INCREASED SENSITIVITY TO OZONE-INDUCED INJURY AND ALTERED PULMONARY MECHANICS IN MICE WITH CHRONIC LUNG INFLAMMATION. EFFECTS OF AGING.

by

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ABSTRACT OF THE DISSERTATION

Increased Sensitivity to Ozone-Induced Injury and Altered Pulmonary Mechanics in Mice with Chronic Lung Inflammation. Effects of Aging.

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Ozone is a ubiquitous urban air pollutant known to damage the lung. Injury is a result of both direct interaction of ozone and its oxidative products with proteins and lipids in the epithelial lining fluid of the lung and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and inflammatory mediators by infiltrating inflammatory cells. Surfactant protein-D (SP-D) is a pulmonary collectin that down-regulates macrophage activation. In these studies we analyzed the effects of progressive pulmonary macrophage inflammation and emphysema associated with aging in mice lacking SP-D on the persistence of ozone-induced injury, macrophage activation, and altered functioning of the lung. We hypothesized that loss of SP-D results in increased sensitivity to ozone. Young (8 wk), middle age (27 wk), and elderly (80 wk)

wild type (WT) and SPD-/- mice were exposed to air or ozone (0.8 ppm, 3 h). Bronchoalveolar lavage fluid (BAL) and tissue were collected 72 h later. Loss of SP-D resulted in increased sensitivity to inhaled ozone at 8 wk and 27 wk of age as observed by increased BAL protein, nitrogen oxides and chemotactic activity. Increased numbers of enlarged, vacuolated macrophages were also present. Aging was associated with increased macrophage numbers, alveolar wall rupture and increases in BAL protein, as well as Type II hyperplasia and expression of proliferating cell nuclear antigen. Heme oxygenase-1+ macrophages together with classically (iNOS+) and alternatively (mannose receptor+, YM-1+, or galectin-3+) activated macrophages also increased in aging SP-D-/- mice. In contrast, while increases in MR+, Ym1+, and galectin-3+ macrophages were observed in WT mice following ozone exposure, no changes were observed in SP-D-/- mice. In both WT and SP-D-/- mice, aging was associated with reduced lung stiffness. Ozone exposure caused alterations in tissue mechanics in WT mice, and both airway and tissue mechanics in SP-D-/- mice. Loss of SP-D led to increased sensitivity to ozone up to 27 wk of age, however at 80 wk, this was overwhelmed by the larger effects of age-related increases in baseline inflammation and lung injury. Understanding how these responses are regulated could improve disease prognosis in those exposed to air pollutants.

DEDICATION

I dedicate this work to my family, friends, and lab mates. You are the amazing people in my life who I strive to be more like. You have made the load seem less heavy and the road less long.

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ABBREVIATIONS

ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage fluid
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
Cst	static compliance
CRD	carbohydrate recognition domain
DAMPS	danger-associated molecular patterns
E_L	pulmonary elastance
EPA	Environmental Protection Agency
η	hysteresivity
FEV-1	forced expiratory volume in 1 second
G	tissue damping
GdCl3	gadolinium chloride
GSG	glutathione
GSSG	oxidized glutathione
H	tissue elastance
HO-1	heme oxygenase-1
IFN-γ	interferon-gamma
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-4	interleukin-4
IL-6	interleukin-6

IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
LDH	lactate dehydrogenase
LPS	lipopolysaccharide non-steroidal anti-inflammatory drugs (NSAIDS)
MARCO	macrophage receptor with collagenous structure
MHC	major histocompatiblilty complex
MMP	matrix metalloproteinases
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NOS	nitric oxide synthase
NRF-2	NF-E2 related factor-2
PAMPS	pathogen associated molecular patterns
PCNA	proliferating cell nuclear antigen
PEEP	positive end-expiratory pressure
PGG2	prostaglandin G2
PGJ2	prostaglandin J2
PPAR-γ	peroxisome proliferator activated receptor-gamma
Pro-SP-C	pro-surfactant protein-C
R_L	pulmonary resistance
Rn	newtonian resistance
RNS	reactive nitrogen species
ROS	reactive oxygen species

SIRPα	signal inhibitory protein alpha
SOD	superoxide dismutase
SP-A	surfactant protein-A
SP-B	surfactant protein-B
SP-C	surfactant protein-C
STAT-1	signal transducers and activation of transcription-1
TGF-β	transforming growth factor-beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha,
Zrs	input impedance

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INTRODUCTION

LUNG STRUCTURE AND CELLS

The primary function of the lung is gas exchange, facilitating the movement of oxygen in and carbon dioxide out of the circulation. Structurally, the lung consists of a series of branching airways, which become more numerous as they travel deeper into the lung, and alveoli which serve as the blood-gas interface between the lung and the pulmonary blood vessels. The lung is divided into two compartments, the conducting zone and the respiratory zone. The conducting zone consists of the trachea, bronchi, bronchioles, and terminal bronchioles. It functions to direct air to and from the gas-exchange regions of the lung. Gas exchange takes place in the respiratory zone, which includes the respiratory bronchioles, the alveolar ducts and the alveolar sacs (West, 2008). The major gas exchange unit of the lung is the alveolus, which is comprised of multiple unique cell populations, each performing specialized functions. These cells include Type I and Type II alveolar epithelial cells, alveolar macrophages, and fibroblasts and blood vessel endothelial cells.

Most of the surface area of the alveolus is comprised of Type I cells, which participate in gas exchange. These cells are well suited for this function as a result of their thin, elongated morphology. Although these large squamous cells account for only ~10% of lung cells, they cover approximately 98% of the internal surface area (Dobbs et al., 2010). In addition to their barrier and gas exchange functions, Type I cells express ion channels and are capable of ion and water transport, suggesting that these cells play an important role in fluid transport in the lung. Additionally, Type I cells have been found to be capable of proliferating in vivo. Further analysis of these functions will

provide new insight into the contribution of Type I cells to lung homeostasis and response to injury (Dobbs et al., 2010). Because of their large surface area, Type I cells are highly sensitive to injury (Herzog et al., 2008).

Type II cells have a cuboidal morphology and are smaller than Type I cells. They are localized primarily at the corners of the alveolar sacs. Type II cells produce, secrete, and recycle the proteins and lipids that comprise pulmonary surfactant, including surfactant protein (SP)-B and SP-C, which ensure that the surface tension across the alveoli remains low, as well as SP-A and SP-D, which are important components of the innate immune system. Type II cells also prevent fluid accumulation in the alveolus and function as progenitor cells for injured type I cells. Additionally, they play an important role in innate immunity by releasing both antimicrobial and inflammatory mediators into the alveolar space including lysozyme, lipocalins, glutathione, complement, reactive nitrogen species (RNS), chemokines, growth factors, and cytokines such as interleukin (IL)-1β, IL-1α, tumor necrosis factor (TNF)-α, and IL-6 (Herzog et al., 2008; Mason, 2006; Whitsett et al., 2010). Deficits in Type II cell functioning disrupt surfactant homeostasis and underlie the pathogenesis of pulmonary disorders such as acute respiratory distress syndrome, interstitial lung disease and pulmonary alveolar proteinosis (Whitsett et al., 2010).

Macrophages play both homeostatic and pathogenic roles in the lung. Several distinct subpopulations have been identified including alveolar macrophages in the alveolus and interstitial macrophages. These populations have distinct locations and functions within the lung. Alveolar macrophages are located within the alveolar space. Here they are exposed to high oxygen concentration and are able to interact with inhaled

toxicants, particulate matter and pathogens, resulting in the production of reactive oxygen species (ROS), RNS and the expression of inflammatory cytokines, a response important for host defense (Franke-Ullmann et al., 1996; Laskin, Weinberger, et al., 2001). Interstitial macrophages are found in the interstitium of the lung, where they are in contact with extracellular matrix and connective tissue (Laskin, Weinberger, et al., 2001). Interstitial macrophages possess immunoregulatory functions, and are important for maintenance of immune homeostasis in the lung (Franke-Ullmann et al., 1996). Upon stimulation, they are capable of interacting with T-cells through major histocompatablilty (MHC) proteins and they express anti-inflammatory cytokines (Bedoret et al., 2009; Gea-Sorli et al., 2011). The ability of both populations of lung macrophages to phagocytose particulate matter and microbes, secrete cytokines, proteases, reactive oxygen and nitrogen species, and act as antigen presenting cells, enable them to initiate robust inflammatory responses and to restore lung homeostasis (Guth et al., 2009). Alveolar macrophages play a major role in this response. Because they continuously encounter inflammatory stimuli and irritants in inhaled air, alveolar macrophages act as pulmonary sentinels and play an important immunosurveillance function (Tomlinson et al., 2012). Turnover of the alveolar macrophage population is relatively slow; their half-life is 30-60 days, resulting in a long lived resident population (Bowden et al., 1980; Maus et al., 2006). Furthermore, a high proportion of resident alveolar macrophages persist in the lung following resolution of an inflammatory response, while recruited macrophages, that accumulate in the lung early during the inflammatory response, are transient (Janssen et al., 2011). Unique features of the lung environment dictate the characteristic phenotype of lung macrophages. Thus, as a consequence of exposure to surfactant proteins

possessing immunoregulatory properties such as SP-A and SP-D, alveolar macrophages exhibit enhanced bacterial uptake and killing in comparison to other macrophage populations (Guth et al., 2009; Wright, 2005).

Fibroblasts secrete extracellular matrix, providing structural support for the alveolus through a complex network of fibrous macromolecules including collagen, elastin, and proteoglycans (Suki et al., 2008). Extracellular matrix has been shown to be capable of modulating immune cell activation, migration, proliferation and differentiation (Sorokin, 2010). Furthermore, inflammatory cytokines and proteases such as matrix metalloproteinases (MMP)s released in inflamed tissues can result in modification of extracellular matrix and tissue remodeling (Morrison et al., 2009). Cleavage fragments of extracellular matrix have been found to attract neutrophils and macrophages in chronically inflamed emphysematous lungs (Houghton et al., 2006). Hyaluronan is produced by fibroblasts and is degraded by oxygen radicals. Low molecular weight hyaluronan fragments interact with toll-like receptors following lung injury, propagating the inflammatory response (D. Jiang et al., 2005). Collagen is the most abundant extracellular matrix component in the lung, providing most of the structural integrity of the alveolar wall (Suki et al., 2008). Collagen forms randomly arranged fibers and fibrils which vary in thickness and play an important load bearing role in the lung (Sobin et al., 1988). Elastic fibers are comprised of elastin, fibrillin and fibullin (Reinhardt et al., 1996). Elastin is composed of flexible cross-linked polypeptides. It is organized into easily extensible fibers that exhibit structural heterogeneity (Suki et al., 2008). The stiffness of elastin is approximately 2 orders of magnitude smaller than collagen. This is likely due to the more ordered structure of collagen fibers and increased fiber diameter

(Fung, 1993). Whereas elastin fibers contribute to lung elasticity at normal breathing volumes, collagen fibers acquire increased importance at larger volumes (Setnikar, 1955). Interaction among these components also contributes to the viscoelastic properties of lung tissue (Suki et al., 2008). As strain increases in the lung, tissue stress, which was originally borne by elastin fibers, is progressively taken up by collagen fibers, which start to straighten from their loose state and assume a load bearing role. The stiffness of the tissue therefore increases as more collagen fibers are recruited. The contribution of the organization and interaction of multiple types of fibers, rather than the individual properties of each fiber type, is considered the major determinant of tissue stiffness (Maksym et al., 1997). Altering the composition of the extracellular matrix through remodeling or direct damage causes a reorganization of stresses in the alveolar wall network affecting the mechanical behavior of the tissue and contributing to further progression of injury. Alveolar wall destruction during emphysema for example can concentrate stresses in this network leading to further fiber disruption (Gefen et al., 1999).

PULMONARY SURFACTANTS

Pulmonary surfactant fluid is composed of 90% lipids, the majority of which are phospholipids, and 10% proteins. Four surfactant proteins have been characterized: SP-A, SP-B, SP-C, and SP-D which perform distinct functions that contribute to the surface active and host defense properties of the surfactant fluid (Fehrenbach, 2001). Surfactant proteins are produced mainly by alveolar Type II cells. SP-B and SP-C function to lower surface tension in the alveolus during the cyclic expansion and compression of breathing

(Gustafsson et al., 2000). This enables efficient ventilation and alveolar stability. SP-A and SP-D have immunoregulatory functions. These proteins can bind to pathogens, damaging microbial membranes and facilitating their phagocytosis. They also regulate inflammatory signaling and apoptotic cell clearance by alveolar macrophages by binding to cell surface receptors including calreticulin, SIRPα, toll-like receptors, and mannose receptor (Chroneos et al., 2010). Reuptake and recycling of surfactant proteins takes place in Type II cells. Surfactant from the lining fluid layer is endocytosed by Type II cells and routed back to lamellar bodies and lysosomes for degradation (Baritussio et al., 1992). SP-C is produced by post-translational modification of pro-SP-C. This involves the addition of palmitic acid residues and cleavage of the C-terminal and N-terminal flanking regions (Beers et al., 2005). The mature SP-C protein is packaged in lamellar bodies which subsequently release their contents into the alveolar space (Weaver et al., 2001). Since pro-SP-C is exclusively produced by Type II cells, and is not secreted from the cell without being modified to produce mature SP-C, pro-SP-C is considered a specific marker for Type II cells (Beers et al., 1992).

Surfactant Protein-D

SP-D is a pulmonary collectin or C-type lectin. It is a 43-kDa protein composed of a short N-terminal collagenous domain, a coiled neck domain, and lectin head C-terminal carbohydrate recognition domain (CRD). It is synthesized by alveolar type II cells and non-cillated bronchoepithelial cells (E. C. Crouch, 2000). The collagenous domain allows for the formation of a triple helix between three monomers, resulting in the production of a trimer in which the head and tail regions are aligned. Four trimers assemble at their tail regions to form a dodecamer, stabilized through interchain disulfide

bonds of two conserved cysteines in the N-terminus tail domain. Additional assembly of subunits results in the formation of large multimeric structures (E. Crouch et al., 1994).

SP-D plays a key role in regulating the innate immune system in the lung. It can bind directly to microorganisms via a CRD region initiating microbicidal activities (E. C. Crouch, 2000). SP-D has been shown to bind LPS from gram negative bacteria, causing agglutination (Kuan et al., 1992). SP-D is also a critical component of pulmonary host defense. Levels of SP-D have been reported to be increased after Pneumocystis carinii infection (O'Riordan et al., 1995). Furthermore, the CRD of SP-D directly binds to a surface glycoprotein complex on Pneumocystis carinii which facilitates its binding to alveolar macrophages (O'Riordan et al., 1995). SP-D also has direct microbicidal effects on bacteria. It promotes the clearance of E. coli by increasing microbial membrane permeability (Wu et al., 2003). SP-D also stimulates the clearance of apoptotic cells by alveolar macrophages (Vandivier et al., 2002). SP-D has a dual function in the lung, engaging in both pro- and anti-inflammatory activities (Gardai et al., 2003). The nature of the response depends on the ability of the head and tail regions of SP-D to bind to different cell surface receptors. The CRD head domain of SP-D binds to signal inhibitory protein alpha (SIRPα) activating SHP-1, which suppresses p38 mitogen activated protein kinase activation and blocks nuclear factor (NF)-κB mediated transcription of inflammatory genes. Conversely, under conditions of oxidative stress, increases in nitric oxide lead to S-nitrosylation of two critical cysteines in SP-D, buried in the tail region of the multimer that are responsible for stabilization of the multimeric structure (Gardai et al., 2003). This disrupts the SP-D multimer and releases trimer subunits that are capable of interacting with receptors at their tail regions. These newly exposed tail regions of SP-

D trimers stimulate pro-inflammatory mediator production by binding to calreticulin/CD91 complexes, resulting in p38 phosphorylation and activation of NF-κB (Gardai et al., 2003). Thus, S-nitrosylated SP-D has been shown to promote inflammatory responses by functioning as a potent chemoattractant (Guo et al., 2008).

Mice deficient in SP-D exhibit altered surfactant homeostasis and abnormal alveolar macrophage and type II cell morphology, although surfactant activity remains intact (Botas et al., 1998). While fetal lungs from SP-D-/- mice appear indistinguishable from wild type control mice, by 3 weeks of age progressive histological changes begin to develop. Infiltrates of enlarged, foamy, and multinucleated macrophages are observed, as well as an accumulation of SP-A and SP-B. Enlarged lamellar bodies are also noted in alveolar regions. Type II cells are enlarged, hyperplastic, and contain enlarged lamellar bodies. At 8 weeks of age, the lungs of SP-D deficient mice exhibit 8-fold greater alveolar phospholipid pools, 4-fold greater bronchoalveolar lavage fluid (BAL) protein concentrations, and 4-fold greater numbers of alveolar macrophages. By 18 weeks of age, phospholipid content and BAL protein concentration remains stable, but by 24 weeks of age alveolar macrophages are increased 10-fold above levels observed in wild type mice (Botas et al., 1998). Subpleural, fibrotic lesions and altered elastin deposition are present at 30 weeks (Wert et al., 2000). These data, along with findings of increased oxidant production, MMP activity, and changes in parenchymal structure are consistent with the idea that the development of chronic pulmonary inflammation in these mice leads to parenchymal remodeling.

Mice lacking SP-D develop progressive pulmonary emphysema (Hawgood et al., 2002; Wert et al., 2000; Yoshida et al., 2001). This is supported by reports that lipid

hydroperoxidase, an indicator of oxidative stress, is increased in the lungs of SP-D-/- mice when compared to wild type mice. Furthermore, lung volume is increased; thus by 3 weeks, an increase in the percentage factional area of airspace compared to respiratory parenchyma is observed. This parenchymal destruction continues to increase with age; thus, by 12 weeks of age, SP-D-/- mice have significantly greater lung volumes then wild type mice (Wert et al., 2000). Macrophages isolated from lungs of SP-D-/- mice also display evidence of increased oxidant production and inducible nitric oxide synthase iNOS activity, consistent with inflammatory lung disease (Atochina et al., 2004). Hydrogen peroxide production, as well as MMP-9 and MMP-12 activity is increased in alveolar macrophages isolated from SP-D-/- mice (Wert et al., 2000). This is associated with increased NF-κB activity, suggesting that SP-D may regulate oxidative stress in the lung by modifying the production of inflammatory mediators generated by alveolar macrophages (Yoshida et al., 2001). Production of MMP, likely enhanced by NF-κB over activation in alveolar macrophages is thought to cause degradation of alveolar walls, leading to emphysema (Wert et al., 2000).

LUNG FUNCTION: RESPIRATORY MECHANICS

The mechanical properties of the lung are influenced by the viscous and elastic structural characteristics of the tissue, as well as forces acting at the air-liquid interface of the alveolar surface, and airway smooth muscle activity (O'Neil et al., 1984). Lung function has been classically measured in laboratory rodents by the parameters of pulmonary resistance (R_L) and elastance (E_L) (Bates et al., 2005; Glaab et al., 2007; Irvin et al., 2003). Resistive forces in the lung act to impede airflow and represent the energy

that is dissipated as air is moved into and out of the lung. Increases in resistance therefore reflect narrowing of the conducting airways. Elastance represents the elastic recoil pressure in the lung. This stored energy provides the driving pressure for expiration and reflects lung stiffness (Faffe et al., 2009). Compliance is the inverse of elastance reflecting the ease of expansion of the lung. For this simple resistive-elastic model, known as the single compartment model, measurements are made based on the assumption that the lung behaves as a single homogeneously ventilated elastic compartment served by a flow resistive pipe. The mechanical behavior of this model is described by relating transpulmonary pressure and airflow to resistance and compliance in the equation of motion. Multiple linear regression is then used to obtain the values of R_L and C_L (Bates et al., 2005; Irvin et al., 2003).

To assess differences between the central airways and the lung periphery, the forced oscillation technique is used. The animal is ventilated with an oscillatory sinusoidal waveform during pauses in regular ventilation. This allows for respiratory input impedance (Zrs), to be measured. Measuring Zrs allows analysis of the magnitude of the respiratory system over a range of frequencies, which represent distinct parts of the lung because different components of the respiratory system are ventilated at different frequencies. Measurements acquired at high frequencies reflect airway mechanics, while low frequencies reflect the properties of the parenchyma and small airways (Petak et al., 1993). Zrs consists of two components: the real part of Zrs which represents the resistance of the respiratory system over the given range of frequencies, and the imaginary part of Zrs which is the reactance and represents lung compliance (Collins et al., 2003; Glaab et al., 2007). These parameters reflect changes in respiratory mechanics,

but provide no information on the nature or location of these changes. Fitting Zrs data to the constant phase model enables one to distinguish between airway and tissue compartments. Changes in pulmonary mechanics representing alterations in either the central airways or the parenchyma can be determined using this technique (Glaab et al., 2007). One parameter derived from this model is Newtonian resistance (Rn), which reflects the resistance of the central airways and changes in response to airway narrowing. Tissue damping (G) which is a measure of viscous energy dissipation in the tissue can also be measured as a reflection of tissue resistance. Tissue elastance (H) reflects the elastive properties of the tissue and is a measurement of the stiffness of the lung. G and H increase in response to either changes in the physical properties of the tissue, to derecruitment as small airways close, or to heterogeneity in airflow. Hysteresivity can also be calculated from this model and is represented by the Greek letter eta (η). Eta is the ratio of G to H ($\eta = G/H$) and is a measure of the relationship between dissipative forces or resistive forces, and elastic forces of the lung. During inhalation, it takes a greater pressure to achieve a certain volume in the lung then it does during exhalation. This difference is hysteresis and represents the energy lost per cycle of respiration (Faffe et al., 2009).

In addition to these dynamic measurements, which provide information on the relationship between pressure and flow during inflation and deflation, the mechanical behavior of the lung can also be assessed for its static properties, when airflow is absent. A pressure volume curve, generated by slowly inflating the lung to total lung capacity and then deflating it to residual volume, is used to measure these static properties and analyze the relationship between pressure and volume (O'Neil et al., 1984). Changes in

the elastic properties of the lung tissue and changes in lung volume can be analyzed using these curves.

Positive end-expiratory pressure (PEEP) is the amount of pressure above atmospheric pressure left in the lung at the end of expiration. Increasing PEEP results in the opening or recruitment of closed lung units (Glaab et al., 2007). Furthermore, recruitment of lung units with increases in PEEP affect the resistive and elastive properties of the lung (S. Ito et al., 2007). The effects of altering PEEP on lung function can be evaluated to allow for assessment of heterogeneity in responsiveness and changes in airway and parenchymal mechanical properties (Massa et al., 2008).

Effects of Lung Injury on Pulmonary Mechanics

Due to the fact that the lung is continuously exposed to physiological stresses resulting from forces acting to keep it inflated during the cyclic act of breathing, the stress-bearing systems of the lung are essential to the functional properties of this organ. These systems, including the gas-liquid interface, the connective tissue matrix, and contractile elements, can be altered during the development of lung pathology, resulting in increasing heterogeneity in the mechanical properties of the lung, and impairments in lung functioning (Faffe et al., 2009). Resistance to expansion of lung units following lung injury, for instance by obstruction of an alveoli or bronchiole, causes the surrounding lung units to exert an increased inflationary stress on the lung unit. Thus, the tension and strain on the connective tissue of this unit alters the interdependence of the elements of the parenchymal network, preventing uniform expansion and increasing mechanical heterogeneity (Hubmayr, 2006). For example, unequal distribution of surface tension resulting from flooded alveoli and trapped air pockets or aberrations in surfactant

properties, also results in heterogeneous expansion of the lung, which affect mechanical behavior (Hubmayr, 2006). Additionally, injury can decrease the number of airspaces capable of expanding during inspiration, increasing the risk of overexpansion of surrounding air spaces leading to further deformation injury (Gattinoni et al., 1987). Moreover, extracellular matrix remodeling during injury alters the amount and ratio of collagen and elastin fibers, which each have different elastic properties, thus shifting the balance of stresses in the lung contributing to mechanical dysfunction (Negri et al., 2000).

A number of studies have reported that lung injury alters the dependence of pulmonary mechanics on changes in PEEP (Massa et al., 2008; Salerno et al., 2007; Shardonofsky et al., 2006). For example, changes in the collagen and elastin content of the lung, which occurs during tissue remodeling in response to inflammation or injury, alter the elastic recoil pressure and airway contractile response, as well as PEEP dependence of airway resistance and tissue elastance (Shardonofsky et al., 2006). Furthermore, these functional changes appear to be the result of alterations in the interaction between airways and parenchyma and reduced parenchymal tethering (Khan et al., 2010). Decreases in parenchymal tethering lead to increases in the magnitude and velocity of airway narrowing (Khan et al., 2007). These studies demonstrate that tissue remodeling during pathological processes alters interactions between airways and their parenchymal attachments which has significant effects on airway responsiveness.

INFLAMMATION AND INFLAMMATORY MEDIATORS

Inflammation is the response of the immune system to injurious or infectious agents. The four cardinal signs of inflammation are swelling, redness, heat, and pain, which reflect the actions of various inflammatory, neurogenic and cell signals. Mediators released from damaged cells and increased vascular permeability initiates the recruitment of leukocytes to sites of injury. One of the first cell types to arrive are neutrophils, which typically appear in the tissue within 6 hr (Yuen et al., 1996). These cells have potent antimicrobial activity and release pro-inflammatory mediators such as ROS, RNS, pro-inflammatory cytokines and proteases which aid in pathogen destruction. Neutrophils are short-lived cells, with a half-life of about 9-12 hr. They are replaced by macrophages, which arrive at sites of injury beginning 24 hr post injury or infection. Macrophages are relatively long-lived cells and can remain at sites of inflammation for prolonged periods of time. Like neutrophils, macrophages are highly phagocytic cells that engulf and destroy pathogens (X. Zhang et al., 2008). The process of inflammation is orchestrated by neutrophil and macrophage-derived mediators including cytokines, ROS, RNS, and pro-inflammatory lipids, and proteases. ROS and RNS are effector molecules generated to eliminate invading pathogens, however dysregulated, excessive production results in oxidative and nitosative stress and tissue injury. Release of proteases by macrophages and neutrophils and the generation of pro-inflammatory lipids have also been implicated in tissue injury. Pro-inflammatory cytokines and chemokines can initiate and intensify inflammatory responses. These mediators can recruit inflammatory cells to the site of inflammation and promote the production of additional inflammatory mediators (Medzhitov, 2010). Once the insult has been eliminated, these mediators can also

orchestrate the switch to resolve inflammation and initiate wound repair. The release of anti-inflammatory cytokines and lipid products works to re-establish homeostasis and stimulate the clearance of dead cells and debris. Growth factors are also produced to stimulate proliferation of fibroblasts and angiogenesis (P. Martin et al., 2005). If this process is dysregulated, the inflammatory response can persist, resulting in the development of a chronic inflammatory state.

Cytokines are cell secreted proteins which have important signaling functions, regulating inflammatory cell activation, promotion of cell growth, and stimulating the release of other inflammatory mediators (Dinarello, 2007). Major pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 are released early in the inflammatory process, amplifying the response. These mediators increase the expression of adhesion molecules to promote inflammatory cell recruitment, stimulate the production of ROS and RNS from macrophages and neutrophils, and augment the release of additional pro-inflammatory mediators (Bradley, 2008; Dinarello, 2007). TNF-α, together with interferon (IFN)-γ, is also able to induce the development of macrophages with a pro-inflammatory phenotype (Mosser et al., 2008). IFN-γ has also been found to enhance TNF-α expression and induce production of nitric oxide (Dinarello, 2000). IL-4, IL-10, and IL-13 are anti-inflammatory cytokines. Production of IL-10 during the inflammatory response acts to limit inflammatory responses by participating in a negative feedback loop, inhibiting the development of Th1 type responses. It can suppress the production of the pro-inflammatory cytokines IL-1 and TNF-α (Saraiva et al., 2010). These cytokines can also alternatively activate macrophages, resulting in a pro-resolution, wound repair phenotype (Mosser et al., 2008).

Eicosanoids are generated from polyunsaturated fatty acids, particularly arachidonic acid, and consist of prostaglandins, thromboxanes, leukotrienes and hydroxy acids. These compounds are important mediators of inflammation (Williams et al., 1988). Eicosanoids are generated during inflammatory reactions via the enzyme cyclooxygenase (COX)-2. COX-2 catalyzes the cyclooxygenation of arachadonic acid to generate prostaglandin (PG)G2. PGG2 is then reduced to PGH2 and several downstream PGs, prostacyclins and thromboxanes by various isomerases (S. F. Kim, 2011). COX-2 expression is induced in most tissues and in macrophages during inflammation by pro-inflammatory mediators such as lipopolysaccharide (LPS) and cytokines (Gilroy et al., 2000). COX-2 activity and expression is regulated by NF-κB, nitric oxide generated via iNOS and ROS (Higdon et al., 2012). Nitric oxide generated via iNOS can S-nitrosylate COX leading to an increase in its activity. Additionally, peroxynitrite generated via the interaction of nitric oxide and superoxide anion can modify COX-2 activity by interacting with either heme or tyrosine residues in the protein. Interaction of peroxynitrite with heme results in an increase in COX activity, whereas modification of key tyrosine residues leads to enzyme inactivation (S. F. Kim, 2011).

Eicosanoids mediate a number of inflammatory processes including vasoconstriction and dilation, coagulation, pain and fever (Funk, 2001). Findings that many of these processes are suppressed by inhibition of COX-2 using non-steroidal anti-inflammatory drugs (NSAIDS) demonstrate that COX-2 is involved in the generation of key inflammatory lipids (Funk, 2001). One major pro-inflammatory end product generated via COX-2 is PGE2. Production of PGE2 is increased in lung macrophages during inflammatory responses following exposure to toxicants such as LPS, ozone and

silica (Eliopoulos et al., 2002; Fakhrzadeh et al., 2002; Mohr et al., 1992). One mechanism by which PGE2 contributes to inflammation is induction of IL-6 (Hinson et al., 1996). In a carrageenan-induced paw inflammation model, neutralization of PGE2 resulted in an abrogation of both inflammation and IL-6 production, suggesting the pro-inflammatory effects of PGE2 involve induction of these cytokines (Portanova et al., 1996). Furthermore, the addition of PGE2 to mouse macrophage cultures induced IL-6 expression (Hinson et al., 1996). Studies with mice deficient in COX and with NSAIDS have also shown an anti-inflammatory role for COX-2 and some COX-2 derived eicosanoids (Gilroy et al., 2000). For example, PGJ2 has been found to bind to and activate peroxisome proliferator activated receptor (PPAR)-γ, a transcription factory important in the suppression of inflammatory mediator production, resulting in the reduction of several pro-inflammatory mediators (Zingarelli et al., 2005). In addition, PGJ2 has been reported to inhibit the synthesis of TNF-α, IL-1β, and IL-6 in monocytes and iNOS expression in macrophages (C. Jiang et al., 1998).

Reactive Oxygen and Nitrogen Species

ROS and RNS are generated by macrophages in response to activating stimuli such as IFN-γ, LPS, and TNF-α, or upon phagocytosis (Mantovani et al., 2004). They are also generated under conditions of oxidative stress, where their production overwhelms cellular protection mechanisms, such as the production of antioxidants. Under these conditions ROS and RNS contribute to the persistence of inflammation and tissue injury (Forman et al., 2001). ROS and RNS also have important physiological functions. These species can act as second messengers within cells to modulate function (Forman et al., 2001; Gow et al., 2004; Mikkelsen et al., 2003).

ROS include superoxide anion, hydroxyl radical, and hydrogen peroxide. In phagocytic cells, superoxide anion is generated from plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Superoxide dismutase catalyzes the formation of hydrogen peroxide from superoxide (Forman et al., 2001). Because hydrogen peroxide does not contain a radical, it has a longer diffusion distance which enables it to act as a second messenger. In the presence of a reduced transition metal hydrogen peroxide can react to form hydroxyl radical, a highly reactive radical, via the Fenton reaction.

Nitric oxide is generated via nitric oxide synthase (NOS) a family of enzymes that function to oxidize L-arginine to citrulline and nitric oxide (Laskin et al., 2010). These enzymes include an inducible form (iNOS), neuronal (nNOS), and endothelial (eNOS). iNOS is induced in inflammatory macrophages and neutrophils, as well as epithelial cells (Pautz et al., 2010). nNOS is constitutively expressed by nerve cells and skeletal muscle and plays a role in nerve signaling. eNOS is constitutively expressed by vascular endothelial cells and has important blood vessel dilation functions (Villanueva et al., 2010). iNOS, is involved in inflammatory responses and is up-regulated by inflammatory mediators including TNF-α, IFN-γ, IL-1β, as well as LPS and oxidative stress (Pautz et al., 2010). iNOS produces greater amounts of nitric oxide than neuronal and endothelial NOS. Regulation of iNOS can occur via modulation of transcription or the control of enzymatic activity (Pautz et al., 2010). The main mechanism of regulation is transcription. The promoter region of the iNOS gene contains binding sites for multiple transcription factors, including NF-κB and STAT-1α (Xie et al., 1994). NF-κB is a major target for regulating iNOS expression. LPS, IL-1β, TNF-α, and oxidative stress up-

regulate iNOS expression through NF-κB (Pautz et al., 2010). After iNOS induction, nitric oxide is continuously produced until the enzyme degrades (MacMicking et al., 1997). Another mechanism of iNOS regulation is through control of iNOS activity. For example, iNOS activity depends on substrate availability. Consumption of arginine by arginase has been shown to decrease iNOS activity (Mori et al., 2000).

Nitric oxide has a range of chemical reactivity, allowing it to participate in a wide variety of biological processes. Nitric oxide and superoxide anion can react to produce peroxynitrite, a highly reactive molecule with cytotoxic potential (Slauch, 2011). Nitric oxide can participate in cellular signaling through posttranslational modification of proteins. This can occur by binding to a metal center, nitrosylation of thiol groups and amines, nitration of tyrosine, and oxidation of thiols and tyrosine (Gow et al., 2004). Nitric oxide also has the ability to nitrosate the thiol side chain on cysteine residues to form an S-nirtrosothiol. This reaction occurs under conditions that promote S-nitrosylation, such as a close proximity of acidic and basic amino acids whose side chains flank the cysteine thiol, and the location of the cysteine in a hydrophobic pocket within the protein (Hess et al., 2005). S-nirtrosothiol formation can be accomplished by autooxidation of nitric oxide to a higher oxide of nitrogen forming a nitrosating intermediate (V. G. Kharitonov et al., 1995). This requires the interaction of two nitric oxide molecules and is therefore dependent on nitric oxide concentration, making it more likely to occur under inflammatory conditions where amounts of nitric oxide are dramatically increased (Gow et al., 2004). S-nitrosylation has been established as a cellular signaling mechanism. Thus, it acts with specificity, reversibility, and by an enzyme-controlled mechanism leading to activation or inhibition. A number of proteins

with varied functions including kinases, transcription factors, and enzymes have been found to be modified via S-nitrosothiol formation (Stamler et al., 1997).

RNS generated via iNOS play a dual role in the pathogenesis of inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (S. A. Kharitonov et al., 1994). Excessive production of nitric oxide can result in S-nitrosylation of a number of proteins including NF-κB. This down regulates its activity and suppresses inflammation (Gaston et al., 2006). Conversely, excessive nitric oxide production resulting from increased iNOS activity has also been reported to promote disease pathogenesis. In COPD, the formation of RNS from iNOS-derived nitric oxide is associated with oxidative stress, inflammation, MMP activation and inactivation of antiproteases (Sugiura et al., 2011). In fact, in patients with COPD, it has been demonstrated that Type II cells, neutrophils, and bronchial epithelial cells expressed increased amounts of iNOS, when compared to healthy subjects (Maestrelli et al., 2003; Ricciardolo et al., 2005). Moreover, mice lacking iNOS activity through selective inhibition or gene knock out are protected from emphysema induced by loss of SP-D or tobacco smoke (Atochina-Vasserman et al., 2007; Seimetz et al., 2011). Similarly, iNOS expression increases in alveolar macrophages following exposure to pulmonary toxicants such as LPS, ozone, silica, asbestos, diesel exhaust particulates and vesicants. (D'Alessio et al., 2012; Fakhrzadeh et al., 2002; Malaviya et al., 2012; Quinlan et al., 1998; Srivastava et al., 2002; V. R. Sunil, Shen, et al., 2012; H. Zhao et al., 2009). Exposure of mice to LPS results in acute pulmonary inflammation, as well as increased expression of iNOS in alveolar macrophages. In contrast, in mice lacking iNOS, early pulmonary injury is attenuated, however resolution of inflammation is impaired and mortality increased

(D'Alessio et al., 2012). Exposure of mice to ozone also results in increased pulmonary inflammation and macrophage accumulation, while mice lacking iNOS are protected from inflammation and injury (Fakhrzadeh et al., 2002). Similarly, exposure of rodents to pulmonary toxicants such as silica, asbestos, diesel exhaust particulates or nitrogen mustard has been reported to result in pulmonary inflammation, as well as iNOS expression and nitric oxide production by alveolar macrophages (Malaviya et al., 2012; Quinlan et al., 1998; Srivastava et al., 2002). Silica induced pulmonary inflammation is reduced in mice lacking iNOS (Srivastava et al., 2002). Inhibition of iNOS with N^G-monomethyl-l-arginine prevents asbestos induced neutrophil influx (Quinlan et al., 1998), while inhibition of iNOS by aminoguanidine reduces nitrogen mustard induced oxidative stress, inflammation and injury (Malaviya et al., 2012). Neutrophil accumulation and production of pro-inflammatory cytokines by alveolar macrophages was reduced in iNOS-/- mice exposed to diesel exhaust particles, when compared to WT mice (H. Zhao et al., 2009). These findings support the idea that iNOS plays an important role in the cytotoxic functioning of macrophages that can potentiate lung injury when dysregulated.

OXIDATIVE STRESS AND ANTIOXIDANTS

ROS and RNS are produced in the body under physiologic conditions by mitochondrial respiration; however these species are also produced during inflammation by immune cells such as macrophages and neutrophils. In order to prevent tissue damage following the generation of these reactive species, an antioxidant defense system is in place to convert these highly reactive radicals to less injurious compounds. There are several major antioxidants in the body that involved in this activity. Superoxide

dismutase (SOD) converts superoxide anion to hydrogen peroxide, while catalase generates O_2 and water from hydrogen peroxide. Glutathione peroxidase also helps to eliminate hydrogen peroxide (Aruoma, 1998). This occurs when glutathone peroxidase catalyzes the conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase then recycles GSSG back to GSH. GSH is also able to eliminate the highly reactive peroxynitrite, by reduction to nitrate (Rahman et al., 2000). Oxidative stress is a condition that develops when the generation of reactive species outweighs these oxidative defense mechanisms in the body. Oxidative stress can result in cell damage and death (Aruoma, 1998). Not only does failure to eliminate ROS and RNS result in damage to proteins, lipids and DNA by these species, transcription factors important in the generation of pro-inflammatory mediators, such as NF-κB and AP-1 are redox sensitive and are activated under these conditions (Rahman et al., 2000).

Heme oxygenase (HO)-1

HO-1 is an inducible oxidative stress defense enzyme that catalyzes the degradation of heme into biliverdin, iron and carbon monoxide. The antioxidant properties of HO-1 are the result of the biological effects of these end products, produced during heme catabolism (Fredenburgh et al., 2007; Gozzelino et al., 2010). Oxidative stress can lead to the release of heme from some hemoproteins, resulting in cytotoxic free heme (Baker et al., 2003). Heme contains a Fe2+ atom capable of producing highly toxic hydroxyl radicals from hydrogen peroxide. HO-1 catabolizes free heme, preventing it from exerting pro-oxidant effects on surrounding cells and tissues, by cleaving the photoporphyrin IX ring, producing biliverdin and free iron (Gozzelino et al., 2010). Free iron, released by HO-1, is sequestered by ferritin, oxidizing Fe2+ to Fe3+,

neutralizing the oxidant activity of iron (Baker et al., 2003). Bilverdin is then converted to bilirubin by biliverdin reductase. Bilirubin is an antioxidant and has been shown to play an anti-inflammatory role in the lung (Kapitulnik et al., 2009). Heme catabolism also results in the production of carbon monoxide, which also exhibits anti-inflammatory properties (Otterbein et al., 2000). The cytoprotective functions of carbon monoxide also result from its ability to interact with Fe2+, as well as promote further HMOX1 gene (which encodes HO-1) transcription. HO-1 is induced by oxidative stress. ROS releases repressors on the promoter region of the HMOX1 gene leading to the degradation of Keap-1, and the translocation of the transcription factor NF-E2 related factor-2 (NRF-2) to the nucleus; this results in HMOX1 transcription (Choi et al., 1996).

In the lung HO-1 is expressed by Type II cells and macrophages in response to pro-inflammatory cytokines, LPS, nitric oxide, hypoxia, hyperoxia, and heme (Fredenburgh et al., 2007). Furthermore HO-1 has been reported to be altered in pulmonary disorders such as COPD, asthma, idiopathic pulmonary fibrosis, and acute respiratory distress syndrome. In COPD, dysregulation in antioxidant defense systems are thought to contribute to disease pathogenesis (Fredenburgh et al., 2007). Macrophages isolated from BAL of patients suffering from COPD express reduced levels of HO-1 when compared to healthy individuals (Maestrelli et al., 2003). Furthermore, over-expression of HO-1 in mice reduces elastase-induced emphysema, attenuating neutrophil migration into the lung and pro-inflammatory cytokine release, while increasing anti-inflammatory cytokines in BAL (Shinohara et al., 2005).

MACROPHAGES

Macrophages are mononuclear phagocytes derived from hematopoietic stem cells in the bone marrow. These cells mature into monocytes in the peripheral blood and differentiate into mature macrophages when they migrate into tissues (Geissmann et al., 2010). Mature macrophage populations are found in all tissues of the body. The largest populations are found in the liver and the lung. Macrophages are highly plastic cells which develop specialized functional properties in response to signals they encounter in their microenvironment. For example, lung macrophages display high microbicidal, and antigen presentation activities, important for host defense against inhaled pathogens (Guth et al., 2009). Kupffer cells, located in hepatic sinusoids are essential for the removal of foreign material from the portal circulation (Laskin, Weinberger, et al., 2001). Thus, these cells are efficient at phagocytosing particles. Kupffer cells are also antigen presenting cells and possess cytotoxic capabilities (Naito et al., 2004). Gluth et al. (2009) showed that the lung environment is responsible for the unique phenotype of alveolar macrophages. These cells are dendritic-like in that they express high levels of CD11c. The observation that peritoneal macrophages or bone marrow derived macrophages adoptively transplanted into airways of donor mice upregulate CD11c, characteristic of alveolar macrophages, provides strong support for the idea that the microenvironment can control macrophage biological activity.

Macrophages act as the sentinels of the innate immune system, providing immediate host defense by recognizing common microbial motifs, engulfing pathogens and foreign materials and initiating inflammatory responses (Mantovani et al., 2004; T. R. Martin et al., 2005; X. Zhang et al., 2008). Macrophages also contribute to tissue

repair and wound healing. Macrophages are active secretory cells releasing an array of mediators that function to attract and activate other cells of the immune system (e.g., chemokines), as well as ROS and RNS, pro- and anti-inflammatory cytokines, proteases, and bioactive lipids that contribute to their functional activity (Mosser et al., 2008). Macrophages are also considered professional antigen presenting cells expressing major histocompatibility complex proteins, and activating T-helper lymphocytes, contributing to the regulation of adaptive immunity.

Evidence suggests that the diverse activities of macrophages are mediated by distinct subpopulations. Macrophages have been generally classified into two broad functional groups, classically activated M1 macrophages and alternatively activated M2 macrophages (Cassetta et al., 2011; Martinez et al., 2008; Mosser et al., 2008). Classically activated M1 macrophages develop in response to stimulation by IFN-γ and either pathogen associated molecular patterns (PAMPS) such as LPS, the pro-inflammatory cytokine TNF-α, or GM-CSF. These cells are highly microbicidal, and generate cytotoxic effector molecules, including ROS and RNS, and pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, and IL-1 (Cassetta et al., 2011; Mantovani et al., 2007; Martinez et al., 2008). Classical macrophage activation is augmented and sustained by IFN-γ generated by T cells and natural killer cells. IFN-γ activates the signal transducers and activation of transcription (STAT)-1 signal transduction pathway resulting in the expression of iNOS and the production of nitric oxide (Mosser et al., 2008).

Although classically activated M1 macrophages release pro-inflammatory cytokines, ROS, proteases and pro-inflammatory lipids to protect the host, excess,

production of these products can exacerbate tissue injury. In the lung exposure to a variety of toxicants results in an accumulation of M1 macrophages which release pro-inflammatory mediators. Furthermore, blocking or preventing their ability to produce these mediators ameliorates tissue injury induced by these agents (Laskin et al., 2011). For example, ozone inhalation results in increased numbers of alveolar macrophages in the lung (Fakhrzadeh et al., 2002). Additionally, production of nitric oxide and superoxide anion, and expression of TNF-α, and IL-1 by these cells is enhanced and correlates with tissue injury (Fakhrzadeh, Laskin, & Laskin, 2004; Pendino et al., 1994). Elimination of M1 macrophages by treatment of animals with gadolinium chloride (GdCl3) abrogates ozone-induced inflammation, reducing expression of iNOS and TNF-α, as well as production of nitric oxide and superoxide anion (Pendino et al., 1995). Moreover, ozone-induced inflammation is abrogated in mice lacking iNOS, a response correlated with decreased numbers of alveolar macrophages. Furthermore these cells do not produce nitric oxide or RNS (Fakhrzadeh et al., 2002). Macrophages also contribute to lung injury following exposure to LPS. Mice lacking M1 macrophage activation receptor CD40 exhibit reduced LPS-induced lung injury; release of TNF-α, IL-1β, and macrophage inflammatory protein (MIP) by macrophages is deficient in these mice following LPS exposure (Hashimoto et al., 2004).

Alternatively activated M2 macrophages, are important in down regulating inflammation and inducing tissue repair and remodeling. M2 macrophages are subdivided into M2a, M2b, and M2c subpopulations. M2a cells develop in response to IL-4 and IL-13 which antagonize the actions of IFN-γ. M2a cells promote fibrogenesis, tissue repair, and proliferation. They are also important in antigen presentation and

express mannose receptor and MHCII (S. Gordon, 2003; Mantovani et al., 2004). M2b cells develop in response to immune complexes and triggering of toll-like receptors (TLR). These cells are involved in immunoregulation, promotion of type II inflammatory responses and release both IL-10 and pro-inflammatory cytokines such as TNF-α and IL-6. M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β), and glucocorticoids. These cells inhibit the production of pro-inflammatory cytokines, down regulating inflammation and promoting tissue repair and regeneration (Mantovani et al., 2004; Martinez et al., 2008).

Alternatively activated macrophages have also been implicated in tissue injury. Prolonged or exaggerated M2 macrophage responses can contribute to and sustain chronic inflammatory conditions (Laskin et al., 2011). For example, in models of silica and bleomycin-induced pulmonary fibrosis, macrophage expression of markers of M2 activation is increased (Hancock et al., 1998; Misson et al., 2004). Inhibition of M2 macrophages by serum amyloid P attenuates bleomycin-induced fibrosis in mice (Murray et al., 2010), while mice deficient in IL-4R, which is required for M2 activation, do not develop silica-induced fibrosis (Migliaccio et al., 2008).

M1 and M2 macrophages can also be distinguished by the differential metabolism of L-arginine. In M1 macrophages arginine is converted to citrulline and nitric oxide via iNOS, which is important in host defense against pathogens. In M2 macrophages, arginine plays an important restorative function. It is converted by arginase to urea and ornithine, a precursor for polyamines and proline, which regulate cell proliferation and collagen production. Since the production of nitric oxide and ornithine depends on the availability of arginine, these activities can be reciprocally regulated in macrophages by

cytokines that influence macrophage polarization. It has been shown that the M1 cytokines IFN-γ and TNF-α induce iNOS and the synthesis of nitric oxide, whereas the M2 cytokines IL-4, IL-13, and IL-10 induce arginase (Mori et al., 2004; Morris et al., 1998; Munder et al., 1998).

PHENOTYPIC MARKERS OF MACROPHAGE ACTIVATION

Several markers have been identified that are specific to either classically or alternatively macrophage activation. Macrophage polarization not only results in expression of different cytokines and inflammatory mediators, but also alters expression of receptors and markers on the cell surface, which aid in the identification of these unique populations (Cassetta et al., 2011). Classical activation of macrophages results in a population that express iNOS and have enhanced pro-inflammatory and microbicidal activity, while macrophages with an alternatively phenotype express increased amounts of YM-1, mannose receptor, and in some cases galectin-3, which have been found to be induced by Th2 cytokines, such as IL-4 and IL-13 (Mosser et al., 2008).

Mannose Receptor

Mannose receptor is a member of the c-type lectin superfamily and was originally described as an endocytic receptor on alveolar macrophages, but is also expressed by macrophages in other tissues and by endothelial cells of the liver and lymphatic system and by kidney mesangial cells (East et al., 2002). Mannose receptor possesses three types of extracellular binding domains. The N-terminal cysteine rich domain is capable of binding sulfated sugars, the fibronectin type II domain is involved in collagen binding, and the c-type lectin-like domains bind sugars that are terminated in D-mannose, L-

frucose, and N-acetyl glucosamine (Gazi et al., 2009). These domains allow mannose receptor to bind to and internalize a variety of exogenous and endogenous materials, including hormones and ligands on immune cells and microorganisms (Taylor et al., 2005). Because of its ability to bind to mannose on the surface of microbes, mannose receptor is considered to be a pattern recognition receptor (Gazi et al., 2009). Its functions as an endocytic receptor also allow mannose receptor to play a role in antigen presentation by routing ligands for MHC processing through the endocytic pathway (Apostolopoulos et al., 2000). Other cellular responses elicited by mannose receptor binding include phagocytosis, cell migration, and intracellular signaling (Gazi et al., 2009; Le Cabec et al., 2005).

It has been suggested that mannose receptor functions not to propagate danger signals but rather to maintain homeostasis by facilitating quiet and anti-inflammatory clearance through activation of multiple cellular functions (Varin et al., 2009). Expression of mannose receptor expression is characteristic of an alternatively activated macrophage phenotype, as this type of activation leads to alterations in receptors associated with modification of phagocytosis and membrane trafficking (Varin et al., 2009). Studies have shown that the anti-inflammatory cytokines which promote alternative macrophage activation including IL-4, IL-13 and IL-10, upregulate mannose receptor expression and endocytosis in these cells, while expression is down regulated by classical macrophage activators such as pro-inflammatory cytokines (Chieppa et al., 2003; Doyle et al., 1994; Stein et al., 1992). Activation of mannose receptor induces the production of IL-10 and IL-1R agonist, while suppressing the expression of pro-inflammatory IL-1β and TNF-α in dendritic cells, a response that dampens inflammation

and inhibit sTh-1 activation (Chieppa et al., 2003). Furthermore, mice lacking mannose receptor have increased levels of glycoproteins associated with inflammation, suggesting that clearance of reactive substances by mannose receptor plays an important role in maintaining homeostasis (S. J. Lee et al., 2002). Interestingly, during chronic inflammation, mannose receptor has also been shown to mediate cell fusion of alternatively activated macrophages. This results in the formation of multinucleated giant cells (McNally et al., 2011). Mannose receptor participates in endoplasmic reticulum mediated phagocytosis that results in macrophage fusion (McNally et al., 2005). Furthermore, the formation of these giant cells is prevented by inhibitors of mannose receptor functioning (McNally et al., 1996).

YM-1

YM-1 is a member of the 18 glycoslyhydrolase family of chitenases and chitenase-like proteins. Unlike true chitenases, which can hydrolyse chiten, YM-1 lacks both a chiten binding domain and critical amino acids in its catalytic site, and is therefore not capable of chitenase enzymatic activity (Kzhyshkowska et al., 2007). YM-1 is a rodent specific protein and an analogous chitenase-like protein has not been identified in humans (C. G. Lee et al., 2011). YM-1 is expressed in the lung, spleen and bone marrow. In the lung, YM-1 is expressed by proximal airway epithelial cells and alveolar macrophages (Homer et al., 2006; Nio et al., 2004). The biological functions of YM-1 have yet to be fully elucidated. Expression of YM-1 by alveolar macrophages increases in response to parasite infection and in eosinophil mediated inflammation (C. G. Lee et al., 2011). YM-1 is expressed by alternatively activated macrophages, and its expression is up-regulated by IL-4 and IL-13 (Raes et al., 2002). This has been shown to occur

through a STAT-6 dependent mechanism (Dasgupta et al., 2011). It has also been reported that the promoter for YM-1 contains a STAT-6 binding region and that YM-1 mRNA expression in macrophages decreased in STAT-6 deficient mice (Welch et al., 2002).

YM-1 may also play a role in mediating inflammatory responses. YM-1 is chemotactic for neutrophils, eosinophils and T cells (Nio et al., 2004). In mouse models of allergic airway inflammation, mRNA and protein expression of YM-1 increases following ova sensitization (Agapov et al., 2009; J. Zhao et al., 2005). Furthermore, treatment of ova sensitized mice with N-acetylcysteine, which prevented activation of NF-κB by iNOS, decreased YM-1 expression (L. Zhang et al., 2009). NF-κB inhibition via plant flavonoids was also found to decrease YM-1 expression in ova sentsitized mouse lungs (Goh et al., 2012). These studies indicate a potential role for this protein in response to oxidative stress and suggest that YM-1 may play an important role in Th2 responses and in chronic inflammation in the lung.

Galectin-3

Galectin-3 is a member of the galectin family that possesses as C-terminal carbohydrate recognition domain. Upon ligand binding to the carbohydrate recognition domain, galectin-3 is capable of forming dimers and higher order oligomers through its N-terminal regulatory domain, resulting in a protein with multivalent carbohydrate recognition domains and cross-linking of oligosaccharides on the cell surface (Ahmad et al., 2004). The ability to dimerize allows galectin-3 to perform a variety of functions. Galectin-3 regulates cell activation and cell adhesion through three different modes of action: receptor clustering, lattice formation and cell-cell interactions (Nieminen et al.,

2007). Galectin-3 can localize in either the cytoplasm or nucleus, or can be released extracellularly. Because of its ability to regulate cell activation, cell migration, and apoptosis, galectin-3 plays an important role in inflammation (Henderson et al., 2009). In this regard, galectin-3 has been shown to contribute to neutrophil and mast cell activation, macrophage chemotaxis, opsonization of apoptotic cells and microbial clearance (Henderson et al., 2009). Furthermore, galectin-3 oligomerization activates cell signaling pathways involved in neutrophil oxidative burst and mast cell degranulation (Frigeri et al., 1993; Yamaoka et al., 1995). Galectin-3 also plays a role in macrophage polarization. However, whether galectin-3 promotes classical or alternative activation depends on the tissue (Henderson et al., 2009). Thus, in the liver galectin-3 expression in response to acetaminophen-induced inflammation is not only correlated with expression of markers of classically activated macrophages, mice lacking galectin-3 exhibit reduced hepatotoxicity and have decreased expression of pro-inflammatory proteins in the liver (Dragomir et al., 2012). In the lung, however, galectin-3 expression is associated with airway remodeling, characteristic of chronic inflammation in mouse models of asthma, as well as expression of FIZZ-1 in alveolar macrophages, an alternative activation marker (Ge et al., 2010). Additionally, exposure of rats to ozone increases markers of alternatively macrophage activation, as well as macrophage expression of galectin-3 (V. R. Sunil, Patel-Vayas, et al., 2012). The ability of IL-4 and IL-13 to induce an alternatively activated phenotype is impaired in alveolar macrophages from mice lacking galectin-3, although classical activation was not affected. Additionally, classical activation of macrophages by IFN-γ and LPS decreased galectin-3 expression, while IL-4 and IL-13 increased galectin-3 expression (MacKinnon et al.,

2008). Galectin-3 is also involved in chronic inflammatory processes. Alternative activation of macrophages is associated with increased fibrogenesis. Studies on galectin-3 in fibrogenesis have shown both increases and decreases in fibrogenesis with induction of galectin-3 expression (Henderson et al., 2006; Lopez et al., 2006). This discrepancy is thought to be the result of the fact that galectin-3 expression was analyzed in different organs with different disease pathogenesis.

AGING AND INNATE IMMUNITY

Aging involves a progressive decline in physiological functioning and is associated with the development of a number of pathologies. The free radical theory of aging and the oxidative stress hypothesis suggest that a loss of control in the production and regulation of reactive oxygen and nitrogen species and reactive lipids play key roles in the development of age-related disease (H. Y. Chung et al., 2009; Franceschi et al., 2000). According to these theories, impairments in antioxidant defense systems with age result in redox imbalance and oxidative stress. Altered redox status leads to increased production of pro-inflammatory mediators and dysregulated immune functioning (H. Y. Chung et al., 2009). Thus, oxidative stress and loss of antioxidant defenses with age result in increased production of ROS and RNS leading to an accumulation of oxidatively modified proteins, lipids, and DNA (Lavrovsky et al., 2000; Squier, 2001; Stadtman, 2004). The interaction of reactive species with proteins, lipids and nucleic acids compromises their functioning, which contributes to age-related physiological declines (Kelly et al., 2003; Squier, 2001; Stadtman, 2004). For example, in human fibroblasts, levels of oxidized proteins are significantly greater in cells from subjects over the age of

60, when compared to young and middle-aged donors. Oxidative modification of the enzyme glucose-6-phosphate dehydrogenase induces heat liability and loss of functional enzyme activity (Oliver et al., 1987).

Antioxidant defense systems have been reported to decrease with age (H. Y. Chung et al., 2009). Age related alterations in HO-1 expression and function have also been described, however the nature of these changes depends on the tissue and cell type. Thus, LPS induced increases in HO-1 expression in alveolar macrophages were found to be impaired with age in mice, however no age related differences were observed in bronchiolar epithelial cells (Y. Ito et al., 2009). Decreases in HO-1 expression with age have also been demonstrated in rat brain hippocampus and substantia nigra, as well as mouse kidney (Ewing et al., 2006; Ferenbach et al., 2011). In contrast, HO-1 expression increases with age in rat livers and Kupffer cells (Bloomer et al., 2009; Lavrovsky et al., 2000). Additionally, senescence accelerated mice express decreased levels of HO-1 compared with normally aging mice (Bayram et al., 2012). These findings suggest an important role for HO-1 in the regulation of oxidative species during aging.

Cellular senescence results in cessation of proliferation and has been reported to contribute to the process of aging (Freund et al., 2010; Harley et al., 1990; Rodier et al., 2011). Senescent cells differ from quiescent cells in that cell cycle arrest is irreversible (Rodier et al., 2011). Senescence is the result of incremental loss of telomeres, the DNA at the ends of chromosomes, with each S phase of cell division. Because DNA polymerases are unidirectional, they cannot prime a new strand at the end of a chromosome, resulting in loss of DNA (Campisi et al., 2007). Senescence can contribute to functional deficits by depleting tissues of progenitor cells. This results in

compromised tissue regeneration and repair (Drummond-Barbosa, 2008). In addition to loss of proliferative capacity, senescent cells acquire a "senescent-associated secretory phenotype", releasing growth factors, chemokines, cytokines, and proteases (Kumar et al., 1992). Many of these proteins are pro-inflammatory, including IL-6, IL-1, GM-CSF, and macrophage chemotactic proteins. Senescent cells are therefore thought to be one factor contributing to chronic inflammation with age (Freund et al., 2010).

Aberrations in the innate immune system are a hallmark of the aging process. Dysfunctional orchestration of the immune response by cells of the innate immune system reduce the ability of the host to defend against pathogens and xenobiotics and initiate wound healing and tissue repair (Lloberas et al., 2002; Plowden et al., 2004; Stout & Suttles, 2005). Macrophage function is known to be altered with age, which has implications for inflammation, the control of ROS and RNS, and the clearance of pathogens (Gomez et al., 2008; Kovacs et al., 2009). The nature of these changes however, depends on the tissue the macrophages are localized in and on how they respond to differentiating and inflammatory signals (Plowden et al., 2004; Sebastian et al., 2005). Alterations in phagocytosis, chemotactic activity, and cytokine production, and the ability of macrophages to respond to Th1 and Th2 stimuli are reported to be altered with age which results in aberrant orchestration of the inflammatory response (Aprahamian et al., 2008; Mahbub et al., 2012; Ortega et al., 2000; Stout & Suttles, 2005). In neutrophils phagocytosis, chemotaxis and free radical production decreases with age (Fulop et al., 2004). Although numbers of natural killer cells increase with age, production of IFN-γ, IL-2, IL-8, macrophage inflammatory protein (MIP) and RANTES decrease (Panda et al., 2009).

The transcription factor NF-κB, is a major regulator of inflammatory gene expression in macrophages (Makarov, 2000). Age-related increases in NF-κB activity have been described in the heart, liver, kidney, and brain (Giardina et al., 2002). Furthermore, ROS and PGE2 production via COX-2 increase with age in macrophages and rat kidney have been shown to result from age related increases in NF-κB activation (H. J. Kim et al., 2000). Additionally, in humans IL-6, TNF-α and IL-1, pro-inflammatory mediators regulated by NF-κB, are increased in serum in aged, when compared to young individuals (Maggio et al., 2006).

Aging is a risk factor for many chronic diseases. Age related dysfunction has been found to contribute to the development of cancer, atherosclerosis, diabetes, arthritis, and others (H. Y. Chung et al., 2009). For example, increasing adiposity with age contributes to the development of obesity (Weiss, 2007). Furthermore, the production of pro-inflammatory cytokines by macrophages and adipocytes may increase systemic inflammation and susceptibility to other age related disorders (H. Y. Chung et al., 2009; Greenberg et al., 2006). Alterations in the regulation of expression of adhesion molecules with age have been found to contribute to the pathogenesis of vascular disorders such as atherosclerosis (Zou et al., 2006). Furthermore, chronic inflammation plays a critical role in the development of atherosclerosis, affecting functions such as leukocyte recruitment to the vascular endothelium, to production of chemotactic proteins by smooth muscle cells (H. Y. Chung et al., 2009).

Effects of Aging on Macrophage Functioning

Macrophages are known to play a key role in both acute and chronic inflammatory pathologies, and their ability to respond to environmental signals has been

reported to become altered with age (Gomez et al., 2008; Kovacs et al., 2009). Age-dependent alterations in chemotaxis, phagocytosis, bactericidal activity, ROS and cytokine production, and phenotype have also been described in macrophages (Brubaker et al., 2011; Stout & Suttles, 2005). However, the nature of these changes is unclear, as varied and conflicting results have been reported (Sebastian et al., 2005). Among the factors that contribute to the varied findings are species, strain, and gender, as well as the tissue origin, maturation state, activating stimulus and the method used to collect the cells.

One of the largest determinates of age related changes in macrophage function is their origin. Tissue environment has been shown to significantly influence macrophage function, affecting antigen presentation, phagocytosis, and cytokine production. (Guth et al., 2009; Laskin & Laskin, 2001; Stout, Jiang, et al., 2005). Studies on the effects of aging on macrophage activity, typically carried out on peritoneal, splenic, alveolar, and bone marrow-derived macrophages, have produced varied results. For example, Kohut et al. (2004) compared LPS-induced production of the pro-inflammatory cytokines IL-1, IL-12 and TNF-α by alveolar, peritoneal and splenic macrophages; these investigators found that while cytokine production was increased in alveolar and splenic macrophages from old mice, when compared to young mice, peritoneal macrophages exhibited decreased responsiveness with age. Similar reports of decreased production of TNF-α and IL-6 have been described in peritoneal macrophages with age (Boehmer et al., 2004; Cecilio et al., 2011). Additionally, while LPS stimulated expression of NOS is decreased in splenic macrophages with age (Mahbub et al., 2012), in peritoneal and alveolar macrophages nitric oxide production and iNOS expression is increased (L. C. Chen et al.,

1996; Kohut et al., 2004; Qureshi et al., 2011; Smallwood et al., 2011). These reports suggest that in vitro stimulation by LPS results in an amplification of pro-inflammatory activity in mouse alveolar macrophages with age. This is supported by a study showing age related increases in the abundance of a number of proteins known to play central roles in promoting macrophage activation towards a classically activated phenotype, as well as a report of reduced expression of the antioxidant response enzyme HO-1 (Y. Ito et al., 2009; Smallwood et al., 2011). In contrast, in mouse peritoneal macrophages stimulated with LPS, there appears to be a general decline in responsiveness. Thus, iNOS and proinflammatory cytokine expression, as well as arginase-1 activity decreases with age (Boehmer et al., 2004; Cecilio et al., 2011; Kissin et al., 1997; Kohut et al., 2004; Smallwood et al., 2011).

Species differences are also observed in the response of macrophages from aged animals to activating stimuli. In contrast to mice, the inflammatory response of rat alveolar macrophages to LPS decreases with age. Thus, expression of TNF-α is reduced, as well as nitric oxide and superoxide anion production (Antonini et al., 2001; Corsini et al., 1999; Hayakawa et al., 1995). A similar dichotomy between mice and rats is observed in peritoneal macrophages; however the response is opposite to alveolar macrophages. Thus, increases, rather than decreases, are observed in TNF-α expression in rat peritoneal macrophages, suggesting that, in rats, pro-inflammatory responses to stimulation are increased with age (Tang et al., 2000).

The nature of the method used to assay macrophage activity may also explain the divergence in the responses observed in macrophages with age. For example, in many studies with peritoneal macrophages, the cells are collected after they are elicited with

thioglycollate, while in others resident macrophages are analyzed (Boehmer et al., 2004; Kohut et al., 2004). Thioglycollate has been shown to not only activate peritoneal macrophages by increasing intracellular ATP, lysosomal hydrolases, and C3 mediated phagocytosis, but also to negatively affect cytolysis capabilities (Shaw et al., 1982). These alterations could impact the cells response to further stimulation by LPS or the other activating stimuli used in these studies. This is supported by findings that age related decreases in iNOS expression are observed in resident peritoneal macrophages, while increases iNOS expression are observed in thioglycollate elicited cells (Kissin et al., 1997; Qureshi et al., 2011). Another contributory factor may be whether the macrophages are stimulated in vitro or in vivo. Peritoneal macrophages from aged mice exposed to LPS in vitro produce decreased levels of the pro-inflammatory cytokines IL-6 and TNF-α, when compared to young mice, while expression of monocyte inflammatory protein and CXC chemokines is increased in cells collected from mice treated intraperitoneally with LPS (Boehmer et al., 2004; Gomez et al., 2007). Additionally, responses of macrophages also appear to vary with the nature of the stimulus. For example, bacterial clearance of Klebsiella pneumoniae was found to increase with age in rat alveolar macrophages, while clearance of Listeria monocytogenes decreases (Antonini et al., 2001; Yokota et al., 1988).

A number of studies have shown that one determining factor of macrophage function and phenotype is the microenvironment of the tissue in which the cells reside (Guth et al., 2009; Stout, Jiang, et al., 2005). Age related changes in these tissues will therefore affect the functioning of these cells. For example, expression of cell adhesion molecules on vascular endothelium decreases with age and responsiveness to VEGF is

reduced, altering interactions between tissue and innate immune cells (Ashcroft et al., 1998). Mahbub et al. (2012) demonstrated that splenic macrophages from elderly mice are less responsive to polarization into classically and alternatively activated phenotypes by LPS, IFN-γ, and TNF-α or IL-4. However when bone marrow progenitor cells are differentiated into macrophages, thus removing the aging microenvironment, their response to cytokine stimulation is similar to bone marrow derived macrophages isolated from young mice. This demonstrates that extrinsic factors may also play a role in altered macrophage functioning with age.

Aging in the lung

In the lung, age related alterations are observed at the structural level and in immune responsiveness. The ratio of Type II cells to Type I cells decreases with age (Pinkerton et al., 1982). Additionally, lung permeability is increased (Tankersley et al., 2003). Aging also results in impairments in the functional and mechanical properties of the lung (Janssens et al., 1999). Excessive production of pro-inflammatory mediators is evident with age which leads to a state of chronic low-level activation of the immune system. This may be due, in part, to over activation of NF-κB. This is supported by findings that the activity of NF-κB is increased in the lungs of senescent mice, when compared to younger mice in response to LPS exposure (Chang et al., 2004; H. J. Kim et al., 2000; Spencer et al., 1997). This is associated with increased production of IL-1β and ROS and expression of iNOS and COX-2 in older mice (Chang et al., 2004; H. J. Kim et al., 2000). Findings that antioxidant defenses are impaired with age are consistent with a pro-inflammatory state in the lungs of the elderly, and supported by findings that glutathione levels decrease in the respiratory tract lining fluid as animals age (Kelly et al.,

2003). Dysregulation of the immune system and declines in protective immunity with age result in increased susceptibility to viral, bacterial and parasitic infections (Gomez et al., 2008). Decreases in the efficacy of influenza vaccines have also been described in the elderly (Grubeck-Loebenstein et al., 2009). This contributes to a greater incidence of chronic and autoimmune diseases and infection in aging individuals (H. Y. Chung et al., 2009). Evidence suggests that the elderly are also more susceptible to the adverse effects of air pollution, in part due to a low level chronic inflammatory state (Ciencewicki et al., 2008). Furthermore, epidemiologic data indicate that individuals with COPD are hypersensitive to air pollutants (e.g., ozone), exhibiting increased morbidity and mortality (Desqueyroux et al., 2002; Zanobetti et al., 2011). Similarly, senescent rats have been reported to be more susceptible to ozone as well as particulate air pollution induced lung injury/inflammation relative to younger controls (Mustafa et al., 1985; Vasanthi R Sunil et al., 2007; Tankersley et al., 2003; Vincent et al., 1996). This is associated with greater IL-6 production and reduced levels of the antioxidant, ascorbate (Vincent et al., 1996). Mortality rates are also greater in older rats exposed to ozone when compared to younger rats supporting the idea that inhalation of ozone causes greater lung injury in older rats (Mustafa et al., 1985). Additionally, senescent mice exhibited increased lung permeability and altered lung compliance after exposure to diesel exhaust particles (Tankersley et al., 2003).

Effects of Aging on Lung Function

Optimum functioning of the respiratory system occurs around 20 years of age in males and 25 years in females. Thereafter, aging results in a progressive decline in lung performance (Janssens et al., 1999). This is associated with enlarged airspaces and

increased lung volumes (Pinkerton et al., 1982). The elastic recoil and the resistance of the lung are also decreased with age, which alters the pressure-volume relationship in the tissue (Janssens et al., 1999). Most age-related changes in lung function are related to three major structural changes that occur in the respiratory system. These include alveolar distension, chest wall stiffening, and decreased strength of respiratory muscles (Janssens, 2005). Alveolar distension is the result of alterations in elastic fibers, which appear to be ruptured and coiled in respiratory bronchioles and alveoli (Verbeken et al., 1992). These changes, which occur around alveolar ducts, promote dilation of alveoli and airspace enlargement (Sprung et al., 2006). A reduction in elastic recoil of the lung parenchyma results from decreases in surface tension forces as alveolar diameter increases (Miller, 2010). This can be explained by LaPlace's law, which in this context, states that the surface tension is greater for a small bubble than a large one (Tenney, 1993). Since studies have failed to show any alterations in the properties of lung surfactant with age, the reduction in elastic recoil is not likely surfactant related (Miller, 2010). Furthermore, since the amount of collagen and elastin in the lung are not altered with age, it appears that increases in alveolar diameters do not result from parenchymal destruction but rather from alterations in the configuration of elastin around the alveolar ducts and in coiling structure (Janssens et al., 1999). Because the extracellular matrix proteins of the elastic fiber system in the lung function throughout the lifetime of the individual without replacement, they can gradually accumulate damage with age (Sherratt, 2009). Studies suggest that the accumulation of ROS mediated damage with age results in fiber remodeling, contributing to their functional deterioration. The normal development of protein cross-links during elastic fiber maturation is essential to strength

of the fiber (Bailey, 2001). However, oxidation of elastic fibers by ROS can reduce these structurally important cross-links with age (Cantor et al., 2006; Umeda et al., 2001). In addition, increases in pathological cross-linking can also occur with age, resulting in the formation of pro-inflammatory advanced glycation end products and aberrant fiber functioning (Sherratt, 2009).

The normal aging process has been referred to as "senile emphysema", while emphysema is thought to be a "disease of accelerated aging. However, there are clear distinctions between these two pathologies. Thus, unlike the development of emphysema, airspace enlargement with aging is evenly distributed and occurs without cellular infiltrates into the alveolar spaces and consequent destruction of alveolar walls. Nevertheless, the end result is a similar change in lung compliance (Janssens et al., 1999). One consequence of a reduction in the support of airways by the surrounding tissues is a tendency for small airway collapse (Janssens, 2005). This more distensible aged tissue results in increases in residual volume; that is the volume that remains in the lung after maximal exhalation due to air trapping. Additionally, closing capacity, the volume at which small airways close, increases with age. As a consequence, these changes are associated with age-related decreases in the vital capacity, the amount air that can be moved into and out of the lung (Miller, 2010). Furthermore, since alveolar distension increases surface area with age, without altering blood supply, ventilation-perfusion mismatch occurs (Sprung et al., 2006). The functional result of these alterations is a deterioration of the gas exchange capacity of the lungs with age.

Emphysema

Emphysema has been described as a disease of accelerated aging (MacNee, 2009). As described above, pathologic mechanisms leading to the development of emphysema are similar to changes observed in the lung with aging, and result in immune system dysregulation. Protease/anti-protease imbalances and impaired antioxidant defenses develop in patients with emphysema and lead to a chronic low grade inflammatory state in the lung (MacNee, 2009; Sharma et al., 2009; Tsuji et al., 2006, 2010). Exposure to cigarette smoke and air pollutants have been reported to result in the development of emphysema (Barnes, 2000). Direct exposure of these injurious agents to the lung leads to the release of danger-associated molecular patterns (DAMPS) and initiates Toll-like receptor signaling and inflammation. This results in the accumulation of inflammatory cells in the lung (Decramer et al., 2012). Macrophages play an important role in the development of emphysema. Patients with emphysema have increased numbers of macrophages in their lungs, which are localized at sites of alveolar destruction (Tetley, 2002). Furthermore, the activity of these cells is altered; thus production of MMPs is increased, causing tissue remodeling and destruction of alveolar walls (Grumelli et al., 2004). Conversely, clearance of apoptotic cells is impaired (Hodge et al., 2003).

Emphysema is a destruction of lung parenchyma distal to terminal bronchioles. The associated loss in alveolar surface area results in impaired gas exchange (Winkler et al., 2011). In patients this can be measured by progressive declines in forced expiratory volume in 1 second (FEV1) (Decramer et al., 2012). The major alteration in respiratory mechanics resulting from the development of emphysema is a decrease in tissue elastance (S. Ito et al., 2005; Vanoirbeek et al., 2010). This is associated with increases in collagen

and decreases in elastin content and alterations in their structure (Faffe et al., 2009; S. Ito et al., 2005). This remodeling results in weakened alveolar walls, which fail at lower stresses. In a mouse model of emphysema, Ito et al. (2006) found that aberrant and weakened collagen structure contribute to the development of emphysema. These collagen abnormalities correlate with deviations in the responsiveness of the lung to pressure and volume changes and decreased tissue stiffness. Furthermore these irregularities result in increased mechanical forces on parenchyma surrounding areas of destruction of the alveolar network, increasing susceptibility to further mechanical injury during respiration (S. Ito et al., 2006).

OZONE

Ozone is a ubiquitous urban air pollutant known to cause significant lung injury, especially in vulnerable populations. In the Clean Air Act of 1970, ozone was designated by the U.S. Environmental Protection Agency (EPA) as a criteria air pollutant (Suh et al., 2000). In 1997 the EPA reduced the ozone standard from 0.12 ppm to 0.08 ppm due to evidence of adverse health effects at these low levels. Symptoms include chest tightness, cough, and wheezing. Adverse effects also include increased incidence of hospital admissions, emergency room visits and chronic illness (Suh et al., 2000). In 2008 the EPA further lowered the National Ambient Air Quality Standard to 0.075 ppm based on evidence of adverse impacts below the 1997 standard (C. S. Kim et al., 2011). For example, a recent study showed decrements in pulmonary function and increases in airway inflammation in healthy adults after exposure to 0.06 ppm ozone for 6.6 hr, a level below the current ozone standard (C. S. Kim et al., 2011).

In the stratosphere, ozone is formed when high energy UV radiation interacts with O_2 molecules causing them to break down generating highly reactive radicals which interact with O_2 forming ozone. In the upper atmosphere, the formation of ozone represents a protective process by which oxygen absorbs damaging UV energy. In contrast, in the troposphere, UV radiation does not have sufficient energy to directly break the bond in O_2 molecules; it is, however, capable of breaking down NO_2 molecules and hydrocarbons, components of air pollution, resulting in the generation of oxygen radicals. These radicals can interact with O_2 molecules resulting in the formation of ozone and photochemical smog (Putman et al., 1997).

Because of its low solubility, inhaled ozone first interacts with the epithelial lining fluid of the lung, which contains lipids and proteins. This interaction can either neutralize potential toxic effects of ozone, or result in the production of ROS which can damage surrounding cells, impair surfactant function, and initiate inflammation (Putman et al., 1997). The epithelial lining fluid layer is thicker in the upper airways and contains greater amounts of antioxidants making it relatively resistant to oxidative injury. In contrast, the lower airways and bronchiolar alveolar regions of the lung are thinner, making epithelial cells in these regions more directly susceptible to ozone-induced damage (Putman et al., 1997). In these regions, ozone can interact with both extracellular surfactant and the epithelial cells of the lung. Interaction with proteins and lipids can result in the formation of ROS which cause lipid peroxidation and protein adduct formation, alterations in signaling molecules, oxidation of proteins and damage to DNA. This leads to inflammation and cell injury. Inflammatory cells are recruited to sites of

injury also release ROS, as well as RNS, adding to the oxidative burden and exacerbating lung inflammation and injury (Ciencewicki et al., 2008).

Ozone-induced injury to the lung is characterized by alveolar epithelial damage, pulmonary edema, blood-air barrier disruption, and airway hyperresponsiveness (D. K. Bhalla, 1999; Foster et al., 2000; Foster et al., 1996). Ozone also disrupts epithelial tight junctions, leading to compromised epithelial defense and increased airway hyperreactivity (D. K. Bhalla, 1999; Foster et al., 2000; Foster et al., 1996). Epithelial barrier disruption has been described in humans after exposure to ambient levels of ozone, as evidenced by increased lung permeability (Foster et al., 1996). Epithelial barrier disruption may increase susceptibility to pulmonary infections and allow entry of other toxic pollutants into the tissue, increasing the risk of airway injury (D. K. Bhalla, 1999).

One marker of alveolar epithelial damage is increases in BAL protein levels and numbers of inflammatory cells, which reflects increased blood/air barrier permeability (D. K. Bhalla, 1999; Fakhrzadeh et al., 2002; Pendino et al., 1995). In humans, exposure to ambient levels of ozone results in inflammatory changes in the lung. For example, exposure to 0.1 or 0.08 ppm ozone for 6.6 hr results in increased numbers of neutrophils in BAL fluid, which is accompanied by increased production of PGE2, fibronectin, IL-6, and lactate dehydrogenase (LDH) (Devlin et al., 1991). Ozone also has direct effects on epithelial cell function. In these cells production of the proinflammatory cytokines IL-6 and IL-8, and fibronectin is increased after exposure (Devlin et al., 1991).

Exposure to ozone also has important implications for the development of lung diseases. Ozone has been shown to exacerbate asthma, increasing airway hyperreactivity

(Kierstein et al., 2008). In addition, ROS and oxidant mediated damage generated as a result of exposure to ozone have been implicated in the pathogenesis of COPD, acute respiratory distress syndrome (ARDS), and cystic fibrosis (Ciencewicki et al., 2008). In mice, chronic exposure to ozone for 3 or 6 wk resulted in the development of chronic inflammation and emphysema (Triantaphyllopoulos et al., 2011). Changes in the lung included increases in caspase-3, IL-13, IFN-γ, and MMP-12. This correlated with decreases in HO-1. Structural changes included increases in lung volume, mean linear intercept, and alveolar space, as well as decreases in epithelial area. Conversely, none of these changes were observed in mice acutely exposed for 3 hr to ozone. This raises the possibility that continuous daily exposure to ozone may be more hazardous than acute exposure, causing more permanent changes and contributing to the development of emphysema and COPD.

Macrophages and Ozone Toxicity

Evidence suggests that macrophages play a significant role in ozone toxicity (Al-Hegelan et al., 2011; Laskin et al., 2011). Thus, in response to ozone-induced oxidative stress and tissue injury, these cells increase in number and become activated (Fakhrzadeh et al., 2002; Pendino et al., 1993; Schwartz et al., 1976). In animal models, ozone inhalation results in increased production of superoxide anion and release of PGE2, TNF-α and IL-1, and expression of iNOS and COX-2, by lung macrophages, but decreased expression of the antioxidant glutathione (Fakhrzadeh et al., 2002; Pendino et al., 1996; Pendino et al., 1994). Ozone-induced increases in iNOS and decreases in glutathione expression are blunted, however, by endotoxin treatment, consistent with reports that microbial defenses in the lung are impaired after exposure to ozone (Gilmour, Park,

Doerfler, et al., 1993; Pendino et al., 1996). In vitro studies with human alveolar macrophages have shown that exposure to ozone results in reduced phagocytosis of immune complexes and blunted superoxide anion production (S. Becker et al., 1991; Devlin et al., 1991). Paradoxically, these cells released increased amounts of PGE2, which may contribute to reduced phagocytosis (Aronoff et al., 2004). In vitro production of TNF-α, IL-1β, IL-6, and IL-8 by alveolar macrophages is increased following ozone (Arsalane et al., 1995), however in response to stimulation with LPS, ozone exposed alveolar macrophages produced significantly less pro-inflammatory cytokines, when compared to air exposed cells (S. Becker et al., 1991). These alterations in the functioning of alveolar macrophages contribute to increased susceptibility to infection after ozone exposure (Becker et al., 1998).

Ozone induced inflammation in the lung is also associated with increased production of TNF-α by macrophages (Pendino et al., 1994). TNF-α is a pro-inflammatory cytokine that promotes macrophage production of ROS and pro-inflammatory mediators such as IL-6 and MMPs (Bradley, 2008). Furthermore, mice lacking TNFR1, the major inflammatory receptor for TNF-α are protected from ozone induced inflammation and injury (Cho et al., 2001). The transcription factor, NF-κB regulates the production of TNF-α following ozone exposure. Thus, after ozone intoxication, increased NF-κB activity is observed in alveolar macrophages (Fakhrzadeh, Laskin, Gardner, et al., 2004; Fakhrzadeh, Laskin, & Laskin, 2004; Laskin et al., 2002). Furthermore, macrophages from mice deficient in NF-κB produce reduced amounts of nitric oxide and TNF-α and are protected from ozone-induced lung injury (Fakhrzadeh, Laskin, & Laskin, 2004). Inhibition of TNF-α in rats with TNF-α antibodies reduced

neutrophil accumulation in the lung and decreased epithelial permeability and pro-inflammatory cytokine expression (Deepak K Bhalla et al., 2002). In human alveolar macrophages, ozone exposure impaired production of TNF-α in response to LPS, when compared to air exposed cells (Susanne Becker et al., 1991). Additionally, it has been demonstrated that the degree of ozone induced decrement in lung function is dependent on polymorphisms in TNF-α suggesting involvement of this cytokine in susceptibility to ozone-induced injury (Yang et al., 2005).

Ozone-induced oxidative stress has also been reported to result in the release of DAMPS, such as low molecular weight hyaluronan, which is produced by injured cells and tissue (Garantziotis et al., 2009). DAMPS activate TLRs which are pattern recognition receptors expressed on the surface of alveolar macrophages (Kleeberger et al., 2001; Lorne et al., 2010). This initiates cell signaling pathways leading to activation of NF-κB and expression of pro-inflammatory proteins such as TNF-α and iNOS (Akira, 2003). Findings that ozone-induced oxidative stress and inflammation are absent in mice with non-functional TLR4 suggests that TLR4 mediated activation of pro-inflammatory functioning in macrophages contributes to ozone-induced injury (Connor et al., 2012; J. W. Hollingsworth, Kleeberger, et al., 2007; Kleeberger et al., 2001). TLR4 also plays a role in ozone induced airway hyperresponsiveness. Mice deficient in TLR4 are protected from airway hyperresponsiveness (J. W. Hollingsworth, 2nd et al., 2004). Ozone-induced airway hyperresponsiveness is thought to be the result of interaction of this receptor with low molecular weight hyaluronan (Garantziotis et al., 2009). Upon exposure to ozone, hyaluronan is oxidized resulting in the release of short soluble fragment that are a ligand for TLR4. Studies have found that these fragments mediate

ozone induced airway hyperresponsiveness ((Garantziotis et al., 2009). Furthermore, TLR4 deficient mice administered low molecular weight hyaluronan were protected from airway hyperresponsiveness (Garantziotis et al., 2010).

Recent studies have demonstrated that lung macrophages develop classically and alternatively activated phenotypes following ozone exposure (V. R. Sunil, Shen, et al., 2012). Thus, exposure of rats to ozone results in increases in iNOS, TNF-α, COX-2, and monocyte chemotactic protein, markers of classical activation (V. R. Sunil, Shen, et al., 2012). Expression of these proteins is biphasic, suggesting that they were expressed by both resident and infiltrating populations (V. R. Sunil, Shen, et al., 2012). Arginase-1, YM-1, and galectin-3, markers of anti-inflammatory, wound repair macrophages, are also expressed early in the inflammatory response to ozone. The observation that this overlapped with expression of classical macrophage markers illustrates that the process of wound repair is initiated early after ozone intoxication. This is supported by findings that ozone exposure leads to increases in populations of alveolar macrophages that express fractalkine receptor (CXCR3), as well as the scavenger receptor, macrophage receptor with collagenous structure (MARCO), markers of anti-inflammatory/ wound repair macrophages (Tighe et al., 2011). (Dahl et al., 2007). Moreover, loss of CXCR3 results in reduced numbers of anti-inflammatory macrophages in the lung. This is associated with increased susceptibility to ozone induced injury, as evidenced by increased airway hyperreactivity, BAL neutrophil content and pro-inflammatory cytokine expression (Tighe et al., 2011). Similarly, mice lacking MARCO exhibit increased susceptibility to ozone-induced pulmonary inflammation, resulting in increased accumulation of neutrophils and oxidized lipids in the lung. These data demonstrate that

anti-inflammatory macrophages play a protective role in the lung by scavenging oxidized lipids generated from exposure of lung lining fluids to ozone (Dahl et al., 2007). Taken together, these studies show that multiple subpopulations of macrophages contribute to the pulmonary response to ozone.

Effects of ozone exposure on lung function

Ozone intoxication has been reported to cause significant alterations in lung functioning both in humans and experimental models. Adverse effects of ozone inhalation include increased bronchial reactivity and exacerbation of pulmonary disorders such as asthma and COPD (Que et al., 2011). Furthermore, susceptibility to these effects is increased in the young and elderly (Li et al., 2013).

In humans, ozone exposure leads to deterioration of pulmonary function, as measured by decreases in respiratory frequency, FEV1, forced vital capacity, and in accordance, increases in airway resistance (Ciencewicki et al., 2008; Que et al., 2011). These deficits are associated with airway hyperreactivity and chest discomfort on inspiration. The severity of these responses depends on both the concentration and the duration of the exposure. Furthermore, physical activity has been found to exacerbate these responses (Bernstein et al., 2004). Children and the elderly also exhibit increased sensitivity to ozone (Li et al., 2013). For example, ozone has been linked with asthma exacerbations in children. Thus increases in ambient ozone concentrations are correlated with increases in emergency room visits and wheeze in asthmatic children (Strickland et al., 2010). This is thought to result from several factors, including the fact that the immune system and lung are not fully developed in children. In addition, peripheral airways are smaller in children relative to adults and hence, more easily occluded

following inflammatory responses. Children are also more active and breathe more air per unit body weight (Bateson et al., 2008; Strickland et al., 2010). In the elderly, ozone increases mortality an additional 1.10% per each 10 ppb increase in concentration in people over 65 years of age (Medina-Ramon et al., 2008). Additionally, increases in ozone concentrations are correlated with elevated hazard ratios for patients with COPD, diabetes, congestive heart failure, and myocardial infarction (Zanobetti et al., 2011). Because exposure to ozone is associated with biomarkers of oxidative stress and with decreases in heart rate variability, ozone exposure places individuals with poor health at greater risk of adverse outcomes (C. Chen et al., 2007; S. K. Park et al., 2005).

In animal models, alterations in airway mechanics resulting from exposure to ozone are known to increase airway resistance and to reduce compliance. Increased airway resistance has been observed not only under physiologic conditions, but also in response to constrictive agents such as methacholine, indicating ozone exposure increases airway hyperresponsiveness (Fouke et al., 1988; Savov et al., 2004; Takahashi et al., 1993). This is ameliorated by inhibition of ROS production, suggesting that free radical generation is involved in the response (Chitano et al., 1995; J. W. Hollingsworth, Kleeberger, et al., 2007; Takahashi et al., 1993). In addition, the pro-inflammatory cytokine TNF promotes airway hyperreactivity following ozone inhalation (Shore et al., 2001). This suggests that inflammation resulting from exposure to ozone may contribute to alterations in airway mechanics. Parenchymal mechanics have also been found to be involved in ozone-induced alterations in lung responsiveness. Increases in tissue resistance in response to methacholine administration are increased in mice exposed to ozone when compared to air exposed mice (Rivera-Sanchez et al., 2004). Although it has

been established that elderly mice and rats have increased susceptibility to ozone induced inflammation and oxidative stress, as well as increases in mortality, when compared to younger animals, (Mustafa et al., 1985; Servais et al., 2005; Vincent et al., 1996), studies have yet to investigate the effects of ozone exposure on lung functioning in elderly animals.

AIMS OF THE DISSERTATION

The overall objective of these studies was to analyze the effects of chronic macrophage inflammation due to loss of SP-D on the persistence of ozone induced injury, inflammation and altered functioning in the lung. We also determined if progressive pulmonary inflammation associated with aging in SP-D-/- mice leads to an exacerbated response to ozone. We hypothesized that loss of SP-D will result in prolonged injury and oxidative stress, as well as alteration of macrophage activation and pulmonary mechanics, in response to ozone. Moreover, these responses will be impaired with age. To test this hypothesis the following specific aims were addressed:

Specific Aim I: Determine if chronic pulmonary inflammation resulting from loss of SP-D alters the sensitivity of mice to ozone.

Specific Aim II: Analyze the effects of increasing pulmonary inflammation and the development of emphysema in aging SP-D-/- mice on the response to inhaled ozone.

METHODS

Mice

Male 8 wk, 27 wk, and 80 wk SP-D-/-mice were bred in the Rutgers University animal facility. Age and sex matched C57BL/6J WT mice, obtained from Jackson Laboratories (Bar Harbor, ME), were used as controls. Animals were housed under pathogen free conditions in microisolation cages and received food and water ad libitum. All animals received humane care in compliance with the institution's guidelines, as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. All procedures were reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee.

Ozone Exposure

Mice were placed in whole body Plexiglas chambers and exposed to 0.8 ppm ozone or air for 3 hr. A Gilmont ozone generator was used to generate ozone from oxygen gas via an ultraviolet light source (Orec, Phonex, AZ). The concentration of ozone within the chamber was maintained by adjusting the light intensity and monitored with an ozone analyzer (model 400E; Teledyne Instruments, San Diego, CA). Mice were sacrificed 24-72 hr post exposure.

Collection and Analysis of Bronchoalveolar Lavage Fluid

Mice were anesthetized and a cannula inserted into the trachea. One ml of PBS was slowly instilled and withdrawn four times using a syringe. BAL was then

centrifuged at 300 x g for 8 min and cell-free supernatant collected. The cell pellet was then resuspended in PBS and enumerated using a Coulter Counter. BAL protein concentration was determined using a microplate BCA assay with bovine serum albumin (BSA) as the standard (Thermo Scientific, Rockford IL). For differential analysis, cytospins were prepared and fixed in methanol. Slides were then stained with Giemsa (Labchem Inc, Pittsburgh, PA). A total of 100 cells were counted per slide.

Histology

After lavage, the left lung was inflation fixed with ice cold 3% paraformaldehyde + 2% sucrose overnight, washed three times with PBS + 2% sucrose, and transferred to tubes containing 50% ethanol. The tissue was then sectioned (6 μm), and stained with hemotoxylin and eosin. Images were examined by light microscopy and acquired using 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 II cell hyperplasia, fibrin deposition, and the appearance of cholesterol and hemoglobin crystals were assessed blindly by a veterinary pathologist (LeRoy Hall, D.V.M., Ph.D.). Semiquantitative grades (0 to 3) were assigned to the tissues, with grade 0 = no changes, grade 1 = minimal or small changes, grade 2 = mild to medium changes, and grade 3 = moderate to extensive changes. One section from 5-6 mice per treatment group was analyzed. Slides were also blindedly scored for severity of inflammation by two

independent observers on a zero to five scale (0 = no inflammation, 5 = severe inflammation) as outlined in Rudmann et al. (1998).

To assess the extent of alveolar damage, radial alveolar counting was performed as previously described by Cooney et al. (1982). 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 two alveoli, a value of 1 was assigned. For each lung section, 14 to 20 respiratory bronchioles were counted.

Quantitation of BAL Nitrogen Oxide (NOx) Levels

Nitric oxide metabolites in BAL were assessed using an Ionics/Sievers Nitric Oxide Analyzer 280 (NOA 280; Ionics Instruments, Boulder, CO). Ten µl of BAL were injected into a solution of 0.4 mM vanadium chloride in 1 M HCl at 95ºC, which reduced oxides of nitrogen with a nitrogen oxidation state greater than +2 to nitric oxide. NOx were then transferred to the analyzer using argon gas. The area under the resultant peak and nitrate standards was used to calculate total NOx concentrations.

Chemotaxis Assay

Chemotactic activity of BAL fluid was assessed using a 96-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) as described by Guo et.al., (2008). BAL, monocyte chemoattractant protein-1 (positive control, 500 ng/ml) or buffer (negative control) (30 µl) 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 hr. The membrane and lower wells were removed and centrifuged, membrane side facing down at 450xg for 5 min to ensure adherence of the migrated cells onto the membrane. The membrane was removed and stained with Diff Quick (CAMCO, Fort Lauderdale, FL). Migrated cells were counted microscopically under oil-immersion. Data are expressed as the average number of migrated cells per 20 high power fields.

Immunohistochemistry

Paraffin embedded lung was sectioned at a thickness of 6 µm and mounted onto slides. Slides were then deparafinized in xylene for 8 min. Slides were rehydrated through a series of gradient alcohols ending in deionized water for 2 min in each solution. Citrate antigen retrieval was then performed; slides were placed in citrate retrieval solution (0.1 M Na Citrate plus 0.1 M Citric Acid) and then microwaved on 10% power for 10 min. After cooling for 20 min, slides were rinsed in deionized water 3 times for 5 min. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 3% H_2O_2 for 10 min followed by washing twice in deionized water for 5 min in 0.3% PBST for 5 min, and then in PBS for 5 min. Tissue was blocked in PBST containing 1.5% normal serum for 2 hr at room temperature by drawing a circle around each section using an immedge pen and pipetting the solution into the circle. This was followed by overnight incubation at 4^oC with rabbit IgG or rabbit polyclonal antibodies to iNOS, (1:1500; Abcam, Cambridge, MA), COX-2 (1:1,000; Abcam, Cambridge, MA), mannose receptor (1:1500; Abcam), YM-1 (1:500; Stem Cell Technologies, Vancouver,

BC), galectin-3 (1;2000; R&D Systems, Minneapolis, MN), HO-1 (1:750; Enzo Life Sciences, Farmingdale, NY), SP-C (1:2000; Milipore, Billerica, MA), or PCNA (1:500; Abcam). Sections were then incubated with biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA) for 30 min. The slides were washed in PBST 3 times for 5 min and then in PBS for 5 min. Subsequently, the slides were incubated in a 1:200 solution of biotinylated secondary antibody from Vectastain in PBS for 30 min at room temperature. The slides were then washed in PBST 3 times for 5 min followed by PBS for 5 min. The sections were then incubated in ABC reagent from Vectastain for 30 min and washed in PBST 3 times for 5 min, and in PBS for 5 min. DAB peroxidase substrate solution was added to the sections until brown staining became evident. Staining was quenched by placing the slides in tap water and rinsing briefly 3 times. The sections were counterstained with hematoxylin for 1 min and rinsed briefly in tap water 3 times and in PBS for 30 sec. Slides were then dehydrated in a series of gradient alcohols for 30 sec in each solution and placed in xylene 3 times for 3 min. The slides were covered with a coverslip using paramount and dried overnight before microscopic examination.

Measurement of Pulmonary Mechanics

Mice were anesthetized with ketamine (240 mg/kg) and xylazine (12 mg/kg) and then ventilated through a tracheal cannula at a frequency of 150 breaths per min and a tidal volume of 10 mL/kg using a flexiVent (SCIREQ, Montreal, Canada). The mice were treated with increasing doses of methacholine (0-96 mg/kg) using a nebulizer. For each dose, respiratory mechanics was measured at PEEPs ranging from 0 cm H_2O to 6 cm H_2O. Baseline measurements were assessed at a PEEP of 3 cm H_2O. Parameters of

dynamic whole lung mechanics, including total dynamic resistance and compliance were determined from a single compartment model using a multiple linear regression. Lung function measurements were also partitioned into parameters representing the properties of either the airways or lung tissue by analyzing Z_L data, generated using an 8 s broadband flow perturbation. A constant-phase model was fitted to the impedance spectra, allowing for calculation of frequency-independent central airway resistance, and a constant-phase tissue compartment containing the coefficients of G and H. Hysteresivity was determined by the ratio of G/H. A pressure volume curve was generated and used to calculate static compliance (Cst). Resistance and elastance spectra were generated from Z_L. Preliminary model fitting of the Z_L spectra to the constant phase model revealed a systematic deviation from the data, which resulted from the need for one mechanical relationship to be characterized by both the real and imaginary portions of the impedance, and is typical of lung pathologies associated with heterogeneous or nonlinear alterations in mechanics. To characterize these spectra with higher fidelity, best fit lines were generated by using linear regression analysis. Parameters representing the key features of the R_L curve were calculated using the equation:

$$\text{Resistance} = (a + bf)/(c + f)$$

where f = frequency, a/c = the low frequency asymptote of the curve, when tissue resistive and viscous effects predominate, and b = the high frequency asymptote, when airway resistive effects predominate. Parameters representing different portions of the E_L curve were calculated using the equation:

$$\text{Elastance} = E_0 + \Delta E\,(1 - e^{-\beta f})$$

where E_0 = elastance at 0 Hz, representing the portion of the elastance spectra at low frequencies, E_0 = intrinsic tissue stiffness, ΔE = the magnitude of elastance, and β = the rate elastance changes with frequency. 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 flexiVent software by integration.

Statistics

All experiments were repeated 3 times using at least 3 animals per treatment group per experiment. Data were analyzed by three-way analysis of variance. Significance for each comparison was calculated using a nonpaired, two-tailed, Student's t test. A p value ≤ 0.05 was considered statistically significant. For non-normally distributed data, a Mann-Whitney Rank Sum test was performed.

Conditions of respiratory mechanical heterogeneity, such as the development of pulmonary pathology, often limit the applicability of mechanical element-based modeling approaches reducing the reliability of estimated lung function parameters and complicating their interpretation. As an alternative to such candidate models, which assume tissue homogeneity, data can be fit using an empirical approach for parameter estimation. Multiple curves whose asymptotic profile resembled those of resistance and elastance spectra were fit to impedance data from air and ozone exposed WT and SP-D-/- mice and compared for their likelihood of fitting the data. This was done to select an

empirical model for analysis of resistance and elastance spectra which best fits the data across these conditions. For model selection, the Akaike Information Criteria (AIC) was utilized because it allows for the simultaneous comparison of >2 models with any number of free parameters and does not require these models to be nested. The AIC was used to determine the relative fitness of each candidate model by using likelihood ratios to compute the relative probability that a test spectra is best characterized by the respective models. The sum of squared residuals, ϕ_M, across all frequencies can be used to compute the AIC for each model, *M*,

$$AIC_M = 2p + n * ln\left(\frac{\phi_M}{n}\right) + \frac{2p(p+1)}{n-p-1}$$

where *p* is the number of free parameters and *n* is the number of data points utilized in the model fit. Relative likelihood of each model was determined from the formula

$$L_M = e^{\frac{1}{2}(\min(AIC) - AIC_M)}$$

which allows computation of model probability to be

$$P_M = \frac{L_M}{sum(L_i)}$$

where L_M and P_M are the likelihoods and probabilities of the empirical model respectively. The model selected had the greatest AIC determined probability, meaning it had a greater likelihood of fitting the data than the other models tested.

In order to compare the effects of strain and ozone exposure on resistance and elastance spectra of 8 wk old mice, significant differences between curves were evaluated by fitting a curve to spectra data using the parameters which defined that of the control group. A coefficient of determination could then be calculated between spectral data and the best fit curve of the appropriate control group. Poor fit of a spectrum to its control

curve indicates that the two curves are dissimilar. To determine if these differences were significant, R^2 values generated from the correlation of these fits were then used to generate t values, for which statistical significance could be determined by setting $\alpha \leq 0.05$.

In further studies, the effects of strain, age and ozone exposure on resistance and elastance spectra were compared. The need to compare effects of strain and treatment conditions across multiple age groups necessitated the use of a more sophisticated nonlinear regression curve fitting approach for hypothesis testing. For two given experimental conditions, the alternative hypothesis was tested that two curvilinear relationships are required to characterize the observed data versus the null hypothesis that one curve is satisfactory. This is accomplished by using the sum of squared residuals from the best fit curves generated for parameter estimation to compute a pooled variance. This is a measure of the combined error in fitting two curves to the observed data and is representative of the evidence for the alternative hypothesis. A separate nonlinear regression curve was then fit to both sets of resistance or elastance spectra data in order to determine the variance associated with the null hypothesis. As the alternative hypothesis corresponds to a more complex model, an F-test was used to weigh the decrease in model variance against a penalty for the addition of 3 free parameters. The reduction in model variance is calculated as the difference between model variances weighted by the degrees of freedom for each model fit. The F-statistic was determined as the ratio of the reduced model variance to the pooled variance. Statistical significance was determined if the F-statistic was greater than F-critical for a given α, number of parameters, and number of data points in the fit. It is inferred from the rejection of the null hypothesis that the

underlying data is generated from two distinct populations of mechanical behavior. For treatment conditions determined significant at the spectral level, subsequent testing of parameter values was performed using t-test for unequal variances at $\alpha = 0.05$ level.

PART I. EFFECTS OF CHONIC PULMONARY INFLAMMATION RESULTING FROM LOSS OF SP-D ON OZONE-INDUCED TOXICITY

Ozone is a ubiquitous urban air pollutant generated as a component of photochemical smog. Inhaled ozone causes ozonation and peroxidation of proteins and lipids in the epithelial lining fluid layer of the lung, resulting in the production of oxidized proteins, aldehydes and free radicals, which can damage surrounding tissue (Al-Hegelan et al., 2011; Ciencewicki et al., 2008). This is accompanied by an accumulation of activated macrophages in the lung and the production of additional cytotoxic and pro-inflammatory mediators including reactive oxygen and nitrogen species which contribute to tissue injury (Laskin et al., 2011). Airway and tissue mechanics are also altered following ozone exposure. Thus in humans, ozone inhalation leads to a deterioration of pulmonary function, as measured by decreases in respiratory frequency, FEV_1, and forced vital capacity, and increases in airway resistance (Ciencewicki et al., 2008; Foster et al., 2000; Que et al., 2011). Ozone has been shown to exacerbate asthma and increase airway hyperreactivity (Foster et al., 2000; C. S. Kim et al., 2011), and to contribute to increased morbidity and mortality in patients with COPD (Desqueyroux et al., 2002; Zanobetti et al., 2011). Similar alterations in lung function and increases in sensitivity to ozone have been described in animal models of asthma and allergic inflammation (Gilmour, Park, & Selgrade, 1993; Kierstein et al., 2008; Rivera-Sanchez et al., 2004).

SP-D is a pulmonary collectin synthesized mainly by alveolar Type II cells, which plays a key role in innate immune defense (E. Crouch et al., 2001; Haczku, 2008). Under homeostatic conditions, SP-D functions as an anti-inflammatory protein, in part by suppressing NF-κB-mediated transcription of inflammatory genes (Gardai et al., 2003).

However, following induction of oxidative stress, modification of critical cysteines in SP-D by reactive species such as nitric oxide leads to a change in its activity to a proinflammatory mediator (Guo et al., 2008). Findings of persistent localization of macrophages in the lung and increased production of ROS and RNS by these cells in mice lacking native SP-D are consistent with its anti-inflammatory function (Atochina et al., 2004; Yoshida et al., 2001). In previous studies we demonstrated that inflammatory macrophages and mediators they release play a key role in lung injury and oxidative stress induced by ozone (Fakhrzadeh et al., 2002; Fakhrzadeh, Laskin, & Laskin, 2004). Based on these observations we hypothesized that persistent macrophage localization and activation in the lung due to loss of SP-D would result in prolonged sensitivity of mice to inhaled ozone; moreover this would be associated with exacerbated functional defects in the lung. To test this hypothesis, we analyzed the effects of ozone on lung injury, oxidative stress, macrophage activation, and pulmonary mechanics in SP-D-/- mice 72 hr post exposure, a time when inflammation and injury have, for the most part, resolved in wild type mice (Fakhrzadeh et al., 2002). We also developed a novel experimental modeling approach to analyze alterations in lung function, with the goals of dissecting the effects of ozone on central airways and parenchyma, and of assessing heterogeneity in lung responsiveness and the propensity for alterations in lung stiffness. Results from our studies, along with previous reports of increases in lung neutrophils in SP-D-/- mice following ozone exposure (Haczku, 2008), highlight the importance of analyzing underlying disease pathology in susceptibility to pulmonary irritants

RESULTS

Effects of Ozone Inhalation on Lung Injury and Macrophage Activation in WT and SP-D-/- Mice

Consistent with previous studies (Fakhrzadeh et al., 2002), we found that ozone-induced lung injury, inflammation and oxidative stress, resolved by 72 hr. Thus, while significant increases in BAL cell number were observed at 24 hr and 48 hr post ozone exposure, and protein concentration and chemotactic activity at 48 hr, by 72 hr post exposure, these changes were largely gone (Figures 1-4). Hence, no significant differences were noted between air and ozone exposed WT mice in BAL cell number, protein or NOx levels (Figure 1). Similarly, BAL chemotactic activity, which has previously been demonstrated correlates with lung inflammatory cell activity (Guo et al., 2008), as well as lung inflammation score were at control levels (Figures 1 and 5). Inactivation of the SP-D gene results in the development of persistent pulmonary inflammation characterized by a predominant macrophage infiltrate, which progresses to emphysema as the animal gets older (Botas et al., 1998; Wert et al., 2000). In further studies we examined whether this change in baseline inflammation, alters the response of mice to ozone. Loss of SP-D by itself, had no significant effect on lung injury, oxidative stress, or inflammatory cell activation, as measured by BAL protein, NOx, and chemotactic activity (Figure 1). In contrast, BAL cell number was increased in SP-D-/- mice relative to WT mice (Figure 1). Inflammation score was also greater in SP-D-/- mice (Figure 5). In SP-D-/- mice, ozone exposure resulted in 5-fold increase at 24 hr and a 3-fold increase at 72 hr in the number of cells recovered in BAL (Figures 1 and 2). This was associated with a 2-fold increase in inflammation score (Figure 5). Lung lavage

had no effect on inflammation score or on the accumulation of these cells in the tissue (Figure 6). In air exposed SP-D-/- mice, but not WT mice, we also noted peribronchial accumulation of enlarged foamy macrophages. This became more pronounced following ozone exposure. BAL macrophage number, protein and NOx levels, as well as BAL chemotactic activity, were also significantly increased in SP-D-/- mice 48 hr and 72 hr after ozone (Figures 1-4).

We next assessed expression of iNOS and COX-2, enzymes important in the generation of proinflammatory RNS and eicosanoids, respectively, and markers of classically activated macrophages (Munder et al., 1998; G. Y. Park et al., 2006; Stout, Jiang, et al., 2005). Low levels of iNOS and COX-2 protein expression were evident in lungs of SP-D-/- mice, but not WT mice (Figure 7). Whereas iNOS was localized in alveolar macrophages, COX-2 was mainly localized in Type II cells. In WT mice, ozone exposure resulted in increased expression of COX-2 in Type II cells, with no effect on iNOS. Quantitative assessment if iNOS and COX-2 expression confirmed these findings (Figure 8). In contrast, in SP-D-/- mice treated with ozone, increases in expression of iNOS in macrophages and COX-2 in Type II cells were noted.

Effects of Ozone Inhalation on Lung Function in WT and SP-D-/- Mice

In order to examine the physiological consequences of ozone-induced injury in SP-D-/- mice, we measured lung function using a series of perturbations including single frequency and broad band forced oscillation, pressure-volume loops, and lung volume measurements. Initially, respiratory mechanics were partitioned into airway and tissue responses by analyzing impedance spectra data using a constant phase model (Hantos et al., 1992). At baseline, R, Rn, and Cst values were greater in SP-D-/- mice, when

compared to WT mice (Table 1). Whereas in WT mice, ozone inhalation resulted in increased Rn, relative to air control, in SP-D-/- mice significant changes were only observed in H and Cst (Table 1). η and G were also measured; neither of these parameters was altered by loss of SP-D or by ozone inhalation (data not shown).

The effects of altering PEEP on lung function were next evaluated to allow for assessment of heterogeneity in responsiveness and changes in airway/parenchymal elastic properties (Massa et al., 2008). In WT mice, increasing PEEP from 3 to 6 cm H_2O resulted in increases in both Rn and Cst (Figure 9). Similar changes were observed in SP-D-/- mice, however, the PEEP-dependent increase in Rn was blunted relative to WT mice. Treatment of mice with ozone had no major effects on Cst at any of the PEEPs examined (Figure 9). In contrast, following ozone exposure both WT and SP-D-/- mice were significantly less responsive to increasing PEEP from 3 to 6 cm H_2O with respect to Rn, while they were more responsive to PEEP below 3 cm H_2O.

In further studies we examined the effects of changes in PEEP on the resistance and elastance spectra derived directly from the forced oscillation technique, which more accurately reflects heterogeneous lung injury (Kaczka et al., 2011). Ozone exposure produced no significant changes in the overall resistance spectra in WT, while a frequency dependent increase in the E_L spectra was observed at PEEPs of 0 and 1 cm H_2O (Figure 10). Elastance spectra were also significantly increased in ozone exposed SP-D-/- mice, relative to air exposed animals, however this response was observed at all PEEP levels. In SP-D-/- mice, increases in R_L spectra were also noted at a PEEP of 0 cm H_2O after ozone exposure. Changes in resistance were most evident at low frequencies. In order to ensure that lung volume related changes did not influence analysis of

resistance and elastance spectra, initial lung volumes were established at 0 PEEP; no significant differences were noted between the groups (Table 2).

To better quantify changes in the resistive and elastic properties of the lung following ozone exposure, resistance and elastance spectra were fit to model equations such that the parameters defining these responses could be estimated (see Methods section). In WT mice ozone exposure produced no significant change in **a**, while **b** was significantly increased at 1 cm and 3 cm H_2O, and **c** decreased at 6 cm H_2O, when compared to air exposed mice (Table 3). A significant increase in ΔE accompanied by a fall in β was observed at low PEEP after exposure of WT mice to ozone, with no effect on the inherent elastance (E_0). Loss of SP-D resulted in a different pattern of changes in these parameters in response to ozone. Thus, at low PEEP **a** increased significantly in SP-D-/- mice following ozone inhalation, while **b** decreased. In contrast, **c** was not altered at any PEEP examined. In SP-D-/- mice, ozone inhalation also produced a significant increase in inherent elastance as shown by elevated $\mathbf{E_0}$ at all PEEPs. In addition, at low PEEPs ozone exposure resulted in increased ΔE and decreased β, indicating a high frequency ventilation-dependent increase in lung stiffness.

In air exposed SP-D-/- mice, there were no significant changes in **a** across all PEEPs when compared to WT mice (Table 3). However, loss of SP-D did result in a significant elevation of **b** at all PEEPs, while at high PEEP, there was a significant decrease in **c**. No significant changes in ΔE were noted in mice lacking SP-D, at any level of PEEP, however, E_0 was decreased at low PEEP. In contrast, loss of SP-D resulted in a significant increase in β at a PEEP of 6 cm H_2O.

DISCUSSION

Accumulating evidence suggests that lung injury induced by ozone is a result of its direct interaction with proteins and lipids in the epithelial lining fluid, and the actions of ROS, RNS and other cytotoxic mediators generated by inflammatory cells (Al-Hegelan et al., 2011; Ciencewicki et al., 2008; Laskin et al., 2011). SP-D plays a key role in regulating inflammatory responses in the lung; specifically, it acts to balance macrophage activation in a manner dependent upon modification by nitric oxide (Gardai et al., 2003; Guo et al., 2008; Haczku, 2008). In the absence of SP-D, mice develop progressive inflammatory disease in the lung, characterized by a predominant peribronchiolar macrophage infiltrate, airspace enlargement, and increases in lung volume, culminating in emphysema (Wert et al., 2000). The present studies demonstrate that persistent localization of activated macrophages in the lung, associated with loss of SP-D, results in prolonged sensitivity of mice to the cytotoxic effects of inhaled ozone, and causes exacerbated alterations in lung function. These data are novel and provide support for a critical protective role of SP-D in regulating macrophage inflammatory responses and oxidative stress within the lung (Atochina et al., 2004; Gardai et al., 2003; Guo et al., 2008).

Previous studies from our laboratory have demonstrated that ozone-induced inflammation, as measured by numbers of alveolar macrophages recovered in BAL, peaks 48 hr after acute exposure, returning to control levels by 72 hr (Fakhrzadeh et al., 2002). Consistent with these results, the present studies show that BAL macrophage number and chemotactic activity, as well as lung inflammation score which were maximally increased 48 hr post-ozone exposure in WT mice and were at control levels 72

hr post-exposure. The observation that BAL NOx and protein levels were also similar in air and ozone exposed WT mice indicates that oxidative stress and alveolar epithelial barrier dysfunction have also resolved by this time. Despite resolution of these responses, WT mice remain functionally compromised 72 hr after ozone inhalation. Thus, in these mice, central airway resistance (Rn) was significantly increased, with no apparent effect on the parenchyma, as measured by H and G. These findings are in accord with reports of hyperreactivity in response to methacholine challenge following ozone exposure (J. W. Hollingsworth, Maruoka, et al., 2007; Que et al., 2011). Interestingly, at PEEPs both above and below the physiological level of 3 cm H_2O, which represent hypo- and hyper-inflation of the lung, respectively, a decrease in central airway resistance was noted following ozone exposure. This observation, however, was not consistent with the lack of change in H and G, as it implies that parenchymal recruitment is affecting central airway resistance. We speculated that these differences may be due to the use of a constant phase model to analyze our data, which assumes that the lung behaves homogeneously in terms of both its resistive and elastic properties. Therefore, to take into account heterogeneity in the response of the lung to ozone, impedance frequency spectra were analyzed. Since different portions of the impedance frequency spectrum represent distinct components of the respiratory system, effects on airway and parenchymal components can readily be distinguished (Petak et al., 1993). Whereas the resistance spectra were not significantly altered in ozone exposed mice, when compared air exposed mice, significant changes in the component parameters, **a**, **b** and **c**, were noted. The nature of these changes indicated that with full recruitment and hyperinflation, there is a reduction in the low frequency component of the resistance

spectrum following ozone exposure, supporting the idea that ozone causes a loss of parenchymal integrity. Consistent with this are our findings that at low PEEPs, ozone inhalation significantly altered the elastance spectra in WT mice. To analyze this, elastance spectra data were fit to a three parameter exponential model (see Methods section). Our observation that ozone had no effect on any of the parameters at a physiological PEEP of 3 cm H_2O is in agreement with the constant phase model. In contrast, the low PEEP spectra showed that there is a lung recruitment/frequency dependent change in the elastic properties of the lung following ozone exposure. These data, together with the changes observed in resistance spectra, indicate a loss of parenchymal tethering resulting and a failure to maintain airway patency at low PEEP, and as the lung inflates (at high PEEP), a loss of lung stiffness. This approach to analyzing functional alterations in the lung is novel and provides new insights into the heterogeneous nature of tissue injury induced by ozone.

In accord with previous reports (Atochina et al., 2004; Botas et al., 1998), we found that mice lacking SP-D exhibited significant pulmonary inflammation relative to WT mice, characterized by increased numbers of macrophages in the lung and in BAL. We also observed that lung macrophages were enlarged in SP-D-/- mice relative to WT mice, and exhibited a foamy appearance suggesting that they are activated (Wert et al., 2000). Treatment of SP-D-/- mice with ozone resulted in significant increases in macrophages in the lung, as reflected by inflammation score and BAL cell number, as well as in BAL protein and NOx levels, and chemotactic activity 48 hr and 72 hr post exposure. These results are novel and indicate that oxidative stress and macrophage localization and activation in the lung following ozone exposure are prolonged in SP-D-/-

mice. Ozone-induced lung injury is characterized by disruption of epithelial tight junctions and increases in blood/air barrier permeability resulting in increases in BAL protein content (D. K. Bhalla, 1999; Foster et al., 1996). Our data demonstrate that persistent pulmonary macrophage inflammation, resulting from loss of SP-D, also results in prolonged sensitivity of the mice to the cytotoxic effects of ozone.

iNOS and COX-2 are key enzymes in the generation of cytotoxic and proinflammatory RNS and eicosanoids implicated in lung injury induced by inhaled irritants (G. Y. Park et al., 2006; Que et al., 2011). In previous studies we reported that ozone inhalation resulted in increased expression of iNOS and COX-2 in lung macrophages and Type II cells, a response that was evident 0-48 hr post exposure (Fakhrzadeh et al., 2002; Fakhrzadeh, Laskin, & Laskin, 2004; Punjabi et al., 1994). The present studies demonstrate that while ozone-induced increases in Type II cell expression of COX-2 persisted for at least 72 hr in WT mice, macrophage expression of iNOS was transient. These findings are consistent with the resolution of macrophage-mediated inflammation at this time (Fakhrzadeh et al., 2002). In SP-D-/- mice however, macrophage expression of iNOS remained elevated above control for at least 72 hr after ozone inhalation. These data suggest the persistence of a proinflammatory macrophage phenotype in SP-D-/- mice. Earlier studies reported increased constitutive expression of iNOS in macrophages and alterations in nitric oxide metabolites in the lungs of SP-D-/- mice (Atochina et al., 2004). Moreover, treatment of these mice with an iNOS inhibitor attenuated the inflammatory responses associated with loss of SP-D (Atochina-Vasserman et al., 2007). It is possible that the favoring of proinflammatory macrophage activation in lungs of SP-D-/- mice, which occurs as a consequence of excessive iNOS

activity, leads to a hypersusceptible state such that ozone mediated injury and oxidative stress are prolonged. Surprisingly, BAL NOx levels were similar in ozone treated WT and SP-D-/- mice. This most likely reflects the fact that NOx is only a measure of the extracellular pool of nitric oxide metabolites. Of note was our observation that ozone-induced increases in Type II cell COX-2 also remained elevated above air control in SP-D-/- mice 72 hr post-ozone exposure. These data provide additional support for a role of Type II cells in the inflammatory response to ozone (Punjabi et al., 1994).

Baseline central airway and parenchymal mechanics have been reported to be altered in SP-D-/- mice when compared to WT mice (Collins et al., 2003). Similarly, we observed alterations in central airway pulmonary mechanics in SP-D-/- mice relative to WT mice, as measured by increases in R, Rn and Cst (Vanoirbeek et al., 2010). Additionally, at all PEEPs measured, R_L was greater at high frequencies, suggesting that persistent pulmonary inflammation in SP-D-/- mice leads to alterations in central airway mechanics. Our findings that these alterations are similar to the effects of ozone in WT mice provide support for this conclusion. We also found that loss of SP-D altered the elastic recoil properties of the lung parenchyma, and caused increases in heterogeneous ventilation, airway resistance and lung stiffness. In contrast to our findings, decreases in elastic recoil of lung parenchyma have previously been described in SP-D-/- mice (Collins et al., 2003). Differences between our findings may be due to differences in the age of the mice analyzed.

Due to preexisting macrophage inflammation, we speculated that ozone-induced alterations in pulmonary mechanics would be more extensive in SP-D-/- mice, when compared to ozone exposed WT mice, and indeed, this was the case. Thus in SP-D-/-

mice ozone inhalation resulted in alterations not only in airway mechanics, but also in the parenchymal integrity of the already compromised lungs. In lungs of SP-D-/- mice, there was an accumulation of inflammatory macrophages, which increased following ozone exposure, and a pronounced lipoproteinosis. This buildup of inflammatory material may cause a stiffening of the lung with a consequent increase in inherent tissue elastance. This is supported by our observation that in the derecruited lung, ozone exposure resulted in a dramatic increase in elastance, a change that was frequency dependent and delayed, when compared to air exposed SP-D-/- mice. Moreover, constant phase model analysis revealed increased baseline tissue elastance, H, which is consistent with a stiffening effect on parenchymal mechanics and indicative of restrictive lung injury. The fact that this was not observed in ozone exposed WT mice suggests that SP-D is important in maintaining the elastic properties of the lung. Increases in both the resistance and elastance spectra at low frequencies suggest that in SP-D-/- mice, ozone exposure results in heterogeneous changes in lung function (Lutchen et al., 1997). The observation that these changes were ameliorated as PEEP increased indicate that ozone exposure, like loss of SP-D, affects parenchymal tethering, which could result in small airway collapse. Functionally, SP-D-/- mice appear similar to ozone-injured WT mice with an apparent loss of parenchymal integrity resulting in derecruitment and a loss of airway tethering. Taken together, these results indicate that lung function is significantly altered by both loss of SP-D and by ozone exposure and that there is an interaction between these proinflammatory stimuli. These results support findings in other models of lung injury that SP-D is important in maintaining normal lung functioning (Atochina et al., 2003; Collins et al., 2003; Erpenbeck et al., 2006; Takeda et al., 2003).

In previous studies, Kierstein et al. (2006) reported increased numbers of neutrophils in BAL 12- 48 hr following exposure of SP-D-/- mice to ozone. However, effects of ozone on macrophage accumulation and activation in the lung were not assessed; lung injury, oxidative stress and pulmonary mechanics were also not evaluated. Thus, results presented herein demonstrating sustained lung injury, oxidative stress, and macrophage activation, and exacerbated alterations in pulmonary mechanics are novel. Also unique to the present studies is the use of a constant-phase model of lung function to assess alterations in central airway mechanics, and analysis of resistance and elastance spectra at various levels of PEEP. This analysis revealed the importance of parenchymal injury in mediating changes in lung function. Since polymorphisms in SP-D have been associated with the development of COPD (Kishore et al., 2005), this study highlights the importance of understanding the role of persistent inflammation caused by loss of this protein on the response of susceptible populations to ozone exposure. It should be pointed out that in the current studies SP-D-/- mice were used as a model of persistent pulmonary macrophage inflammation. At present it is unclear if it is the absence of SP-D or the underlying inflammation that is responsible for the observed differences in ozone responses between WT and SP-D-/- mice. Further studies utilizing an experimental model of conditional loss of SP-D would enable the specific effects of deficiency in this protein on lung responses to ozone to be assessed.

Figure 1. Effects of ozone inhalation on lung inflammation and injury. BAL was collected 72 hr after exposure of WT and SP-D-/- mice to air or ozone and analyzed for cell, protein and NOx content, and chemotactic activity. BAL chemotactic activity was assessed using the RAW 264.7 mouse macrophage cell line (ATCC, Manassas, VA) as previously described (E. Crouch et al., 2001). Hank's balanced salt solution was used as the negative control. Chemotaxis data are expressed as the average number of migrated cells per 20 high power fields. The value range for these experiments is 0-30 migrated cells per high power field. Each bar represents the mean $\pm$ SEM of 3 experiments (n = 4-9 mice/treatment group/experiment; n = 12-27 mice/group total). Data were analyzed by two-way ANOVA. [a]Significantly different ($p \leq 0.05$) from WT mice. [b]Significantly different ($p \leq 0.05$) from air control.

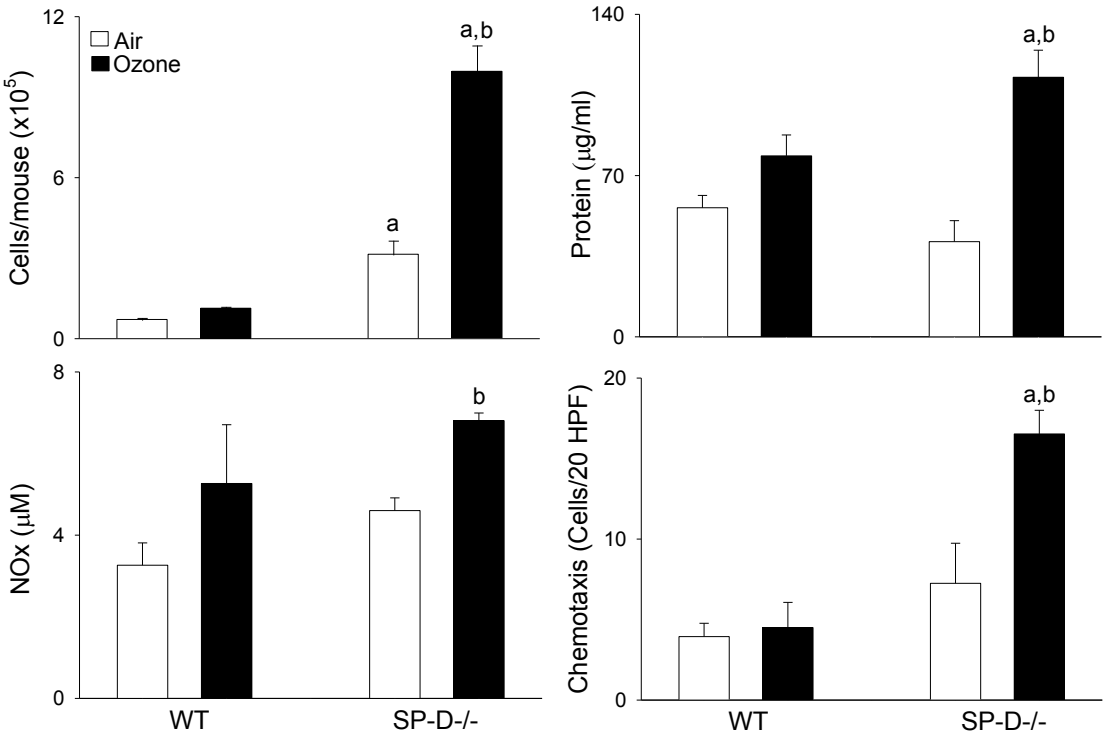

Air
Ozone
Cells/mouse (x10^5)
12
6
0
a
a,b
Protein (μg/ml)
140
70
0
a,b
NOx (μM)
8
4
0
b
Chemotaxis (Cells/20 HPF)
20
10
0
a,b
WT
SP-D-/-
WT
SP-D-/-

Figure 2. Effects of ozone inhalation on lung inflammation. BAL was collected 24-72 hr after exposure of WT and SP-D-/- mice to air or ozone and analyzed for cell number. Each bar represents the mean $\pm$ SEM (n = 3-5 mice/treatment group). Data were analyzed by two-way ANOVA. [a]Significantly different ($p \leq 0.05$) from WT mice. [b]Significantly different ($p \leq 0.05$) from air control.

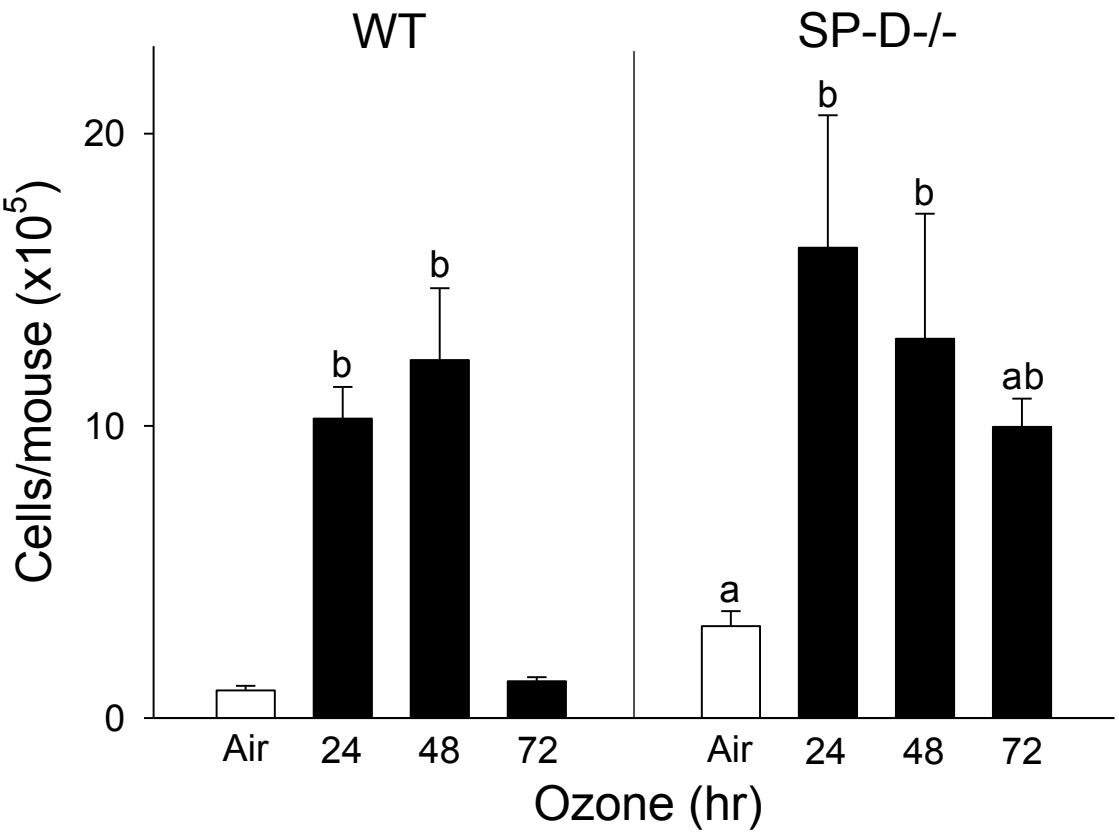

WT
SP-D-/-
Cells/mouse (x10^5)
20
10
0
b
b
a
b
b
ab
Air
24
48
72
Air
24
48
72
Ozone (hr)

Figure 3. Effects of ozone inhalation on lung injury. BAL was collected 24-72 hr after exposure of WT and SP-D-/- mice to air or ozone and analyzed for protein content. Each bar represents the mean $\pm$ SEM (n= 3-5 mice/treatment group). Data were analyzed by two-way ANOVA. [a]Significantly different ($p \leq 0.05$) from air control.

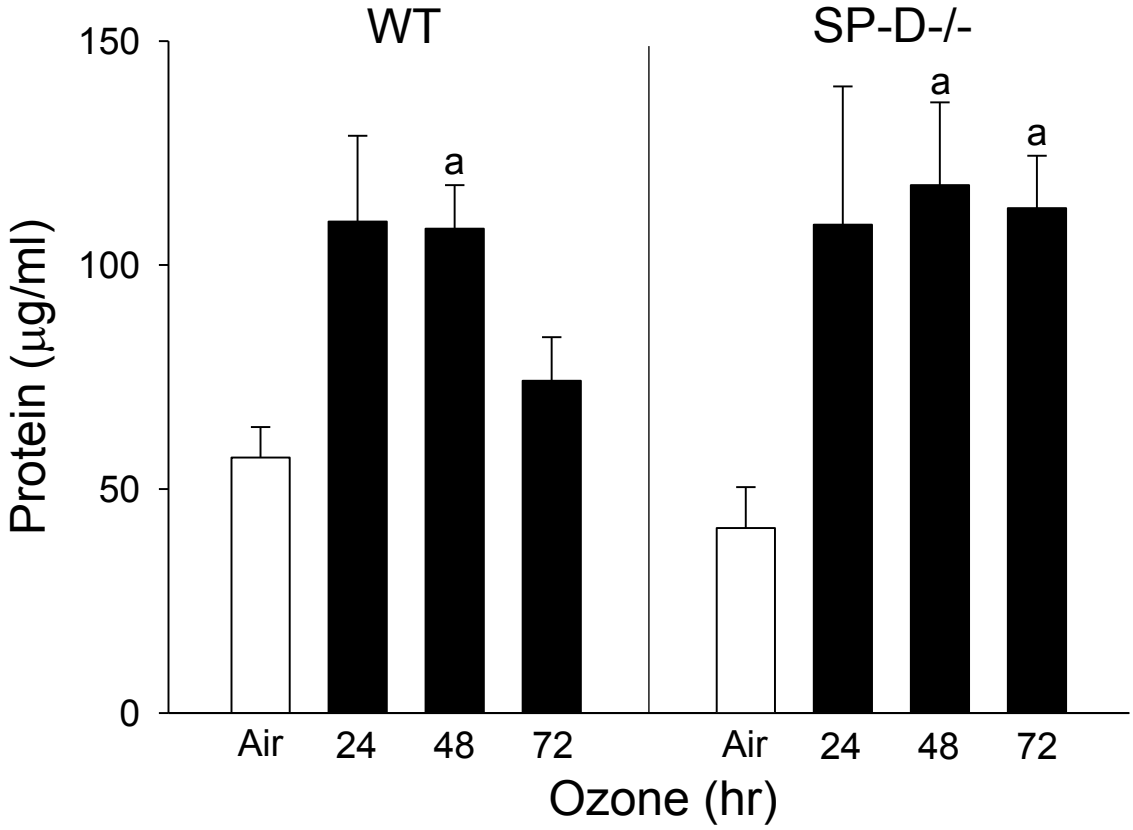

WT
SP-D-/-
150
100
50
0
Protein (μg/ml)
a
a
a
Air
24
48
72
Air
24
48
72
Ozone (hr)

Figure 4. Effects of ozone inhalation on lung inflammation. BAL was collected 48-72 hr after exposure of WT and SP-D-/- mice to air or ozone and analyzed for chemotactic activity. BAL chemotactic activity was assessed using the RAW 264.7 mouse macrophage cell line (ATCC, Manassas, VA) as previously described (E. Crouch et al., 2001). Hank's balanced salt solution was used as the negative control. Chemotaxis data are expressed as the average number of migrated cells per 20 high power fields. The value range for these experiments is 0-30 migrated cells per high power field. Each bar represents the mean $\pm$ SEM (n = 4-9 mice/treatment group). Data were analyzed by two-way ANOVA [a]Significantly different ($p \leq 0.05$) from WT mice. [b]Significantly different ($p \leq 0.05$) from air control.

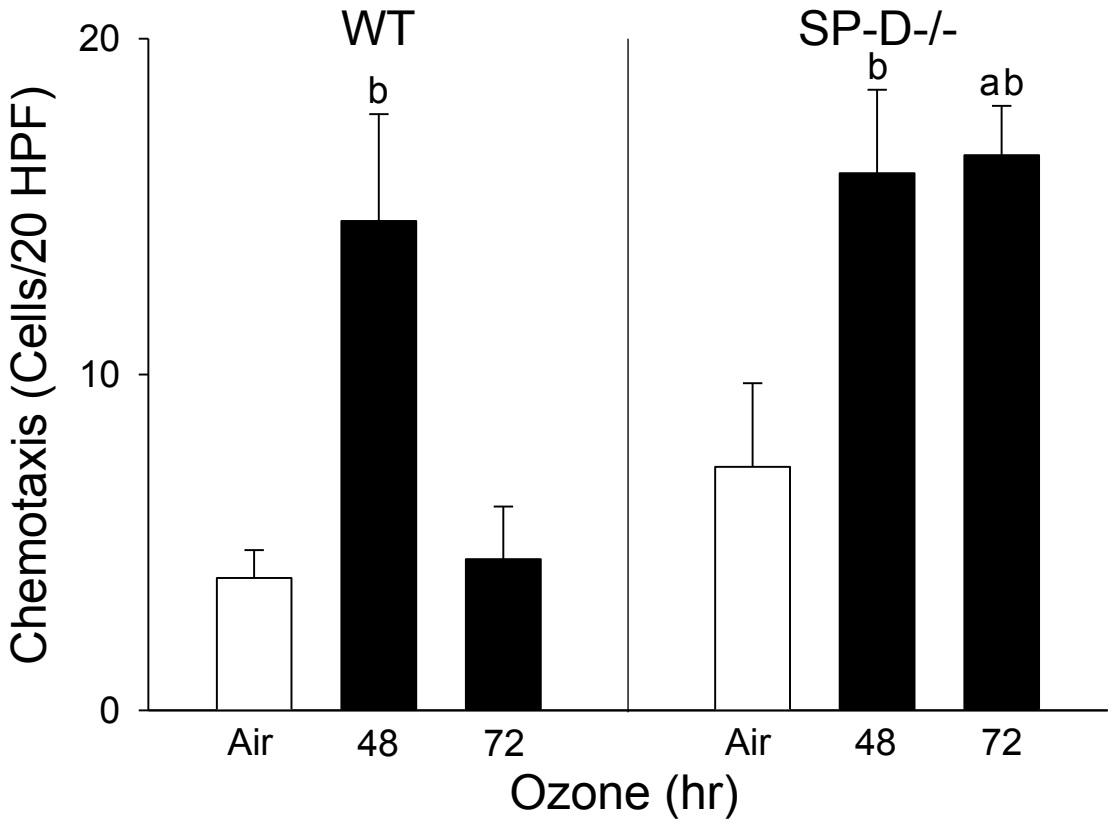
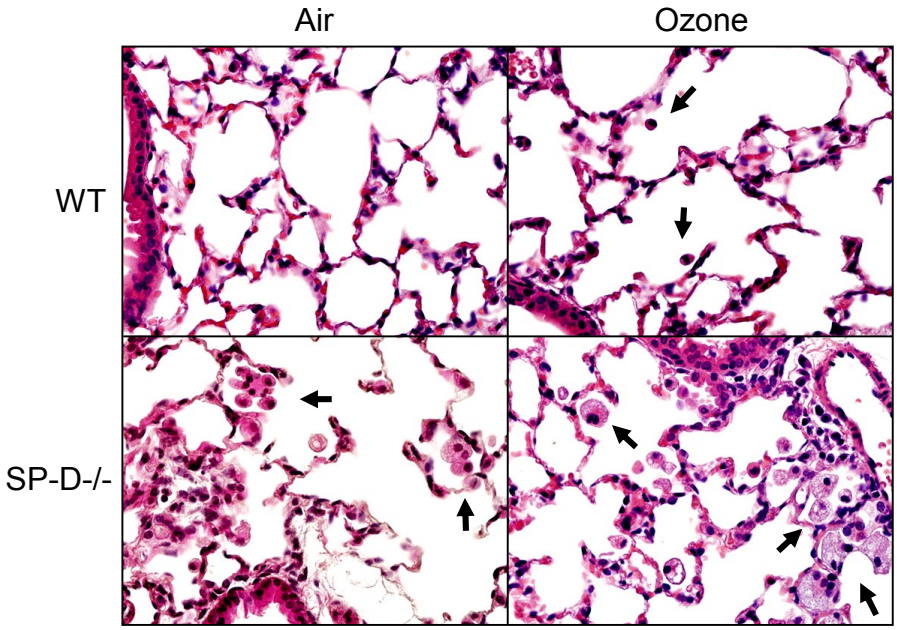

WT
SP-D-/-
20
10
0
Chemotaxis (Cells/20 HPF)
b
b
ab
Air
48
72
Air
48
72
Ozone (hr)

Figure 5. Effects of ozone inhalation on lung structure and inflammation score. *Upper panel*: Lung sections, prepared 72 hr after exposure of WT and SP-D-/- mice to air or ozone, were stained with hematoxylin and eosin. Arrows show alveolar macrophages. Original magnification, 600x. *Lower panel*: Slides were scored blindly by two independent observers for severity of inflammation on a zero to five scale (0 = no inflammation, 5 = severe inflammation). Each bar represents the median of 3 experiments (n = 4-9 mice/treatment group/experiment; n = 12-27 mice/group total). Significance was assessed by ANOVA based on the ranks. . [a]Significantly different ($p \leq 0.05$) from WT mice. [b]Significantly different ($p \leq 0.05$) from air control.


Air
Ozone
WT
SP-D-/-

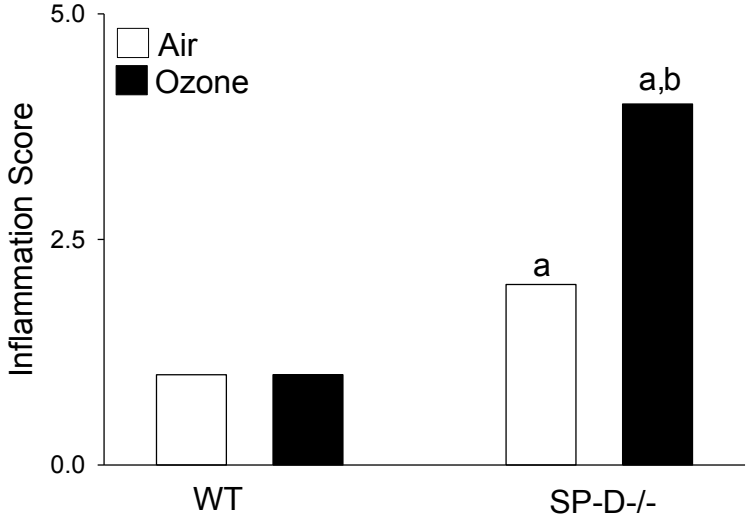

5.0
2.5
0.0
Inflammation Score
Air
Ozone
a
a,b
WT
SP-D-/-

Figure 6. Effects of bronchoalveolar lavage on lung histology. Lung sections from lavaged and non-lavaged WT and SP-D-/- mice were stained with hematoxylin and eosin. Original magnification, 400x.

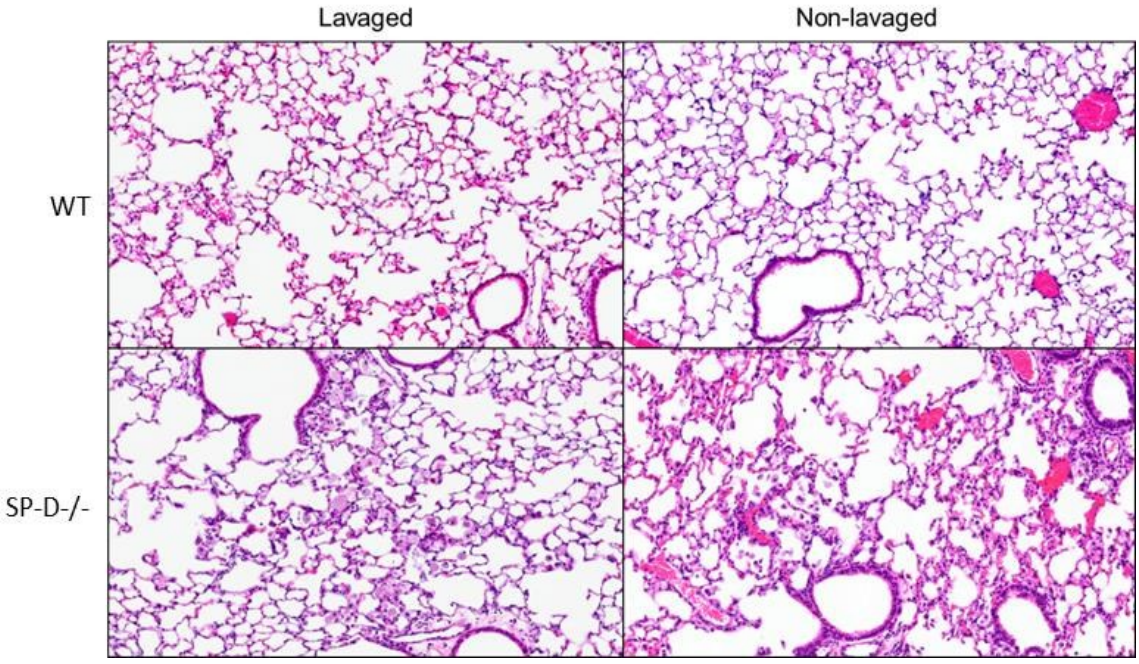

Lavaged
Non-lavaged
WT
SP-D-/-

Figure 7. Effects of ozone inhalation on lung expression of iNOS and COX-2. Lung sections, prepared 72 hr after exposure of WT and SP-D-/- mice to air or ozone, were stained with a 1:100 dilution of antibody to iNOS (upper panels), a 1:1000 dilution of antibody to COX-2 (lower panels), or IgG control followed by a 1:200 dilution of biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. Insets show iNOS or COX-2 positive cells. One representative section is shown from three separate experiments (n = 3 mice/treatment group/experiment). Original magnification, 600x; Inset, 1000x.

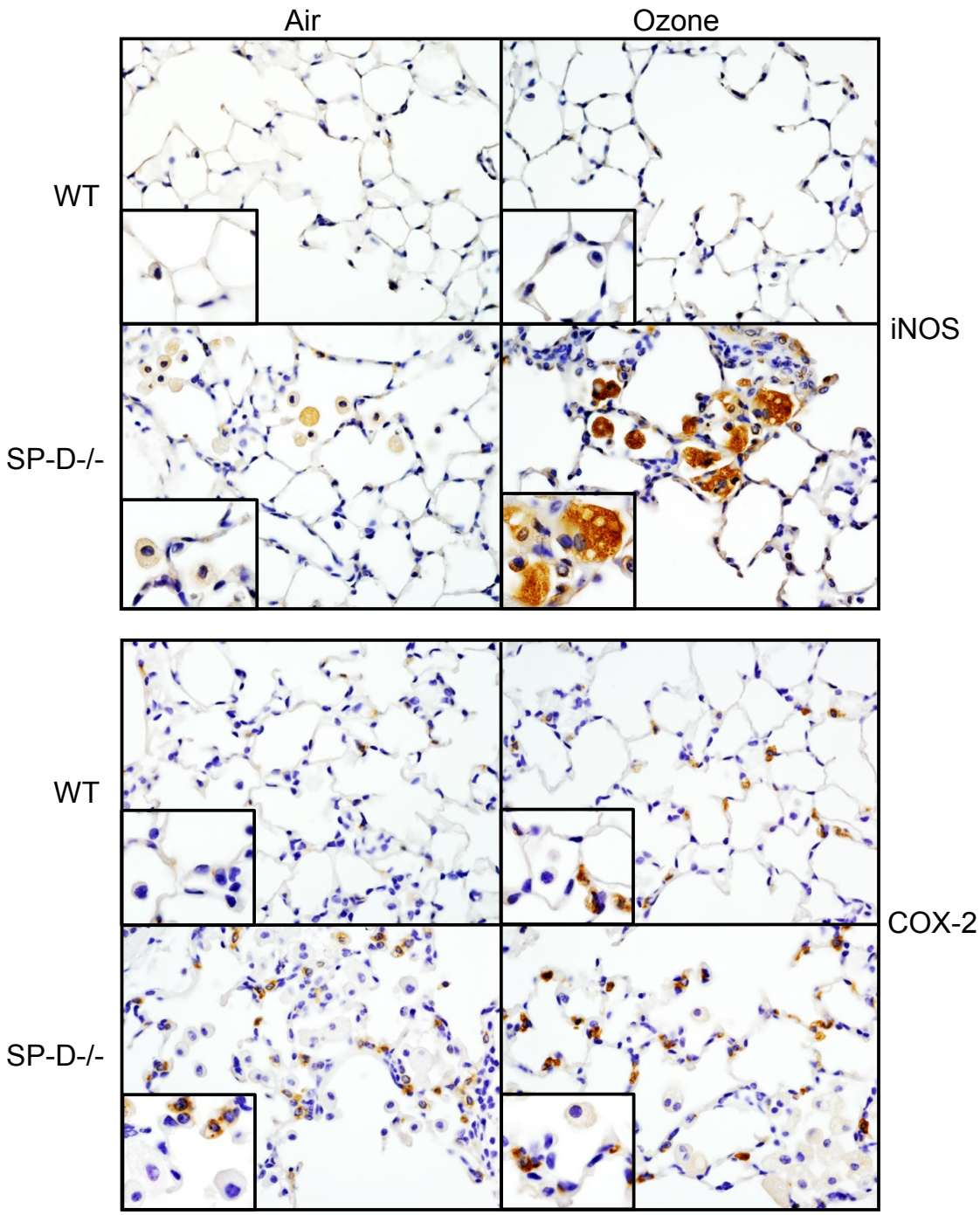

Air
Ozone
WT
SP-D-/-
iNOS
WT
SP-D-/-
COX-2

Figure 8. Quantitative analysis of iNOS and COX-2 expression. Lung sections, prepared 72 hr after exposure of WT and SP-D-/- 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 on a scale of 0 = no staining, 1 = light staining, 2 = medium staining, 3 = dark staining. 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 WT mice. [b]Significantly different ($p \leq 0.05$) from air control.

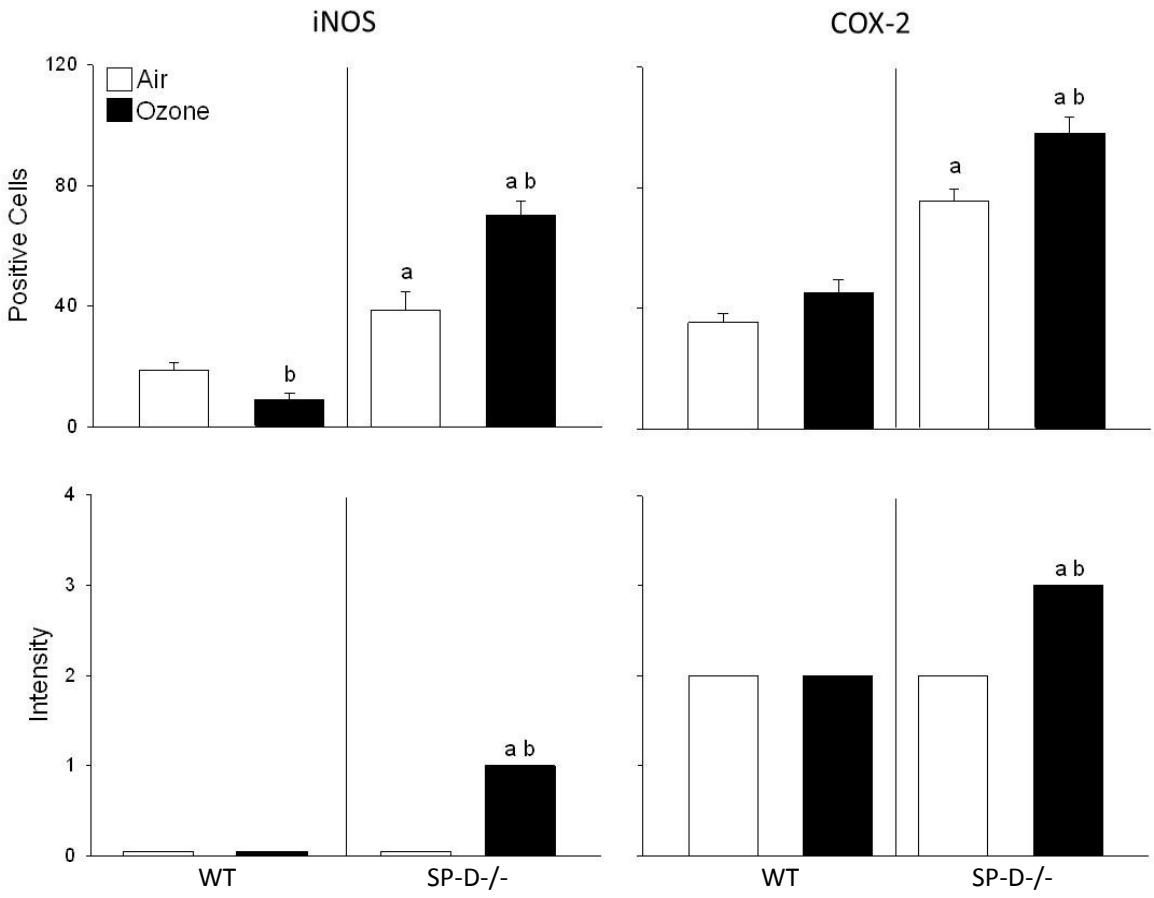

iNOS
COX-2
Air
Ozone
Positive Cells
120
80
40
0
a b
a
b
a b
a
a b
Intensity
4
3
2
1
0
a b
a b
WT
SP-D-/-
WT
SP-D-/-

Table 1. Effects of ozone on baseline lung function in WT and SP-D-/- mice

	WT		SP-D-/-	
	Air	Ozone	Air	Ozone
R (cm H_2Oxs/ml)	0.97 ± 0.03	1.08 ± 0.03	1.37 ± 0.05[b]	1.35 ± 0.11[b]
Rn (mm H_2Oxs/ml)	24.42 ± 1.0	44.83 ± 2.2[a]	49.39 ± 2.2[b]	46.60 ± 1.3
C (ml/mm H_2O)	1.88 ± 0.0	1.79 ± 0.1	1.84 ± 0.1	1.51 ± 0.1b
Cst (ml/mm H_2O)	2.84 ± 0.1	2.78 ± 0.1	3.33 ± 0.2[b]	2.53 ± 0.2[a]
H (cm H_2O/ml)	56.44 ± 1.51	59.48 ± 2.34	53.15 ± 3.38	65.24 ± 3.73[a]

Definition of abbreviations: R, dynamic resistance; Rn, central airway resistance; C, compliance; Cst, static compliance; H, tissue elastance.

Lung function was measured 72 hr after exposure of WT and SP-D-/- mice to air or ozone at a PEEP of 3 cm H_2O. Each measurement was performed in triplicate. Values are means ± SEM of 3 experiments (n = 4-6 mice/treatment group/experiment; n = 12-18 mice/group total). Data were analyzed by two-way ANOVA.

[a]Significantly different ($P < 0.05$) from air control.

[b]Significantly different ($P < 0.05$) from WT mice.

Figure 9. Effects of ozone inhalation on Rn and Cst in WT and SP-D-/- mice. Lung function was measured 72 hr after exposure of WT (circles) and SP-D-/- (squares) mice to air (open symbols) or ozone (closed symbols). The lung was subjected to increasing PEEP. *Upper panel*: For Rn, impedance spectra were measured using the forced oscillation technique and results analyzed using the constant phase model. *Lower panel*: Cst was calculated from pressure-volume loops. Measurements were performed in triplicate at each PEEP. For each sample at each PEEP, Rn and Cst values were normalized to PEEP=3, the physiological pressure for mice. Each point represents the mean $\pm$ SEM of 3 experiments (n = 4-6 mice/treatment group/experiment; n = 12-27 mice/group total). Data were analyzed by two-way ANOVA and a nonpaired two-tailed Student's t test. *Significantly different ($p \leq 0.05$) from air or WT control.

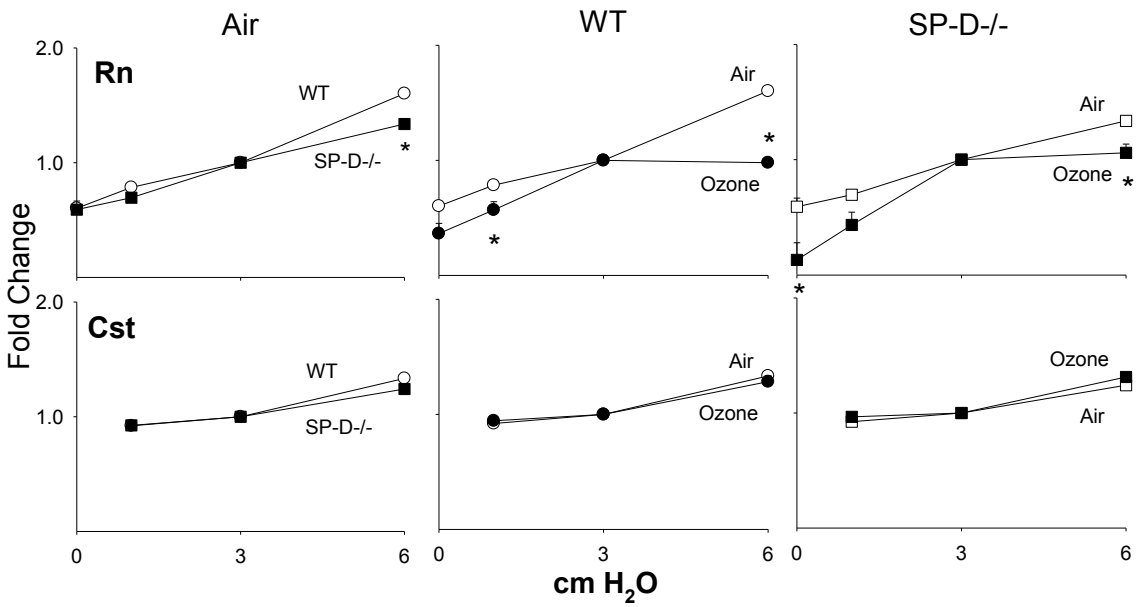

Air
WT
SP-D-/-
Rn
Cst
WT
SP-D-/-
Air
Ozone
Air
Ozone
Ozone
Air
Fold Change
2.0
1.0
0
3
6
cm H2O
*

Figure 10. Effects of ozone inhalation on R_L and E_L spectra in WT and SP-D-/- mice. Lung function was measured 72 hr after exposure of WT and SP-D-/- mice to air or ozone. Lungs were subjected to increasing PEEP. Impedance spectra were generated using the forced oscillation technique. Resistance and elastance spectra were then derived from Z_L. Measurements were performed in triplicate at each PEEP. Each point represents the mean $\pm$ SEM of 3 experiments (n = 4-6 mice/treatment group/experiment; n = 12-27 mice/group total). Data were analyzed by non-linear regression.

*Significantly different ($p \leq 0.05$) from air control.

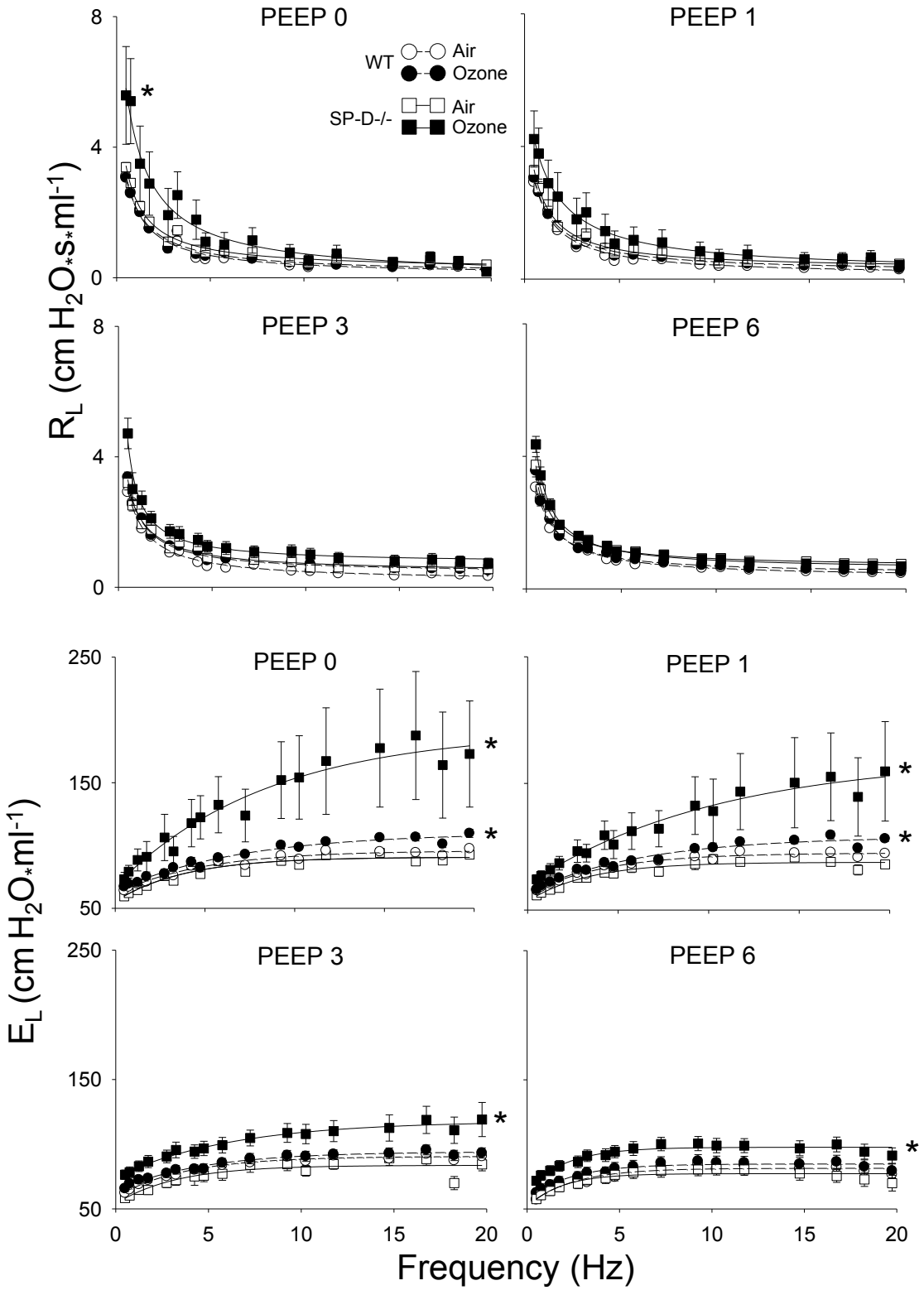

PEEP 0
PEEP 1
PEEP 3
PEEP 6
WT
Air
Ozone
SP-D-/-
Air
Ozone
R_L (cm H_2O*s*ml^{-1})
E_L (cm H_2O*ml^{-1})
Frequency (Hz)
*

Table 2. Starting lung volumes in WT and SP-D-/- mice

	Air	Ozone
WT	0.091 ± 0.019	0.101 ± 0.022
SP-D	0.111 ± 0.028	0.086 ± 0.018

Starting lung volumes were measured at 0 PEEP 72 hr after exposure of WT and SP-D-/- 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.

Table 3. Effects of ozone on R_L and E_L spectra in WT and SP-D-/- mice

		WT		SP-D-/-	
	PEEP	Air	Ozone	Air	Ozone
a	0	3.39 ± 0.11	3.62 ± 0.19	3.69 ± 0.16	8.71 ± 3.41
	1	3.50 ± 0.05	3.53 ± 0.19	3.52 ± 0.32	8.09 ± 3.76
	3	3.20 ± 0.07	2.91 ± 0.22	3.32 ± 0.20	3.12 ± 0.71
	6	3.20 ± 0.13	2.59 ± 0.18	2.89 ± 0.19	3.16 ± 0.25
b	0	0.08 ± 0.01	0.13 ± 0.04	0.24 ± 0.04[b]	-0.06 ± 0.09[a]
	1	0.10 ± 0.02	0.20 ± 0.04[a]	0.29 ± 0.04[b]	0.13 ± 0.04[a]
	3	0.19 ± 0.01	0.43 ± 0.02[a]	0.45 ± 0.01[b]	0.73 ± 0.17
	6	0.34 ± 0.01	0.45 ± 0.01	0.66 ± 0.01[b]	0.57 ± 0.06
c	0	0.58 ± 0.03	0.70 ± 0.06	0.62 ± 0.05	0.76 ± 0.19
	1	0.67 ± 0.03	0.68 ± 0.04	0.60 ± 0.04	1.05 ± 0.33
	3	0.61 ± 0.01	0.45 ± 0.05	0.67 ± 0.13	0.31 ± 0.16
	6	0.58 ± 0.02	0.30 ± 0.02[a]	0.37 ± 0.03[b]	0.29 ± 0.03
E_0	0	61.36 ± 1.21	63.75 ± 1.35	54.90 ± 0.96[b]	66.46 ± 4.92
	1	62.08 ± 1.11	64.04 ± 1.22	56.62 ± 1.95[b]	69.26 ± 2.40[a]
	3	59.18 ± 1.31	64.04 ± 2.43	53.66 ± 2.15	73.88 ± 4.20[a]
	6	52.45 ± 1.35	57.37 ± 3.41	51.19 ± 2.89	65.24 ± 3.25[a]
ΔE	0	34.57 ± 0.85	46.92 ± 2.75[a]	36.55 ± 0.83	130.54 ± 56.58[a]
	1	32.99 ± 0.65	44.60 ± 1.59[a]	31.16 ± 1.75	115.18 ± 60.07[a]
	3	31.43 ± 0.77	30.61 ± 1.73	30.12 ± 2.69	57.01 ± 19.96
	6	29.02 ± 0.81	27.52 ± 1.92	26.18 ± 2.29	32.59 ± 2.46
β	0	0.20 ± 0.00	0.15 ± 0.02[a]	0.27 ± 0.05	0.18 ± 0.03[a]
	1	0.24 ± 0.01	0.16 ± 0.02[a]	0.30 ± 0.03	0.14 ± 0.03[a]
	3	0.27 ± 0.01	0.22 ± 0.02	0.30 ± 0.03	0.21 ± 0.04
	6	0.41 ± 0.01	0.44 ± 0.03	0.56 ± 0.05[b]	0.48 ± 0.03

Definition of abbreviations: a, b and c are parameters of R_L; a and c reflect R_L at low frequencies; b, reflects R_L at high frequencies. E_0, ΔE and β are parameters of E_L. E_0 represents E_L at 0 Hz. ΔE reflects the magnitude of E_L, and β the rate of E_L change with frequency. Lung function was measured 72 hr after exposure of WT and SP-D-/- mice to air or ozone. Each measurement was performed in triplicate. Values are means ± SEM of 3 experiments (n= 4- 6 mice/treatment group/experiment; n= 12-18 mice/group total). Data were analyzed by two-way ANOVA.

[a]Significantly different ($P< 0.05$) from air control.

[b]Significantly different ($P< 0.05$) from WT mice.

PART II. AGE-RELATED INCREASES IN OZONE-INDUCED INJURY AND ALTERED PULMONARY MECHANICS IN MICE WITH PROGRESSIVE LUNG INFLAMMATION

Aging is associated with dysregulation of the immune system resulting in low level chronic inflammation (Brubaker et al., 2011; H. Y. Chung et al., 2009; Kovacs et al., 2009). This pathology, along with impairments in antioxidant defense, are thought to contribute to exacerbated responses of the elderly to tissue injury and infection, and the development of inflammatory diseases such as COPD (Brubaker et al., 2011; Franceschi et al., 2000; Kovacs et al., 2009; Sarkar et al., 2006). Macrophages are known to play a key role in chronic inflammation, and their ability to respond to environmental signals has been reported to be altered with age. Thus, macrophages exhibit age related increases in chemotaxis and the generation of reactive oxygen and nitrogen species (Smallwood et al., 2011). Macrophages from elderly rodents also release increased quantities of cytokines which amplify their proinflammatory activity (Kohut et al., 2004). This is correlated with increases in the abundance of a number of proteins that promote macrophage activation towards a classically activated proinflammatory phenotype (Smallwood et al., 2011).

In the lung, macrophage activity is regulated by SP-D, a pulmonary collectin synthesized mainly by alveolar type II cells (E. Crouch et al., 2001; Haczku, 2008). Under homeostatic conditions, SP-D functions as an anti-inflammatory protein, suppressing NF-κB mediated transcription of macrophage inflammatory genes (Gardai et al., 2003). However, following induction of oxidative stress, increased production of nitric oxide results in S-nitrosylation of critical cysteines in SP-D leading to a change in

its activity to a proinflammatory mediator (Guo et al., 2008). Findings that mice lacking SP-D exhibit chronic pulmonary inflammation, characterized by the presence of activated macrophages in the lung, and that this increases with age, are consistent with an important function of SP-D in innate immunity (Wert et al., 2000). In previous studies we demonstrated that low level pulmonary inflammation accompanying loss of SP-D in young mice, is associated with prolonged injury, oxidative stress, and altered pulmonary mechanics in response to inhaled ozone (Groves et al., 2012). The effects of increasing pulmonary inflammation and the development of emphysema in aging SP-D-/- mice on the response to inhaled ozone are unknown and were investigated. For these studies, we analyzed the response to ozone 72 hr post exposure, a time when lung injury and inflammation have, for the most part, resolved in young (8-10 wk old) WT mice (Fakhrzadeh et al., 2002). This allowed us to directly assess the impact of progressive lung inflammation and injury, as a consequence of loss of SP-D, on sensitivity to ozone. Surprisingly, we found that the ability of WT mice to repair ozone-induced lung injury was unaffected by increasing age. In contrast, while loss of SP-D resulted in increased ozone toxicity in 8 wk and 27 wk old mice, by 80 wk these effects were overwhelmed by extensive baseline injury and inflammation. These data provide additional support for a role of activated macrophages in the development of chronic lung diseases in the elderly (Hodge et al., 2003; Sharma et al., 2009).

RESULTS

Effects of increasing age on lung histology in WT and SP-D-/- mice

In initial studies we analyzed the effects of increasing age on lung structure. In SP-D-/- mice, but not WT mice, age-related alterations were noted in lung histology (Figure 11). Thus, consistent with previous reports (Botas et al., 1998; Wert et al., 2000), at 8 wk of age, minimal to mild multifocal infiltrations of macrophages and lymphocytes were observed; macrophages were predominately localized in perivascular regions, while lymphocytes were concentrated around bronchioles and in the interstitium (Figure 11). Histological scoring of the tissue confirmed increased numbers of macrophages and lymphocytes in the tissue of SP-D-/- mice (Table 4). Some of the macrophages were enlarged and vacuolated, and exhibited a ground-glass-like granular cytoplasm, while others contained an eosinophilic cytoplasm, suggestive of phagocytized surfactant (Figure 11). 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 (Figure 11 and Table 4). Similar changes were noted in 27 wk old SP-D-/- mice, however they were more pronounced; thus macrophage infiltration into perivascular and peribronchiolar regions was greater, becoming mild to moderate (Figure 11 and Table 4). 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 pathologic changes had

become severe and included mild to marked macrophage and lymphocyte inflammation (Figure 11 and Table 4). There was also an abundance of multinucleated "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 II 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 interstitum and in some alveoli, necrotic debris was visible.

Radial alveolar counting supported age related increases in alveolar epithelial destruction in SP-D-/- mice. Thus, a decrease was observed in radial alveolar counts in SP-D-/- mice relative to WT mice (Figure 13). This was evident at 27 wk, persisting for at least 80 wk. In 80 wk old SP-D-/- mice, significant increases in BAL protein levels were also observed (Figure 14). BAL cell number was increased in SP-D-/- 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 (Figures 13 and 14).

In response to lung injury, Type II cells become enlarged and begin to proliferate in order to repair damaged alveolar epithelium (G. Y. Park et al., 2006; Prokhorova et al., 1998). Pro-SP-C is a specific marker of Type II cells (Glasser et al., 1991). Low constitutive levels of pro-SP-C were detected in Type II cells in young SP-D-/- mice, and to a lesser extent in young WT mice (Figure 15). In Type II cells from SP-D-/- mice, this was associated with low levels of expression of the proliferation marker, proliferating cell

nuclear antigen (PCNA) (Figure 16). In both WT and SP-D-/- mice, the number of Type II cells expressing pro-SP-C and PCNA increased with increasing age. This response was more pronounced in SP-D-/- mice, relative to WT mice.

Aging is associated with oxidative stress (Finkel et al., 2000); therefore, we analyzed the effects of increasing age on expression of the antioxidant enzyme HO-1, which is highly responsive to oxidative stress (Haines et al., 2012). Consistent with previous reports (Fredenburgh et al., 2007), HO-1 was found to be constitutively expressed by alveolar macrophages (Figure 17). In WT mice, increasing age was associated with decreased expression of HO-1 in alveolar macrophages. In contrast, in SP-D-/- mice, the number of HO-1 positive macrophages increased significantly as the mice grew older, a response which was most prominent at 80 wk of age.

COX-2 has been shown to increase during the development of chronic pulmonary inflammation, such as COPD (Xaubet et al., 2004). Low constitutive levels of COX-2 were noted in Type II cells in young SP-D-/- mice, but not WT mice (Figure 18). In SP-D-/- mice, increasing age was associated with increased expression of COX-2 in 27 wk and 80 wk old mice, while in WT mice aging had no effect.

We next characterized the phenotype of the macrophages in the lungs of WT and SP-D-/- mice by analyzing expression of markers of classical and alternative activation. iNOS is an enzyme mediating the generation of proinflammatory RNS and is a prototypical marker of classically activated macrophages (Laskin et al., 2011; G. Y. Park et al., 2006). In 8 wk old SP-D-/- mice, but not WT mice, low numbers of iNOS expressing macrophages were present in the lung (Figure 19). In SP-D-/- mice, the number of iNOS positive macrophages increased significantly with age. Mannose

receptor, YM-1 and galectin-3 are markers of alternatively activated macrophages that display anti-inflammatory activity and participate in wound repair (Laskin et al., 2011; Raes et al., 2002; Stein et al., 1992). Macrophage expression of each of these markers was increased at 27 wk of age relative to 8 wk of age in WT mice (Figures 20-22). In contrast, at 80 wk of age, macrophage expression of mannose receptor and YM-1was decreased, while galectin-3 remained elevated. Loss of SP-D 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 SP-D-/- mice, the number 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.

Effects of increasing age on pulmonary mechanics in WT and SP-D-/- mice

The effects of aging on lung function in WT and SP-D-/- mice were analyzed by examining pulmonary mechanics. Increasing age from 8 wk to 27 wk significantly altered resistance and elastance spectra in both WT and SP-D-/- mice. Thus, decreases in the resistance and elastance spectra were noted in SP-D-/- mice, and in the elastance spectra in WT mice. The resistance spectra was also decreased in WT mice at 27 wk, but only at low frequencies; at high frequencies, it was increased (Figure 23). Whereas in WT mice no changes in the elastance spectra were noted at 80 wk, relative to 27 wk old mice, in 80 wk old SP-D-/- mice, the elastance spectra was reduced. We also found that the elastance spectra in 8 wk old SP-D-/- mice was significantly decreased relative to 8 wk old WT mice. While loss of SP-D had no effect on the elastance spectra at 27 wk of age, at 80 wk it was increased. In 80 wk old SP-D-/- mice, the resistance spectra was

also increased at low frequencies relative to WT mice. To better define the nature of these age related changes, respiratory mechanics were 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 wk and 80 wk of age (Figure 26). Altered pulmonary elastance was also noted in 27 wk and 80 wk old WT mice, which was evident from decreases in the frequency dependent change, ΔE, as well as in the low frequency parameter, E_0. Loss of SP-D resulted in a significant increase in **b** in 8 wk old mice, but did not alter any other parameter at this age. Whereas no major differences were noted between WT and SP-D-/- mice at 27 wk of age, at 80 wk, **a/c** was significantly increased, and E_0 and ΔE were significantly decreased in SP-D-/- mice, relative to WT mice. In SP-D-/- mice, increasing age from 8 wk to 27 wk was associated with decreases in the resistance parameters, **a/c** and **b**, and the elastance parameters E_0 and ΔE. By 80 wk of age, however, parameters representing the resistance and elastance spectra were similar to 8 wk old SP-D-/- mice.

Effects of Increasing Age on the Response of WT and SP-D-/- Mice to Inhaled Ozone

In our next series of studies we analyzed the effects of inhaled ozone on aging WT and SP-D-/- mice. Consistent with our previous studies (Groves et al., 2012), no significant histologic changes were observed in the lungs of 8 wk old WT mice 72 hr following ozone exposure (Figure 12); radial alveolar counts were also unaltered in these mice (Figure 13). Increasing age had no significant effect on the sensitivity of WT mice to ozone, in terms of changes in lung structure. In contrast, in SP-D-/- mice, exposure to

ozone resulted in increased numbers of lymphocytes in the lung, a response that was prominent at 8 wk of age, with no effects on macrophages (Figure 12 and Table 4). Type II cell and bronchial epithelial hyperplasia were also increased in the lungs of SP-D-/- mice, however, this was not observed until 27 wk and 80 wk of age, respectively. Conversely, alveolar fibrin deposition was decreased in 80 wk old ozone treated SP-D-/- mice, relative to air exposed mice. Ozone exposure also resulted in increases in BAL protein and cell number in 8 wk old SP-D-/- mice, with no effect in 27 wk or 80 wk old SP-D-/- mice, or in WT mice (Figure 14). In contrast, expression of pro-SP-C was reduced in Type II cells after ozone inhalation in 8 wk old SP-D-/- mice, as well as in WT mice at 27 wk and 80 wk of age (Figure 15). Additionally, in both WT and SP-D-/- mice, Type II cell expression of PCNA increased following ozone exposure, but only at 8 wk of age, with no effect in 27 wk or 80 wk old mice (Figure 16). Ozone exposure resulted in increased macrophage HO-1 expression in 8 wk old WT mice, while in 8 wk SP-D-/- mice, HO-1 decreased (Figure 17). In contrast, ozone had no effect on HO-1 expression in 27 wk or 80 wk old WT or SP-D-/- mice. Increased COX-2 expression was also observed in Type II cells at all ages in WT mice following ozone intoxication, and in 8 wk and 27 wk old SP-D-/- mice (Figure 18).

The effects of ozone on macrophage phenotype were also analyzed in aging WT and SP-D-/- mice. In WT mice, increased expression of iNOS in macrophages, as well as Type II cells was observed following ozone exposure at 80 wk of age, with no effects at 8 wk or 27 wk. In contrast, in SP-D-/- mice, increases in the number of iNOS positive macrophages were noted at 8 wk and 27 wk of age, with no further change relative to air control at 80 wk (Figure 19). Following ozone exposure, the number of YM-1, mannose

receptor and galectin-3 positive macrophages was increased, when compared to air control in 8 wk old, but not in 27 wk or 80 wk old WT mice (Figures 20-22). Conversely, ozone caused no changes in expression of these markers in SP-D-/- mice in any age group.

We next assessed the effects of ozone on pulmonary mechanics in young, middle aged and elderly WT and SP-D-/- mice. In WT mice, ozone exposure significantly increased resistance spectra at 8 wk of age and both resistance and elastance spectra at 27 wk of age, with no effects at 80 wk (Figures 24 and 25). Analysis of the component parameters revealed that these changes were associated with significant increases in **b** in 8 wk old mice and ΔE in 27 wk old mice (Figure 26). In 8 wk SP-D-/- mice, ozone exposure resulted in significant increases in both resistance and elastance spectra, which correlated with increases in **b** and E_0, respectively. Similarly, in 27 wk old SP-D-/- mice, resistance and elastance spectra were also significantly increased following ozone exposure. Despite the increase in resistance spectra, no individual component was significantly altered in SP-D-/- mice at this age; the increased elastance observed corresponded to increases in both E_0 and ΔE. In 80 wk old SP-D-/- 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 (Al-Hegelan et al., 2011; Ciencewicki et al., 2008). Epidemiologic data indicate that individuals with diseases such as asthma or COPD are hypersensitive to ozone, exhibiting increased morbidity and mortality (Desqueyroux et al., 2002; Zanobetti et al., 2011). Similar results have been reported in the elderly (Ciencewicki et al., 2008). The role of progressive chronic inflammation in this increased sensitivity is unknown and was assessed in the present studies using young (8 wk old), middle aged (27 wk old) and elderly (80 wk old) WT and SP-D-/- mice. Unexpectedly, we found that increasing age was not associated with increases in numbers of inflammatory cells in the lungs of WT mice, and did not significantly alter their ability to repair ozone-induced injury. These results were surprising since in humans, aging is associated with low level chronic inflammation and reduced responsiveness to infectious agents (H. Y. Chung et al., 2009; Sarkar et al., 2006). These data suggest a potential limitation with the use of healthy mouse models in studies of aging. In contrast, in SP-D-/- mice, aging was associated with progressive inflammation; moreover, this resulted in increased sensitivity to ozone, but only up to 27 wk of age. By 80 wk of age, it appears that lung inflammation and injury have reached maximal levels, such that ozone cannot cause further damage.

SP-D has been shown to regulate macrophage inflammatory activity in the lung (Gardai et al., 2003; Guo et al., 2008). Consistent with this function are findings that loss of SP-D results in chronic pulmonary inflammation, characterized by elevated numbers of activated macrophages in the lung which progressively increases as the animal age (Botas et al., 1998; Wert et al., 2000). Similarly, we found evidence of enlarged granular

macrophages in the lungs of young SP-D-/- mice, and increased numbers of these cells in BAL. These changes became more pronounced as the mice aged. Thus, greater numbers of enlarged macrophages were present in the tissue and in BAL relative to 8 wk old SP-D-/- mice. In addition in older mice multinucleated giant cells were observed. By 80 wk of age, many of the macrophages also contained cholesterol crystals, suggestive of phagocytized surfactant. Deficiency in SP-D has been shown to result in increased accumulation of surfactant lipids in alveolar spaces (Botas et al., 1998; Ikegami et al., 2005). Clearance of surfactant is accomplished either by reuptake and recycling in Type II cells or macrophage phagocytosis and degradation (Hawgood et al., 2002). Our observations of Type II cell hyperplasia and cholesterol crystals in macrophages indicate that by 80 wk of age, there are significant aberrations in surfactant homeostasis. This is in accord with previous studies showing increases in SP-A and SP-B production and the development of large lamellar bodies in Type II cells in SP-D-/- mice as they aged (Botas et al., 1998).

Evidence suggests that pathologic changes in the lungs of SP-D-/- mice are progressive, resulting in the development of emphysema that becomes pronounced at 24 wk of age (Wert et al., 2000; Yoshida et al., 2001). Similarly, in 27 wk old SP-D-/- mice, we found significant alveolar rupture in the lung and decreases in radial alveolar counts, which are characteristic features of emphysema. Moreover, this continued to progress for at least 80 wk; this has not been reported previously. Additionally, at 27 wk of age interstitial thickening was present and by 80 wk of age, fibrin deposition in the alveolar spaces was evident. We also observed significant increases in Type II cell expression of pro-SP-C and PCNA, a typical response to lung injury (Mason, 2006; Prokhorova et al.,

1998). This was associated with increases in BAL protein levels in 80 wk old SP-D-/- mice, confirming the persistence of epithelial injury. Type II cell expression of COX-2 was also increased with age, suggesting that these cells contribute to chronic inflammation in these mice. In contrast, no significant age related alterations in lung structure or inflammation were observed in WT mice, demonstrating the importance of SP-D in protecting the lung from the development of progressive inflammatory pathology. We did, however, note increases in Type II cell expression of pro-SP-C and PCNA in aging WT mice. Type II cells proliferate in response to lung injury, and are important for the maintenance of the alveolar epithelium (Herzog et al., 2008; Mason, 2006). Increased expression of pro-SP-C and PCNA in Type II cells in middle aged and elderly WT mice suggests some low level submicroscopic alveolar epithelial injury.

Oxidative stress and impaired antioxidant defense play important roles in chronic inflammatory diseases such as COPD. Reactive oxygen species generated by inflammatory cells add to the increased oxidative burden, and have been reported to contribute to parenchymal destruction (K. F. Chung et al., 2008). HO-1 is a cytoprotective antioxidant enzyme that is highly responsive to oxidative stress (Haines et al., 2012; Herzog et al., 2008). It also has anti-inflammatory activity and has been shown to suppress macrophage proinflammatory responses (Weiss, 2007). In 8 wk old WT mice, constitutive expression of HO-1 was evident in alveolar macrophages; expression decreased with increasing age, confirming impairment in antioxidant defenses. In contrast, in SP-D-/- mice, numbers of HO-1 positive macrophages increased with age, which may function to reduce their proinflammatory activity.

The diverse activities of macrophages are thought to be mediated by distinct subpopulations that develop in response to inflammatory signals they encounter in their microenvironment. Thus, following exposure to Th1 stimuli such as interferon-γ and toll like receptor-4 agonists including LPS, macrophages acquire a classically activated proinflammatory/cytotoxic phenotype, while exposure to Th2 cytokines like interleukin IL-4, IL-13, and IL-10 results in an alternatively activated phenotype, characterized by anti-inflammatory and wound repair activity (S. Gordon, 2003; Hodge et al., 2003; X. Zhang et al., 2008). In WT mice, aging had no effect on numbers of classically activated iNOS-positive macrophages in the lung, while increases in numbers of alternatively activated mannose receptor and YM-1 positive macrophages were observed at 27 wk of age and galectin-3 positive macrophages at 27 wk and 80 wk of age. In contrast in SP-D-/- mice, age related increases in both classically activated and alternatively activated macrophages were noted. Increases in alternatively activated macrophages in the lung as these mice age may reflect a compensatory attempt to limit tissue injury in the absence of SP-D. Of note was our observation that peak accumulation of galectin-3 positive macrophages was observed in 27 wk old SP-D-/- mice, while mannose receptor and YM-1 positive macrophages continued to increase up to 80 wk of age. These data highlight the complexity of macrophage phenotype and support the idea that there are multiple subpopulations of alternatively activated macrophages involved in the response to chronic tissue injury (Laskin et al., 2011; Martinez et al., 2008).

We also found that pulmonary mechanics was altered with aging in both WT and SP-D-/- mice. In WT mice decreases in pulmonary resistance at low frequencies, as well as elastance spectra were observed in 27 wk and 80 wk old mice, relative to 8 wk old

mice, indicating that lung stiffness decreases with age. These findings are in accord with previous reports of age related decreases in lung elastance (Janssens, 2005). Analysis of the component spectral parameters revealed that although resistance and elastance of the tissue decreased with age, resistance of the airways increased. These results are consistent with decreases in parenchymal integrity with age leading to a loss of lung rigidity, and hence a fall in overall elastance and resistance. These changes would also produce a reduction in parenchymal tethering of the airways, which may explain the rise in airway resistance.

Loss of SP-D exacerbated the effects of aging on pulmonary mechanics. Thus, decreases in the elastance spectra were noted in 8 wk old SP-D-/- mice relative to WT mice. Similar decreases in elastance have been observed in other mouse models of emphysema (S. Ito et al., 2006; S. Ito et al., 2005). Elastance spectral changes correlated with age related decreases in parameters reflecting inherent tissue elastance, E_0, and frequency dependence, ΔE suggesting a loss of parenchymal integrity. Reversal of many of these trends in 80 wk old SP-D-/- mice is indicative of lung stiffening, which is most likely the result 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 SP-D-/- mice up to 13 wk of age (Collins et al., 2003). Our findings that lung inflammation and structural abnormalities continue to increase up to 80 wk are novel, and consistent with reports that inflammation contributes to reduced pulmonary function (Hakansson et al., 2012; Sharma et al., 2009).

Evidence suggests that the elderly are more susceptible to the adverse effects of air pollution, in part due to a low chronic inflammatory state (Ciencewicki et al., 2008;

Franceschi et al., 2000). In further studies we analyzed the effects of increasing age on susceptibility to ozone-induced lung injury in WT mice and mice lacking SP-D. Since pulmonary inflammation and macrophage accumulation and activation increase markedly in SP-D-/- mice as they age, we speculated that these mice would be hypersensitive to ozone, and indeed this was the case. Thus, in SP-D-/- mice, but not WT mice, ozone inhalation resulted in significant Type II cell hyperplasia at 27 wk, and bronchiolar epithelial cell hyperplasia at 80 wk, which is consistent with the response of young WT mice early (24-48 hr) after ozone exposure (Castleman et al., 1980; Prokhorova et al., 1998; Schuller-Levis et al., 1995). In contrast, ozone inhalation caused no further alterations in radial alveolar counts, or in BAL cell number or protein content in middle aged or elderly SP-D-/- mice. These data suggest that extensive baseline injury and inflammation in senescent SP-D-/- mice may reduce their ability to respond to an environmental insult.

Although in WT mice increasing age had no significant effect on ozone-induced injury or inflammation, increases in macrophage HO-1 expression were blunted. These findings are in accord with reports of age related defects in HO-1 expression in response to LPS stimulation in alveolar macrophages (Y. Ito et al., 2009). In SP-D-/- mice, ozone caused decreases in alveolar macrophage HO-1 expression in young, but not middle aged or elderly mice. This may contribute to increased numbers of proinflammatory macrophages in the lungs of SP-D-/- mice treated with ozone (Weis et al., 2009).

Consistent with previous studies (Prokhorova et al., 1998), we found that PCNA expression was increased in Type II cells 72 hr following exposure of young WT mice to ozone, reflecting persistent tissue repair. This response was not altered by loss of SP-D,

suggesting that similar repair processes are activated in these mice following ozone intoxication. This is supported by findings that in both WT and SP-D-/- mice, increasing age resulted in a blunted PCNA response to ozone. This may be due to the greater baseline proliferative response in Type II cells as the mice age. We also found that pro-SP-C expression was suppressed in 27 wk and 80 wk old WT mice and 8 wk old SP-D-/- mice following ozone exposure, which may be indicative of reduced surfactant activity. This is supported by findings of abnormal surfactant function in mice following acute lung injury induced by LPS (Ingenito et al., 2001). Our findings that both PCNA and pro-SP-C expression were unaltered by ozone exposure, relative to air control in 27 wk and 80 wk old SP-D-/- mice indicate that while SP-D does not appear to regulate the ability of Type II cells to proliferate in response to ozone-induced injury, it may contribute to the maintenance of surfactant homeostasis in older animals.

Ozone exposure has also been shown to increase COX-2 expression in the lung (V. R. Sunil, Patel-Vayas, et al., 2012). Similarly, we found that ozone inhalation increased the number of COX-2+ Type II cells in WT mice of all ages, and in SP-D-/- mice at 8 wk and 27 wk of age. At 80 wk of age, it appears that maximum COX-2 expression is already present in the majority of these cells. These findings support previous reports that Type II cell expression of COX-2 is induced by ozone exposure and also demonstrate that this response is not impaired with age or the presence of chronic pulmonary inflammation.

Age related alterations in macrophage phenotype were also observed in the lung after ozone exposure. Whereas in young (8 wk old) WT mice, increases in alternatively activated macrophages were observed, which is consistent with our PCNA data and the

resolution of inflammation and injury 72 hr post ozone exposure (Fakhrzadeh et al., 2002), in elderly (80 wk old) WT mice, increases in iNOS positive classically activated macrophages were noted, with no change in numbers of mannose receptor, YM-1, or galectin-3 positive alternatively activated macrophages. These data suggest a shift in macrophage activation in elderly WT mice toward a proinflammatory, classically activated phenotype (Smallwood et al., 2011). 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 (L. C. Chen et al., 1996; Kohut et al., 2004). In SP-D-/- mice, ozone inhalation also resulted in increased numbers of iNOS positive classically activated macrophages; however this was only observed in young and middle aged mice. By 80 wk, it appears that maximum numbers of iNOS positive macrophages are already present in the lung. Previous studies have shown that lung injury is blunted in mice lacking iNOS demonstrating a key role of reactive nitrogen species in ozone toxicity (Fakhrzadeh et al., 2002). Increased iNOS expression in SP-D-/- mice at 8 wk and 27 wk of age may contribute to their increased sensitivity to ozone. In SP-D-/- mice, ozone had no effects further effect on numbers of macrophages expressing mannose receptor, YM-1, or galectin-3. These data provide additional support for the idea that the pathological effects of loss of SP-D are greater than those of ozone.

In accord with previous reports (Groves et al., 2012), ozone-induced functional alterations in lung mechanics including increases in airway resistance persisted for at least 72 hr post exposure in young WT mice, despite the resolution of injury and inflammation. Interestingly, these functional changes were blunted at 27 wk and 80 wk

of age. This may reflect the fact that ozone-induced changes in airway mechanics are not discernible from the more significant age-related increases in these activities. In SP-D-/- mice, ozone caused significant alterations in pulmonary mechanics in both 8 wk and 27 wk old mice. In young mice this was characterized by increases in resistance and elastance. These changes were the result of alterations in both tissue and airway mechanics and represented ozone-induced stiffening of the lung, likely resulting from the buildup of inflammatory cells. In 27 wk old SP-D-/- mice, ozone-induced changes in lung resistance and elastance spectra were similar to those observed in young mice, but were less pronounced. Analysis of the component spectral parameters showed that changes in lung elastance resulted from increases in the inherent elastance of the tissue, as well as its frequency dependence. By 80 wk of age, ozone had no significant effect on respiratory mechanics, 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 (van Hoek et al., 2012).

In summary, the present studies provide novel data on changes in lung structure and function in healthy mice as they age, and the impact of loss of SP-D on this response. We also compared structural and functional alterations in the lungs of young, middle aged and elderly mice following ozone intoxication. Our data 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 SP-D-/- mice, which correlates with prolonged responsiveness to ozone. However, in

elderly mice, this increased sensitivity is overcome by significant baseline inflammation and injury. Since the development of immune dysregulation with age has been shown to contribute to pulmonary disorders such as COPD (MacNee, 2009), our findings demonstrate the importance of understanding how regulation of innate immune functioning could improve disease prognosis in susceptible populations exposed to air pollutants.

Figure 11. Lung histology in WT and SP-D-/- mice following exposure to air. Lung sections, prepared 72 hr following exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air, were stained with hematoxylin and eosin. Arrows, macrophages; arrowheads, lymphocytes; E, macrophages containing eosinophilic cytoplasm; G, macrophages containing granular cytoplasm; M, multi-nucleated macrophages; AR, alveolar rupture; IT, interstitial thickening; T2, Type II cell hyperplasia; HC, hemoglobin crystals. Original magnification, 80x (upper panels); 200x (lower panels).

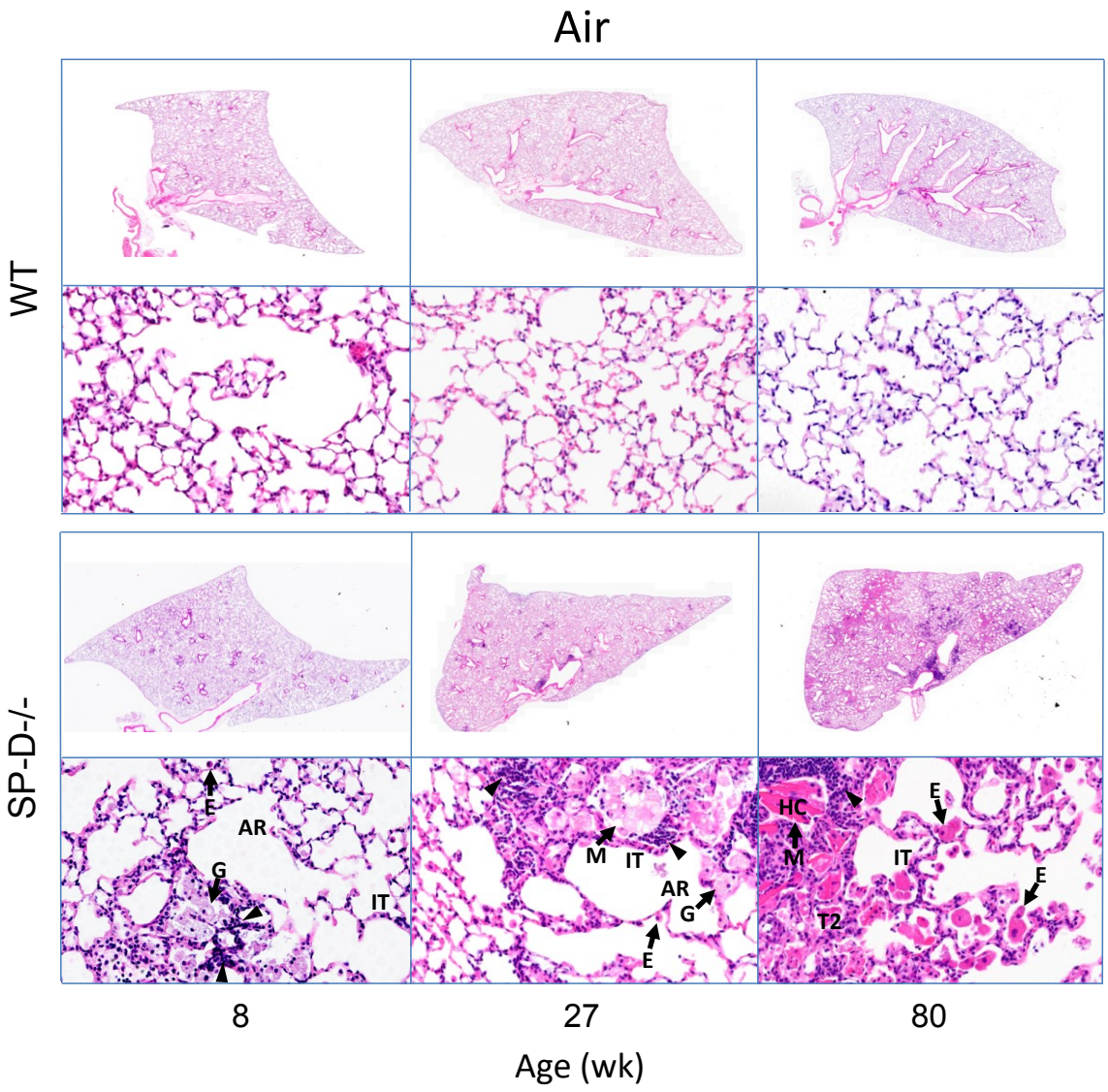

Air
WT
SP-D-/-
E
AR
G
IT
M
IT
AR
G
E
HC
M
E
IT
E
T2
8
27
80
Age (wk)

Figure 12. Lung histology in WT and SP-D-/- mice following exposure to ozone. Lung sections, prepared 72 hr following exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to ozone, were stained with hematoxylin and eosin. Arrows, macrophages; arrowheads, lymphocytes; E, macrophages containing eosinophilic cytoplasm; G, macrophages containing granular cytoplasm; M, multi-nucleated macrophages; AR, alveolar rupture; IT, interstitial thickening; T2, Type II cell hyperplasia; HC, hemoglobin crystals. Original magnification, 80x (upper panels); 200x (lower panels).

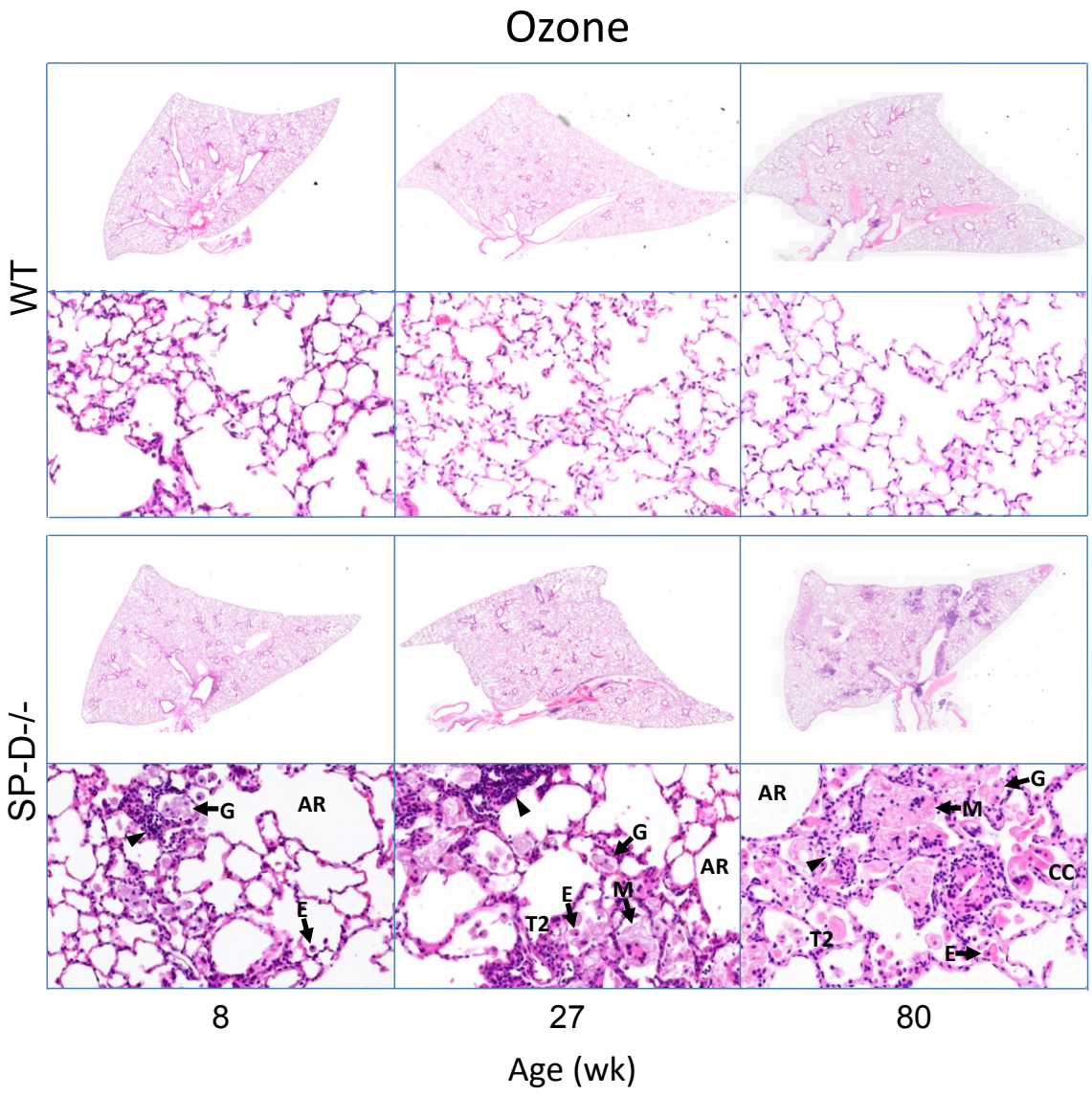

Ozone
WT
SP-D-/-
G
AR
E
G
AR
E
M
T2
AR
G
M
CC
T2
E
8
27
80
Age (wk)

Table 4. Effects of increasing age on pulmonary pathology in SP-D-/- mice

	8 wk		27 wk		80 wk	
	Air	Ozone	Air	Ozone	Air	Ozone
Macrophages	1.2 ± 0.2[a]	1.7 ± 0.3	2.2 ± 0.2[a,b]	2.0 ± 0.0	2.8 ± 0.3[a,b]	2.8 ± 0.3[a]
Lymphocytes	1.0 ± 0.0[a]	2.0 ± 0.0[c]	1.5 ± 0.2[a,b]	2.0 ± 0.0	2.5 ± 0.2[a,b]	2.8 ± 0.3[a]
Alveolar rupture	1.0 ± 0.0[a]	1.7 ± 0.3	1.3 ± 0.3[a]	2.0 ± 0.0	2.2 ± 0.3[a,b]	3.0 ± 0.0[a]
Interstital thickening	0.4 ± 0.2	0.0	1.0 ± 0.3[a]	0.0[c]	2.2 ± 0.3[a,b]	2.3 ± 0.3
Pleural thickening	0.0	0.0	0.3 ± 0.3	0.0	1.5 ± 0.5	1.0 ± 0.6
Type II cell hyperplasia	0.0	1.0 ± 0.6	0.0	1.7 ± 0.3[c]	1.8 ± 0.2[a,b]	1.7 ± 0.9
Alveolar fibrin deposition	0.0	0.0	0.0	0.0	1.7 ± 0.4[a,b]	0.0[c]
Cholesterol crystals	0.0	0.3 ± 0.3	0.0	0.0	1.7 ± 0.3[a,b]	1.3 ± 0.3
Hemoglobin crystals	0.0	0.0	0.0	0.0	0.8 ± 0.5	1.0 ± 0.6
Bronchial epithelia hyperplasia	0.0	0.0	0.0	0.0	0.0	1.7 ± 0.3[c]

The extent of pulmonary pathology was analyzed using semiquantitative scores; 0, no changes; 1, minimal or small changes; 2, mild to medium changes; and 3, moderate to extensive changes. Values are means ± SEM (n = 5-6). [a]Significantly different from WT mice, not shown. [b]Significantly different from young mice. [c]Significantly different from air control.

Figure 13. Radial alveolar counts in WT and SP-D-/- mice. Lung sections, prepared 72 hr following exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with hematoxylin and eosin. Each bar represents the mean $\pm$ SE (n = 3 mice). [a]Significantly different from WT mice. [b]Significantly different from 8 wk old mice.

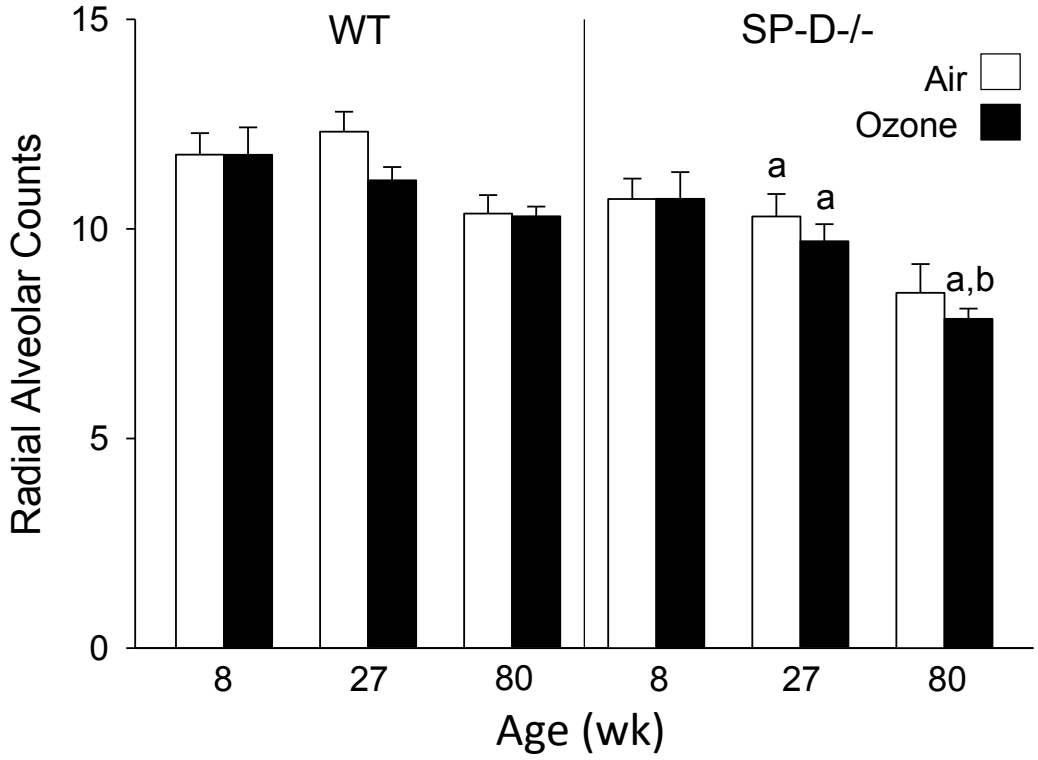

WT
SP-D-/-
Air
Ozone
Radial Alveolar Counts
15
10
5
0
a
a
a,b
8
27
80
8
27
80
Age (wk)

Figure 14. BAL protein and cell counts in WT and SP-D-/- mice. BAL, collected 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, was analyzed for cell and protein content. Each bar represents the mean $\pm$ SE (n = 4-9 mice/treatment group/experiment). [a]Significantly different from WT mice. [b]Significantly different from 8 wk old mice. [c]Significantly different from air control.

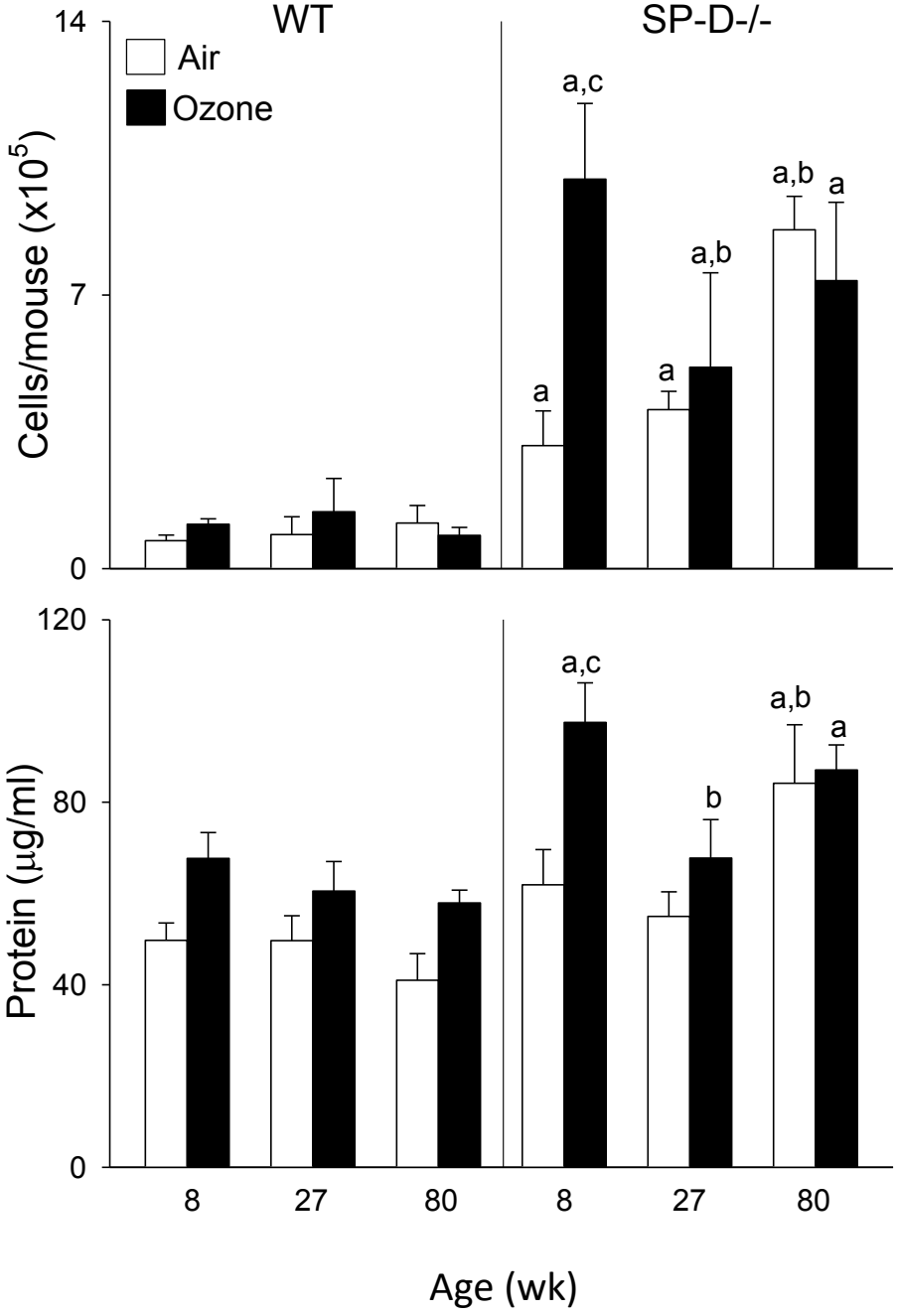

WT
SP-D-/-
Air
Ozone
Cells/mouse (x10^5)
14
7
0
a,c
a,b
a,b
a
a
a
Protein (μg/ml)
120
80
40
0
a,c
a,b
a
b
8
27
80
8
27
80
Age (wk)

Figure 15. Expression of pro-SP-C in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to pro-SP-C or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

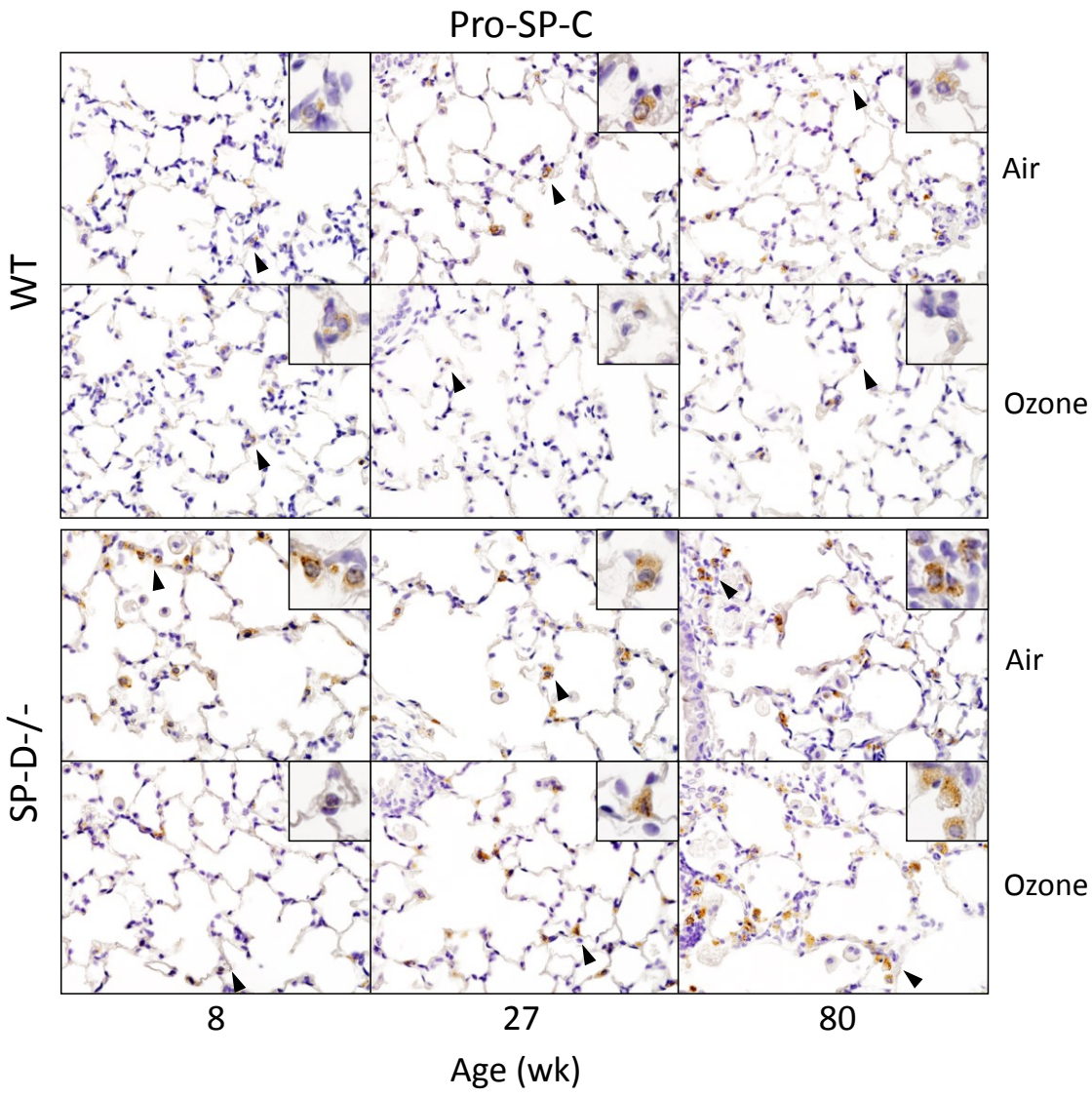

Pro-SP-C
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 16. Expression of PCNA in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to PCNA or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

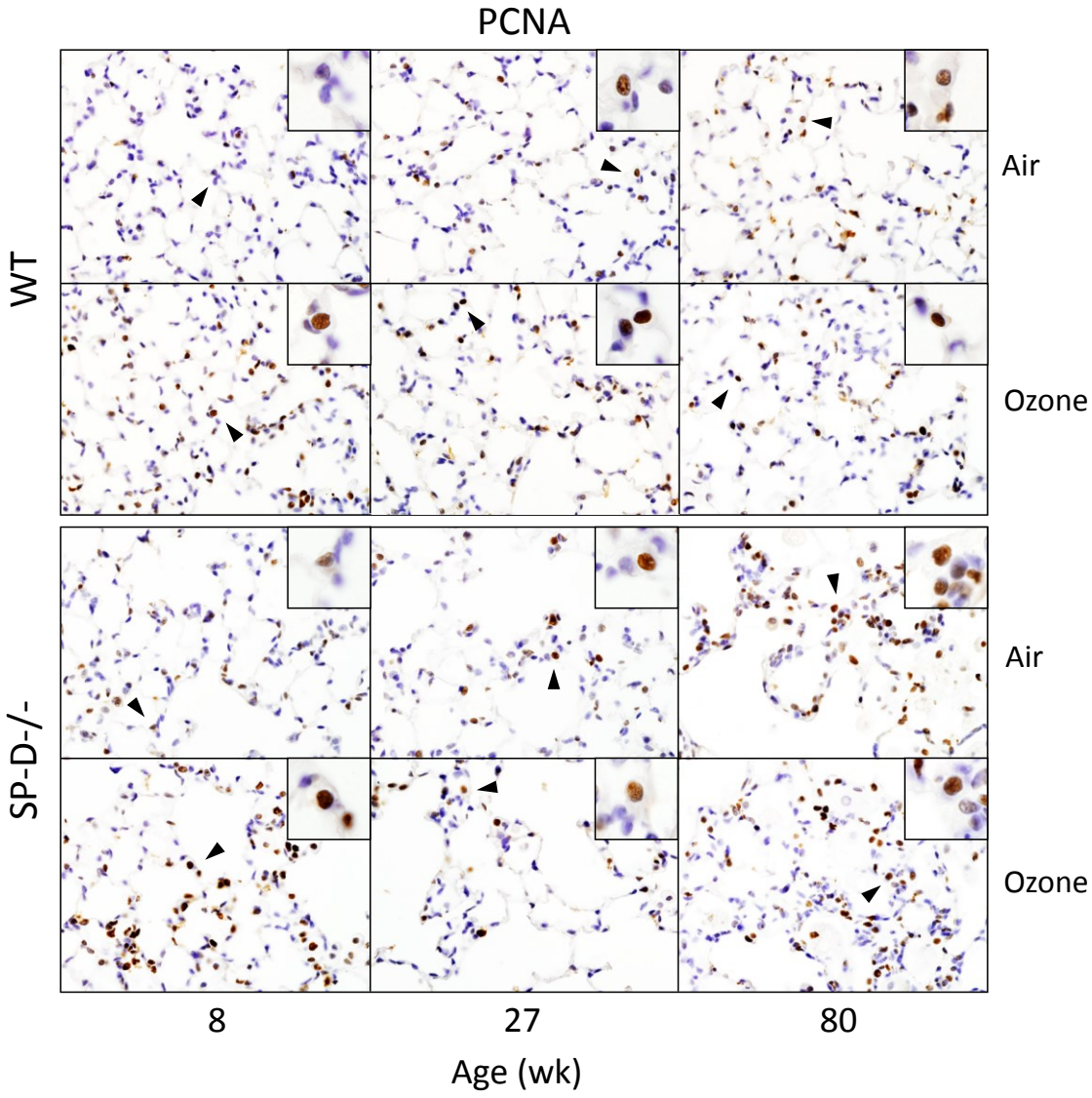

PCNA
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 17. Expression of HO-1 in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to HO-1 or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

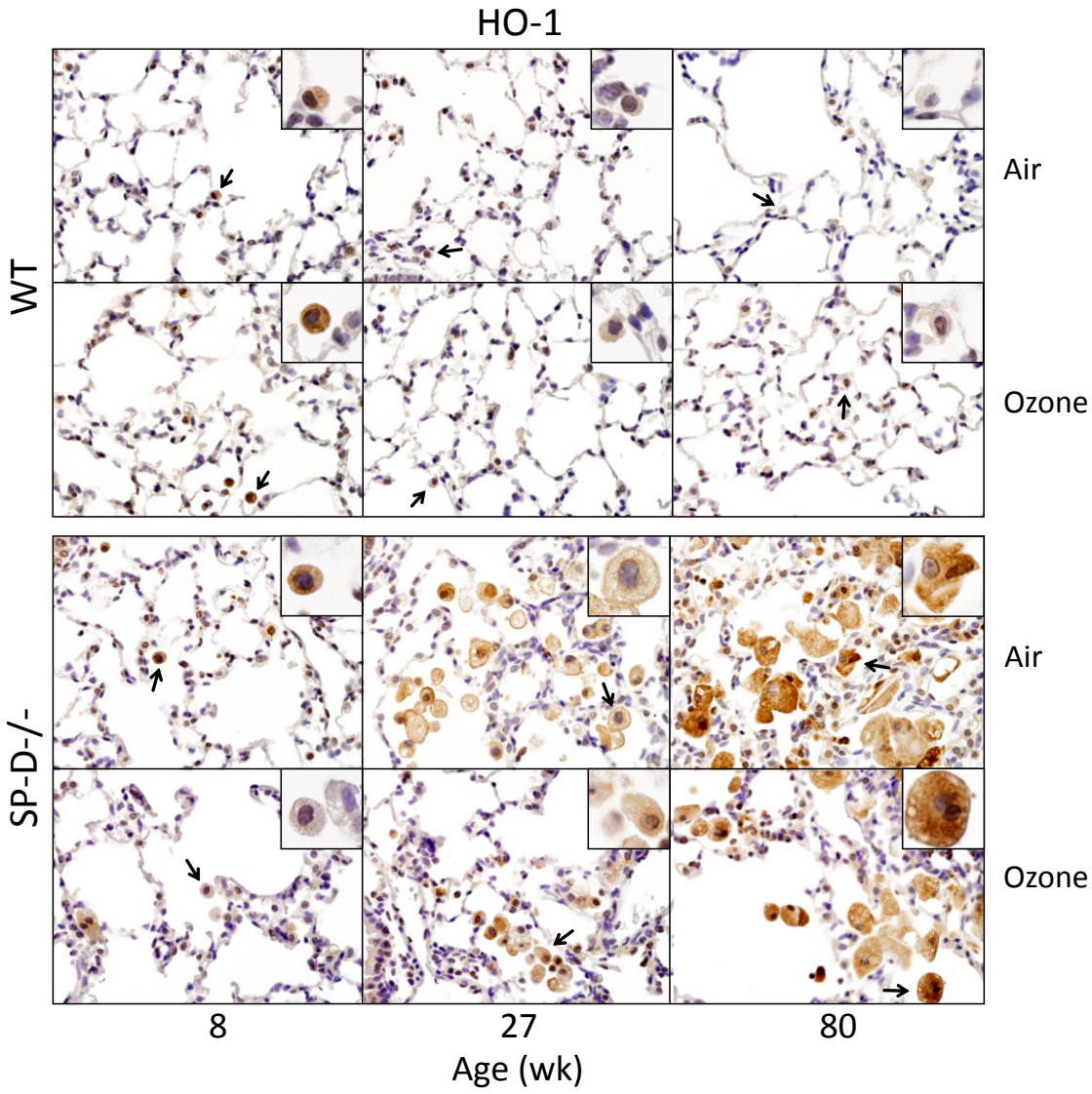

HO-1
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 18. Expression of COX-2 in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to COX-2 or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

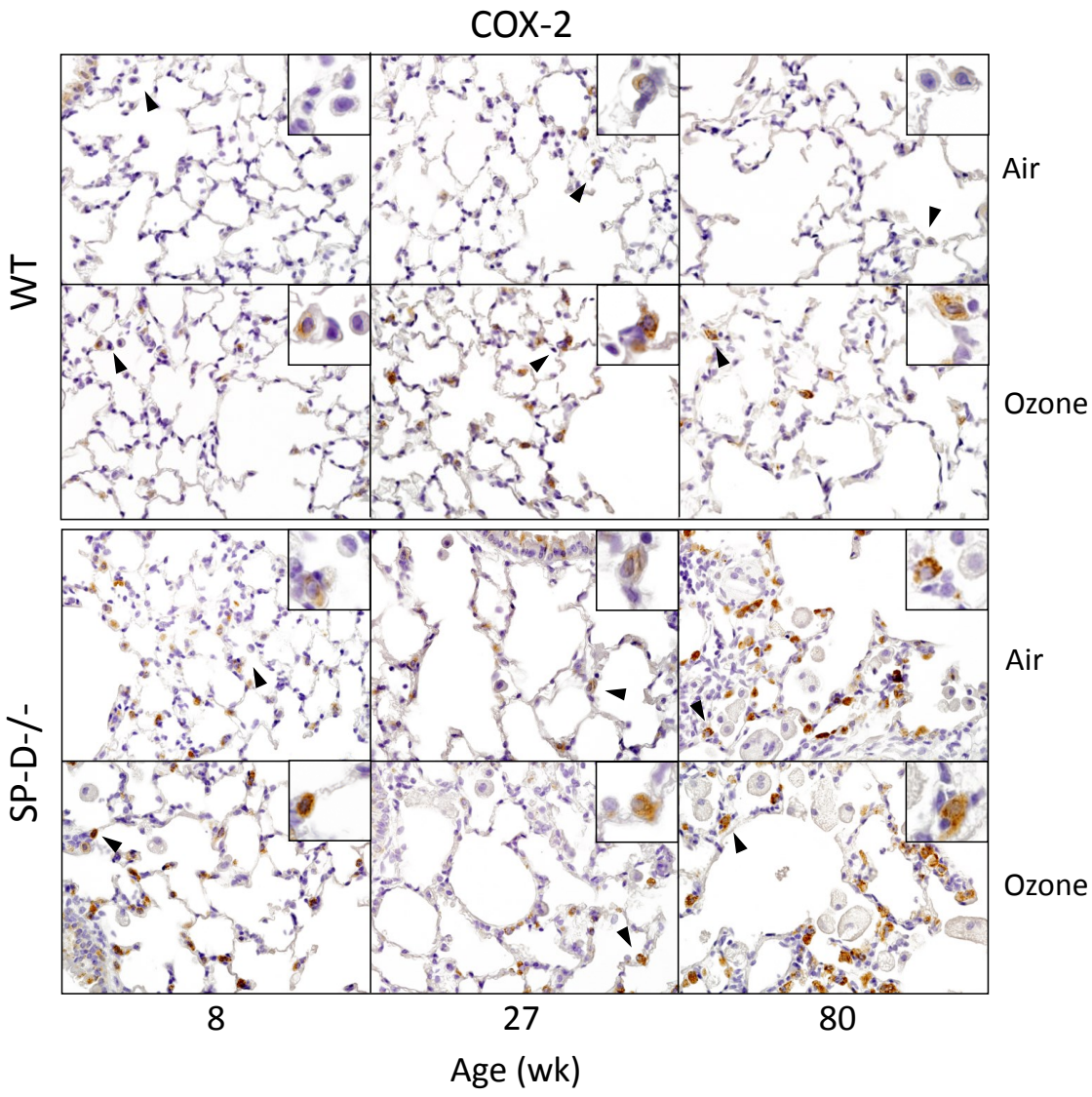

COX-2
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 19. Expression of iNOS in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to iNOS or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

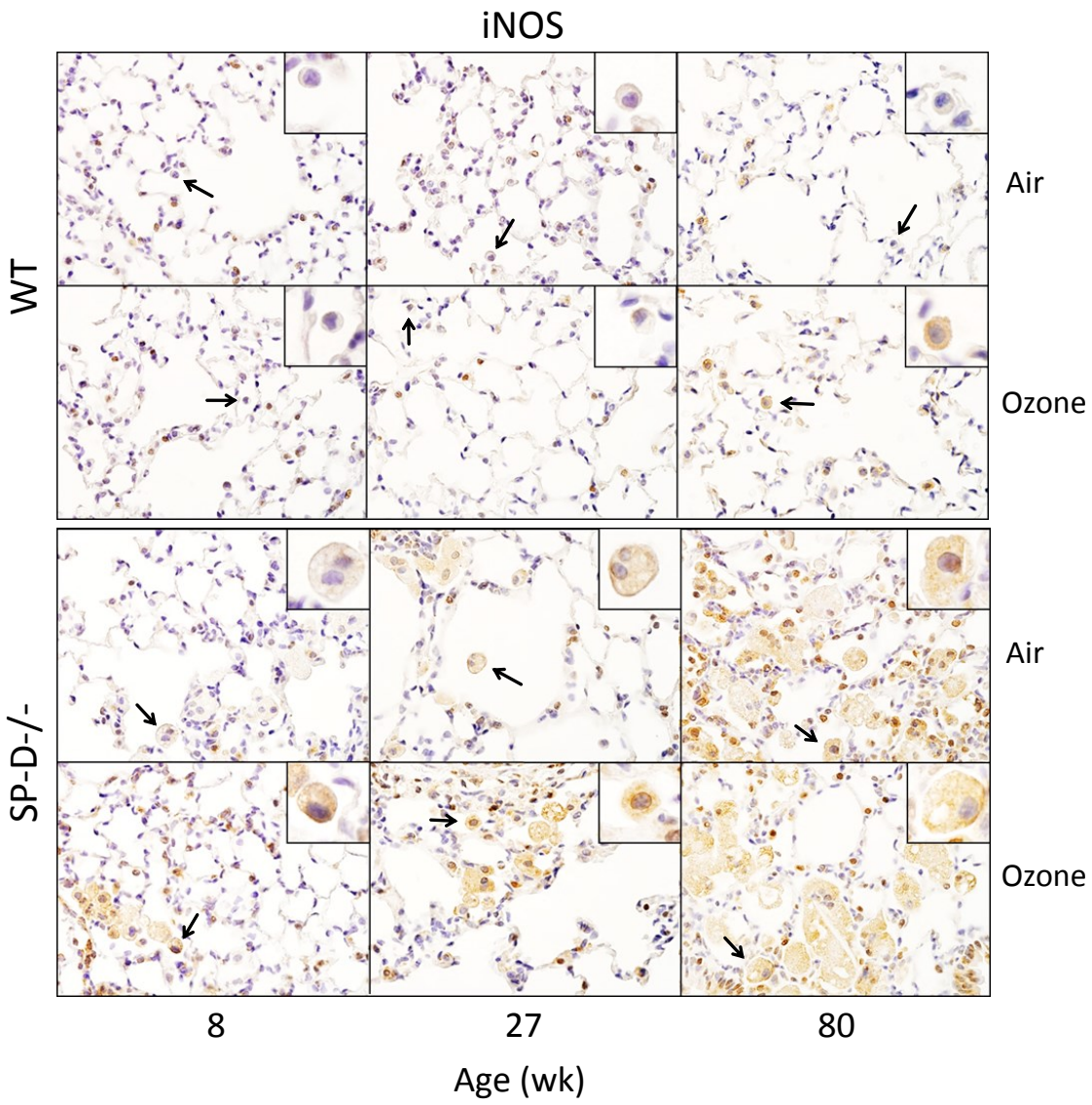

iNOS
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 20. Expression of mannose receptor in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to mannose receptor or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

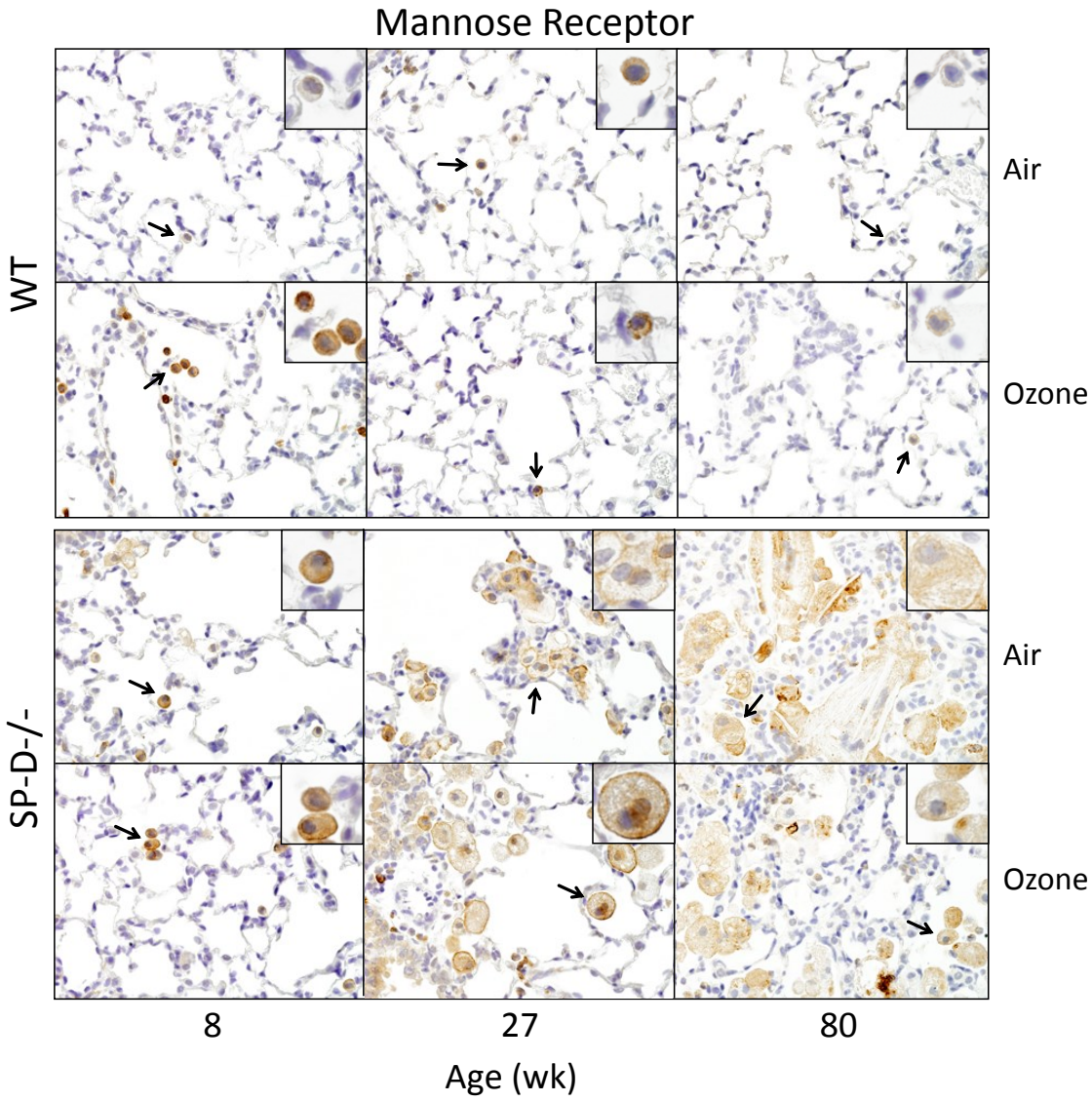

Mannose Receptor
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 21. Expression of YM-1 in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to YM-1 or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

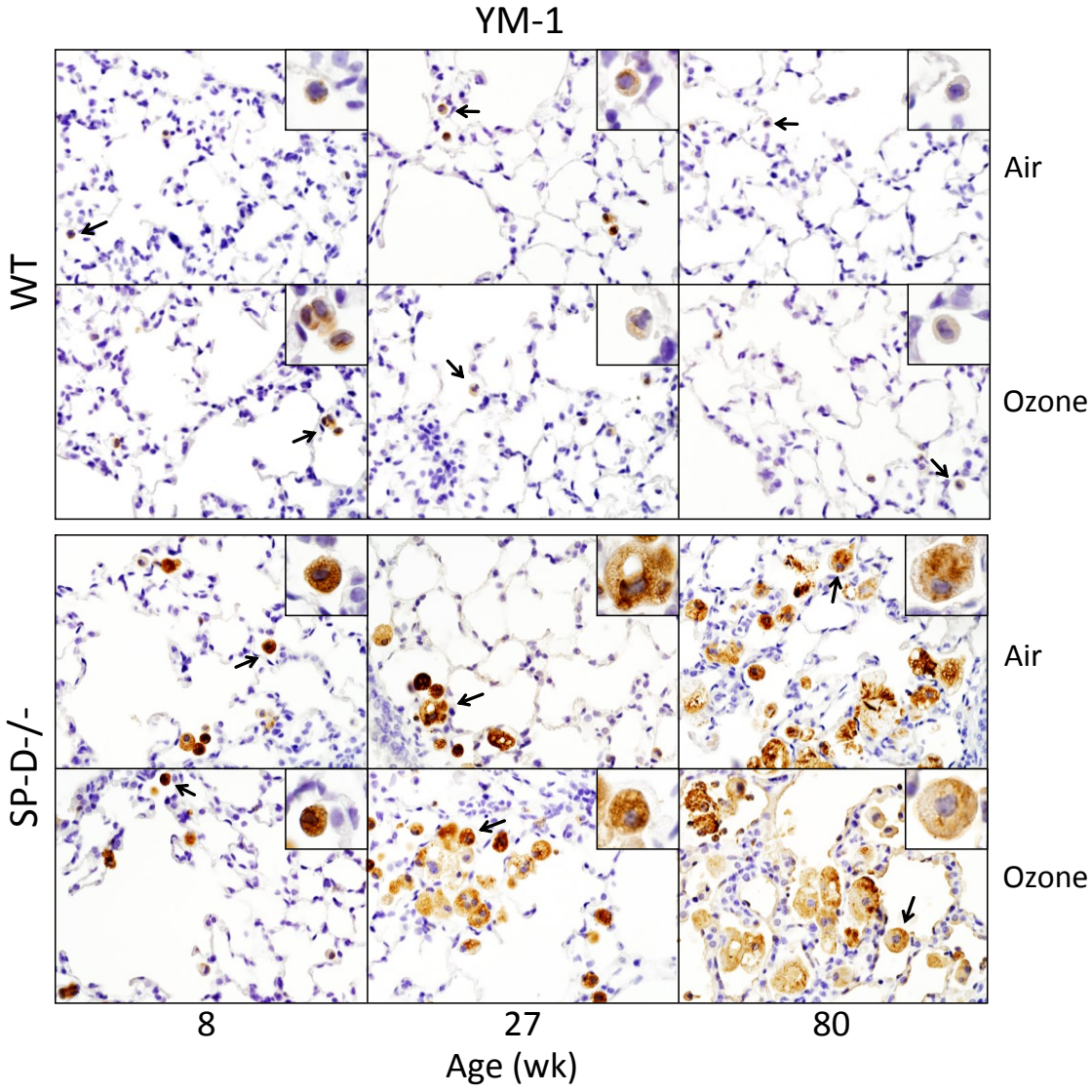

YM-1
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 22. Expression of galectin-3 in WT and SP-D-/- mice. Lung sections, prepared 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone, were stained with antibody to galectin-3 or IgG control followed by biotinylated secondary antibody. Binding was visualized using a peroxidase substrate DAB kit. One representative section from three separate experiments is shown (n = 3 mice/treatment group). Original magnification, 600x.

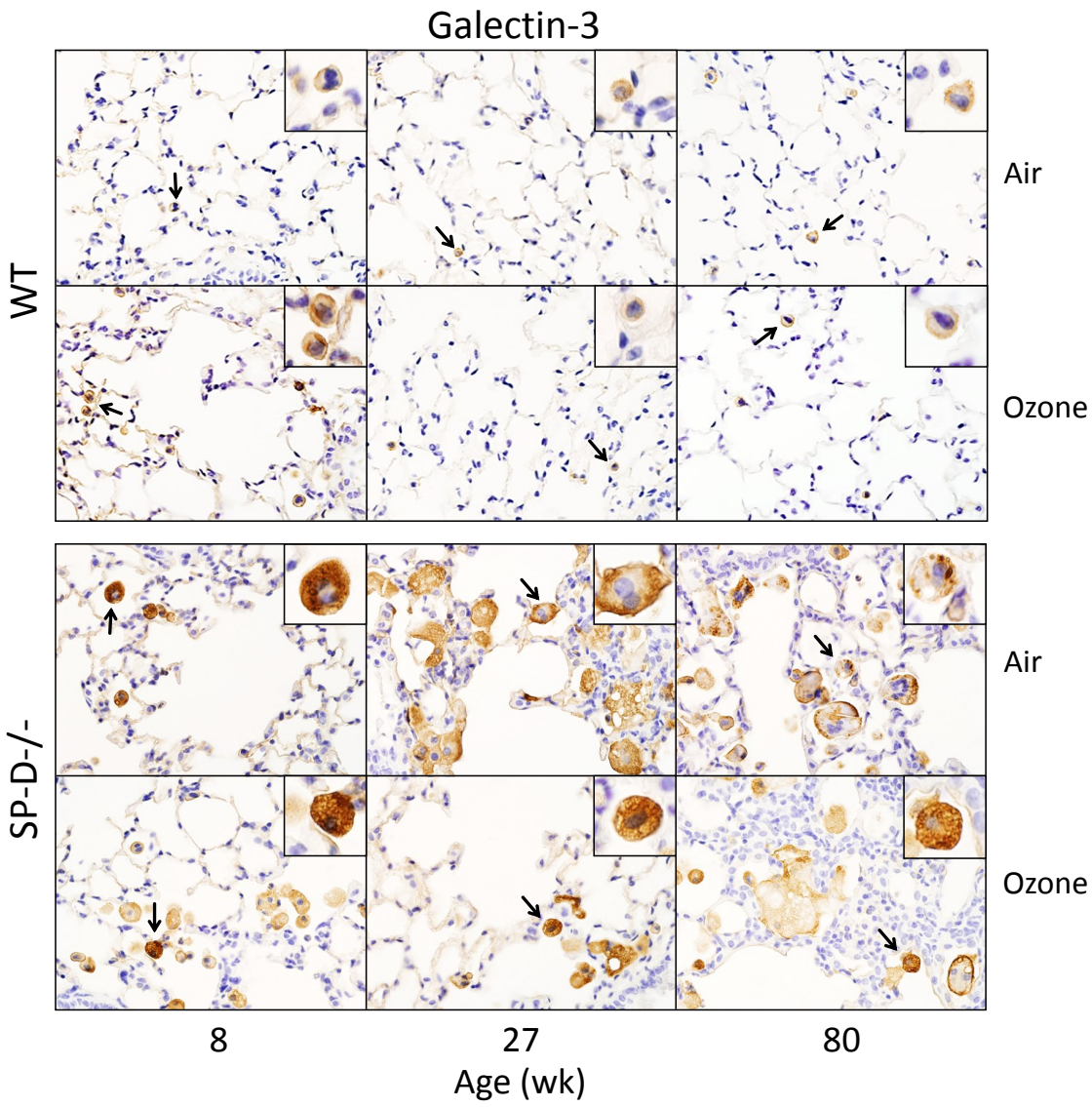

Galectin-3
WT
SP-D-/-
Air
Ozone
Air
Ozone
8
27
80
Age (wk)

Figure 23. Lung resistance and elastance spectra in WT and SP-D-/- mice. Pulmonary mechanics were measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. Impedance spectra were generated using the forced oscillation technique. Resistance and elastance were then derived from input impedance data. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

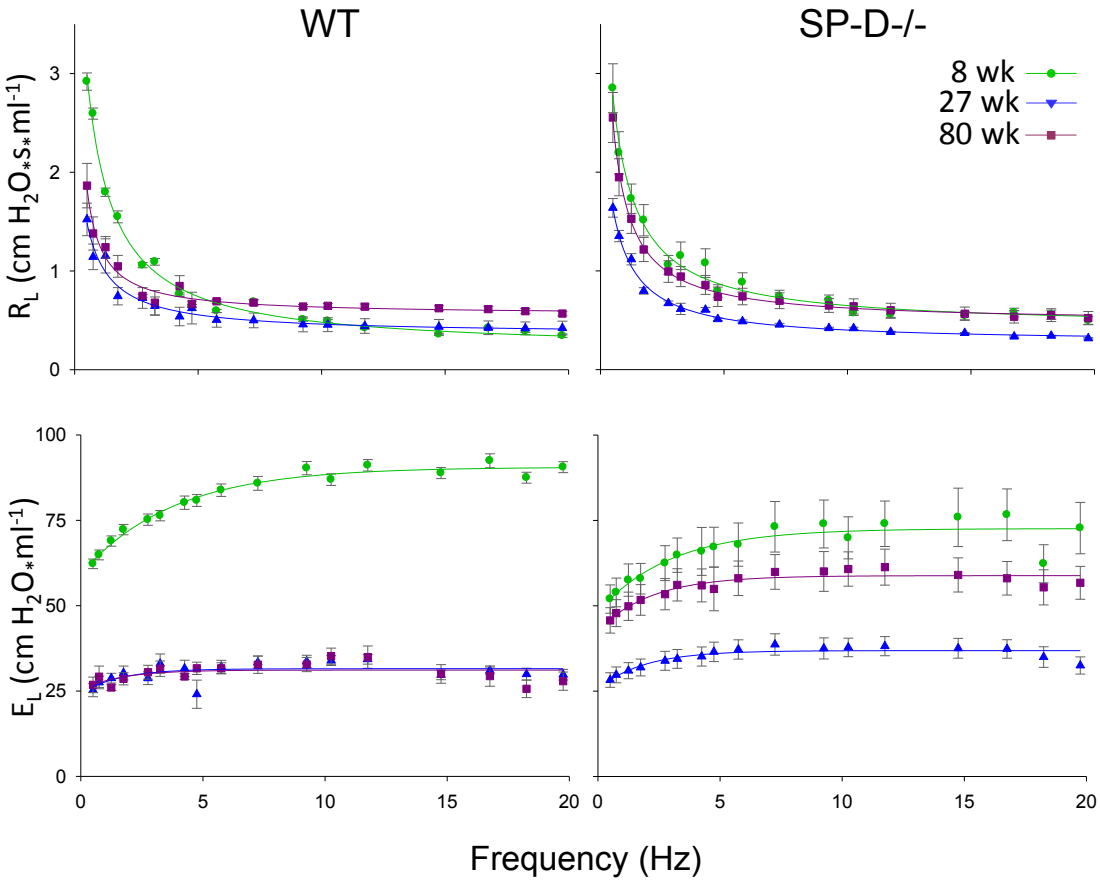

WT
SP-D-/-
8 wk
27 wk
80 wk
R_L (cm H_2O*s*ml^{-1})
E_L (cm H_2O*ml^{-1})
Frequency (Hz)

Figure 24. Effects of ozone on lung resistance spectra. Lung function was measured 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone. Impedance spectra were generated using the forced oscillation technique. Resistance was derived from input impedance data. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

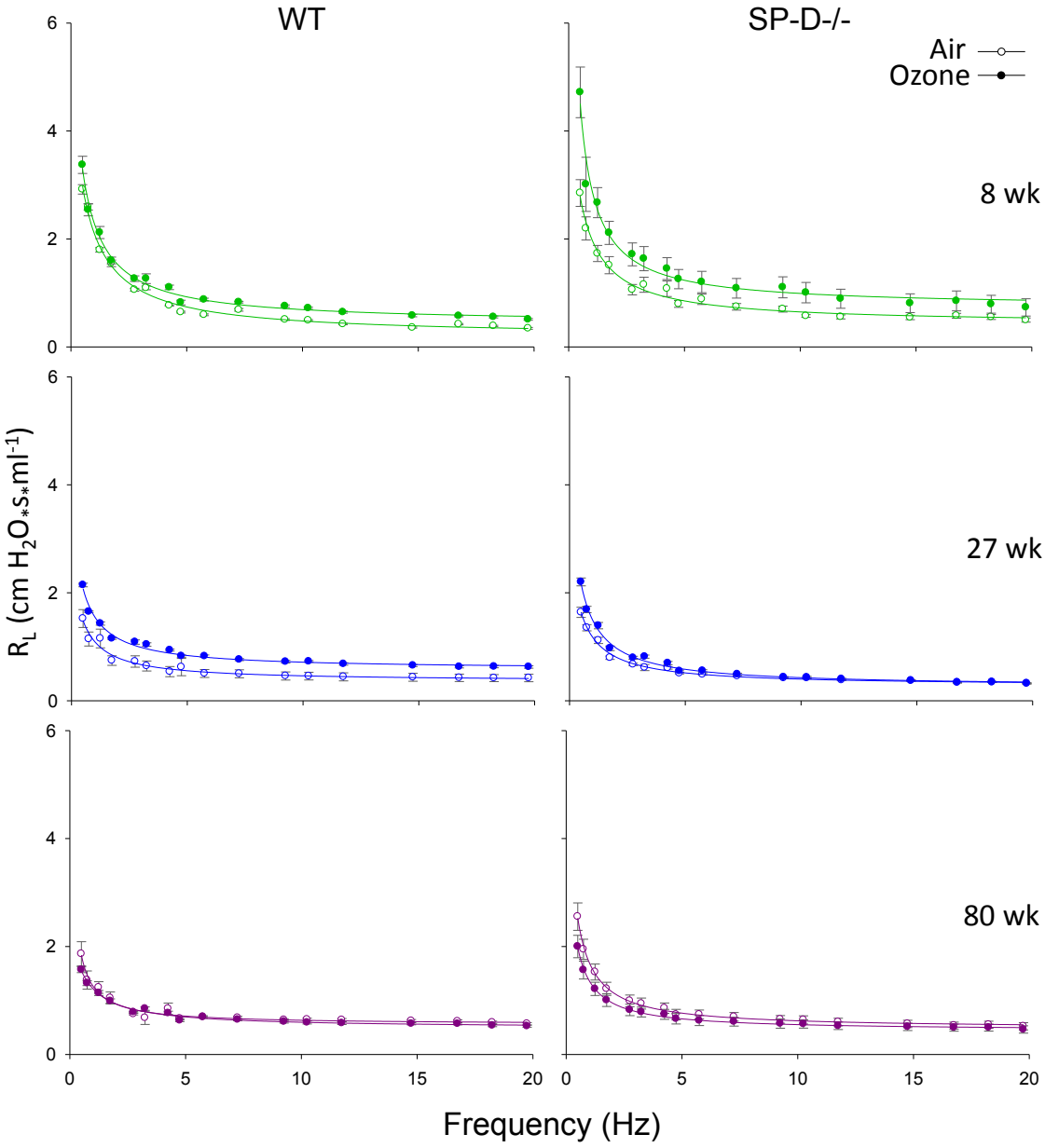

WT
SP-D-/-
Air
Ozone
8 wk
27 wk
80 wk
R_L (cm H_2O*s*ml^{-1})
Frequency (Hz)
0
2
4
6
0
5
10
15
20

Figure 25. Effects of ozone on lung elastance spectra. Lung function was measured 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone. Impedance spectra were generated using the forced oscillation technique. Elastance was derived from input impedance data. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

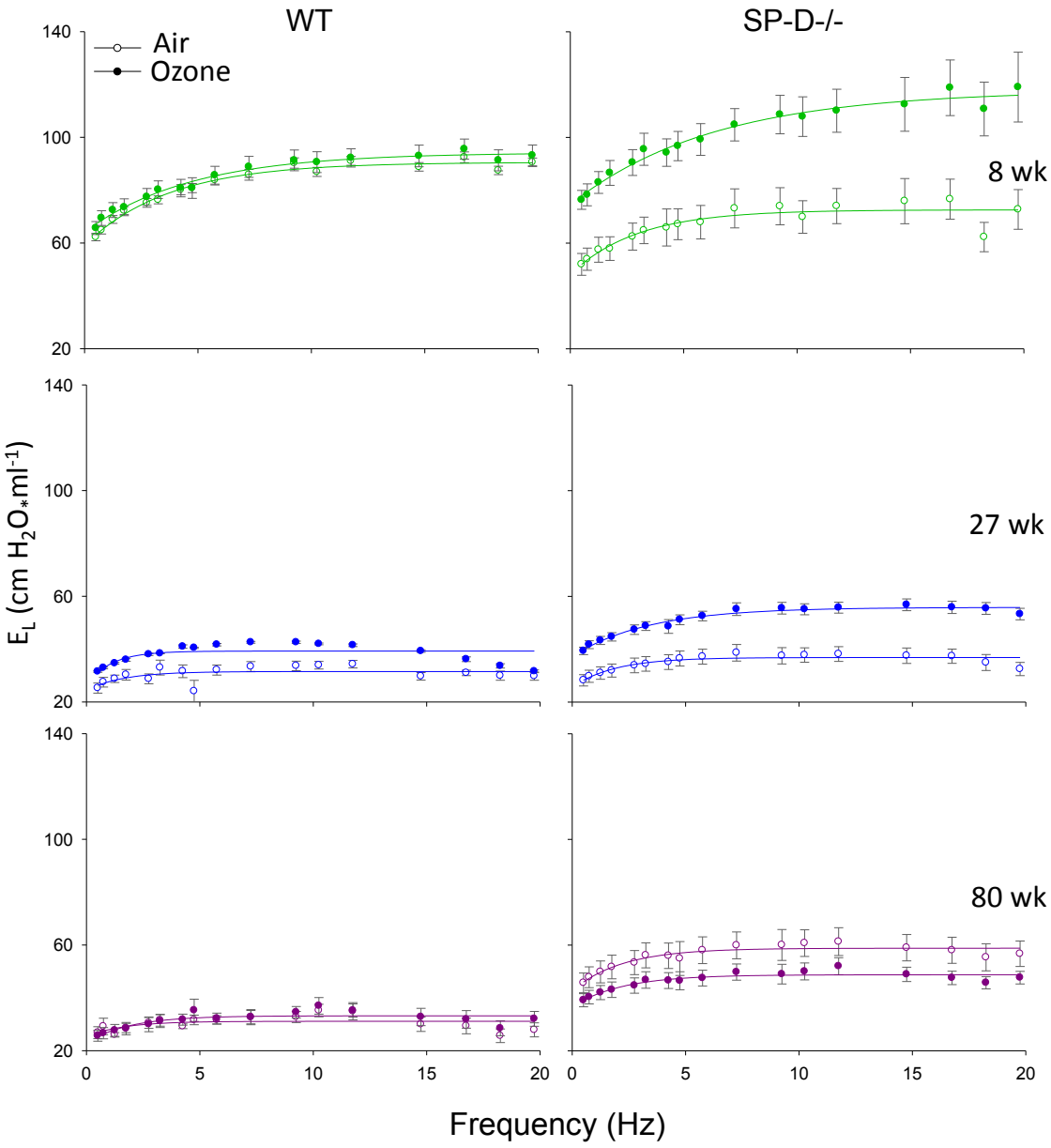
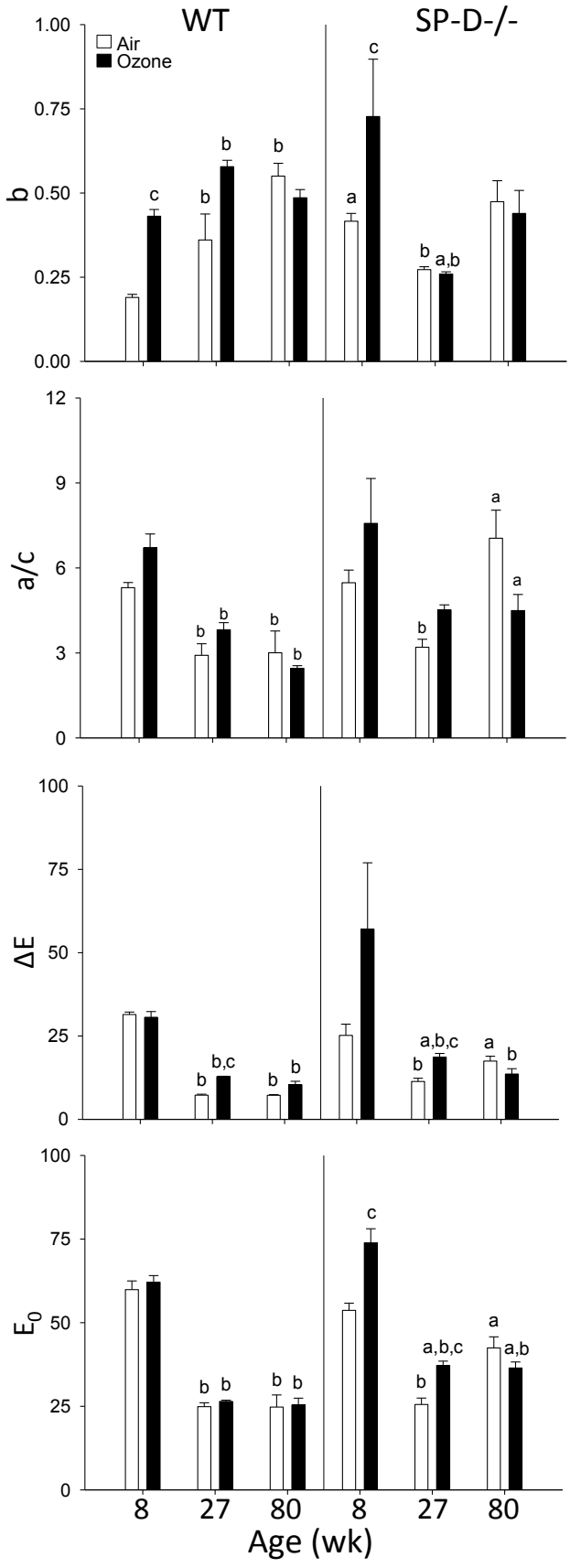

WT
SP-D-/-
Air
Ozone
8 wk
27 wk
80 wk
E_L (cm $H_2O_*ml^{-1}$)
Frequency (Hz)
140
100
60
20
0
5
10
15
20

Figure 26. Effects of ozone on lung resistance and elastance spectral parameters. Lung function was measured 72 hr after exposure of 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice to air or ozone. **a**, **b**, and **c** are parameters of resistance; **a** and **c** reflect resistance at low frequencies, **b** reflects resistance at high frequencies. E_0, ΔE, and β are parameters of elastance; E_0 represents elastance at 0 Hz, ΔE reflects the magnitude of elastance, and β the rate of elastance change with frequency. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).


WT
SP-D-/-
Air
Ozone
b
a/c
ΔE
E0
Age (wk)
8
27
80

PART III. EFFECTS CHRONIC PULMONARY INFLAMMATION, EMPHYSEMA AND OZONE ON PULMONARY MECHANICS

Resistive and elastive forces in the lung are influenced by the viscous and elastic structural characteristics of the tissue, as well as forces acting at the air-liquid interface of the alveolar surface, and airway smooth muscle activity (O'Neil et al., 1984). These characteristics affect how energy that is dissipated as air is moved into and out of the lung, and how its storage provides the driving pressure for expiration during elastic recoil of the lung tissue (Faffe et al., 2009). The stress-bearing systems of the lung are essential to the functional properties of the tissue due to the fact that the lung is continuously exposed to physiological stresses resulting from forces acting to keep it inflated during the cyclic act of breathing. The development of lung pathology can alter these systems, including the gas-liquid interface, the connective tissue matrix, and contractile elements, resulting in increasing heterogeneity in the mechanical properties of the lung, and impairments in lung functioning (Faffe et al., 2009). Obstruction of an alveoli or bronchioles as a result of inflammation or aberrations in surfactant properties causes the surrounding lung units to exert an increased inflationary stress on the lung unit, altering the tension and strain on the connective tissue network, preventing uniform expansion and increasing mechanical heterogeneity (Hubmayr, 2006). Additionally, decreases in the number of airspaces capable of expanding during inspiration as a result of pulmonary injury increases the risk of overexpansion of surrounding air spaces leading to further deformation injury (Gattinoni et al., 1987). Moreover, extracellular matrix remodeling during injury results in alterations in the ratio of collagen and elastin fibers, which each

have different elastic properties, thus shifting the balance of stresses in the lung contributing to mechanical dysfunction (Negri et al., 2000).

In addition to measuring the mechanical properties of the lung under normal respiratory conditions, assessing the responsiveness of respiratory mechanical properties to an applied stress provides insight into the nature of the forces influencing lung functioning. For example, methacholine is a bronchoconstrictor in the lung. Thus, assessing the degree of change in resistance of central airways in response to administration of methacholine is a measure of airway hyperreactivity (Birnbaum et al., 2007). Assessment of the reactivity of airways is important in determining the role of airway dysfunction in functional impairments to respiration. Increased sensitivity to methacholine challenge is observed in individuals with asthma and COPD (Postma et al., 1998). PEEP is the amount of pressure above atmospheric pressure left in the lung at the end of expiration. Increasing PEEP results in the recruitment or of opening closed lung units (Glaab et al., 2007). Increasing PEEP and recruiting lung units affects the resistive and elastive properties of the lung (S. Ito et al., 2007). Furthermore, the effects of altering PEEP on lung function can be evaluated to allow for assessment of heterogeneity in responsiveness and changes in the mechanical properties of the airway and parenchyma (Massa et al., 2008).

Lung injury alters the dependence of pulmonary mechanics on changes in PEEP (Massa et al., 2008; Salerno et al., 2007; Shardonofsky et al., 2006). Tissue remodeling in response to inflammation or injury changes the collagen and elastin content of the lung and reduces parenchymal tethering. Alterations in the interaction between airways and parenchyma alter elastic recoil pressure and airway contractile response, as well as the

PEEP dependence of airway resistance and tissue elastance (Khan et al., 2010; Shardonofsky et al., 2006).

Aging also results in a progressive decline in lung performance (Janssens et al., 1999). This is associated with enlarged airspaces and increased lung volumes (Pinkerton et al., 1982). The elastic recoil and the resistance of the lung are also decreased with age, which alters the pressure-volume relationship in the tissue (Janssens et al., 1999). Age-related changes in lung function have been related to alveolar distension, chest wall stiffening, and decreased strength of respiratory muscles (Janssens, 2005). The appearance of ruptured and coiled elastic fibers in respiratory bronchioles and alveoli around alveolar ducts results in alveolar distension, promoting the dilation of alveoli and airspace enlargement (Sprung et al., 2006; Verbeken et al., 1992).

In mice, loss of SP-D results in the accumulation of activated macrophages in the lung and the development of chronic pulmonary inflammation, which increases with age and results in the development of emphysema (Wert et al., 2000). In previous studies we demonstrated that pulmonary inflammation in SP-D-/- mice, is associated with altered pulmonary mechanics in response to inhaled ozone and that this response is affected by age and the development of emphysema (Groves et al., 2012). How these conditions affect the responsiveness of pulmonary mechanics to alterations in the respiratory system, including bronchoconstriction and recruitment, were next investigated. This allowed us to assess the impact of inflammation, injury and emphysema on heterogeneity in lung responsiveness.

RESULTS

Previous studies demonstrated that ozone-induced inflammation and injury are most apparent 24 hr-48 hr post exposure, and are, for the most part, resolved by 72 hr (Fakhrzadeh et al., 2002). To determine the relationship between ozone induced inflammation and alterations in lung functioning, the constant phase model was used to assess pulmonary mechanics in 27 wk SP-D-/- mice 24 hr-72 hr following ozone inhalation. The most significant alterations induced by ozone were in Rn; the largest increases occurred 24 hr and 72 hr post exposure. In contrast, R, C and Cst were only minimally altered by ozone at all time points (Figure 27).

The effects of age, chronic inflammation and ozone exposure on methacholine responsiveness were next assessed in 8 wk old young and 27 wk old middle aged WT and SP-D-/- mice 72 hr after exposure to air or ozone. In WT mice, aging increased the dependency of G on methacholine concentration, while Cst decreased (Figure 28). In contrast, no apparent differences in methacholine dependency were observed with age in SP-D-/- mice. While ozone exposure decreased the methacholine responsiveness of G and H in WT mice, this response did not develop until the mice were 27 wk of age (Figure 29). Conversely, in SP-D-/- mice, ozone-induced decreases in methacholine responsiveness were observed in H and Rn at 8 wk of age (Figure 30). By 27 wk of age, however, ozone-induced changes in G and H in response to methacholine was lost, while Rn responsiveness was increased.

To assess the impact of age, inflammation and ozone on lung function heterogeneity, the PEEP dependence of resistance and elastance spectra was assessed in air and ozone exposed 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. In WT mice,

increasing age had minimal effects on the PEEP dependence of resistance and elastance spectra (Figures 31 and 32). This was not altered by ozone exposure in 8 wk and 80 wk mice, however recruitment-related decreases in both resistance and elastance spectra were observed following ozone inhalation in 27 wk old mice. In contrast to WT mice, aging resulted in decreased elastance in response to increasing PEEP in SP-D-/- mice, a response that was evident in 27 wk old mice (Figure 32). Resistance spectra, however, were not affected. Additionally, ozone induced decreases in elastance spectra in response to recruitment were observed in 8 wk old mice and persisted through 27 wk of age, however resistance spectra were not altered (Figures 31 and 32).

Parameters representing the high and low frequency portions of the resistance and elastance spectra were also assessed to determine differences in functional changes to the airways and parenchyma. In WT mice, increases in the high frequency resistance parameter **b** with PEEP were observed in 8 wk old mice, a response that was reversed in 27 wk and 80 wk old mice (Figure 33). Representing low frequencies, **c** decreased with PEEP in both 8 wk and 27 wk old mice, while no alterations in **a** were observed with age (Figures 34 and 35). For elastance spectra, alterations in PEEP responsivenes were observed in β, which was increased in 80 wk old mice, however the other parameters were not altered with age (Figures 36-38). In SP-D-/- mice, the increases in **b** with PEEP observed in 8 wk mice were abrogated by 27 wk of age (Figure 33). Neither **a** or **c** exhibited PEEP responsiveness in any age group (Figures 34 and 35). E_0 decreased with PEEP in 27 wk but not 8 wk or 80 wk mice, while decreases in ΔE and increases in β with PEEP were observed by 27 wk of age (Figures 36-38). Ozone exposure in WT mice reduced the PEEP responsiveness of **c** at 27 wk of age, but did not alter any other

resistance or elastance parameters (Figures 33-38). In SP-D-/- mice, ozone inhalation resulted in a decrease in **a** and ΔE in response to PEEP at 8 wk of age, but had no other effects on resistance or elastance parameters.

DISCUSSION

In these studies we analyzed responsiveness of pulmonary mechanics to bronchoconstriction and recruitment. G has been shown to increase not only with increases in the resistive properties of lung parenchyma, but with closure of small airways (Faffe et al., 2009). Methacholine is used to measure airway hyperreactivity because of its brochoconstrictive properties (Birnbaum et al., 2007). In WT mice, therefore, the development of increased responsiveness of tissue resistance, G to methacholine with age may be a reflection of an increased sensitivity for airway closure in response to bronchoconstricton with age. The fact that SP-D-/- mice exhibit reduced responsiveness of G to brochoconstriction may be the result of alterations in the constrictive capacities of small airways in these mice. This is supported by findings that destruction of alveolar septae as a result of emphysema pathogenesis results in reductions in parenchymal tethering which leads to aberrations in the magnitude and velocity of airway narrowing (Khan et al., 2007).

Increased airway resistance following ozone inhalation has been observed not only under physiologic conditions, but also in response to constrictive agents such as methacholine, indicating ozone exposure increases airway hyperresponsiveness (Fouke et al., 1988; Savov et al., 2004; Takahashi et al., 1993). This is ameliorated by inhibition of ROS production, suggesting that free radical generation is involved in the response (Chitano et al., 1995; J. W. Hollingsworth, Kleeberger, et al., 2007; Takahashi et al., 1993). Ozone induced alterations in lung functioning were noted 24 hr-72 hr post exposure in SP-D-/- mice. In WT mice, inflammation is resolved by 72 hr (Fakhrzadeh et al., 2002), while in SP-D-/- mice inflammation is persistent (Groves et al., 2012).

Altered lung functioning was therefore apparent throughout the inflammatory response induced by ozone. The most prominent alterations in lung function were increases in airway hyperreactivity, as evident by increased sensitivity of Rn to methacholine. At 72 hr post exposure this response was still evident in 27 wk SP-D-/- mice, while in young healthy WT mice, ozone exposure had no effect on methacholine responsiveness. This demonstrates that ozone induced inflammation and injury affect methacholine responsiveness, a conclusion supported by findings of increased airway hyperreactivity at earlier time points following ozone exposure, when inflammation is still present (L.-Y. Zhang et al., 1995). In contrast to young WT mice, ozone-induced alterations in the responsiveness of tissue resistance and elastance parameters to methacholine were reduced in middle aged WT mice. Although resolution of inflammation is not altered with age in WT mice, ozone-induced increases in COX-2 expression and Type II cell proliferation increase with age, while the antioxidant defense enzyme HO-1 decreases, which could result from a low-level epithelial injury in older WT mice at this time point (Groves, unpublished findings). Taken together, this suggests that an increased susceptibility to ozone injury develops with age and that this has functional consequences. Additionally, age does not affect ozone-induced alterations in methacholine responsiveness in SP-D-/- mice since at all ages inflammation is present.

Recruitment of lung units has been demonstrated to be altered with lung injury and the development of emphysema (Ghadiali et al., 2011; S. Ito et al., 2006; Salerno et al., 2007). In SP-D-/- mice, PEEP induced increases in **b**, which represents airway resistance, were ameliorated with the progression of chronic inflammation and emphysema in older mice. This could reflect a decrease in parenchymal tethering with the

development of emphysema. Alterations in airway resistance with PEEP have been reported in a model of empysema using tight skin and pallid mice (S. Ito et al., 2006). Results of these studies demonstrated that airway resistance decreases with PEEP in both emphysemic and control mice, however there was no difference in the PEEP reponsiveness between these two groups. Differences in our findings could reflect differences in the pathogenesis of emphysema in these models, as emphysema resulting from loss of SP-D is accompanied by a state of chronic inflammation. Application of PEEP reduced elastance spectra in SP-D-/- at 27 wk of age, but did not affect resistance or elastance in 8 wk and 80 wk old mice or in WT mice. This was correlated with decreases with PEEP in the parameter representing inherent tissue elastance, E_0, in these mice. This may be due to an increase in closed lung units in SP-D-/- mice, compared with 8 wk mice or WT mice that can be recruited with PEEP. This is supported by model simulation of recruitment in mice with acute lung injury. Elastance is increased in mice with acute lung injury due to a reduced number of lung units. The application of PEEP opens these units and decreases elastance (Massa et al., 2008). The fact that this was not observed in 80 wk old SP-D-/- mice with more developed emphysema may reflect that the almost complete consolidation of many alveolar spaces by macrophages and lymphocytes, (Groves, unpublished findings), prevents recruitment with PEEP.

Ozone exposure results in alveolar epithelial injury, which affects its integrity, and induces the development of pulmonary inflammation (D. K. Bhalla, 1999; Foster et al., 2000; Foster et al., 1996; Putman et al., 1997). Additionally, ozone exposure results in a deterioration in pulmonary function and has been shown to increase bronchial reactivity in humans (Ciencewicki et al., 2008; Foster et al., 2000; Que et al., 2011).

Therefore, to further elucidate the consequences of ozone induced inflammation and injury on lung functioning, the effects of ozone on PEEP responsiveness was also investigated. Ozone inhalation amplified decreases in elastance spectra in response to recruitment in both WT mice and SP-D-/- mice at 8 wk and 27 wk of age, however this response was more prominent in SP-D-/- mice. Because recruitment of lung units decreases PEEP, these results suggest that ozone-intoxication could result in airway collapse and that SP-D-/- mice are more susceptible to this injury. The correlation with increased PEEP responsiveness of ΔE in SP-D-/- mice also indicates that airway components contribute to this response.

Taken together, these results demonstrate that alterations in the responsiveness of respiratory mechanics to methacholine and PEEP-induced changes in respiratory conditions are associated with pathogenic process in the lung resulting from inflammation and injury. Additionally, the nature of these alterations provide insight into the how these processes influence lung functioning. Increases in ozone concentrations are correlated with elevated hazard ratios for patients with COPD, diabetes, congestive heart failure, and myocardial infarction (Zanobetti et al., 2011). In support of this, these findings also suggest that chronic inflammation and the development of emphysema increase susceptibility to ozone-induced decrements in lung functioning.

Figure 27. Effects of ozone inhalation on resistance and compliance in WT and SP-D-/- mice in response to methacholine challenge. Lung function was evaluated in response to increasing doses of methacholine 24 hr-72 hr after exposure of WT and SP-D-/- mice to air or ozone. Impedance spectra were measured using the forced oscillation technique and results analyzed using the constant phase model. Cst was calculated from pressure-volume loops. Measurements were performed in triplicate at each methacholine dose. Values were normalized to 0 mg/kg methacholine. Each point represents the mean $\pm$SEM (n= 2-4 mice/treatment group).

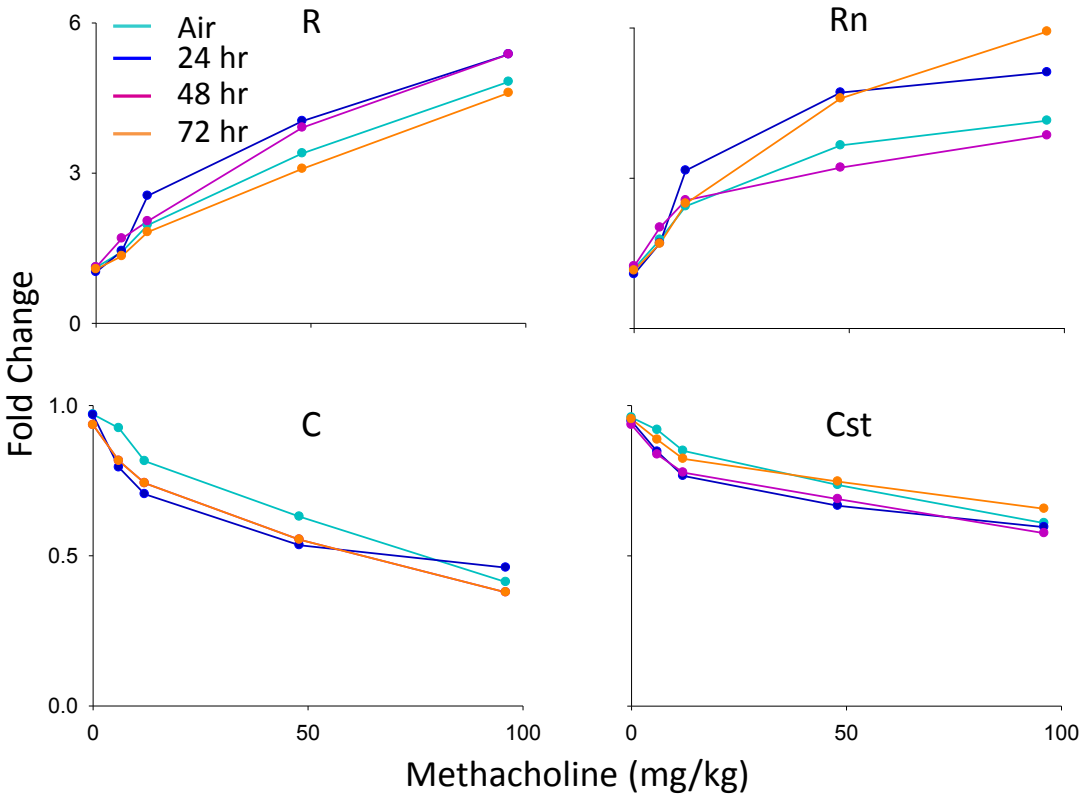

Air
24 hr
48 hr
72 hr
R
Rn
C
Cst
Fold Change
Methacholine (mg/kg)
6
3
0
1.0
0.5
0.0
0
50
100

Figure 28. Effects of loss of SP-D on methacholine responsiveness. Lung function was measured in WT and SP-D-/- mice. Lung function was evaluated in response to increasing doses of methacholine 72 hr after exposure of WT and SP-D-/- mice to air or ozone. Impedance spectra were generated using the forced oscillation technique. Resistance and elastance spectra were then derived from Z_L. For Rn, G and H, impedance spectra were measured using the forced oscillation technique and results analyzed using the constant phase model. Cst was calculated from pressure-volume loops. Measurements were performed in triplicate at each methacholine dose. Values were normalized to 0 mg/kg methacholine. Each point represents the mean $\pm$ SEM (n = 3-6 mice/treatment group). Data were analyzed by non-linear regression.

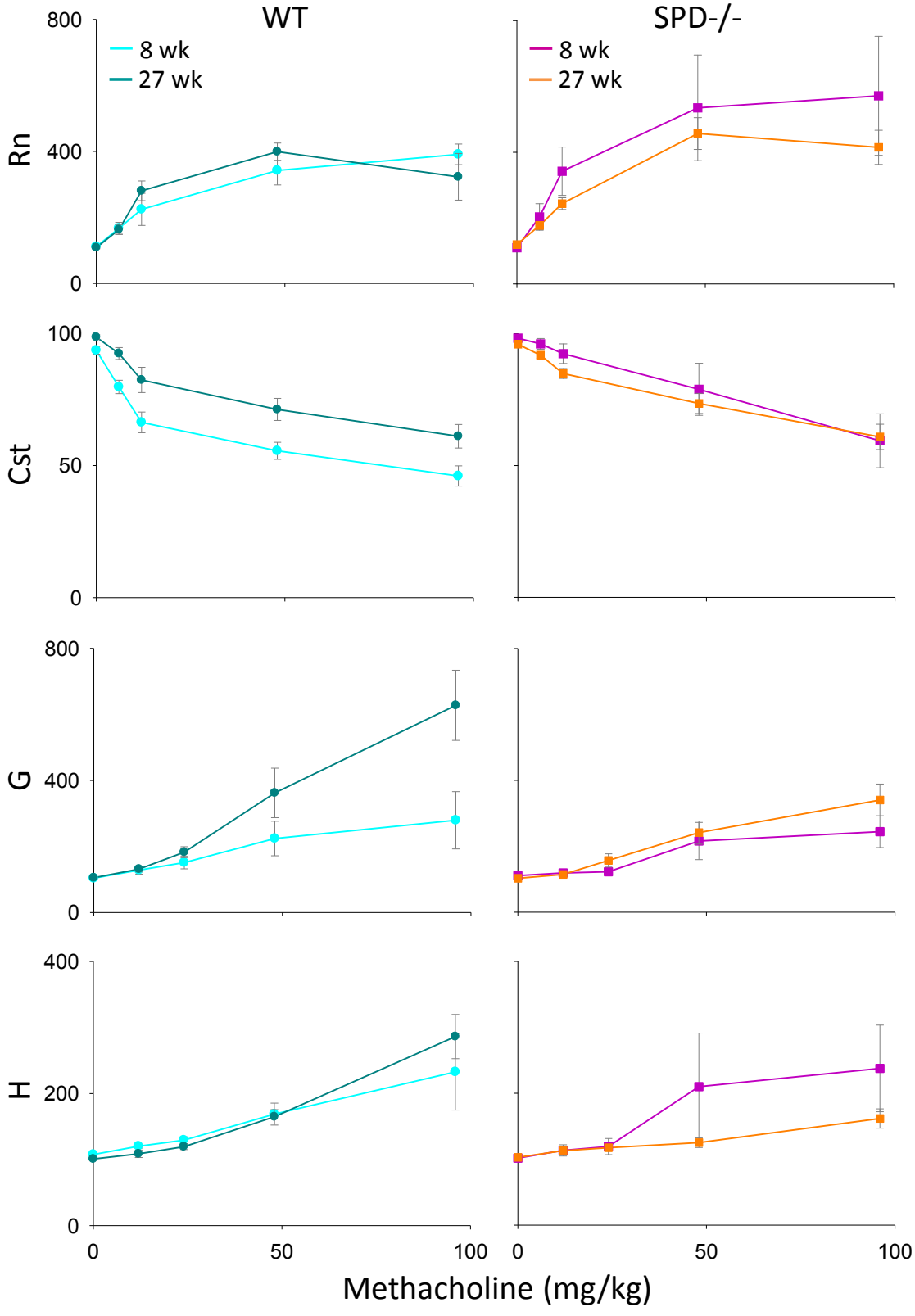

WT
SPD-/-
8 wk
27 wk
8 wk
27 wk
Rn
Cst
G
H
800
400
0
100
50
0
800
400
0
400
200
0
0
50
100
0
50
100
Methacholine (mg/kg)

Figure 29. Effects of ozone inhalation on methacholine responsiveness in WT mice. Lung function was was evaluated in response to increasing doses of methacholine 72 hr after exposure WT mice to air or ozone. Lungs were subjected to 0-96 mg/kg methacholine. Impedance spectra were generated using the forced oscillation technique. Resistance and elastance spectra were then derived from Z_L. For Rn, G and H, impedance spectra were measured using the forced oscillation technique and results analyzed using the constant phase model. Cst was calculated from pressure-volume loops. Measurements were performed in triplicate at each methylcholine dose. Values were normalized to 0 mg/kg methacholine. Each point represents the mean $\pm$ SEM (n = 3-6 mice/treatment group). Data were analyzed by non-linear regression.

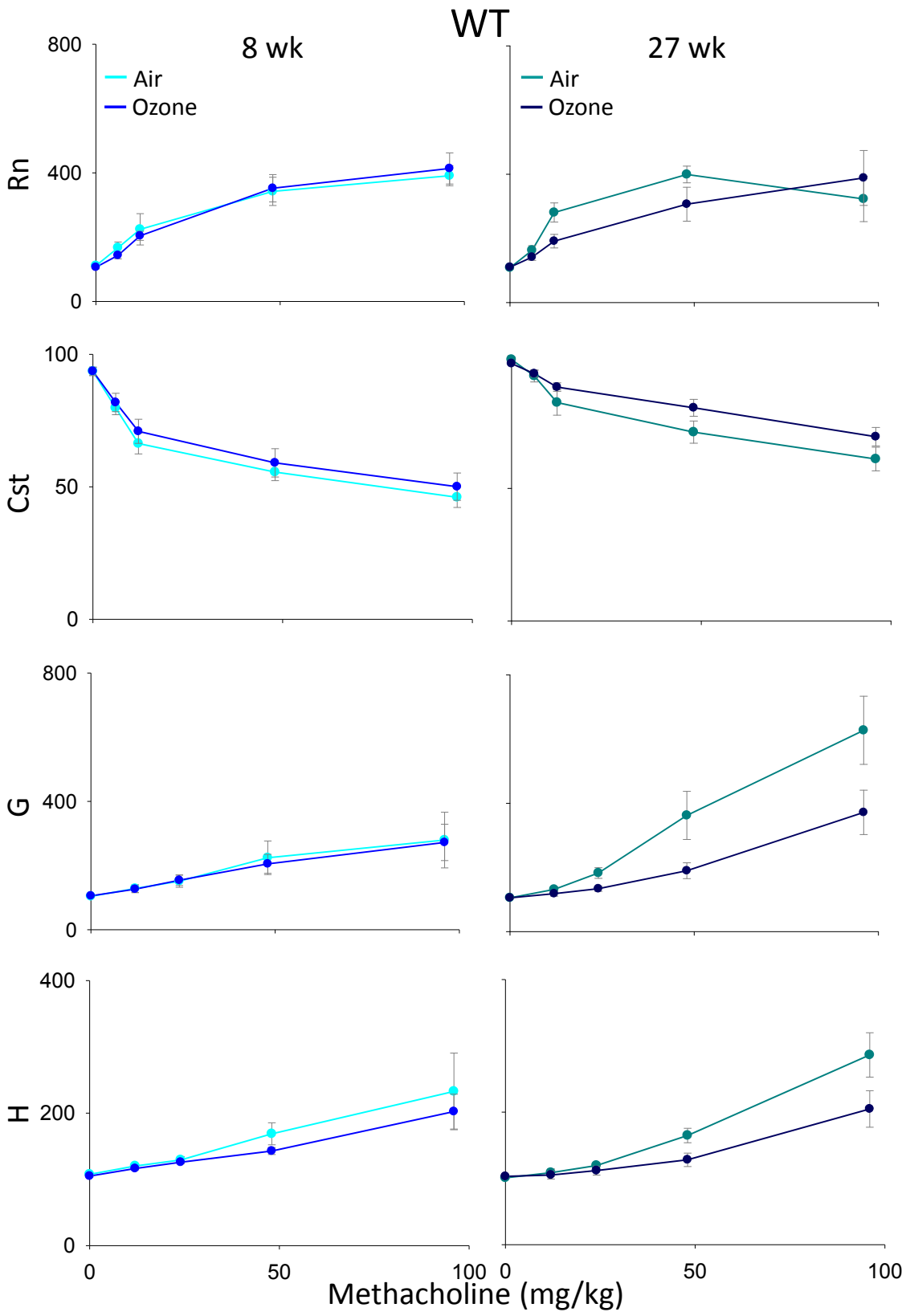

WT
8 wk
27 wk
Air
Ozone
Air
Ozone
Rn
Cst
G
H
800
400
0
100
50
0
800
400
0
400
200
0
0
50
100
0
50
100
Methacholine (mg/kg)

Figure 30. Effects of ozone inhalation on methacholine responsiveness in SP-D-/- mice. Lung function was was evaluated in response to increasing doses of methacholine 72 hr after exposure of SP-D-/- mice to air or ozone. Lungs were subjected to 0-96 mg/kg methacholine. Impedance spectra were generated using the forced oscillation technique. Resistance and elastance spectra were then derived from Z_L. For Rn, G and H, impedance spectra were measured using the forced oscillation technique and results analyzed using the constant phase model. Cst was calculated from pressure-volume loops. Measurements were performed in triplicate at each methylcholine dose. Values were normalized to 0 mg/kg methacholine. Each point represents the mean $\pm$ SEM (n = 3-6 mice/treatment group). Data were analyzed by non-linear regression.

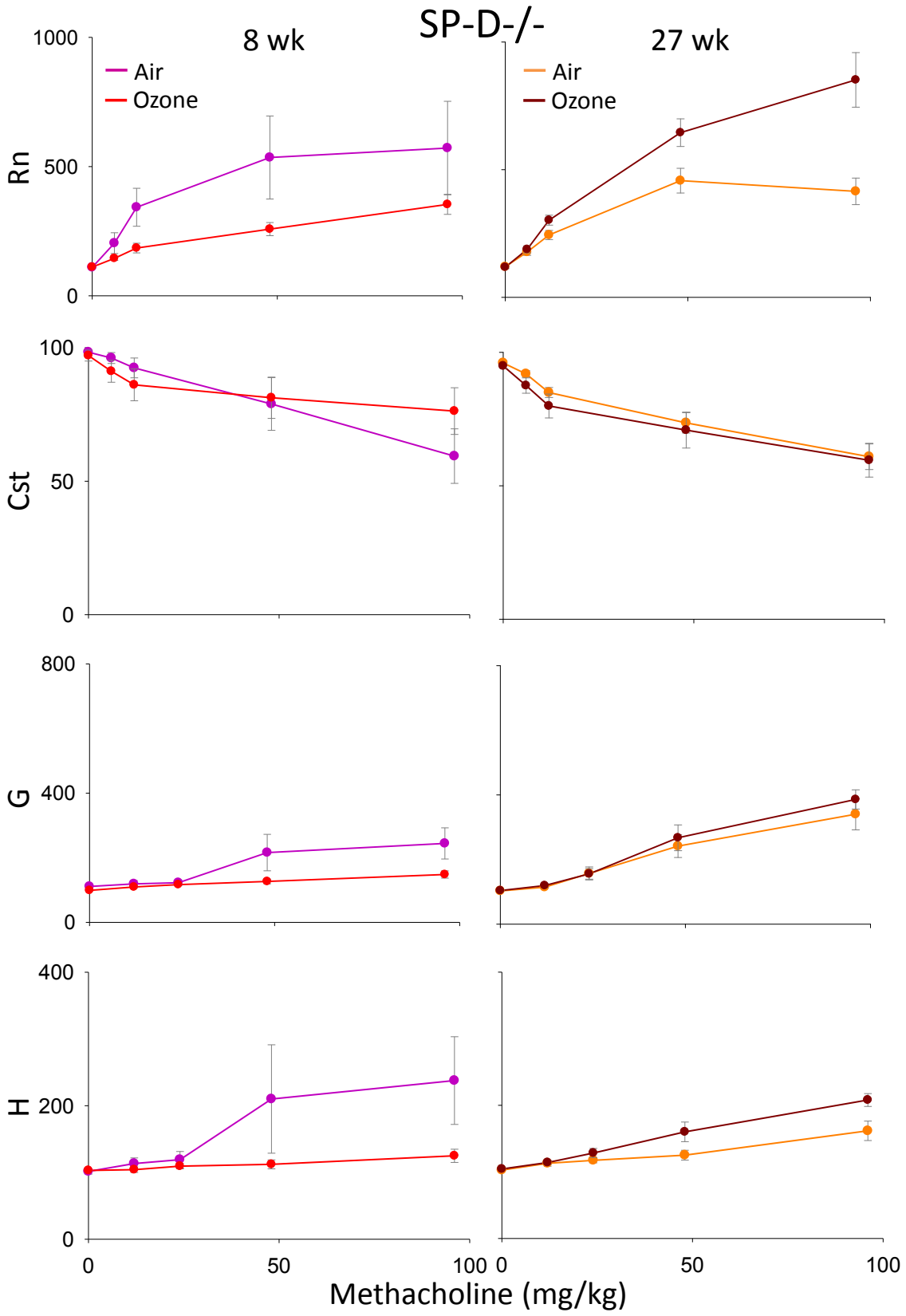

SP-D-/-
8 wk
27 wk
Air
Ozone
Air
Ozone
Rn
Cst
G
H
1000
500
0
100
50
0
800
400
0
400
200
0
0
50
100
0
50
100
Methacholine (mg/kg)

Figure 31. Effects of age on lung resistance spectra in WT and SPD-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. Impedance spectra were generated using the forced oscillation technique. Resistance was derived from input impedance data. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

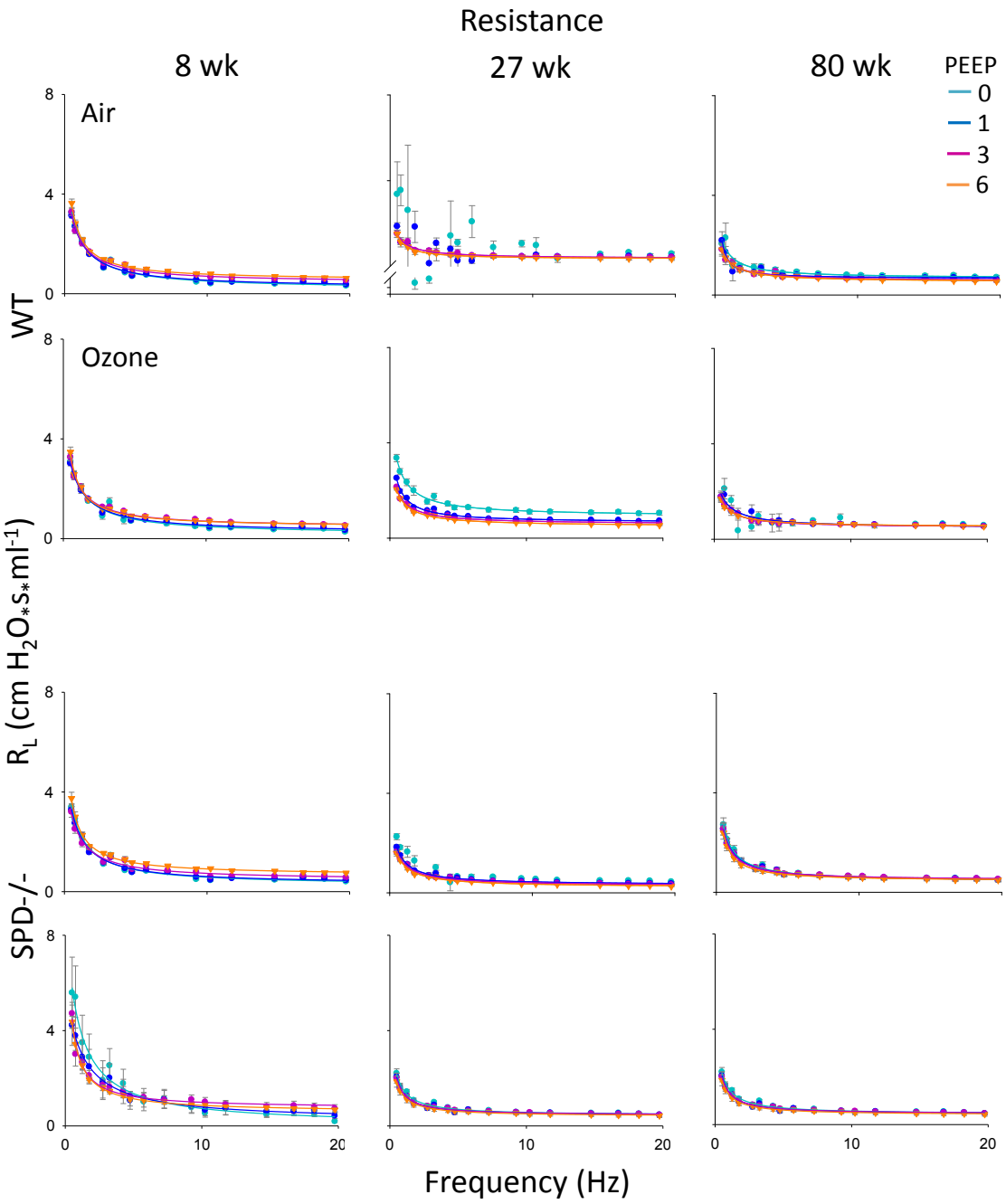

Resistance
8 wk
27 wk
80 wk
PEEP
0
1
3
6
Air
Ozone
WT
SPD-/-
R_L (cm H_2O*s*ml^{-1})
Frequency (Hz)
0
4
8
10
20

Figure 32. Effects of age on lung elastance spectra in WT and SPD-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. Impedance spectra were generated using the forced oscillation technique. Elastance was derived from input impedance data. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

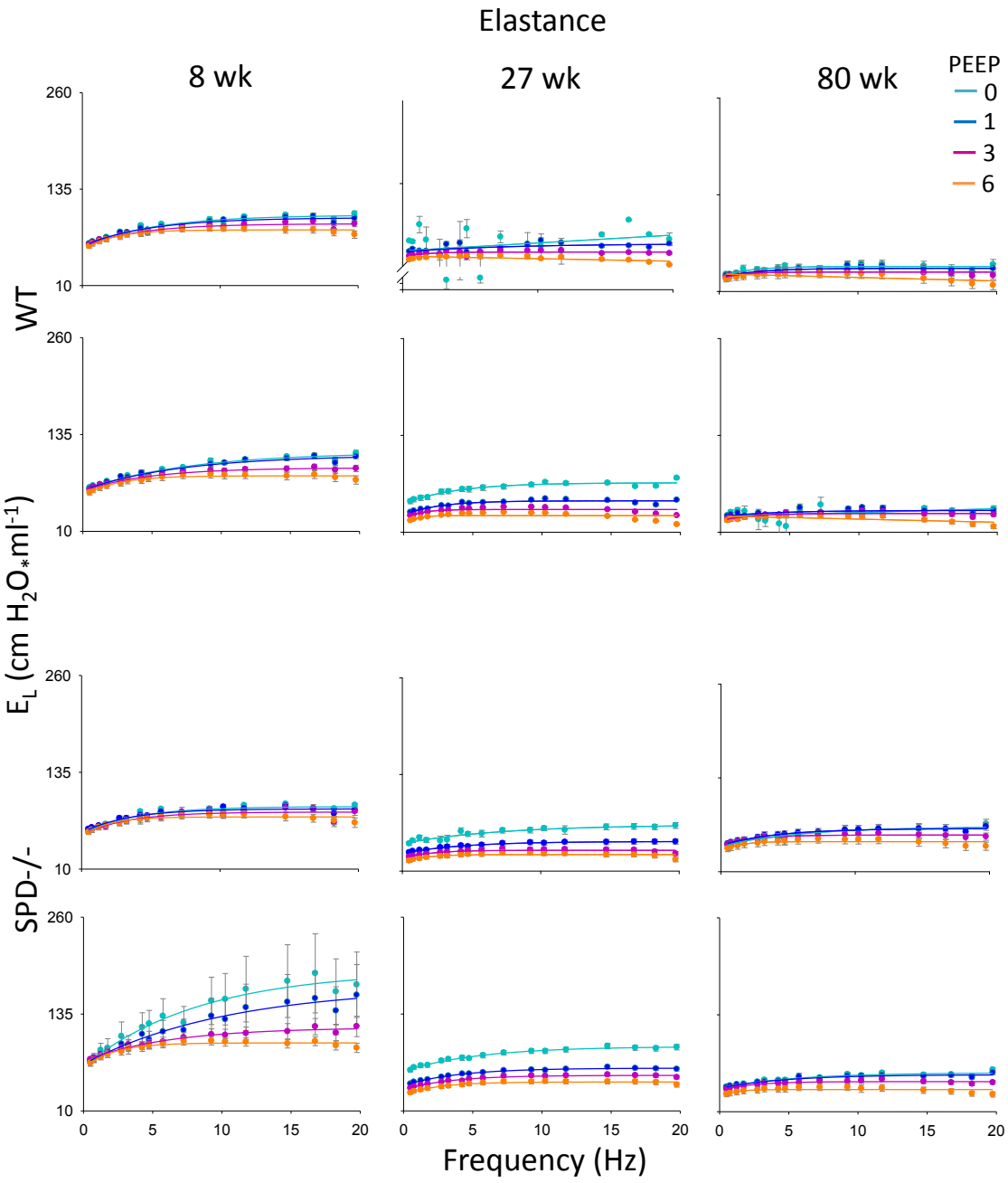

Elastance
8 wk
27 wk
80 wk
PEEP
0
1
3
6
WT
SPD-/-
E_L (cm H_2O*ml^{-1})
260
135
10
0
5
10
15
20
Frequency (Hz)

Figure 33. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. **b** reflects resistance at high frequencies. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

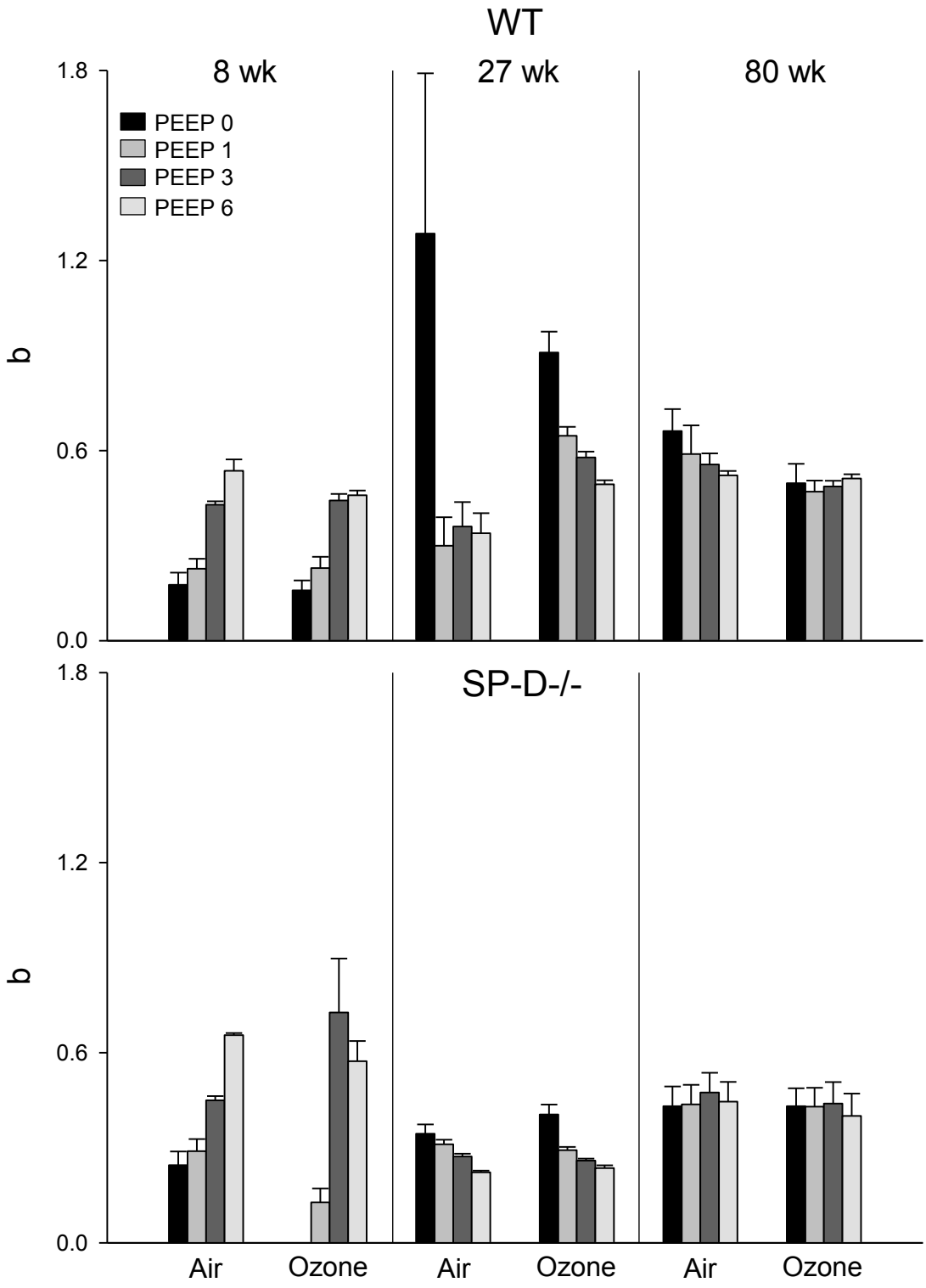

WT
8 wk
27 wk
80 wk
PEEP 0
PEEP 1
PEEP 3
PEEP 6
b
1.8
1.2
0.6
0.0
SP-D-/-
Air
Ozone
Air
Ozone
Air
Ozone

Figure 34. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. **a** reflects resistance at low frequencies. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

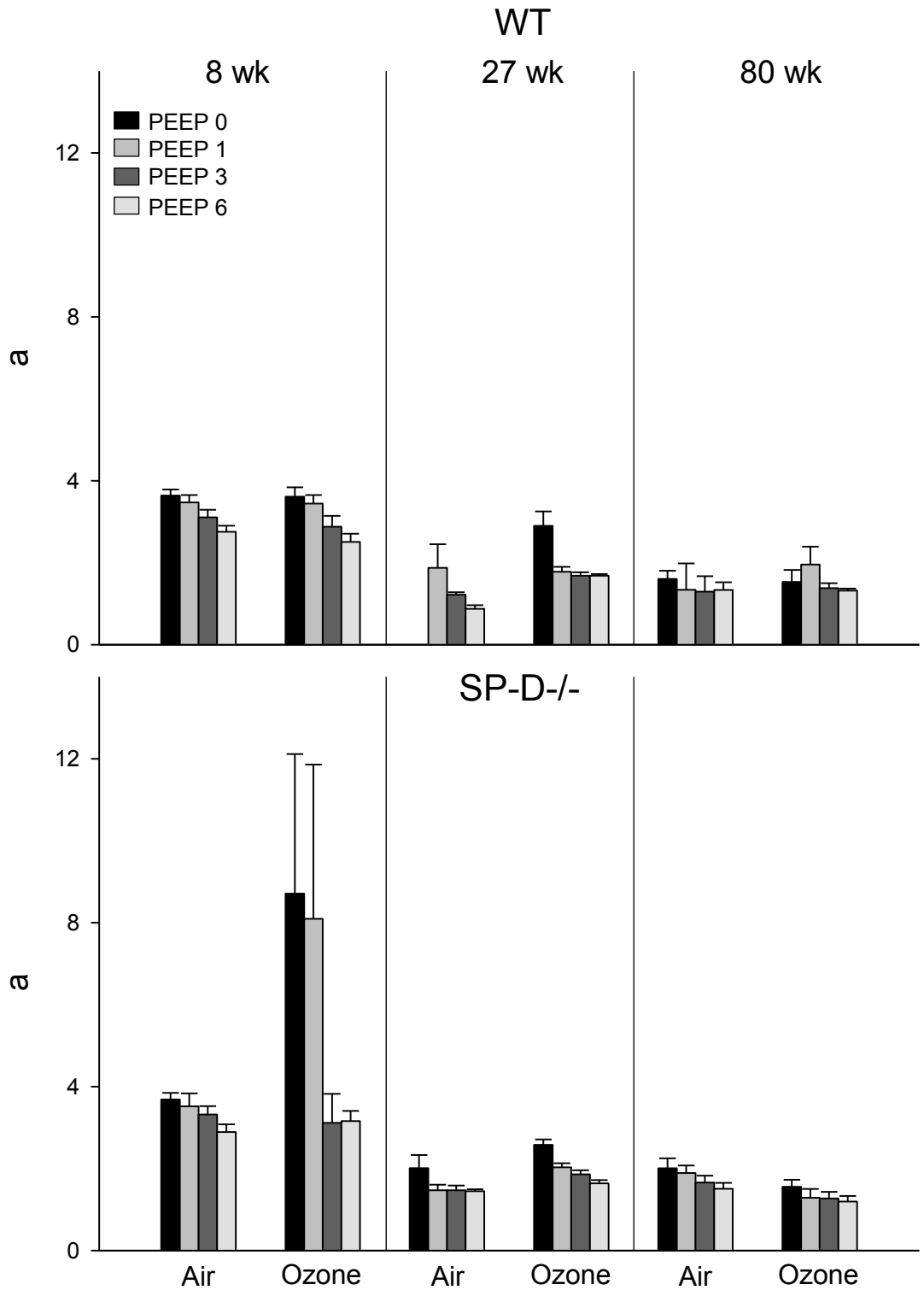

WT
8 wk
27 wk
80 wk
PEEP 0
PEEP 1
PEEP 3
PEEP 6
12
8
4
0
a
SP-D-/-
12
8
4
0
a
Air
Ozone
Air
Ozone
Air
Ozone

Figure 35. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. **c** reflects resistance at low frequencies. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

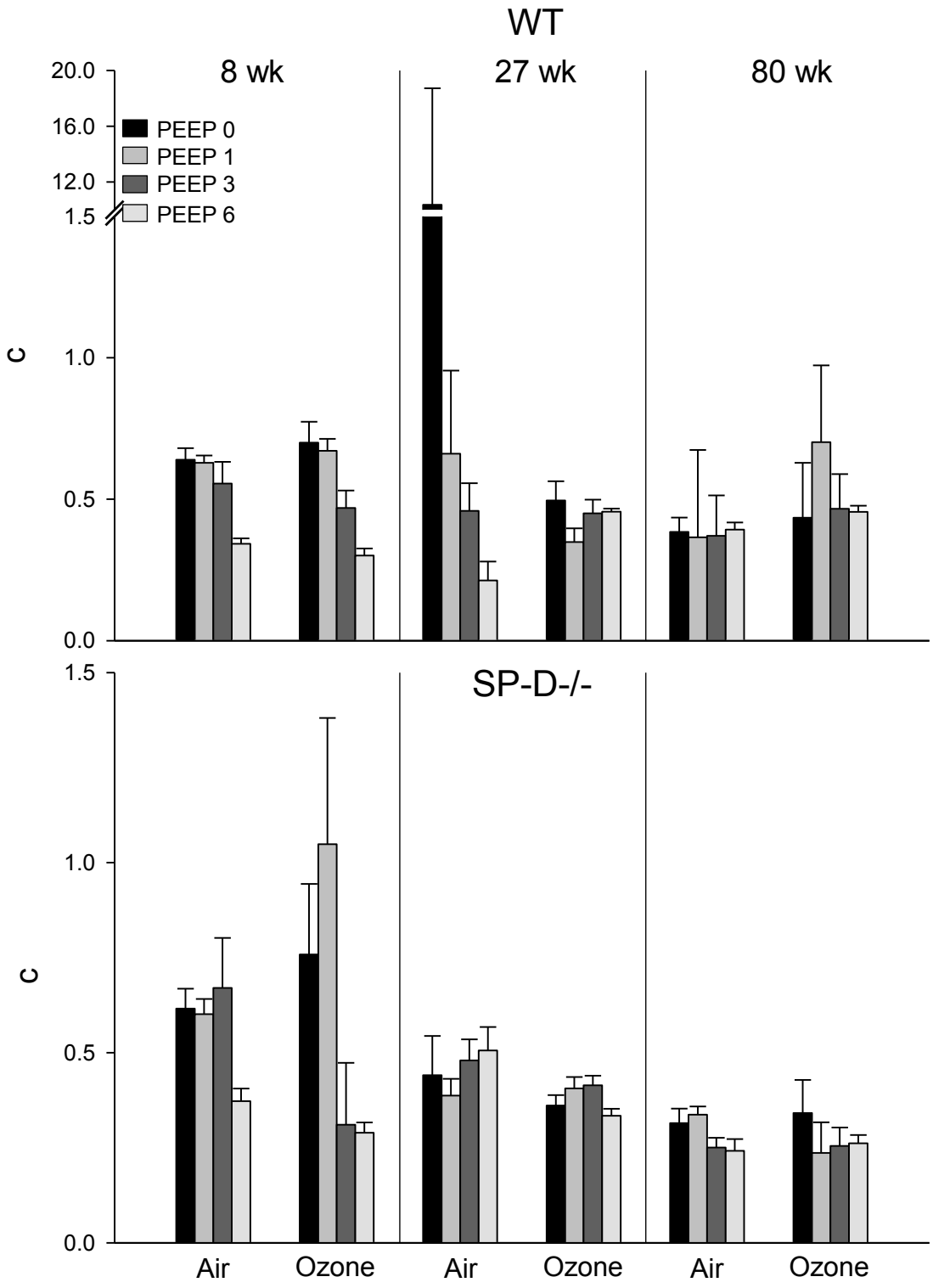

WT
8 wk
27 wk
80 wk
20.0
16.0
12.0
1.5
1.0
0.5
0.0
PEEP 0
PEEP 1
PEEP 3
PEEP 6
C
SP-D-/-
1.5
1.0
0.5
0.0
C
Air
Ozone
Air
Ozone
Air
Ozone

Figure 36. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. E_0 represents elastance at 0 Hz. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

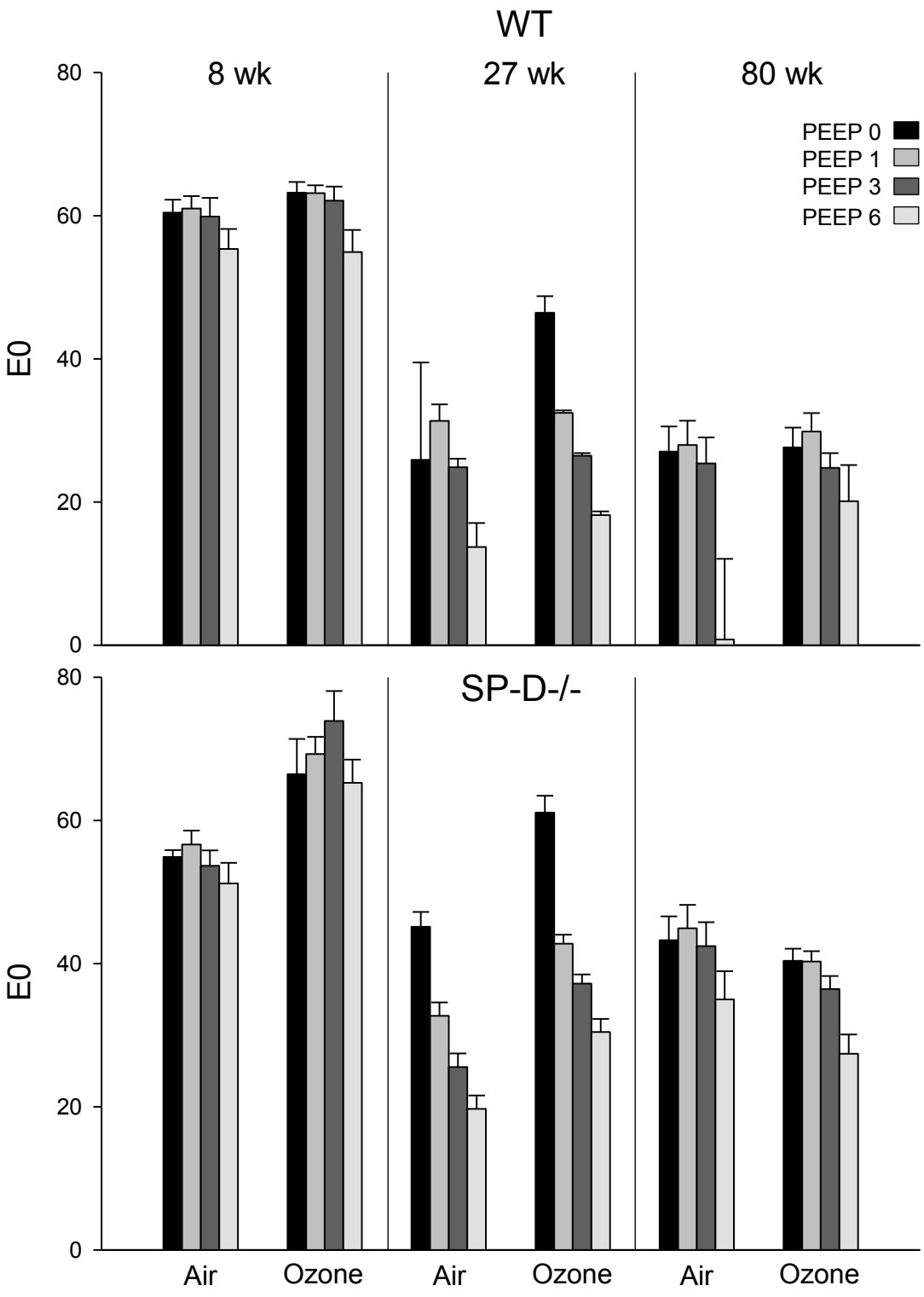

WT
8 wk
27 wk
80 wk
PEEP 0
PEEP 1
PEEP 3
PEEP 6
E0
80
60
40
20
0
SP-D-/-
Air
Ozone
Air
Ozone
Air
Ozone

Figure 37. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. ΔE reflects the magnitude of elastance. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean $\pm$ SE (n = 4-10 mice/treatment group).

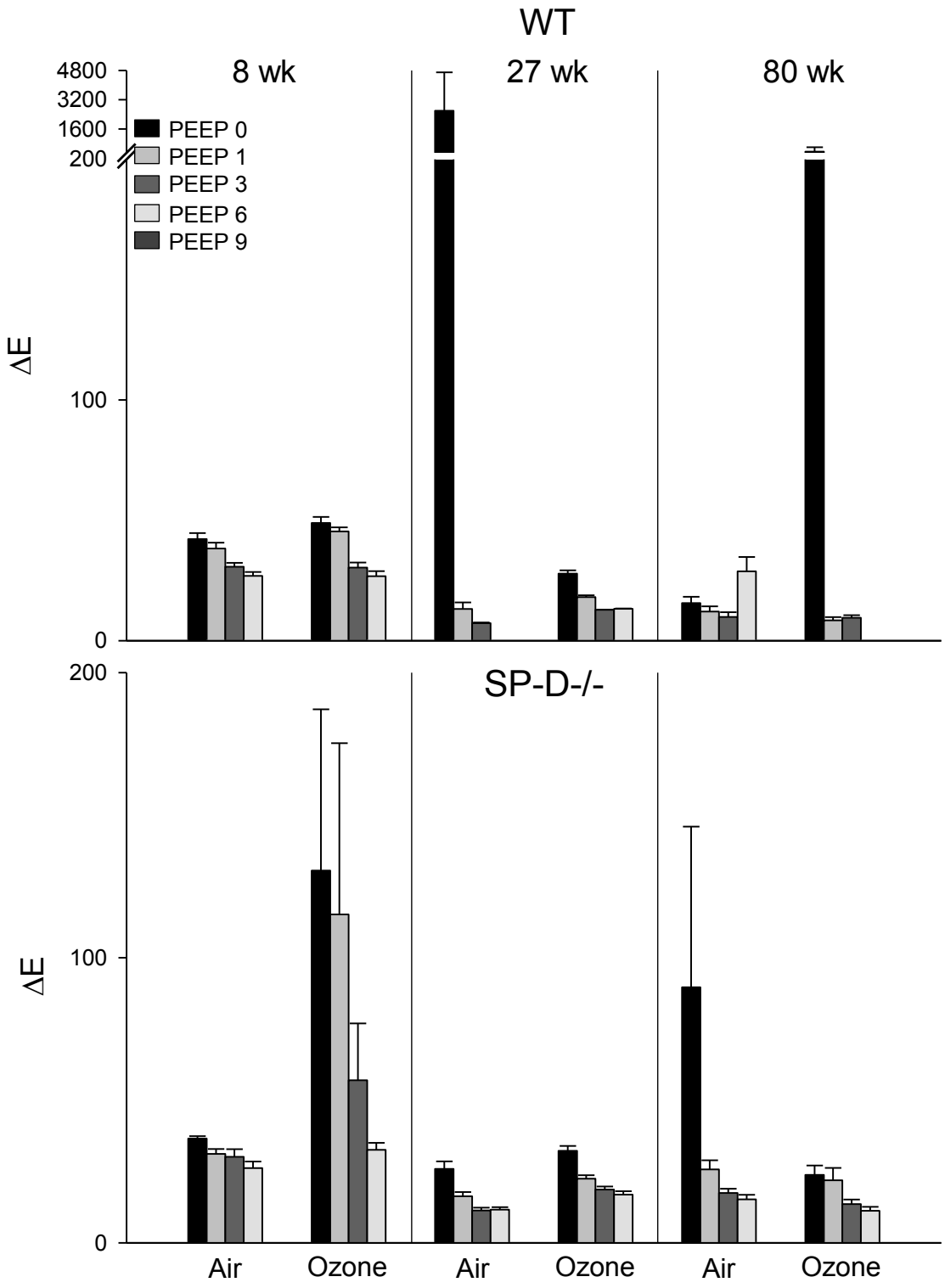
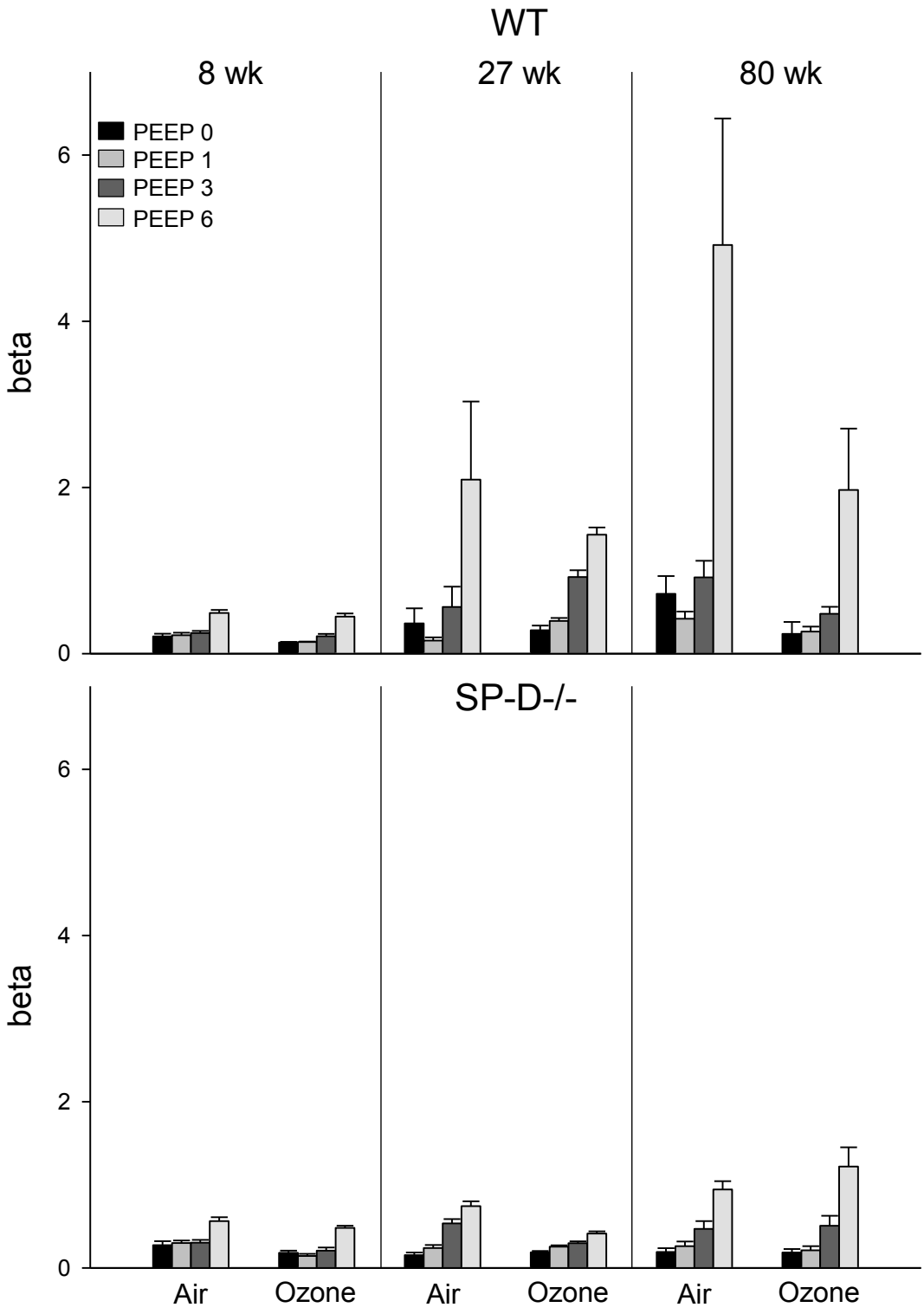

WT
8 wk
27 wk
80 wk
4800
3200
1600
200
100
0
PEEP 0
PEEP 1
PEEP 3
PEEP 6
PEEP 9
ΔE
SP-D-/-
200
100
0
ΔE
Air
Ozone
Air
Ozone
Air
Ozone

Figure 38. Effects of age on lung resistance and elastance spectral parameters in WT and SP-D-/- mice. Lung function was measured in 8 wk, 27 wk and 80 wk old WT and SP-D-/- mice. β reflects the rate of elastance change with frequency. Measurements were performed in triplicate at a PEEP of 3 cm H_2O. Each point represents the mean ± SE (n = 4-10 mice/treatment group).


WT
8 wk
27 wk
80 wk
PEEP 0
PEEP 1
PEEP 3
PEEP 6
beta
0
2
4
6
SP-D-/-
beta
0
2
4
6
Air
Ozone
Air
Ozone
Air
Ozone

SUMMARY AND CONCLUSIONS

The overall objective of these studies was to analyze the effects of progressive pulmonary macrophage inflammation and emphysema associated with aging in mice lacking SP-D on the persistence of ozone-induced injury, macrophage activation, and altered functioning in the lung. We hypothesized that SP-D-/- mice would exhibit prolonged injury and oxidative stress, as well as alterations to macrophage activation and pulmonary mechanics, in response to ozone, and that these responses would be altered with age. The results of these studies demonstrated that mice lacking SP-D exhibited significant pulmonary inflammation relative to WT mice, characterized by increased numbers of macrophages in the lung and in BAL. We also found that pulmonary mechanics were altered with loss of SP-D. Thus, increases in R, Rn and Cst were observed in SP-D-/- mice relative to WT mice. Additionally, at all PEEPs measured, resistance spectra was greater at high frequencies, suggesting that persistent pulmonary inflammation in SP-D-/- mice leads to alterations in central airway mechanics. Loss of SP-D also altered the elastic recoil properties of the lung parenchyma, and caused increases in heterogeneous ventilation, airway resistance and lung stiffness.

Results also demonstrated that chronic pulmonary inflammation resulting from loss of SP-D results in prolonged sensitivity of mice to inhaled ozone and is associated with exacerbated functional defects in the lung. Thus, mice lacking SP-D exhibited significant pulmonary inflammation relative to WT mice. Increased BAL protein and nitrogen oxides were observed 72 hr following ozone inhalation indicating prolonged lung injury and oxidative stress. Increased numbers of vacuolated, activated macrophages were also present in BAL and in histologic sections from SP-D-/- mice,

which was associated with increased BAL chemotactic activity as well as lung inflammation score. Additionally, macrophage and Type II cell expression of iNOS and COX-2 remained elevated above control for at least 72 hr after ozone inhalation in SP-D-/- mice, suggesting that a pro-inflammatory macrophage phenotype persists in these mice and that Type II cells may contribute to the inflammatory response. In both WT and SP-D-/- mice, inhalation of ozone was associated with functional alterations in the lung. One major finding is that despite resolution of inflammatory responses, WT mice remain functionally compromised 72 hr after ozone exposure. We found that this was limited to central airway mechanics using the constant phase model. Further analysis of the dependence of the constant phase parameters, as well as resistance and elastance spectra, on PEEP revealed that there is a lung recruitment change in the elastic properties of the lung following ozone exposure. The nature of these changes demonstrate a loss of parenchymal tethering, supporting the idea that ozone causes a loss of parenchymal integrity. Due to preexisting chronic inflammation, we speculated that ozone-induced alterations in pulmonary mechanics would be more extensive in SP-D-/- mice, when compared to WT mice following ozone exposure. In support of this, we found that in SP-D-/- mice, ozone inhalation resulted in alterations not only in airway mechanics, but also in the parenchymal integrity of the already compromised lungs. Findings that in the derecruited lung, ozone exposure resulted in a frequency dependent increase in elastance, suggest that the buildup of inflammatory material may cause a stiffening of the lung. Furthermore, increases in both the resistance and elastance spectra at low frequencies that were ameliorated with PEEP suggest that in SP-D-/- mice, ozone exposure affects

parenchymal tethering, which could result in small airway collapse, and results in heterogeneous changes in lung function.

Another aspect of these studies was to evaluate the effects of increasing pulmonary inflammation and the development of emphysema in aging SP-D-/- mice on the response to inhaled ozone. This was examined using young (8 wk), middle age (27 wk), and elderly (80 wk) WT and SP-D-/- mice. We observed age-related increases in macrophage inflammation, along with alveolar wall rupture and Type II cell hyperplasia, in SP-D/- mice. Additionally, macrophage expression of markers of both classical and alternative activation were observed to increase with age. Unexpectedly, increasing age was not associated with increases in numbers of inflammatory cells in the lungs of WT mice; moreover macrophage expression of markers of alternative activation, but not classical activation increased. Although age-related effects of pulmonary mechanics were exacerbated with loss of SP-D, in both WT and SP-D-/- mice, increasing age from 8 wk to 27 wk resulted in reduced lung stiffness, as reflected by decreases in resistance and elastance spectra, and alterations in their component parameters, suggesting a loss of parenchymal integrity. This response was blunted in 80 wk old SP-D-/- mice, however, and is indicative of lung stiffening, likely resulting from the large numbers of inflammatory cells in the tissue and excessive fibrin deposition.

Resolution of ozone-induced lung injury was also unaffected by increasing age in WT mice, although aging resulted in a shift in markers of macrophage activation, toward a pro-inflammatory, classically activated phenotype, following ozone inhalation. In contrast, mice lacking SP-D exhibited increased ozone-induced toxicity at 8 wk and 27 wk of age, however by 80 wk these effects were overwhelmed by extensive baseline

injury and inflammation. Thus, while ozone exposure resulted in increased numbers of classically activated macrophages in young and middle aged mice, by 80 wk, maximum numbers of these cells appear to be already present in the lung. In young and middle aged WT and SP-D-/- mice ozone caused increases in lung resistance and elastance spectra, consistent with alterations in tissue mechanics in WT mice, and both airway and tissue mechanics, representing an ozone-induced stiffening of the lung, likely resulting from the buildup of inflammatory cells, in SP-D-/- mice. In contrast, ozone had no effect on pulmonary mechanics in 80 wk old mice. These results suggest that in SP-D-/- mice, ozone-induced toxicity is overwhelmed by the larger effects of age-related increases in baseline inflammation and lung injury in these mice and that aging and chronic inflammation reduce the ability of the immune system to respond to additional inflammatory insults.

FUTURE STUDIES

Our studies suggest that increased iNOS expression by macrophages may contribute to chronic inflammation in aging SP-D-/- mice. This is supported by earlier studies reporting that treatment of these mice with an iNOS inhibitor attenuated the inflammatory responses associated with loss of SP-D (Atochina-Vasserman et al., 2007). Characterizing age-related alterations in pulmonary inflammation and alterations in lung function in mice lacking both iNOS and SP-D, or alternatively by administering an iNOS specific inhibitor such as 1400W or aminoguanidine to SP-D-/- mice (Atochina-Vasserman et al., 2007; Malaviya et al., 2012), would further clarify the role of iNOS in the development of chronic inflammation and emphysema in mice lacking SP-D. The current studies demonstrated that ozone exposure increases iNOS expression in both WT and SP-D-/- mice; since previous studies have also shown that ozone-induced lung injury is blunted in mice lacking iNOS, this lead us to speculate that the increased iNOS expression in SP-D-/- mice may contribute to their increased sensitivity to ozone. Assessing the response of iNOS/SP-D double knockout mice to ozone would help clarify this question. It will also be of interest to analyze the effects of aging on double knockout mice, since ozone-induced macrophage expression of iNOS was increased in older mice.

These present studies also evaluated how macrophage activation into classically and alternatively activated phenotypes was altered with chronic pulmonary inflammation, age and ozone exposure, however whether these populations consisted of resident or infiltrating populations was not determined. Flow cytometry and antibodies for resident and infiltrating macrophages could be used to characterize alveolar macrophages isolated

from young and old WT and SP-D-/- mice after exposure to air or ozone. Infiltrating populations would be characterized as $Ly6C^{hi}$,$CD11b^{+}$ and $CD62L^{+}$, whereas resident populations would be $Ly6C^{lo}$, $CD62L^{-}$ and $CXG3CR1^{hi}$ (Siamon Gordon et al., 2005).

In further studies, macrophage inhibitors could be used to elucidate the precise role of classically and alternatively activated macrophages on inflammation, injury and changes in lung function following ozone exposure. For example, young and old WT and SP-D-/- mice could be administered gadolinium chloride, to deplete pro-inflammatory macrophages (Pendino et al., 1995), or clodronate liposomes, to deplete anti-inflammatory wound repair macrophages (Elder et al., 2004) before ozone exposure, and inflammation and injury characterized. Alternatively, alveolar macrophages could be isolated from healthy mice, cultured and differentiated into classically or alternatively activated phenotypes using IFN-γ or IL-4 and IL-13, respectively. These activated macrophages could then be adoptively transferred into the lungs of young and old WT and SP-D-/- mice and inflammation and injury characterized, as well as how these exogenous macrophages affect inflammatory responses of these mice to ozone. Using this same adoptive transfer technique, macrophages from SP-D-/- mice could be transferred into WT mice which would then be exposed to ozone. Findings that the presence of these cells increased inflammation and injury would provide additional support for our findings that chronic macrophage activation increases susceptibility to ozone-induced lung injury. Similarly, increased ozone-induced inflammation and injury in young mice that have received macrophages from older mice would demonstrate and age related dysfunctions in alveolar macrophage activity would also result in increased sensitivity to ozone. Additionally, determining the contribution of specific macrophage

populations to alterations in lung function is of value, since no work has previously been conducted on this subject.

The results of our studies show that a large determinate of lung functioning is age and that this effect is often greater than alterations in respiratory mechanics induced by ozone exposure. Since the mechanical properties of the lung are influenced by the viscous and elastic structural characteristics of the tissue, as well as forces acting at the air-liquid interface of the alveolar surface, it would be useful to determine if alterations in respiratory mechanics resulting from chronic inflammation, emphysema in SP-D-/- mice resulted from alterations in the properties of the parenchyma or to surfactant dysfunction. Since collagen and elastin fibers each have different elastic properties, alterations in the amount and ratio of these fibers during inflammation induced extracellular matrix remodeling will shift the balance of stresses in the lung contribute to mechanical dysfunction. Therefore, histological sections of young and old WT and SP-D-/- mice could be stained with orcein, or aldehyde fuchsin to identify elastic fibers and masson trichrome or picrosirius red to identify collagen fibers and changes in the ratio of these fibers determined using image analysis software such as Image J. Since macrophage induced MMP activation is associated with parenchymal destruction and tissue remodeling during emphysema pathogenesis, immunohistochemical or western blotting techniques should also be used to evaluate expression of MMP's in alveolar macrophages isolated from young and old WT and SP-D-/- mice. Since macrophage MMP expression has been shown to increase following ozone exposure, it would also be interesting to determine if age and chronic inflammation alters this response. To determine the contribution of surfactant-related properties to alterations in lung functioning related to

age and loss of SP-D, a phospholipid assay could be performed on surfactant containing BAL collected from these mice. Taken together, these studies will allow us to elucidate the mechanisms by which macrophages contribute to inflammatory resulting from loss of SP-D and ozone inhalation as well as to determine how inflammatory changes affect lung airway and tissue properties and lead to changes in respiratory mechanics.

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