ONLINE DATA SUPPLEMENT

Prolonged Injury and Altered Lung Function after Ozone Inhalation in Mice with Chronic Lung Inflammation

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MATERIALS AND METHODS

Chemotaxis Assay

Chemotactic activity of BAL fluid was assessed using a 96-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) as described previously (E1). Briefly, 30 μl of BAL, monocyte chemoattractant protein-1 (positive control, 500 ng/ml) or buffer (negative control) were placed in the lower wells of the chemotactic chamber. A 5 μM pore size polycarbonate filter was placed over the wells, and the upper chamber secured into place. RAW 264.7 mouse macrophages (ATCC, Manassas, VA, 100 μl, $1x10^6$ cells/ ml) were added to the upper wells, and the chamber incubated at 37ºC for 3 h. The lower chamber with the membrane attached was then centrifuged (450 x g, 5 min), the membrane removed and stained with Diff Quick (CAMCO, Fort Lauderdale, FL). Cells migrated through the filter were counted microscopically under oil-immersion. Data are expressed as the average number of migrated cells per 20 high power fields.

Assessment of Lung Morphometry

Lung morphometry was analyzed by the radial alveolar count method (E2). Measurements were performed on hemotoxylin and eosin stained sections from respiratory bronchioles to the edge of the acinus (pleura or connective tissue septum). Respiratory bronchioles were identified under 100x magnification and a perpendicular line drawn from the center of the bronchial to the closest acinus. The number of alveoli transected by this line was enumerated. When the line traversed the common wall of 2 alveoli, a value of 1 was assigned. For each lung section, 14 to 20 respiratory bronchioles were counted.

Quantitative Analysis of COX-2 and iNOS Expression

The number of macrophages and Type II cells staining for iNOS and COX-2 was quantified microscopically. Each positively stained cell was assigned a staining intensity score on a scale of 0= no staining, 1= light staining, 2= medium staining, 3= dark staining.

Generation of Pressure/Volume Loops; Calculation of Cst and Starting Lung Volumes

Quasi-static pressure/volume loops were generated from functional residual capacity by setting PEEP to 0 cm H_2O and delivering 7 stepwise inflations of inspiratory volume to a total volume of 0.8 ml, followed by 7 equal expiratory steps, pausing for 1 s at each step. Plateau cylinder pressure was measured during each pause and plotted against piston displacement. Calculation of quasi-static compliance was performed by integration using flexiVent software. In order to establish that differences in starting lung volumes did not affect analysis of R_L and E_L, starting lung volume values at 0 PEEP were determined as the minimum inspiratory volume generated by the pressure/volume loops (Table E1).

Statistics

For each R_L and E_L spectrum (Figure 5), a best fit line was generated using non-linear regression. To test for significant differences between control and treated groups, the spectra data from the treated groups were fit to a curve generated using the values of the constraints of the best fit line of the control group. A coefficient of determination was then calculated to assess the goodness of fit of the data to the regression curve. To determine if differences between spectral curves obtained from control and treated mice were significant, the R^2 values were used to generate t values. Significance was determined by setting α to 0.05.

Online Supplement References:

E1. Guo CJ, Atochina-Vasserman EN, Abramova E, Foley JP, Zaman A, Crouch E Beers MF, Savani RC, Gow AJ. S-nitrosylation of surfactant protein-D controls inflammatory function. PLoS Biol 2008;6:2414-2423.

E2. Cooney TP, Thurlbeck WM. The radial alveolar count method of Emery and Mithal: a reappraisal 1- Postnatal lung growth. Thorax 1982;37:572-579.

SUPPLEMENTAL FIGURES

Figure E1

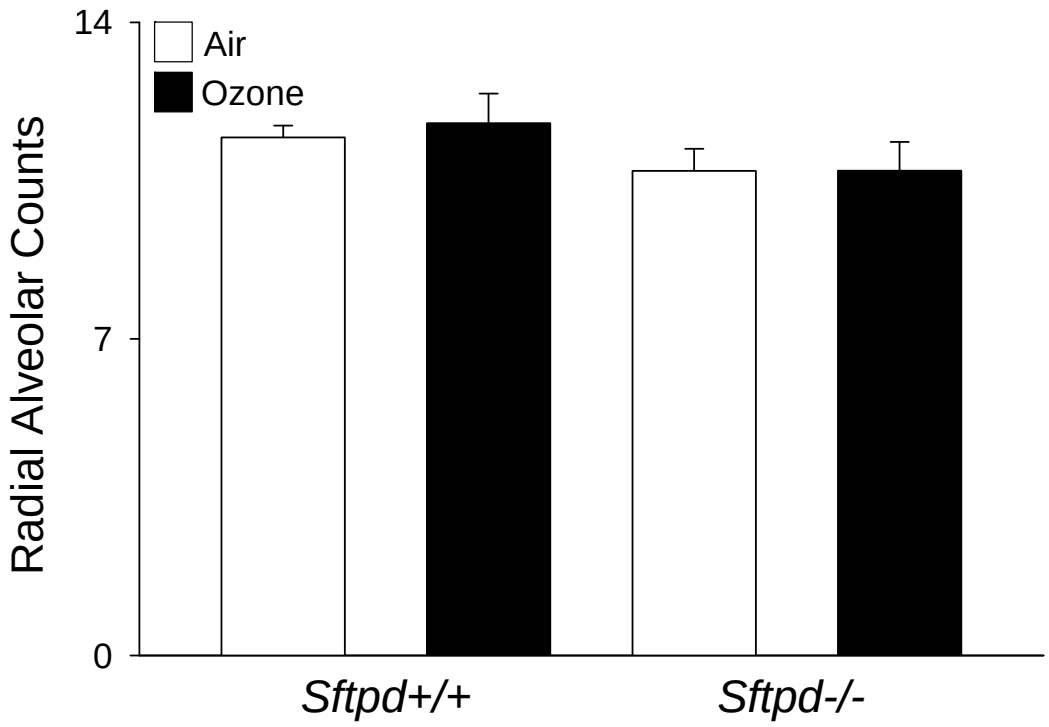

Figure E1. Effects of loss of *Sftpd* on lung structure. Lung sections from *Sftpd+/+* and *Sftpd-/-* mice were stained with hematoxylin and eosin and radial alveolar counts performed as described in the Supplemental Methods section. Each bar represents the mean $\pm$ SE (n=3 mice/treatment group). Data were analyzed by two-way ANOVA. No significant differences were observed between groups.

Figure E2

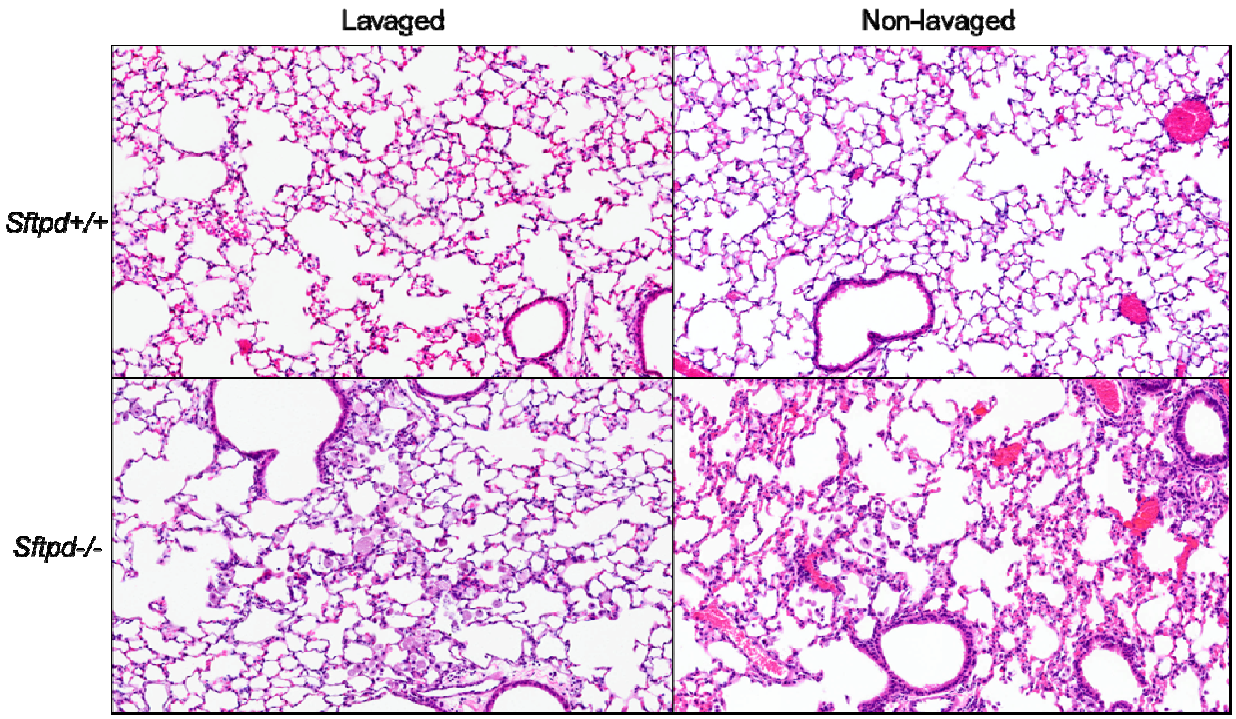

Figure E2. Effects of bronchoalveolar lavage on lung histology. Lung sections from lavaged and non-lavaged *Sftpd*+/+ and *Sftpd*-/- mice were stained with hematoxylin and eosin. Original magnification, 400x.

Figure E3

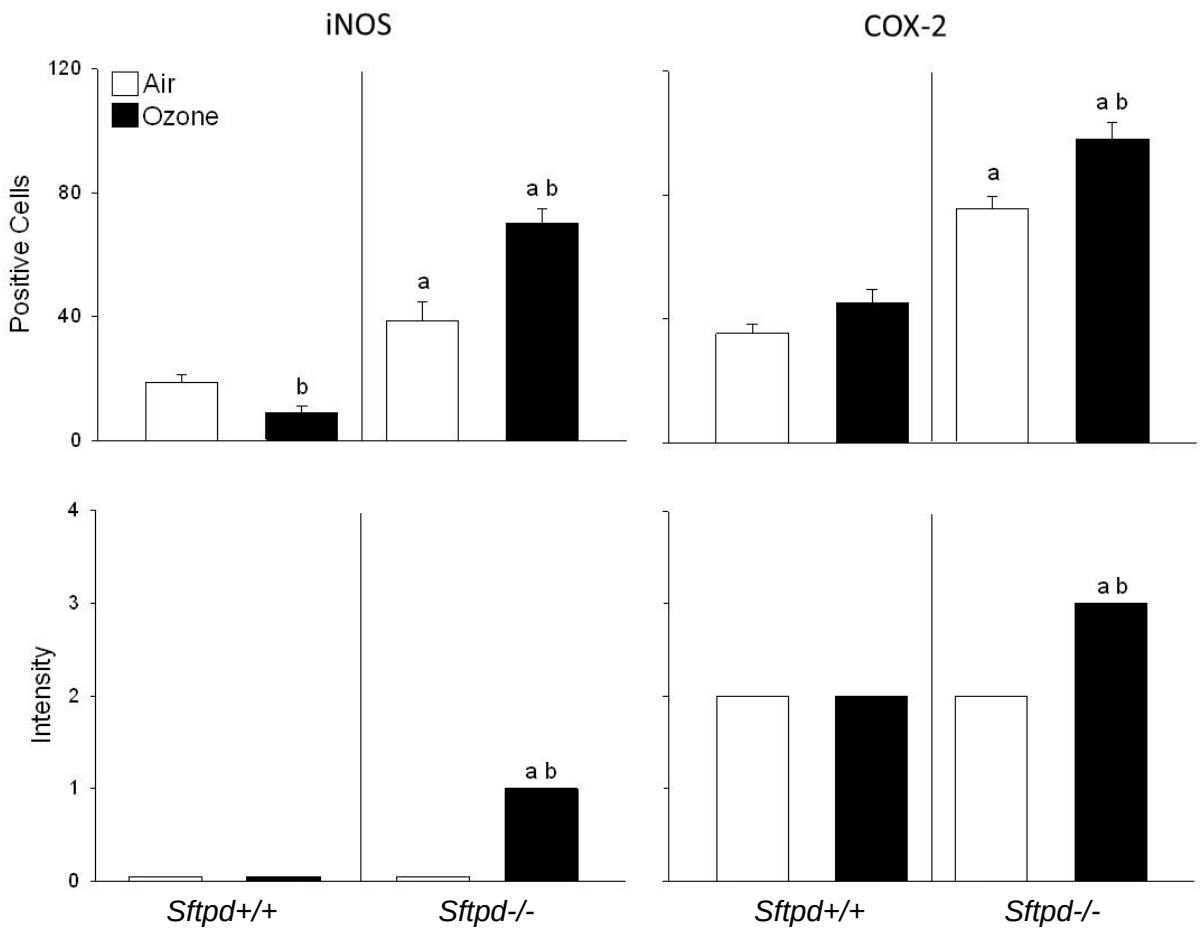

Figure E3. Quantitative analysis of iNOS and COX-2 expression. Lung sections, prepared 72 h after exposure of *Sftpd+/+* and *Sftpd-/-* mice to air or ozone, were stained for iNOS or COX-2. For iNOS quantitation, macrophages in ten 10x fields were counted and assigned a staining intensity score as described in the Supplementary Methods section. Data are presented as percentage iNOS positive macrophages. For COX-2, positively stained Type II cells were counted in ten 20x fields and assigned a staining intensity score. Each bar represents the mean $\pm$ SE (n=3 mice/treatment group). Data were analyzed by ANOVA based on the ranks. [a]Significantly different ($p \leq 0.05$) from *Sftpd+/+* mice. [b]Significantly different ($p \leq 0.05$) from air control.

Table E1

Table E1. Starting lung volumes in *Sftpd*+/+ and *Sftpd*-/- mice

	Air	**Ozone**
***Sftpd*+/+**	0.091 ± 0.019	0.101 ± 0.022
***Sftpd*-/-**	0.111 ± 0.028	0.086 ± 0.018

Starting lung volumes were measured at 0 PEEP 72 h after exposure of *Sftpd*+/+ and *Sftpd*-/- mice to air or ozone. Each measurement was performed in triplicate. Values are means ± SE (n= 4- 6 mice/treatment group). Data were analyzed by two-way ANOVA. No significant differences were observed between groups.