Walter A. Rosenblith New Investigator Award

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# Novel Mechanisms of Ozone-Induced Pulmonary Inflammation and Resolution, and the Potential Protective Role of Scavenger Receptor BI

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with a Critique by the HEI Review Committee

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Research Report 204

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# ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives balanced funding from the U.S. Environmental Protection Agency and the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 340 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in more than 260 comprehensive reports published by HEI, as well as in more than 2,500 articles in the peer-reviewed literature.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Review Committee are widely disseminated through HEI's website (www.healtheffects.org), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

# ABOUT THIS REPORT

Research Report 204, *Novel Mechanisms of Ozone-Induced Pulmonary Inflammation and Resolution, and the Potential Protective Role of Scavenger Receptor BI*, presents a research project funded by the Health Effects Institute and conducted by Dr. Kymberly M. Gowdy of Ohio State University College of Medicine, Columbus, Ohio, and her colleagues. This research was funded under HEI's Walter A. Rosenblith New Investigator Award Program, which provides support to promising scientists in the early stages of their careers. The report contains three main sections.

**The HEI Statement**, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Review Committee's comments on the study.

**The Investigators' Report,** prepared by Gowdy and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

**The Critique,** prepared by members of the Review Committee with the assistance of HEI staff, places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.

# HEI STATEMENT

Synopsis of Research Report 204

# Effects of Ozone on Acute Lung Inflammation and Injury in Mice

**BACKGROUND**

Ozone is one of the six criteria pollutants regulated by the U.S. Environmental Protection Agency under the Clean Air Act. Given that importance, investigation into what biological mechanisms underlie ozone's effects continues to be of scientific and policy interest. Currently, the precise mechanisms by which acute exposure to ambient ozone triggers inflammation in the airways are not well understood.

In this study, Dr. Kymberly Gowdy, a recipient of HEI's Walter A. Rosenblith New Investigator Award, and her colleagues evaluated how acute exposure to ozone affected markers of inflammation and injury in the lung, both during initiation and resolution of the response. Gowdy and colleagues were particularly interested in evaluating two features of the resolution phase: the role of specialized pro-resolving mediators, lipid mediators that act as a key signal to switch to the resolution phase; and efferocytosis, the process by which cells that have been activated during the inflammatory response and are facing cell death (apoptosis) are removed by macrophages. The latter process helps the lung return to baseline (homeostasis), preventing detrimental effects if inflammation were to continue (see Statement Figure).

**APPROACH**

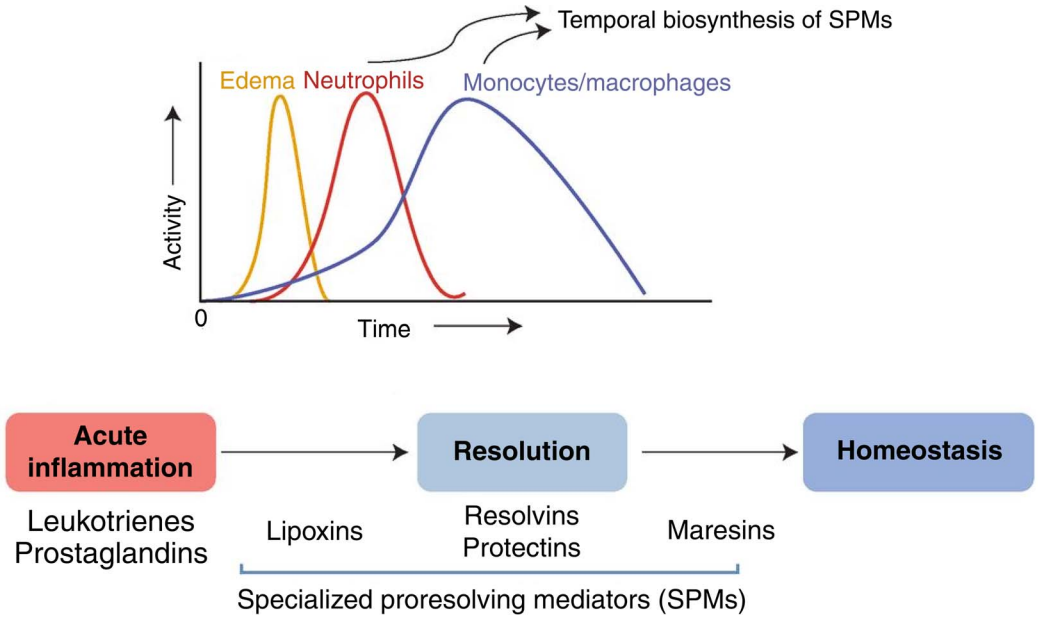
The investigators exposed mice to 1 ppm ozone for 3 hours and evaluated effects in the lungs mostly at 24 hours after the end of exposure. They used standard techniques to measure markers of inflammation — including levels of leukocytes, macrophages, and cytokines and chemokines — as well as injury (protein leak) in lung fluid and tissue. They also developed a sensitive high-performance liquid chromatography tandem mass spectrometry technique to measure low levels of specialized lipid mediators in homogenized lung tissue. Because the rapid reaction of ozone with components of the respiratory tract produces oxidized phospholipids, which in turn can induce an inflammatory response, the investigators also measured levels of oxidized phospholipids in lung homogenates.

To assess efferocytosis, Gowdy and colleagues gave mice an easily visualized T cell line undergoing cell death by apoptosis by dispensing the cells into the back of the throat 24 hours after ozone exposure

## What This Study Adds

- The study evaluated how acute exposure of mice to ozone affects initiation and resolution of the inflammatory response in the lung.
- It is the first to examine the role of specialized pro-resolving mediators, key lipids of the resolution phase of inflammation, in how lung cells are activated and later removed by macrophages.
- Acute ozone exposure resulted in changes in levels of specialized pro-resolving mediators and other markers of lung inflammation and injury in mice. Supplementation of mice with specialized pro-resolving mediators before ozone exposure decreased some of those markers.
- This provides a rationale for future research to evaluate whether supplementation with specialized pro-resolving mediators may mitigate human conditions that involve chronic inflammation, such as chronic respiratory and cardiovascular diseases.

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Kymberly M. Gowdy at East Carolina University, Greenville, North Carolina (currently at the Ohio State University College of Medicine, Columbus, Ohio), and colleagues. Research Report 204 contains both the detailed Investigators' Report and a Critique of the study prepared by the Institute's Review Committee.




**Statement Figure. Key events in the induction and resolution of the inflammatory response.** (Adapted with permission from Serhan CN, Chiang N, Dalli J, Levy BD. 2014. Lipid mediators in the resolution of inflammation. Cold Spring Harb Perspect Biol 7:a016311; © Cold Spring Harbor Laboratory Press.)

so that they would move directly into the airspace. The investigators then isolated alveolar macrophages from these mice and microscopically evaluated what percentage of macrophages had taken up the T cell line. Because oxidized phospholipids bind to scavenger receptor (SR)-BI that is expressed on macrophages and many other cells, the investigators also evaluated mice lacking the SR-BI receptor (SR-BI knockout mice). They also administered specialized pro-resolving mediators prior to ozone exposure and measured their levels as well as markers of lung inflammation and injury.

## KEY RESULTS AND INTERPRETATION

Exposure to 1 ppm ozone for 3 hours resulted in increases in some of the expected markers of a standard inflammatory response at 24 hours. For example, Gowdy and colleagues reported increases in numbers of macrophages and neutrophils in lung fluid, as well as in levels of pro-inflammatory cytokines and chemokines in lung tissue. Ozone exposure did not consistently affect lung injury; protein leak increased 2-fold, but only in male mice.

Levels of specialized lipid mediators increased at 6 hours, decreased at 24 hours, and were back to baseline levels 72 hours after mice were exposed to ozone, compared with mice exposed to filtered air. Levels of oxidized phospholipids increased approximately 2- to 3-fold at 6 hours after ozone exposure. Effects of ozone on efferocytosis were difficult to interpret: one experiment in female mice showed a decrease, but effects in male mice were variable.

Pretreatment of mice with specialized pro-resolving mediators prior to ozone exposure decreased levels of some markers of the inflammatory response, such as neutrophils and macrophages in lung fluid, and of some pro-inflammatory cytokines and chemokines in lung tissue compared with levels of those markers in mice that had not been pretreated. Thus, pretreatment appeared to mitigate some of the inflammatory effects of ozone exposure.

The effects of ozone on the inflammatory response and efferocytosis were similar in mice that either expressed or did not express the receptor SR-BI, apart from an increase in neutrophils in mice lacking SR-BI.

**HEI REVIEW COMMITTEE EVALUATION**

In its independent review, the HEI Review Committee considered the work by Gowdy and colleagues in ozone-exposed mice to be an interesting new approach for evaluating the events involved in both the early and later phases of the inflammatory response (reflecting initiation and resolution) after acute exposure to ozone. In particular, the study was a valuable initial attempt to understand the role of different types of lipid mediators produced during those early and later phases of inflammation.

The investigators successfully used a sensitive technique to identify and quantify low levels of oxidized phospholipids that were generated early in the response to ozone, as well as specialized pro-resolving lipid mediators that play a key role in the resolution of the inflammatory response. In addition, the investigators showed that pre-treating mice with lipid mediators prior to ozone exposure partially mitigated the resulting inflammatory response. These results offer the possibility that pretreatment with lipid mediators can be used in a clinical or dietary setting to offset inflammation induced by air pollutants or perhaps even pathogens.

The investigators concluded that the SR-BI receptor plays a protective role in either ozone-induced inflammation or resolution of the response. However, the Committee disagreed, based on findings that most markers of the inflammatory and injury response were similar in mice that did or did not express SR-BI, and that the effect of ozone on efferocytosis was almost identical in these two sets of mice.

The Committee noted several important limitations in the study design that reduced confidence in the generalizability of the results. One major limitation was that the investigators used only one concentration of ozone, 1 ppm. Based on uptake of radioactive ozone by lung cells of rodents and humans, this exposure concentration of 1 ppm in mice was estimated to correspond to a fairly high human exposure concentration of 200 ppb (or 42.8 $\mu g/m^3$) of ozone. A further limitation of the study design was that the investigators measured only a limited set of markers of inflammation without performing histopathology, which would have shown both the extent of inflammatory damage and injury to multiple lung cell types, and how the damage and injury might have resolved. The Committee also considered the results and interpretations of the efferocytosis assay to be of uncertain significance, because the system used to evaluate efferocytosis did not clearly model the process in the body by which activated cells are removed during the inflammatory response.

In summary, the Committee thought this study provided a good foundation for further research to assess the role of specialized lipid mediators in mitigating inflammatory responses. Given that exposure to ozone exacerbates chronic inflammatory conditions such as asthma and cardiovascular disease, it will be worth exploring whether ozone affects the resolution of inflammation in these conditions, and whether enhancement of lipid mediator levels through diet or other interventions may be clinically useful in mitigating such conditions.

# INVESTIGATORS' REPORT

## Novel Mechanisms of Ozone-Induced Pulmonary Inflammation and Resolution, and the Potential Protective Role of Scavenger Receptor BI

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## ABSTRACT

***Introduction.*** Increases in ambient levels of ozone ($O_3$*), a criteria air pollutant, have been associated with increased susceptibility and exacerbations of chronic pulmonary diseases through lung injury and inflammation. $O_3$ induces pulmonary inflammation, in part by generating damage-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and scavenger receptors (SRs). This inflammatory response is mediated in part by alveolar macrophages (AMs), which highly express PRRs, including scavenger receptor BI (SR-BI). Once pulmonary inflammation has been induced, an active process of resolution occurs in order to prevent secondary necrosis and to restore tissue homeostasis. The processes known to promote the resolution of inflammation include the clearance by macrophages of apoptotic cells, known as efferocytosis, and the production of specialized pro-resolving mediators (SPMs). Impaired efferocytosis and production of SPMs have been associated with the pathogenesis of chronic lung diseases; however, these impairments have yet to be linked with exposure to air pollutants.

***Specific Aims.*** The primary goals of this study were: Aim 1 — to define the role of SR-BI in $O_3$-derived pulmonary inflammation and resolution of injury; and Aim 2 — to determine if $O_3$ exposure alters pulmonary production of SPMs and processes known to promote the resolution of pulmonary inflammation and injury.

***Methods.*** To address Aim 1, female wild-type (WT) and SR-BI-deficient, or knock-out (SR-BI KO), mice were exposed to either $O_3$ or filtered air. In one set of experiments mice were instilled with an oxidized phospholipid (oxPL). Bronchoalveolar lavage fluid (BALF) and lung tissue were collected for the analyses of inflammatory and injury markers and oxPL. To estimate efferocytosis, mice were administered apoptotic cells (derived from the Jurkat T cell line) after $O_3$ or filtered air exposure.

To address Aim 2, male WT mice were exposed to either $O_3$ or filtered air, and levels of SPMs were assessed in the lung, as well as markers of inflammation and injury in BALF. In some experiments SPMs were administered before exposure to $O_3$ or filtered air, to determine whether

This Investigators' Report is one part of Health Effects Institute Research Report 204, which also includes a Critique by the Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Kymberly M. Gowdy, Division of Pulmonary, Critical Care and Sleep Medicine, Ohio State University College of Medicine, 473 W. 12th Ave. Columbus, OH 43210; e-mail: *Kymberly.Gowdy@osumc.edu*. No potential conflict of interest was reported by the authors.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

* A list of abbreviations and other terms appears at the end of this volume.

SPMs could mitigate inflammatory or resolution responses. Efferocytosis was measured as in Aim 1.

***Results.*** For Aim 1, SR-BI protein levels increased in the lung tissue of mice exposed to $O_3$, compared with mice exposed to filtered air. Compared with WT controls, SR-BI KO mice had a significant increase in the number of neutrophils in their airspace 24 hours post $O_3$ exposure. The oxPL levels increased in the airspace of both WT and SR-BI KO mice after $O_3$ exposure, compared with filtered air controls. Four hours after instillation of an oxPL, SR-BI KO mice had an increase in BALF neutrophils and total protein, and a nonsignificant increase in macrophages compared with WT controls. $O_3$ exposure decreased efferocytosis in both WT and SR-BI KO female mice.

For Aim 2, mice given SPM supplementation before $O_3$ exposure showed significantly increased AM efferocytosis when compared with the $O_3$ exposure control mice and also showed some mitigation of the effects of $O_3$ on inflammation and injury. Several SPMs and their precursors were measured in lung tissue using reverse-phase high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). At 24 hours after $O_3$ exposure 14R-hydroxydocosahexaenoic acid (HDHA) and 10,17-dihydroxydocosahexaenoic acid (diHDoHE) were significantly decreased in lung tissue, but at 6 hours after exposure, levels of these SPMs increased.

***Conclusions.*** Our findings identify novel mechanisms by which $O_3$ may induce pulmonary inflammation and also increase susceptibility to and exacerbations of chronic lung diseases.

## BACKGROUND

### HEALTH EFFECTS OF ACUTE INCREASES IN AMBIENT $O_3$

Despite increasing regulations, exposure to air pollution remains a serious health concern, globally leading to 9 million premature deaths each year (Landrigan 2017). $O_3$ is a central component of air pollution and is associated with more than 3,000 deaths annually in the United States (Hubbell et al. 2005). Acute increases in ambient $O_3$ levels are associated with cardiopulmonary complications, which include decreased pulmonary function, acute lung injury, and exacerbation of pre-existing pulmonary and cardiovascular diseases (Basu 2009; Liu et al. 2009; Srebot et al. 2009). The mechanisms for these acute effects, with the exception of functional physiological changes, have not been well characterized. These detrimental health effects occur partially through $O_3$-induced pulmonary inflammation and injury as defined by epithelial cell damage, inflammatory cytokine and chemokine release, and inflammatory cell influx into the airspace (Hollingsworth et al. 2007; Pendino et al. 1995; Tighe et al. 2011). Currently, the mechanisms triggering $O_3$-induced inflammatory responses during acute increases in ambient $O_3$ levels are ill-defined, and there are no effective methods to prevent $O_3$-induced pulmonary inflammation and injury.

In addition to acute health effects that occur with increases in ambient $O_3$ levels, recent evidence indicates that individuals living in areas with heightened $O_3$ levels have a greater incidence of lung diseases (HEI 2020). Increases in ambient $O_3$ levels have been linked to the development of asthma, idiopathic pulmonary fibrosis, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (Conti et al. 2018; Hansel et al. 2016; Reilly et al. 2019; Rhee et al. 2019). These associations emphasize the need to further investigate the relationship between $O_3$ and the mechanisms known to increase susceptibility to chronic lung diseases.

### MECHANISMS OF $O_3$-INDUCED PULMONARY INFLAMMATION

When $O_3$ is inhaled, it targets the epithelial lung lining fluid resulting in the generation of secondary oxidation products that function as DAMPs. DAMPs produced as a result of $O_3$ exposure include oxPLs, such as 1-palmitoyl-2-glycerophosphocholine (PON-GPC) and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC) (Bromberg 2016). Recognition of oxPL by PRRs (including TLRs and SRs) promotes pulmonary inflammation and injury (Bluml et al. 2005; Erridge et al. 2008; Seimon et al. 2010) through downstream effects, which include the release of proinflammatory cytokines and chemokines (Bluml et al. 2005; Erridge et al. 2008; Seimon et al. 2010). The production of these proinflammatory mediators drives the influx of immune cells, including neutrophils, which can damage tissue and induce microvascular injury. This response can decrease the physiological function of the lung and enhance airway hyperresponsiveness.

### MECHANISMS LEADING TO THE RESOLUTION OF PULMONARY INFLAMMATION

Following tissue injury or inflammation, resolution of inflammation occurs through multiple active and dynamic processes to restore tissues to homeostasis. During resolution of inflammation, pulmonary lipid metabolism shifts from the production of pro-inflammatory lipid mediators (i.e., prostaglandins and leukotrienes) to the production of SPMs (Levy et al. 2001). SPM production drives secretion of anti-inflammatory cytokines (e.g., interleukin 10,

transforming growth factor β) and macrophage efferocytosis, which prevents secondary necrosis. If the production of SPMs does not occur, inflammation persists, contributing to chronic inflammation and disease (Basil and Levy 2016). Therefore, understanding these resolution components in the context of $O_3$ exposure offers the opportunity to investigate whether the processes that are important in the resolution of inflammation are impaired.

SPMs are a family of endogenously produced bioactive lipid mediators that include resolvins, protectins, and maresins, which are synthesized from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and lipoxins, which are synthesized from arachidonic acid. DHA is metabolized through three pathways that result in DHA-derived mediators. Through the cyclooxygenase 2 (COX-2) pathway, 17R-hydroperoxy(Hp)DHA is produced and is then metabolized by peroxidase to produce 17R-HDHA, which is then further metabolized via 5-lipoxygenase (LOX) and hydrolase to produce D-series resolvins. Through the 15-LOX pathway, DHA is metabolized to 17S-HpDHA, which leads to protectin D1 (10(R),17(S)-DiHDoHE) production (Serhan and Petasis 2011). Through the 12-LOX pathway, DHA is metabolized to 14S-HpDHA, which is further metabolized to 14S-HDHA, leading to downstream maresin production (Kuda 2017). SPMs regulate pro-resolving molecular pathways through complex signaling mechanisms via the G-protein-coupled receptors leukotriene B4 receptor 1, lipoxin A4/formyl peptide receptor 2 (lipoxin A4/FPR2), and chemokine-like receptor 1 (ChemR23) (Serhan and Chiang 2013). ChemR23 and lipoxin A4/FPR2 signaling enhances macrophage efferocytosis, decreases pro-inflammatory cytokine production, and increases anti-inflammatory cytokine production (Serhan and Chiang 2013).

### SR-BI

The SR-BI receptor has recently been reported to be critical for recognizing $O_3$-induced DAMPs and for the production of SPMs known to promote efferocytosis. SR-BI has previously been reported to bind and recognize oxPLs (Gao et al. 2010; Komai et al. 2017). Previous reports have indicated that SRs (SR-A, macrophage receptor with collagenous structure [MARCO], and CD36) can alter $O_3$-induced pulmonary inflammation (Dahl et al. 2007; Robertson et al. 2013). Recent studies have also reported that SR-BI expression is decreased in lung epithelial cells and murine lung tissue after $O_3$ exposure (Sticozzi et al. 2018). However, it is currently unknown if SR-BI expression is altered in the lung after $O_3$ exposure and if SR-BI has a role in $O_3$-induced pulmonary inflammation, the resolution of inflammation, or both.

### OVERALL HYPOTHESIS AND AIMS

We do not know the exact mechanism by which $O_3$ exposure induces pulmonary inflammation and if exposure disrupts processes important in resolving pulmonary inflammation. The central hypothesis of this study was that $O_3$-induced pulmonary health effects are the result of delayed clearance of DAMPs and the dampening of processes essential for resolving inflammation and promoting tissue homeostasis. Given that macrophage efferocytosis is known to be important in the resolution of inflammation, we decided to focus on SR-BI. This hypothesis was tested by addressing two specific aims:

1. Define the role of SR-BI in $O_3$-derived pulmonary inflammation and resolution of injury.
2. Determine if $O_3$ exposure alters pulmonary production of SPMs and processes known to promote the resolution of pulmonary inflammation and injury.

## AIM 1: ROLE OF SCAVENGER RECEPTOR BI IN $O_3$-INDUCED PULMONARY INFLAMMATION AND THE RESOLUTION OF INJURY

### INTRODUCTION

#### $O_3$ and Pattern Recognition Receptors

Short- and long-term exposure to $O_3$ has been associated with increased susceptibility or exacerbations of chronic pulmonary diseases through lung injury and inflammation. When $O_3$ is inhaled, its primary target is the epithelial lung lining fluid. This interaction results in the generation of secondary oxidation products, which function as DAMPs. Known $O_3$-induced DAMPs include oxPLs, low molecular weight hyaluronan, and oxysterols (Bromberg 2016). These DAMPs are then recognized by PRRs, such as TLRs and SRs, which promote pulmonary inflammation and injury (Bauer et al. 2011; Dahl et al. 2007; Komai et al. 2017; Roh and Sohn 2018). Adequate clearance of these DAMPs is critical in decreasing inflammation and injury and for the restoration of the lung to homeostasis.

***Scavenger Receptors.*** SRs are PRRs known to recognize and clear DAMPs, such as oxidized lipids and oxidized proteins, but some PRRs also facilitate the clearance of apoptotic cells (Brouckaert et al. 2004). Currently,

10 classes of SR have been identified (classes A–H); however, few have been examined in the context of $O_3$-induced lung inflammation and injury (PrabhuDas et al. 2017). Two class A SRs — MARCO and SR-AI/II — have been found to scavenge oxPLs and cholesterols that are generated by $O_3$ (Arredouani et al. 2007; Dahl et al. 2007). The class B SR CD36 has been shown to potentiate the inflammatory response and mediate vascular dysfunction in mice exposed to $O_3$ (Robertson et al. 2013). However, more studies are needed to understand the importance of these receptors in the context of $O_3$-induced lung inflammation.

***Scavenger Receptor BI.*** SR-BI, a class B SR, is a membrane-bound receptor that has mostly been studied in vascular biology because of its role in cholesterol ester uptake from high-density lipoprotein (HDL) (Krieger and Kozarsky 1999; Linton et al. 2017; Zhang et al. 2005). In addition to recognizing HDL, SR-BI has also been reported to bind a broad array of ligands, both endogenous (oxPLs, serum amyloid A, α-1 antitrypsin) and exogenous (pathogens, lipopolysaccharide) (Cai et al. 2005, 2012; Shen et al. 2018a). SR-BI has been shown to activate the MAPK/GULP/Rac1 pathway to mediate efferocytosis in Sertoli cells (Osada et al. 2009). Likewise, SR-BI KO peritoneal macrophages have an impaired efferocytic response in atherosclerotic lesions through suppressed activation of the Src/PI3K/Rac1 pathway and increased secretion of pro-inflammatory cytokines (Tao et al. 2015). Taken together, these data indicate that SR-BI is critical in processes known to bind DAMPs and resolve tissue inflammation and injury.

Although multiple studies have investigated the role of class A SRs in lung diseases, the role of SR-BI in the lung is understudied. In the airspace, SR-BI is expressed on AMs and alveolar epithelial cells where it mediates the uptake of HDL and vitamin E (Kolleck et al. 2000; Santander et al. 2017; Valacchi et al. 2007). Recent studies from our lab indicate that SR-BI is critical in the pulmonary host-defense response during bacterial pneumonia (Gowdy et al. 2015). However, the role of SR-BI in oxidant-induced lung diseases that generate DAMPs, such as pulmonary inflammation and injury noted after $O_3$ exposure, is beginning to be defined. Additionally, it is unknown if SR-BI is critical in resolving lung injury and inflammation. To address this, the first part of the study described in this report utilized a whole-body SR-BI KO murine strain to evaluate the role of SR-BI in $O_3$-induced pulmonary inflammation and injury. This model was used to determine if the inflammatory response is dependent on the SR-BI-mediated clearance of DAMPs and if SR-BI participates in the resolution of lung injury.

## METHODS AND STUDY DESIGN

The first part of the study used female WT and SR-BI KO mice. Mice were exposed to either $O_3$ or filtered air. In some experiments, mice were instilled with oxPLs. BALF and lung tissue were collected for the analyses.

### Animals

C57BL/6J (WT or SR-BI$^{+/+}$) and B6;129S2-Scarb1$^{tm1Kri}$/J (SR-BI-deficient or SR-BI KO) female mice, 8–13 weeks old and weighing 18–22g, were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in house. SR-BI KO breeders were maintained on a probucol-supplemented diet (Sigma, St. Lois, MO) to combat female infertility as previously described (Miettinen et al. 2001), whereas pups were fed a normal chow diet after weaning. SR-BI KO mice were backcrossed more than six generations onto C57BL/6J before use. We chose to use female mice for our experiments given that litters produced by the SR-BI KO breeders yielded more females than males. Previous experiments conducted using both littermate WT and commercial WT (C57BL/6J) controls confirmed very similar responses (Gowdy et al. 2015). Mice were killed with an intraperitoneal (i.p.) injection of a ketamine–xylazine cocktail (90 mg/kg & 10 mg/kg).

All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of East Carolina University.

### Murine In Vivo Exposures

***$O_3$ or Filtered Air.*** WT and SR-BI KO mice were placed in stainless steel wire exposure chambers inside a metal Hinners chamber and exposed either to filtered air or to 1 ppm $O_3$ for three hours. The dose of 1 ppm was chosen based on previous literature that determined that this dose in rodents is equivalent to 200 ppb exposure in humans (Hatch et al. 1994; Wiester et al. 1988). The dose and timing of dosage was chosen to reflect what is noted during an $O_3$ action day, which is defined by the Environmental Protection Agency as the ozone levels in exceedance of 75 ppb averaged over the course of 3 years. This allows comparisons of these data to other mechanistic studies evaluating how exposure drives pulmonary inflammation and injury (Dahl et al. 2007; Hollingsworth et al. 2007; Robertson et al. 2013; Tighe et al. 2011, 2018).

$O_3$ was generated in the chamber by directing 100% oxygen through an ultraviolet (UV) light generator using a Teledyne T703 $O_3$ calibrator (Teledyne API, San Diego, CA) and then mixed with the filtered air supply. Temperature

and humidity of the chamber air were monitored continuously, as was the $O_3$ concentration, with a Teledyne T400 UV light photometer (Teledyne API).

***Instillation of oxPL and BALF Analyses.*** To evaluate the role of $O_3$-induced oxPLs in pulmonary inflammation, either phosphate buffered saline (PBS), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), or oxPAPC (Hycult Biotech, Wayne, PA; 200 μg/kg) were instilled oropharyngeally (o.p.) into WT and SR-BI KO female mice, and the BALF was analyzed for total protein (see Table 1 for oxPL formula names). The BALF was analyzed for cellular differentials and total protein. The dose of oxPAPC instilled in mice was based on a previous publication (Dahl et al. 2007) and yielded a pulmonary injury response comparable to that of 1 ppm $O_3$ exposure.

### BALF Collection and Analysis

BALF was collected immediately after the mice were killed by lavaging the right lung lobes three times with three volumes of 0.9% saline solution (Braun Medical Inc., Irvine, CA). The lavage volume was based on body weight (26.25 mL/kg body weight) (Kilburg-Basnyat et al. 2018) and did not compromise the alveolar barrier integrity (Tighe et al. 2018). The resulting lavage was centrifuged (600*g*, 6 min, at 4°C).

An aliquot of the supernatant was removed and used to measure total protein using the bicinchoninic acid assay (BCA) Protein-Assay Kit (Thermo Scientific, Hercules, CA). The cell pellets were suspended in 1 mL of red blood cell ammonium chloride potassium lysis buffer, vortexed, and incubated for 1 minute. To stop the reaction, 4 mL of 1 × PBS was added. The cells were then centrifuged again at 600*g* for 6 minutes and aspirated. Then 1 mL of 1 × PBS + 10% fetal bovine serum (FBS) was added to the cells.

Total cell counts in the BALF of the right lobe were obtained by manually counting with a hemocytometer (Hausser Scientific, Horsham, PA). Each sample (120 μL) was centrifuged onto slides using a Cytospin 4 (ThermoFisher, Waltham, MA) and subsequently stained with Diff Quik solution (ThermoFisher) for differential cell counts, with at least 200 cells counted from each slide (Kilburg-Basnyat et al. 2018).

### Lung Tissue Sampling

Mice were necropsied either 6, 24, or 48 hours after exposure to $O_3$ or filtered air. The left lungs were removed, flash frozen, and stored at −80°C. The tissue was used for ribonucleic acid (RNA) isolation and Western Blot analyses. These time points were chosen because they represent the initiation (6 hr) of pulmonary inflammation (as represented by airspace neutrophil infiltration), the beginning of resolution (24 hr), and the stabilization of resolution (48 hr) post $O_3$ exposure.

### Reverse-Phase HPLC-MS/MS Measurement of oxPLs in BALF

First, 100 μL of each BALF sample was added to 1 mL of water and mixed with 1.8 mL methanol containing 3% acetic acid and 0.01% butylated hydroxytoluene (BHT). Then 5.3 μL of 0.5 μM [2H4]C16:0-platelet activating factor ([2H4]PAF) was added as an internal standard and the mixture vortexed. Then 4 mL of heptane with 0.01% BHT was added, and the sample was vortexed and centrifuged at 1,750*g* for 10 minutes. The upper phase was then removed

**Table 1.** oxPL Formula Names

| Abbreviation | Formula Name |
|---|---|
| C18 Kodia-PC | carbon 18- 5-keto-6-octendioic acid esters of 2-lyso-phosphocholine |
| oxPAPC | oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine |
| PAzPC | 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine |
| PGPC | 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine |
| PONPC | 1-palmitoyl-2-(9-oxononanayl)-phosphocholine |
| POVPC | 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine |
| SAzPC | 1-stearoyl-2-azelaoyl-sn-glycerophosphocholine |
| SGPC | 1-stearoyl-2-glutaroyl-sn-glycero-3-phosphocholine |
| SONPC | 1-stearoyl-2-(9-oxononanayl)-phosphocholine |
| SOVPC | 1-stearoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine |

by aspiration and discarded, and the lower phase washed two more times with heptane–BHT. To extract oxidized phosphatidylcholines (PC), a mixture of 4 mL of chloroform with 0.01% BHT and 1 mL of 0.7 M formic acid was added to the lower phase and vortexed well. After centrifugation, the resulting lower (chloroform) phase was collected and dried under nitrogen gas, and the sample was reconstituted in 100 μL of 85% methanol with 0.1% formic acid, sonicated, filtered through a 0.2 μm filter, and then transferred to autosample vials for reverse-phase HPLC-MS/MS.

Reverse-phase HPLC-MS/MS was carried out using Waters Acquity ultra-performance liquid chromatography coupled to AB Sciex QTRAP6500. Solvent A was 0.1% formic acid in water, solvent B was 0.1% formic acid in methanol, and the HPLC column was Waters BEH C18 1.7 μm 2.1 × 50 mm column. Initial conditions were a flow rate of 0.2 mL/min at 50% solvent A for 3 minutes, followed by a linear gradient ramp to 15% solvent A for 2 minutes; then a gradient ramp to 0% solvent A over 2 minutes along with a ramp of flow rate to 0.4 mL/min; after a 2-minute hold at these conditions, flow and solvent were returned to initial conditions and allowed to equilibrate for 1.5 minutes prior to starting the next injection. The mass spectrometer was operated in multiple reaction monitoring positive ion mode, with ion source spray voltage set at 5,500 V, temperature at 400°C. Nitrogen was used for the ion source gas 1 and gas 2 at pressures of 30 and 50 arbitrary units, respectively. For each analyte, transition reactions to the m/z 184.1 product ion at −35 eV collision energy were monitored. Precursor ions for these transitions were as follows: PGPC, m/z 610.3; SGPC, m/z 638.3; POVPC, m/z 594.3; SOVPC, m/z 622.3; PONPC, m/z 650.3; SONPC, m/z 678.3; PAzPC, m/z 666.3; SAzPC, m/z 694.3; and [2H4]PAF internal standard, m/z 528.3. Values for each analyte were calculated using the ratio of peak areas for the analyte vs [2H4]PAF times the amount of internal standard added (2.7 pmol) divided by sample volume. Example chromatographs of standards are shown in Supplemental Figure 1 in Additional Materials 1 (available on the HEI website).

### AM Efferocytosis Assay in BALF

Jurkat T cells (ATCC CRL-2899) were grown based on manufacturer's instructions, plated, and UV irradiated (Stratalinker UV Crosslinker model 1800) at 60 mJ/cm$^2$ to induce apoptosis. After irradiation, Jurkat T cells were incubated for 4 hours at 37°C with 5% $CO_2$. Apoptosis was confirmed via Annexin V (positive staining) and propidium iodide (negative staining) (Trevigen, Gaithersburg, MD) by flow cytometry (analyzed on BD Biosciences LSRII). Cells were approximately 75% apoptotic (Annexin V$^+$/PI$^-$). Then 1 × 10$^6$ cells were instilled o.p., and mice were killed and BALF harvested for cytospins 1.5 hours after dosing. The efferocytic index was calculated based on the number of AMs that phagocytized apoptotic Jurkat T cells compared with the number of AMs without apoptotic cell uptake out of a total 100 macrophages.

### RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction in Lung Tissue

RNA was isolated from lung tissue samples using the RNEasy kit (Qiagen, Venlo, Netherlands). Complementary deoxyribonucleic acids (cDNA) were generated from purified RNA using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the HT7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers were purchased from Applied Biosystems. Fold changes in expression for messenger RNA (mRNA) quantities were calculated using cycle threshold (Ct) values and the $2^{-\Delta\Delta Ct}$ method. Samples were normalized to 18S as previously described (Kilburg-Basnyat et al. 2018). See Table 2 for primer information.

**Table 2.** Primer Sequences for Mouse Cytokines and Chemokines[a]

| Taqman Primers | |
|---|---|
| Primer | Assay ID |
| Euk 18s rRNA | Hs99999901_s1 |
| IL-6 | Mm00446190_m1 |
| CXCL1 | Mm04207460_m1 |
| CXCL2 | Mm00436450_m1 |
| CCL3 | Mm00441259_g1 |
| TNF-α | Mm00443258_m1 |
| IL-1β | Mm00434228_m1 |

CCL = C-C motif chemokine ligand; CXCL = C-X-C motif chemokine ligand; Euk 18s rRNA = eukaryotic, which has ribosomal RNA size 18S; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha.

[a] Primer sequences obtained from Taqman for quantitative RT-PCR of chemokines and cytokine gene expression in whole lung homogenate.

### Western Blot Analyses in Lung Tissue

Total protein was extracted from lung tissue samples using a radio immunoprecipitation assay lysis buffer (Thermo Scientific, Rockford, IL) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA) and sodium fluoride at 1M. Lysis buffer was added to each sample according to weight (50 μL/mg) and homogenized using a bead mill 4 homogenizer (Fisherbrand, Waltham, MA). Protein concentrations were quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Then 30 μg of protein was mixed with an equal volume of 2 × sample buffer, loaded on a 12% sodium dodecyl sulfate–polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane by a transfer system (Bio-Rad Laboratories, Inc. Des Plaines, IL). The membrane was blocked with 5% BSA in Tris-buffered saline with Tween 20 and incubated with a primary anti-rabbit polyclonal SR-BI (82 kDa) antibody (1:1000) at 4°C overnight. A goat anti-rabbit lgG (H+L) secondary antibody (1:8000) was used to incubate the membrane at room temperature for 1 hour followed by incubation for 1 minute with clarity western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc. Des Plaines, IL). The blot was then imaged in a molecular imager with β-actin (42 kDa) used as an internal control. The intensity of the blot band was quantified and analyzed by Image Lab.

### Statistical Analysis

Data were pooled from three experiments to compare $O_3$ and filtered air exposure. For the oxPAPC instillation studies, data were pooled from two separate experiments. Data are expressed as the mean value ± the standard error of the mean (SEM). Due to small sample sizes ($n < 20$), data were analyzed using nonparametric one-way analysis of variance (ANOVA) (Kruskal-Wallis test) followed by comparison using a Dunn multiple comparisons test to correct for multiple comparisons using statistical hypothesis testing in GraphPad Prism 7.00 (San Diego, CA). With comparisons of two groups, an unpaired nonparametric *t* test (Mann-Whitney test) was utilized. When more than one variable was being assessed, a two-way ANOVA with a post hoc test for multiple comparisons (Sidak or Tukey) was used to determine whether or not the variables interacted with each other and to assess multiple comparisons within the different groups. For all statistical analyses, a value of $P < 0.05$ was considered significant.

## RESULTS

### Effect of $O_3$ Exposure on SR-BI Gene Expression

To elucidate the impact of $O_3$ exposure on SR-BI expression, we determined if SR-BI expression and production were altered after exposure. This was investigated by exposing WT female mice to filtered air or 1 ppm $O_3$. When compared with filtered air, SR-BI gene expression in the lung tissue of mice exposed to $O_3$ was slightly increased, although not statistically significant, at 6 hours, but then significantly decreased at 24 and 48 hours post $O_3$ exposure (Figure 1A). In contrast, when compared with filtered air, SR-BI protein levels in lung tissue of mice exposed to $O_3$ increased 4- and 5-fold at both 6 and 24 hours post $O_3$ exposure, respectively, and were still 2-fold higher at 48 hours post $O_3$ exposure (Figures 1B and C). Taken together, these data indicate that $O_3$ exposure alters SR-BI expression and production in whole lung tissue.

### Effect of SR-BI on $O_3$-Induced Neutrophilic Inflammation

Given that SR-BI expression was modulated by $O_3$ exposure, it was of interest to understand the role of this SR in $O_3$-induced pulmonary inflammation and injury. WT and SR-BI KO female mice were exposed to 1 ppm of $O_3$ as previously described and were necropsied 24 and 48 hours post exposure to assess pulmonary inflammation and injury.

When compared with WT controls, SR-BI KO mice had a significant increase in the number of neutrophils in their airspace 24 hours post $O_3$ exposure (Figure 2A). However, SR-BI KO mice had no difference in BALF protein, an indicator of alveolar–epithelial barrier permeability (Figure 2C). Additionally, SR-BI KO mice exposed to $O_3$ did not show any difference in the pulmonary expression of cytokines and chemokines known to induce neutrophil chemotaxis when compared with WT $O_3$-exposed mice (Figure 2D). To determine if these effects persisted past 24 hours, markers of pulmonary inflammation and injury were assessed 48 hours post $O_3$ exposure. Compared with WT controls, SR-BI KO mice had a slight, although not significant, increase in the number of neutrophils in their airspace (Figure 2B) and no difference in BALF protein 48 hours post exposure (Figure 2C).

### Levels of oxPL in Airspace of SR-BI KO Mice After $O_3$ Exposure

To determine if enhanced levels of oxPLs in the airspace drives the increased pulmonary inflammation in SR-BI KO mice, we utilized reverse-phase HPLC-MS/MS to measure known oxPLs in BALF. We focused on the PC oxPLs because of the abundance of PCs in the lung lining fluid (Almstrand et al. 2015). Several oxPLs including PGPC,

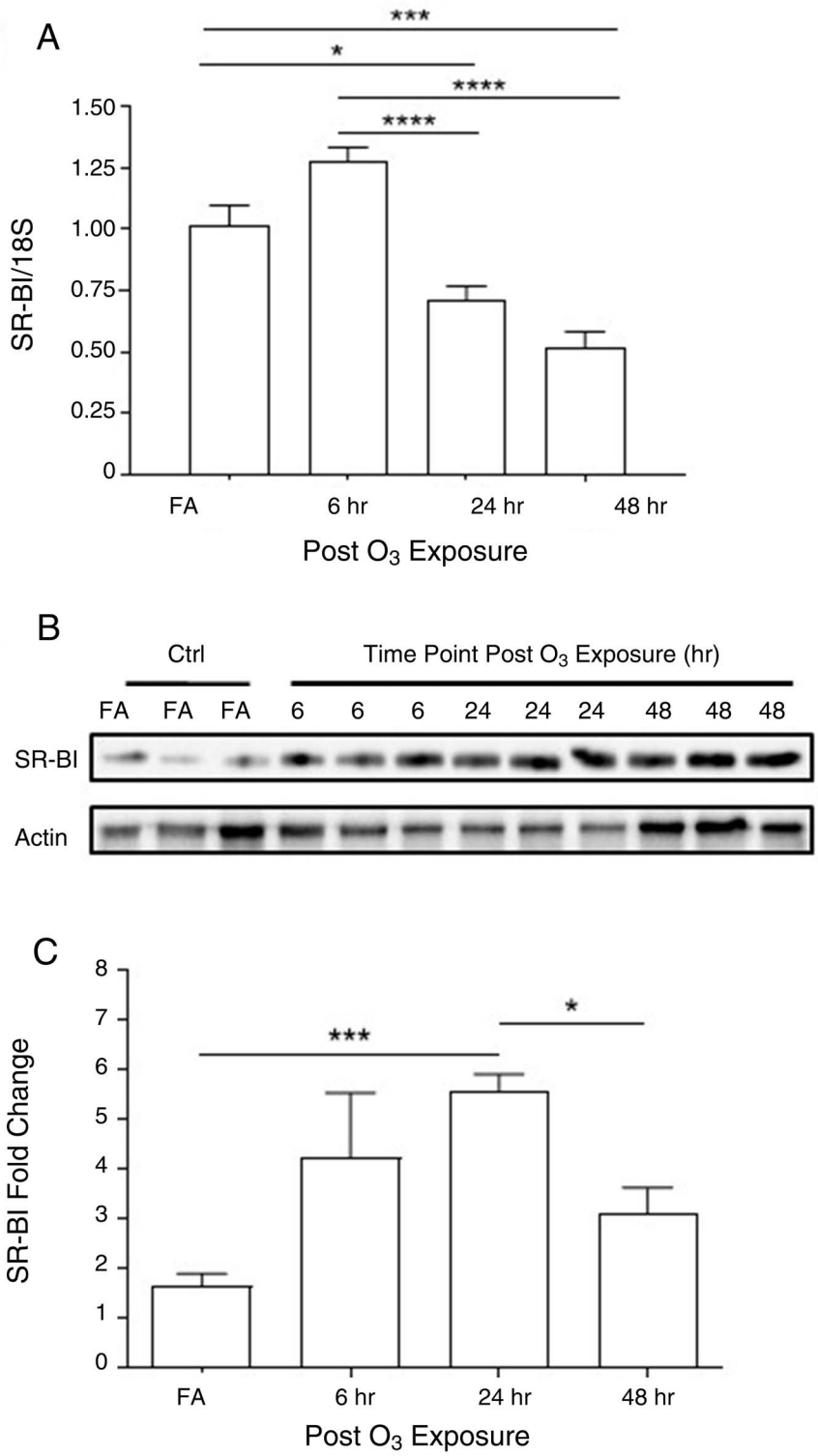


**Figure 1. SR-BI expression in lung tissue of WT female mice at different times after $O_3$ exposure. (A)** SR-BI RNA expression using quantitative RT-PCR, and **(B)** SR-BI protein expression by western blot; **(C)** quantification of densitometry from western blots. The SR-BI band was normalized to actin. For real time PCR, gene expression was normalized to 18S ribosomal RNA. $n$ = 5–8 per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. FA = filtered air.

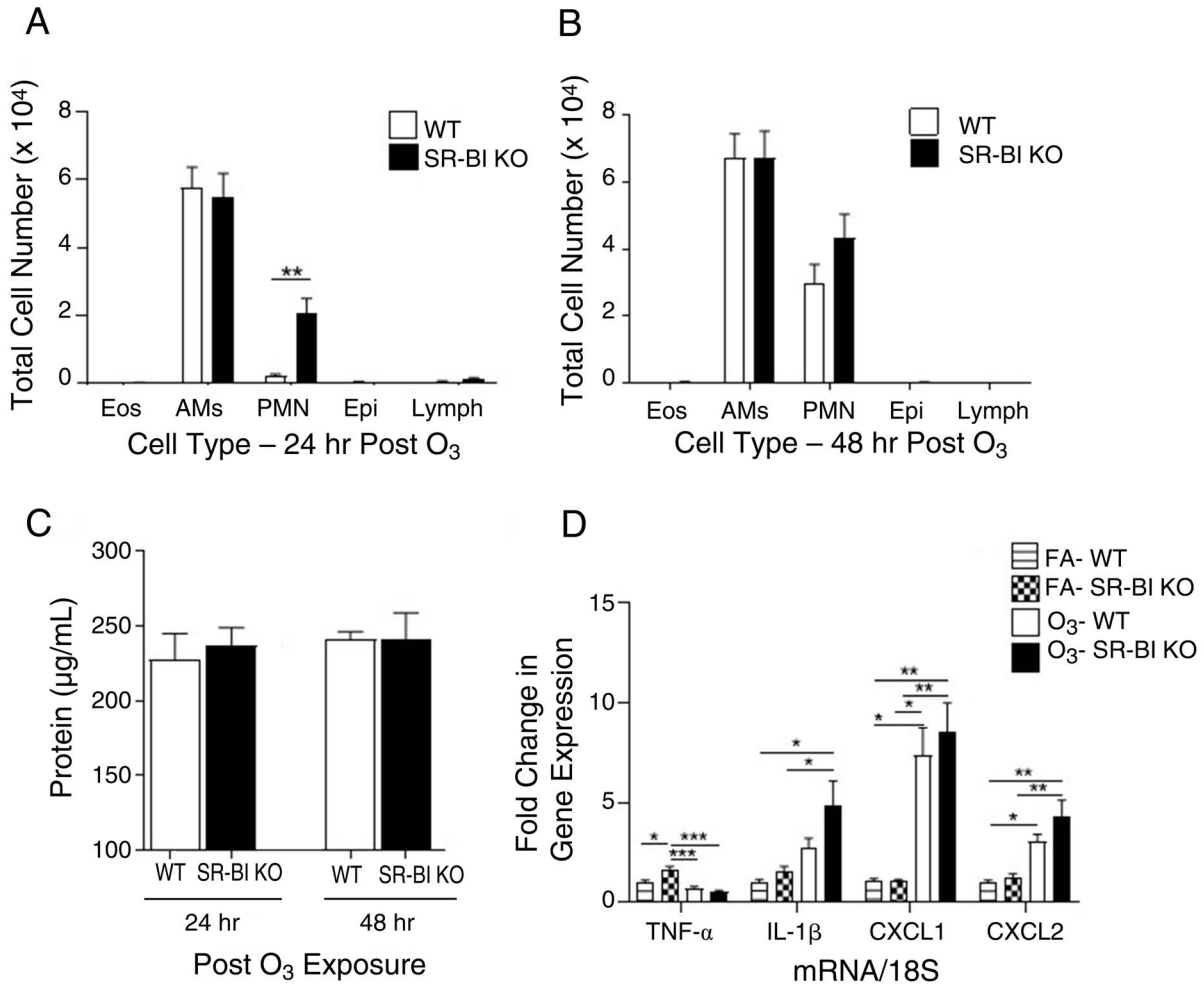


**Figure 2. Effects of SR-BI expression on markers of $O_3$-induced inflammation and injury.** Female WT and SR-BI KO mice were exposed to 1 ppm $O_3$ for 3 hours and necropsied 24 and 48 hours later. BALF was analyzed for (**A**) cell differentials at 24 hours, (**B**) cell differentials at 48 hours, (**C**) total protein at 24 and 48 hours. Lung tissue was analyzed for (**D**) fold change in gene expression of pro-inflammatory cytokines and chemokines TNF-α, IL-1β, CXCL1, and CXCL2 at 24 hours. $n$ = 6–9 per group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. AMs = alveolar macrophages; CXCL1 and CXCL2 = C-X-C motif chemokine ligands 1 and 2; Eos = eosinophils; Epi = epithelial cell; FA = filtered air; IL-1β = interleukin 1 beta; Lymph = lymphocytes; PMN = polymorphonuclear leukocytes (neutrophils); TNF-α = tumor necrosis factor alpha.

SGPC, POVPC, SOVPC, PONPC, SONPC, PAzPC, SAzPC, and C18Kodia-PC were measurable in the BALF of both WT and SRB1 KO mice (Figure 3 and Table 1; also Supplemental Figure 2, available on the HEI website in Additional Materials). At baseline, PONPC was significantly decreased in the airspace of SR-BI KO mice when compared with WT controls, whereas the other PC-derived oxPLs were not significantly different in the two sets of mice (Figure 3A). At 6 hours post $O_3$ exposure, oxPL levels were increased in the airspace of both WT and SR-BI KO mice when compared with filtered air controls (Figure 3B). These PC-derived oxPL levels in the airspace returned to baseline 24 hours post exposure (Figure 3C). After ozone exposure, oxPL concentrations were not significantly different in BALF from WT or SR-BI KO mice, (Figure 3C).

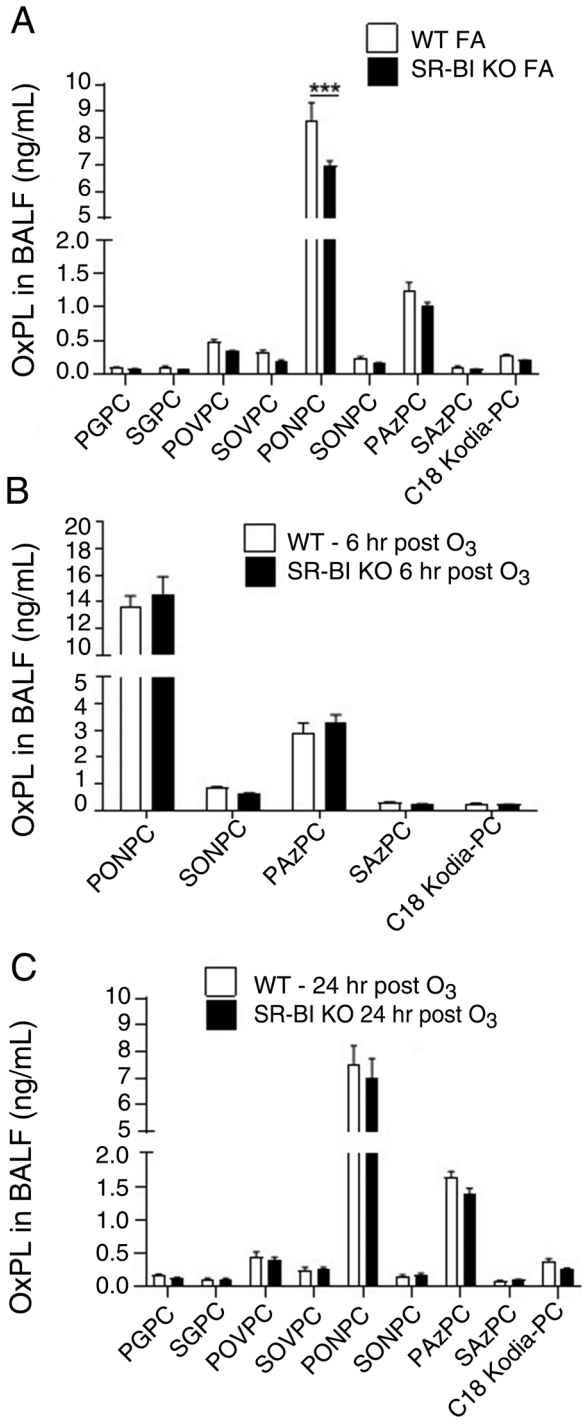


**Figure 3. OxPL levels in BALF of WT and SR-BI KO mice after $O_3$ exposure.** Female WT and SR-BI KO mice were exposed to filtered air or 1 ppm $O_3$ for 3 hours and necropsied 6 or 24 hours after exposure. BALF was analyzed for oxPLs by HPLC-MS/MS. $n = 5$ per group. **(A)** filtered air (FA); **(B)** $O_3$ at 6 hours; **(C)** $O_3$ at 24 hours. $^{***}P < 0.001$. (See Abbreviations and Other Terms at the end of this volume for the formula names spelled out.)

### Effect of SR-BI on oxPL-Induced Pulmonary Neutrophilia and Microvascular Injury

The oxPLs generated in the airspaces are known to increase pulmonary inflammation and injury (Dahl et al. 2007), but it is unclear if SR-BI alters the pulmonary inflammatory response to oxPLs. To determine the impact of oxPL on pulmonary inflammation and injury, we instilled o.p. PBS, PAPC (an unoxidized phospholipid control), or oxPAPC (an oxPL found in the airspace after $O_3$ exposure) into WT mice. At 4 hours after dosing, total BALF protein was increased only in the WT mice given oxPAPC, whereas PBS and PAPC did not induce pulmonary injury (Figure 4A). To determine whether SR-BI protects against oxPLs generated after $O_3$ exposure, we gave o.p. oxPAPC to WT and SR-BI KO mice. Four hours after dosing, SR-BI KO mice had an increase in BALF neutrophils and a nonsignificant increase in macrophages (Figure 4B) and in BALF total protein (Figure 4C) compared with WT controls.

### Effect of SR-BI Deficiency and $O_3$ Exposure on AM Efferocytosis

Recently, SR-BI was shown to bind phosphatidyl serine on dead cells and facilitate macrophage efferocytosis in atherosclerotic lesions (Tao et al. 2015). Given the enhanced pulmonary neutrophilia noted in $O_3$-exposed SR-BI KO mice, we considered that AM efferocytosis in these mice might be impaired. Thus, to evaluate AM efferocytosis, we exposed WT and SR-BI KO mice to filtered air or $O_3$, and then o.p. instilled apoptotic Jurkat T cells into them 24 hours post exposure, when neutrophilia was beginning to resolve. Animals were killed 1.5 hours after instillation. BALF cells were harvested and AM efferocytosis evaluated using the efferocytic index. Regardless of genotype, $O_3$ exposure decreased the efferocytic capabilities of AMs (Figure 5A). However, even in the absence of $O_3$ exposure, it was apparent that efferocytosis was significantly decreased in AMs isolated from the airspace of SR-BI KO mice compared with AM from WT mice (Figure 5A). Representative images of AMs engulfing apoptotic cells from each treatment group are indicated by black arrows and AMs that did not engulf apoptotic cells are designated by white arrows (Figure 5B).

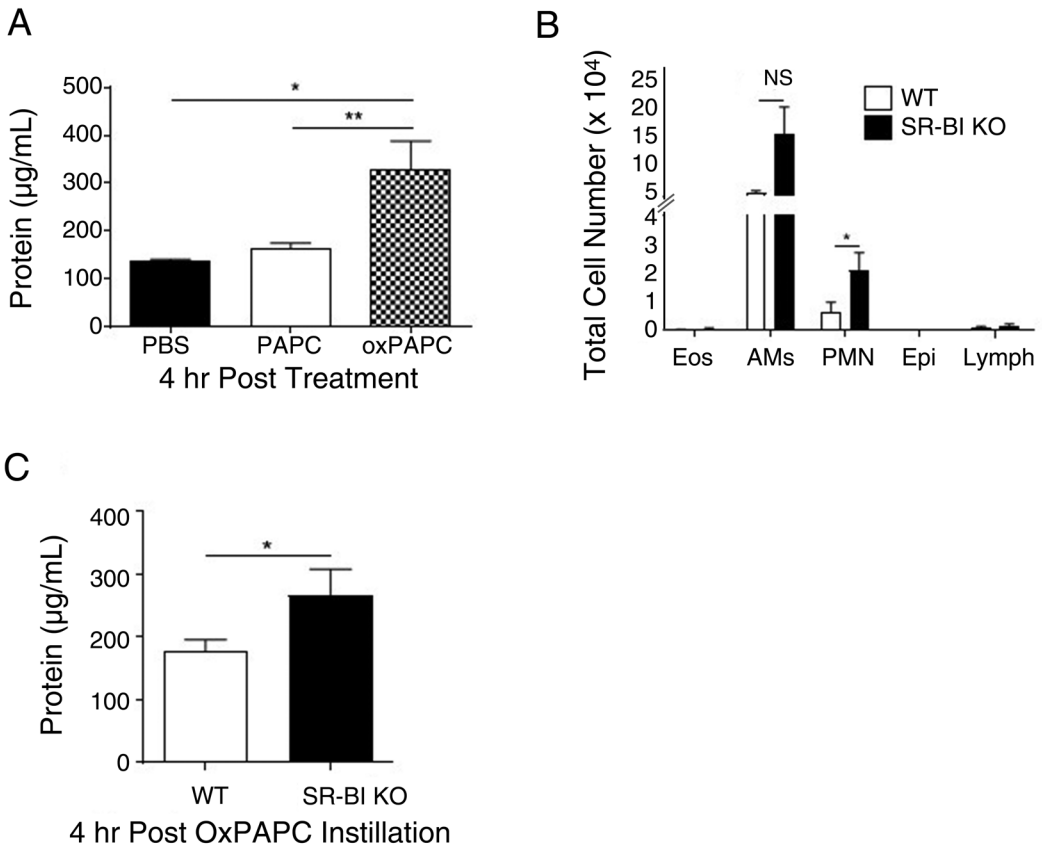


**Figure 4. Effects of instillation of oxPL or oxPAPC on pulmonary inflammation and injury in WT and SR-BI KO mice. (A)** Female WT mice were administered PBS, 200 μg/mL PAPC, or 200 μg/mL oxPAPC o.p., and BALF was analyzed for total protein 4 hours later. Female WT and SR-BI KO mice were administered 200 μg/mL oxPAPC o.p. and BALF was analyzed 4 hours later for **(B)** cellular differentials and **(C)** total protein. $n = 5$ per group. $^{*}P < 0.05$; $^{**}P < 0.01$. AM = alveolar macrophages; Eos = eosinophils; Epi = epithelial cells; Lymph = lymphocytes; NS = not significant; PMN = polymorphonuclear leukocytes (neutrophils).

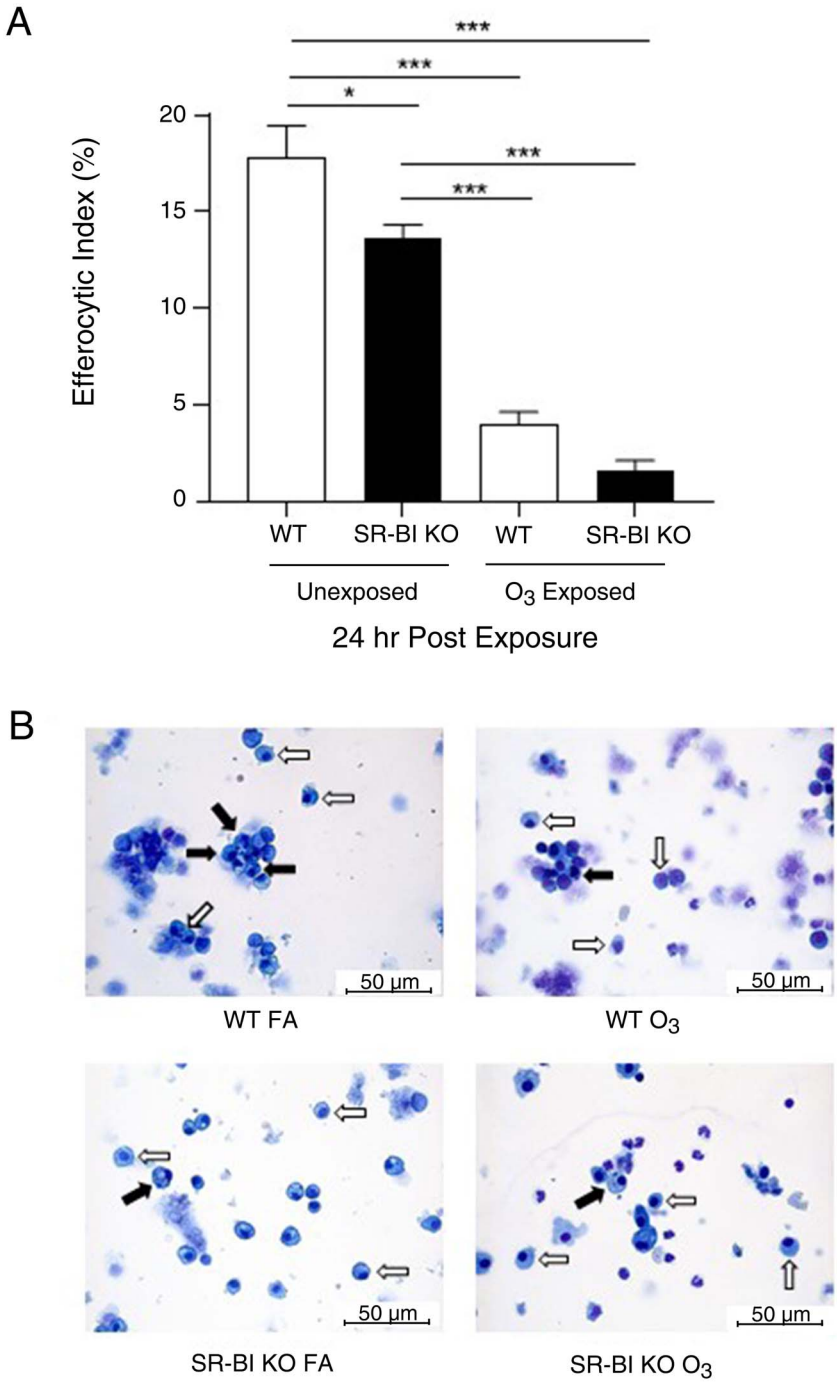


**Figure 5. Effect of SR-BI expression on AM efferocytosis in unexposed and $O_3$-exposed mice.** Female WT and SR-BI KO mice exposed to either filtered air (FA) or 1 ppm $O_3$ for 3 hours were administered apoptotic (Annexin $V^+/PI^-$) Jurkat T cells i.p. 24 hours later. Then 90 minutes after dosing, BALF was assessed for AM efferocytosis. **(A)** Efferocytic index 24 hours after FA or $O_3$ exposure in WT and SR-BI KO mice; $^*P < 0.05$; $^{***}P < 0.001$. $n = 4–7$/group. **(B)** Representative images of AM used for calculation of efferocytic index. Black arrows show alveolar macrophages that have engulfed apoptotic cells and white arrows represent macrophages that did not.

## DISCUSSION

Increases in ambient levels of $O_3$ have been associated with increased susceptibility to and exacerbations of chronic pulmonary diseases. These detrimental pulmonary consequences are thought to occur as a result of enhanced lung inflammation and injury (Chen et al. 2015). In the first part of the study, we found that the pathogen recognition receptor SR-BI is protective against $O_3$-induced pulmonary inflammation by dampening airspace neutrophilia and maintaining effective AM macrophage efferocytosis. Additionally, SR-BI KO mice had augmented pulmonary inflammation and injury when dosed with oxPAPC, an $O_3$-induced DAMP, indicating that this PRR may protect the lung from DAMP-induced inflammation. Collectively, these data indicate a protective role for SR-BI in $O_3$-induced pulmonary inflammation and the resolution of this response.

We also observed that acute $O_3$ exposure modulates pulmonary SR-BI expression and production. Our data add to a growing body of literature indicating that $O_3$ can modulate SR-BI expression, although the mechanisms by which this may occur are not completely understood (Sticozzi et al. 2018; Valacchi et al. 2007). $O_3$-induced alterations in SR-BI expression and production could reflect differential regulation of transcription factors known to drive SR-BI expression (Cao et al. 1997; Liu et al. 2009; Shen et al. 2018a; Speen et al. 2016). $O_3$ is known to inactivate LXR, a transcription factor that regulates SR-BI expression (Bromberg 2016; Shen et al. 2018a, 2018b; Speen et al. 2016). Furthermore, increased pulmonary SR-BI production may be in response to $O_3$-induced DAMPs known to be SR-BI ligands, including high-mobility group box 1 protein (HMGB1) and oxPLs (Banerjee et al. 2011; Bromberg 2016; Cao et al. 1997; Gillotte-Taylor et al. 2001; Lockett et al. 2015). Lastly, $O_3$ exposure may increase the cholesterol demand of the lung, thus requiring increased cholesterol trafficking and SR-BI expression and production (Dai et al. 2012; Fessler 2017). These data suggest an important role of SR-BI in mitigating the $O_3$-induced pulmonary inflammatory response.

Although $O_3$ produces DAMPs such as oxPL that induce pulmonary inflammation and injury (Bauer et al. 2011; Bromberg 2016; Dahl et al. 2007), we found that SR-BI deficiency did not alter the amount of oxPLs in the airspace after $O_3$ exposure. This suggests that SR-BI is not involved in the generation or clearance of PC-derived oxPLs in the airspace after $O_3$ exposure.

We did find, however, that SR-BI KO mice instilled with oxPAPC showed significantly increased lung injury and airspace neutrophilia compared with WT mice, supporting our hypothesis that SR-BI is essential in regulating the pulmonary immune response to oxPLs. It is known that oxPLs are ligands for SR-BI, TLR2, and TLR4, and that they produce a pulmonary inflammatory response via NF-κB signaling (nuclear factor kappa-light-chain-enhancer of activated B cells) (Imai et al. 2008; Kadl et al. 2011). However, we speculate that the increased injury and inflammation in SR-BI KO mice may be the result of several factors: the first factor being diminished cellular uptake pathways, which may temper subsequent TLR signaling. Although not characterized with SR-BI, class A SRs are known to facilitate clearance of oxidized lipids and cholesterol (Dahl et al. 2007). The second factor may be decreased suppressive effects of HDL in the lung. SR-BI KO mice are known to have altered lipid profiles, and their HDL does not produce nearly as many antioxidants as that of WT mice, which results in increased oxidative stress in SR-BI KO mice (Rigotti et al. 1997; Van Eck et al. 2007). It would be of interest to confirm whether or not these factors play a role in the protective effects of SR-BI in DAMP-induced pulmonary inflammation and injury.

Several studies led us to the hypothesis that the enhanced airspace neutrophilia noted in SR-BI KO mice after $O_3$ exposure may reflect a defect in AM efferocytosis. In a mouse model of diet-induced atherosclerosis, SR-BI KO macrophages in atherosclerotic lesions have less apoptotic cell uptake (Tao et al. 2015).

We elucidated the role of SR-BI in mediating AM efferocytosis, regardless of exposure. Our results support the body of literature that indicates that lack of expression of SR-BI is associated with a defect in AM efferocytosis. We found, surprisingly, that $O_3$ exposure suppressed AM efferocytosis, which has not been previously reported. However, $O_3$ exposure has been reported to decrease AM phagocytosis of pathogens (Gilmour et al. 1991; Mikerov et al. 2008). Phagocytosis is an essential macrophage function that provides protection in the host defense against pathogens and promotes the production of cytokines and chemokines important in recruiting additional immune cells to the site of infection (Ren et al. 2017). Unlike phagocytosis, efferocytosis in macrophages results in the production of anti-inflammatory cytokines (including interleukin 10 and transforming growth factor β) and the restoration of tissue homeostasis (Gheibi Hayat et al. 2019). Additionally, impaired efferocytosis has been linked to the progression of multiple chronic lung diseases, including asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (McCubbrey and Curtis 2013).

Our data indicate that SR-BI is critical in AM efferocytosis, potentially promoting prolonged pulmonary inflammation and decreased resolution of inflammation after ozone exposure. We therefore decided to pursue an avenue of research to evaluate how acute $O_3$ exposure might alter processes important in the resolution of lung injury. This line of research is described next in Aim 2.

## AIM 2: ROLE OF $O_3$ IN THE RESOLUTION OF LUNG INFLAMMATION

### INTRODUCTION

#### Mechanisms of Resolving Pulmonary Inflammation

The lungs are constantly exposed to environmental insults, including air particulates, viruses, bacteria, and oxidant gases that trigger pulmonary inflammation (Puttur et al. 2019). These insults can compromise gas exchange and induce irreversible tissue injury (Bhattacharya and Westphalen 2016). After tissue inflammation, resolution processes must take place to mitigate further insult to tissue and promote tissue homeostasis. The resolution of inflammation is an active, dynamic process mediated by tissue, cellular, and temporally specific signaling mediators. One cellular process that is crucial to returning tissue homeostasis is macrophage efferocytosis. Efferocytosis is an anti-inflammatory process whereby macrophages clear out apoptotic cells and debris as well as produce multiple anti-inflammatory mediators (Angsana et al. 2016; Ariel and Ravichandran 2016). Recently, the progression of chronic lung diseases has been associated with defects in efferocytosis (Angsana et al. 2016; Grabiec et al. 2017; Hamon et al. 2014; Kim et al. 2018; Vandivier et al. 2009).

In addition to cellular events driving resolution processes, the production of soluble mediators is crucial to returning tissue to homeostasis. During resolution of inflammation, pulmonary lipid metabolism shifts from the production of proinflammatory lipid mediators to the production of SPMs (Levy et al. 2001). If this shift does not occur, inflammation persists and can contribute to inflammatory and chronic lung diseases (Basil and Levy 2016).

***Specialized Pro-Resolving Mediators.*** SPMs are a family of endogenously produced bioactive lipid mediators that include resolvins, protectins, and maresins, which are synthesized from EPA and DHA, and lipoxins, which are synthesized from arachidonic acid. DHA-supplemented diets have been shown to increase SPM precursors 14-HDHA, 17-HDHA, and the SPM protectin D1 systemically (Levy and Serhan 2014). SPMs regulate pro-resolving molecular pathways through complex signaling mechanisms, including the G-protein-coupled receptors leukotriene B4 receptor 1, lipoxin A4/FPR2, and ChemR23. ChemR23 and lipoxin A4/FPR2 signaling enhances macrophage efferocytosis, decreases pro-inflammatory cytokine production, and increases anti-inflammatory cytokine production (Serhan and Chiang 2013).

***SPMs and Lung Disease.*** Many SPMs and SPM precursors have also been shown to aid in the resolution of lung disease. In rodent models of allergic airway inflammation or asthma, administration of SPMs — including lipoxin A4, lipoxin B4, resolvin E1, resolvin D1, and protectin D1 — has been reported to mitigate pulmonary inflammation and airway hyperresponsiveness (Angsana et al. 2016; Grabiec et al. 2017; Hamon et al. 2014; Kim et al. 2018; Levy and Serhan 2014; Vandivier et al. 2009). Additionally, the SPM resolvin D1 has been shown to prevent airspace enlargement and reduce macrophage and neutrophil infiltration during chronic cigarette smoke exposure (Croasdell et al. 2015; Hsiao et al. 2013). Similarly, dietary administration of DHA that increases tissue levels of the D resolvins has been shown to reduce neutrophilic infiltration, pro-inflammatory cytokines, and inflammation in mice intranasally challenged with dust from a commercial swine operation (Nordgren et al. 2014). Together, these studies reveal a crucial role for SPMs in mitigating pulmonary disease progression. However, to our knowledge, no studies have examined the role of SPMs in the pulmonary inflammatory and injury response after $O_3$ exposure.

#### Summary and Objective of Study

$O_3$ exposure causes pulmonary inflammation and injury and impairs AM phagocytosis, but how exposure alters resolution processes and the restoration of tissue homeostasis remain poorly defined. SPMs and AM efferocytosis are known to promote resolution of pulmonary inflammation in models such as cigarette-induced pulmonary inflammation, allergic asthma, and pneumonia (Aoki et al. 2008; Croasdell et al. 2015; Seki et al. 2010). However, the role of SPMs and AM efferocytosis in $O_3$-induced inflammatory and resolution responses is not known. Given our data that acute $O_3$ exposure impairs AM efferocytosis (Figure 5), we tested the hypothesis that the resolution of $O_3$-induced pulmonary inflammation is driven by a decrease in SPM production leading to diminished AM efferocytosis and prolonged inflammation.

### METHODS AND STUDY DESIGN

The second part of the study used male WT mice. Mice were exposed to either $O_3$ or filtered air. To measure the efferocytic index, some mice were given apoptotic Jurkat T cells after $O_3$ or filtered air exposure; in some experiments, mice were given SPMs before exposure. BALF, lung tissue, and blood were collected for analyses.

### Animals

C57BL/6J (WT or SR-BI$^{+/+}$) male mice, 8–12 weeks old and weighing 20–25g, were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice were chosen for these experiments to enable comparisons of the findings of this study to the findings of other mechanistic studies — primarily performed in male mice — investigating how $O_3$ induces pulmonary inflammation (Dahl et al. 2007; Hollingsworth et al. 2007; Robertson et al. 2013; Tighe et al. 2011, 2018). Mice were killed with an i.p. injection of a ketamine–xylazine cocktail (90 mg/kg & 10 mg/kg).

All experiments were performed in accordance with the Animal Welfare Act and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of East Carolina University.

### Murine In Vivo Exposures

WT male mice were placed in stainless steel wire exposure chambers inside a metal Hinners chamber and exposed either to filtered air or 1 ppm $O_3$ for 3 hours. The dose of 1 ppm was chosen based on previous literature that determined that this dose in rodents is equivalent to 200 ppb exposure in humans (Hatch et al. 1994; Wiester et al. 1988). The dose and timing of dosage was chosen to reflect what is noted during an $O_3$ action day, which is defined by the Environmental Protection Agency as the ozone levels in exceedance of 75 ppb averaged over the course of 3 years. This allows comparisons of these data to other mechanistic studies evaluating how exposure drives pulmonary inflammation and injury (Dahl et al. 2007; Hollingsworth et al. 2007; Robertson et al. 2013; Tighe et al. 2011, 2018).

$O_3$ was generated in the chamber by directing 100% oxygen through a UV light generator using a Teledyne T703 $O_3$ calibrator (Teledyne API, San Diego, CA) and then mixed with the filtered air supply. Temperature and humidity of the chamber air were monitored continuously, as was the $O_3$ concentration, with a Teledyne T400 UV light photometer (Teledyne API).

### BALF Collection and Analysis

BALF was collected immediately after the mice were killed by lavaging the right lung lobes 3 times with 3 volumes of 0.9% saline solution (Braun Medical Inc., Irvine, CA). The lavage volume was based on body weight (26.25mL/kg body weight) (Kilburg-Basnyat et al. 2018) and did not compromise the alveolar barrier integrity (Tighe et al. 2018). The resulting lavage was centrifuged (600*g*, 6 min, at 4°C).

An aliquot of the supernatant was removed and used to measure total protein using the BCA Protein-Assay Kit (Thermo Scientific, Hercules, CA). The cell pellets were suspended in 1 mL of red blood cell ammonium chloride potassium lysis buffer, vortexed, and incubated for 1 minute. To stop the reaction, 4 mL of 1 × PBS was added. The cells were then centrifuged again at 600*g* for 6 minutes and aspirated. Then 1 mL of 1 × PBS + 10% FBS was added to the cells.

Total cell counts in the BALF of the right lobe were obtained by manually counting with a hemocytometer (Hausser Scientific, Horsham, PA). Each sample (120μL) was centrifuged onto slides using a Cytospin 4 (ThermoFisher, Waltham, MA) and subsequently stained with Diff Quik solution (ThermoFisher) for differential cell counts, with at least 200 cells counted from each slide (Kilburg-Basnyat et al. 2018).

### AM Efferocytosis Assay in BALF

Jurkat T cells (ATCC CRL-2899) were grown based on manufacturer's instructions, plated, and UV irradiated (Stratalinker UV Crosslinker model 1800) at 60 mJ/cm$^2$ to induce apoptosis. After irradiation, Jurkat T cells were incubated for 4 hours at 37°C with 5% $CO_2$. Apoptosis was confirmed via Annexin V (positive staining) and propidium iodide (negative staining) (Trevigen, Gaithersburg, MD) by flow cytometry (analyzed on BD Biosciences LSRII). Cells were approximately 75% apoptotic (Annexin V$^+$/PI$^-$). Then 1 × 10$^6$ cells were instilled o.p., and mice were killed and BALF harvested for cytospins 1.5 hours after dosing. The efferocytic index was calculated based on the number of AMs that phagocytosed apoptotic Jurkat T cells compared with the number of AMs without apoptotic cell uptake out of a total 100 macrophages.

### Lung Tissue Sampling and Lipid Mediator Sample Preparation

Mice were necropsied 6, 24, or 48 hours after exposure to $O_3$ or filtered air. Following removal, the left lungs were flash frozen and stored at −80°C until they were able to be shipped on dry ice for lipid mediator analysis. Each whole lung was placed in a pre-chilled 2 mL Qiagen tube with a stainless-steel bead and 500 μL of pre-chilled methanol. Each lung was then homogenized in its respective tube at 50 hertz for 10 minutes before centrifuging at 4°C at 18,000 × *g* for 10 minutes. The supernatant was then removed and the samples pre-treated for solid phase extraction (SPE). The homogenate was subsequently pretreated. Pretreatment included tissue homogenate equivalent to 500 μg of protein brought up to a volume of 1 mL in 10% methanol along with 10 μL of 10 pg/μL internal

standard solution (resolvin D2-d5, LTB4-d4, and 9(S)-HODE-d4 in ethanol) (Armstrong et al. 2020). The homogenate was then analyzed for SPE as previously described (Armstrong et al. 2020). See Table 3 for information on standards used.

Lipid mediators in the pretreated lung tissue homogenate were isolated by SPE as previously described (Deems et al. 2007) using Strata-X 33 μm 30 mg/1 mL SPE columns (Phenomenex, Torrance, CA) on a Biotage positive pressure SPE manifold. Methanol (1 mL) and internal standard solution (10 μL) were added, the sample vortexed and stored overnight at −20°C.

### Reverse-Phase HPLC-MS/MS Measurement of SPMs in Lung Tissue

All standards and internal standards used for reverse-phase HPLC-MS/MS were purchased from Cayman Chemicals (Ann Arbor, MI, USA). All HPLC solvents and extraction solvents were HPLC grade or better. Quantitation of lipid mediators was performed using reverse-phase HPLC-MS/MS as previously described (Armstrong et al. 2020). Briefly, the HPLC system consisted of an Agilent 1260 autosampler (Agilent Technologies, Santa Clara, CA), an Agilent 1260 binary loading pump (pump 1), an Agilent 1260 binary analytical pump (pump 2), and a 6-port switching valve.

Reverse-phase HPLC-MS/MS was performed on an Agilent 6490 triple quadrupole mass spectrometer in negative ionization mode. Data for lipid mediators were acquired in dynamic MRM mode using experimentally optimized collision energies obtained by flow injection analysis of authentic standards. Calibration standards for each lipid mediator were analyzed over a range of concentrations from 0.25 to 250 pg on column. Calibration curves for each lipid mediator were constructed using Agilent MassHunter quantitative analysis software. Samples of lung tissue homogenate were quantitated using the calibration curves to obtain the column concentration, followed by multiplication of the results by the appropriate dilution factor to obtain the concentration in picograms per microgram of protein (tissue homogenates) as previously described (Armstrong et al. 2020).

### RNA Isolation and Quantitative RT-PCR in Lung Tissue

RNA was isolated from lung tissue samples using the RNEasy kit (Qiagen, Venlo, Netherlands). cDNA were generated from purified RNA using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Quantitative RT-PCR was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the HT7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers were purchased from Applied Biosystems. Fold changes in expression for mRNA quantities were calculated using Ct values and the $2^{-\Delta\Delta Ct}$ method. Samples were normalized to 18S as previously described (Kilburg-Basnyat et al. 2018). See Table 2 for primer information.

### Evaluation of SPM Supplementation in Lung Tissue

The 14(S)-HDHA, 17(R)-HDHA, and 10,17-DiHDoHE were purchased diluted in ethanol from Cayman Chemicals (Ann Arbor, MI). Each was then diluted to a concentration of 1,000 ng in PBS for each i.p. injection. This dose of

**Table 3.** Multiple Reaction Monitoring Parameters, Retention Times, and Associated Internal Standards for Lipid Mediators by Reverse-Phase HPLC-MS/MS[a]

| Compound | MRM | CE | RT | Internal standard |
|---|---|---|---|---|
| Resolvin D2-d5 (IS) | 380.2 ≥ 141.0 | 13 | 9.30 | NA |
| Resolvin D2 | 375.2 ≥ 141.0 | 13 | 9.35 | Resolvin D2-d5 |
| Resolvin D1 | 375.2 ≥ 141.0 | 13 | 10.13 | Resolvin D2-d5 |
| 10,17-DiHDoHE | 359.2 ≥ 153.0 | 13 | 12.95 | LTB4-d4 |
| 7S Maresin-1 | 359.2 ≥ 341.2 | 9 | 12.95 | LTB4-d4 |
| 7R Maresin-1 | 359.2 ≥ 250.0 | 17 | 13.02 | LTB4-d4 |
| 17(S)-HDHA | 343.2 ≥ 281.2 | 9 | 17.40 | 9(S)-HODE-d4 |
| 14(S)-HDHA | 343.2 ≥ 205.0 | 9 | 17.73 | 9(S)-HODE-d4 |

CE = collision energy; IS = internal standard; HPLC-MS/MS = high performance liquid chromatography with tandem mass spectrometry; MRM = multiple reaction monitoring; NA = not applicable; RT = retention time.

[a] Protectin D1 could not be quantified because the standard was not available.

SPMs–SPM precursors was used in other studies and shown to increase SPM levels in lung tissue during an influenza infection (Kosaraju et al. 2017). Each SPM injection was given separately to each mouse, with a total of three i.p. injections per mouse. Control mice were given the vehicle (PBS comparably diluted in ethanol) without SPM. Stringent precautions were taken to prevent oxidation of the SPMs, including nitrogen purging of samples and storage in amber vials at −80°C (Kosaraju et al. 2017).

### Evaluation of Systemic Inflammation in Blood

Immediately after the mice were killed, blood was collected from the right ventricle with a 25-gauge needle into an EDTA tube. Complete blood counts (CBCs) were determined by a Coulter LH 755 hematology analyzer, and differentials were determined with blood smears.

### Statistical Analysis

Data were pooled from two initial exposure experiments to compare $O_3$ and filtered air exposure. For the SPM and vehicle i.p. injections prior to $O_3$ or filtered air exposure, data were pooled from three separate exposure experiments. Data are expressed as mean ± SEM. Due to small sample sizes ($n < 20$), data were analyzed using nonparametric one-way ANOVA (Kruskal-Wallis test) followed by comparison using a Dunn multiple comparisons test to correct for multiple comparisons using statistical hypothesis testing in GraphPad Prism 7.00 (San Diego, CA). Where deemed appropriate, unpaired nonparametric *t* tests (Mann-Whitney tests) were utilized to analyze data. When more than one variable was being assessed, a two-way ANOVA with a post hoc test for multiple comparisons (Sidak or Tukey) was used to determine whether or not the variables interacted with each other and to assess multiple comparisons within the different groups. For all statistical analyses, a value of $P < 0.05$ was considered significant.

## RESULTS

### AM Efferocytosis at 24 and 48 Hours Post $O_3$ Exposure

$O_3$ exposure has previously been reported to induce pulmonary inflammation as well as to decrease AM phagocytosis (Arsalane et al. 1995; Hotchkiss et al. 2009; Pendino et al. 1995). While evaluating the role of SR-BI in $O_3$-induced pulmonary inflammation, we noted that AM efferocytosis was decreased 24 hours after exposure to $O_3$. (Figure 5). To further explore this phenomenon, WT male mice were exposed for 3 hours to 1 ppm $O_3$, and efferocytosis was measured at 24, 48, and 72 hours later (Figure 6). The time points were chosen to evaluate time points where pulmonary inflammation, as measured by airspace neutrophilia, had resolved. $O_3$ exposure significantly decreased the efferocytic index 24 and 48 hours after exposure, but not at 72 hours post exposure.

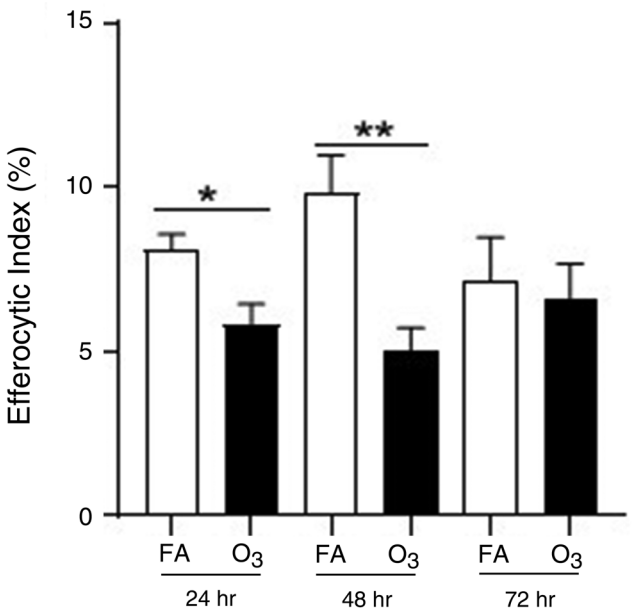


**Figure 6. AM efferocytosis at 24, 48, and 72 hours after $O_3$ exposure.** Male WT mice exposed to either filtered air (FA) or $O_3$, were given apoptotic (Annexin $V^+/PI^-$) Jurkat T cells o.p. 24, 48, or 72 hours after exposure. BALF was assessed for AM efferocytosis 90 mins after dosing. *$P < 0.05$; **$P < 0.01$; $n = 8–11$ per group.

### SPM Production in the Lung (After $O_3$ Exposure)

Given the decrease in AM efferocytosis noted after $O_3$ exposure, it was of interest to determine the molecular mechanism that drives this. Increased inflammation and decreased macrophage efferocytosis have been associated with decreased SPM production (Cai et al. 2016; Duvall et al. 2017), but it is unclear if $O_3$ exposure alters SPM production or levels. To address this, SPMs and their precursors were measured in lung tissue after exposure to $O_3$ or filtered air using reverse-phase HPLC-MS/MS. SPMs, including resolvin D1, resolvin D2, 7S maresin-1, lipoxin A4, and 7R maresin-1, were below the limit of detection in the lung tissue of filtered-air- and $O_3$-exposed mice. However, several DHA-derived lipid mediators were measurable, including 14-HDHA, 17-HDHA, and 10,17-DiHDoHE at 6, 24, 48, and 72 hours after $O_3$ exposure. At 6 hours post $O_3$ exposure, 14-HDHA, 17-HDHA, and 10,17-DiHDoHE were significantly increased in lung tissue (Figure 7A). At 24 hours, 14-HDHA and 10,17-DiHDoHE concentrations were significantly decreased in $O_3$-exposed mice compared to controls, and 17-HDHA levels trended downward but did

not reach statistical significance ($P = 0.09$; Figure 7B). There were no significant differences in 14-HDHA, 17-HDHA, or 10,17-DiHDoHE levels in $O_3$-exposed or control mice at 48 hours (Figure 7C) and 72 hours (Figure 7D) post exposure.

### Effect of SPM Supplementation Prior to $O_3$ Exposure on Pulmonary 14-HDHA, 17-HDHA, and 10,17-DiHDoHE Levels

To determine if SPM levels decreased by $O_3$ exposure could be restored in the lungs, mice were supplemented 1 hour prior to filtered air or $O_3$ exposure with 14-HDHA, 17-HDHA, and 10,17-DiHDoHE, for a total of three injections per mouse. Resolvin D1, resolvin D2, and lipoxin A4 were below the level of detection in the lungs of all treatment groups. In supplemented filtered-air-exposed mice, there were no changes in pulmonary SPM production. In contrast, SPM supplementation increased 14-HDHA, 17-HDHA, and 10,17-DiHDoHE levels in $O_3$-treated mice (Figure 8). Interestingly, 7R maresin-1 was not detectable in the lungs of any treated group, except for $O_3$+SPM-treated mice (Figure 8). This suggests that 7R maresin-1 was produced only in response to SPM supplementation after $O_3$ exposure.

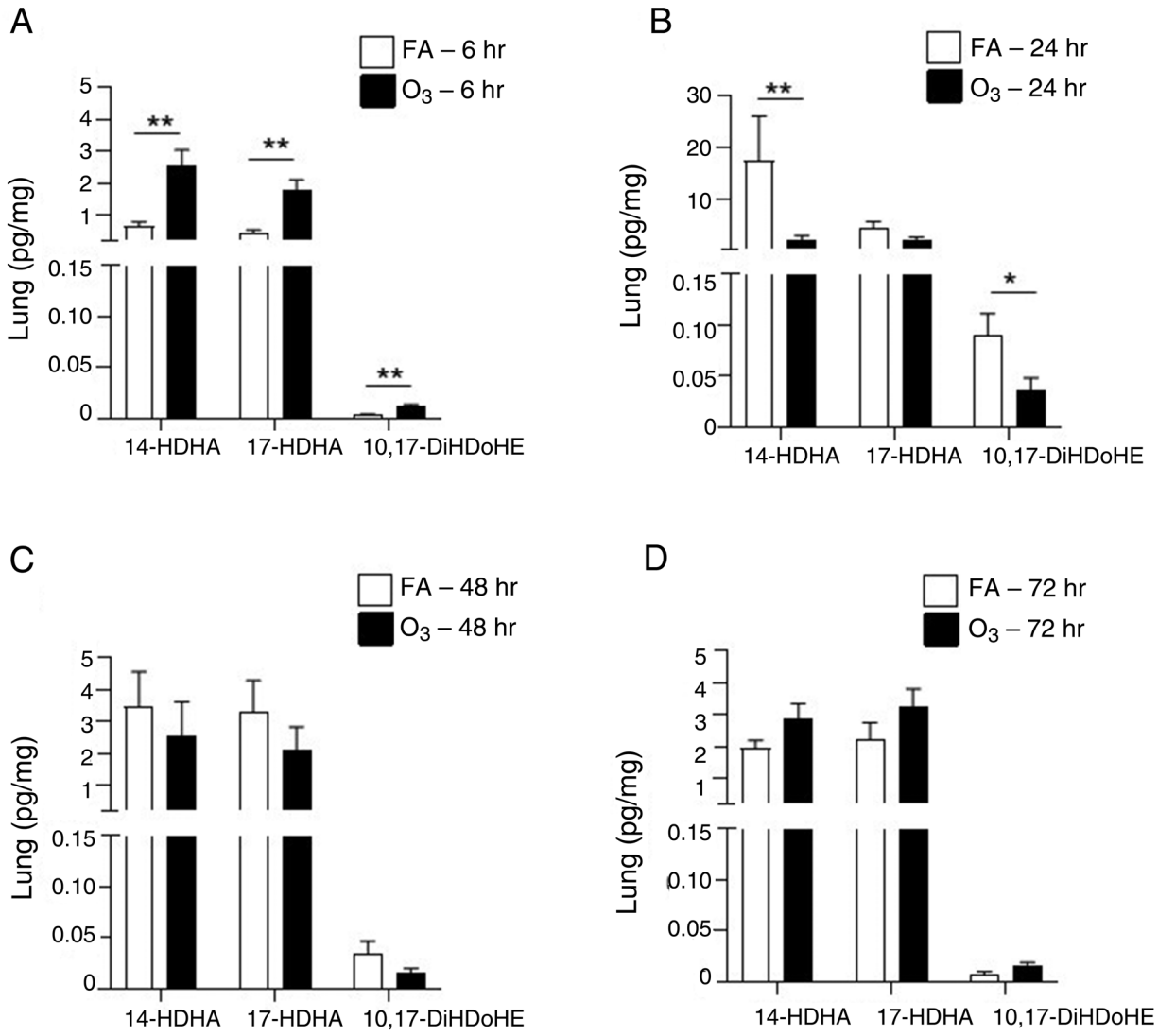


**Figure 7. SPM levels in the lung after $O_3$ exposure.** Male WT mice were exposed to filtered air or 1 ppm $O_3$ for 3 hours and necropsied **(A)** 6 hours, **(B)** 24 hours, **(C)** 48 hours, or **(D)** 72 hours later for whole lung homogenate levels of the SPMs 14-HDHA, 17-HDHA, and 10,17-DiHDoHE. $*P < 0.05$; $**P < 0.01$; $n = 6$ per group.

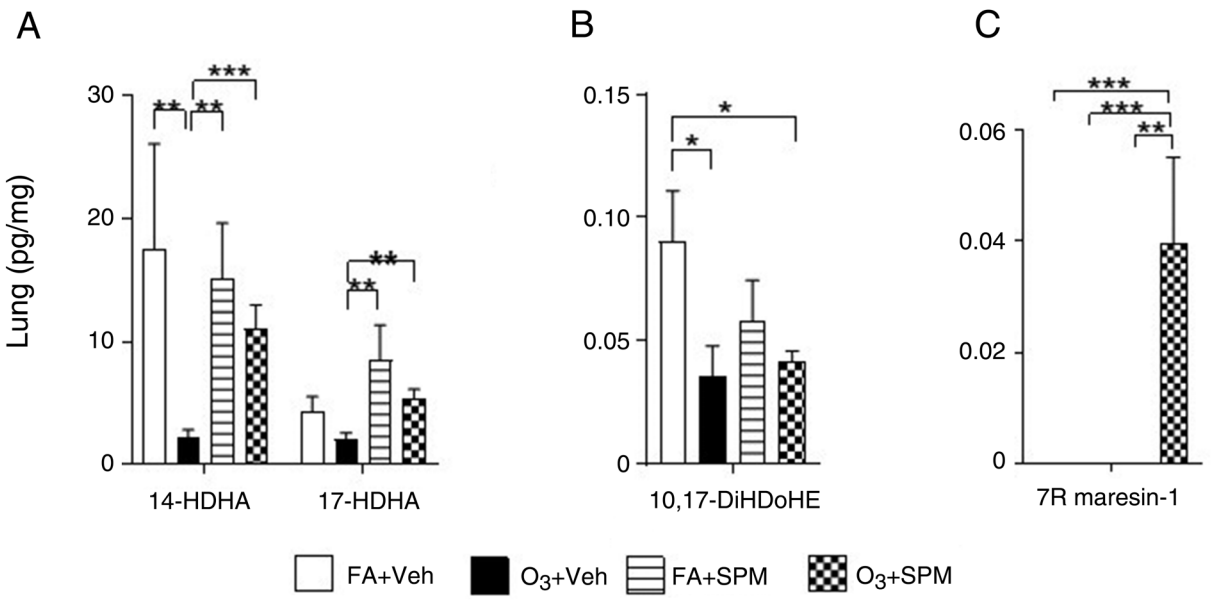


**Figure 8. Effect of SPM supplementation before $O_3$ exposure on pulmonary 14-HDHA, 17-HDHA, 10,17-DiHDoHE, and 7R maresin-1 levels.** Male mice were given 14-HDHA, 17-HDHA, and 10,17-DiHDoHE in three separate injections, 1 hour before FA or $O_3$ exposure. Lung tissue was harvested for SPM analysis via HPLC-MS/MS 24 hours after exposure. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 5$–6 per group. Veh = vehicle.

### Effect of SPM Supplementation Prior to $O_3$ Exposure on Pulmonary Inflammation and Markers of Injury

Previous studies demonstrated that SPM supplementation decreased pulmonary inflammation and restored immune cell function in models of allergic asthma and bacterial pneumonia (Haworth et al. 2008; Ishizuka et al. 2008; Seki et al. 2010). To determine if SPM supplementation could prevent $O_3$-induced pulmonary and systemic inflammation, mice were supplemented with SPMs and exposed to filtered air or $O_3$. Cellular inflammation, injury, and cytokine production were measured 24 hours after exposure. Compared with controls, BALF macrophage and neutrophil cell counts were decreased in SPM-supplemented, $O_3$-exposed mice (Figure 9A). SPM supplementation did not lower BALF protein concentrations or BALF albumin concentrations, suggesting that SPM supplementation did not prevent $O_3$-induced lung injury (Figures 9B and C). $O_3$ exposure increased proinflammatory cytokines and chemokines, but relative to the $O_3$ vehicle control the $O_3$-exposed mice supplemented with SPMs showed a decrease in the proinflammatory cytokines interleukin 6 and interleukin 1β, and the neutrophil chemoattractant C-X-C motif chemokine ligand 1 (Figure 9D).

White blood cell (WBC) counts were assessed to determine if SPM supplementation alters systemic as well as pulmonary inflammation. WBC levels remained unchanged in filtered-air-exposed mice supplemented with SPMs, whereas SPM supplementation with $O_3$ exposure showed increased WBC counts that fell short of significance ($P = 0.053$; Figure 9E). Compared with the $O_3$ control, $O_3$+SPM-supplemented mice showed significantly increased circulating monocytes (Figure 9F). $O_3$+SPM supplementation also increased lymphocyte counts, although this did not reach statistical significance ($P = 0.061$).

### Effect of SPM Supplementation Prior to $O_3$ Exposure on AM Efferocytosis

Given that SPM supplementation restored pulmonary SPM levels and reduced $O_3$-induced inflammation at a time when AM efferocytosis was impaired, it was of interest to determine if pulmonary efferocytic defects were mitigated by SPM pre-supplementation. Compared with the vehicle plus filtered air exposure, $O_3$ exposure decreased AM efferocytosis, but not statistically significantly (Figure 10). In filtered-air-exposed mice supplemented with SPMs, the AM efferocytic response remained unchanged when compared with the filtered-air control. Mice given SPM supplementation before $O_3$ exposure showed significantly increased AM efferocytosis when compared with the $O_3$ vehicle exposure control group.

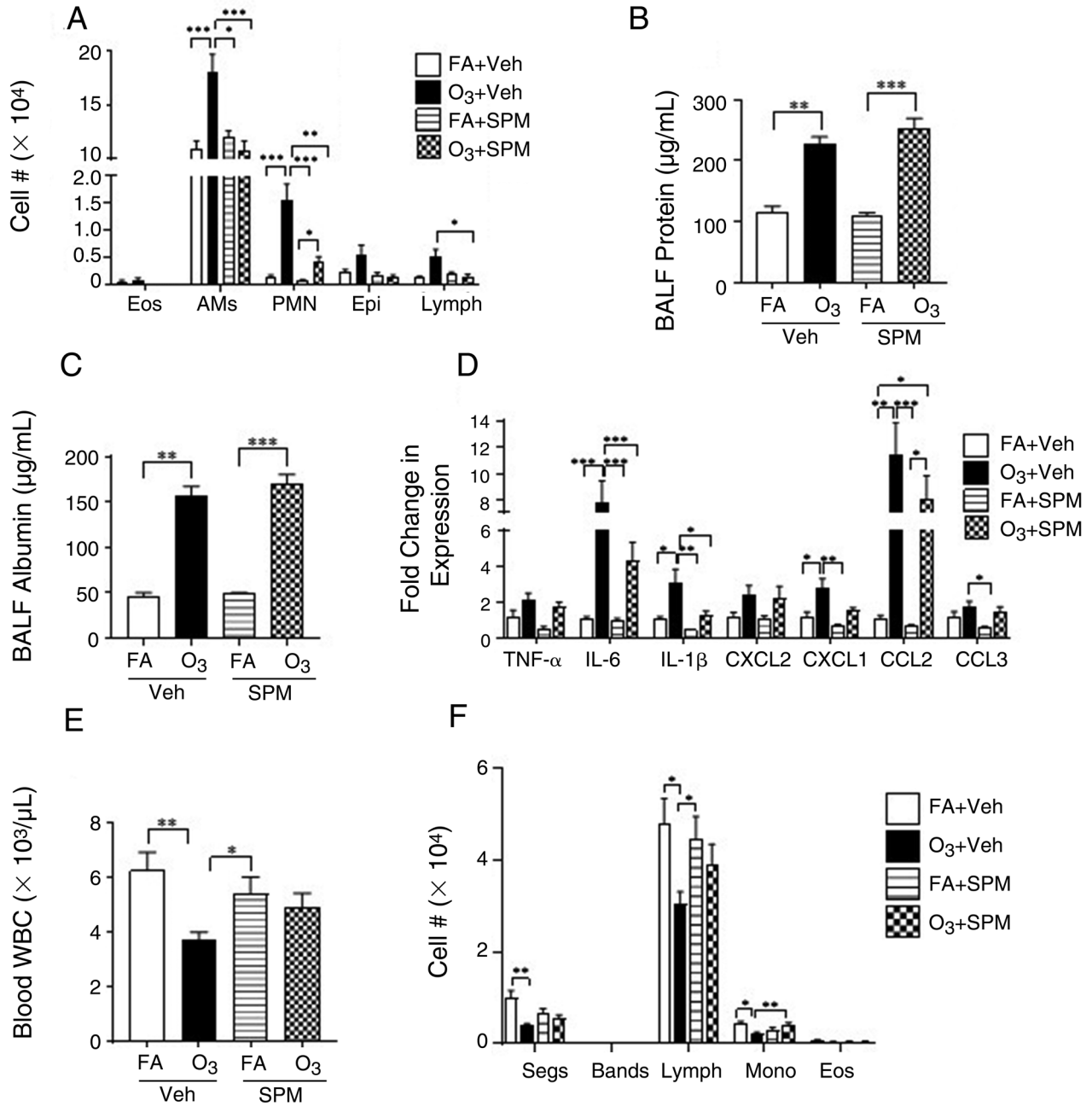


**Figure 9. Effect of SPM supplementation before $O_3$ exposure on pulmonary inflammation, markers of injury, and circulating immune cells.** Male WT mice were given 14-HDHA, 17-HDHA, and 10,17-DiHDoHE in three separate injections, 1 hour before FA or $O_3$ exposure. Markers of inflammation and injury, and levels of cytokines and chemokines were measured 24 hours after exposure. **(A)** BALF differential count ($n$ = 10–11 per group); **(B)** BALF protein ($n$ = 10–11 per group); **(C)** BALF albumin ($n$ = 5–6 per group); **(D)** whole lung homogenate cytokines and chemokines, normalized to 18S using quantitative RT-PCR ($n$ = 5 per group); **(E)** circulating total WBC ($n$ = 10–11 per group); and **(F)** circulating differential WBC count ($n$ = 10–11 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Bands = banded nucleus (immature neutrophils); CXCL1 and CXCL2 = C-X-C motif chemokine ligands 1 and 2; CCL2 and CCL3 = C-C motif chemokine ligands 2 and 3; Eos = eosinophils; Epi = epithelial cells; FA = filtered air; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; Lymph = lymphocytes; Mono = monocytes; PMN = polymorphonuclear leukocytes (neutrophil)s; Segs = segmented neutrophils; TNF-α = tumor necrosis factor alpha; Veh = vehicle.

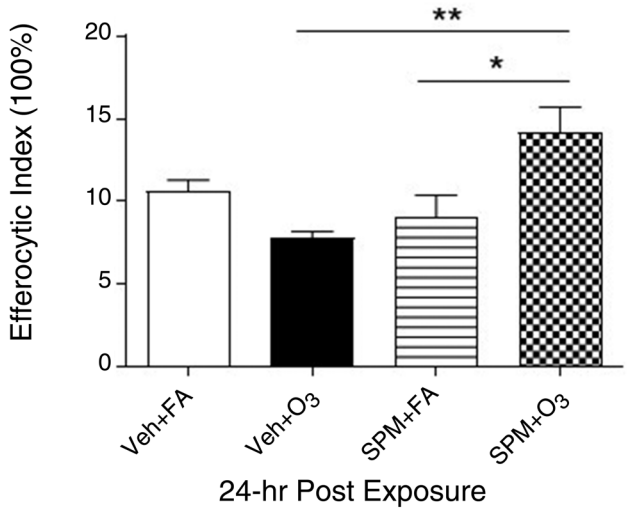


**Figure 10. Effect of SPM supplementation before $O_3$ exposure on AM efferocytosis.** Male mice were given 14-HDHA, 17-HDHA, and 10,17-DiHDoHE in three separate injections, 1 hour before FA or $O_3$ exposure. Then 24 hours after exposure mice were given apoptotic (Annexin $V^+/PI^-$) Jurkat T cells. BALF was harvested 90 mins after dosing, and AM efferocytosis was assessed. $n = 5–7$ per group. $^*P < 0.05$; $^{**}P < 0.01$. FA = filtered air; Veh = vehicle.

## DISCUSSION

$O_3$ exposure causes pulmonary and systemic inflammation and injury, but the mechanisms leading to the resolution of inflammation and injury remain poorly defined. SPMs are known to promote resolution of inflammation in models such as cigarette-induced pulmonary inflammation, allergic asthma, and pneumonia (Croasdell et al. 2015; Ishizuka et al. 2008; Seki et al. 2010). However, the role of SPMs in $O_3$-induced inflammatory responses was unknown. In this study, we demonstrated that (1) $O_3$ exposure increased pulmonary inflammation, and decreased select pulmonary SPM levels and AM efferocytosis, and (2) SPM supplementation prevented $O_3$-induced pulmonary inflammation while increasing pulmonary SPMs and rescuing AM efferocytic defects.

Ambient $O_3$ is a significant public health burden that causes both pulmonary and systemic inflammation and exacerbates pre-existing cardiopulmonary diseases. In addition to studies showing that exposure to $O_3$ exacerbates existing cardiopulmonary diseases, recent studies have shown that individuals living in areas with elevated ambient $O_3$ levels are more susceptible to developing chronic lung diseases than are individuals living where $O_3$ levels are lower (Conti et al. 2018; Hansel et al. 2016; Reilly et al. 2019; Rhee et al. 2019). Given that impaired resolution processes have been linked to the development of chronic lung diseases, it was of interest to investigate if $O_3$ impairs the resolution of pulmonary inflammation.

We first evaluated AM efferocytosis at multiple time points post exposure. Previous studies have shown that acute $O_3$ exposure impairs AM phagocytosis of bacterial pathogens (Gilmour et al. 1991). Our data indicate that a single acute $O_3$ exposure impairs AM efferocytosis up to 48 hours post exposure. This defect could be explained by multiple mechanisms. One mechanism could be the creation of $O_3$-induced DAMPs, such as oxPLs and HMGB1, which are known to disrupt macrophage efferocytosis by competing with apoptotic cells for engulfment (Bochkov et al. 2002; Linton et al. 2016). Another mechanism leading to impaired AM efferocytosis could be that $O_3$ disrupts lipid class switching, leading to enhanced production of proinflammatory lipid mediators and suppressing SPMs. Previous studies have shown that blocking COX-2 reverses $O_3$-induced impairments in phagocytosis (Canning et al. 1991). Additionally, the data we present here show that 24 hours post $O_3$ exposure, DHA-derived SPMs are suppressed in the lung, and exogenous administration of these SPMs rescues SPM levels in the lung, decreases airway neutrophilia, decreases expression of select cytokines and chemokines, and restores AM efferocytosis. However, we did note defects in AM efferocytosis at 48 hours, at which

pulmonary SPM levels had returned to baseline, indicating that other mechanisms besides impaired SPM production may be altered after exposure. Taken together, these data indicate that acute $O_3$ exposure impairs soluble and cellular processes that promote tissue homeostasis.

It has previously been reported that $O_3$ exposure increases bioactive lipids in the lung that are known to modulate inflammatory responses. Data from studies by Miller and colleagues (Miller et al. 2016a,b) demonstrated that $O_3$ exposure alters peripheral lipid metabolism in humans and rodents. A limitation of these studies is that they did not consider how $O_3$ alters pulmonary lipid metabolism, specifically SPMs. We demonstrated that, at 24 hours after $O_3$ exposure, levels of 14-HDHA and 10,17-DiHDoHE decreased; however, we did not examine whether this was due to decreased SPM production or increased SPM degradation. Prior work has determined that pro-inflammatory lipid metabolites (prostaglandins and leukotrienes) are elevated in the lung after $O_3$ exposure (Alexis et al. 2000; Hazucha et al. 1996), but no data exist about pro-resolution lipids. Our data suggest that $O_3$ exposure alters lipid metabolism, resulting in a reduction of SPMs. Altered lipid mediator class switching has been described in the context of inflammatory conditions including sepsis, pneumonia, and atherosclerosis (Serhan and Petasis 2011; Serhan and Chiang 2013). Together, this suggests that decreased SPM production and increased pro-inflammatory lipid mediator production may contribute to pulmonary inflammation after $O_3$ exposure.

Although $O_3$ exposure directly targets the lung, systemic effects of $O_3$ exposure are increasingly implicated in systemic and vascular diseases. $O_3$ exposure alters systemic metabolism, resulting in vascular dysfunction and atherogenesis (Miller et al. 2016b; Robertson et al. 2013). Previous studies have noted that $O_3$ alters circulating factors and increases pro-inflammatory cytokine expression in vasculature (Miller et al. 2016b; Robertson et al. 2013). Additionally, $O_3$ levels correlated with incidence and severity of cardiovascular diseases (Brook et al. 2004; Goldberg et al. 2001).

In our study, $O_3$ exposure suppressed systemic WBC counts, particularly circulating lymphocytes and monocytes. The decrease in circulating immune cells could be the result of activation of the cells, resulting in increased adherence to or migration through the vasculature. The $O_3$-induced alterations in circulating WBCs were associated with decreased systemic production of SPMs (Kilburg-Basnyat et al. 2018), which has been associated with systemic and vascular inflammation in models of cardiovascular disease (Fredman et al. 2016). It is still unknown how $O_3$ exposure might decrease systemic SPM production that could contribute to pollution-driven cardiovascular disease.

$O_3$ exposure increases pulmonary inflammation, suppresses AM efferocytosis, and decreases SPM levels in the lung, which may contribute to $O_3$-induced pulmonary inflammation. Supplementing with 14-HDHA, 17-HDHA, and 10,17-DiHDoHE prevents pulmonary inflammation and prevents suppression of AM efferocytosis. Overall, the driving force of $O_3$-induced inflammation may be a decrease in select SPMs leading to impaired efferocytosis and a failure to resolve inflammation. SPM supplementation may be a novel therapeutic angle to improve $O_3$-induced inflammation and chronic pulmonary consequences.

## OVERALL CONCLUSIONS

$O_3$ exposure is known to induce pulmonary inflammation, but the mechanisms driving inflammation and whether $O_3$ exposure alters resolution of inflammation remain poorly defined. One potential mechanism of inflammation resolution is efferocytosis, a process by which macrophages clear out dead cells and debris after tissue injury or inflammation in order to restore tissue homeostasis. However, the impact of $O_3$ exposure on altering efferocytosis and the molecular mechanisms driving the resolution response were previously unknown. In this study we demonstrate that (1) SR-BI protects the lung from $O_3$-induced inflammation and promotes efferocytosis, (2) $O_3$ exposure decreases SPM levels, and (3) administration of certain SPMs and SPM precursors reduces $O_3$-induced pulmonary inflammation and augments AM efferocytosis. These findings indicate that $O_3$-induced inflammation may be perpetuated by reductions in resolution processes, and that strategies to augment resolution prior to $O_3$ exposure may prevent acute health effects and decrease susceptibility to chronic lung diseases.

This report summarizes a two-part study investigating the impact of $O_3$-induced inflammation on alterations in resolving mechanisms. The first part of the study (Aim 1) evaluated the role of SR-BI in $O_3$-induced pulmonary inflammation. We found sustained neutrophilia in the airspace in SR-BI KO mice after exposure, which led us to evaluate AM efferocytosis in WT and SR-BI KO mice. We report two novel findings: (1) SR-BI KO mice have a baseline defect in AM efferocytosis, and (2) $O_3$ exposure diminishes AM efferocytosis. We decided to investigate the role of $O_3$ in modulating mechanisms that resolve inflammation. Aim 2 of this report shows that $O_3$ alters pulmonary SPM levels, leading to impaired AM efferocytosis as well as lung inflammation and injury. Although our research focus shifted, we discovered a novel research angle elucidating the mechanisms by which $O_3$ exposure may increase the incidence and severity of chronic lung diseases.

Aims 1 and 2 both deal with lipid metabolism and AM efferocytosis. Both SR-BI deficiency and $O_3$ exposure decreased AM efferocytosis. In Aim 2, we found that increasing the levels of DHA-derived SPMs rescued $O_3$-induced reductions in AM efferocytosis. However, we did not measure pulmonary SPMs in SR-BI KO mice or determine if SPM replacement rescues the decreased AM efferocytosis. SR-BI is known to bind and facilitate HDL transport but has also been shown to be important in fatty acid uptake (Krieger and Kozarsky 1999). Therefore, it is possible that defects in AM efferocytosis noted with SR-BI deficiency could be a result of impaired SPM production. Additionally, if environmental exposures alter SR-BI expression, the lung's ability to resolve inflammation may be impaired. Given the genetic and pharmacological alterations in SR-BI that have been identified in the human population, variations in SR-BI expression could contribute to the pulmonary pathogenesis following $O_3$ exposure (Helgadottir et al. 2018; Vickers and Rodriguez 2014). Further studies will be needed to identify SR-BI cell-specific contributions and the signaling mechanisms by which SR-BI may play a role in efferocytosis and SPM production.

## LIMITATIONS

Although the two-part study resulted in novel findings, we acknowledge there were limitations. The first part of this study, which evaluated the potential role of SR-BI in the pulmonary response to $O_3$ exposure, focused mainly on pulmonary inflammation and injury at two time points — 24 and 48 hours post $O_3$ exposure — whereas the efferocytic response in SR-BI KO mice was examined only at 24 hours post $O_3$ exposure. Future research measuring the efferocytic response and pulmonary inflammation at additional time points post exposure may determine if these impairments persist or if efferocytic responses are restored. Additionally, we measured only one class of oxPL — phosphatidylcholines — after $O_3$ exposure, chosen because it is the most abundant class of phospholipids in the airspace (Agassandian and Mallampalli 2013; Karnati et al. 2018). However, other phospholipid classes, including phosphatidylinositols and phosphatidylglycerols, are known to stimulate innate immune responses (Voelker and Numata 2019). The SR-BI KO mice we used are deficient in SR-BI expression in all tissues. Unfortunately, this did not allow us to evaluate cell-specific SR-BI contributions. Future studies using cell-specific downregulation of SR-BI (in particular, in macrophages) may be able to better decipher the role of expression of SR-BI in specific cell types in protecting the lung from $O_3$ exposure.

A limitation of the second aim of this study — to determine if $O_3$ exposure alters pulmonary production of SPMs and processes known to promote the resolution of pulmonary inflammation and injury — is that we did not consider or determine how $O_3$ alters pulmonary lipid metabolism, specifically SPMs. We demonstrated that $O_3$ exposure decreased 14-HDHA and 10,17-DiHDoHE; however, it has not yet been examined whether this was due to decreased SPM production, lipid mediator class switching, availability of the parent fatty acid, or increased SPM degradation. In the future, we will be designing studies to further investigate the origin of the decreased SPM production after $O_3$ exposure. Additionally, we acknowledge that we utilized one laboratory to analyze lipid mediator production in lung tissue and a different laboratory to measure oxPLs in airspace. The lipidomic field has published multiple articles emphasizing the need to standardize techniques across laboratories, make shared reference materials available, and implement the reporting of standards for dissemination of lipidomics data (Liebisch et al. 2017; Triebl et al. 2020; Wolrab et al. 2020). In an effort to conform to these new standards, future samples will be validated by another lipidomic laboratory that uses commercially available standards in their lipidomic sample analyses to identify lipid species.

Last, we would like to highlight that the sex of the mice used in the two parts of the study was different. The work reported in Aim 1 was performed exclusively in female mice because the cholesterol deficiency in SR-BI KO mice increases the number of females born per litter (Miettinen et al. 2001; Santander et al. 2013). In addition, male pups have a high incidence of hydrocephalus and are difficult to breed. We performed a preliminary study with male SR-BI KO mice exposed to $O_3$, and noted an increase in airspace neutrophilia compared with WT controls (data not shown).

By contrast, we used exclusively male mice in Aim 2 of this study, for two reasons: (1) the estrous cycle has been reported to influence $O_3$-induced pulmonary inflammation (Fuentes et al. 2019), and (2) most studies have been performed in male mice, so using them would allow our findings to be compared with other murine studies examining the $O_3$-induced pulmonary inflammation. We are currently evaluating whether sex alters pulmonary SPM production and AM efferocytosis.

## IMPLICATIONS OF FINDINGS

Our data suggest a new paradigm that $O_3$ exposure alters processes important in resolving the inflammatory response. These data indicate that exposure may alter how

the lung returns to homeostasis and how long it takes. Understanding the kinetics and time course of these findings may be important in understanding how increases in ambient $O_3$ levels drive exacerbations of chronic lung diseases and increase the incidence of chronic lung diseases. Additionally, given that SPM precursor administration or dietary DHA supplementation can be used to increase SPM levels, these may lead to novel therapeutic approaches for preventing $O_3$-induced inflammation.

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## MATERIALS AVAILABLE ON THE HEI WEBSITE

Supplemental tables and figures not included in the main report can be found in Additional Materials, available on the HEI website, *www.healtheffects.org/publications.*

Appendix A. Data Tables

Appendix B. Supplemental Figures.

## ABOUT THE AUTHORS

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# Research Report 204, *Novel Mechanisms of Ozone-Induced Pulmonary Inflammation and Resolution, and the Potential Protective Role of Scavenger Receptor BI*, K.M. Gowdy et al.

## INTRODUCTION

Ozone is a reactive oxidant gas formed, in the presence of sunlight, through complex photochemical reactions among pollutants emitted from anthropogenic and natural sources. Although ozone in the stratosphere protects the planet from harmful ultraviolet radiation, human exposure to increased levels of ozone at ground level produces adverse health effects. Because the effects of ozone exposure are prevalent and important, ozone is one of the six criteria pollutants regulated by the U.S. Environmental Protection Agency (U.S. EPA*) under the Clean Air Act. Given that importance, investigation into what biological mechanisms underlie ozone's effects continues to be of scientific and policy interest.

Upon inhalation, ozone deposits rapidly in the respiratory tract, interacting with components of the airway lining fluid to generate multiple oxidation products. Because the airway lining fluid is rich in phospholipids, interaction with ozone generates oxidized phospholipids, among other products. Oxidized phospholipids in turn interact with receptors expressed on lung cells, including alveolar macrophages and epithelial cells. Scavenger receptor BI (SR-BI) is one of the receptors expressed on lung cells that binds oxidized phospholipids.

Activation of lung cells, together with the activation of nerve fibers in the respiratory tract, leads to the development of an inflammatory response in the airways and changes in respiration. Ozone-induced changes in the airways can thence result in effects outside the lungs, particularly in the heart and blood vessels (U.S. EPA 2020). Nonetheless, the precise mechanisms by which acute exposure to ozone trigger inflammation in the airways are still currently not well understood.

To better understand the molecular pathways involved in the ozone-activated inflammatory response and the possible role played in this response by SR-BI, Dr. Kymberly Gowdy, then of East Carolina University, Greenville, North Carolina, submitted an application "Scavenger receptor BI regulates oxidized lipid driven pulmonary and vascular inflammation after ozone exposure," in response to HEI's Request for Applications 14-4, the Walter A. Rosenblith New Investigator Award. This award was established to provide support for an outstanding new investigator at the assistant professor level to conduct research in the area of air pollution and health and is unrestricted with respect to the topic of research.

Dr. Gowdy proposed that SR-BI mediated the clearance (removal) of oxidized phospholipids generated in the lung during ozone exposure, and that a lack of this clearance would lead to enhanced inflammation in the lungs, with potential consequences outside the lungs (i.e., systemically). To evaluate this hypothesis, Gowdy proposed to examine in detail characteristics of the inflammatory response in both normal and SR-BI-deficient mice to acute exposure to ozone, with particular focus on the lipid molecules generated. HEI funded Gowdy's application, considering it a new area worthy of study in understanding the mechanisms by which oxidizing air pollutants, such as ozone, might act.

This Critique provides the HEI Review Committee's independent evaluation of the study. It is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

## SCIENTIFIC AND REGULATORY BACKGROUND

### HEALTH EFFECTS OF OZONE

Ozone has both short- and long-term adverse effects on human health, with the strongest evidence for decreasing pulmonary function (U.S. EPA 2020). Ozone exposure has

Dr. Gowdy's 3-year study, "Scavenger receptor BI regulates oxidized lipid driven pulmonary and vascular inflammation after ozone exposure," began in November 2015. Total expenditures were $448,309. The draft Investigators' Report from Gowdy and colleagues was received for review in September 2019. A revised report, received in May 2020, was accepted for publication in June 2020. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and the Review Committee's Critique.



* A list of abbreviations and other terms appears at the end of this volume.

been associated with worsening of asthma symptoms (acute effects), increases in deaths and hospital admissions for respiratory illnesses such as chronic obstructive pulmonary disease and asthma (both acute and chronic effects), reduced lung growth, and a higher risk of developing asthma (chronic effects). Short-term exposure to ozone may also result in inflammation and injury in the airways (discussed in detail in the next section).

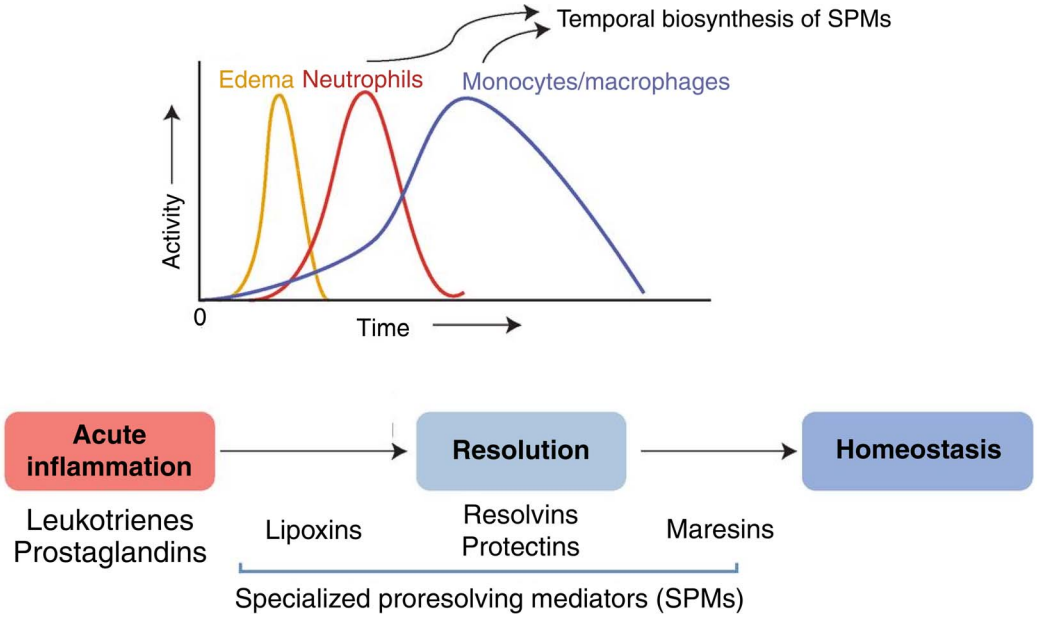
There is some evidence that short-term exposure to ozone is associated with adverse cardiovascular outcomes, including an increased risk of cardiovascular mortality. However, controlled short-term exposures of older humans to low levels of ozone (70 and 120 ppb) confirmed respiratory but not cardiovascular effects of ozone in healthy older adults (Frampton et al. 2017). Currently, the U.S. National Ambient Air Quality Standard for ozone remains at an 8-hour average concentration of 70 ppb (U.S. EPA 2020).

## THE INFLAMMATORY RESPONSE — FROM ACTIVATION TO RESOLUTION

### Activation of the Inflammatory Response

Inflammation is a crucial part of the body's early response to pathogens, such as bacteria. However, nonpathogenic stimuli such as ozone can also activate inflammation by generating oxidized phospholipids in lung lining fluid that then induce damage to lung cells. Oxidized phospholipids express patterns of molecules not found on normal cells or products. These damage-associated molecular patterns are recognized by receptors — for example, the family of toll-like receptors and scavenger receptors — expressed by cells of the innate immune system, and in particular, the phagocytic cell types (macrophages and polymorphonuclear leukocytes [neutrophils] [PMN]). Damaged cells and activated lung-lining components, as well as pathogens, trigger a cascade of events that result in an inflammatory response, see Critique Figure.

The earliest stage in the inflammatory response is characterized by tissue edema (influx of water) caused by increased blood flow and microvascular permeability. Lipid mediators — leukotrienes and prostaglandins — are released that attract PMN, which infiltrate the tissue. Activated PMN generate reactive oxygen species that, in the response to a pathogen, aid in the degradation of the pathogen. These activated PMN also release pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and chemokines that in turn attract blood monocytes into the tissue and activate tissue-resident macrophages (the tissue counterpart of monocytes in blood). Epithelial cell injury and disruption of the epithelial barrier at the surface of the lung also occur. The




**Critique Figure. Key events in the induction and resolution of the inflammatory response.** (Adapted with permission from Serhan et al. 2014, Figure 1, © Cold Spring Harbor Laboratory Press.)

cytokines and chemokines produced in this phase of the inflammatory response activate multiple cell types throughout the body, resulting for example in fever and the so-called acute phase response in the circulation.

**Resolution of the Inflammatory Response**

Most inflammatory responses are self-limiting (i.e., the inflammation resolves): after a few days, the pathogen is cleared or the damaged cells removed, and the tissue returns to its baseline internal state (homeostasis). In some situations, such as asthma, however, the inflammatory response does not resolve, and chronic inflammation results.

Studies over the last 20 years, particularly by Serhan and colleagues (Bannenberg and Serhan 2010; Chiang and Serhan 2020; Serhan 2014), have established that the resolution of inflammation is an active process. One key event is a so-called lipid-mediator class switch, when the synthesis of lipid mediators switches from the early pro-inflammatory set of prostaglandins and leukotrienes to a later set of specialized pro-resolving mediators, which are derived from polyunsaturated fatty acids (e.g., omega-3 fatty acids).

Specialized pro-resolving mediators, divided into structurally distinct subsets (lipoxins, resolvins, protectins, and maresins), have several important overlapping functions that promote the return to homeostasis. First, they inhibit further PMN recruitment into the tissue. Second, they stimulate a change in phenotype and function of macrophages — which initially produce the pro-inflammatory cytokines TNF-α and IL-1 β — to a later, pro-resolving phenotype that synthesizes the cytokines IL-10 and transforming growth factor β. Many studies suggest that these differences in macrophage function and phenotype are mediated by distinct subsets of macrophages called M1, pro-inflammatory, and M2, pro-resolving (reviewed in Laskin et al. 2019).

Third, specialized pro-resolving mediators promote efferocytosis. In efferocytosis, PMN that are activated during the inflammatory response and that subsequently become apoptotic (moribund), are phagocytized and hence removed by the pro-resolving M2 subset of macrophages (Arandjelovic and Ravichandran 2015). Removal of apoptotic PMN by efferocytosis returns the tissue to homeostasis. Studies also indicate that efferocytosis plays a crucial part in everyday homeostasis — billions of cells turn over inside the body each day and have to be removed (e.g., developing thymocytes and neurons, and senescent red blood cells). Recent data suggest that defects in efferocytosis may lead to the persistence of inflammation, playing a significant role in the induction of chronic inflammatory and autoimmune diseases, such as Crohn's disease and ulcerative colitis (Doran et al. 2019; Morioka et al. 2019).

**The Multiple Roles of SR-BI**

SR-BI is expressed in many tissues and cell types, including hepatocytes, epithelial cells, smooth muscle cells, monocytes and macrophages, and neurons. It binds a wide array of native or modified phospholipids and lipoproteins (Shen et al. 2018), which can be derived from either endogenous body components or from exogenous ligands.

As described in the introduction, SR-BI expressed by lung cells such as macrophages and epithelial cells binds oxidized phospholipids, generated by the interaction of ozone with phospholipids in the lung lining fluid (Gao et al. 2010; Komai et al. 2017). SR-BI also promotes the uptake of lipid-soluble vitamins such as vitamin E into host cells. SR-BI also plays a key role in cholesterol degradation and thus cardiovascular responses: expressed on hepatocytes and endothelial cells, it acts as a receptor for high-density lipoprotein (HDL) cholesterol. It removes cholesterol from HDL and returns the lipid-poor lipoprotein to the circulation (Shen et al. 2018).

This process — reverse cholesterol transport — drives the movement of cholesterol from peripheral tissues toward the liver, and is protective against the development of atherosclerosis. Variations in the human gene, SCARB1, may contribute to genetic susceptibility to coronary heart disease (Zeng et al. 2017). Expression of SR-BI by macrophages is also relevant in atherosclerosis: in a mouse model, the absence of SR-BI on macrophages was found to be pro-atherosclerotic, increasing necrosis and the inflammatory response and decreasing efferocytosis (Tao et al. 2015).

SR-BI also plays a role in responses to pathogens: it binds lipopolysaccharide, the major cell-wall component of gram-negative bacteria, and it is a receptor for the hepatitis C virus that allows the virus to enter and infect cells. Furthermore, Gowdy and colleagues showed previously that SR-BI is important in the response to bacterial pneumonia: SR-BI knockout mice lacking the gene for SR-BI that were infected with Klebsiella pneumoniae had impaired phagocytic killing and increased mortality, as well as increased numbers of PMN in lung compared with wild-type controls (Gowdy et al. 2014).

In the current report, Gowdy and colleagues wanted to extend the information on the role of SR-BI in bacterial infection to the evaluation of the role of SR-BI in the effects of ozone on the inflammatory response. They also wanted to evaluate inflammatory events that occurred after ozone exposure during the initiation and resolution phases.

## SUMMARY OF THE STUDY

### SPECIFIC AIMS

The original goal of the study was to evaluate whether SR-BI mediated the clearance (removal) of oxidized phospholipids generated in the lung during ozone exposure, and whether a lack of this clearance would lead to enhanced inflammation, with potentially systemic consequences. As the study developed, however, it evolved to include the evaluation of how exposure to ozone affected the synthesis of additional lipid mediators — specialized pro-resolving mediators — that play a role in the resolution of inflammation, and whether SR-BI also had a role in the resolution of inflammation.

Thus, the investigators' central hypothesis during the study was that ozone-induced pulmonary health effects are the result of delayed clearance of damage-associated molecular patterns and the dampening of processes essential for resolving inflammation and promoting tissue homeostasis. The study had two specific aims. Aim 1 was to define the role of SR-BI in ozone-induced pulmonary inflammation and resolution of injury. Aim 2 was to determine if ozone exposure alters pulmonary production of specialized pro-resolving mediators and the resolution of pulmonary inflammation and injury.

For Aim 1, the investigators exposed normal (wild-type) and SR-BI knockout mice to ozone and compared their responses by measuring multiple parameters of inflammatory and injury responses and levels of oxidized phospholipids in lung lavage fluid and homogenized lung tissue, as well as efferocytosis by alveolar macrophages. Some experiments compared the effects of administering an oxidized phospholipid into the lungs of wild-type and SR-BI knockout mice.

For Aim 2, wild-type mice were exposed to ozone and evaluated for levels of specialized pro-resolving mediators, the extent of inflammatory and injury responses, and macrophage efferocytosis. The investigators also assessed whether these responses were mitigated by the administration of lipid mediators prior to ozone exposure.

### METHODS

#### Animals and Inhalation Exposures

For Aim 1, the investigators used female C57BL/6J (wild-type) mice and female mice in which the SR-BI gene had been knocked out (SR-BI knockout mice). For Aim 2, they used only male C57BL/6J (wild-type) mice.

The investigators exposed mice to ozone at a concentration of 1 ppm, or to filtered air, for 3 hours in a multi-tiered inhalation (Hinners) chamber. Ozone was generated in the chamber by directing 100% oxygen through an ultraviolet light generator using an ozone calibrator and then mixing it with the filtered air supply.

#### Pretreatment with Oxidized Phospholipids or Lipid Mediators

To evaluate the role of oxidized phospholipids in pulmonary inflammation, the investigators instilled groups of female mice oropharyngeally with 200 μg/kg oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC); the phospholipid PAPC (as an unoxidized control); or phosphate buffered saline (the solution that the compounds were delivered in). The 200 μg/kg dose of oxPAPC was intended to yield "a comparable pulmonary injury response" that was provided by inhaling 1 ppm ozone, based on a previous study (Dahl et al. 2007).

To evaluate the role of specialized pro-resolving mediators and their precursors in reducing the effects of ozone-induced pulmonary effects, individual mice were injected intraperitoneally in three separate injections with 1 μg each of 10,17-dihydroxydocosahexaenoic acid (DiHDoHE) (a lipid mediator) and of 14-hydroxydocosahexaenoic acid (HDHA) and 17-HDHA (both precursors of specialized pro-resolving mediators), 1 hour before ozone or filtered air exposure. Because the compounds were solubilized in ethanol, control groups were injected with a solution of ethanol diluted in phosphate buffered saline.

#### Lung Tissue and Fluid Collection and Analysis

Animals were killed immediately after the 3-hour exposure to ozone or filtered air. Bronchoalveolar lavage fluid was collected immediately afterward by lavaging the lung lobes with saline. The resulting lavage was centrifuged, and an aliquot of the supernatant was removed and used to measure total protein, a measure of cell injury (leakage from injured cells). In some experiments, levels of the protein albumin in the supernatant were used as an additional marker of cell injury. The report does not describe how this was done. For tissue analyses, lungs were homogenized in 10% methanol and treated as described in Kosaraju and colleagues (2017).

The cell pellet was resuspended in buffer that lysed (broke down) red blood cells. Total cell counts in the lavage fluid of the right lobe were obtained by manually counting with a hemocytometer. Each sample was centrifuged onto slides and subsequently stained for differential cell counts of PMN, macrophages, lymphocytes, and epithelial cells.

Gowdy and colleagues also measured the following markers of inflammation in lung tissue: the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, and the chemokines CXCL1 and CXCL2, which are both synthesized by macrophages and are chemotactic for PMN. They also measured CCL2, a chemokine produced by many cells (including macrophages) that regulates migration of monocytes and macrophages, and CCL3, which is produced by multiple cells in the inflammatory response (including macrophages and epithelial cells) and which recruits PMN.

### Alveolar Macrophage Efferocytosis Assay

The Jurkat T cell line was irradiated by ultraviolet light to induce apoptosis (cell death) and then incubated for 4 hours at 37°C with 5% $CO_2$. After staining the cells with Annexin V and propidium iodide, the investigators judged them to be approximately 75% apoptotic or moribund (what the investigators refer to as Annexin$V^+/PI^-$). One million Jurkat cells were instilled oropharyngeally into mice 24 hours after exposure to ozone or filtered air. The mice were killed 1.5 hours later and their lungs lavaged.

The investigators harvested lung lavage fluid and evaluated by microscopy whether alveolar macrophages had phagocytized the apoptotic Jurkat T cells. Gowdy and colleagues then calculated an efferocytic index, that is, the number of alveolar macrophages that contained phagocytized apoptotic cells compared with the number of macrophages without apoptotic cells. A total of 100 macrophages was evaluated. In this Critique we use the term efferocytosis to indicate this efferocytic index.

### Measurement of Oxidized Phospholipids

The investigators measured oxidized phospholipids in lung lavage fluid, focusing on oxidized phosphatidylcholines. In brief, samples were extracted with a mixture of methanol, acetic acid, and heptane plus 0.01% butylated hydroxytoluene. The organic phase was then mixed with chloroform, 0.01% butylated hydroxytoluene, and 0.7 M formic acid, and the chloroform phase collected and dried under nitrogen gas. The sample was then reconstituted in 85% methanol plus 0.1% formic acid for evaluation by reverse phase high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS).

### Measurement of Lipid Mediators

The investigators assessed specialized pro-resolving lipid mediators in lung homogenates prepared from the left lung lobe that were suspended in 10% methanol. They quantified lipid mediators using reverse phase HPLC-MS/MS as described in their publication, Kosaraju and colleagues (2017). Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer in negative ionization mode.

### Statistical Analysis

The investigators used standard statistical approaches to analyze the data. To compare effects after exposure to ozone and filtered air they pooled data from three experiments for Aim 1 and two experiments for Aim 2. For the oxPAPC studies, they pooled data from two experiments. For the specialized pro-resolving mediator and vehicle injections prior to ozone or filtered air exposure, data were pooled from three separate exposure experiments. Due to small sample sizes ($n < 20$), they used a nonparametric one-way analysis of variance (ANOVA) test (Kruskal-Wallis) followed by a Dunn multiple comparisons test. For comparisons of two groups, they used an unpaired nonparametric $t$ test (Mann-Whitney test). When more than one variable was assessed, they used a two-way ANOVA with a post hoc test for multiple comparisons (Sidak or Tukey) to determine whether the variables interacted with each other and to assess multiple comparisons among the groups. For all statistical analyses, a value of $P < 0.05$ was considered significant.

## SUMMARY OF MAIN RESULTS

### Assessment of Lipid Mediators

***Levels of Specialized Lipid Mediators and Their Precursors*** The investigators detected lung tissue levels of the lipid mediator 10,17-DiHDoHE and the precursors 14-HDHA and 17-HDHA in the range of 0.003–17.5 picograms per milligram lung tissue of wild-type mice. The effects of ozone exposure varied at different times after the end of exposure. At 6 hours, levels of 10,17-DiHDoHE, 14-HDHA, and 17-HDHA increased more than 2-fold compared with filtered-air-exposed controls, whereas at 24 hours after exposure, levels of 14-HDHA and 10,17-DiHDoHE (but not 17-HDHA) decreased by about 50%, compared with filtered air controls. However, at 24 hours, levels of 14-HDHA in air-exposed mice increased 10-fold compared with levels at all other time points examined. At 48 and 72 hours after exposure, no differences were found in lipid mediator levels between filtered air-exposed and ozone-exposed mice. These results can be found in Investigators' Report Figure 7.

***Levels of Oxidized Phospholipids*** The investigators detected and identified multiple oxidized phospholipids in the range of 0.1–14.6 nanograms per milliliter of lung lavage fluid in wild-type and SR-BI knockout mice exposed to filtered air or to ozone at various times after ozone exposure. In wild-type mice, levels of two oxidized phospholipids were increased approximately 2-fold at 6 hours after

the end of ozone exposure. These results are shown in Investigators' Report Figure 3. As described later in Ozone Response in Mice Lacking SR-BI Expression, levels of individual oxidized phospholipids after ozone treatment were very similar in mice that did express SR-BI versus those that did not.

### Ozone Effects in Wild-type Mice

Most of the results reported by Gowdy and colleagues were obtained at 24 hours after the end of a 3-hour exposure to 1 ppm ozone.

***Increases in Some Markers of Inflammation and Injury in the Lung*** Assessing results of ozone exposure in male and female mice, Gowdy and colleagues reported increases in numbers of macrophages and PMN in lung fluid, as well as in levels of the cytokines IL-6 and IL-1β and the chemokines CXCL1, CXCL2 and CCL2 in lung tissue in one or both sexes. Protein and albumin levels were increased 2-fold and 3-fold respectively (measured in male mice only). These results are shown in Investigators' Report Figures 2 and 9. Overall, these changes are consistent with ozone inducing some of the expected markers of a standard inflammatory response at 24 hours, but not consistently affecting injury.

***Variable Effects on Efferocytosis*** Exposure to ozone had variable effects on efferocytosis. In the only set of experiments to evaluate a time-course of effects (in male mice), Gowdy and colleagues reported that ozone exposure decreased efferocytosis by approximately 25% at 24 hours after exposure and by approximately 60% at 48 hours, compared with filtered air controls at the same time point. However, at 72 hours after exposure, levels of efferocytosis were the same in macrophages from ozone- and filtered-air-exposed animals, and were similar to those observed pre-exposure in filtered-air-exposed mice (at baseline). In a second set of experiments in male mice, ozone exposure had no effect on efferocytosis at 24 hours after exposure. Gowdy and colleagues measured efferocytosis in female mice at only one time point. At 24 hours, exposure to ozone decreased efferocytosis by approximately 75% compared with filtered-air-exposed mice. These results are shown in Investigators' Report Figures 5, 6, and 10.

***Ozone Effects after Pretreatment with a Lipid Mediator or Precursors*** The investigators reported the following results for a lipid mediator (10,17-DiHDoHE) and precursors (14-HDHA and 17-HDHA) administered to mice before they were exposed to ozone: pretreatment somewhat decreased the levels of PMN and macrophages in lung lavage fluid, and of IL-6, IL-1β, and CXCL1 in lung tissue compared with ozone exposure alone. However, pretreatment did not generally reduce levels of these inflammatory markers compared with levels in filtered-air-exposed mice. The investigators stated that these findings suggest that pretreatment with specialized pro-resolving mediators and their precursors reduced, that is, mitigated, some features of the inflammatory response after ozone exposure. However, no effects were seen on markers of ozone-induced lung injury — bronchoalveolar lavage protein or albumin levels — or on levels of CXCL2 (another chemokine synthesized by macrophages that is chemotactic for PMN).

Levels of 14-HDHA and 17-HDHA (but not 10,17-DiHDoHE) were increased 24 hours after ozone exposure in the lungs of mice that were pre-treated, compared with ozone-exposed mice that were not pretreated. Overall, the results suggest that pre-administration of specialized pro-resolving mediators before ozone exposure can boost their levels at 24 hours after exposure. Also, pre-treatment with lipid mediators increased efferocytosis in ozone-exposed mice compared with filtered-air-exposed mice. These results are shown in Investigators' Report Figures 8, 9, and 10.

### Ozone Response in Mice Lacking SR-BI Expression

Gowdy and colleagues focused mainly on evaluating responses at 24 hours after mice were exposed to 1 ppm ozone for 3 hours, comparing responses in wild-type and SR-BI knockout mice. They reported that levels of several inflammatory markers in lavage fluid were higher in knockout mice compared with wild-type mice. For example, they observed approximately 4-fold more PMN in lung lavage fluid and 2-fold higher levels of IL-1β in lung tissue. The comparative increase in PMN numbers was observed at 24 hours but not at 48 hours post-exposure.

However, many other markers of inflammation and injury measured in lung tissue and lavage fluid after ozone exposure did not differ between wild-type and SR-BI knockout mice. For example, levels of macrophages, protein, TNF-α, and CXCL1 and CXCL2 (chemokines chemotactic for PMN) were not different. Collectively, these results suggest that the major effect of the absence of SR-BI on the inflammatory response to ozone at 24 hours was an increase in PMN in the knockout mice compared with wild-type mice.

Gowdy and colleagues found no difference in efferocytosis of alveolar macrophages obtained from wild-type and SR-BI knockout mice. Twenty-four hours after exposure to ozone, efferocytosis was decreased by approximately 75% in both types of mice. However, they reported that baseline efferocytosis was 25% lower in macrophages obtained

from SR-BI knockout mice compared with wild-type mice. These results are shown in Investigators' Report Figure 5.

Levels of oxidized phospholipids in lung lavage fluid did not differ between wild-type and SR-BI knockout mice after ozone exposure. Thus, the lack of expression of SR-BI — a receptor for oxidized phospholipids that is expressed on many cell types in the lung — did not affect the levels of those phospholipids found in the lung after ozone exposure.

The investigators did not evaluate specialized pro-resolving mediator levels in SR-BI knockout mice after ozone exposure, so no comparisons could be made with lipid mediator levels in wild-type mice.

### Summary of the Investigators' Conclusions

Gowdy and colleagues stated the following conclusions based on the results presented in their report:

1. Ozone exposure decreases levels of certain specialized pro-resolving mediators and reduces alveolar macrophage efferocytosis. Based on these findings, the investigators further concluded that ozone inhibits the resolution of inflammation.
2. SR-BI protects the lung from ozone-induced inflammation and promotes efferocytosis.
3. Administration of specialized pro-resolving mediators or their precursors reduces ozone-induced pulmonary inflammation and augments efferocytosis of alveolar macrophages.

However, for reasons discussed in the next section, the HEI Review Committee thought that the findings presented in this report provided only limited support for the investigators' conclusions.

## HEI REVIEW COMMITTEE EVALUATION

The HEI Review Committee considered the topics and the goals of the study — to investigate the molecular mechanisms involved in both the initiation and the resolution of the inflammatory response after exposure of mice to ozone — to be important. Furthermore, the Committee considered this to be an initial and groundbreaking lipidomics study, that is, a study designed to identify, quantify, and assess the role of low-level lipid mediators in the pulmonary inflammatory and injury response after ozone exposure. In addition, evaluating the potential roles of the multifunctional receptor, SR-BI, and of efferocytosis — a critical homeostatic mechanism to remove moribund cells — in the response to ozone was novel.

The Committee thought that the investigators had successfully measured low levels of multiple lipid mediators. The Committee also concluded that the data provided some support for the finding that pre-administration of precursors of lipid mediators may mitigate some markers of ozone-induced pulmonary inflammation. On the other hand, the Committee noted several limitations of the experimental design and methodology that reduced confidence in other conclusions drawn by the investigators and in the generalizability of the results.

### IDENTIFICATION OF LIPID MEDIATORS

The Committee noted that Gowdy and colleagues have conducted a valuable initial attempt to identify and assess the role of various lipid mediators in a mouse model of pulmonary inflammation and injury after acute exposure to ozone. They successfully developed and used a sensitive technique, HPLC-MS/MS, to identify and quantify low levels of lipid mediators in lung tissue and lung lavage fluid of mice. Many oxidized phospholipids could be measured at levels between 0.1 and 14.6 nanograms per milliliter of lavage fluid, and specialized pro-resolving lipid mediators at levels between 0.003 and 17.5 picograms per milligram of lung tissue. As the investigators acknowledge, these respective measurements were made in two different laboratories, and standardization of lipid assays across laboratories has not been optimized (Triebl et al. 2020; Wolrab et al. 2020).

From the results presented in the report, however, it is not clear that either kind of lipid mediator will be useful markers of ozone exposure, at least at the time points evaluated in this study. Acute ozone exposure increased levels of both oxidized phospholipids and specialized pro-resolving mediators by only 2- to 3-fold at their maximum (which occurred 6 hours post-exposure). In addition, the investigators reported substantial background levels of both sets of lipid mediators in samples collected from filtered-air-exposed mice, the origin of which was not defined. This suggests that some endogenous inflammation was proceeding in the lungs of these mice. Acute exposure to a fairly high concentration — 1 ppm ozone — minimally enhanced the levels of mediators above this background level.

Gowdy and colleagues indicated that one part of the central hypothesis of the study was that ozone-induced pulmonary health effects result from "delayed clearance of damage-associated molecular patterns." However, although the investigators did measure levels of such patterns after ozone exposure (i.e., levels of various oxidized phospholipids), they did not provide any experimental evidence of "delayed clearance" (i.e., presumably lower levels of oxidized phospholipids in ozone-exposed versus filtered-air-exposed mice). Therefore, the Committee

thought that support for this part of the hypothesis was lacking.

As the investigators also acknowledge, they focused on phosphatidylcholine-derived oxidized phospholipids because of their abundance in the lung lining fluid (Almstrand et al. 2015). Future studies are encouraged to assess levels of other oxidized lipids, particularly those derived from phosphatidylserine and phosphatidylinositol.

## PRETREATMENT WITH SPECIALIZED LIPID MEDIATORS

The finding that pretreatment of mice with specialized lipid mediators prior to ozone exposure mitigated some markers of the inflammatory response (without affecting lung injury) indicates that such supplementation may be useful in mitigating the inflammatory effects in humans of exposure to ozone and other airborne pollutants that generate inflammatory responses. These findings provide a further rationale for studies to evaluate supplementation of specialized lipid mediators as a way to reduce chronic inflammatory conditions (Serhan 2014).

As specialized lipid mediators are derived from three omega-3 fatty acids, it is possible that the human cardiovascular benefits resulting from diets that are rich in these fatty acids (such as fish oil) may result from the pro-resolving functions of the lipid mediators. Thus, dietary supplementation with omega-3 fatty acids or fish oils may boost production of the lipid mediators and prevent adverse effects caused by exposure to ozone. However, in rats supplemented with dietary fish oil and exposed to ozone, Gowdy and colleagues found that fish oil supplementation was cardioprotective but increased pulmonary inflammation and injury (Snow et al. 2018).

Recent data from ozone-exposed epithelial cells in vitro suggest a possible reason for these adverse effects: that ozone was reacting with polyunsaturated fatty acids to increase the formation of lipid hydroperoxides, which have inflammatory effects (Corteselli et al. 2020). These findings suggest that caution will be needed in attempting the use of dietary supplements to mitigate the effects of ozone exposure in humans.

## LIMITATIONS IN ASSESSING THE KINETICS OF THE OZONE RESPONSE

All experiments were performed using a single 3-hour exposure of mice to ozone at one concentration (1 ppm). The investigators chose this exposure concentration as the equivalent of a human exposure to ozone of about 200 ppb (or 42.8 $\mu g/m^3$). The Committee considered this to be a reasonable assumption, based on experiments of radioactive ozone uptake by lung lavage cells of humans and rodents that support a 4- to 5-fold concentration difference in sensitivity between rodents and humans (Hatch et al. 2013). Nonetheless, a human acute exposure to 200 ppb ozone — well above the current 8-hour U.S. National Ambient Air Quality Standard of 70 ppb — would be found only in highly polluted areas or on an ozone action day in the United States.

In addition, most of the readouts of ozone effects were evaluated at only one time point, at 24 hours after exposure. The investigators chose the 24-hour time point so they could compare their results with other mouse studies of the induction of the ozone response, and because they considered this to be the "beginning of resolution" of the inflammatory response. However, 24 hours post-exposure may be too early to measure resolution; collecting extensive data at later time points such as 48 hours and beyond would likely have been more informative.

In fact, the Committee noted that the data collected at time points other than 24 hours post exposure in some of the experiments did present a more complete picture of the kinetics of the various endpoints measured than those made at 24 hours, allowing for different interpretations. Considering the whole time course after exposure, the Committee concluded that the investigators showed that ozone exposure had increased levels of specialized pro-resolving mediators 2-fold early in the response (at 6 hours), but provided only limited evidence for a decrease in such levels later in the response (at 24 hours).

The Committee recognized that evaluating responses after a single acute exposure to ozone is a necessary initial step in understanding the potential roles of mediators in the inflammatory pathways. Nonetheless, real-world human exposure to ambient ozone is repeated and episodic, and reports have described that multiple exposures to ozone in humans (and rodents) can result in adaptation, that is, diminished inflammatory and respiratory responses (e.g., Wiester et al. 2000). Future experiments will be needed to determine whether multiple exposures to ozone will show a different pattern of inflammatory changes.

## LIMITATIONS OF USING LUNG FLUID AND LUNG TISSUE MEASUREMENTS

The Committee considered that a more complete picture of the extent of the pulmonary pathobiological and physiological responses after exposure to ozone would have been obtained if the investigators had also evaluated histopathology of the potentially affected areas of the lung, to assess epithelial cell injury and proliferation, and the status of other key lung cell types such as goblet cells and

club cells. The investigators did obtain limited histological data on the effects of specialized pro-resolving mediator supplementation on the lung (Kilburg-Basnyat et al. 2018), but those data were not presented in the current report. The Committee recommends that future studies include an evaluation of macrophage subpopulations (such as M1 and M2) by immunostaining and by assessing their cytokine production. In addition, the Committee noted that collecting lung lavage fluid for biochemical and cellular responses in the airspace would sample only parts of the lung and would mix material from ozone-reactive and nonreactive sites in the lung, thereby diluting the effects of ozone. Similarly, of the total cellular material in the lung tissue homogenate, 10%–15% at most would be from the sites of injury and inflammation. Thus, the investigators may have missed some effects by limiting their measurements to lung lavage fluid and homogenized tissue without assessing lung histology.

### INTERPRETATION OF THE EFFEROCYTOSIS ASSAY

Efferocytosis plays a key role in the resolution phase of the inflammatory response, in which macrophages phagocytize apoptotic (dying) neutrophils to return the tissue to homeostasis. However, the Committee was not clear whether the in vivo assay, an artificial system to assess efferocytosis in the current study, was standardized sufficiently to be considered an accurate assessment of efferocytosis. In addition, the Committee had concerns about the interpretation of the results.

First, only a small percentage (around 10% in most experiments) of alveolar macrophages were capable of efferocytosis in this assay, so small differences could have a potentially exaggerated impact on percentage changes in the response. Second, the effect was variable. One experiment in macrophages collected from male mice showed no change after ozone exposure, and a second experiment exhibited variation in efferocytosis in macrophages collected from mice exposed to filtered air at various time points that made interpretation of an ozone effect difficult.

Third, ozone decreased efferocytosis by 75% in macrophages from female mice at 24 hours after exposure, but the significance of this decrease in efferocytosis is unclear. It is possible that this result represents a true sex difference in the efferocytosis response to ozone. It should be noted that there have been sex differences in other airway inflammatory responses to ozone (e.g., Cabello et al. 2015, Cho et al. 2019), so further experiments to clarify this finding would be useful.

However, the Committee considered that this finding might have resulted from a technical issue. By administering apoptotic T cells at 24 hours after ozone exposure, most of the alveolar macrophages present would still be the pro-inflammatory M1 subpopulation, whose function does not include efferocytosis — a key function of pro-resolving M2 macrophages, which develop later in the response. In effect, the protocol may have interrupted or prematurely pushed the sequential transition of macrophages from the M1 to the M2 phenotype. As a consequence, the efferocytosis in macrophages from ozone-exposed mouse lungs would appear to be decreased in comparison with the response in macrophages from filtered-air-exposed lungs, which could be more easily activated in this assay to develop into M2 cells capable of efferocytosis.

For these reasons, the Committee found it difficult to conclude that exposure to ozone consistently decreased efferocytosis, disagreeing with the investigators' conclusion. Future experiments would be useful to more directly determine the efferocytotic ability of the macrophages present, and also the phenotype and functional capabilities of the subtypes of macrophages present; for example, their specific cytokine production could be assessed to further understand these mechanisms.

### LACK OF SUPPORT FOR OZONE-INHIBITING RESOLUTION OF INFLAMMATION

Gowdy and colleagues concluded that ozone delayed or inhibited the resolution of inflammation. However, as described above, the Committee considered that the variability in the results precluded any clear-cut conclusions about ozone decreasing efferocytosis by alveolar macrophages, and so inhibiting resolution of the inflammatory response. In addition, ozone exposure increased the levels of two lipid pro-resolving mediators early in the inflammatory response (6 hours), suggesting that at that point in time, ozone (the inducer of the inflammatory response) induced key signals that are required for the resolution of the response. Therefore, the Committee concluded that ozone was not impairing the resolution of the inflammatory response that it was inducing. Indeed, the Committee considered it to be counterintuitive that an agent, such as ozone, that reacts rapidly with the body's components and induces acute inflammation was somehow actively inhibiting the resolution of the inflammatory response hours, if not days, later.

Nonetheless, the Committee considered that it would be worth further investigation to determine whether exposure to ozone affects the resolution of the inflammatory response in chronic conditions such as chronic obstructive pulmonary disease and asthma (U.S. EPA 2020). In those conditions, ozone exposure may add an inflammatory stimulus to ongoing disease processes, potentially pushing

the balance in lung tissues toward pro-inflammatory rather than pro-resolving pathways. In this ozone-exposure scenario, reductions in both lipid mediator production and efferocytosis might be anticipated.

## NO CONVINCING ROLE OF SR-BI IN OZONE RESPONSE

SR-BI has been shown to play an important role in different mouse models, for example, in atherosclerosis and in the response to Klebsiella pneumoniae (Gowdy et al. 2014). However, the current study indicates that the effects of acute ozone exposure were broadly similar in mice that either expressed SR-BI (wild-type) or did not express it (SR-BI knockout mice). The only major difference was a higher number of PMN in the knockout mice. From all these results, the Committee found it difficult to conclude that SR-BI plays a major role in the inflammation and injury response to ozone, and so suggests that receptors other than SR-BI are important in the airway response to ozone.

## SUMMARY AND CONCLUSIONS

The Committee considered the work by Gowdy and colleagues in ozone-exposed mice to be an interesting new approach for evaluating the events involved in both the early and later phases of the inflammatory response (reflecting initiation and resolution) after acute exposure to ozone. In particular, the study was a valuable initial attempt to understand the role of different types of lipid mediators produced during those early and later phases of inflammation.

To this end, the investigators successfully used a sensitive technique, reverse HPLC-MS/MS, to identify and quantify low levels of oxidized phospholipids that were generated early in the response to ozone, as well as specialized pro-resolving lipid mediators that play a key role in the resolution of the inflammatory response. In addition, the investigators showed that pre-administering lipid mediators to mice prior to ozone exposure mitigated some effects of the resulting inflammatory response. These results offer the possibility that pre-administration of lipid mediators can be used in a clinical or dietary setting to offset the inflammatory effects induced by stimuli such as air pollutants or even pathogens.

The investigators concluded that the SR-BI receptor plays a protective role in either ozone-induced inflammation or resolution of the response. However, the Committee disagreed based on findings that most markers of the inflammatory and injury response were similar in mice that did or did not express SR-BI, and that the effect of ozone on efferocytosis was almost identical in these two sets of mice.

The Committee noted several important limitations in the study design that reduced confidence in the generalizability of the results. One major limitation was that the investigators used only one concentration of ozone, 1 ppm. Based on uptake of radioactive ozone by lung cells of rodents and humans, this exposure concentration of 1 ppm in mice was estimated to correspond to a fairly high human exposure concentration of 200 ppb (or 42.8 $\mu g/m^3$) ozone. A further limitation of the study design was that the investigators measured only a limited set of markers of inflammation, focusing on counts of leukocyte populations and epithelial cells and levels of pro-inflammatory cytokines and protein (as a marker of injury). The investigators did not perform histopathology, which would have shown both the extent of inflammatory damage and injury to multiple lung cell types, and how the damage and injury might have resolved. The Committee also considered the results and interpretations of the efferocytosis assay to be of uncertain significance.

In summary, the Committee thought the current report provided a good foundation for further studies to assess the role of specialized lipid mediators in mitigating inflammatory responses. Given that exposure to ozone exacerbates chronic inflammatory conditions such as asthma and cardiovascular disease, it will be worth exploring whether ozone affects the resolution of inflammation in these conditions, and whether enhancement of lipid mediator levels through diet or other interventions may be clinically useful in mitigating such conditions.

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# ABBREVIATIONS AND OTHER TERMS

| | |
|---|---|
| AM | alveolar macrophage |
| ANOVA | analysis of variance |
| BALF | bronchoalveolar lavage fluid |
| BCA | bicinchoninic acid assay |
| BHT | butylated hydroxytoluene |
| C18Kodia-PC | carbon 18- 5-keto-6-octendioic acid esters of 2-lyso-phosphocholine |
| CBC | complete blood count |
| CD36 | cluster of differentiation 36 |
| cDNA | complementary deoxyribonucleic acid |
| ChemR23 | chemokine-like receptor 1 |
| COX-2 | cyclooxygenase 2 |
| Ct | cycle threshold |
| DAMP | damage-associated molecular pattern |
| DHA | docosahexaenoic acid |
| DiHDoHE | dihydroxydocosahexaenoic acid |
| EPA | eicosapentaenoic acid |
| FBS | fetal bovine serum |
| HDHA | hydroxydocosahexaenoic acid |
| HDL | high-density lipoprotein |
| HMGB1 | high-mobility group box 1 protein |
| HpDHA | hydroperoxyDHA |
| HPLC-MS/MS | high performance liquid chromatography with tandem mass spectrometry |
| IL | interleukin |
| i.p. | intraperitoneal |
| lipoxin A4/FPR2 | lipoxin A4/formyl peptide receptor 2 |
| LOX | lipoxygenase |
| MARCO | macrophage receptor with collagenous structure |
| mRNA | messenger ribonucleic acid |
| o.p. | oropharyngeally |
| $O_3$ | ozone |
| oxPAPC | oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine |
| oxPL | oxidized phospholipid |
| PAF | platelet activating factor |
| PAPC | 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine |
| PAzPC | 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |
| PGPC | 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine |
| PMN | polymorphonuclear leukocyte (neutrophil) |
| PONPC | 1-palmitoyl-2-(9-oxononanayl)-phosphocholine |
| POVPC | 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine |
| PRR | pattern recognition receptor |
| RNA | ribonucleic acid |
| RT-PCR | real-time polymerase chain reaction |
| SAzPC | 1-stearoyl-2-azelaoyl-sn-glycero-phosphocholine |
| SEM | standard error of the mean |
| SGPC | 1-stearoyl-2-glutaroyl-sn-glycero-3-phosphocholinene |
| SONPC | 1-stearoyl-2-(9-oxononanayl)-phosphocholine |
| SOVPC | 1-stearoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine |
| SPE | solid phase extraction |
| SPM | specialized pro-resolving mediator |
| SR | scavenger receptor |
| SR-BI | scavenger receptor BI |
| SR-BI KO | SR-BI-deficient or knock-out |
| TLR | toll-like receptor |
| TNF-α | tumor necrosis factor alpha |
| UV | ultraviolet |
| U.S. EPA | U.S. Environmental Protection Agency |
| WBC | white blood cell |
| WT | wild-type |

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