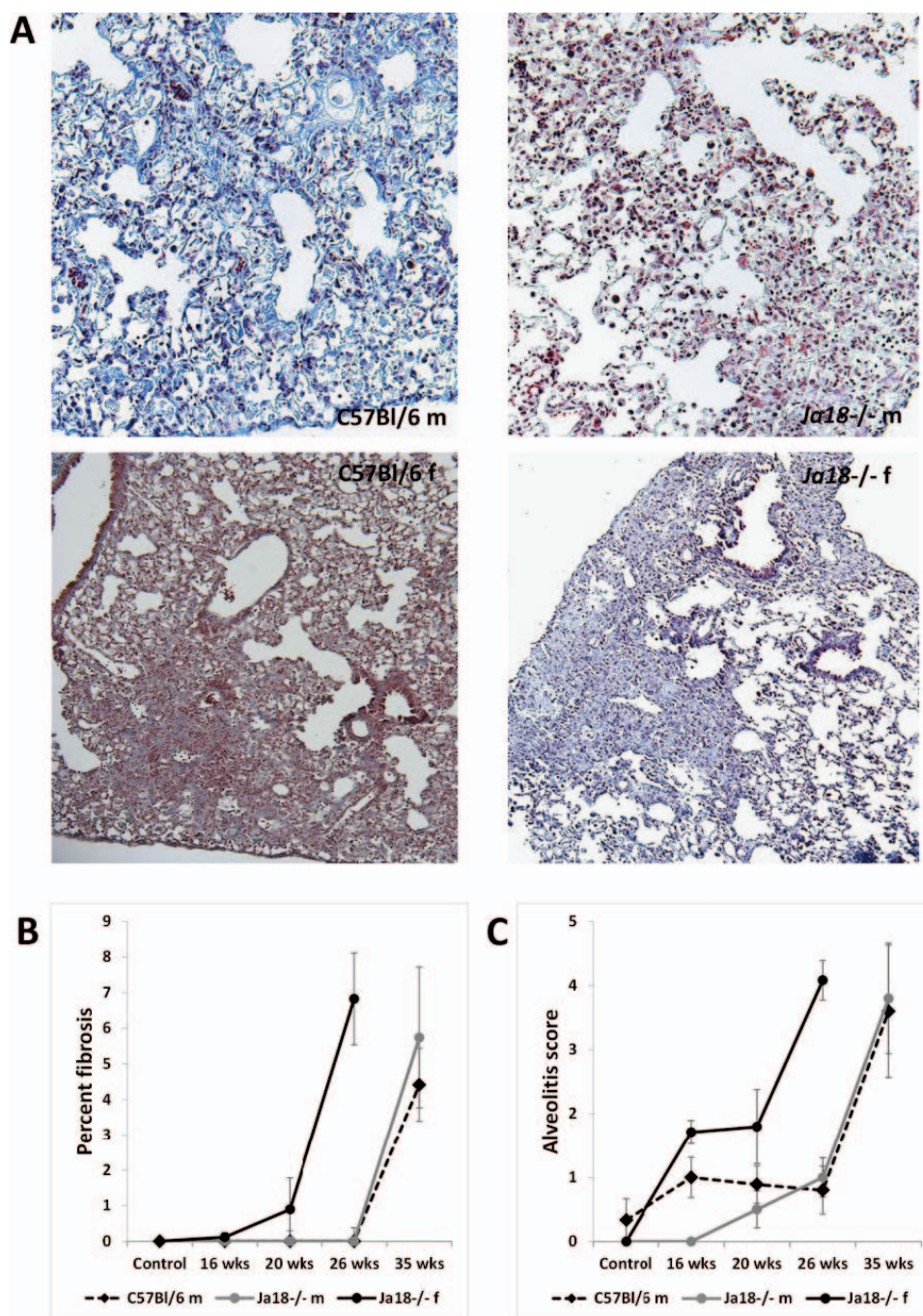


FIG. 3. Radiation-induced lung phenotype development in *Ja18^{-/-}* and C57BL/6J mice. After a single dose of 18 Gy radiation to the thorax, surviving C57BL/6J WT and *Ja18^{-/-}* mice were euthanized at 16, 20, 26 or 35 weeks postirradiation. Panel A: Images of Masson's trichrome-stained lung sections from strains, euthanized when in distress (22 weeks postirradiation for females, 32 weeks for males), indicating fibrosis responses to whole-thorax irradiation, 200 $\times$ magnification. Panel B: Percentage of fibrotic lung tissue in trichrome-stained histological sections. Control values are from nonirradiated mice. Panel C: Alveolitis scores derived from semi-quantitative evaluation of histological sections. Phenotypes are presented as mean $\pm$ SE for groups of 4–8 mice.

shown in Fig. 3, the onset of both alveolitis and fibrosis was later in male mice compared to female mice, in agreement with the survival data. There was no difference in degree of alveolitis or fibrosis between *Ja18^{-/-}* and C57BL/6J males after irradiation at any time point.

Lavage Phenotype of *Ja18^{-/-}* Mice

Bronchoalveolar lavage cell differentials were obtained from mice euthanized in the time course experiment to assess the contribution of inflammatory cells to the development of lung disease. Thoracic irradiation resulted

TABLE 1
Total Bronchoalveolar Lavage Cell Count ($\times 10^4/\text{ml}$) in C57BL/6 and iNKT-Deficient Mice

	C57BL/6J males	<i>Jal8</i> ^{-/-} males	<i>Jal8</i> ^{-/-} females
Control	3.48 $\pm$ 1.03	5.65 $\pm$ 1.02	3.70 $\pm$ 0.44
16 weeks	3.81 $\pm$ 1.00	7.01 $\pm$ 1.10	7.61 $\pm$ 0.11
20 weeks	7.81 $\pm$ 2.07	10.00 $\pm$ 3.12	41.08 $\pm$ 9.30
26 weeks	10.60 $\pm$ 5.62	17.20 $\pm$ 3.96	40.94 $\pm$ 5.40
35 weeks	21.10 $\pm$ 4.07	24.90 $\pm$ 3.89	

Note. Results are shown as mean $\pm$ SEM for groups of 4–8 mice.

in 6–10 fold increases in the number of cells infiltrating the lungs in all mice succumbing to distress (Table 1), with a significant increase compared to control starting at 16 and 20 weeks post-treatment in *Jal8*^{-/-} and C57BL/6J mice, respectively. We observed no significant differences in cell counts between C57BL/6J and *Jal8*^{-/-} male mice at any of the time points ($P > 0.08$) whereas *Jal8*^{-/-} female mice had more cells in their BAL compared to males at 20 and 26 weeks post-treatment ($P < 0.03$).

As shown in Fig. 4 the numbers of polymorphonuclear neutrophil (PMN) cells in BAL samples peaked at the distress time point in all strains ($P < 0.03$ compared to controls) although the increase was minimal in *Jal8*^{-/-} males. Higher PMN numbers were measured in both distressed BL/

6J male and *Jal8*^{-/-} female mice compared to male *Jal8*^{-/-} mice ($P < 0.05$). Numbers of lavage macrophages were also increased in all strains compared to controls, with greatest values in *Jal8*^{-/-} females while macrophage numbers did not differ between *Jal8*^{-/-} and BL/6J males throughout the time course experiment ($P > 0.08$). Lymphocyte counts were increased in female *Jal8*^{-/-} mice, peaking at 26 weeks ($P = 0.006$ compared to controls) at which time they exceeded the numbers in *Jal8*^{-/-} males ($P = 0.007$).

DISCUSSION

Through an assessment of *Jal8*^{-/-} mice we demonstrate that radiation-induced pulmonary fibrosis is not dependent

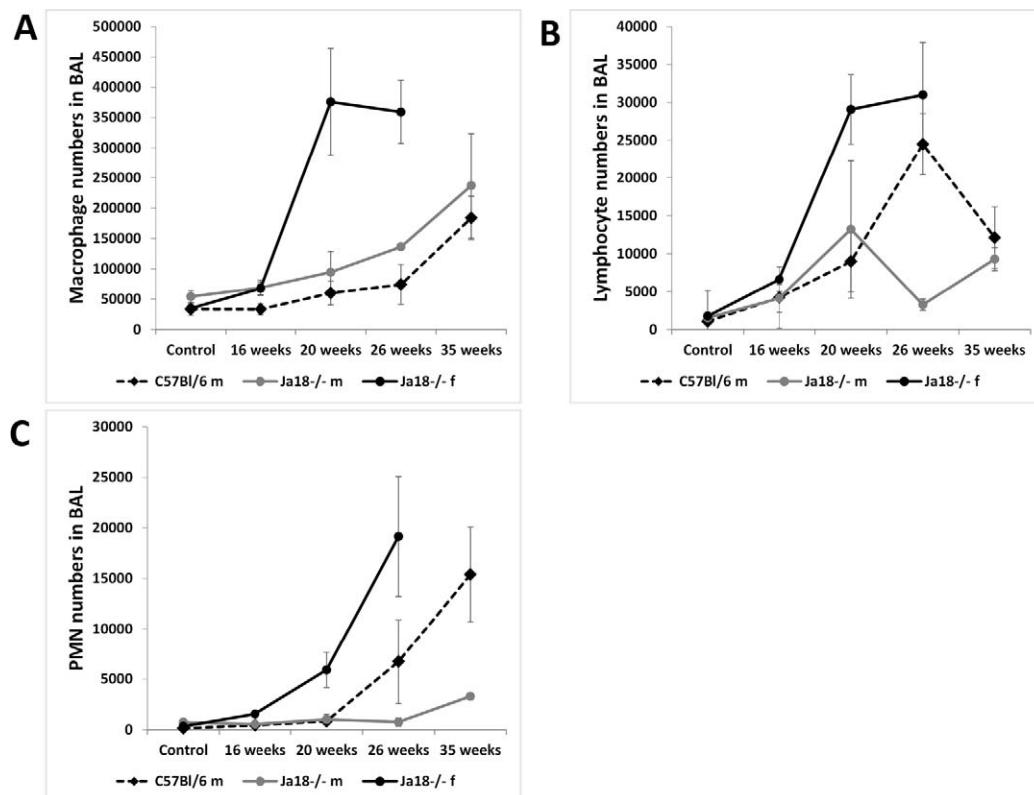


FIG. 4. Postirradiation bronchoalveolar lavage cell numbers of *Jal8*^{-/-} and C57BL/6J mice. After a single dose of 18 Gy radiation to the thorax, surviving C57BL/6J WT and *Jal8*^{-/-} mice were euthanized at 16, 20, 26 or 35 weeks postirradiation. Bronchoalveolar lavage samples were collected at necropsy and cells were morphologically identified from cytospin preparations. Results are shown as mean $\pm$ SE for groups of 4–8 mice. PMN = polymorphonuclear cell.

on the presence of iNKT cells. The wild-type and NKT deficient mice studied developed similar degrees of alveolitis and fibrosis and did not differ in their survival after 18 Gy irradiation to the thorax.

An imbalance between pro- and anti-fibrotic mediators contributes to the development of pulmonary fibrosis after various stimuli including radiation and bleomycin (28, 29) and given the wide spectrum of cytokines produced by iNKT cells (9, 10), they were hypothesized herein to be a candidate cell type associated with fibrosis. That their reduced numbers, however, did not affect the onset or severity of radiation-induced lung disease in C57BL/6J mice suggests there to be other cellular cytokine sources important to the development of this trait. For example, NKT cells can be induced to produce the pro-fibrotic cytokine interleukin-17 (10), but additional cellular sources of this cytokine, which have also been implicated in fibrosis development, include $\gamma\delta$ T cells and T helper cells (29, 30). Further, the potential involvement of Cd1d-dependent type II or type III NKT cells was not evaluated here and may also affect radiation-induced lung disease.

The contribution of NKT cells to radiation-induced lung disease may be influenced, in part, by the latency over which this pathology develops. Specifically, in the acute response model of fibrosis induced by bleomycin within weeks of treatment, Kim *et al.* (16) reported that both NKT-deficient *Cd1d*^{-/-} and *Jal8*^{-/-} mice developed increased levels of pulmonary fibrosis and decreased survival post-treatment compared to C57BL/6J mice, and that these results were due to the absence of anti-fibrotic interferon- γ produced by NKT cells. Thus a fibrotic response in excess of that of C57BL/6J mice required an early mitigating effect of NKT cells whereas postirradiation, any mitigating effect of NKT cells was not evident on fibrosis developing over six months. Supporting a putative latency effect is the fact that although male mice of both strains developed similar radiation-induced disease, when the animals were in respiratory distress we found increased PMN numbers in the lungs of C57BL/6J mice but not *Jal8*^{-/-} mice, which suggests the existence of alternative pathways to fibrosis in the male mice. Given that neutrophil chemotaxis is dictated by the cytokines present in the lung, it is possible that the lower PMN numbers observed in *Jal8*^{-/-} males are due to a different cytokine environment compared to C57BL/6J mice. For example NKT cell products IL13 and IL4 (13) can reduce neutrophilia (31), possibly by inhibiting production of neutrophil chemoattractant, IL17 (32), but are both potent pro-fibrotic mediators (33, 34). Thus, a higher concentration of these cytokines in *Jal8*^{-/-} mice could trigger a fibrotic response in the absence of neutrophilia.

In summary, our studies with *Jal8*^{-/-} mice reveal that a deficiency in iNKT cells does not alter the survival time or the degree of histological disease of C57BL/6 mice exposure to radiation, suggesting that these cells are not essential for the development of radiation-induced pulmonary fibrosis in this strain.

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