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Lung Iron Overload Does Not Exacerbate the Fibrotic Response to Bleomycin in a Mouse Model of Pulmonary Fibrosis

To the Editor:

Idiopathic pulmonary fibrosis (IPF) is the most common of the idiopathic interstitial pneumonias, with a prevalence of 10–60 cases per 100,000 in North America and Europe (1). With a dismal prognosis of 2–4 years median survival from diagnosis and its associated hospitalizations and healthcare costs, IPF carries a heavy cost for both the individual patient and for society (2).

One promising area of IPF research is the role of iron in the pathogenesis of pulmonary fibrosis. Multiple translational studies have shown increased amounts of iron in IPF lung, including in BAL fluid (3) and alveolar macrophages (4), and the association of iron concentrations with severity of disease and the development of associated pulmonary vascular disease (5). One study of patients with IPF found a significantly increased incidence of *HFE* (homeostatic iron regulator) allelic variants compared with normal control subjects, with increased amounts of iron-dependent reactive

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This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

oxygen species (ROS) in patients with *HFE* allelic variants as compared with patients with wild-type alleles and IPF (4). These findings suggest that these patients have a genetic predisposition to IPF from iron dysregulation, possibly driven by increased generation of ROS by Fenton's reaction (6, 7). Another group recently proposed that a decreased proportion of transferrin receptor 1 (CD71)-expressing alveolar macrophages in patients with IPF may lead to impaired iron sequestration and ultimately worse survival (8). Building on preexisting clinical and translational data, we used mouse models of iron overload and pulmonary fibrosis to examine the causal relationship between iron and pulmonary fibrosis.

Hepcidin-knockout (HKO) mice (9) were backcrossed onto the C57BL/6J background, then bred to produce HKO and wild-type (WT) mice as age-matched and sex-matched littermates. Hepcidin deficiency causes unregulated intestinal iron absorption and systemic iron overload, with secondary lung iron loading (10). Male and female HKO mice were further placed on a high-iron diet (5,000 parts/million iron; Teklad diet TD.14064; Envigo) at weaning for 2.5–4.5 weeks to augment iron loading and then placed on standard chow for the remainder of the protocol. WT mice were maintained on standard chow throughout. At 7–9.5 weeks of age, mice were treated with bleomycin 1–1.5 U/kg (Fresenius Kabi) in sterile saline through oropharyngeal aspiration and then analyzed at 2 or 3 weeks after bleomycin administration. A subset of HKO and WT mice was harvested at 7.5–10 weeks of age and served as untreated control animals.

Blood hemoglobin (Hgb) and mean corpuscular volume (MCV) were measured using a HemaVet blood analyzer (Drew Scientific). Lung and liver tissue nonheme iron concentrations were determined with a colorimetric assay (Sekisui Diagnostics). For iron localization, formalin-fixed and paraffin-embedded lung tissues underwent enhanced Perls' stain using potassium ferrocyanide 2% and HCl 4% and the Vector SG Peroxidase Substrate kit (SK-4700; Vector Laboratories) and were then counterstained with 0.5% neutral red solution. Lung tissue sections were also treated with Masson's trichrome staining at the University of California Los Angeles Translational Pathology Core Laboratory, then scanned for digital analysis. Lung collagen quantification was performed using the Sircol Soluble Collagen Assay (Biocolor) according to the manufacturer's protocol. Lung tissue *Nqo1* was measured by qRT-PCR, as previously described (11). For a listing of primer sequences, see Table E1 in the data supplement.

SigmaPlot (Systat Software) was used for all statistical analyses. Normally and nonnormally distributed data were analyzed using the *t* test or Mann-Whitney rank-sum test, respectively. Multivariate analyses were performed using two-way ANOVA.

Immediately before bleomycin treatment, HKO mice and WT mice underwent hematologic measurements. As expected, iron-overloaded HKO mice had a trend toward increased Hgb (Figure 1A; median HKO Hgb, 12.9 g/dl vs. WT Hgb, 14.5 g/dl; $P = 0.20$) as well as increased MCV values (Figure 1B; mean HKO MCV, 61.8 fl vs. WT MCV, 54.5 fl; $P < 0.05$) as compared with their WT littermates. Tissue iron was measured to confirm a differential in the iron status of the WT and HKO groups. Both liver and lung iron were dramatically increased in the treated HKO mice compared with their genotype control animals, at more than 33-fold and 6-fold the concentrations of WT mice (Figures 1C and 1D; $P < 0.001$ and $P < 0.001$), reflecting effective iron loading of both tissue

stores and the lung. Interestingly, liver and lung iron concentrations increased in HKO mice after bleomycin treatment, with a trend toward concurrent increase in WT mice after treatment. Although the etiology is unclear, this observation suggests that the iron loading phenotype in IPF lung may be partially secondary to the inflammatory or fibrotic process. A representative image of Perls' staining of bleomycin-treated HKO mouse lung illustrates the architectural distortion, septal thickening, and inflammatory cell infiltration that is characteristic of the model (Figure 1E). The distribution of iron staining is diffuse, including the airway epithelium and areas of intense punctate staining that are potentially sequelae of microhemorrhages.

To quantify the degree of fibrosis, lung collagen was measured in both untreated and treated mice at 2 and 3 weeks after bleomycin administration (Figure 1F). As expected, collagen concentrations for all treated groups were increased compared with untreated groups, confirming the development of fibrosis with our model. However, there were no significant differences in collagen concentrations between the genotypes at either time point. Of note, lung *Nqo1* measurements showed that iron-mediated ROS levels increased after bleomycin treatment (Figure 1G; mean WT untreated Δ CT [Δ cycle threshold], -4.3 vs. WT 3-wk Δ CT, -2.8 ; mean HKO untreated Δ CT, -3.9 vs. HKO 3-wk Δ CT, -1.6 ; $P < 0.05$ and $P < 0.001$, respectively) but without a significant difference between genotypes at each time point, indicating that iron overload alone is not sufficient to increase ROS generation and subsequent fibrotic damage in response to bleomycin. Representative digital images of Masson's trichrome-stained bleomycin-treated mouse lungs demonstrate the characteristic patchy collagen deposition of pulmonary fibrosis in both WT and HKO mice (Figure 1H). Weight loss was also tracked as a systemic marker of disease severity (Figure 1I). Although there was significant weight loss in the treated mice as compared with their own baselines (data not shown), there was no difference in the weight loss among any of the treated groups.

A recent study reported increased pulmonary fibrosis in transferrin receptor 2 mutant mice after bleomycin treatment (12). Of note, the study used collagen deposition around small airways as the primary biochemical parameter of fibrosis severity. Despite a much more severe iron overload phenotype in our HKO mice, we did not observe a significant difference between WT and transgenic mice in response to the bleomycin model, using conventional measurements of whole-lung collagen. The potential reasons for the divergent conclusions include the different outcome measures and inherent differences in the various genetic and dietary models of iron overload. The strengths of our study include the severity of the iron overload in our model, the use of a robust global measure of pulmonary fibrosis, and the inclusion of body weight as a measure of general health. ■

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Figure 1. Severe iron (Fe) overload does not augment the response to bleomycin in a mouse model of pulmonary fibrosis. Hematologic measurements prior to bleomycin treatment show (A) a trend toward increased hemoglobin and (B) increased mean corpuscular volume (MCV) in hepcidin-knockout (HKO) mice ($n = 4/\text{genotype}$). (C) Liver Fe and (D) lung Fe measurements at 2 weeks after bleomycin treatment confirm severe Fe overload in HKO mice ($n = 6\text{--}9/\text{group}$). (E) Representative enhanced Perl's stain of bleomycin-treated HKO mouse lung demonstrates Fe deposition. (F) Collagen quantification of whole-lung tissue shows no difference between the genotypes at either 2 weeks or 3 weeks ($n = 2\text{--}5$ for untreated groups; $n = 4\text{--}9$ for treated groups). (G) Lung *Nqo1* increases in both genotypes after bleomycin treatment but without showing a difference between genotypes at each time point ($n = 4\text{--}10/\text{group}$). (H) Representative digital image of whole-lung Masson's trichrome stain of WT and HKO mice. (I) Mouse weights show no difference in weight loss among any of the treated groups ($n = 4\text{--}9/\text{group}$). Box-plot graphs depict the median (solid line) $\pm$ 25th/75th percentile and/or the mean (dashed line); dot density plots depict the mean (solid line). * $P < 0.05$ and ** $P < 0.001$ by (B) t test or (C and D) two-way ANOVA as indicated or (F and G) two-way ANOVA for comparison of each treated group with its untreated genotype control group. CT = cycle threshold; NS = not significant; WT = wild type. Scale bar, 50 μm .

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Retraction: Obesity-induced Endoplasmic Reticulum Stress Causes Lung Endothelial Dysfunction and Promotes Acute Lung Injury

This article (1) has been retracted by the authors. It was discovered that the same immunoblot images were used to represent two different experimental conditions. This includes Figure 1D in which a blot of GRP78 is identical to a blot labeled as β -catenin in another paper (2), and Figure 2F in which a blot of GAPDH is identical to a portion of a GAPDH image used in that paper (2). In addition, anomalies were identified in the Western blot images in Figures 1D, 2B, 2F, 2H, 3A, 3E, 3F, and 5B. Given that the scientific

integrity of this study has been compromised, the authors believe that retraction of this paper is the best course of action. All the authors have agreed to this decision.

The authors apologize to the *Journal* and its readers. ■

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Retraction: Chronic Alcohol Ingestion In Rats Alters Lung Metabolism, Promotes Lipid Accumulation, and Impairs Alveolar Macrophage Functions

An article published in the December 2014 issue of the *Journal* (1) has been retracted by the authors. It was discovered that the GAPDH band in Figure 4C is incorrect and is identical to that used in Figure 4D. Moreover, the images used to represent acetyl-CoA synthase in Figure 4D and pAMPK in Figure 4C are the same. In addition, anomalies were identified in the Western blot images for AMPK (Figure 4C) and FASN (Figure 4D). Based on these findings, although the authors continue to stand behind the conclusions reached, the scientific integrity of the manuscript has been compromised. All the authors have agreed to this decision, and they apologize to the *Journal* and its readers. ■

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