Age-related increases in ozone-induced injury and altered pulmonary mechanics in mice with progressive lung inflammation

Angela M. Groves,1 Andrew J. Gow,1 Christopher B. Massa,1 LeRoy Hall,2 Jeffrey D. Laskin,3 and Debra L. Laskin1

1Department of Pharmacology and Toxicology, Rutgers University, Ernest Mario School of Pharmacy, Piscataway, New Jersey; 2Drug Safety Sciences, Janssen Research & Development, Raritan, New Jersey; and 3Department of Environmental and Occupational Medicine, Rutgers University, Robert Wood Johnson Medical School, Piscataway, New Jersey

Submitted 28 January 2013; accepted in final form 12 August 2013

Groves AM, Gow AJ, Massa CB, Hall L, Laskin JD, Laskin DL. Age-related increases in ozone-induced injury and altered pulmonary mechanics in mice with progressive lung inflammation. Am J Physiol Lung Cell Mol Physiol 305: L555–L568, 2013. First published August 30, 2013; doi:10.1152/ajplung.00027.2013.—In these studies we determined whether progressive pulmonary inflammation associated with aging in surfactant protein D (Sftpdp−/−) mice leads to an exacerbated response to ozone. In Sftpdp−/− mice, but not wild-type (WT) mice, age-related increases in numbers of enlarged vacuolated macrophages were observed in the lung, along with alveolar wall rupture, type 2 cell hyperplasia, and increased bronchoalveolar lavage protein and cell content. Numbers of heme oxygenase + macrophages also increased with age in Sftpdp−/− mice, together with classically (iNOS +) and alternatively (mannose receptor +, YM-1+, or galectin-3+) activated macrophages. In both WT and Sftpdp−/− mice, increasing age from 8 to 27 wk was associated with reduced lung stiffness, as reflected by decreases in resistance and elastance spectra; however, this response was reversed in 80-wk-old Sftpdp−/− mice. Ozone exposure (0.8 ppm, 3 h) caused increases in lung pathology, alveolar epithelial barrier dysfunction, and numbers of iNOS + macrophages in 8- and 27-wk-old Sftpdp−/−, but not WT mice at 72 h postexposure. Conversely, increases in alternatively activated macrophages were observed in 8-wk-old WT mice following ozone exposure, but not in Sftpdp−/− mice. Ozone also caused alterations in both airway and tissue mechanics in Sftpdp−/− mice at 8 and 27 wk, but not at 80 wk. These data demonstrate that mild to moderate pulmonary inflammation results in increased sensitivity to ozone; however, in senescent mice, these responses are overwhelmed by the larger effects of age-related increases in baseline inflammation and lung injury.

ozone; macrophages; aging; emphysema; surfactant protein D

correlated with increases in the abundance of a number of proteins that promote macrophage activation toward a classically activated proinflammatory phenotype (44).

In the lung, macrophage activity is regulated, in part, by surfactant protein D (Sftpdp), a pulmonary collectin synthesized mainly by alveolar type 2 cells (10, 20). Under homeostatic conditions, Sftpdp functions as an anti-inflammatory protein, suppressing NF-κB-mediated transcription of macrophage pro-inflammatory genes (15). However, following induction of oxidative stress, increased production of nitric oxide results in S-nitrosylation of critical cysteines in Sftpdp, leading to a change in its activity to a proinflammatory mediator (19). Earlier reports demonstrated that mice lacking Sftpdp exhibit chronic low-level pulmonary inflammation, characterized by the presence of activated macrophages in the lung (3). Moreover, macrophage inflammation increases with age in these mice, findings consistent with an important regulatory role of Sftpdp in innate immunity (48). In previous studies we showed that low-level pulmonary inflammation accompanying loss of Sftpdp in young mice is correlated with prolonged injury, oxidative stress, and altered pulmonary mechanics in response to inhaled ozone (18). The effects of chronic and progressive inflammation and the development of emphysema, which mimics biological responses in humans (6), on the response to inhaled ozone are unknown and were investigated in aging Sftpdp−/− mice. For these studies, we analyzed the response to ozone 72 h postexposure, a time when lung injury and inflammation have, for the most part, resolved in young (8- to 10-wk-old) wild-type (WT) mice (12). This allowed us to directly assess the impact of progressive lung inflammation and injury on ozone sensitivity.

MATERIALS AND METHODS

Animals and exposures. Male C57BL/6J WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Sftpdp−/− mice were generated on a C57BL/6 background (3) and bred at Rutgers University. Animals were housed under pathogen-free conditions in microisolation cages and provided food and water ad libitum. All procedures were reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee. Mice were euthanized 72 h following exposure (3 h) to ozone (0.8 ppm) or air in whole body Plexiglas chambers. Ozone was generated with a Gilmont generator (Orec, Phoenix, AZ); concentrations in the chamber were measured with an ozone analyzer (Teledyne Technologies, Thousand Oaks, CA).

BAL protein and cell content. Bronchoalveolar lavage fluid (BAL) was collected by slowly instilling and withdrawing 1 ml of Ca2+/Mg2+-free phosphate-buffered saline (PBS) into the lung four times via a 1-ml syringe. BAL was centrifuged (300 g, 8 min, 4°C) and
protein concentrations in supernatants were quantified by the BCA protein assay (Thermo Scientific, Rockford, IL) with bovine serum albumin (BSA) as the standard. Cell pellets were washed twice (300 g, 8 min, 4°C), resuspended in Ca²⁺/Mg²⁺-free PBS, and enumerated by using a Coulter Counter (Beckman Coulter, Brea, CA). For differential analysis, cytopsins were prepared, fixed in methanol, and then stained with Giemsa (Labchem, Pittsburgh, PA). A total of 100 cells were counted per slide.

Histology and immunohistochemistry. Following lavage, the left lobe of the lung was inflation fixed overnight in ice-cold 3% paraformaldehyde containing 2% sucrose. The tissue was then washed three times with PBS/2% sucrose and transferred to tubes containing 50% ethanol. Tissue sections (6 μm) were prepared, stained with hematoxylin and eosin, and examined by light microscopy. Images were acquired via a VS120 Virtual Microscopy System (Olympus, Center Valley, PA). The extent of inflammatory and structural changes in the lung and the airways, including macrophage, neutrophil, and lymphocyte localization, alterations in alveolar epithelial barriers, interstitial and pleural thickening, bronchial and type 2 cell hyperplasia, fibrin deposition, and the appearance of cholesterol and hemoglobin crystals, were assessed blindly by a board-certified veterinary pathologist (LeRoy Hall, D.V.M., Ph.D.). Semi-quantitative grades (0 to 3) were assigned to the tissues, with grade 0 = no changes, grade 1 = minimal or small changes, grade 2 = mild to moderate changes, and grade 3 = moderate to extensive changes. One section from each of five to six mice per treatment group was analyzed. To assess the extent of alveolar damage, radial alveolar counting was performed as previously described (9). Respiratory bronchioles were identified under ×100 magnification, and a perpendicular line was drawn from the center of the bronchus to the closest acinus. The number of alveoli transected by this line was enumerated. When the line transversed the common wall of two alveoli, a value of 1 was assigned. For each lung section, 14–20 respiratory bronchioles were counted.

For immunohistochemistry, tissue sections were deparaffinized and incubated in citrate buffer (0.1 M Na citrate plus 0.1 M citric acid, pH 6.0) for 30 min followed by 3% H₂O₂ for 10 min, and then 1.5% normal rabbit serum for 2 h. This was followed by overnight incubation at 4°C with rabbit IgG or rabbit polyclonal antibodies to inducible nitric oxide synthase (iNOS, 1:1,500; Abcam, Cambridge, MA), mannose receptor (1:1,500; Abcam), YM-1 (1:500; Stem Cell Technologies, Vancouver, BC), galectin-3 (1:2,000; R&D Systems, Minneapolis, MN), heme oxygenase-1 (HO-1, 1:750; Enzo Life Sciences, Valley Stream, NY), pro-surfactant protein (SP)-C (1:2,000; Millipore, Billerica, MA), or PCNA (1:500; Abcam). Sections were then incubated in biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA) for 30 min. A DAB Peroxidase Substrate Kit (Vector Labs) was used to visualize binding. The number of positively staining macrophages and type 2 cells was quantified microscopically for each slide, as previously described (18). Each enumerated cell was assigned a staining intensity score on a scale of 0–4 with 0 = no staining, 1 = light staining, 2 = medium staining, 3 = moderate staining, and 4 = dark staining. Three slides per treatment group were analyzed.

Measurement of pulmonary mechanics. Pulmonary mechanics was measured at positive end-expiratory pressures (PEEP) ranging from 0 to 6 cmH₂O by use of a flexiVent (SCIREQ, Montreal, PQ, Canada). Mice were ventilated at a frequency of 150 breaths/min and a tidal volume of 10 ml/kg. Baseline measurements were assessed at a PEEP of 3 cmH₂O. Input impedance data (ZL) were generated by using an 8-s broadband flow perturbation. Resistance and elastance spectra were generated from ZL. To partition measurements of respiratory mechanics into parameters representing the properties of either the airways or lung tissue, parameters representing the key features of the resistance spectra (Rl) curve were calculated with the equation

\[ R_l = \frac{a + bf}{c + f} \]

where f = frequency, a/c is a measure of the low-frequency asymptote of the curve, when tissue resistive and viscous effects predominate, and b is a measure of the high-frequency asymptote, when airway resistive effects predominate. Parameters representing different portions of the elastance spectra (El) curve were calculated with the equation

\[ E_l = E_0 + \Delta E(1 - e^{-bf}) \]

where \( E_0 \) represents the theoretical elastance at 0 Hz and thus is a measure of inherent tissue stiffness, \( \Delta E \) is the magnitude of the frequency-dependent change in elastance, and \( \beta \) is a measure of the rate elastance changes with frequency.

Statistical analysis. Experiments were repeated at least three times using four to nine mice per treatment group per experiment. Data were analyzed by two-way ANOVA and a nonparametric Student’s t-test. For nonparametric data, a Mann-Whitney rank sum test was performed. For each resistance and elastance spectra, a best fit line was generated as previously described (18). For hypothesis testing using the mechanical impedance data, the residual error from curve fitting with nonlinear regression was used as a measure of goodness of fit. Comparison between two experimental conditions was made by evaluating the reduction in model error when two independent curves were fit to the data (alternative hypotheses fit), vs. the residual error when only one curve was used to fit the data (null hypothesis fit). This reduction in error allowed for the calculation of an F statistic, which was used to determine statistical significance. For treatment conditions determined significant at the spectral level, subsequent testing of parameter values was performed by using the t-test for unequal variances at the \( \alpha = 0.05 \) level.

RESULTS

Effects of increasing age on lung histology and pulmonary mechanics in WT and Sftpd⁻/⁻ mice. In initial studies we analyzed the effects of increasing age on lung structure. In Sftpd⁻/⁻ mice, but not WT mice, age-related alterations were noted in lung histology (Fig. 1). Thus, consistent with previous reports (3, 48), at 8 wk of age, minimal to mild multifocal infiltration of macrophages and lymphocytes was observed; macrophages were predominately localized in perivascular regions, whereas lymphocytes were concentrated around bronchioles and in the interstitium (Fig. 1A). Histological scoring of the tissue confirmed increased numbers of macrophages and lymphocytes in the tissue of Sftpd⁻/⁻ mice (Table 1). Some of the macrophages were enlarged and vacuolated and exhibited a ground-glass-like granular cytoplasm, whereas others contained an eosinophilic cytoplasm, suggestive of phagocytized surfactant (Fig. 1A). In these multifocal areas of inflammation, interstitial thickening of the alveolar walls was also observed, along with some neutrophils. Minimal multifocal rupture of the alveolar walls was also evident (Fig. 1A and Table 1). Similar changes were noted in 27-wk-old Sftpd⁻/⁻ mice; however, they were more pronounced. Thus macrophage infiltration into perivascular and peribronchiolar regions was greater, becoming mild to moderate (Fig. 1A and Table 1). Many of the macrophages appeared as multinucleated “giant” cells containing either eosinophilic or ground-glass granular cytoplasm. Lymphocytes, plasma cells, and Russell bodies were also noted in perivascular and peribronchiolar regions, as well as neutrophils adjacent to degenerating macrophages. Minimal to mild multifocal rupture of the alveolar walls and interstitial thickening was present throughout the tissue. By 80 wk, the pathological changes had become severe and included mild to marked macrophage and lymphocyte inflammation (Fig. 1A and Table 1). There was also an abundance of multinucleated

Downloaded from http://ajplung.physiology.org/ by 10.220.33.6 on November 6, 2017
“giant” macrophages, some of which were large enough to fill multiple alveoli. Hemoglobin and cholesterol crystals were also frequently observed in these cells. Multifocal aggregates of lymphocytes were also present, together with minimal to mild proliferation of type 2 cells, interstitial thickening of the alveolar walls, and fibrin deposition. Widespread rupture of the alveolar epithelial walls was evident, ranging from minimal to moderate in severity. Plasma cells and Russell bodies were also present in the interstitium, and in some alveoli necrotic debris was visible.

Fig. 1. Lung histology in wild-type (WT) and Sftpd−/− mice following exposure to air or ozone. Lung sections, prepared 72 h following exposure of 8-, 27-, and 80-wk-old WT and Sftpd−/− mice to air (A) or ozone (B), were stained with hematoxylin and eosin. Arrows, macrophages; arrowheads, lymphocytes; E, macrophages containing eosinophilic cytoplasm; G, macrophages containing granular cytoplasm; M, multinucleated macrophages; AR, alveolar rupture; IT, interstitial thickening; T2, type 2 cell hyperplasia; HC, hemoglobin crystals; CC, cholesterol crystals. Original magnification, ×80 (top); ×200 (bottom).
Radial alveolar counting supported age-related increases in alveolar epithelial destruction in Sftpd−/− mice. Thus radial alveolar counts were decreased in 27- and 80-wk-old Sftpd−/− mice relative to WT mice (Fig. 2). In 80-wk-old Sftpd−/− mice, significant increases in BAL protein levels were also observed (Fig. 3). BAL cell number was increased in Sftpd−/− mice at all age groups relative to WT mice. In contrast, no significant changes in BAL protein or cell number or in radial alveolar counts were observed in WT mice with increasing age (Figs. 2 and 3).

In response to lung injury, type 2 cells become enlarged and begin to proliferate to repair damaged alveolar epithelium (27, 40). Pro-SP-C is a precursor of SP-C expressed by type 2 cells (16). In young Sftpd−/− mice, and to a lesser extent young WT mice, low-level expression of pro-SP-C was noted in type 2 cells (Fig. 4). In Sftpd−/− mice, this was associated with low-level expression of the proliferation marker, PCNA (Fig. 5). In both WT and Sftpd−/− mice, numbers of type 2 cells expressing pro-SP-C and PCNA increased with increasing age. This response was more pronounced in Sftpd−/− mice, relative to WT mice (Table 2).

HO-1 is an antioxidant enzyme constitutively expressed by alveolar macrophages (14). In WT mice, increasing age was correlated with significantly decreased constitutive expression of HO-1 in alveolar macrophages (Fig. 6 and Table 2). In contrast, in Sftpd−/− mice, macrophage HO-1 expression was increased in 27- and 80-wk-old mice, relative to 8-wk-old animals.

We next characterized the phenotype of the macrophages in the lungs of WT and Sftpd−/− mice by analyzing expression of markers of classical and alternative activation (32). iNOS is an enzyme mediating the generation of proinflammatory reactive nitrogen species and is a prototypical marker of classically activated macrophages (32). In 8-wk-old Sftpd−/− mice, but not WT mice, low numbers of iNOS-expressing macrophages were present in the lung (Fig. 7). Whereas in WT mice aging had no effect on iNOS+ macrophages in the lung, in Sftpd−/− mice, numbers of iNOS+ macrophages increased with increasing age.
mice numbers of these cells increased significantly with age (Table 2). Mannose receptor, YM-1, and galectin-3 are markers of alternatively activated macrophages that display anti-inflammatory activity and participate in wound repair (32, 41, 45). Macrophage expression of each of these markers was increased at 27 wk of age compared with 8 wk of age in WT mice (Figs. 8, 9, and 10, and Table 2). In contrast, at 80 wk of age, macrophage expression of mannose receptor and YM-1
The resistance and elastance spectra in reaching a peak at 27 wk for galectin-3, and at 80 wk for mice, numbers of macrophages expressing these alternative was associated with a marked increase in numbers of macrophages in ten stained Type II cells in ten fields. Galectin-3 remained elevated. Loss of Sftpd decreased, whereas galectin-3 remained elevated. Loss of Sftpd was associated with a marked increase in numbers of macrophages expressing mannose receptor, YM-1, and galectin-3, relative to WT mice, in all age groups. Moreover, in Sftpd mice, numbers of macrophages expressing these alternative macrophage activation markers increased with increasing age, reaching a peak at 27 wk for galectin-3, and at 80 wk for mannose receptor and YM-1.

The effects of aging on lung function in WT and Sftpd mice were analyzed by examining pulmonary mechanics. Increasing age from 8 to 27 wk was associated with decreases in the resistance and elastance spectra in Sftpd mice, and in the elastance spectra in WT mice (Fig. 11). The resistance spectra was also decreased in WT mice at 27 wk, but only at low frequencies; at high frequencies, the resistance spectra was increased (Fig. 11). Whereas in WT mice the elastance spectra was similar in 27- and 80-wk-old mice, in 80-wk-old Sftpd mice the elastance spectra was reduced compared with 27-wk-old Sftpd mice. We also found that the elastance spectra in 8-wk-old Sftpd mice was significantly decreased relative to 8-wk-old WT mice. Although no differences between the genotypes were noted in the elastance spectra at 27 wk of age, at 80 wk it was increased in Sftpd mice. The resistance spectra was also increased at low frequencies in 80-wk-old Sftpd mice relative to WT mice (Fig. 12).
partitioned into airway and tissue compartments. In WT mice, decreases in \( a_c \), which represents the low-frequency portion of the resistance curve, where tissue effects predominate, and increases in the high-frequency parameter \( b \), which reflects airway changes, were observed at 27 and 80 wk of age (Fig. 13). Altered pulmonary elastance was also noted in 27- and 80-wk-old WT mice, as reflected by decreases in the frequency-dependent change, \( \Delta E \), as well as in the low-frequency parameter, \( E_0 \).

Fig. 6. Expression of heme oxygenase-1 (HO-1) in WT and Sftpdl\(^{-/-}\) mice. Lung sections, prepared 72 h after exposure of 8-, 27-, and 80-wk-old WT and Sftpdl\(^{-/-}\) mice to air or ozone, were stained with antibody to HO-1 or IgG control followed by biotinylated secondary antibody. Binding was visualized by use of a peroxidase substrate DAB kit. One representative section from 3 separate experiments is shown (\( n = 3 \) mice/treatment group). Original magnification, \( \times 600 \).

Fig. 7. Expression of inducible nitric oxide synthase (iNOS) in WT and Sftpdl\(^{-/-}\) mice. Lung sections, prepared 72 h after exposure of 8-, 27-, and 80-wk-old WT and Sftpdl\(^{-/-}\) mice to air or ozone, were stained with antibody to iNOS or IgG control followed by biotinylated secondary antibody. Binding was visualized by use of a peroxidase substrate DAB kit. One representative section from 3 separate experiments is shown (\( n = 3 \) mice/treatment group). Original magnification, \( \times 600 \).
The high-frequency parameter $b$ was significantly increased in $Sftp^{−/−}$ mice, compared with WT mice at 8 wk of age, with no differences in any of the other parameters. Whereas no major differences were noted between WT and $Sftp^{−/−}$ mice at 27 wk of age, at 80 wk $\alpha/c$ was significantly increased and $E_0$ and $\Delta E$ were significantly decreased in $Sftp^{−/−}$ mice, relative to WT mice. In $Sftp^{−/−}$ mice, increasing age from 8 to 27 wk was associated with decreases in the resistance.

![Mannose Receptor expression](image1.png)

**Fig. 8.** Expression of mannose receptor in WT and $Sftp^{−/−}$ mice. Lung sections, prepared 72 h after exposure of 8-, 27-, and 80-wk-old WT and $Sftp^{−/−}$ mice to air or ozone, were stained with antibody to mannose receptor or IgG control followed by biotinylated secondary antibody. Binding was visualized by use of a peroxidase substrate DAB kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, $×600$.

![YM-1 expression](image2.png)

**Fig. 9.** Expression of YM-1 in WT and $Sftp^{−/−}$ mice. Lung sections, prepared 72 h after exposure of 8-, 27-, and 80-wk-old WT and $Sftp^{−/−}$ mice to air or ozone, were stained with antibody to YM-1 or IgG control followed by biotinylated secondary antibody. Binding was visualized by use of a peroxidase substrate DAB kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, $×600$. 
parameters $a/c$ and $b$ and the elastance parameters $E_0$ and $\Delta E$. By 80 wk of age, however, parameters representing the resistance and elastance spectra were similar to those of 8-wk-old Sftpd$^{-/-}$ mice.

Effects of increasing age on the response of WT and Sftpd$^{-/-}$ mice to inhaled ozone. In our next series of studies we analyzed the effects of inhaled ozone on aging WT and Sftpd$^{-/-}$ mice. Consistent with our previous studies (18), no
significant histological changes were observed in the lungs of 
8-wk-old WT mice 72 h following ozone exposure (Fig. 1B); 
radial alveolar counts were also unaltered in these mice (Fig. 
2). Increasing age had no significant effect on the sensitivity of WT mice to ozone, in terms of lung structure. In contrast, in 
Sftpd−/− mice, exposure to ozone resulted in increased num-
bers of lymphocytes in the lung, with no effect on macrophages 
and Table 1). Type 2 cell and bronchial epithelial 
hyperplasia were also increased in the lungs of 
air-exposed mice. In 8-wk-old 
Conversely, in ozone-exposed 
2 cells was observed following ozone exposure at 80 wk of 
age, with no effects at 8 or 27 wk (Fig. 7 and Table 2). 
numbers of iNOS+ macrophages were noted at 8 and 27 wk of 
age, with no further change relative to air control at 80 wk. 
Whereas in WT mice numbers of YM-1+, mannose recep-
tor+, and galectin-3+ macrophages increased, compared with 
air control in 8-wk-old, but not in 27- or 80-wk-old animals, in 
Sftpd−/− mice ozone caused no changes in numbers of cells 
expressing alternative macrophage activation markers in any 
age group (Figs. 8, 9, and 10 and Table 2). 
The effects of ozone on pulmonary mechanics were assessed 
next. In WT mice, ozone exposure resulted in a significant 
increase in the resistance spectra at 8 wk of age, and both 
resistance and elastance spectra at 27 wk of age, with no effects 
at 80 wk (Fig. 12). Analysis of the component parameters 
showed that this was associated with significant increases in 
b in 8-wk-old WT mice and ∆E in 27-wk-old WT mice (Fig. 13). 
In 8-wk-old Sftpd−/− mice, ozone caused significant increases 
in both resistance and elastance spectra, which correlated with 
increases in b and E0, respectively. Similarly, in 27-wk-old 
Sftpd−/− mice, resistance and elastance spectra were also 
significantly increased following ozone exposure. However, no 
individual component parameters of the resistance spectra were 
altered. In contrast, increases in both E0 and ∆E were observed. 
In 80-wk-old Sftpd−/− mice, ozone had no effect on resistance 
or elastance spectra, or on their component parameters.

DISCUSSION
Ozone is a ubiquitous urban air pollutant known to cause 
damage to the alveolar epithelium (1, 7). Epidemiological data 
indicate that individuals with chronic inflammatory diseases 
such as asthma or COPD are hypersensitive to ozone, exhib-
Lung function was measured 72 h after exposure of 8-, 27-, and 80-wk-old WT mice to air or ozone.

**Fig. 13. Effects of ozone on lung resistance and elastance spectral parameters.**

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>WT</th>
<th>Sftpδ−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td><img src="Air.png" alt="Graph" /></td>
<td><img src="Air.png" alt="Graph" /></td>
</tr>
<tr>
<td>Ozone</td>
<td><img src="Ozone.png" alt="Graph" /></td>
<td><img src="Ozone.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

Each point represents mean ± SEM (n = 4–10 mice/treatment group).

**AJP-Lung Cell Mol Physiol** doi:10.1152/ajplung.00027.2013 • www.ajplung.org

Evidence suggests that pathological changes in the lungs of Sftpδ−/− mice are progressive, resulting in the development of emphysema by 24 wk of age. In 27-wk-old Sftpδ−/− mice, we observed alveolar rupture and decreases in radial alveolar counts, which are characteristic features of emphysema. Moreover, this continued to progress for at least 80 wk of age. These data support the idea that there are multiple subpopulations of alternatively activated macrophages involved in the response to chronic tissue injury (32, 34).

Evidence suggests that pathological changes in the lungs of Sftpδ−/− mice are progressive, resulting in the development of emphysema by 24 wk of age (48, 51). Similarly, in 27-wk-old Sftpδ−/− mice, we observed alveolar rupture and decreases in radial alveolar counts, which are characteristic features of emphysema. Moreover, this continued to progress for at least 80 wk of age, an observation that has not been reported previously. Additionally, we noted interstitial thickening at 27 wk of age and, at 80 wk, an observation that has not been reported previously.

Macrophages contribute to acute and chronic inflammatory responses, releasing both pro- and anti-inflammatory mediators, as well as angiogenic, mitogenic, and, under pathological conditions, profibrotic proteins (38). Evidence suggests that the diverse activities of macrophages are mediated by distinct subpopulations that develop in response to inflammatory signals in their microenvironment. Two major functionally distinct subpopulations of macrophages have been identified: classically activated/proinflammatory macrophages and alternatively activated anti-inflammatory/wound repair macrophages (17, 34, 53). Sftpδ has been reported to play a role in negatively regulating classically activated macrophage function in the lung (15, 19). Consistent with this activity are findings that loss of Sftpδ results in increased numbers of enlarged iNOS+ proinflammatory macrophages in the lung (3, 48). The present studies demonstrate that alternatively activated anti-inflammatory/wound repair macrophages are also negatively regulated by Sftpδ. Thus, in addition to increases in iNOS+ macrophages in lungs of Sftpδ−/− mice, we observed increases in YM-1+, mannose receptor+, and galectin-3+ macrophages.

Moreover, as observed with classically activated macrophages, numbers of alternatively activated macrophages increased with advancing age. Of note, peak accumulation of galectin-3+ macrophages was observed in 27-wk-old Sftpδ−/− mice, whereas mannose receptor+ and YM-1+ macrophages continued to increase up to 80 wk of age. These data support the idea that there are multiple subpopulations of alternatively activated macrophages involved in the response to chronic tissue injury (32, 34).

Evidence suggests that pathological changes in the lungs of Sftpδ−/− mice are progressive, resulting in the development of emphysema by 24 wk of age (48, 51). Similarly, in 27-wk-old Sftpδ−/− mice, we observed alveolar rupture and decreases in radial alveolar counts, which are characteristic features of emphysema. Moreover, this continued to progress for at least 80 wk of age, an observation that has not been reported previously. We also noted interstitial thickening at 27 wk of age and, at 80 wk of age, fibrin deposition in the alveolar spaces, as well as increases in type 2 cell expression of pro-SP-C and PCNA, typical responses to lung injury (23, 35, 40); BAL protein levels were also increased in 80-wk-old Sftpδ−/− mice, confirming persistent epithelial injury. In contrast, no significant...
age-related alterations in lung structure or inflammation were observed in WT mice, consistent with the idea that Sftpd contributes to protection of the lung from the development of progressive inflammatory pathology. We did, however, note increases in type 2 cell expression of pro-SP-C and PCNA in middle-aged and elderly WT mice, indicating the presence of some low-level submicroscopic alveolar epithelial injury. This is supported by our findings of increased numbers of alternatively activated mannose receptor+ and YM-1+ macrophages in 27-wk-old WT mice, and galectin-3+ macrophages in 27- and 80-wk-old WT mice, cells known to be involved in tissue repair (17).

Despite lack of major changes in lung structure and inflammation in aging WT mice, significant alterations in pulmonary mechanics were observed. Thus decreases in pulmonary elastance and resistance spectra at low frequencies were noted in 27- and 80-wk-old mice relative to 8-wk-old mice, indicating a decrease in lung stiffness. These findings are in accord with previous reports of age-related decreases in lung elastance (28). In contrast, resistance of the airways increased with aging, results consistent with a reduction in parenchymal tethering of the airways (29). The effects of aging on pulmonary mechanics were exacerbated in mice lacking Sftpd. Hence decreases in elastance spectra were more pronounced between 8- and 27-wk-old Sftpd−/− mice. This was correlated with decreases in inherent tissue elastance, as measured by E0 and ΔE, suggesting a loss of parenchymal integrity. Similar decreases in elastance have been described in other mouse models of emphysema (26, 27). Reversal of these trends in 80-wk-old mice indicates the presence of lung stiffening, most likely a consequence of the large numbers of inflammatory cells in the tissue and excessive fibrin deposition. Age-related alterations in respiratory mechanics including decreases in pulmonary elastance have previously been described in Sftpd−/− mice up to 13 wk of age (8). Our findings that lung inflammation and structural abnormalities continue to increase up to 80 wk of age are novel and in accord with reports that inflammation contributes to reduced pulmonary function (22, 43).

Evidence suggests that the elderly are more susceptible to the adverse effects of air pollution, in part because of chronic low-level pulmonary inflammation (7, 13). Consistent with this notion, in Sftpd−/− mice, but not WT mice, we found evidence of persistent ozone-induced injury, as measured by increases in BAL protein and cells at 8 wk of age, and type 2 cell hyperplasia and bronchiolar epithelial cell hyperplasia in 27- and 80-wk-old mice, respectively. Numbers of iNOS+ proinflammatory macrophages were also increased at 8 and 27 wk of age in Sftpd−/− mice. Previous studies have shown that lung injury is blunted in mice lacking iNOS, demonstrating a role of reactive nitrogen species in ozone toxicity (12). Increased numbers of iNOS+ macrophages in 8- and 27-wk-old Sftpd−/− mice may contribute to their increased sensitivity to ozone. In contrast, BAL cell number and protein content were similar in air- and ozone-exposed 27- and 80-wk-old Sftpd−/− mice, along with radial alveolar counts, type 2 cell PCNA expression, and numbers of classically and alternatively activated macrophages, suggesting that progressive baseline injury and inflammation in aging Sftpd−/− mice may reduce their ability to respond to an environmental insult.

In WT mice, we observed a different pattern of macrophage activation. Thus, in young (8-wk-old) WT mice, alternatively activated macrophages were increased in the lung, a finding consistent with our PCNA data and the resolution of inflammation and injury 72 h following ozone exposure (12). Conversely, in elderly (80-wk-old) WT mice, increases in iNOS+ classically activated macrophages were noted, with no change in numbers of mannose receptor+, YM-1+, or galectin-3+ alternatively activated macrophages. These data suggest a shift in macrophage activation in elderly WT mice toward a proinflammatory, classically activated phenotype (44). This is in accord with previous reports of increased iNOS expression in peritoneal macrophages and increased nitric oxide production by alveolar macrophages from aged mice in response to inflammatory stimuli (5, 30).

HO-1 is a cytoprotective antioxidant enzyme that is acutely responsive to oxidative stress (21). It also has potent anti-inflammatory activity and its persistent expression in macrophages is thought to be important in suppressing their proinflammatory responses (47). In WT mice, numbers of HO-1+ macrophages decreased with age, which may contribute to increases in iNOS+ macrophages in lungs of elderly mice. Conversely, in Sftpd−/− mice, aging was associated with in-

Table 3. Summary of effects of aging and ozone on WT and Sftpd−/− mice

<table>
<thead>
<tr>
<th></th>
<th>WT Mice</th>
<th>Sftpd−/− Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation and injury</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging: no effect</td>
<td>Aging:</td>
<td></td>
</tr>
<tr>
<td>Ozone: 8 wk resolved</td>
<td>Ozone: 8 wk relative to air</td>
<td></td>
</tr>
<tr>
<td>27 wk: resolved</td>
<td>27 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td>80 wk: resolved</td>
<td>80 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td><strong>Macrophage phenotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging: ↑ alternatively activated</td>
<td>Aging: ↑ alternatively activated</td>
<td></td>
</tr>
<tr>
<td>Ozone: 8 wk</td>
<td>Ozone: 8 wk</td>
<td></td>
</tr>
<tr>
<td>27 wk: ↑ alternatively activated</td>
<td>27 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td>80 wk: ↑ classically activated</td>
<td>80 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td><strong>Lung architecture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging: Type II cell hyperplasia</td>
<td>Aging: ↑ Type II cell hyperplasia and alveolar rupture</td>
<td></td>
</tr>
<tr>
<td>Ozone: 8 wk</td>
<td>Ozone: 8 wk</td>
<td></td>
</tr>
<tr>
<td>27 wk: resolved</td>
<td>27 wk: ↑ Type II cell hyperplasia</td>
<td></td>
</tr>
<tr>
<td>80 wk: resolved</td>
<td>80 wk: ↑ Type II cell hyperplasia</td>
<td></td>
</tr>
<tr>
<td><strong>HO-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging: ↓ HO-1 in macrophages</td>
<td>Aging: ↓ HO-1 in macrophages</td>
<td></td>
</tr>
<tr>
<td>Ozone: 8 wk</td>
<td>Ozone: 8 wk</td>
<td></td>
</tr>
<tr>
<td>27 wk: no change relative to air</td>
<td>27 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td>80 wk: no change relative to air</td>
<td>80 wk: no change relative to air</td>
<td></td>
</tr>
<tr>
<td><strong>Lung function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging: ↓ lung stiffness</td>
<td>Aging: ↓ lung stiffness, reversed by 80 wk</td>
<td></td>
</tr>
<tr>
<td>Ozone: 8 wk</td>
<td>Ozone: 8 wk</td>
<td></td>
</tr>
<tr>
<td>27 wk: no change relative to air</td>
<td>27 wk: response blunted relative to 8 wk</td>
<td></td>
</tr>
<tr>
<td>80 wk: no change relative to air</td>
<td>80 wk: response blunted relative to 8 wk</td>
<td></td>
</tr>
</tbody>
</table>

All measurements were made 72 h following exposure to air or ozone.
increased numbers of HO-1+ macrophages, which correlated with increased numbers of alternatively activated macrophages in the lung, suggesting a potential mechanism contributing to their appearance in the tissue. Whereas in WT mice HO-1+ macrophages increased after ozone exposure, in Sftpd+/−/ mice they decreased; however, this was only observed in young animals, a time when classically activated macrophages were increased in Sftpd+/−/ mice and alternatively activated macrophages were increased in both WT and Sftpd+/−/ mice. These data suggest that factors in addition to HO-1 regulate macrophage phenotype in the lung following exposure to ozone.

In accord with previous reports (18), we found that ozone-induced alterations in airway resistance persisted for at least 72 h postexposure in young WT mice, despite the resolution of injury and inflammation. Although these airway changes were small, they are likely to be significant since early stages of airway disease may be present before an abnormality is detected by resistance measurements (49). Of note, ozone-induced functional changes in WT mice were blunted at 27 and 80 wk of age, a response that appeared to be overwhelmed by the more significant increases in these activities as a function of increasing age. In 8-wk-old, and to a lesser extent 27-wk-old, Sftpd+/−/ mice, ozone caused significant alterations in pulmonary mechanics. This was characterized by increases in resistance and elastance, which appeared to be due to alterations in both tissue and airway mechanics. The observation that inherent elastance of the tissue increased, as well as its frequency dependence, suggests ozone-induced stiffening of the lung, likely resulting from the build up of inflammatory cells. By 80 wk of age, ozone had no significant effect on respiratory mechanics in Sftpd+/−/ mice, an age at which we also observed a loss of macrophage responsiveness to ozone-induced activation. This suggests that aging and chronic inflammation reduce the ability of the immune system to respond to additional inflammatory insults. This is supported by findings that patients with chronic respiratory diseases are at increased risk for pneumococcal infection (46).

The present studies provide novel data on changes in lung structure and function in healthy mice as they age, and the impact of chronic progressive inflammation due to loss of Sftpd on the response to ozone. The data are summarized in Table 3. Our results demonstrate that aging has minimal effects on lungs of WT mice and their ability to resolve ozone-induced injury. In contrast, significant age-related alterations in structure and function occur in lungs of Sftpd+/−/ mice through 27 wk of age, which correlates with prolonged responsiveness to ozone. By 80 wk of age it appears that this increased sensitivity is overcome by significant baseline inflammation and injury. However, we cannot exclude the possibility that these differences result from extrapulmonary effects of loss of Sftpd. It is also possible that the development of pulmonary pathology in aging Sftpd+/−/ mice is the result of differences in the dose of inhaled ozone that reaches distinct parts of the respiratory tract.

Patients with COPD have been reported to have decreased pulmonary Sftpd levels (50); moreover, a single nucleotide polymorphism in Sftpd is associated with emphysema pathogenesis (25). Additionally, decreased levels of Sftpd have been detected in BAL from smokers (2, 37). The development of immune dysregulation with age has been shown to contribute to pulmonary disorders such as COPD (24, 33). Our findings demonstrate the importance of understanding how regulation of innate immune functioning could improve disease diagnosis in susceptible populations exposed to air pollutants.

GRANTS
This research was supported by National Institute of Health Grants R01ES004738, R01CA132624, R01HL086621, U54AR055073, P30ES005022, and T32ES007148.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


