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# Genetic Deficiency in Complement Component 4b Does Not Alter Radiation-Induced Lung Disease in Mice

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Previous investigations have shown altered levels of complement components to be associated with radiation-induced lung disease. In this study we aimed to determine whether a deficiency in complement component 4b alters the lung response to irradiation of C57BL/6 mice. The pulmonary phenotype of C57BL/6 *C4b*<sup>-/-</sup> mice and their wild-type littermates was assessed following an 18 Gy single dose to the thoracic cavity. The assessed end points included, survival time postirradiation, bronchoalveolar lavage cell differential, hydroxyproline measures and histological evidence of alveolitis and fibrosis. The lung phenotype of *C4b*-deficient mice did not differ from that of wild-type mice in terms of survival time postirradiation, tissue hydroxyproline levels or by histological evidence of alveolitis or fibrosis. No differences in bronchoalveolar cell differential counts were evident among the irradiated mice grouped by *C4b* genotype. We concluded that a deficiency in *C4b* does not alter radiation-induced lung disease in the C57BL/6 mouse model. © 2013 by

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complement in the pulmonary phenotype includes a proteomic analysis that reveals complement component C3 and C4b binding protein alpha chain and vitronectin of 76 proteins analyzed to be more highly expressed in the plasma of lung cancer patients with radiation-induced lung toxicity grade  $\geq 2$  compared to those without (4). Studies in animal models that exhibit radiation-induced alveolitis and fibrosis phenotypes with similar temporal and histological patterns to the clinical response (1, 2) also suggest a complement contribution to lung disease. Specifically, we (5–7) and others (8, 9) have reported that C57BL/6J mice respond to high-dose whole-thorax irradiation by developing alveolitis and atelectatic regions of fibrosis at approximately 6 months after radiation treatment. We also defined the pulmonary response to 18 Gy whole-thorax irradiation, at the expression level in mice of three strains, A/J, C3H/HeJ (alveolitis response) and C57BL/6J [fibrosis response, ref. (10)] and identified the pathway of complement signaling to be significantly represented only in the fibrosis responding strain.

In this study, we exposed *C4b* knockout mice (11) to whole-thorax irradiation to determine whether a deficiency in this complement component altered the development of radiation-induced lung disease.

## INTRODUCTION

Thoracic cavity radiotherapy can produce serious inflammatory (alveolitis) or fibrotic (fibrosing alveolitis) side effects in the lung (1, 2). Pulmonary fibrosis is characterized by cellular proliferation and progressive accumulation of extracellular constituents, which result in remodeling of the lung interstitium (3). Alveolitis, or pneumonitis, is an inflammatory response associated with cellular infiltration of the airspace and thickening of the alveolar walls (3).

The exact mechanisms, which lead to both alveolitis and fibrosis, are as yet unknown, but a component of the innate immune system, complement, may be involved in the lung response to thoracic irradiation. Clinical implication of

## MATERIALS AND METHODS

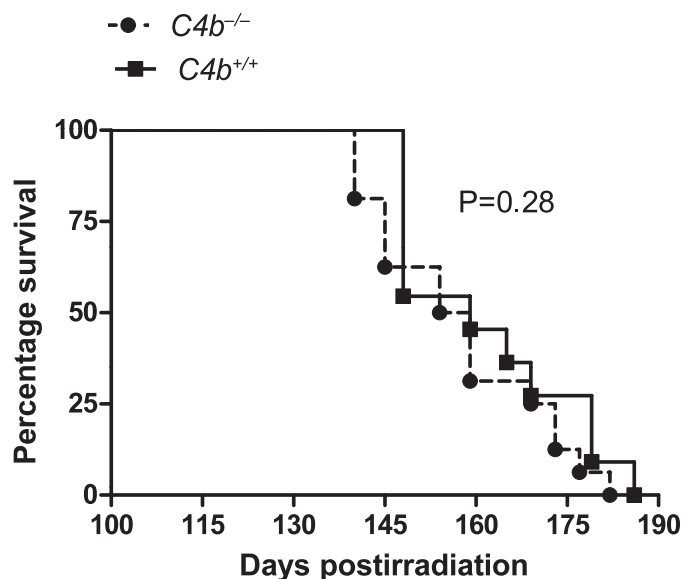
### *Mice*

C57BL/6-*C4b* knockout mice (strain name B6.129S4-*C4b*<sup>tm1Crr</sup>/J; genetic background C57BL/6J, Jackson Laboratory stock no. 003643) and wild-type littermates were obtained from the Jackson Laboratory and were bred and housed in the animal facility of the Meakins-Christie Laboratories. The mice were genotyped for *C4b* using the protocol available at [www.jax.org](http://www.jax.org). Lung tissue was procured from untreated mice and the expression of *C4b* was assessed by RT-PCR using Applied Biosystem's Assay-on-Demand™ Mm00437890-m1 and a previously described method (10). The expression of *C4b* in the lungs of C57BL/6-*C4b* knockout mice was 15% of the level measured in wild-type littermates,  $P < 0.002$ ;  $n = 8$  mice per group. All mice were handled according to guidelines and regulations of the Canadian Council on Animal Care, as approved by McGill University.

### *Radiation Treatment*

Female mice were treated at 8 weeks of age. Lung damage was elicited by whole-thorax radiation exposure using a GammaCell

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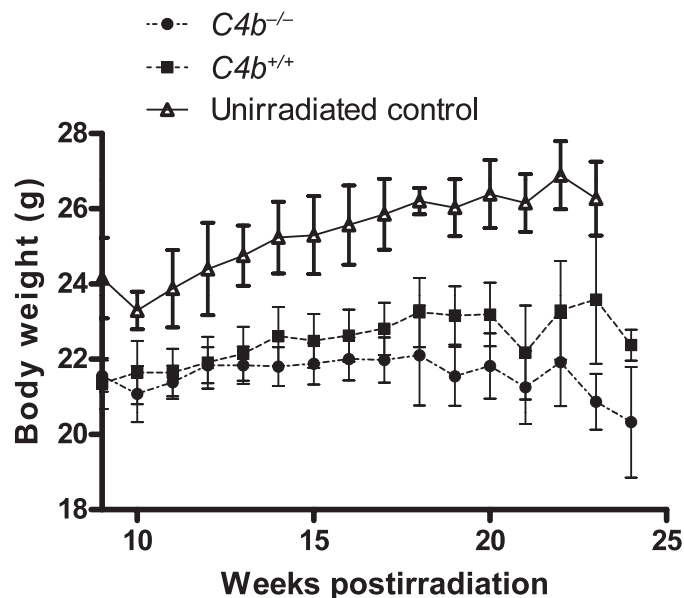


**FIG. 1.** Postirradiation survival time of wild-type and *C4b*-deficient mice. Mice ( $n = 11$ – $15$  per group) were exposed to a single dose of 18 Gy whole-thorax radiation and euthanized when in respiratory distress.

Cesium-137 unit, as previously described (5, 7). A dose of 18 Gy delivered at 0.6 Gy/min, was given to elicit a lung disease response in the majority of the animals based on the known response of the background strain (5–7, 10). The rest of the body was shielded with 3 cm of lead to reduce the beam strength to 3% in this area. The irradiated mice were humanely euthanized when moribund. For this assessment the mice were weighed weekly beginning nine weeks after irradiation; animals that lost >20% of their body weight within a period of 2 weeks and exhibited distress through ruffled fur, accelerated breathing and hunched posture were euthanized, as described previously (5). The survival time of a mouse was either the time at euthanization due to moribund symptoms, which were evident in the majority of mice, or the time at which the animal was found dead. Differences in survival between strains of mice grouped by *C4b* genotype were assessed by the log-rank test. The control mice were not treated and were sacrificed at the 15–24 week time points.

#### Histology

At necropsy, bronchoalveolar lavage was performed, as previously described (5, 7). The lungs were then removed and the single left lobe of each mouse was perfused with 10% neutral buffered formalin and submitted for histological processing. Lung sections were stained with Masson's trichrome, and the area of fibrosis in the left lung lobe was determined from a user drawn region (Image-Pro Plus Software) and compared to the area of the entire lobe to yield the percentage of pulmonary fibrosis for individual mice (12). To assess alveolitis, H&E stained left lung sections were evaluated through semi-quantitative histology (5). Alveolitis was scored subjectively on a scale of 0–6, with 0 representing no alveolitis and 6 representing extreme alveolitis characterized by excessive thickening of the alveolar walls with cellular infiltration and diffuse exudates throughout the alveolar spaces. One section per mouse was scored. All scoring was completed by a user blinded to mouse genotype and treatment. Differences in fibrosis phenotype between mice grouped by genotype were assessed with Student's *t* test, and a Mann-Whitney test was used to evaluate alveolitis differences.



**FIG. 2.** Postirradiation body weight of wild-type and *C4b*-deficient mice. Mice were exposed to a single dose of 18 Gy whole-thorax radiation and body weight was recorded beginning at week 9 after irradiation, mean ( $\pm$  standard error). There were no significant differences in body weight in irradiated mice grouped by *C4b* genotype,  $P > 0.16$ .

#### Hydroxyproline Assay

The hydroxyproline content of the right mouse lungs was determined using previously described standard methods (13–15).

#### Bronchoalveolar Lavage Fluid (BAL) Analysis

The BAL fluid was centrifuged (302g for 10 min at 4°C) and the supernatant was removed and stored at  $-85^{\circ}\text{C}$ . The cellular pellet was resuspended in 0.25 mL PBS. Inflammatory cell counts were performed (400 $\times$  magnification) on cytocentrifuged cells (214.2g for 3 min) after staining with a hematoxylin-eosin kit (Hema-3 Stain Set by Protocol), as previously described (5, 7).

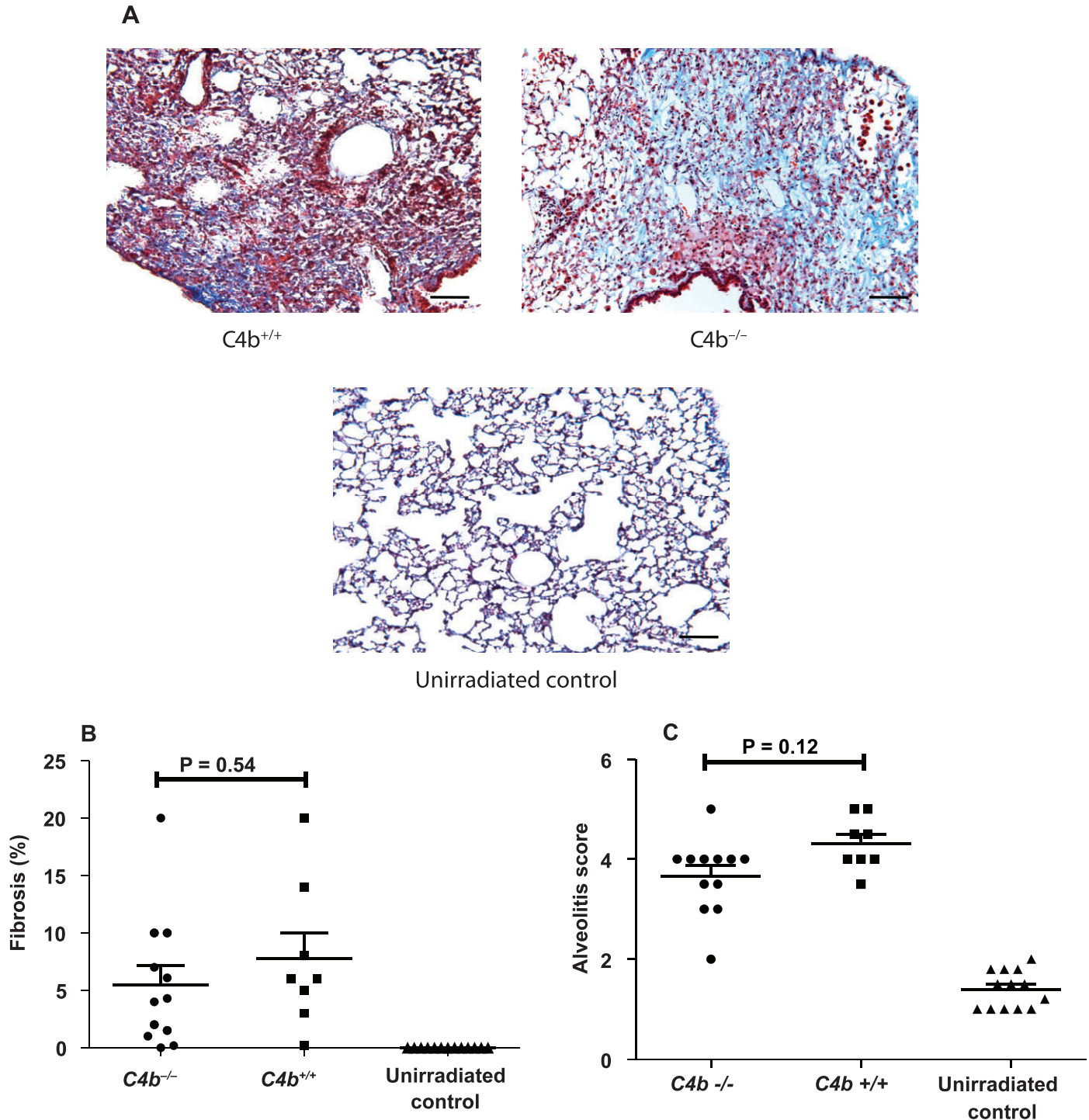
## RESULTS

#### Survival Time and Weight Change Phenotype

To determine whether mice deficient in *C4b* differ from wild-type littermates in their response to whole-lung irradiation, mice of each genotype were exposed to a dose of radiation of 18 Gy and sacrificed upon presentation of respiratory distress. As shown in Fig. 1, postirradiation survival time of *C4b*<sup>-/-</sup> mice did not differ from that of wild-type littermate mice ( $P = 0.28$ ). Further, the onset of distress was similar in the groups of mice, as indicated by postirradiation weight loss as shown in Fig. 2.

#### Histological and Biochemical Phenotype

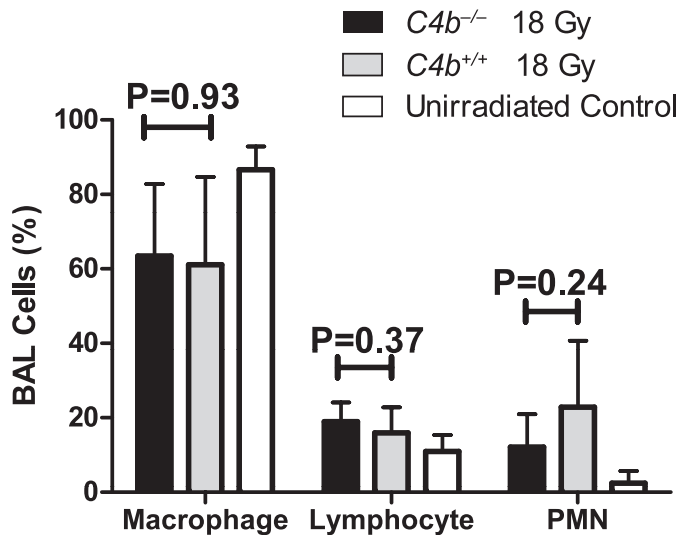
Histological evaluation of the lungs, which was completed at the times indicated in Fig. 1, revealed that the respiratory distress of the irradiated mice was due to



**FIG. 3.** Radiation-induced lung disease of wild-type and *C4b*-deficient mice. Mice of each group were exposed to 18 Gy whole-thorax radiation and euthanized when in respiratory distress. Panel A: Histological sections illustrate subpleural regions of fibrosing alveolitis (blue streaks indicate collagen deposition) in radiation treated, but not in control, mice. Masson's trichrome stain, 400 $\times$  magnification, bars = 100  $\mu$ m. Panel B: Fibrosis scores based on image analysis of histological sections. Panel C: Alveolitis scores derived from semi-quantitative evaluation of histological sections. The average  $\pm$  standard error of scores is indicated. The average fibrosis in untreated control mice was 0% and the average alveolitis score of 1.4 in untreated mice was not altered by *C4b* genotype.

fibrosing alveolitis (Fig. 3) and is in agreement with previous reports of the wild-type strain (5–10). Image analysis of histological sections demonstrated that *C4b*<sup>-/-</sup> mice also developed significant fibrosis after thoracic

irradiation, but was similar to the level observed in wild-type mice (Fig. 3B). As a second indicator of fibrosis, we measured the hydroxyproline levels in the lungs of untreated and irradiated mice. The tissue level of hydroxy-



**FIG. 4.** Bronchoalveolar lavage cell differential of wild-type and  $C4b$ -deficient mice following whole-thorax radiotherapy. Mice were treated with 18 Gy to the lung and sacrificed upon presentation of respiratory distress. Bronchoalveolar lavage samples were collected at necropsy and cells were morphologically identified from cytospin preparations. The average percentage of each cell type ( $\pm$ std) is presented. There were no significant differences in lavage cell differential in irradiated mice grouped by  $C4b$  genotype,  $P > 0.21$ .

proline did not differ between  $C4b^{-/-}$  and wild-type mice either after irradiation (263  $\mu\text{g}/\text{lung}$  vs. 259  $\mu\text{g}/\text{lung}$ ;  $P = 0.79$ ) or in the untreated control condition (195  $\mu\text{g}/\text{lung}$  vs. 193  $\mu\text{g}/\text{lung}$ ;  $P = 0.93$ ). Finally, the extent of alveolitis, assessed through semi-quantitative scoring of histological sections, did not differ among groups of irradiated mice segregated by genotype (Fig. 3C).

#### Inflammatory Cell Counts

To determine whether the inflammatory response to whole-thorax irradiation is altered by a  $C4b$  deficiency, we quantified the bronchoalveolar cell types. There were radiation-induced increases in total bronchoalveolar lavage cell counts (data not shown) in agreement with previous reports of the wild-type strain (5, 7), but as shown in Fig. 4, there was no difference in lavage cell differential by  $C4b$  genotype in radiation-treated mice. The lavage differential of unirradiated mice did not differ by  $C4b$  genotype ( $P > 0.25$ , data not shown).

## DISCUSSION

Complement signaling can influence innate and adaptive immune responses (16, 17), both of which have been implicated in the development of radiation-induced lung disease (18, 19). In this study, we demonstrate that a deficiency in complement component C4b does not accelerate the onset of respiratory distress nor the amount of lung disease in mice exposed to whole-thorax irradiation. Therefore, complement may not be a key constituent of the

radiation-induced lung injury in C57BL/6J mice, or the C4b component specifically is not involved.

Created by gene targeting and shown to lack C4 in the serum (11), this model of C4b deficiency has been studied for complement involvement in collagen-induced arthritis (20), B cell function (21) and the development of glomerulonephritis (22) among other traits. The response of these mice to thoracic irradiation had not been documented, but, as evaluated here using standard markers of radiation-induced lung disease (5, 23, 24), no differences in response from wild-type littermates were evident. Similar studies in genetically modified animals have been completed to investigate how particular variations affect the radiation response of the lung. As in the present study, the principal phenotypes evaluated have been time to onset of distress and the histological indication of alveolitis or fibrosis that contributed to the distress (18, 23, 25). In one investigation, Yang *et al.* (25) showed post-thoracic irradiation survival to be enhanced in cc chemokine ligand 3-deficient ( $Ccl3^{-/-}$ ) mice or in mice deficient for receptor  $Ccr1^{-/-}$ , relative to wild-type, while mice lacking chemokine (C-C motif) receptor 5 were not protected from radiation-induced injury and fibrosis. With these findings, the authors concluded that the selective interaction of Ccl3 with its receptor, Ccr1, and not with Ccr5, was critical for radiation-induced lung inflammation and fibrosis. Similarly, we reported that the lung phenotype of toll-like receptor-4 ( $Tlr4$ )-deficient and  $Tlr2$ -deficient mice did not differ from that of wild-type mice in terms of survival time postirradiation, or by histological evidence of alveolitis or fibrosis. However, we found that mice deficient in both receptors developed respiratory distress at an earlier time than did wild-type mice and presented an enhanced fibrotic response (18), indicating a deficiency in both receptors to produce an effect that was not evident in mice lacking either gene alone.

In summary, we evaluated the effect of  $C4b$  deficiency on radiation-induced lung disease in a mouse model and show that the deficiency does not significantly alter survival time nor the extent of histological disease evident in distressed mice. These results therefore indicate that radiation-induced lung disease is not affected by a  $C4b$  deficiency in C57BL/6J mice.

## ACKNOWLEDGMENTS

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