

**MECHANISMS MEDIATING MACROPHAGE ACCUMULATION AND
ACTIVATION IN THE LUNG DURING THE PATHOGENESIS OF OZONE-
INDUCED LUNG INJURY**

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ABSTRACT OF THE DISSERTATION

Mechanisms Mediating Macrophage Accumulation and Activation in the Lung during the
Pathogenesis of Ozone-induced Lung Injury

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Classically and alternatively activated macrophages and inflammatory mediators they release play a key role in the pathogenesis of ozone-induced lung injury. In these studies, we investigated the origin of these cells and mechanisms regulating their accumulation in the lung following ozone exposure. We hypothesized that macrophages originate in the bone marrow and the spleen, and that chemokine receptors CCR2 and CX3CR1 mediate their migration to the lung; moreover, macrophage activation is controlled, in part, by the nuclear receptor FXR. To test this hypothesis, we analyzed the effects of ozone on splenectomized mice, CCR2 knockout mice and FXR knockout mice. Following ozone exposure, increased numbers of pro-inflammatory CD11b⁺Ly6C^{Hi} and anti-inflammatory CD11b⁺Ly6C^{Lo} macrophages were observed in lungs of control (CTL) mice.

Splenectomy resulted in decreases in pro-inflammatory macrophages in the lung and down regulation of CCR2, CCL2, and CCL4, but increases in CD11b⁺Ly6C^{Lo} anti-inflammatory macrophages. After ozone exposure, we also observed increases in lung macrophages staining positively for CCR2, a chemokine receptor known to mediate

trafficking of pro-inflammatory macrophages from the bone marrow to sites of injury. Loss of CCR2 was associated with reduced numbers of CD11b⁺Ly6C^{Hi} and iNOS⁺ pro-inflammatory macrophages in the lung and decreased expression of the pro-inflammatory cytokines, IL-1 β and TNF α . Decreases in proinflammatory/cytotoxic lung macrophages in SPX and CCR2^{-/-} mice were correlated with reduced ozone toxicity and oxidative stress, demonstrating that these cells originate in both the spleen and bone marrow. To further investigate macrophage trafficking from the bone marrow, we generated GFP⁺ chimeric mice by adoptive transfer of 2x10⁶ bone marrow (BM) cells from GFP⁺ mice into irradiated CTL mice. After 4 weeks, approximately 98% of BM cells were GFP⁺, while only 5% of lung macrophages were GFP⁺. Ozone exposure resulted in an increase in pro-inflammatory GFP⁺CD11b⁺Ly6C^{Hi} and anti-inflammatory GFP⁺CD11b⁺Ly6C^{Lo} macrophages in the lung at 24 h. Whereas GFP⁺Ly6C^{Hi} macrophages remained elevated for 72 h, increases in GFP⁺Ly6C^{Lo} macrophages were transient. These studies suggest that bone marrow contributes both pro- and anti-inflammatory macrophages to lung macrophage pools responding to ozone. This was confirmed using CX3CR1^{+/GFP} reporter mice and by staining lung macrophages for CCR2. These data suggest that multiple macrophage subpopulations play distinct roles in ozone-induced lung injury.

To investigate potential mechanisms regulating macrophage activation, we used transgenic mice lacking FXR, a nuclear receptor with anti-inflammatory activity. Treatment of WT mice with ozone resulted in increased FXR expression in the lung, most notably in macrophages. Loss of FXR resulted in increased numbers of pro-inflammatory Ly6C^{Hi} macrophages in the lung and prolonged up-regulation of iNOS, indicating chronic inflammation and macrophage activation. Conversely, numbers of

MR⁺, YM1⁺, and Arg I⁺ anti-inflammatory macrophages were decreased. These data indicate that FXR plays a role in limiting lung inflammatory responses to ozone.

Taken together, these studies demonstrate that multiple mechanisms contribute to pro-inflammatory and anti-inflammatory lung macrophage accumulation and activation in the lung following ozone exposure. Identification of the origin of inflammatory macrophages and of mechanism mediating their activation may be important in the development of novel therapeutics aimed at selectively targeting these cells and reducing inflammatory lung injury.

DEDICATION

This work is dedicated to my mother, Suzy, who always loved me unconditionally. I could not have done this without you. This work is also dedicated to my husband, David, who was so supportive during the challenges of graduate school and life. I am truly thankful for having them in my life.

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ABBREVIATIONS

4-HNE	4-Hydroxy-2-Nonenal
AA, ω -6	Arachidonic Acid
ABCA1	ATP-Binding Cassette Transporter A1
ABCB11	ATP-Binding Cassette Transporter 11
ABCB4	ATP-Binding Cassette Transporter 4
ABCG1	ATP-Binding Cassette Transporter G1
ADAM	A Disintegrin and Metalloprotease
ADAM10	A Disintegrin and Metalloprotease 10
ADAM17	A Disintegrin and Metalloprotease 17
AKRs	Aldo-Keto Reductases
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog 1
ALDH	Aldehyde Dehydrogenase
AP-1	Activator Protein 1
ApoE	Apolipoprotein E
ASC	Apoptosis Associated Speck-like Protein
AT1R	Angiotensin 2 Type 1 Receptor
BAL	Bronchoalveolar Lavage
BARE	Bile Acid Response Elements
CCL17	Chemokine (C-C) Motif Ligand 17
CCL18	Chemokine (C-C) Motif Ligand 18
CCL19	Chemokine (C-C) Motif Ligand 19
CCL2	Chemokine (C-C motif) Ligand 2

CCL22	Chemokine (C-C) Motif Ligand 22
CCL24	Chemokine (C-C) Motif Ligand 24
CCL5	Chemokine (C-C motif) Ligand 5
CCR2	Chemokine (C-C) Motif Receptor 2
CD11c	Cluster of Differentiation 11c
CD36	Cluster of Differentiation 36
CD38	Cluster of Differentiation 38
CD4	Cluster of Differentiation 4
CD62L/L-selectin	Cluster of Differentiation 62 Ligand
CD8	Cluster of Differentiation 8
cIAP1	Cellular Inhibitor of Apoptosis 1
cIAP2	Cellular Inhibitor of Apoptosis 2
COX-2	Cyclooxygenase-2
Csf1r/CD115	Colony Stimulating Factor-1 Receptor
CX3CL1/Fractalkine	Chemokine (C-X3-C) Motif Ligand 1
CX3CR1	Chemokine (C-X3-C) Motif Receptor 1
CXCL10	Chemokine (C-X-C) Motif Ligand 10
CXCL12	Chemokine (C-X-C) Motif Ligand 12
CXCL13	Chemokine (C-X-C) Motif Ligand 13
CXCL9	Chemokine (C-X-C) Motif Ligand 9
Cypb5	Cytochrome <i>b</i> ₅
DAMPs	Damage Associated Molecular Patterns
DHN	4-Dihydroxy-2-Nonene

DNA	Deoxyribonucleic Acid
eNOS	Endothelial Nitric Oxide Synthases
EPA	United States Environmental Protection Agency
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas-Associated Protein with Death Domain
FEV1	Forced Expiratory Volume in One Second
FGF-19	Fibroblast Growth Factor-19
Fizz1	Resistin Like Alpha
FVC	Forced Vital Capacity
FXR	Farnesoid X Receptor
Gal-3	Galectin-3
GdCl ₃	Gadolinium Chloride
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GSH	Glutathione
GSSG	Oxidized Glutathione
GSTs	Glutathione-S-Transferases
HNA	4-Hydroxy-2-Nonenoic Acid
HNF4/NR2A1	Hepatic Nuclear Factor 4
HO-1	Heme Oxygenase-1
ICAM	Intercellular Adhesion Molecule
IFN- γ	Interferon Gamma
IL-10	Interleukin-10
IL-10R	Interleukin-10 Receptor

IL-13	Interleukin-13
IL-13R α 1	Interleukin-13 Receptor α 1
IL-13R α 2	Interleukin-13 Receptor α 2
IL-15R	Interleukin-15 Receptor
IL-1R	Interleukin -1 Receptor
IL-1 α	Interleukin -1 α
IL-1 β	Interleukin -1 β
IL-4	Interleukin-4
IL-4R α	Interleukin-4 Receptor α
IL-6	Interleukin -6
IL-6R	Interleukin-6 Receptor
iNOS	Inducible Nitric Oxide Synthases
INT-747	Obeticholic Acid
IRF	Interferon Regulatory Factors
IRF-1	Interferon Regulatory Factors-1
IRF-8	Interferon Regulatory Factors-8
Jak1	Janus kinase 1
Jak2	Janus kinase 2
LA, ω -6	Oleic Acid
LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharides
LRH-1/NR5A2	Liver Receptor Homolog-1
Ly6C	Lymphocyte Antigen 6C

MCP-1	Monocyte Chemoattractant Protein-1
MDPs	Macrophage/Dendritic Cell Progenitor
MHC	Major Histocompatibility Complex
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF- κ B	Nuclear Factor- κ B
NLRP3	Nucleotide-Binding Domain and Leucine-Rich Repeat Contain
Protein 3	
NLRs	Nucleotide-Binding-Domain and Leucine-Rich-Repeat-Containing
Receptors	
nNOS	Neuronal Nitric Oxide Synthases
NO ₂	Nitrogen Dioxide
NOS	Nitric Oxide Synthases
ONOO ⁻	Peroxynitrite
PAMPs	Pathogen Associated Molecular Patterns
PPAR γ	Peroxisome Proliferator Activated Receptor- γ
PRR	Pattern Recognition Receptors
PUFAs	Polyunsaturated Fatty Acids
RIP-1	Receptor Interacting Protein-1
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SN-2	Nucleophilic Substitution-2
SOCS1	Suppressor of Cytokine Signaling 1
SOCS3	Suppressor of Cytokine Signaling 3

SOD	Superoxide Dismutase
STAT1	Signal Transducers and Activators of Transcription 1
STAT-1 α	Signal Transducers and Activators of Transcription-1 α
STAT6	Signal Transducers and Activators of Transcription 6
TACE	Tumor-Necrosis-Factor- α Converting Enzyme
TGF- β	Tumor Growth Factor β
TLR4	Toll-Like Receptor 4
TLRs	Toll-Like Receptors
TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
TNF- α	Tumor Necrosis Factor- α
TRADD	Tumor Necrosis Factor Receptor Type 1-Associated DEATH Domain Protein
TRAF-1	Tumor-Necrosis-Factor-Receptor Associated Factor 1
TRAF-2	Tumor-Necrosis-Factor-Receptor Associated Factor 2
TRAF-3	Tumor-Necrosis-Factor-Receptor Associated Factor 3
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VOCs	Volatile Organic Compounds
Ym1	Chitinase-Like 3

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INTRODUCTION

RESPIRATORY TRACT STRUCTURE AND CELLS

The respiratory tract includes the nose, mouth, throat and pharynx (upper); the respiratory airways, larynx, trachea, bronchi, bronchioles (middle) and the lung (lower). Beginning with the trachea, it consists of a series of branching airways; thus the trachea branches into two main bronchi - the left and right superior lobar bronchus, which branch into secondary and tertiary bronchi. These branch into the airways and subsequently the gas exchange units. Exchange of oxygen and carbon dioxide takes place in the respiratory bronchioles, the alveolar ducts and the alveolar sacs. Inhaled oxygen enters the lung and reaches the alveoli. The cells lining the alveoli and the surrounding capillaries are one layer thick, which facilitates their close contact. Oxygen passes quickly through the air-blood barrier in the alveoli to the surrounding capillaries. Carbon dioxide passes from the blood into the alveoli, which is exhaled. Alveoli are comprised of multiple cell populations each of which performs functions essential for gas exchange. These cells include Type I and Type II alveolar epithelial cells, and alveolar macrophages.

Type I cells are involved in gas exchange. These cells are well suited for this function as a result of their thin, elongated structure. Although these large squamous cells account for ~10% of lung cells, they cover approximately 98% of the internal surface area making them highly sensitive to injury (Dobbs et al., 2010). Evidence suggests that Type I cells are terminally differentiated from Type II cells (Weiss et al., 2014). In addition to their barrier and gas exchange functions, Type I cells possess ion channels (Na/K-ATPase) and are capable of ion and water transport, suggesting that these cells play an important role in fluid reabsorption in the lung (Johnson *et al.*, 2006).

Additionally, Type I cells are capable of proliferating *in vivo*. This suggests that Type I

cells may contribute to lung homeostasis and the response to injury. Type II cells function as progenitors for injured Type I cells (Fujino *et al.*, 2011). They have a cuboidal morphology and are smaller than Type I cells. They are localized primarily at the corners of the alveolar sacs. Type II cells produce, secrete, and recycle the proteins and lipids that comprise pulmonary surfactant, including surfactant protein (SP)-B and SP-C. These function to ensure that the surface tension across the alveoli remains low. SP-A and SP-D are also produced by Type II cells. These are pulmonary collectins important in regulating the

innate immune system. Collectively, Type II cells allow effective gas exchange and prevent pulmonary edema. In addition, Type II cells are involved in alveolar defense by secreting antimicrobial and inflammatory mediators. These include lysozyme, beta defensins 2, cathelicidin, lipocalins, glutathione, complement, reactive nitrogen species (RNS), chemokines such as CCL2 and CCL5, growth factors, and cytokines such as interleukin (IL)-1 β , IL-1 α , tumor necrosis factor (TNF)- α , and IL-6 into the alveolar space (Herzog *et al.*, 2008; Mason, 2006; Whitsett *et al.*, 2010). Deficits in Type II cell functioning disrupt surfactant homeostasis and underlie the pathogenesis of pulmonary disorders such as acute respiratory distress syndrome, interstitial lung disease and pulmonary alveolar proteinosis (Vece and Young, 2016).

Macrophages are present throughout the lung. Several distinct subpopulations have been identified including alveolar macrophages in the alveolus and interstitial macrophages in the lung parenchyma. Alveolar macrophages function as sentinels of the immune system, rapidly responding to inhaled pathogens, particulate matter and environmental toxins (Hiraiwa and van Eeden, 2013). Alveolar macrophages also have

the ability to present antigen to lymphocytes, and to release cytokines, chemokines, oxidants, proteases, and bioactive lipids with pro-inflammatory or anti-inflammatory activity. These include reactive oxygen species (ROS), reactive nitrogen species (RNS), inflammatory cytokines, proteases and bioactive lipids (Lumeng and Saltiel, 2011). Interstitial macrophages possess immunoregulatory functions, and are important for maintenance of immune homeostasis in the lung (Bedoret *et al.*, 2009). Interstitial macrophages are smaller and more uniform in size than alveolar macrophages; they appear more similar to peripheral monocytes (Landsman *et al.*, 2007; Misharin *et al.*, 2013). Interstitial macrophages are in contact with extracellular matrix and connective tissue. While alveolar macrophages are responsible for removing particles from the alveoli, interstitial macrophages are involved in antigen presentation. Upon stimulation, interstitial macrophages interact with T-cells through major histocompatibility (MHC) proteins and they express anti-inflammatory cytokines (Bedoret *et al.*, 2009) (Gea-Sorli *et al.*, 2011). The ability of both alveolar and interstitial macrophages to phagocytize particulate matter and microbes, to secrete cytokines, proteases, ROS and RNS, and to act as antigen presenting cells, enable them to initiate robust inflammatory responses and to restore lung homeostasis (Guth *et al.*, 2009).

Unique features of the lung environment dictate the characteristic phenotype of lung macrophages. In mice, alveolar macrophages are distinguished from interstitial macrophages by their high CD11c expression (Zaynagetdinov *et al.*, 2013). Alveolar macrophages from mice are poor antigen presenting cells and have reduced phagocytosis compared to other macrophages. In addition, unlike other macrophages, alveolar macrophages express peroxisome proliferator-activate receptor- γ , which suggests that

they contribute to lipid metabolism and airway homeostasis (Gautier *et al.*, 2012; Malur *et al.*, 2011).

INFLAMMATION

Inflammation is the response of the immune system to injurious or infectious agents. The four cardinal signs of inflammation are swelling, redness, heat, and pain. These are due to vascular dilation and permeability and increase hydrostatic pressure across the vascular bed (Butterfield *et al.*, 2006). The goal of the inflammatory response is to restrict tissue injury and infection, and to initiate wound healing.

Inflammatory responses to infection and injury are distinct. In infection-driven inflammation, neutrophils and macrophages recognize molecular structures present on the surface of pathogens, called pathogen associated molecular patterns (PAMPs) which bind to pattern recognition receptors (PRR). These PRRs include toll-like receptors (TLRs), which are highly conserved cell surface and extracellular receptors containing leucine rich-repeats (Mogensen, 2009). These leucine repeats are involved in recognition and signaling. Ligand binding to TLR initiates intracellular signaling leading to activation of nuclear factor- κ B (NF- κ B), a transcription factor which regulates the production of pro-inflammatory mediators. Inflammation can also be triggered by injury, a process referred to as sterile inflammation. Stressed or damaged cells secrete damage associated molecular patterns (DAMPs). These include molecules such as hyaluronin and glycosaminoglycan (Nikitovic *et al.*, 2015). These DAMPs activate PRR such as TLR on responding inflammatory phagocytes. Intracellular nucleotide binding domain and

leucine-rich-repeat-containing receptors (NLRs) also respond to DAMPs (Proell *et al.*, 2008)

In the acute inflammatory response, resident macrophages are the first to recognize DAMPs and PAMPs. After PPR binding, these cells become activated, releasing multiple inflammatory mediators that initiate signaling cascades. For example, vasoactive amines such as histamine and eicosanoids are secreted to interact with the local endothelium and induce vasodilation (Medzhitov, 2008). Vasodilation allows greater blood flow to increase accessibility to the site of inflammation for immune cells. Increased access to leukocytes that are normally restricted to the blood vessels is essential in the promotion of inflammation. Activated resident macrophages also release mediators that upregulate cell adhesion molecules such as intercellular adhesion molecule (ICAM), P-selectin, and E-selectin on endothelial cells which promote leukocyte localization at sites of injury (Suárez *et al.*, 2010). Endothelial cell surface selectins and adhesion molecules bind to their respective receptors on leukocytes and cause leukocyte rolling, adhesion, and transmigration. Activated endothelial cells and macrophages release lipid mediators and chemokines to attract immune cells toward the site of inflammation. Chemotactic factors can also be released from pathogens or injured tissue.

Neutrophils are the first leukocyte population to arrive at the site of injury/infection. Once neutrophils are localized, they become activated and undergo a respiratory burst and release ROS, antimicrobial peptides such as defensins and proteinases, elastases and other cytotoxic molecules (Faurischou and Borregaard, 2003; Kolaczowska and Kubes, 2013). Infiltrating neutrophils are important at the initial stage of inflammation and pathogen clearance. However, they quickly undergo apoptosis

(Kolaczowska and Kubes, 2013). Subsequently, circulating monocytes are recruited to injured tissues and mature into macrophages. These mononuclear phagocytes are longer lived than neutrophils and remain at sites of inflammation (Zhang and Mosser, 2008). Macrophages assist in the clearance of remaining microbes and damaged cells by producing cytotoxic mediators such as ROS and RNS. Macrophages are also critical in maintaining the inflammatory response by releasing pro-inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 , while also promoting leukocyte recruitment by secreting chemokines (Zhang and Mosser, 2008).

Acute inflammatory responses should result in elimination of the infectious or injurious agent followed by a resolution and repair phase. Resolution of inflammation is mediated by resident and recruited macrophages. The switch in lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins is crucial for the transition from inflammation to resolution. Therefore, the production of pro-inflammatory mediators by macrophages is suppressed while anti-inflammatory/wound repair molecules are released. Several anti-inflammatory mediators have been identified including apolipoprotein E (ApoE) and transforming growth factor beta ($\text{TGF}\beta$) (Zhang *et al.*, 2011). Phagocytosis is also essential for the resolution to inflammation as macrophages play a key role in clearing tissues from harmful exposure to inflammatory and immunogenic contents of dying cells (Serhan *et al.*, 2008).

When acute inflammation doesn't subside, chronic inflammation develops. Chronic inflammation is characterized by the persistence of activated macrophages and lymphocytes at the site of injury or infection. For example, chronic inflammation can be a result from persistent pathogens that were not properly cleared. Chronic inflammation is

a result of exacerbated inflammatory cell activity; this results in perpetuation of tissue injury and potentially fibrosis (Murakami and Hirano, 2012).

INFLAMMATORY MEDIATORS

Reactive Oxygen and Nitrogen Species

ROS and RNS are generated in low amounts during normal metabolic processes and they play physiological roles in signal transduction, activation of transcription factors, mitochondrial electron transport, and iron homeostasis (Mladenka et al., 2009; Ray et al., 2012). Oxidative stress occurs when there is an imbalance between production of ROS and RNS and the activity of antioxidants. Inflammatory macrophages and neutrophils contribute to oxidative stress by generating ROS and RNS in response to stimuli such as IFN- γ , LPS, and TNF- α (Laskin *et al.*, 2011a). Oxidative stress results in damage to proteins, lipids and DNA, and contributes to the pathogenesis of diseases, including sepsis, cardiovascular disease, ischemia-reperfusion injury, cancer, and diabetes (Valko *et al.*, 2007).

ROS include superoxide anion, hydroxyl radicals, hydroperoxy, and hydrogen peroxide (Bayir, 2005). ROS are formed by the addition of an electron to molecular oxygen, leading to the generation of superoxide anion. Superoxide anion cannot cross cell membranes and reacts with surrounding molecules. In the presence of transitional metals such as Fe (II) or Cu (I), superoxide anion form highly reactive hydroxyl radicals via the Fenton reaction. Oxygen radicals include peroxyl radicals and hydroperoxyl, which can initiate cytotoxicity and lipid peroxidation. The enzyme myeloperoxidase is another source of ROS present in neutrophils. In stimulated macrophages, superoxide anion is generated from plasma membrane nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase. Production of ROS contributes to the progression to inflammation and promotes endothelial dysfunction (Thannickal and Fanburg, 2000).

RNS consist of nitric oxide and other oxides of nitrogen including peroxynitrite. Nitric oxide is a reactive gaseous molecule with an unpaired electron that acts as a cellular and intracellular signaling molecule (Gow, 2006). Nitric oxide is generated via nitric oxide synthases (NOS), a family of enzymes that oxidize L-arginine to citrulline and nitric oxide (Laskin et al., 2010). Three isoforms have been identified: inducible (iNOS), neuronal (nNOS), and endothelial (eNOS) (Pautz *et al.*, 2010). nNOS is expressed by nerve cells and plays a role in neuronal signaling. eNOS is constitutively expressed by vascular endothelial cells and has important blood vessel dilation functions (Villanueva and Giulivi, 2010). nNOS and eNOS are constitutively present in certain cell types (Kobayashi, 2010). In contrast, iNOS, is an inducible enzyme, expressed in large amounts by macrophages, that is involved in inflammatory responses (Pautz et al., 2010). Nitric oxide and nitric-oxide-derived RNS are highly reactive, interacting with protein thiols, amine, or hydroxyl groups (Gow *et al.*, 2004). In the presence of superoxide anion, nitric oxide can form peroxynitrite (ONOO^-) and nitrogen dioxide (NO_2). Stimuli that increase ROS production can also influence iNOS expression. iNOS levels are dependent on transcription of its gene. The promoter region of the iNOS gene contains binding sites for multiple transcription factors, including NF- κ B and STAT-1 α (Chen *et al.*, 2015; Qidwai and Jamal, 2010). LPS, IL-1 β , TNF- α , and oxidative stress up-regulate iNOS expression through NF- κ B (Pautz, Art, Hahn, Nowag, Voss and Kleinert, 2010). After iNOS induction, nitric oxide is continuously produced until the enzyme degrades (MacMicking *et al.*, 1997). Another mechanism of iNOS regulation is through control of

iNOS activity. For example, iNOS activity depends on substrate availability.

Consumption of arginine by arginase has been shown to decrease iNOS activity (Mori and Gotoh, 2000).

Cytokines

Cytokines are small proteins that are important in regulating inflammatory cell activity, promoting of cell growth, and stimulating the release of other inflammatory mediators (Dinarello, 2007). Cytokines include lymphokines, monokines, chemokines, and interleukins. Chemokines induce directed migration of target cells. Interleukins are cytokines that are quickly secreted in a response to a stimulus to alter cellular behavior. Collectively, these cytokines can influence the cells that secrete them, nearby cells, or distant cells. Cytokines comprise a large overlapping network in which different cells can secrete the same cytokine or a cytokine can act on various cell types (Dinarello, 2007). This allows flexibility to coordinate the diverse cells in the immune system. To enable communication between cell types, cytokines can act both synergistically or antagonistically to induce a response to a stimulus. Cytokines can be produced during physiological and inflammatory states by a number of different cell types. However, macrophages and helper T cells are the major source of cytokines (Duque et al., 2014).

Pro-inflammatory cytokines are involved in activation of inflammatory responses. TNF- α and IL-1 β are pro-inflammatory cytokines released early in the inflammatory process to promote the inflammatory response. They upregulate expression of adhesion molecules promoting inflammatory cell recruitment and they stimulate the production of other pro-inflammatory mediators (Bradley, 2008; Dinarello, 2007).

TNF- α is a cytokine produced mainly by macrophages. TNF- α is a glycoprotein, originally identified as inducible by LPS, that promoted necrosis of tumor cells. While TNF- α is not detectable under homeostatic conditions, its expression is upregulated during inflammation. TNF- α is synthesized as a 26 kD cell-surface associated protein and cleaved by metalloproteinases, such as tumor necrosis factor- α converting enzyme (TACE), which results in the release of 17 kD soluble TNF- α (Bradley, 2008). TNF- α upregulates the expression of cell adhesion molecules including intercellular adhesion molecule 1 (ICAMs), P-selectin, and E-selectin on endothelial cells (Suárez, 2010).

The effects of TNF- α are mediated by two surface receptors: TNFR1 and TNFR2. TNFR1 mediates the majority of pro-inflammatory and programmed cell death effects of TNF- α . TNFR1 binding to TNF- α recruits receptor interacting protein-1 (RIP-1) and TNFR-associated factor 2 (TRAF-2). TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2 rapidly activate NF- κ B as complex I. Signaling through TNFR1 also initiates apoptotic pathways. TRADD and RIP1 associate with FADD and caspase-8 to form complex II. When NF- κ B is activated by complex I, complex II blocks caspase-8 activity, which allows cell survival. However, if NF- κ B fails to be activated by TNFR1, then complex II will dominate resulting in cell death. TNFR2 signaling promotes cell migration, proliferation, and tissue repair (Aggarwal, 2003). TNFR2 signaling also requires TRAF2 and activation of NF- κ B for transcription of pro-survival genes. TRAF2 subsequently binds to TRAF1, TRAF3, cIAP1, and cIAP2 which promotes cell survival signaling (Cabal-Hierro *et al.*, 2014). TNF- α plays a role in containment of infections, however, excessive amounts of TNF- α contribute to inflammatory diseases. TNF α can stimulate the production of IL-1 and TNF α , as well as other chemokines.

IL-1 β is a cytokine originally discovered as a fever inducing-factor. During inflammation, IL-1 β is cleaved by a multiprotein oligomer called an inflammasome, which consists of nucleotide-binding domain and leucine-rich repeat contain protein 3 (NLRP3), apoptosis associated speck-like protein (ASC), and caspase 1. This complex is responsible for the conversion of pro-caspase 1 to active caspase 1, the enzyme responsible for cleavage of inactive IL-1 β (Lukens *et al.*, 2012). IL-1 β upregulates expression of pro-inflammatory proteins, such as TNF- α , IL-6, chemokines, COX-2, iNOS, adhesion molecules and matrix metalloproteinases (Dinarello, 2005). The biological effects of IL-1 β are mediated by binding to cell surface receptor, IL-1R, which has common signaling proteins as the TLR pathway.

Anti-inflammatory cytokines including IL-4, IL-10, and IL-13 act as specific cytokine inhibitors that function to down regulate the immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Macrophages and monocytes are the main sources of IL-10; while granulocytes and lymphocytes produce IL-4 and IL-13. However, IL-10, IL-4, and IL-13 can also activate macrophages, towards a pro-resolution, wound repair phenotype (Mosser and Edwards, 2008). These cytokines suppress the production of the pro-inflammatory cytokines (IFN- γ , IL-1 β , and TNF- α) (Saraiva and O'Garra, 2010).

Chemokines

Chemokines are cytokines mediating the trafficking of immune and inflammatory cells to sites of injury and infection. Chemokines share a similar organized tertiary structure around cysteine (C) residues. There are four main groups: CC, CXC, CX₃C, and

C. CC chemokines contain two adjacently positioned cysteine residues, whereas CXC chemokines contain two cysteines which are separated by a single amino acid. These two chemokine groups account for the majority of known members of the chemokine family (Allen *et al.*, 2007; Mantovani and Sica, 2010).

Chemokines exert their effects by binding to specific G-protein coupled receptors on responsive cells. Ligand-receptor interactions within the chemokine family are nonspecific. For instance, a chemokine can bind to multiple chemokine receptors and a receptor can be activated by multiple chemokines. This flexibility is important for directing leukocyte trafficking during physiological and inflammatory states. Cells that express chemokine receptors are guided by a chemokine gradient (Callewaere *et al.*, 2007). Chemokines play important roles in physiological and pathological conditions and are functionally classified into “homeostatic” and “inflammatory” chemokines. Homeostatic chemokines control lymphocyte homing and organogenesis under physiological conditions; they are generated constitutively in specific tissues or cells, and include: CXCL12, CXCL13, CCL18, and CCL19 (Zlotnik and Yoshie, 2012). In contrast, inflammatory chemokines are produced in inflamed tissues by resident and infiltrating cells. These chemokines contribute to early inflammatory responses to PRRs on epithelial and immune cells. These inflammatory chemokines are responsible for the recruitment of neutrophils, monocytes, natural killer cells, and lymphocytes to sites of injury/infection (Rot and von Andrian, 2004).

Monocyte chemoattractant protein-1 (MCP-1 or CCL2) is a member of the C-C chemokine family important in migration of monocytes and macrophages, as well as memory T lymphocytes, and natural killer cells. CCL2 can be produced constitutively or

induced by oxidative stress, cytokines, or growth factors. Many cell types, including endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells produce CCL2. These cells are important for antiviral immune responses in the peripheral circulation and in tissues. Because of its involvement with various cell types, CCL2 has been pursued as a potential therapeutic in various diseases, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, and diabetes (Sorensen *et al.*, 2004). CCL2 binds to most CC chemokine receptors, which are expressed on various leukocytes. One chemokine receptor, CCR2 is restricted to monocytes and macrophages (Vestergaard *et al.*, 2004). In mice, CCR2 has been shown to be required for bone marrow egress of pro-inflammatory Ly6C^{Hi} monocytes into the circulation and for monocyte trafficking to sites of inflammation (Tsou *et al.*, 2007). The CCL2-CCR2 axis has also been observed to initiate negative feedback mechanisms that restrict T-cell activation in different inflammatory processes (Flaishon *et al.*, 2004).

Fractalkine (CX3CL1) is the only known member of the CX3C chemokine family. It is produced as a membrane-anchored form that is cleaved to a soluble form by a metalloprotease, a disintegrin and metalloprotease (ADAM). Different proteases from ADAMs are activated during the inflammatory state. ADAM10 constitutively sheds the chemokine, whereas ADAM17 is important under inflammatory conditions (Bourd-Boittin *et al.*, 2009). CX3CL1 is constitutively expressed by multiple cell types including lymphocytes, neurons, microglia, osteoblasts, endothelial and epithelial cells. CX3CL1 binds to CX3CR1⁺ cells, such as NK cells, monocytes, cytotoxic effector T cells, B cells, neurons, microglia, smooth muscle cells, and tumor cells. An important role for the CX3CL1/CX3CR1 axis in inflammation is regulating Ly6C^{Lo} anti-inflammatory

monocytes trafficking. This has been demonstrated in renal diseases, myocardial infarction, and inflammatory bowel diseases (Bain *et al.*, 2013; Peng *et al.*, 2015; Taylor *et al.*, 2014). In atherosclerosis, genetic deletion of CX3CL1/CX3CR1, reduced monocyte accumulation in the artery walls subsequently leading to the development of lesions. However, CX3CL1/CX3CR1 axis is also involved in the pathogenesis of various B cell malignancies, which are induced by inflammation (Corcione *et al.*, 2012).

OXIDATIVE STRESS AND ANTIOXIDANTS

Excessive levels of ROS and RNS that are generated under inflammatory states contribute to injury. These highly reactive cytotoxic species are generated following tissue injury; they are also produced by inflammatory cells such as macrophages and neutrophils. Oxidative stress is a condition that develops when the generation of reactive species overwhelms protective anti-antioxidant defense mechanisms. ROS and RNS can damage proteins, lipids and DNA (Phaniendra *et al.*, 2015). In addition, transcription factors important in the generation of pro-inflammatory mediators, such as NF- κ B and AP-1 are redox sensitive and are activated under conditions of increased oxidative stress (Rahman and MacNee, 2000).

Antioxidant defense mechanisms include enzyme such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. SODs converts superoxide anion to hydrogen peroxide, while catalase generates O₂ and water from hydrogen peroxide (Valko *et al.*, 2007). Glutathione (GSH) is an antioxidant in the cytosol and mitochondria; it reacts with ROS and forms oxidized glutathione (GSSG), which accumulates inside cells. GSH can also act as a cofactor for glutathione peroxidase,

which catalyzes the conversion of reduced GSH to GSSG. Glutathione reductase then recycles GSSG back to GSH. GSH is also able to eliminate highly reactive peroxynitrite, by reduction to nitrate (Rahman et al., 2000). Regulation of reactive species is critical for the resolution of inflammation.

Heme oxygenase-1

Heme oxygenase (HO)-1 is an intracellular enzyme that acts as a defense against heme, a mediator of inflammation (Wagner *et al.*, 2000). High concentrations of heme, which arise as a result of heme catabolism, injure tissues and cells. Heme catabolism is induced by oxidative stress, which drives the breakdown of hemoproteins to free heme (Baker *et al.*, 2003). Heme contains iron (Fe)²⁺ atom that is capable of producing hydroxyl radicals from hydrogen peroxide (Fredenburgh *et al.*, 2007; Gozzelino *et al.*, 2010). HO-1 is upregulated following oxidative stress to catabolize free heme leading to the formation of free iron and biliverdin. Free iron is subsequently neutralized (Baker, Anderson and Baker, 2003). Biliverdin is converted to bilirubin by biliverdin reductase. Bilirubin is an antioxidant that plays an anti-inflammatory roles in the lung. In the lung, HO-1 is expressed by Type II cells and macrophages in response to pro-inflammatory mediators (Fredenburgh *et al.*, 2007).

4-Hydroxy-2-nonenal

4-hydroxy-2-nonenal (4-HNE) is an end product of lipid peroxidation that can alter essential signaling processes. Overproduction of free radicals from ROS induces oxidation of the membrane lipid bilayers, such as the polyunsaturated fatty acids

(PUFAs). In presence of free radicals, cellular membranes break down and form lipid hydroperoxides (Benedetti *et al.*, 1980; Shoeb *et al.*, 2014). Subsequently, hydroperoxides decompose to form reactive lipid electrophiles. The decomposition of hydroperoxide of ω -6 PUFAs at the sn-2 position of glycerophospholipids in cellular membranes leads to the production of 4-HNE. Phospholipids that contain linoleic acid (LA, ω -6) or arachidonic acid (AA, ω -6) within cytoplasmic membranes are considered to be a major source of 4-HNE (West and Marnett, 2006). Among these diverse oxidized lipid products, 4-HNE is one of the most bioactive and well-studied lipid. 4-HNE has the potential to form covalent adducts with nucleophilic functional groups in proteins, nucleic acids, and membrane lipids. There are three major detoxification pathways to convert 4-HNE to a less reactive chemical. These include the formation of adducts with GSH by glutathione-S-transferases (GSTs), the reduction of 4-HNE to 1,4-dihydroxy-2-nonenone (DHN) by aldo-keto reductases (AKRs), or the oxidation of 4-HNE to 4-hydroxy-2-nonenic acid (HNA) by aldehyde dehydrogenase (ALDH) (West and Marnett *et al.*, 2006).

Cytochrome b_5

Cytochrome b_5 (CypB5) is a heme protein that functions as an electron carrier. CypB5 is involved as an electron transfer component in a number of oxidative reactions in biological tissues. These processes include the anabolic metabolism of fats and steroids and catabolism of xenobiotics or endogenous metabolism. CypB5 is an electron donor to NADPH-cytochrome P450 reductase or NADH-cytochrome b_5 reductase. Two isoforms of Cypb5, a microsomal membrane-bound form and a cytoplasmic form, are produced by

alternative splicing. Recently, CypB5 has been detected in the lung following injury (Menoret *et al.*, 2012; Sunil *et al.*, 2015). Thus, after inhalation of *S. aureus* enterotoxin A or ozone, which cause increased capillary permeability, cell damage, and increased protein in the BAL, increases of CypB5 were observed, indicative of acute lung injury

MACROPHAGES

Macrophages are mononuclear phagocytes localized in all tissues of the body. They play a key role in innate immune defense: phagocytizing debris and pathogens, initiating inflammatory responses to infection or injury, and activating the adaptive immune response by processing and presenting antigen.

Resident tissue macrophages perform specialized functions depending on their anatomical location and environment. For instance, osteoclasts are bone marrow macrophages that are involved in bone resorption, whereas alveolar macrophages in the lung function to remove inhaled particles and pathogens. Alveolar macrophages possess a number of unique characteristics; they express high levels of CD11c and have a dendritic appearance. The lung environment is responsible for the distinctive phenotype of alveolar macrophages. Findings that bone marrow cells transplanted into the airways upregulate CD11c expression, support the idea that the microenvironment can control resident macrophage phenotype (Gluth *et al.*, 2009).

Macrophages express numerous cell surface and intracellular receptors that are important in their functioning. These include pattern recognition receptors (PRRs), scavenger receptors, and phagocytosis-related receptors, as well as receptors for cytokines and chemokines. The most prominent PRRs expressed by macrophages are

TLRs. Other PRRs expressed by macrophages include mannose receptor, which binds mannose and fucose-containing structures on bacteria and fungi (Verschoor *et al.*, 2012). Macrophages also express scavenger receptors, such as scavenger receptor-A and CD36, which mediate recognition and phagocytosis of bacteria, apoptotic cells, and endogenous oxidized low-density lipoproteins (Rios *et al.*, 2013; Silverstein *et al.*, 2010). In addition, macrophages express ATP-binding cassette transporter (ABC)A1 and ABCG1, which are responsible for promoting cholesterol efflux. These transporters influence the inflammatory responses to atherosclerosis in mice (Guo *et al.*, 2006b; Wang *et al.*, 2007).

Resident and monocyte-derived macrophages are involved in both the pro-inflammatory/ cytotoxic phase and the anti-inflammatory/wound healing phase of the inflammatory response. During the initial phase, macrophages are activated to release pro-inflammatory/cytotoxic mediators including ROS, RNS, proteases and cytokines to clear pathogens or the injurious agent. Once this is accomplished macrophages modify their phenotype towards an anti-inflammatory and wound healing state. Anti-inflammatory monocytes can also be recruited to inflammatory sites. Resolution of inflammation and the return of tissue homeostasis are mediated by anti-inflammatory/ immunosuppressive (IL-10) and wound healing factors (TGF- β), as well as omega fatty acids-derived local-acting mediators (e.g., lipoxins, resolvins and protectins) released in large part by macrophages. It is thought that the outcome of inflammatory responses depends on the balance between the number and activity of proinflammatory/cytotoxic macrophages and anti-inflammatory/wound healing macrophages (Mantovani and Sica, 2010).

Macrophage Activation

Activated macrophages have been generally classified into two groups, classically activated (M1) macrophages and alternatively activated (M2) macrophages (Cassetta *et al.*, 2011; Mosser and Edwards, 2008). M1 macrophages promote inflammation and mediate antimicrobial defense, while M2 macrophages down regulate inflammation and initiate wound healing. Macrophages adopt an M1 or M2 phenotype as a consequence of exposure to mediators in their microenvironment. Evidence suggests that macrophages are highly flexible and switch from one functional phenotype to another in response to local signaling (Murray and Wynn, 2011).

Pro-inflammatory mediators that polarize macrophages into a classically activated phenotype are defined based on their ability to promote an inflammatory response. Three groups have been identified that induce M1 macrophages: cytokines, pathogen recognition receptors (PRR) agonists, and granulocyte macrophage colony-stimulating factor (GM-CSF). T-helper-1 cells, and to a lesser extent, natural killer cells and macrophages also produce interferon- γ (IFN- γ) during inflammatory responses which drives macrophages to express genes associated with a pro-inflammatory phenotype. Cellular responses to IFN- γ are dependent on IFN- γ receptor 1 and receptor 2. IFN- γ signaling through the IFN γ -receptor results in the recruitment of Janus kinase (Jak)1 and Jak2 adaptors that activate signal transducers and activators of transcription1 (STAT1) and interferon regulatory factors (IRF), such as IRF-1 and IRF-8. Nuclear translocation and activation of STAT1 induces transcription of iNOS, CCL5, CXCL9, and CXCL10 genes (Mosser and Edwards, 2008). IFN- γ also controls specific pro-inflammatory gene expression such as cytokine receptors (IL15R and IL6R) and cell activation markers

(CD36 and CD38). Macrophages derived from mice that lack IFN- γ have reduced production of anti-microbial products; these mice are susceptible to *Mycobacterium bovis* and *Listeria monocytogenes* (Dussurget *et al.*, 2014; Eirin *et al.*, 2015). M1 pro-inflammatory macrophage polarization can also occur in response to bacterial-derived products (Martinez *et al.*, 2008). Pathogens are recognized by PRR on leukocytes which induce pro-inflammatory activities. These responses are due, in large part, to activation of the NF- κ B pathway, which is responsible for upregulating expression of proinflammatory cytokines, such as TNF- α , IL-6, IL-12, and chemokines.

GM-CSF also classically activates macrophages. GM-CSF is produced by a variety of cells, including macrophages. Increased production GM-CSF leads to activation of JAK2/STAT5, NF- κ B, extracellular signal-regulated kinase (ERK), and V-Akt murine thymoma viral oncogene homolog 1 (AKT) (Krausgruber *et al.*, 2011). In addition to similar downstream signaling via IFN- γ and PRR signaling pathways, GM-CSF promotes macrophage antigen presentation, phagocytosis, and microbicidal capacity (Hansen *et al.*, 2008). GM-CSF signaling can also stimulate macrophage production of IL-6, TNF, and IL-1 β .

M1 pro-inflammatory macrophages generate cytotoxic effector molecules, including ROS, RNS, and pro-inflammatory cytokines to protect the host (Martinez and Gordon, 2014). However, excess numbers of M1 macrophages or over production of these mediators can result in tissue injury. In the lung, exposure to ozone has been reported to result in an accumulation of M1 macrophages in the tissue, and the release of pro-inflammatory mediators (Sunil *et al.*, 2012). Elimination of M1 macrophages by treatment of animals with gadolinium chloride (GdCl₃) abrogates ozone-induced

inflammation, reducing expression of iNOS and TNF- α , as well as production of nitric oxide and superoxide anion (Pendino *et al.*, 1995).

An alternative pathway of macrophage activation leads to the generation of M2 macrophages. This is the result of stimulation of macrophages with IL-4 and IL-13, or with IL-10 and decreased levels of pro-inflammatory stimuli (Mosser and Edwards, 2008). Subpopulations of these alternatively activated macrophages can also develop in response to glucocorticoids or immune complexes in combination with LPS (Edwards *et al.*, 2006; Laskin, 2009). M2 macrophages contribute to the resolution of inflammation through multiple macrophage subpopulations. These subpopulations differ in signals that activate them and their phenotype. M2a macrophages are stimulated by IL-4 and IL-13; they are highly phagocytic and proliferative which promotes tissue repair. These macrophages are characterized by increased expression of arginase, mannose receptor, Ym1, Fizz1 in mice and they secrete CCL17, CCL22, CCL24, and TGF- β . M2b macrophages are activated by TLR and IL-1 β complexes and are similar to M2a macrophages. M2b macrophages are also essential for antigen presentation, which leads to increased expression of major histocompatibility complex II. M2c macrophages are stimulated by IL-10 and TGF- β . These cells are immunosuppressive and promote wound repair and tissue remodeling. This population has been reported to express vascular endothelial growth factor (VEGF) and anti-inflammatory mediators (e.g., IL-10) (Martinez *et al.*, 2009; Mantovani *et al.*, 2014; Laskin *et al.*, 2009; Laskin *et al.*, 2011).

IL-10 signals through a specific cell surface receptor (IL-10R) (Sica and Mantovani, 2012). Receptors for IL-4 and IL-13 share a common subunit, IL-4R α . This subunit can dimerize with a gamma subunit to form the type I receptor, or with the IL-

13R α 1 to form the type II receptor. IL-4 can bind to both receptor types, while IL-13 only recognizes the type II receptor. A second IL-13 receptor chain, IL-13R α 2, has been identified, and is thought to represent a decoy receptor for the cytokine (Martinez *et al.*, 2014). Binding of IL-4 and IL-13 to their respective receptors results in activation and nuclear translocation of the transcription factor STAT6, while IL-10R engagement signals through STAT3, leading to upregulation of expression of genes characteristic of alternatively activated macrophages. Peroxisome proliferator activated receptor- γ (PPAR γ) and PPAR δ are a family of transcription factors that control expression of enzymes mediating the synthesis of antiinflammatory lipids associated with alternative macrophage activation. Interestingly, activation of these pathways also leads to expression of molecules important in negative regulation of classical activation-specific genes. Thus, STAT6 activation by IL-4/IL-13 results in upregulation of suppressor of cytokine signaling 1 (SOCS1), which inhibits STAT1-dependent gene expression; IL-4 induces the expression of the transcription factor IRF4, which prevents IRF5-dependent induction of proinflammatory cytokines. Moreover, during classical activation, one of the genes induced via STAT1 is SOCS3, which prevents STAT3-mediated expression of alternative activation genes (Sica and Mantovani, 2012).

Alternatively activated macrophages have also been implicated in tissue injury. Prolonged or exaggerated M2 macrophage responses can contribute to and sustain chronic inflammatory conditions (Laskin *et al.*, 2011). For example, in models of silica and bleomycin-induced pulmonary fibrosis, macrophage expression of markers of M2 activation is increased (Hamilton *et al.*, 2008). Inhibition of M2 macrophages by serum amyloid P attenuates bleomycin-induced fibrosis in mice, while mice deficient in IL-4R,

which is required for M2 activation, do not develop silica-induced fibrosis (Migliaccio *et al.*, 2008; Murray and Wynn, 2011).

M1 and M2 macrophages can also be distinguished by the differential metabolism of L-arginine. In M1 macrophages arginine is converted to citrulline and nitric oxide via iNOS, which is important in host defense against pathogens. In M2 macrophages, arginine plays an important restorative function. It is converted by arginase to urea and ornithine, a precursor for polyamines and proline, which regulate cell proliferation and collagen production. Since the production of nitric oxide and ornithine depends on the availability of arginine, these activities can be reciprocally regulated in macrophages by cytokines that influence macrophage polarization. It has been shown that the M1 cytokines IFN- γ and TNF- α induce iNOS and the synthesis of nitric oxide, whereas the M2 cytokines IL-4, IL-13, and IL-10 induce arginase.

Origin and development of macrophages

The origin of macrophages varies according to developmental stage. During early fetal development in rodents, macrophages are first observed in the yolk sac (Pollard, 2009). Later, myeloid precursors populate the liver, which becomes the primary hematopoietic site (Gordon and Taylor, 2005). Tissue resident macrophages, such as alveolar macrophages, are embryonically established. Resident macrophages express CX3CR1 during development, but subsequently lose expression after birth (Yona and Jung, 2010). Postnatally, the bone marrow becomes the main source of macrophage precursors. Hematopoietic stem cells are the myeloid progenitors in the bone marrow that undergo several commitment stages, including macrophage/dendritic cell progenitor (MDPs) (Gordon and Taylor, 2005). These precursor cell gives rise to monocytes, which

are released into the blood and migrate into tissues where they mature into macrophages (Geissmann *et al.*, 2010). Bone marrow cellularity plays an important role in physiology. This is evident in *op/op* mice, which have a mutation in the *csf1* gene and deformities in growth and skeletal structure (Ryan *et al.*, 2001; Wiktor-Jedrzejczak *et al.*, 1990).

During homeostatic and inflammatory states, the development and survival of monocytes and macrophages is controlled by the growth factor receptor *Csf1r* (colony stimulating factor-1 receptor, CD115), which is expressed on all macrophages and their precursors. *Csf1r* has two ligands, M-CSF and IL-34 (Auffray *et al.*, 2009; Chitu and Stanley, 2006; Lin *et al.*, 2008a). Murine monocytes released from the bone marrow express high levels of the surface antigen, Ly6C. In response to injury or infection these pro-inflammatory Ly6C^{Hi} monocytes localize at inflammatory sites and develop into M1 macrophages (Strauss-Ayali *et al.*, 2007). Under homeostatic conditions, Ly6C^{Hi} monocytes mature into Ly6C^{Lo} anti-inflammatory monocytes, which remain in the blood, functioning to patrol the endothelium (Auffray *et al.*, 2007). CCR2 is a chemokine receptor that mediates monocyte egress from the bone marrow; it also plays a key role in monocyte localization at sites of injury and infection (Crane *et al.*, 2009). Whereas CCR2 is only expressed on myeloid cells, its ligand CCL2 is widely expressed and is upregulated and released at sites of inflammation. This leads to Ly6C^{Hi} monocyte/macrophage recruitment.

The spleen is a lymphoid organ that functions to filter blood and remove aging erythrocytes; it is also involved in immune homeostasis. Evidence suggests that the spleen also functions as an extramedullary site of hemopoiesis. Following injury or infection, splenic monocytes are rapidly deployed (Swirski *et al.*, 2009). Monocytes

originating from bone marrow and spleen are distinct. Thus, bone marrow derived monocytes have been shown to be less mature when compared to their splenic counterparts (Wang *et al.*, 2013). Monocytes are also deployed from the bone marrow and spleen through distinct mechanisms. Thus, while bone marrow monocytes depend on CCR2/CCL2, spleen monocytes migration is mediated by angiotensin-II released at sites of injury or infection, and its receptor, angiotensin receptor (AT1R) expressed on spleen monocytes (Swirski *et al.*, 2009).

Monocyte-derived macrophages in inflammation

Monocytes and resident tissue macrophages were originally considered to originate from the same source. However, it is now known that monocytes and resident macrophages are unique cell types (Hashimoto *et al.*, 2013; Kurihara *et al.*, 1997). Thus, monocytes represent a source of newly recruited macrophages responding to inflammatory triggers (Robbins and Swirski, 2010). They mediate pathological and inflammatory processes. Monocytes and monocyte-derived macrophages have been implicated in various inflammatory diseases. Increased numbers of monocytes and monocytosis are indicators of an inflammatory response. Identification of monocyte involvement has led to potential therapies for cardiovascular diseases, autoimmune disorders, and inflammatory bowel diseases (Getts *et al.*, 2014; Nicholson *et al.*, 2009).

Like macrophages, monocytes are a heterogeneous and plastic population of cells whose activity varies according to signals in the microenvironment (Nahrendorf *et al.*, 2007). In mice, two main subpopulations of monocytes have been identified, based on the expression levels of the surface marker Ly6C (Nahrendorf *et al.*, 2007). The exact

function of Ly6C in monocytes is unclear, but it is a cell surface glycoprotein expressed on CD4⁺ T cells during infection, and it may play a role homing of memory CD8⁺ T cells to lymph nodes (Hanninen *et al.*, 2011; Hu *et al.*, 2015). Ly6C^{Hi} monocytes are considered pro-inflammatory. Low numbers of Ly6C^{Hi} monocytes are observed in the lungs during homeostatic states (Gibbons *et al.*, 2011). Experiments that track Ly6C^{Hi} monocytes have provided evidence that these monocytes have a short life span and are preferentially recruited into tissues where they develop into macrophages (Geissmann *et al.*, 2010). Ly6C^{Hi} also expresses high levels of CCR2 and L-selectin (CD62L), but low levels of CX3CR1. Ly6C^{Lo} monocytes are anti-inflammatory; they express low levels of CCR2 and CD62L, but high levels of CX3CR1. In contrast to Ly6C^{Hi} monocytes, Ly6C^{Lo} monocytes have a longer lifespan. Ly6C^{Hi} monocytes are thought to be more immature, which represent cells which have been recently released into the blood; Ly6C expression is downregulated as monocytes mature (Sunderkotter *et al.*, 2004).

Monocytes arise from myeloid precursors in lymphoid organs. In mice, inflammatory states can stimulate production of monocytes from bone marrow and spleen (Robbins and Swirski, 2010; Yona and Jung, 2010). The bone marrow houses Ly6C^{Hi} monocytes during homeostatic states; these egress during inflammatory responses. Ly6C^{Hi} monocyte migration is dependent on CCR2 (Serbina and Pamer, 2006). Recent evidence demonstrates that the spleen stores monocytes as a reservoir for inflammatory conditions (Swirski *et al.*, 2009). It is unclear if the bone marrow and spleen contribute to different monocyte pools during inflammation. Once localized in inflamed tissue, Ly6C^{Hi} monocytes differentiate into classically activated pro-inflammatory macrophages. CCR2-dependent influx of Ly6C^{Hi} monocytes into the brain is associated with increased

mortality during West Nile virus-induced encephalitis, diabetic renal injury, and myocardial infarction (Awad *et al.*, 2011; Dutta *et al.*, 2015; Getts *et al.*, 2008). Ly6C^{Hi} macrophages have also been implicated in hepatotoxicity induced by acetaminophen and ozone-induced lung injury (Dragomir *et al.*, 2012) (Sunil *et al.*, 2015).

OZONE

Ozone is present in low concentrations throughout the Earth's atmosphere. In the stratosphere, ozone plays a protective role by absorbing harmful solar UV. In contrast, high levels of ozone formed in the troposphere when air pollutants such as nitrogen oxides (NO_x) and volatile organic compounds (VOCs) are broken down by high energy UV radiation, generate radicals that can induce pulmonary toxicity. As ozone has limited solubility in water, most inhaled ozone does not affect the upper respiratory tract. Rather, the majority of inhaled ozone reaches the lower respiratory tract and dissolves in the thin layer of epithelial lining fluid in the conducting airways. Ozone is known to alter lung function (Alexeeff *et al.*, 2007; Suh *et al.*, 2000). Increases in ozone levels are associated with increased respiratory-related emergencies, especially in vulnerable and elderly populations. Regulations have been established which limit air quality standards for ground-level ozone. In 1997, the EPA reduced the National Ambient Air Quality Standard for ozone from 0.12 ppm to 0.08 ppm for an exposure from one hour per year due to evidence of adverse health effects, such as chest tightness, cough, and wheezing. In 2008, the EPA further lowered the standard to 0.075 ppm based on evidence that 0.08 ppm reduced lung function and increased airway inflammation in healthy adults (Kim *et al.*, 2011).

Based on the continued correlation between ozone and lung inflammation, EPA recently revised the Ambient Air Quality Standard to 0.07 ppm. Ozone is known to oxidize phospholipids resulting in the formation of cytotoxic oxysterols (Pulfer *et al.*, 2005; Uhlson *et al.*, 2002). Ozone can also react with proteins and lipids leading to the generation of reactive oxygen species (ROS) which cause lipid peroxidation and protein adduct formation, alterations in cell signaling, oxidation of proteins, damage to DNA, and activation of redox sensitive transcription factors. The lower airways and bronchiolar alveolar regions of the lung are thinner; making epithelial cells in these regions more susceptible to ozone-induced damage (Muller *et al.*, 2013). Oxidative species generated via ozone can directly injure lung epithelial cells initiating a cascade of reactions that result in tissue damage, inflammation, alterations in host defense and alveolar-barrier dysfunction (Migdal and Serres, 2011). Inflammatory cells are recruited to sites of injury which contribute to further injury.

Alveolar epithelial damage and blood-air barrier disruption are characteristic of ozone-induced lung injury (Bhalla, 1999; Foster *et al.*, 1996; Lippmann, 1989). Epithelial barrier disruption has been described in humans after exposure to ambient levels of ozone which may increase susceptibility to pulmonary infections and airway injury (Bhalla, 1999; Foster *et al.*, 1996). A marker of alveolar epithelial damage is increases in bronchoalveolar lavage fluid (BAL) protein content and cell number.

Pulmonary Function and Ozone-Induced Lung Injury

Lung function is an objective marker of the type and severity of respiratory pathologies (Pellegrino *et al.*, 2015). Ozone inhalation has been shown to affect pulmonary function and result in airway hyperreactivity and compromised immune function. Increased incidence and exacerbation of lung disease (asthma and chronic obstructive pulmonary disease) has been reported in areas with high ozone (Que *et al.*, 2011).

A widely utilized method to assess lung function is spirometry which provides information on lung capacity by forced vital capacity (FVC) and forced expiratory volume in one second (FEV1). In humans, ozone exposure can result in airway hyperreactivity and resistance, as well as decreases in respiratory frequency, FEV1, and FVC (Que *et al.*, 2011). The severity of these responses depends on both the concentration and the duration of the ozone exposure. Physical activity can exacerbate these responses (Bernstein *et al.*, 2004). In addition to the deterioration of pulmonary function, ozone exposure also increases bronchial reactivity in humans (Foster *et al.*, 1996)..

In animal models, ozone exposure results in increased airway resistance and reduced compliance. Following ozone exposure, increased airway resistance and airway hyperresponsiveness have been observed at baseline and in response to constrictive agents such as methacholine (Savov *et al.*, 2004). In lungs of ozone-treated mice, airway hyperreactivity is observed which correlates with persistent inflammation. This is evident most prominently in mice lacking surfactant protein D, a pulmonary collectin known to suppress macrophage inflammatory activity in the lung, (Groves *et al.*, 2013; Groves *et*

al., 2012b); these data demonstrate that alterations in pulmonary mechanics correlate with lung inflammation.

Macrophages and Ozone Toxicity

Evidence suggests that macrophages play a role in ozone toxicity (Al-Hegelan *et al.*, 2011). These cells accumulate in the lung and become either classically or alternatively activated which contributes to promoting or resolving injury. Macrophages produce pro-inflammatory cytokines, such as TNF- α and IL-1 β , after in vitro exposure to ozone (Arsalane *et al.*, 1995). Similarly, following exposure of rats to ozone, lung macrophages produce increased amounts of pro-inflammatory cytokines, including IL-1 and TNF- α (Pendino *et al.*, 1994; Laskin *et al.*, 2010). TNF- α can promote ROS production which is thought to contribute to lung injury (Bradley, 2008). Findings that mice lacking TNFR1, the receptor for TNF- α , are protected from ozone induced inflammation and injury demonstrate the importance of this cytokine in toxicity (Chitu and Stanley, 2006). The transcription factor, NF- κ B, regulates the production of TNF- α . Macrophages from mice deficient in NF- κ B produce reduced amounts of nitric oxide and TNF- α ; this is associated with attenuated ozone-induced lung injury (Fakhrzadeh *et al.*, 2004). Accumulating data indicate that macrophages can also play a protective role following ozone-induced lung injury by clearing pathogens and debris by phagocytosis and releasing cytokines and chemokines to modulate the inflammatory response (Dahl *et al.*, 2007). They also augment lung antioxidant activity and release mediators that suppress inflammation and initiate wound repair (Laskin *et al.*, 2011).

Ozone-induced injury results in the release of DAMPS, such as hyaluronan, from injured cells and tissue (Garantziotis *et al.*, 2009). Oxidized fragments of hyaluronan are ligands for TLR4. TLR4 deficient mice treated with hyaluronan were protected from airway hyperresponsiveness and lung inflammation (Garantziotis *et al.*, 2009).

Hyaluronan-activated TLR4 initiates cell signaling pathways which lead to activation of NF- κ B and increased expression of TNF- α and iNOS (Akira and Hemmi, 2003; Lorne *et al.*, 2010). Macrophages and inflammatory mediators involved in the pathogenesis of ozone-induced lung injury have been reported to be suppressed in mice with non-functional TLR4, a response associated with reduced toxicity (Conner *et al.*, 2012).

Galectin-3 (Gal-3) is a glycoprotein involved in promoting inflammation.

Increased numbers of Gal-3⁺ macrophages have been observed in the lung after ozone exposure in mice (Sunil *et al.*, 2015). Moreover, loss of Gal-3 results in a decrease in pro-inflammatory macrophages and an increase in anti-inflammatory macrophages, a response correlated with reduced lung injury. Ozone exposure has also been shown to lead to increases in populations of lung macrophages that express fractalkine receptor (CX3CR1), a marker of anti-inflammatory/ wound repair macrophages (Dahl *et al.*, 2007). Loss of CX3CR1 results in reduced numbers of anti-inflammatory macrophages in the lung, which is associated with increased lung injury. These data demonstrate that anti-inflammatory macrophages play a protective role in the lung by scavenging oxidized lipids and promoting wound repair generated from exposure of lung lining fluids to ozone (Dahl *et al.*, 2007). Taken together, these studies show that multiple subpopulations of macrophages contribute to the pulmonary response to ozone.

FARNESOID X RECEPTOR (FXR)

FXR is an important regulator of metabolic pathways (Fuchs et al., 2013). It is highly expressed in the liver, intestine, kidney, and adrenals. Recent studies have shown that FXR contributes to global signaling pathways involved with bile acids (Lien et al., 2014). Bile acids are steroid-like molecules that are products of cholesterol degradation. Bile acids play a role in glucose and lipid metabolism, energy homeostasis, and immune responses.

Bile acids are involved in bile formation and of cholesterol metabolism. They are synthesized in the liver and transported to the intestines. Bile and bile acids help with the absorption of dietary fats and lipid-soluble molecules. Bile acids are returned to the liver after absorption via the enterohepatic circulation (Matsubara et al., 2014). Transport of bile acids is tightly regulated. In excess, bile acids can induce hepatocyte cytotoxicity. However, bile acids are also essential to promote liver regeneration and repair after injury and for maintaining homeostasis (Modica et al., 2010; Li and Chiang, 2014). Tight regulation of bile acids and bile lipids are accomplished by nuclear receptors. FXR is a member of the nuclear receptor family that is activated at a high concentrations of bile acids. Livers from mice that lack FXR exhibit increased cytotoxicity, DNA oxidative damage, inflammation, and NF- κ B activation (Perez and Briz, 2009). FXR activation by bile acids leads to bile acid synthesis, conjugation, and transport.

Bile acid synthesis begins with CYP7A1 mediated hydrolysis of cholesterol to 7 α -hydroxycholesterol, which is subsequently converted into cholic acid and chenodeoxycholic acid (Russel, 2003). Bile acid synthesis and movement are highly regulated (Lefebvre et al., 2009). CYP7A1 is the first rate-controlling enzyme and is

responsible for negative bile acid-dependent feedback loop. CYP7A1 is regulated by activation of a bile acid response elements (BARE) in its promoter (Russel and Setchell, 1992; Chiang et al., 2000). FXR activation induces expression of nuclear receptor small heterodimer partner (SHP or NR0B2), which can repress CYP7A1 expression through the BARE region (Lu et al., 2000; Goodwin et al., 2000). SHP repression of CYP7A1 gene transcription occurs by promoting the dissociation of coactivators linked to hepatic nuclear factor 4 (HNF4 or NR2A1) and the liver receptor homolog-1 (LRH-1 or NR5A2). FXR also regulates CYP7A1 expression by induction of fibroblast growth factor-19 (FGF-19) expression. FGF-19 subsequently activates the hepatic FGF receptor-4 to downregulate CYP7A1 (Yu et al., 2000; Holt et al., 2003). FXR is also involved with bile acid transport. FXR activation increases bile acid efflux from the liver by upregulating ABCB11 and ABCB4 transporters (Modica and Moschetta, 2006).

Activation of FXR by elevated bile acid concentrations has been shown to accelerate liver regeneration and wound healing; whereas, decreased bile acids and loss of FXR inhibit liver growth. Mice that lack FXR spontaneously develop liver tumors as they age; bile acid-induced DNA damage limits liver regeneration (Guodong and Guo, 2015). Hepatic inflammation is linked to hepatocarcinogenesis. Mice that lack FXR suffer from liver inflammation and spontaneous liver tumors. Therefore, the mutual suppression between FXR and NF- κ B may be essential for prevention of tumorigenesis and chronic inflammation. Targeting FXR pharmacologically may provide a novel approach to tissue regeneration and wound healing (Kong et al., 2012; Bhushan et al., 2013).

Farnesoid X Receptor and Macrophages

Nuclear receptors are recognized to play a role in macrophage activation and function. Activation of PPAR γ and the liver X receptor by lipid mediators antagonizes the expression of inflammatory cytokines, which limit macrophage activation by pathogen-associated molecular patterns (Ricote et al., 1998; Joseph et al., 2003). FXR has also been shown to down regulate inflammation; this due in part to deactivation of the transcription factor NF- κ B, which is known to regulate inflammatory gene expression (Liu et al., 2012; Carr et al, 2015). Several macrophage genes are inhibited by FXR ligands; these are established targets for NF- κ B and AP-1, which are regulators in cells of innate immunity and adaptive immunity (Taylor et al., 1998). This is supported by findings of increased levels of inflammatory cytokines in FXR null mice relative to wild type mice and that the FXR agonist, GW4064 inhibits NF κ B regulated genes (Nam et al, 2007; Wang et al., 2008). Treating BALB/c mice with another FXR agonist, INT-747, protects against the development of intestinal inflammation by reducing cytokines IL-1 β and IL-6 (Strober et al., 2002).

FXR activation suppresses pro-inflammatory TLR4 gene expression and TLR4 regulated genes (Chan et al., 2005; Vavassori et al., 2009). In addition, FXR regulates lung tissue repair after LPS-induced injury (Zhang et al., 2012). FXR activation strongly suppresses the activity of NF- κ B. FXR may suppress p65 by decreasing its DNA binding activity. Bile acids induce hepatic inflammation and can activate NF- κ B; this is accomplished by increasing DNA binding to NF- κ B (Li et al., 2004; Shah et al., 2006). This has been observed in cholestasis, where bile acid movement is obstructed. Therefore, FXR can decrease bile acid–induced hepatotoxicity through the reduction and

transport of bile acids. Surprisingly, FXR does not suppress the NF- κ B-regulated anti-apoptotic genes. This selective control by FXR suggests a key role in hepatoprotection.

FXR is expressed on macrophages and affects macrophage lipid activity (Guo et al., 2006). CD36, a scavenger receptor responsible for uptake of oxidized LDL, present on macrophage is regulated by FXR (Zhang et al., 2006). FXR has also been shown to play a role in macrophage activation. Activation of FXR in macrophages reduces pro-inflammatory cytokine production induced by LPS (Bishop-Bailey et al., 2004; Mencarelli et al., 2009). Alterations in FXR expression might have impact in the pathogenesis of human diseases.

RATIONALE

Ozone is a ubiquitous urban air pollutant generated as a component of photochemical smog. It is a highly reactive molecule that induces cytotoxicity through the generation of free radicals and oxidation of proteins, lipids, and DNA (Vallyathan and Shi, 1997; Mudway and Kelly, 2000). Inhalation of ozone results in disruption of alveolar-epithelium barrier function, leading to compromised epithelial defense and increased airway hyperactivity (Vincent et al., 1997). Evidence suggests that macrophages accumulating in the lung after ozone exposure contribute to both lung injury and repair (Pendino et al., 1995; Fakhrzadeh et al., 2002; Groves et al., 2013; Sunil et al., 2015). These activities are mediated by distinct populations broadly classified as pro-inflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages (Byers and Holtzman, 2011; Laskin et al., 2011). The present studies are focused on tracking macrophage accumulation in the lung and identifying their origin and activity.

Initially, we analyzed the spleen as an extramedullary source of inflammatory cells responding to ozone-induced lung injury. The spleen plays a role in the maturation and deployment of monocytes in response to inflammatory signals (Swirski et al, 2009). In contrast to bone marrow derived monocytes, splenic monocyte migration is mediated by angiotensin II receptor (ATR-1 α) and angiotensin II (AT-2) released from damaged tissues (Nataraj et al., 1999; Leuschner et al., 2011). We speculated that following ozone exposure, cells accumulating in the lung originate from the spleen and experiments were designed to test this hypothesis. Identification of inflammatory cell populations that

participate in the pathological response to ozone and their origin may lead to the development of novel approaches for treating oxidative lung injury.

The bone marrow has been established as a major source of inflammatory monocytes that accumulate at sites of tissue injury. Migration of these cells is regulated by chemokines and chemokine receptors that direct their trafficking to sites of inflammation. The present studies demonstrate that the bone marrow functions as a source of both pro-inflammatory and anti-inflammatory macrophages that contribute to ozone toxicity in the lung. The chemokine receptor, CCR2 plays an essential role in monocyte egress from the bone marrow and localization at sites of injury (Tsou et al., 2007). Pro-inflammatory CCR2⁺ bone marrow monocyte migration occurs in response to the chemokine CCL2 released at sites of injury. Once localized at these sites, CCR2⁺ monocytes, differentiate into pro-inflammatory/cytotoxic macrophages. Similar to CCR2⁺ monocytes, these cells express high levels of the surface antigen, Ly6C (Gordon and Taylor, 2005). Trafficking of anti-inflammatory/wound repair macrophages is dependent on CX₃CR1 and its ligand, fractalkine. CX₃CR1 influences macrophage accumulation both in the steady state and following injury (Geissmann et al., 2003). In contrast to CCR2⁺ pro-inflammatory macrophages, CX₃CR1⁺ anti-inflammatory monocytes and macrophages, express low levels of Ly6C and contribute to tissue repair (Tighe et al., 2011; Jacquelin et al., 2013). The outcome of the response to tissue injury depends on the balance between pro- and anti-inflammatory macrophages.

FXR is a nuclear receptor important in bile homeostasis; it has also been shown to down regulate inflammation, a response due in part to deactivation of the transcription

factor NF- κ B and toll like receptor-4, which are known to regulate inflammatory gene expression (Liu et al., 2012; Carr et al, 2015; Chan et al., 2005). TLR4 has been reported to play a role in ozone-induced lung injury and inflammation (Connor et al., 2012). Activation of FXR in macrophages reduces pro-inflammatory cytokine production induced by LPS (Bishop-Bailey et al., 2004; Mencarelli et al., 2009). It has also been identified in peritoneal macrophages and in pulmonary endothelial cells (Guo et al., 2006a; Zhang et al., 2012b). In addition to regulating lipid metabolism and transport, FXR has been shown to exert anti-inflammatory activity, a response thought to be due to deactivation of NF- κ B in macrophages, and down regulation of key pro-inflammatory genes (Lawrence, 2009; Stojancevic et al., 2012). We speculate that FXR activation is involved with anti-inflammatory activities following ozone exposure. Understanding mechanisms underlying macrophage activation can lead to targeted therapies.

AIMS OF THE DISSERTATION

Exposure to toxic levels of ozone causes damage to the lower lung. This is associated with an accumulation of activated macrophages in the lung, which contribute to both tissue injury and repair. We hypothesize that distinct activated macrophage subpopulations contribute to these processes and that these cells are derived from both bone marrow and spleen. Moreover, lung macrophage activation following ozone exposure involves activation of farnesoid x receptor (FXR). To test this hypothesis, studies were designed to:

SPECIFIC AIM 1: Analyze the origin of macrophages accumulating in the lung following ozone exposure.

SPECIFIC AIM 2: Investigate monocyte trafficking originating from the bone marrow and chemokine receptor regulation of monocyte migration.

SPECIFIC AIM 3: Analyze the role of farnesoid x receptor (FXR) in macrophage activation and trafficking.

MATERIALS AND METHODS

Mice

Female specific pathogen-free C57Bl6/J control (CTL), which included sham-operated and wild type (WT) mice, splenectomized (SPX) mice, C57Bl6/J wild type (WT), B6.129S4-Ccr2^{tm1lf_c}/J (CCR2^{-/-}) mice, and C57BL/6-Tg (CAG-EGFP) 131Osb/LeySopJ (GFP-transgenic) mice, (8-11 weeks; 17-22 g) were obtained from The Jackson Laboratories (Bar Harbor, ME). Female 129-Fxr^{tm1Gonz} (FXR^{-/-}) mice were backcrossed to a C57BL/6 genetic background for 10 generations and genotyped by a PCR-based method (a gift from Dr. Grace Guo, Rutgers University, NJ) (Guo et al., 2006). B6.129P-Cx3cr1^{tm1Litt1} (CX3CR1-GFP) mice were a gift from Dr. Long-Jun Wu (Rutgers University, New Brunswick, NJ). Animals were housed under pathogen free conditions in microisolation cages and received food and water ad libitum. All animals received humane care in compliance with the institution's guidelines, as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. All procedures were reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee.

Generation of bone marrow chimeric mice

WT mice were subjected to whole body irradiation using the Faxitron Torrex X-ray system, model TRX2800, located in the Life Sciences building on Busch Campus. Six unanesthetized mice, at a time, were placed, unrestrained, in a sterile 20 cm x 20 cm x 3 cm plexiglass container and placed in the irradiation chamber. The chamber

containing the mice was placed 13 cm below the source. The X-ray power was set at 100 kV with the aluminum attenuator in place and the mice subjected to whole body radiation (a total of 12 Gy) over 45 min. The dose rate under these conditions is approximately 0.27 Gy/min. GFP-transgenic mice were euthanized with Nembutal (200 mg/kg) and bone marrow collected from the left and right femurs and tibiae. Cells were washed twice with PBS, and viable cells enumerated using a hemocytometer with trypan blue. Bone marrow cells were injected i.v. into irradiated WT mice (2×10^6 /mouse). Chimeric mice were used four weeks later.

Ozone Exposure

Mice were placed in whole body Plexiglas chambers and exposed to 0.8 ppm ozone or air for 3 h. A Gilmont ozone generator was used to generate ozone from oxygen gas via an ultraviolet light source (Orec, Phonex, AZ). The concentration of ozone within the chamber was maintained by adjusting the light intensity and monitored with an ozone analyzer (model 400E; Teledyne Instruments, San Diego, CA). Mice were euthanized 24-120 h post exposure.

Collection and Analysis of Bronchoalveolar Lavage Fluid (BAL)

Mice were anesthetized and a cannula inserted into the trachea. One ml of PBS was slowly instilled and withdrawn four times using a syringe. BAL was then centrifuged at $300 \times g$ for 8 min and cell-free supernatant collected. The cell pellet was then resuspended in PBS and enumerated using a Coulter Counter. BAL protein

concentration was determined using a microplate BCA assay with bovine serum albumin (BSA) as the standard (Thermo Scientific, Rockford IL).

Histology

After lavage, the left lung was inflation fixed with ice cold 3% paraformaldehyde + 2% sucrose overnight, washed three times with PBS + 2% sucrose, and transferred to tubes containing 50% ethanol. The tissue was then sectioned (6 μ m), and stained with hemotoxylin and eosin. Images were examined by light microscopy and acquired using a VS120 Virtual Microscopy System (Olympus, Center Valley, PA).

Immunohistochemistry

The lung was fixed *in situ* via the trachea with PBS containing 3% paraformaldehyde and 2% sucrose solution. After overnight incubation at 4°C, the tissue was washed 3 times in PBS/2% sucrose and then transferred to 50% ethanol. Tissue sections (5 μ m) were deparaffinized with xylene (4 min, x 2) followed by decreasing concentrations of ethanol (100% - 50%) and then, water. After antigen retrieval using citrate buffer (10.2 mM sodium citrate, pH 6.0) and quenching of endogenous peroxidase with 3% H₂O₂ for 10 min, sections were incubated for 2 h at room temperature (RT) with 10-100% normal goat or rabbit serum to block nonspecific binding. This was followed by overnight incubation at 4°C with rabbit antibody to inducible nitric oxide synthase (iNOS, 1:750; Abcam), mannose receptor-1 (MR, 1:1500; Abcam), arginase-1 (Arg1, 1:1500; Abcam), angiotensin type 1 receptor (AT1R, 1:500, Abcam), YM1 (1:500; Abcam), cytochrome b5 (Cypb5, 1:500; Abcam, Cambridge, MA), FXR (1:100, Santa

Cruz, Dallas, TX), matrix metalloproteinase-9 (MMP-9, 1:150; Santa Cruz Biotechnology), ADAM17/TACE (1:50; R&D Systems, Minneapolis, MN), heme oxygenase-1 (HO-1, 1:500; Enzo Life Sciences, Farmingdale, NY), or with goat anti-4-hydroxynoneal (4-HNE, 1:250; Abcam) antibody, or appropriate IgG controls (ProSci, Poway, CA). Sections were then incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at RT. Binding was visualized using a Peroxidase Substrate Kit DAB (Vector Labs). Three random sections from each animal were analyzed.

Flow cytometry

Cells were incubated for 10 min at 4°C with anti-mouse CD16/32 (1:200, clone 93; Biolegend, San Diego, CA) to block non-specific binding and then with FITC-conjugated anti-mouse CD11b (1:200, clone M1/70; Biolegend), PE-conjugated anti-mouse Ly6C (1:200, clone HK1.4; Biolegend), PE/Cy7-conjugated anti-mouse F4/80 (1:200, clone BM8; Biolegend), AF700-conjugated anti-mouse CD11c (1:200, clone N418; Biolegend), and AF647-conjugated anti-mouse Ly6G (1:200, clone 1A8; Biolegend) antibodies or appropriate isotypic controls for 30 min at 4°C followed by incubation with eFluor 780-conjugated fixable viability dye for 30 min at 4°C (1:1000, eBioscience, San Diego, CA). In some experiments, blood and bone marrow cells were incubated with AF 647-conjugated anti-mouse CCR2 (1:100, clone SA203G11; Biolegend), followed by eFluor 780-conjugated fixable viability dye. Cells were then analyzed on a Gallios flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed using Beckman Coulter Kaluza version 1.2 software. Viable cells were initially analyzed

for expression of CD11b, followed sequentially by Ly6G, Ly6C, F4/80 and CD11c. Inflammatory cell subpopulations were identified as described previously (Sunil *et al.*, 2015).

Immunofluorescence

Lungs were inflated with OCT medium (ThermoFisher Scientific, Wilmington, DE) containing 30% sucrose, and then snap frozen in liquid nitrogen-cooled isopentane and embedded. Tissue sections (6 μ m) were prepared, fixed in 90% acetone/10% methanol and air dried. Sections were then stained directly with PE-conjugated anti-mouse Ly6C (1:100, Biolegend; San Diego, CA), AF647-conjugated anti-mouse CCR2 (1:100, Biolegend), or indirectly with anti-rabbit anti-heme oxygenase-1 (HO-1, 1:500; Enzo Life Sciences, Farmingdale, NY), YM1 (1:500; Abcam, Cambridge, MA), CX3CR1 (1:250; Abcam), or with goat anti-Nurr77 (1:100; Abcam) followed by isotype specific goat anti-rabbit conjugated AF555- or mouse anti-goat AF568-conjugated secondary antibody (Thermofisher Scientific; Waltham, MA). Images were acquired using a Leica TCS SP5 confocal microscope with hybrid detectors (Leica Microsystems, Wetzlar, Germany). Identical laser power, gain, and offset settings were used for all analyses. A Pearson's correlation coefficient was used to analyze macrophage co-localization of GFP⁺ or CX3CR1^{+/GFP} cells and Ly6C⁺, CCR2⁺, YM1⁺, Nur77⁺, CX3CR1⁺, or HO-1⁺. Three randomly selected fields/slide from three slides/treatment group were analyzed. Data were analyzed using Leica Application Suite Advanced Fluorescence 2.4 software.

Western Blot Analysis

BAL proteins were separated using native and reducing gel electrophoresis as previously reported (Atochina-Vasserman, 2012; Guo *et al.*, 2008). Briefly, denatured and native aliquots of BAL proteins were fractionated on denaturing 4-12% Novex Bis-Tris gels or 4-16% Bis-Tris NativePAGE gels (Invitrogen, San Diego, CA), respectively, and then transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation of the blots with 10% non-fat milk for 1 h at room temperature. The blots were then incubated overnight at 4°C with a mixture of rabbit polyclonal anti-SP-D antibody DV117 (a gift from Dr. Amy Pastva, Duke University, NC; 1:10,000) and rabbit polyclonal anti-SP-D antibody from Chemicon (Millipore, Billerica, MA; 1:4,000), washed and then incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:5000), diluted in Tris-buffered saline/Tween-20. Immunoreactive bands were visualized using an ECL detection system (GE Healthcare Biosciences, Piscataway, NJ).

To assess surfactant protein content, reducing NuPAGE was performed using individual BAL samples. Gels were transferred to BioRad Immun-blot PDVF membranes, incubated with SP-D or SP-B antibody (M.F. Beers, University of Pennsylvania), goat anti-rabbit linked to horseradish peroxidase, and imaged using Amersham ECL Prime Western Blotting Detection Reagent (Buckinghamshire, United Kingdom). Whole BAL was used for SP-D and SP-B analysis; and sample load was normalized to sample volume (AtochinaVasserman *et al.*, 2009).

Phospholipid analysis

To estimate phospholipid content, BAL was fractionated into small and large aggregate portions by centrifugation at 17,000g for 1 h at 4°C. The supernatant contained the protein-rich small aggregate fraction. The pellet, containing the lipid-rich large aggregate fraction was re-suspended in 35 ml of saline. Inorganic phosphate in the lipid-rich fractions was measured as an estimate of the phospholipid content (Atochina-Vasserman et al., 2009).

Measurement of Pulmonary Mechanics

Mice were anesthetized with ketamine (115 mg/kg) and xylazine (30 mg/kg) and then ventilated through a tracheal cannula at a frequency of 150 breaths per min and a tidal volume of 10 mL/kg using a flexiVent (SCIREQ, Montreal, Canada). Respiratory mechanics was measured at PEEPs ranging from 0 cm H₂O to 9 cm H₂O. Baseline measurements were assessed at a PEEP of 3 cm H₂O. A constant-phase model was fitted to the impedance spectra, allowing for calculation of constant-phase tissue compartment containing the coefficients of G (tissue dampening) and H (tissue elastance). Quasi-static pressure/volume loops were generated from functional residual capacity by setting PEEP to 0 cm H₂O and delivering 7 stepwise inflations of inspiratory volume to a total volume of 0.8 ml, followed by 7 equal expiratory steps, pausing for 1 s at each step. Plateau cylinder pressure was measured during each pause and plotted against piston displacement. Best fit lines were generated by using linear regression analysis.

Real-time PCR

Total RNA was isolated from macrophages using an RNeasy kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's directions. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler (Applied Biosystems, Foster City, CA). All PCR primer pairs were generated using Primer Express 2.0 (Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are presented in Table 1. For each sample, fold changes were relative to GAPDH.

Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using student's t-test and one-way ANOVA. A p value of ≤ 0.05 was considered statistically significant.

TABLE 1. Primer sequences used for RT-PCR.

Gene Target	Primer Forward	Primer Reverse
AT1R	CCATTGTCCACCCGATGAA	TGACTTTGGCCACCAGCAT
CCL4	TGCTCGTGGCTGCCTTCT	GAGGGTCAGAGCCCATTGG
TNF- α	AGGGATGAGAAGTTCCCAAATG	TGTGAGGGTCTGGGCCATA
CCL2	TTGAATGTGAAGTTGACCCGTAA	GCTTGAGGTTGTGGAAAAG
IL-1 β	AGTTGACGGACCCCAAAAGAT	GGACAGCCCAGGTCAAAGG
CCL3	TCTTCTCAGCGCCATATGGA	TCCGGCTGTAGGAGAAGCA
CCR2	TCCACGGCATACTATCAACATCTC	GGCCCCCTTCATCAAGCTCTT
CCR5	TGATAAGCTGCAAAAAGCTGAAGA	GTCAGAGATGGCCAGGTTGAG
CCR1	CTGAGGGCCCCGAAGTGTAC	GGCTAGGGCCCCAGGTGAT
iNOS	GGCAGCCTGTGAGACCTTTG	TGAAGCGTTTCGGGATCTG
CX3CL1	GCACAGGATGCAGGGCTTAC	TGTCAGCCGCCTCAAAACT
CX3CR1	TCGGTCTGGTGGGAAATCTG	GGCTTCCGGCTGTTGGT
Nur77	TCTGCTCAGGCCTGGTACTACA	ATGTTGTCAATCCAATCACCAAAG
SHP	GCCTGGCCCGAATCCT	AAGGGTGCCTGGAATGTTCTT
VLDLR	GTTCCCCCAAAAGGGACTTC	GCCAATTCCTCCACATCAAGTAG
APOE	GCAGGCGGAGATCTTCCA	TGTCTTCCACTATTGGCTCGAA
ABCA1	GGCAATGAGTGTGCCAGAGTTA	TAGTCACATGTGGCACCGTTTT
ABCG1	G TTCAGGAGGCCATGATGGT	CTCGTCTGCCTTCATCCTTCTC
LXR α	GCCTGCAGGACAAAAAGCTT	CACTCGTGGACATCCCAGATC
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG

PART I. ROLE OF SPLEEN-DERIVED MACROPHAGES IN OZONE-INDUCED LUNG INFLAMMATION AND INJURY

Ozone is a ubiquitous urban air pollutant produced as a component of photochemical smog. It is a highly reactive molecule that induces cytotoxicity via its ability to generate free radicals and oxidize a variety of cellular components including proteins, lipids and DNA (Mudway and Kelly, 2000; Vallyathan and Shi, 1997). This leads to structural damage, predominately in alveolar epithelial regions of the lung, and to alterations in pulmonary mechanics (Alexeef *et al.*, 2007). Evidence suggests that macrophages accumulating in the lung after ozone exposure contribute to both tissue injury and repair (Hollingsworth *et al.*, 2007; Pendino *et al.*, 1995; Sunil *et al.*, 2015; Tighe *et al.*, 2011a). These activities are mediated by distinct macrophage subsets broadly classified as pro-inflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages (Byers and Holtzman, 2011). The present studies were aimed at analyzing the origin of these cells, with a focus on the spleen as an extramedullary source.

The spleen is a lymphoid organ that functions to remove aging erythrocytes; it is also involved in immune homeostasis (Mebius and Kraal, 2005). The spleen has been shown to act as a reservoir of inflammatory monocytes that rapidly deploy to sites of injury (Kim *et al.*, 2014; Swirski *et al.*, 2009). Once localized at these sites, spleen monocytes differentiate into macrophages and become activated to release inflammatory mediators, which have been implicated in tissue injury (Swirski *et al.*, 2009). Unlike bone marrow monocytes which depend on the chemokine receptor, CCR2, and its ligand, CCL2, to localize at inflammatory sites, splenic monocyte trafficking is mediated by

angiotensin type I receptor (AT1R) and angiotensin II (AII) released from damaged tissues (Mellak *et al.*, 2015). The present studies demonstrate that the spleen is a source of inflammatory lung macrophages responding to ozone-induced toxicity, and that in their absence, anti-inflammatory and wound repair macrophages predominate, resulting in attenuated tissue injury and oxidative stress. Elucidating the origin of cytotoxic/pro-inflammatory macrophages and mechanisms mediating their accumulation in the lung may lead to the development of novel approaches for reducing oxidant-induced lung injury.

RESULTS

Effects of ozone on spleen myeloid cells

Treatment of control mice with ozone resulted in an increase in the total number of spleen cells at 48 h post exposure, with no change in cell viability (Table 1 and not shown). To characterize these cells, we used techniques in flow cytometry, focusing on myeloid inflammatory cells, which were identified on the basis of forward and side scatter and expression of the β_2 integrin, CD11b (Gordon and Taylor, 2005). CD11b⁺ myeloid cells were found to consist of Ly6G⁻ (monocytic) and Ly6G⁺ (granulocytic) subpopulations (Figure 1). The majority of the cells (~90%) were monocytic. We next analyzed their expression of Ly6C, a monocyte/macrophage activation marker, and F4/80, a marker of maturity (Mitchell *et al.*, 2014). Figure 2 shows that Ly6G⁻F4/80⁺ spleen monocytes consisted of three subpopulations that expressed high (Ly6C^{Hi}), low (Ly6C^{Lo}), and intermediate (Ly6C^{Int}) levels of Ly6C, which is characteristic of pro-inflammatory, anti-inflammatory, and transitional monocytes, respectively (Gordon and Taylor, 2005). Ozone exposure resulted in a significant increase in all three Ly6C positive monocyte subpopulations at 48 h (Figure 2, left panels). In contrast to Ly6G⁻ spleen myeloid cells, the majority (>99%) of the Ly6G⁺ cells were highly positive for Ly6C, suggestive of a MDSC phenotype (Fig. 1, lower panels) (Youn *et al.*, 2008). Both F4/80⁻ granulocytic (G-MDSC) and F4/80⁺ monocytic (M-MDSC) populations were identified; both of these cell types increased 48 h after ozone (Figure 3).

Effects of splenectomy on lung macrophage subpopulations

Earlier studies showed that both pro- and anti-inflammatory macrophages accumulate in the lung after ozone exposure (Fakhrzadeh *et al.*, 2002; Pedino *et al.*, 1995; Sunil *et al.*, 2015; Tighe *et al.*, 2011a). In further studies we used SPX mice to determine if the spleen contributes to these inflammatory cell populations. In air exposed CTL mice, differential staining of BAL cells revealed that >98% were macrophages. These cells were identified as CD11b⁺Ly6C^{Lo}F4/80⁺CD11c⁺, which is consistent with a resident alveolar macrophage phenotype (Guth *et al.*, 2009; Misharin *et al.*, 2013). Ozone inhalation had no effect on resident alveolar macrophages in either CTL or SPX mice (Figure 4, upper panels). In contrast, a significant increase in the total number of CD11b⁺ cells were observed in the lung after ozone (Figure 4, lower panels) (Sunil *et al.*, 2015). As observed in the spleen, monocytic (Ly6G⁻) and granulocytic (Ly6G⁺) subpopulations of CD11b⁺ cells were identified in the lung (supplemental data). However, in the lung only Ly6C^{Hi} pro-inflammatory and Ly6C^{Lo} anti-inflammatory macrophages were detected within the Ly6G⁻ population (Sunil *et al.*, 2012). The majority (>70%) of these macrophage subpopulations were F4/80⁺CD11c⁺, indicative of a mature phenotype (Misharin *et al.*, 2013; Zaynagetdinov *et al.*, 2013). Increases in both pro-inflammatory and anti-inflammatory macrophages were observed in the lungs of CTL mice following ozone exposure; this was most prominent 24 h post exposure (Figure 5). Splenectomy resulted in a significant decrease in Ly6C^{Hi} pro-inflammatory macrophages accumulating in the lung in response to ozone. Conversely, an increase in Ly6C^{Lo} anti-inflammatory macrophages was observed in lungs of SPX mice relative to CTL mice at 72 h post ozone (Figure 5). Ly6G⁺Ly6C⁺ MDSCs were also identified in the lung, which consisted of G-MDSC and M-MDSC subpopulations (Figure 3). In CTL

as well as SPX mice, ozone caused a significant increase in both MDSC subpopulations. While reduced numbers of G-MDSCs were observed in lungs of SPX mice 24 h and 72 h after ozone relative to CTL mice, M-MDSCs were unchanged.

In further studies we analyzed the effects of splenectomy on pro-inflammatory MMP-9⁺ and anti-inflammatory MR-1⁺ macrophages in histologic sections of the lung. In CTL mice, ozone inhalation resulted in increased numbers of MMP-9⁺ macrophages in the lung at 24 h and 48 h post exposure (Figure 6). These cells were reduced in SPX mice. Ozone also caused an increase in MR-1⁺ macrophages in lungs of CTL mice. Although numbers of MR-1⁺ macrophages were constitutively greater in SPX mice than in CTL mice, they were largely unaffected by ozone (Figure 7).

Effects of splenectomy on ozone-induced expression chemokines and chemokine receptors involved in monocyte/macrophage trafficking

In CTL mice, chemokines CCL3 and CCL4 and chemokine receptors, CCR1 and CCR2 were upregulated in the lung 24 h post ozone (Figure 8). In contrast, ozone had no effect on expression of CCL2, CCR5, or CX3CR1. Splenectomy resulted in increased expression of CCR2 and CCL2, when compared to CTL mice, at all post ozone exposure times. Conversely, ozone-induced upregulation of CCR1, CCL3, and CCL4 was blunted in SPX mice. Splenectomy had no significant effect on CCR5 or CX₃CR1 gene expression, or on their response to ozone (Fig. 8). We also analyzed expression of AT1R, which is important in monocyte trafficking from the spleen (Swirski *et al.*, 2009). In CTL mice, increased numbers of AT1R⁺ cells were observed in the lung at 24 h and 48 h after ozone (Figure 9); this was correlated with increased AT1R mRNA expression

(Figure 8). Ozone-induced increases in AT1R⁺ macrophages were delayed in SPX mice relative to CTL mice. Whereas increases in AT1R mRNA expression were also delayed in SPX mice treated with ozone, levels remained upregulated for 72 h.

Effects of splenectomy on bone marrow monocytes and MDSCs

We next analyzed the effects of ozone on bone marrow monocytes in CTL and SPX mice. Three subpopulations of CD11b⁺ monocytes expressing high, low, and intermediate levels of Ly6C were identified in the bone marrow (Figure 2, right panels). The majority of these cells were immature F4/80⁻ (data not shown). In CTL mice, ozone inhalation resulted in an increase in Ly6C^{Hi} monocytes at 48 h and 72 h post exposure (Figure 3). Conversely, Ly6C^{Lo} monocytes increased in the bone marrow after ozone exposure, while Ly6C^{Int} transitional monocytes were unchanged. Whereas splenectomy resulted in a decrease in Ly6C^{Hi} monocytes, increases in Ly6C^{Lo} and Ly6C^{Int} monocytes were noted at 72 h post ozone. Monocytic (F4/80⁺) and granulocytic (F4/80⁻) Ly6G⁺Ly6C⁺ MDSCs were also identified in the bone marrow (Figure 3). Significantly greater numbers of these cells were observed in SPX mice; however, they were not altered by ozone exposure.

Effects of splenectomy on ozone-induced lung injury and oxidative stress

In additional studies we determined if ozone-induced changes in inflammatory cells in the lungs of SPX mice were correlated with alterations in toxicity. Treatment of CTL mice with ozone resulted in significant increases in BAL cell and protein content (Figure 10). This was reduced in SPX mice. Ozone-induced oxidative stress, as measured by the lipid peroxidation end product 4-HNE was also reduced in SPX mice

relative to CTL mice (Figure 11). In contrast, splenectomy had no effect on ozone-induced increases in total SP-D levels in BAL or on structural alterations in SP-D, as reflected by the presence of multiple lower molecular weight SP-D bands in reducing gels (Figure 12).

DISCUSSION

Earlier studies have shown that the spleen is a source of inflammatory monocytes that rapidly accumulate at sites of tissue injury (Bao *et al.*, 2010; Swirski *et al.*, 2009). Once localized at these sites, they develop into macrophages with cytotoxic and pro-inflammatory activities, contributing to the pathogenesis of acute ischemic brain injury and myocardial infarction (Bao *et al.*, 2010; Swirski *et al.*, 2009). Similarly, the present studies demonstrate that the spleen functions as a reservoir of pro-inflammatory monocytes that contribute to ozone toxicity in the lung. This is based on our findings that ozone-induced lung injury and oxidative stress are reduced in SPX mice, a response associated with decreased numbers of pro-inflammatory macrophages in the lung. Conversely, increased numbers of a subset of anti-inflammatory macrophages are observed in lungs of SPX mice after ozone exposure. These data suggest that reduced ozone toxicity in SPX mice is a result of a shift in the balance from pro-inflammatory/cytotoxic macrophages to anti-inflammatory/wound repair macrophages.

Splenic Ly6C^{Hi} monocytes have been reported to be pro-inflammatory/cytotoxic, while Ly6C^{Lo} monocytes function to suppress inflammation and promote wound healing (Mitchell *et al.*, 2014; Swirski *et al.*, 2009). Following ozone exposure, we found that both Ly6C^{Hi} and Ly6C^{Lo} monocyte subpopulations increased in the spleen. However, this response was transient, occurring at 48 h, a time consistent with an increase in the total number of spleen cells. Similar transient increases in monocytes in the spleen have been described in mice after thermal injury to the skin, and after *Plasmodium chabaudi* exposure, suggesting that this may be a general response to tissue injury or infection (Helmby *et al.*, 2000; Noel *et al.*, 2007). Conversely, a reduction in splenic Ly6C^{Hi} pro-

inflammatory monocytes has been reported after post-ischemic brain injury in mice (Bao *et al.*, 2010; Kim *et al.*, 2014). This may be due to egress of these cells from the spleen after injury. Immature Ly6C^{Int} monocytes were also identified in the spleen. These cells are thought to be in transition from a Ly6C^{Hi} to a Ly6C^{Lo} phenotype (Lin *et al.*, 2009). The fact that this monocyte subpopulation increased in the spleen 48 h after ozone exposure suggests that these cells may be important in replenishing pools of anti-inflammatory monocytes deployed from the spleen after lung injury. This is supported by findings that the spleen rapidly replenishes its monocytic reservoir after injury (Swirski *et al.*, 2009).

Consistent with previous findings, ozone inhalation resulted in increased numbers of CD11b⁺ infiltrating myeloid cells in the lung (Sunil *et al.*, 2015; Tighe *et al.*, 2011a). The majority of the cells were Ly6G⁻, indicating a monocytic phenotype; this is in accord with our findings that >98% of BAL cells are macrophages. In CTL mice, maximal accumulation of Ly6C^{Hi} pro-inflammatory and Ly6C^{Lo} anti-inflammatory macrophages in the lung was observed 24 h and 48 h post ozone exposure. The observation that this occurs earlier than peak accumulation of monocytic precursors in the spleen indicates that transient increases in the spleen may mainly function to replace depleted reservoirs. Splenectomy resulted in reduced numbers of Ly6C^{Hi}, as well as MMP-9⁺ pro-inflammatory macrophages in the lung. These findings are consistent with previous reports demonstrating that the spleen is a source of pro-inflammatory monocytic precursors that respond to tissue injury (McKim *et al.*, 2016). In contrast, a reduction in Ly6C^{Hi} macrophages has been described in cardiac tissue after splenectomy in a model of myocardial infarction (Swirski *et al.*, 2009). These data suggest that the contribution of

splenic monocytes to injury and infection likely depends on the disease model and the nature of the pathology. Following ozone exposure, Ly6C^{Lo} anti-inflammatory macrophages increased in the lungs of SPX mice relative to CTL mice, indicating that the origin of these cells is distinct from pro-inflammatory macrophages. Interestingly, MR⁺ anti-inflammatory macrophages were constitutively greater in SPX mice relative to CTL mice, however, these cells were unaffected by ozone exposure. These findings provide support for the notion that there are multiple subpopulations of anti-inflammatory macrophages and that their origin is distinct (Gordon and Taylor, 2005; Wynn *et al.*, 2013). Decreases in pro-inflammatory lung macrophages responding to ozone in SPX mice resulted in a predominance of anti-inflammatory macrophages in the lung. A similar shift in the phenotype of macrophages responding to ozone and reduced toxicity has been described in mice lacking galectin-3 (Sunil *et al.*, 2015). Conversely, in the absence of CX3CR1⁺ M2 macrophages, mice have been reported to be hypersensitive to ozone, a response correlated with increased numbers of pro-inflammatory M1 macrophages in the lung (Tighe *et al.*, 2011a). Taken together, these data support the idea that ozone toxicity is due, in part, to an imbalance between pro-inflammatory and anti-inflammatory macrophages in the lung (Laskin *et al.*, 2011a).

Following ozone exposure, increases in immature pro-inflammatory Ly6C^{Hi} monocytes were also observed in the bone marrow, consistent with enhanced myelopoiesis in response to tissue injury (Courties *et al.*, 2015). In SPX mice, pro-inflammatory Ly6C^{Hi} monocytes decreased in the bone marrow, while anti-inflammatory Ly6C^{Lo} and transitional Ly6C^{Int} monocytes increased following ozone exposure. Increased numbers of anti-inflammatory macrophages in the lungs of SPX mice may be a

consequence of increased release of these cells from the bone marrow. This may be a compensatory response to the loss of the splenic myeloid reservoir (Tripp *et al.*, 1997). A similar decrease in bone marrow monocytes has been reported in patients who have undergone splenectomy (Franco *et al.*, 1996; Tripp *et al.*, 1997).

MDSCs are a heterogeneous population of leukocytes known to negatively regulate inflammatory responses to injury and infection (Nagaraj and Gabrilovich, 2010). Previous studies have shown that MDSCs increase in the lung after *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, or *Pneumocystis pneumonia* infection (Obregon-Henao *et al.*, 2013; Rieber *et al.*, 2013; Zhang *et al.*, 2012a). Similarly, we observed increased numbers of G-MDSC and M-MDSC in the lung, as well as the spleen after ozone exposure. Whereas splenectomy had no effect on lung M-MDSCs, G-MDSCs were reduced, suggesting distinct origins of these MDSC subpopulations. Our findings are consistent with reports that the spleen is a source of G-MDSCs (Levy *et al.*, 2015). Elevated numbers of G-MDSCs and M-MDSCs were also observed in bone marrow of SPX mice, when compared to CTL mice, which may be due to a compensatory increase in the generation of these cells in the absence of the spleen. The role of MDSCs in ozone-induced injury and inflammation is unknown. Lung MDSCs have been implicated in the resolution of inflammation via downregulation of toll-like receptor (TLR) 4 (Bunt *et al.*, 2009). TLR4 has been reported to play a role in ozone-induced lung injury and inflammation (Connor *et al.*, 2012). MDSCs may act to reduce TLR4 in the lung after ozone, which contributes to down regulating inflammation and promoting wound repair; however, this remains to be investigated.

Macrophage trafficking to sites of injury is regulated by locally released chemokines and chemokine receptors present on responding cells. We found that ozone-induced upregulation of chemokines CCL4 and CCL3, and the chemokine receptor CCR1 was correlated with an accumulation of pro-inflammatory macrophages in the lung, suggesting potential signaling pathways mediating their trafficking. Splenectomy resulted in a blunted response of CCR1, CCL4, and CCL3 to ozone, but an increased response of CCR2 and CCL2. Evidence suggests that CCR2 and CCL2 are required for pro-inflammatory Ly6C^{Hi} monocyte egress from the bone marrow (Serbina and Pamer, 2006). Decreases in pro-inflammatory Ly6C^{Hi} monocytes in the bone marrow of SPX mice may be due to upregulation of CCR2 on these cells, which facilitates their release from the bone marrow. AT1R is involved in splenic monocyte migration to sites of injury (Mellak *et al.*, 2015). Splenic AT1R⁺ monocytes have been reported to play a role in the pathogenesis of myocardial infarction; thus, reduced monocyte accumulation in the myocardium was observed in mice treated with angiotensin-converting enzyme inhibitor (Nahrendorf *et al.*, 2010). Following ozone exposure, increases in AT1R⁺ macrophages were observed in the lungs in CTL mice. Findings that ozone-induced accumulation of these cells in the lungs and expression of AT1R mRNA were delayed in SPX mice indicate that the early responding AT1R⁺ macrophages originate in the spleen. The origin of the AT1R⁺ appearing in the lung at later times post ozone in SPX mice remains to be determined.

Decreases in pro-inflammatory macrophages in the lungs of ozone treated SPX mice were correlated with reduced oxidative stress, as measured by the appearance of 4-HNE, a lipid peroxidation end product generated by free radicals following ozone

exposure (Grimsrud *et al.*, 2008; Kirichenko *et al.*, 1996). BAL protein content, a marker of alveolar epithelial barrier dysfunction, was also decreased in ozone-exposed SPX mice. These data support the idea that the spleen is a source of cytotoxic/pro-inflammatory precursors and/or mediators that contribute to ozone-induced lung injury and inflammation. Conversely, ozone-induced structural alterations in SP-D were unaffected by splenectomy. These results were surprising since previous studies showed that reactive nitrogen species (RNS) released by pro-inflammatory macrophages mediate post-translational modifications of SP-D (Atochina-Vasserman, 2012; Groves *et al.*, 2012a; Malaviya *et al.*, 2015). These data suggest that there are alternative sources of RNS in the lungs of SPX mice that modify SP-D. In this regard, earlier studies have shown that Type II pneumocytes generate nitric oxide in response to inflammatory cytokines following ozone exposure (Punjabi *et al.*, 1994). Moreover, RNS generated from lung epithelial cells can contribute to SP-D modifications (Bove and van der Vliet, 2006).

In conclusion, the present studies demonstrate that the spleen contributes a subpopulation of inflammatory macrophages to pools of lung leukocytes responding to ozone. Findings that splenectomy protected mice from ozone-induced injury and oxidative stress are consistent with the idea that the spleen is a source of pro-inflammatory monocytes. Identification of the origin of inflammatory macrophages contributing to ozone toxicity may be important in the development of novel therapeutics aimed at selectively targeting these cells and reducing lung injury.

TABLE 2. Effects of Ozone on Total Number of Spleen Cells. Spleen cells were collected 24-72 h after exposure of mice to air or ozone and viable cells enumerated using a hemocytometer with trypan blue dye exclusion. Data are the mean \pm SE (n = 3-4 mice). ^aSignificantly different (p <0.05) from air-exposed animals.

Time	Spleen Cells x 10⁶
Air	17.6 ± 1.7
24 h	21.8 ± 1.2
48 h	57.8 ± 6.3 ^a
72 h	19.4 ± 0.4

FIGURE 1. Effects of ozone on spleen granulocytes and MDSCs. Spleen cells, collected 24-72 h after exposure of CTL mice to air or ozone, were immunostained with antibodies to CD11b, Ly6G and Ly6C and then analyzed by flow cytometry. One representative histogram from 3-4 mice/treatment group is shown. ***Upper panel:*** CD11b⁺ spleen cells expressing Ly6G. ***Lower panel:*** CD11b⁺Ly6G⁺ spleen cells expressing Ly6C

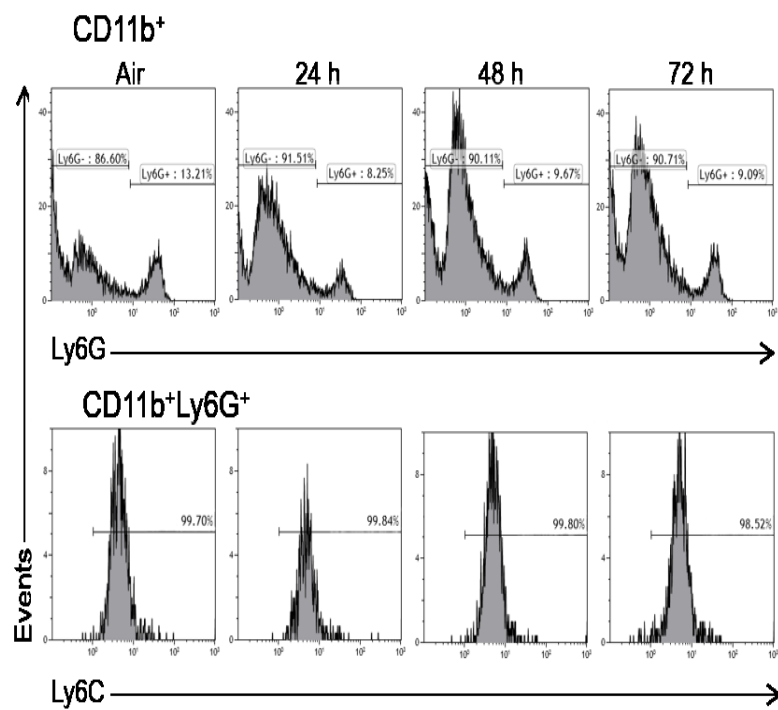


FIGURE 2. Effects of ozone on spleen and bone marrow monocytes. Cells, collected 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, and F4/80 or isotypic controls, and analyzed by flow cytometry. Monocytes were defined as CD11b⁺Ly6G⁻F4/80⁻ and Ly6C^{Hi} (pro-inflammatory), Ly6C^{Lo} (anti-inflammatory), or Ly6C^{Int} (transitional). Bars, mean \pm SE (n = 3-4 mice/treatment group). ^aSignificantly different (p <0.05) from air-exposed animals.

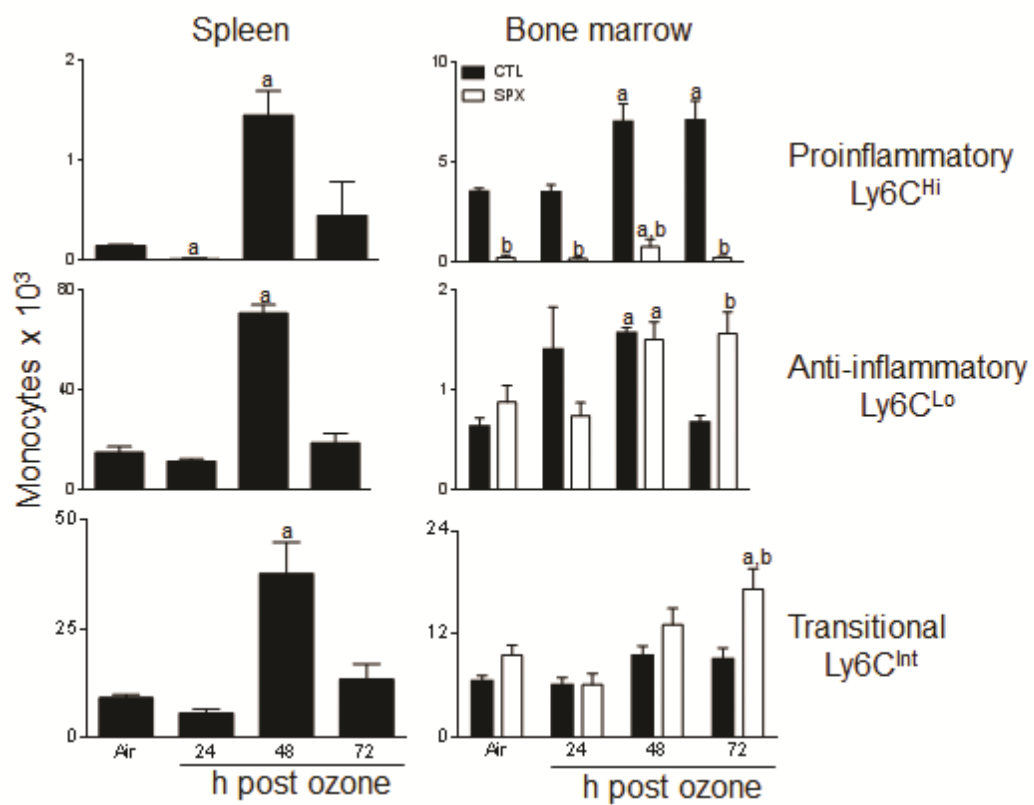


FIGURE 3. Effects of ozone on spleen, lung, and bone marrow MDSCs. Cells, collected 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, and F4/80 or isotypic controls, and analyzed by flow cytometry. MDSCs were defined as CD11b⁺Ly6G⁺ and F4/80⁻ (granulocytic) or F4/80⁺ (monocytic). Bars, mean \pm SE (n = 3-10). ^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from CTL.

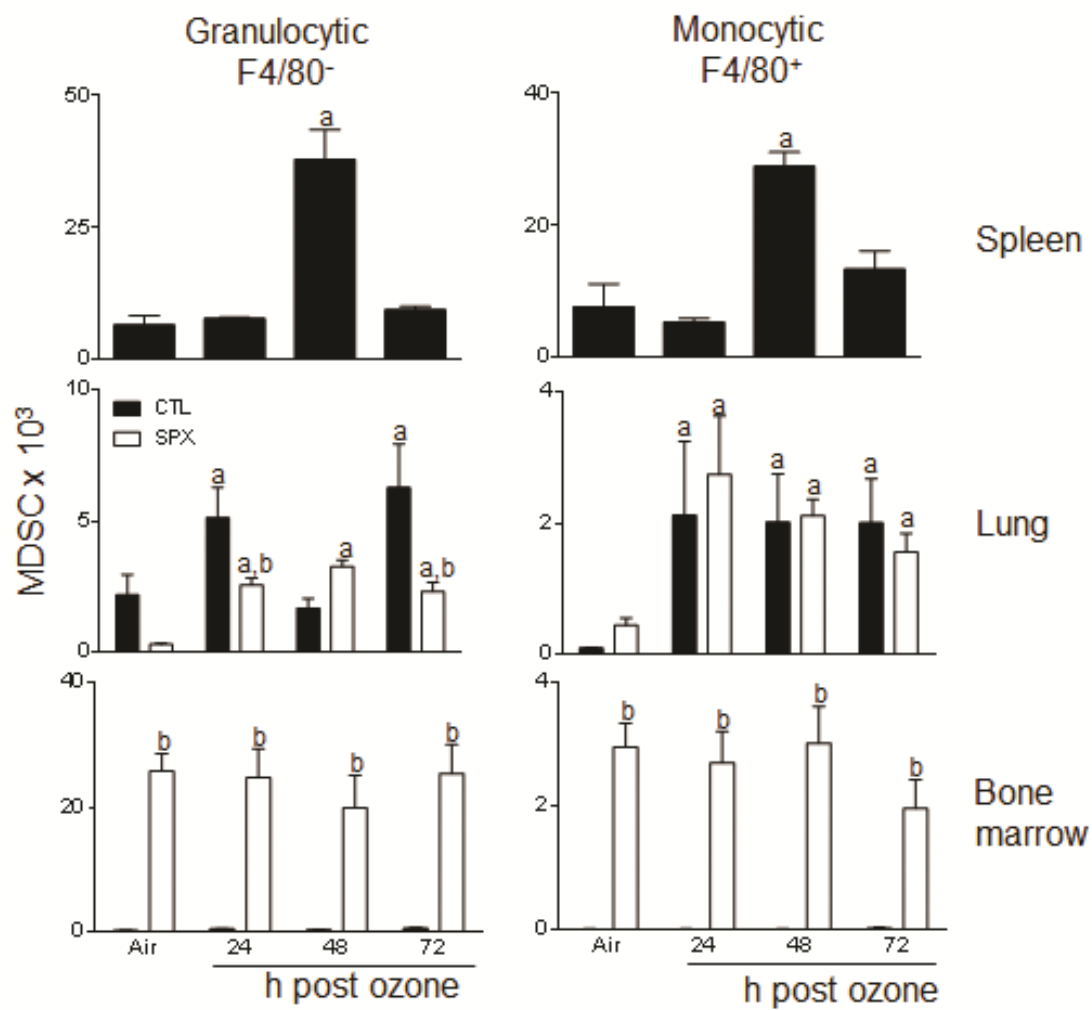


FIGURE 4. Effects of ozone on lung macrophage subpopulations. BAL cells, collected 24-72 h after exposure of CTL mice to air or ozone were stained with antibodies to CD11b, Ly6G, Ly6C, F4/80 and CD11c or isotypic controls, and analyzed by flow cytometry. *Upper Panel:* CD11b⁻Ly6G⁻ cells expressing Ly6C, F4/80 and CD11c. *Lower Panel:* CD11b⁺Ly6G⁻ cells expressing Ly6C, F4/80 and CD11c. One representative dot plot is shown. The percentages represent the mean \pm SE from 3-4 mice/treatment group.

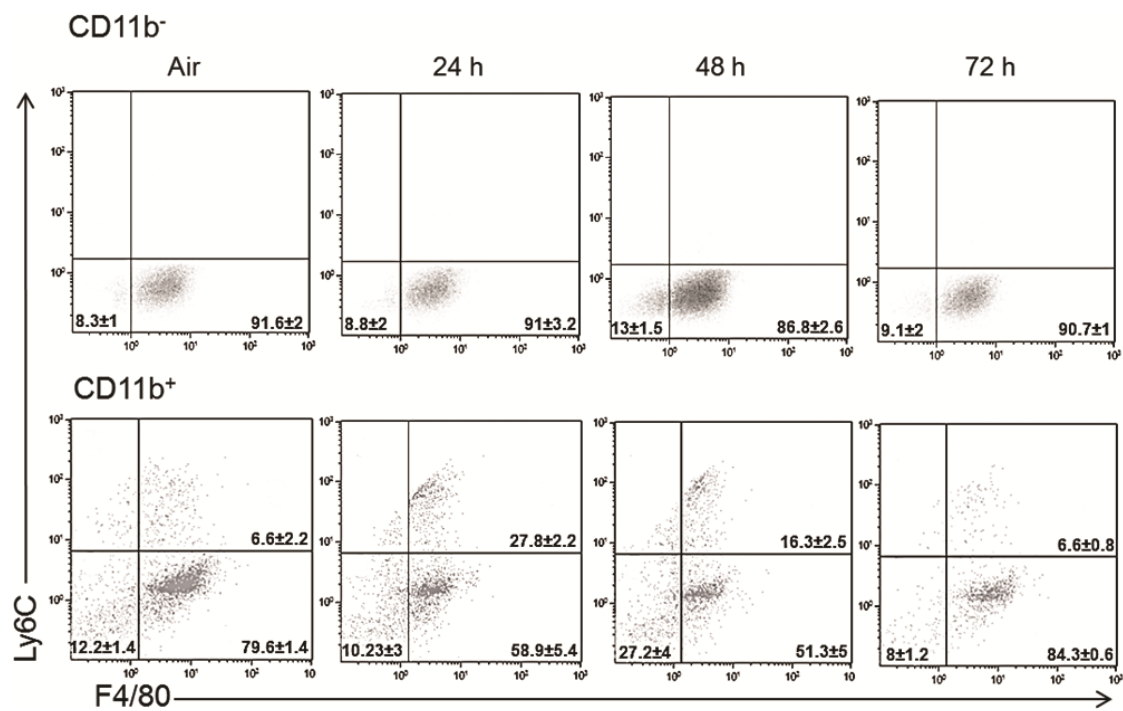


FIGURE 5. Effects of splenectomy on lung macrophages responding to ozone. BAL cells, collected 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, F4/80, and CD11c or isotypic controls and analyzed by flow cytometry. Macrophages (MP) were defined as CD11b⁺Ly6G⁻F4/80⁺CD11c⁺ and Ly6C^{Hi} (pro-inflammatory) or Ly6C^{Lo} (anti-inflammatory). Bars, mean \pm SE (n = 3-10/treatment group). ^aSignificantly different (p < 0.05) from air-exposed animals; ^bSignificantly different (p < 0.05) from CTL.

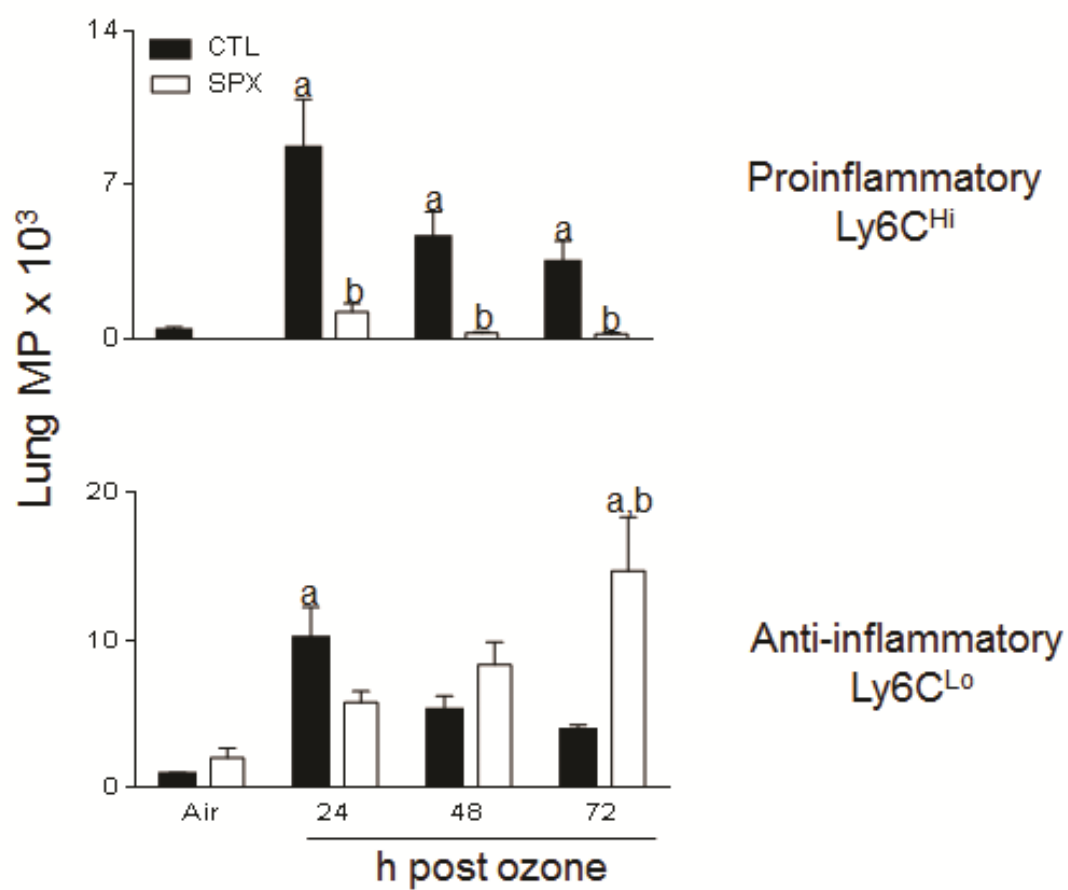


FIGURE 6. Effects of splenectomy on ozone-induced MMP-9 expression. Lung sections, prepared 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibody to MMP-9. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3 separate experiments is shown (n = 3 mice/treatment group). Original magnification, x600.

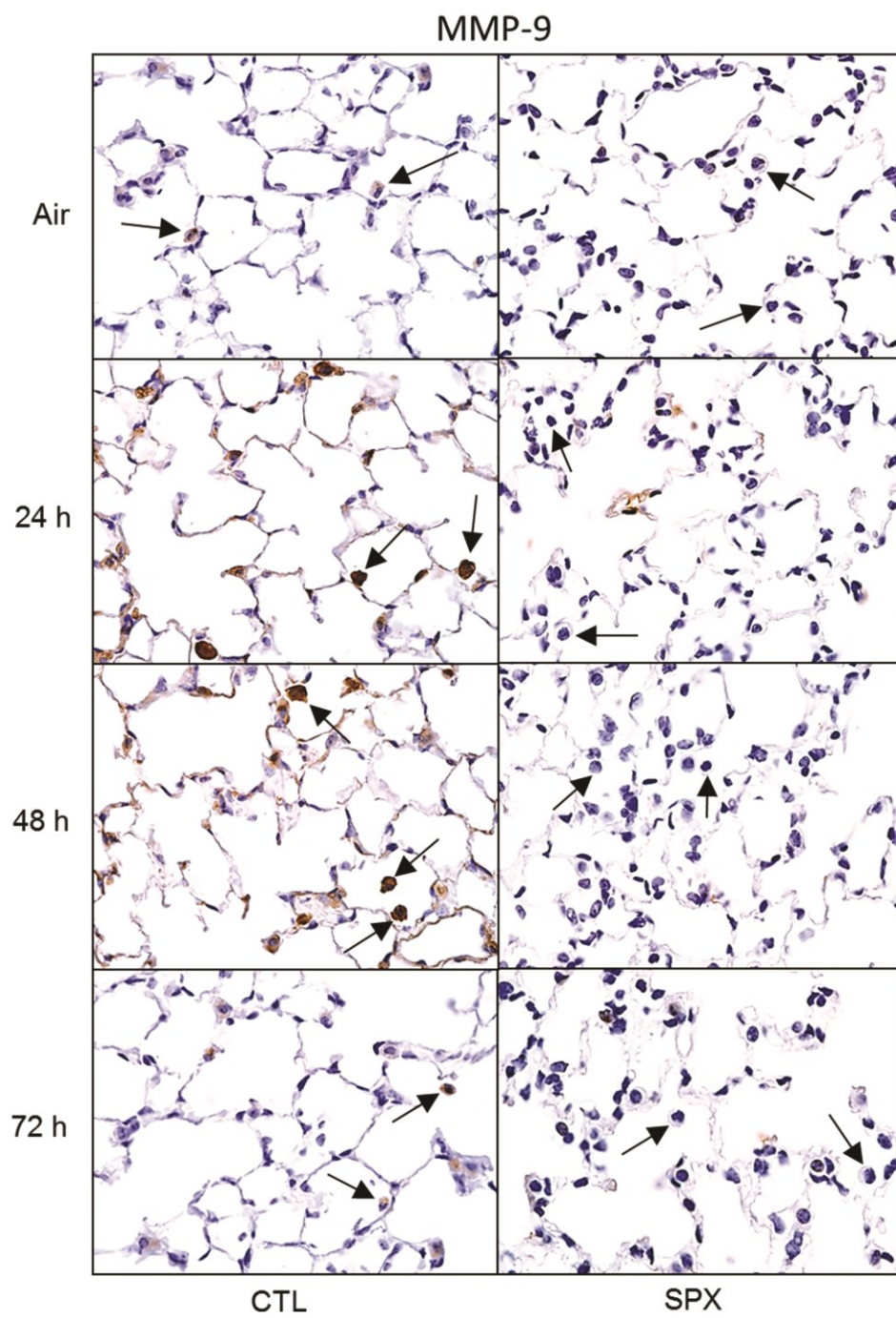


FIGURE 7. Effects of splenectomy on ozone-induced expression of MR-1. Lung sections, prepared 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibody to mannose receptor. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3 separate experiments is shown (n = 3 mice/treatment group). Original magnification, x600.

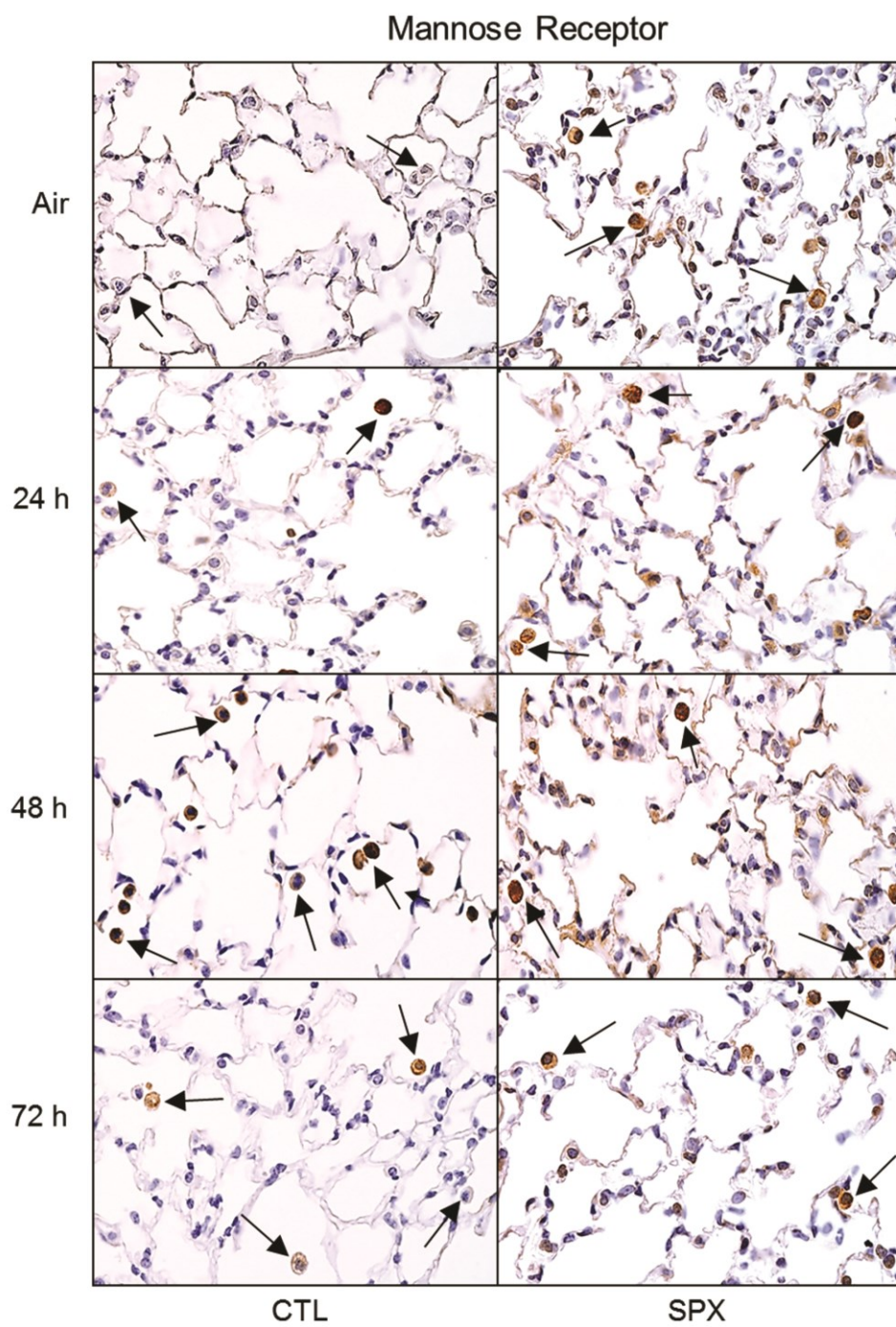


FIGURE. 8. Effects of splenectomy on ozone-induced expression of inflammatory genes. mRNA, prepared from lungs 24-72 h after exposure of CTL and SPX mice to air or ozone, was analyzed by real-time PCR. Data are presented as fold change relative to GAPDH. Bars, mean \pm SE (n = 3-8 mice/treatment group). ^aSignificantly different (p < 0.05) from air-exposed animals. ^bSignificantly different (p < 0.05) from CTL mice.

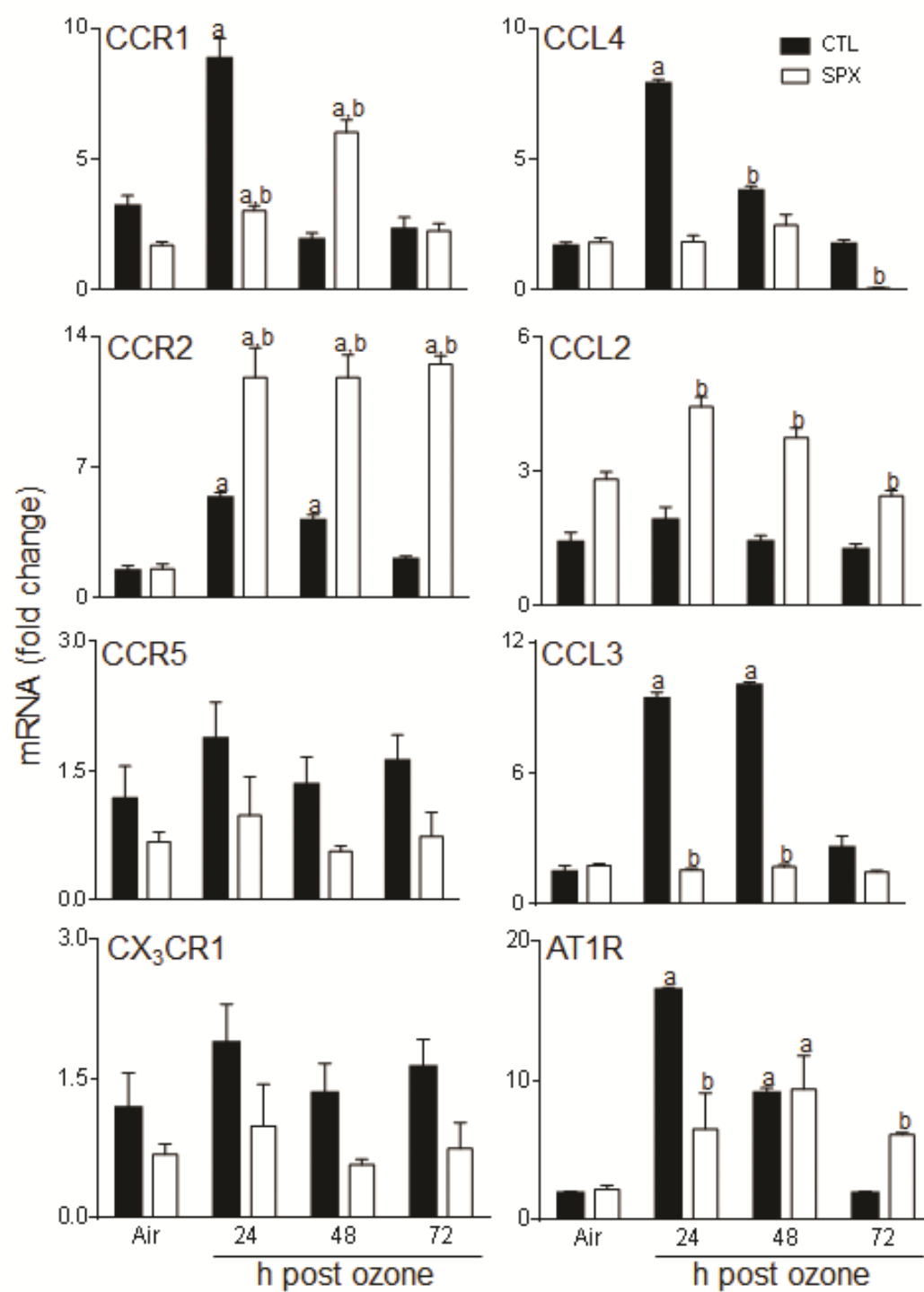


FIGURE 9. Effects of splenectomy on ozone-induced expression of AT1R. Lung sections, prepared 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibody to AT1R. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, $\times 600$.

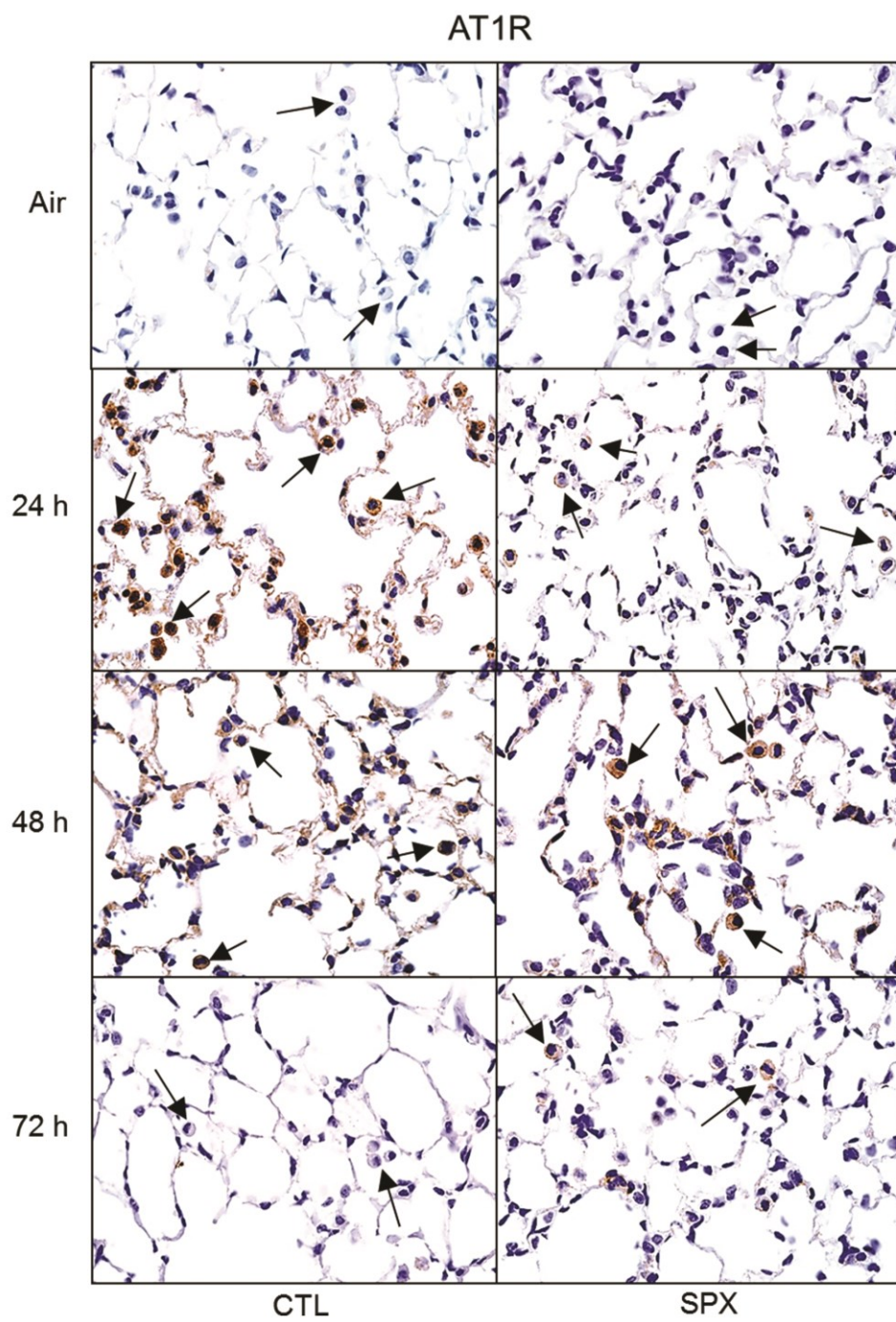


FIGURE 10. Effects of splenectomy on ozone-induced alterations in BAL cell number and protein. BAL was collected 24-72 h after exposure of CTL and SPX mice to air or ozone. *Upper panel:* Viable cells were enumerated using trypan blue due exclusion. *Lower panel:* Cell-free supernatants were analyzed in triplicate for protein using a BCA protein assay kit. Bars, mean \pm SE (n = 3-8 mice). ^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from CTL.

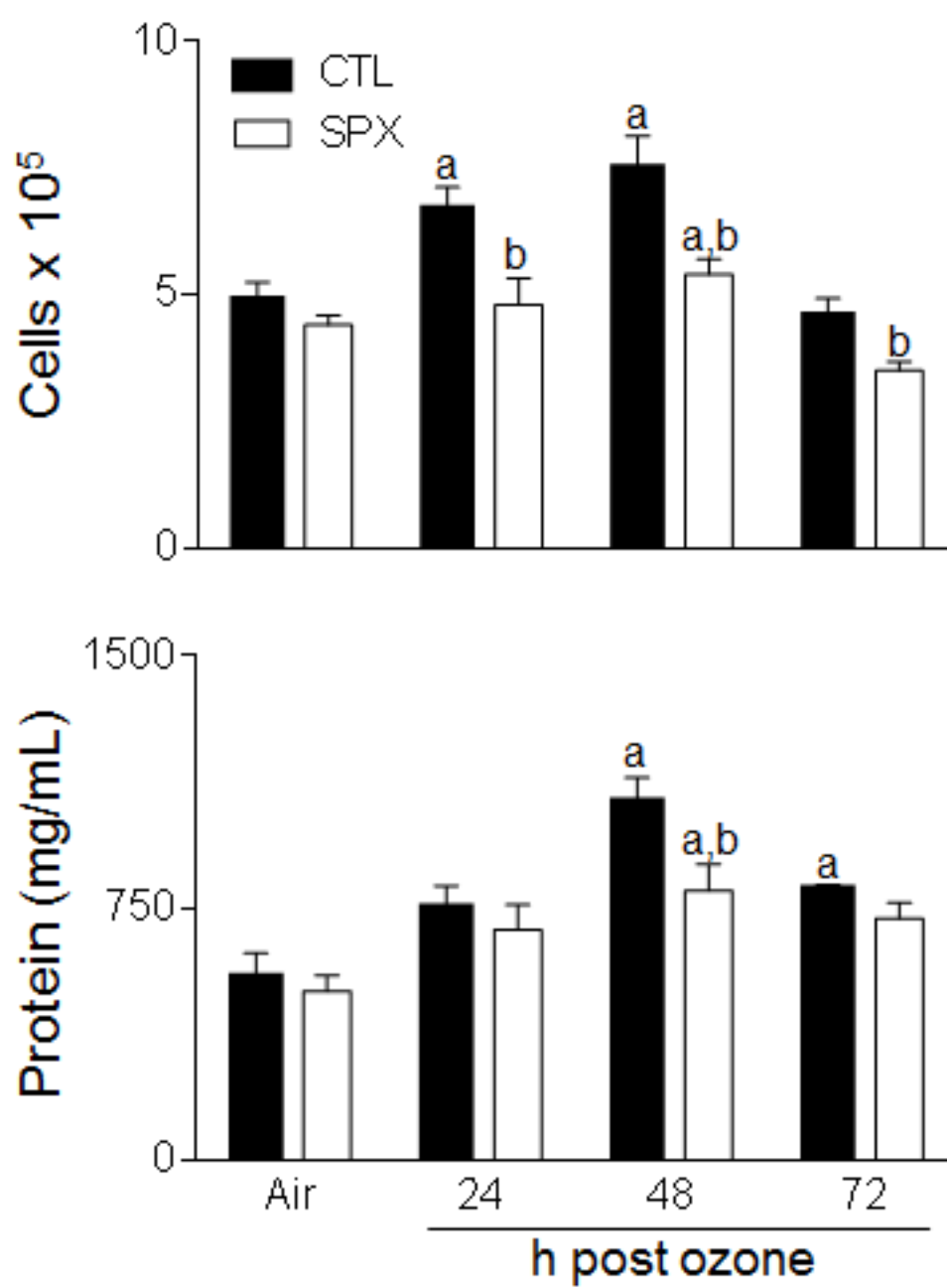


FIGURE 11. Effects of splenectomy on ozone-induced 4-HNE. Lung sections, prepared 24-72 h after exposure of CTL and SPX mice to air or ozone, were stained with antibody to 4-HNE. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown (n = 3 mice/treatment group). Original magnification, x600.

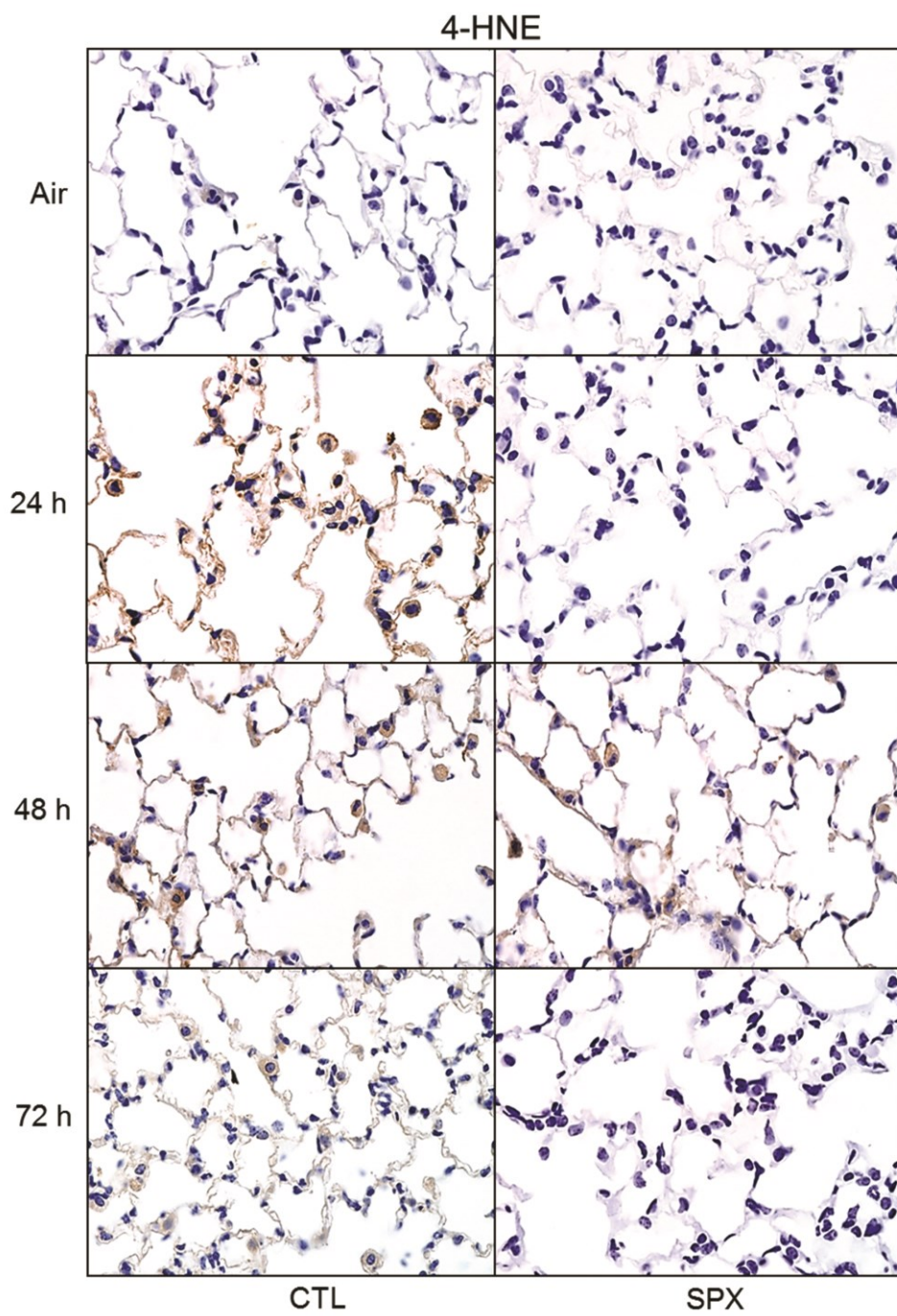
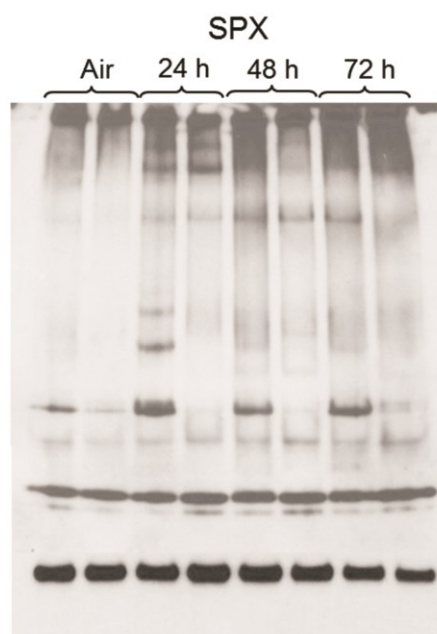
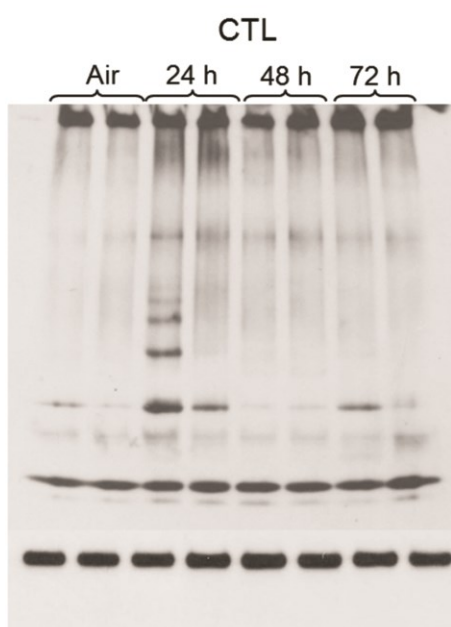
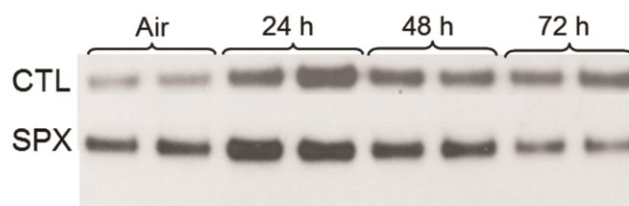


FIGURE 12. Effects of splenectomy on ozone-induced alterations in BAL SP-D structure. BAL was collected 24-72 h after exposure of CTL and SPX mice to air or ozone. BAL SP-D protein was analyzed by western blotting. *Upper panel:* SP-D protein analyzed in denatured gels. *Lower panels:* SP-D protein analyzed in native gels. Each lane represents one animal.



PART II. CCR2 REGULATES INFLAMMATORY CELL ACCUMULATION IN THE LUNG AND TISSUE INJURY FOLLOWING OZONE EXPOSURE

Ozone is a ubiquitous urban air pollutant and a major public health concern, especially in the elderly and in individuals with existing lung disease (Ciencewicki *et al.*, 2008; Uysal and Schapira, 2003). Ozone causes oxidation of membrane lipids and proteins resulting in damage to the respiratory epithelium and the alveolar epithelial layer (Mudway and Kelly, 2000; Pryor and Church, 1991). This is associated with an accumulation of pro-inflammatory macrophages in the lung, which have been implicated in the pathogenesis of ozone toxicity (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011b). Macrophage trafficking to sites of tissue injury depends on chemokines released at these sites and chemokine receptors present on responding cells (Melgarejo *et al.*, 2009). One of the most potent chemokines identified for monocytes and macrophages is macrophage chemotactic protein (MCP)-1 or CCL2, which acts by binding to the chemokine receptor, CCR2. CCL2 levels have been shown to be elevated in lungs of rodents after exposure to pulmonary irritants including ozone, nitrogen dioxide, silica, bleomycin and diesel exhaust, a response correlated with a macrophage-rich pulmonary inflammatory response (Johnston *et al.*, 2000; Okuma *et al.*, 2004; Provoost *et al.*, 2012; Williams *et al.*, 2007). Moreover, mice genetically deficient in CCR2 or treated with a CCR2 antagonist, exhibit significantly reduced recruitment of macrophages to the lung in a number of experimental disease models (Han *et al.*, 2015; Lin *et al.*, 2008b; Lin *et al.*, 2011; Osterholzer *et al.*, 2013; Yang *et al.*, 2010). The CCL2/CCR2 signaling pathway has also been implicated in inflammation-driven lung diseases in humans including asthma, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, acute respiratory distress syndrome, and

bronchiolitis obliterans (Barnes, 2008; Belperio *et al.*, 2001; Moore *et al.*, 2001; Rose *et al.*, 2003; Yadav *et al.*, 2010).

In the present studies, we analyzed the role of CCR2 in macrophage trafficking to the lung in response to ozone-induced injury. Our findings that pro-inflammatory macrophage accumulation in the lung is blunted in mice lacking CCR2, and that this is correlated with reduced injury and oxidative stress provide novel mechanistic insights into inflammatory mechanisms contributing to tissue injury induced by ozone. These data may be useful in the development of new strategies for reducing lung injury induced by air pollutants, and potentially for other inflammatory lung diseases.

RESULTS

In initial studies, we evaluated the effects of ozone on the accumulation of CCR2⁺ cells in the lung by confocal microscopy. Relatively low numbers of CCR2⁺ cells were noted in lungs of air exposed WT mice (Figure 1). Treatment of mice with ozone resulted in an increase in CCR2⁺ cells in the lung, a response which peaked 24 h post exposure. Increased numbers of CCR2⁺ blood monocytes were also observed 24 h post exposure, with no effect on bone marrow monocytes (Figure 2).

Next we used CCR2^{-/-} mice to assess the role of CCR2 in inflammatory cell trafficking to the lung in response to ozone-induced injury. Loss of CCR2 was associated with a significant reduction in CD11b⁺ inflammatory cells in the lung at all times following ozone exposure (Table 1). To determine if this was specific for inflammatory cell subpopulations, we analyzed expression of Ly6G, Ly6C, F4/80, and CD11c on CD11b⁺ cells (Sunil *et al.*, 2015). Mature (F4/80⁺CD11c⁺) infiltrating macrophages (CD11b⁺Ly6G⁻) were identified as Ly6C^{Hi} pro-inflammatory or Ly6C^{Lo} anti-inflammatory (Figures 3 and 4). Treatment of mice with ozone resulted in an increase in the percentage of Ly6C^{Hi} pro-inflammatory macrophages in the lungs of WT mice, most notably at 24 h; these cells were significantly reduced in CCR2^{-/-} mice (Figure 4). We also noted that the percentage of Ly6C^{Lo} anti-inflammatory macrophages was significantly greater in lungs of air-exposed CCR2^{-/-} mice, than in lungs of WT mice; these cells were also reduced after ozone exposure (Figure 4).

Ozone-induced increases in pro-inflammatory Ly6C^{Hi} macrophages in the lung were correlated with increased numbers of pro-inflammatory iNOS⁺ macrophages in histological sections, and with upregulation of iNOS gene expression at 24 h and 48 h

post exposure (Figures 5 and 6). Loss of CCR2 significantly blunted these responses. Following ozone exposure, mannose receptor⁺ anti-inflammatory macrophages also increased in the lung within 24 h (Figure 7); this response was delayed in CCR2^{-/-} mice. The effects of loss of CCR2 on ozone-induced inflammatory gene expression was also analyzed. In WT mice, the pro-inflammatory cytokines, IL-1 β and TNF α , were upregulated in the lung 24 h post ozone; this was significantly reduced in CCR2^{-/-} mice (Figure 6). Expression of the anti-inflammatory genes, CX3CL1, CX3CR1 and NUR77 was greater in air-exposed CCR2^{-/-} mice, when compared to WT mice. While exposure of CCR2^{-/-} mice to ozone resulted in increased expression of CX3CL1 and CX3CR1, expression of NUR77 was decreased. We also observed that expression of ADAM17, a protein important in CX3CL1 release (Garton *et al.*, 2011), was upregulated in lungs of WT mice 24 h following ozone exposure (Figure 8); in CCR2^{-/-} mice, ADAM17 was constitutively upregulated, however, ozone had no significant effect on its expression.

In further studies we determined if changes in inflammatory cell subpopulations in the lungs of CCR2^{-/-} mice were associated with alterations in ozone-induced injury and oxidative stress. Treatment of WT mice with ozone resulted in a significant increase in BAL protein content, a marker of alveolar epithelial injury (Bhalla, 1999) (Figure 9). This was blunted in CCR2^{-/-} mice at 24 h and 48 h post exposure. Similarly, ozone-induced increases in the oxidative stress marker, Cypb5 (Menoret *et al.*, 2012), and the lipid peroxidation end product, 4-HNE (Kirichenko *et al.*, 1996), were reduced in lungs of CCR2^{-/-} mice, when compared to WT mice (Figure 10 and 11). Additionally, ozone-

induced upregulation of the antioxidant, HO-1 was attenuated in lungs of CCR2^{-/-} mice at 24 h and 48 h post exposure (Figure 12).

DISCUSSION

Inflammatory macrophages have been implicated in tissue injury induced by diverse pulmonary toxicants including ozone (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011). Tissue injury is thought to be mediated by cytotoxic/pro-inflammatory mediators released from these cells (Italiani and Boraschi, 2014; Liu *et al.*, 2014). The present studies demonstrate that CCR2 is important in trafficking of inflammatory macrophages to the lung after ozone exposure and that these cells promote oxidative stress and tissue injury. This is based on our findings that ozone-induced alveolar epithelial barrier dysfunction and expression of Cypb5, 4-HNE and HO-1 are reduced in mice lacking CCR2, responses associated with decreased numbers of pro-inflammatory/cytotoxic macrophages in the lung. These data provide new insights into mechanisms regulating macrophage localization in the lung after ozone exposure and their contribution to toxicity.

Treatment of mice with ozone resulted in increased numbers of CCR2⁺ macrophages in the lung. These findings are in accord with reports that CCL2, a major ligand for CCR2, is upregulated in the lung following ozone exposure (Williams *et al.*, 2007). Earlier studies demonstrated increased expression of CCR2 in the lung in experimental models of pulmonary injury induced by diesel exhaust particles, and mustard vesicants (Pryhuber *et al.*, 2003; Tighe *et al.*, 2011b; Venosa *et al.*, 2016), as well as in models of infection (Lin *et al.*, 2011) and fibrosis (Okuma *et al.*, 2004). These data suggest that CCR2⁺ inflammatory macrophage accumulation in the lung may be a general response to injury and infection. We also found that the percentage of CCR2⁺ blood monocytes increased 24 h post ozone exposure, consistent with the notion that these cells originate within the bone marrow (Jung *et al.*, 2015). The fact that no changes

were noted in CCR2⁺ monocytes in the bone marrow may be due to rapid replacement of these cells as they exit the tissue (Wang *et al.*, 2009).

CCR2 has been shown to be required for inflammatory monocyte egress from the bone marrow and accumulation at sites of tissue injury (Serbina and Pamer, 2006; Tsou *et al.*, 2007). We found that loss of CCR2 resulted in a significant reduction in CD11b⁺ infiltrating macrophages in the lung at all post ozone exposure time points analyzed, demonstrating that these cells are derived from blood and bone marrow precursors. As previously reported (Sunil *et al.*, 2015), CD11b⁺ cells responding to ozone were found to be comprised of subpopulations of mature (F4/80⁺CD11c⁺) macrophages exhibiting a proinflammatory (Ly6C^{hi}) or anti-inflammatory (Ly6C^{Lo}) phenotype. Following ozone exposure, pro-inflammatory Ly6C^{hi} macrophages increased in the lung at 24 h, a time consistent with peak accumulation of CCR2⁺ macrophages in the tissue; this response was blunted in CCR2^{-/-} mice. Pro-inflammatory Ly6C^{hi} macrophages accumulating in the lung in response to infection have been shown to express CCR2 and to promote pulmonary injury (Chen *et al.*, 2013) (Lin *et al.*, 2008b). Our findings of a similar correlation between the presence of CCR2⁺ and Ly6C^{hi} macrophages in the lung and ozone-induced tissue injury provide additional support for the cytotoxic/pro-inflammatory activity of these cells (Shi and Pamer, 2011).

Loss of CCR2 was also associated with reduced numbers of iNOS⁺ macrophages in the lung and down regulation of iNOS gene expression, indicating that these cells also traffic to the lung via the CCL2/CCR2 signaling pathway. As expression of iNOS is a characteristic feature of pro-inflammatory/cytotoxic macrophages (Davis *et al.*, 2013) (Laskin *et al.*, 2011), we speculate that CCR2-dependent pro-inflammatory/cytotoxic

macrophages accumulating in the lung in response to ozone are both Ly6C^{Hi} and iNOS⁺. This is supported by previous studies demonstrating pro-inflammatory Ly6C^{Hi} macrophages express iNOS and release cytotoxic oxidants (Dragomir *et al.*, 2012). In macrophages, iNOS mediates the generation of nitric oxide from L-arginine. Nitric oxide is known to rapidly react with oxygen radicals generating additional highly reactive species, which are thought to be important in the ability of pro-inflammatory macrophages to promote tissue injury (Kobayashi, 2010; Wink *et al.*, 2011). Previous studies have demonstrated that macrophage iNOS is key to ozone toxicity (Fakhrzadeh *et al.*, 2002; Kleeberger *et al.*, 2001). Thus, mice lacking iNOS were unable to generate reactive nitrogen species, a response correlated with reduced ozone toxicity. The present studies suggest that pro-inflammatory macrophages are primary contributors of iNOS-derived reactive nitrogen species during the pathogenesis of ozone-induced lung injury, and that these cells accumulate in the lung in a CCR2-dependent manner. A similar dependence of iNOS activity on CCR2 has previously been described in a model of noninfectious lung injury, as well as in a model of allergic contact dermatitis (Chong *et al.*, 2014; Okuma *et al.*, 2006).

Decreases in pro-inflammatory macrophages in lungs of CCR2^{-/-} mice in response to ozone were associated with a reduction in expression of the pro-inflammatory genes, IL-1 β and TNF α . These findings are in accord with studies demonstrating that proinflammatory macrophages are a major source of these cytokines (Arango Duque and Descoteaux, 2014; Herold *et al.*, 2011). Similar decreases in TNF α and IL-1 β have been reported in mice lacking CCR2 in experimental models of skin injury and lung injury (Chong *et al.*, 2014; Tighe *et al.*, 2011a). IL-1 and TNF α have been implicated in lung

injury induced by diverse irritants including ozone (Cho *et al.*, 2007; Johnson *et al.*, 2009). Protection against ozone-induced lung injury and oxidative stress in CCR2^{-/-} mice may be due to decreased release of these proinflammatory mediators. This is supported by previous reports demonstrating that mice lacking TNFR1, the major proinflammatory receptor for TNF α , or NF κ B, a transcription factor known to regulate IL-1 and TNF α production, are protected from ozone toxicity (Fakhrzadeh *et al.*, 2004) (Fakhrzadeh *et al.*, 2008).

Further analysis of lung macrophage subpopulations revealed significantly greater numbers of mature CD11b⁺Ly6G⁺Ly6C^{Lo} anti-inflammatory macrophages in air exposed CCR2^{-/-} mice, when compared to WT mice, along with increased expression of Nur77, a nuclear transcription factor known to regulate macrophage anti-inflammatory/pro-resolution responses (Laskin *et al.*, 2011). This may be due to a loss of Ly6C^{Hi} proinflammatory macrophages to counterbalance their activity (Moore *et al.*, 2013; Wang *et al.*, 2014). As observed with Ly6C^{Hi} pro-inflammatory macrophages, in the absence of CCR2, reduced numbers of Ly6C^{Lo} anti-inflammatory macrophages were observed in the lung after ozone exposure; Nur77 expression was also down regulated. These findings suggest that Ly6C^{Lo} anti-inflammatory macrophages are the main cell type expressing Nur77 in the lung after ozone, and that they are derived, at least in part, from blood and bone marrow precursors (Ginhoux and Jung, 2014). Alternatively, decreases in numbers of Ly6C^{Lo} anti-inflammatory macrophages in CCR2^{-/-} mice treated with ozone may be a consequence of reduced numbers of pro-inflammatory macrophages available for phenotypic switching (Italiani and Boraschi, 2014; Wang *et al.*, 2014). We also found that ozone-induced accumulation of mannose receptor⁺ anti-inflammatory

macrophages was delayed in CCR2^{-/-} mice relative to WT mice. These results are consistent with the idea that there are multiple subpopulations of anti-inflammatory/wound repair macrophages responding to ozone-induced tissue injury, and that mechanisms regulating their activity are distinct (Boorsma *et al.*, 2013). The delayed appearance of mannose receptor⁺ macrophages in the lung may reflect the time required for the generation of additional chemoattractants for these cells. This is supported by our findings that CX3CL1 and ADAM17, an enzyme important in CX3CL1 release (Garton *et al.*, 2011), are increased in lungs of CCR2^{-/-} mice following ozone exposure.

CX3CR1 is a chemokine receptor expressed on anti-inflammatory monocytes/macrophages which is thought to be important in their maturation/development (Shi and Pamer, 2011; Yang *et al.*, 2014a). Upregulation of CX3CR1 in lungs of CCR2^{-/-} mice following ozone exposure may represent an attempt to compensate for the loss of anti-inflammatory macrophages responding to ozone in the absence of CCR2. In this regard, Tighe *et al.* (2011a) identified a population of repair macrophages in the lung after ozone exposure that are dependent on CX3CR1, and that in their absence, oxidative stress is exacerbated. Although Ly6C^{Lo} macrophages were reduced in lungs of CCR2^{-/-} after ozone exposure, they still outnumbered Ly6C^{Hi} macrophages. This suggests that tissue repair processes in CCR2^{-/-} mice are more prominent, which is in accord with our findings that in the absence of CCR2, ozone toxicity and oxidative stress are reduced. These data provide support for the idea that the outcome of the pathogenic response to ozone depends on a balance between pro-inflammatory and anti-inflammatory macrophages (Laskin *et al.*, 2011).

Ozone-induced oxidative stress, as measured by expression of 4-HNE, Cypb5, and HO-1 were markedly reduced in lungs of CCR2^{-/-} mice, relative to WT mice, demonstrating the key contribution of CCR2-dependent inflammatory cells to the oxidative burden in the lung. Previous studies have described a population of CX3CR1-dependent inflammatory macrophages in lungs of mice exposed to ozone that function to limit pathological responses to ozone, presumably because of their ability to scavenge oxidants and/or manage oxidant balance (Tighe *et al.*, 2011a). Our results suggest that Ly6C^{Lo} and mannose receptor⁺ inflammatory macrophages also contribute to these activities in the lung after ozone exposure.

In summary, the present studies demonstrate a key role of CCR2 in regulating pro-inflammatory macrophage accumulation in the lung in response to ozone, and that these cells contribute to lung injury and oxidative stress. Elucidating specific subpopulations of inflammatory cells responding to ozone and mechanisms regulating their trafficking and activity may lead to the development of more efficacious approaches for mitigating oxidant-induced pulmonary toxicity and disease pathogenesis.

FIGURE 13. Effects of ozone on CCR2⁺ cells in the lung. Sections, prepared 24-72 h after exposure of WT mice to air or ozone, were stained with AF647-conjugated anti-mouse CCR2 antibody. CCR2⁺ cells in 3 randomly selected fields/slide were enumerated microscopically at 63x. Bars, mean \pm SE (n=3). ^aSignificantly different (p <0.05) from air exposed animals.

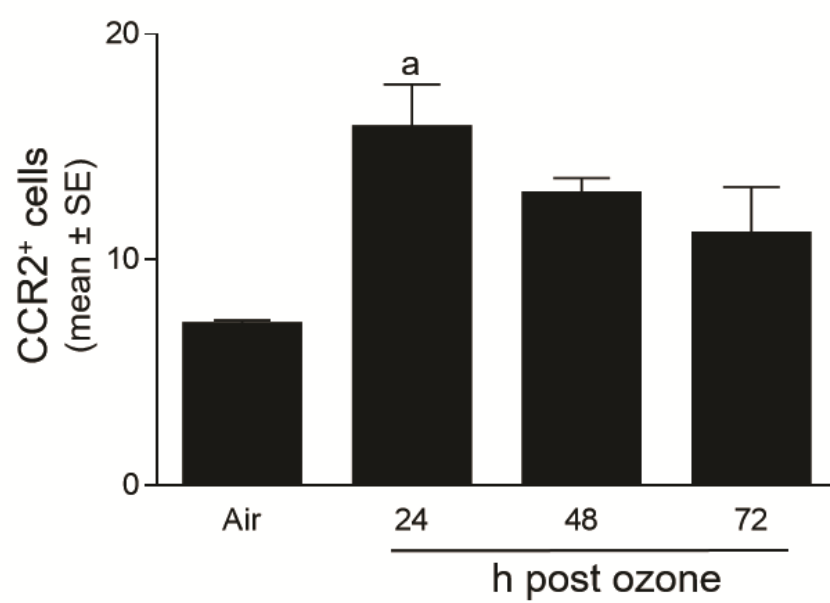


FIGURE 14. Effects of ozone on blood and bone marrow monocyte CCR2

expression. Cells, collected 24-72 h after exposure of WT mice to air or ozone, were immunostained with antibodies to CCR2 and analyzed by flow cytometry as described in the Materials and Methods. One representative histogram from 3-4 mice/treatment group is shown.

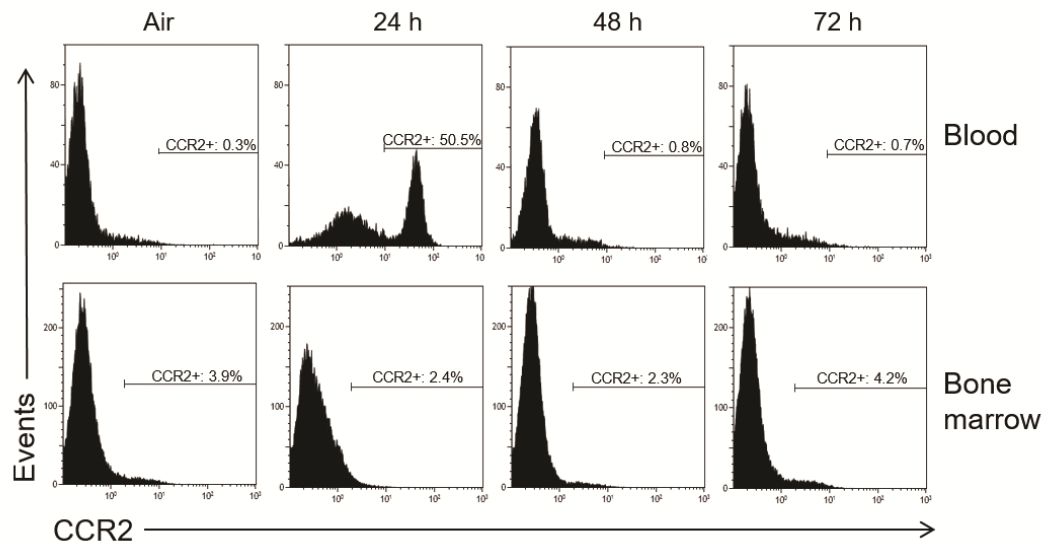


TABLE 3. Effects of loss of CCR2 on ozone-induced increases in CD11b⁺ myeloid cells in the lung. BAL cells, collected 24–72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were immunostained with antibodies to CD11b and analyzed by flow cytometry as described in the Materials and Methods. Values are the mean ± S.E (n=4-8 mice). ^aSignificantly different from air. ^bSignificantly different from WT (p<0.05).

Time (h)	% CD11b ⁺ Cells	
	WT	CCR2 ^{-/-}
Air	1.6 ± 0.3	0.5 ± 0.1
24	4.7 ± 1.0	1.4 ± 0.3 ^b
48	11.2 ± 1.3 ^a	1.0 ± 0.1 ^b
72	5.1 ± 1.4	1.5 ± 0.2 ^b

FIGURE 15. Effects of loss of CCR2 on ozone-induced increases in CD11b⁺ infiltrating macrophages in the lung. BAL cells, collected 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, and F4/80, CD11c or isotypic controls, and analyzed by flow cytometry as described in the Materials and Methods. One representative dot plot from of 3-4 mice/treatment group is shown.

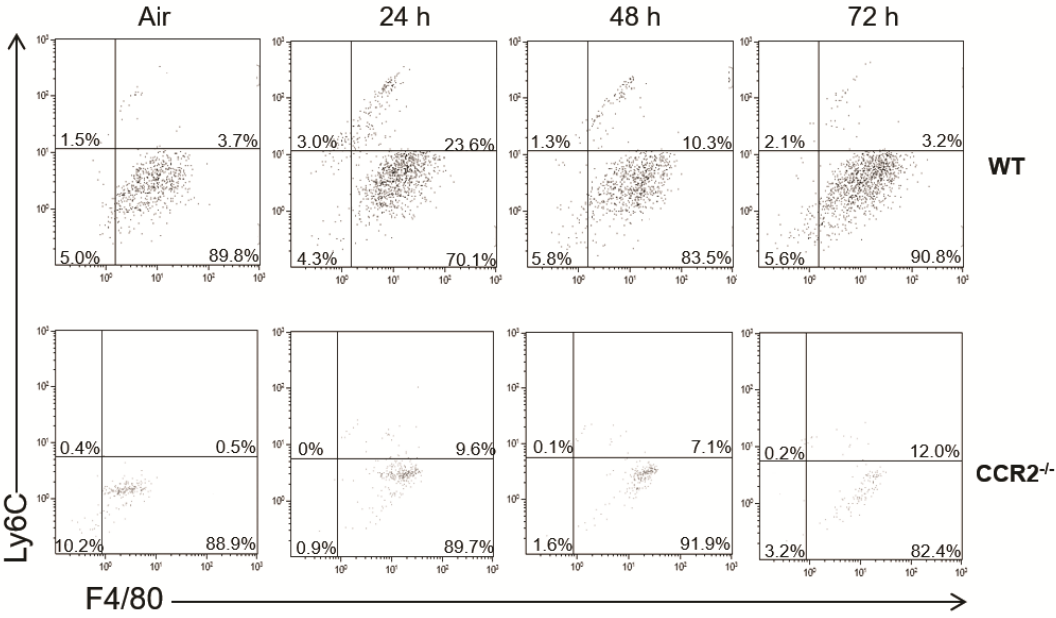


FIGURE 16. Effects of loss of CCR2 on lung macrophage subpopulations

responding to ozone. BAL cells, collected 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, and F4/80 or isotypic controls, and analyzed by flow cytometry. Bars, mean \pm SE (n = 3-10).

^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from WT. ND, not detected.

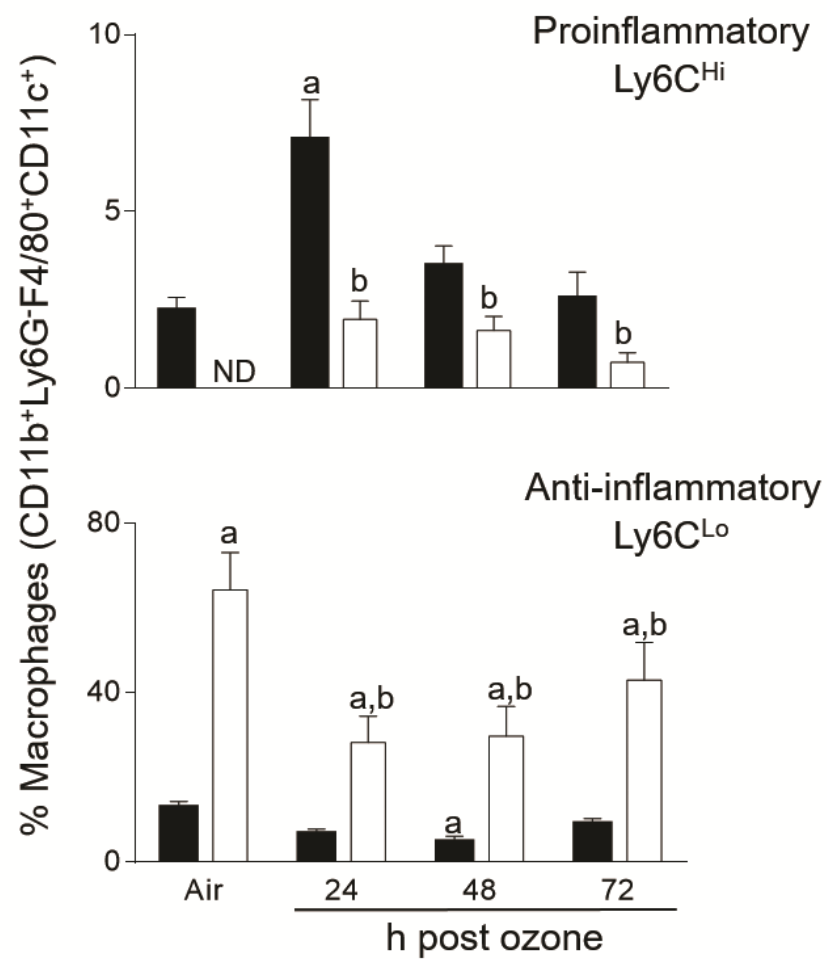


FIGURE 17. Effects of loss of CCR2 on ozone-induced iNOS expression. Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to iNOS. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

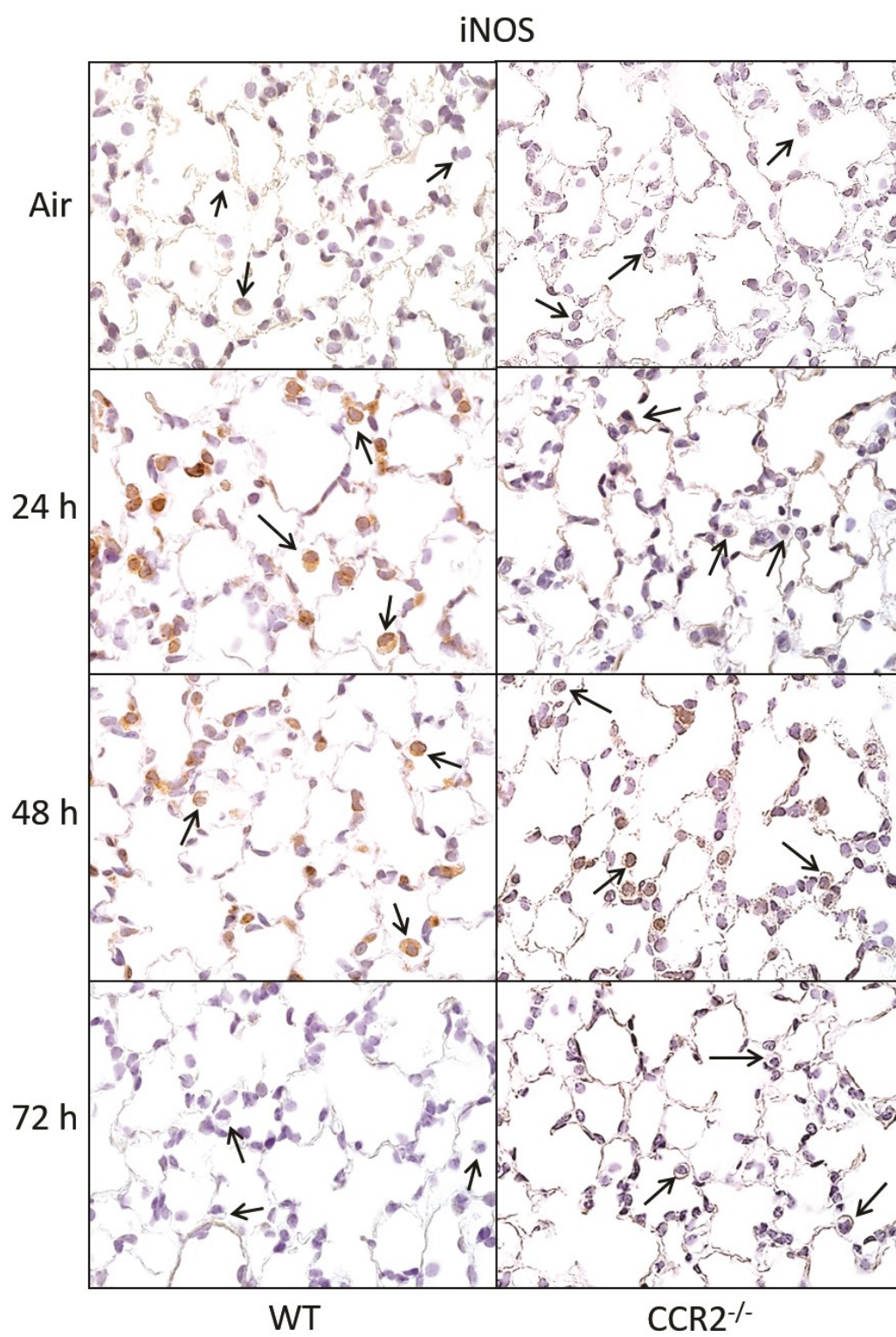


FIGURE 18. Effects of CCR2 on ozone-induced gene expression. Lungs, collected 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were analyzed by real-time PCR. Data were normalized to GAPDH. Bars, mean \pm S.E (n = 3-4 mice). ^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from WT.

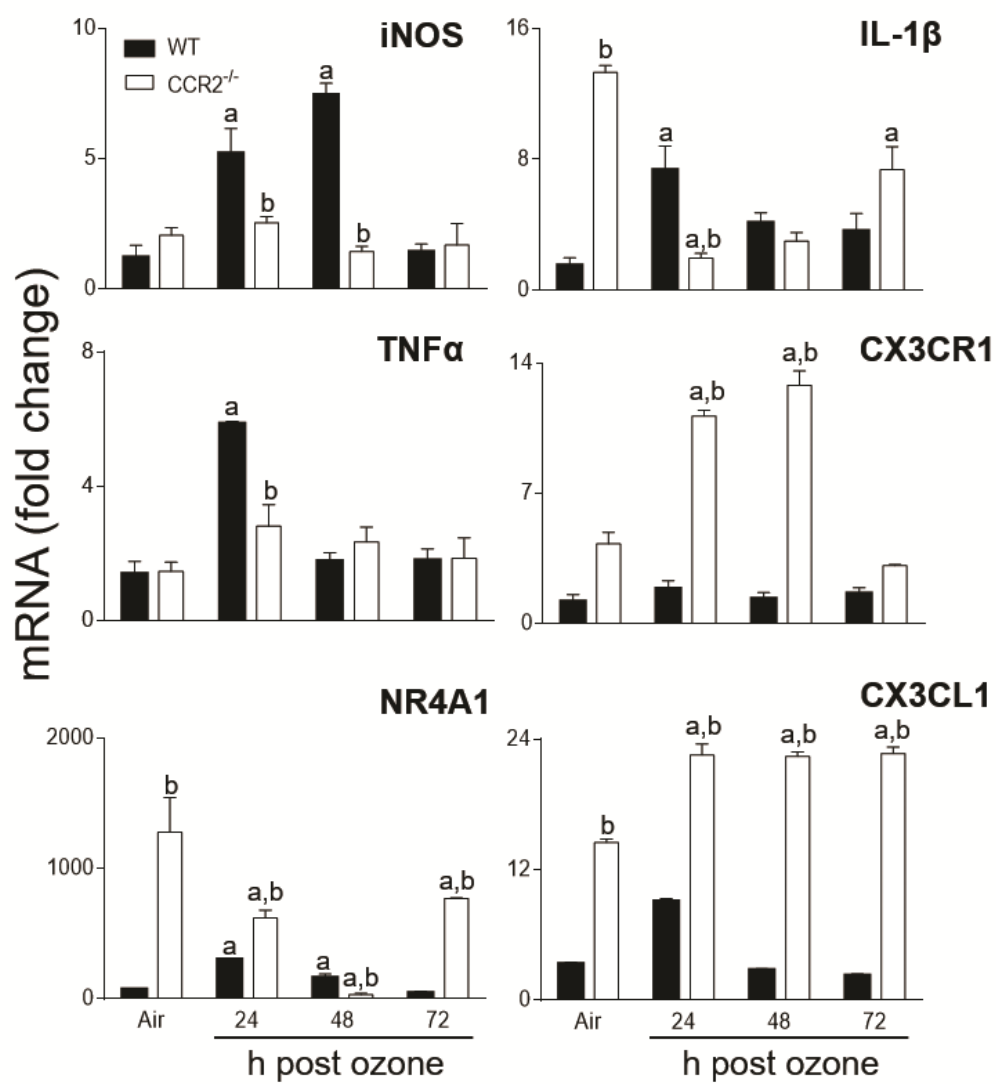


FIGURE 19. Effects of loss of CCR2 on ozone-induced mannose receptor

expression. Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to mannose receptor. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

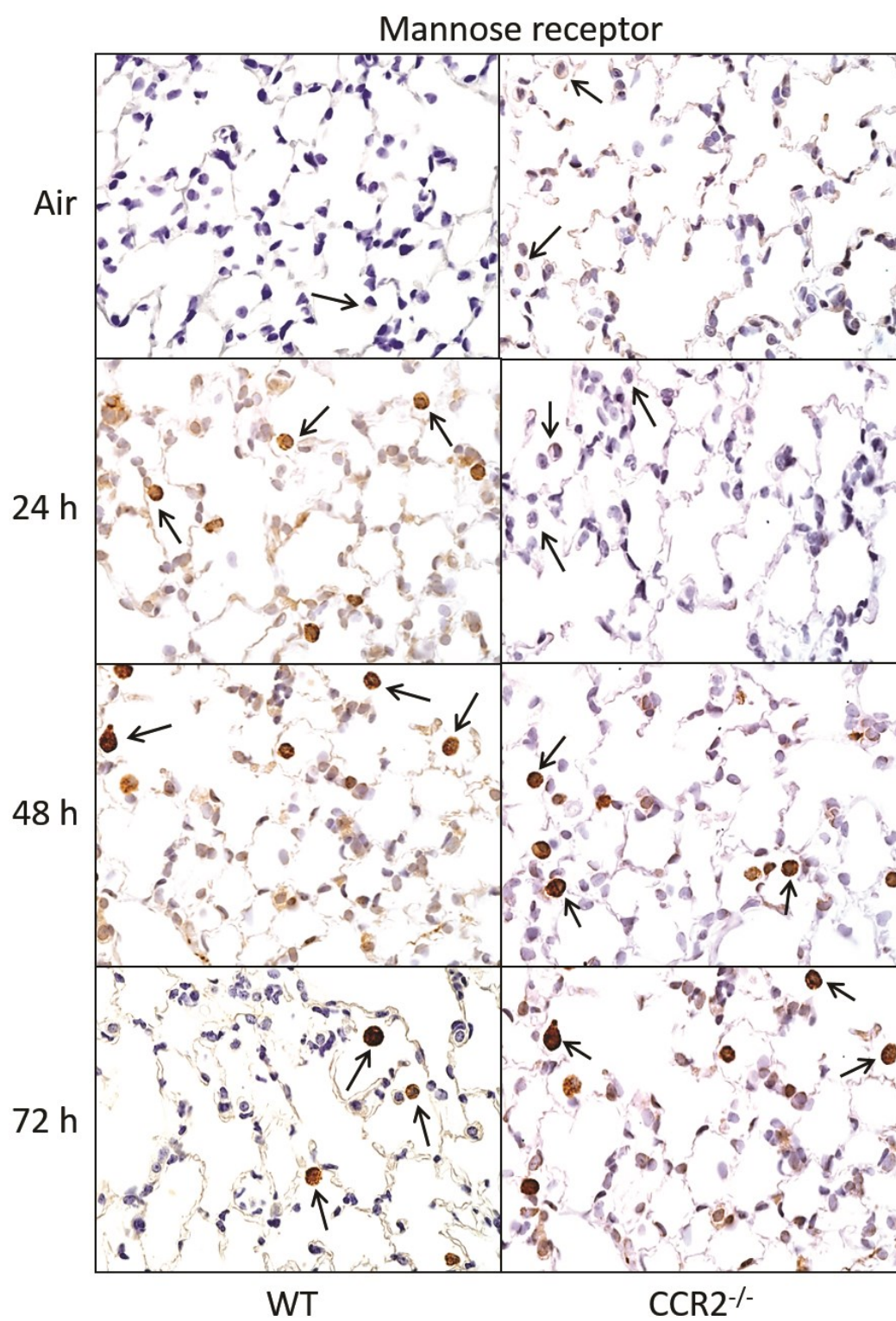


FIGURE 20. Effects of loss of CCR2 on ozone-induced expression of ADAM17.

Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to ADAM17. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown (*n* = 3 mice/treatment group). Original magnification, x600.

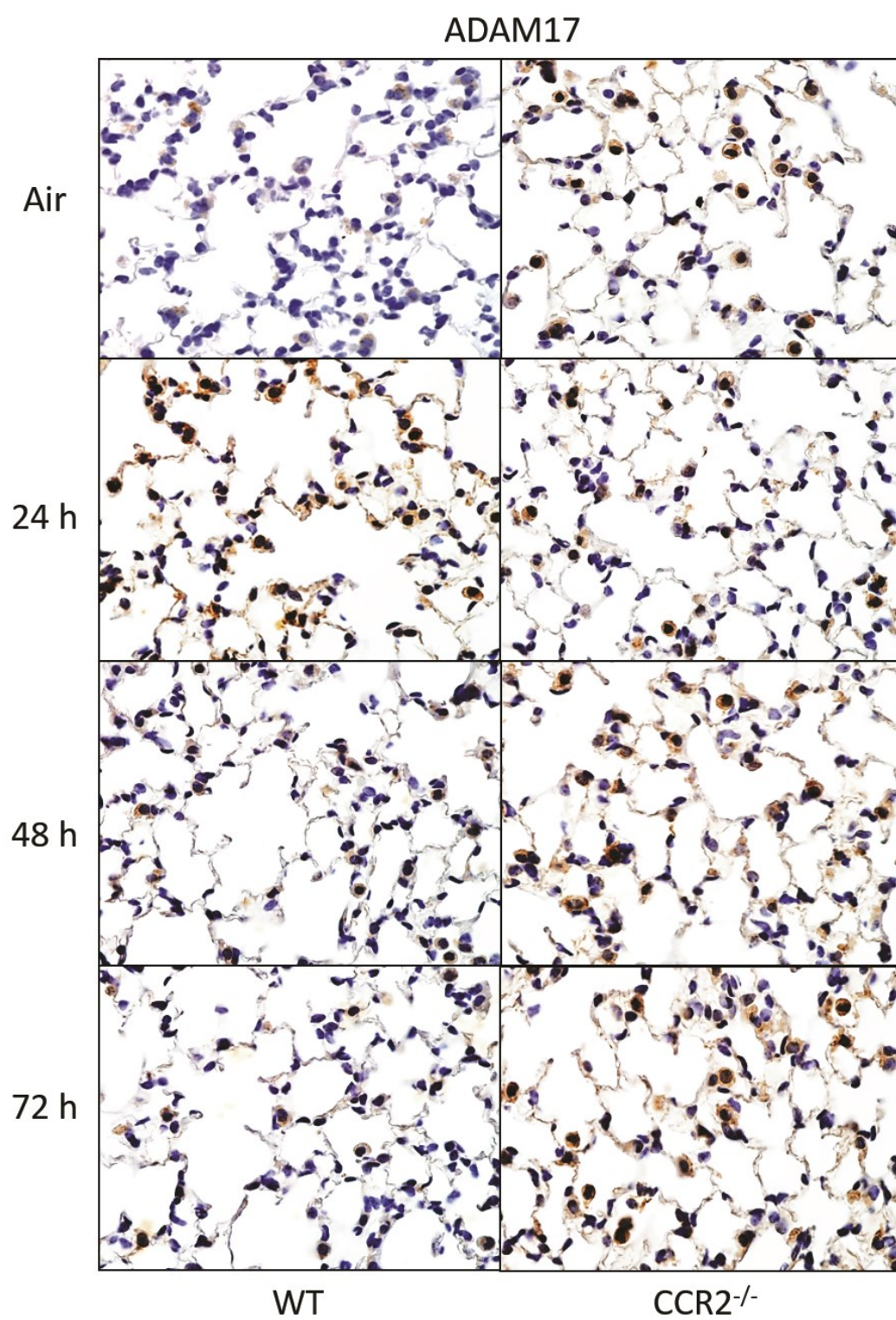


FIGURE 21. Effects of loss of CCR2 on ozone-induced alterations in BAL protein.

BAL was collected 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone. Cell-free supernatants were analyzed in triplicate for protein using a BCA protein assay kit.

Bars, mean \pm SE (n = 3-8 mice). ^aSignificantly different (p < 0.05) from air-exposed animals; ^bSignificantly different (p < 0.05) from WT.

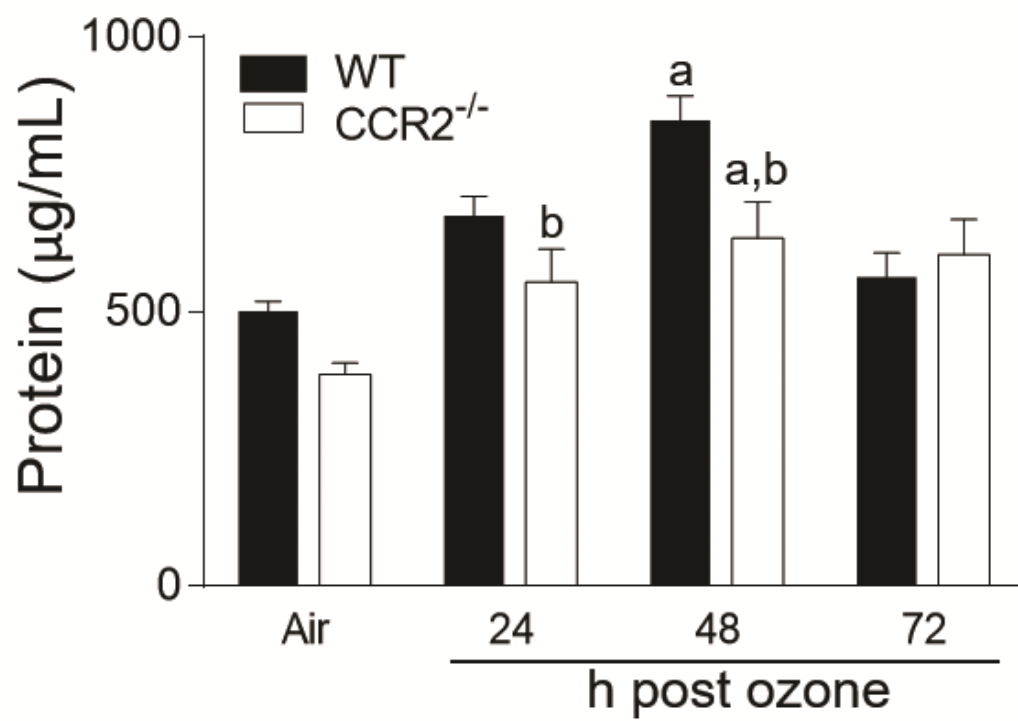


FIGURE 22. Effects of loss of CCR2 on ozone-induced expression of cytochrome

b5. Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to cytochrome b5. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

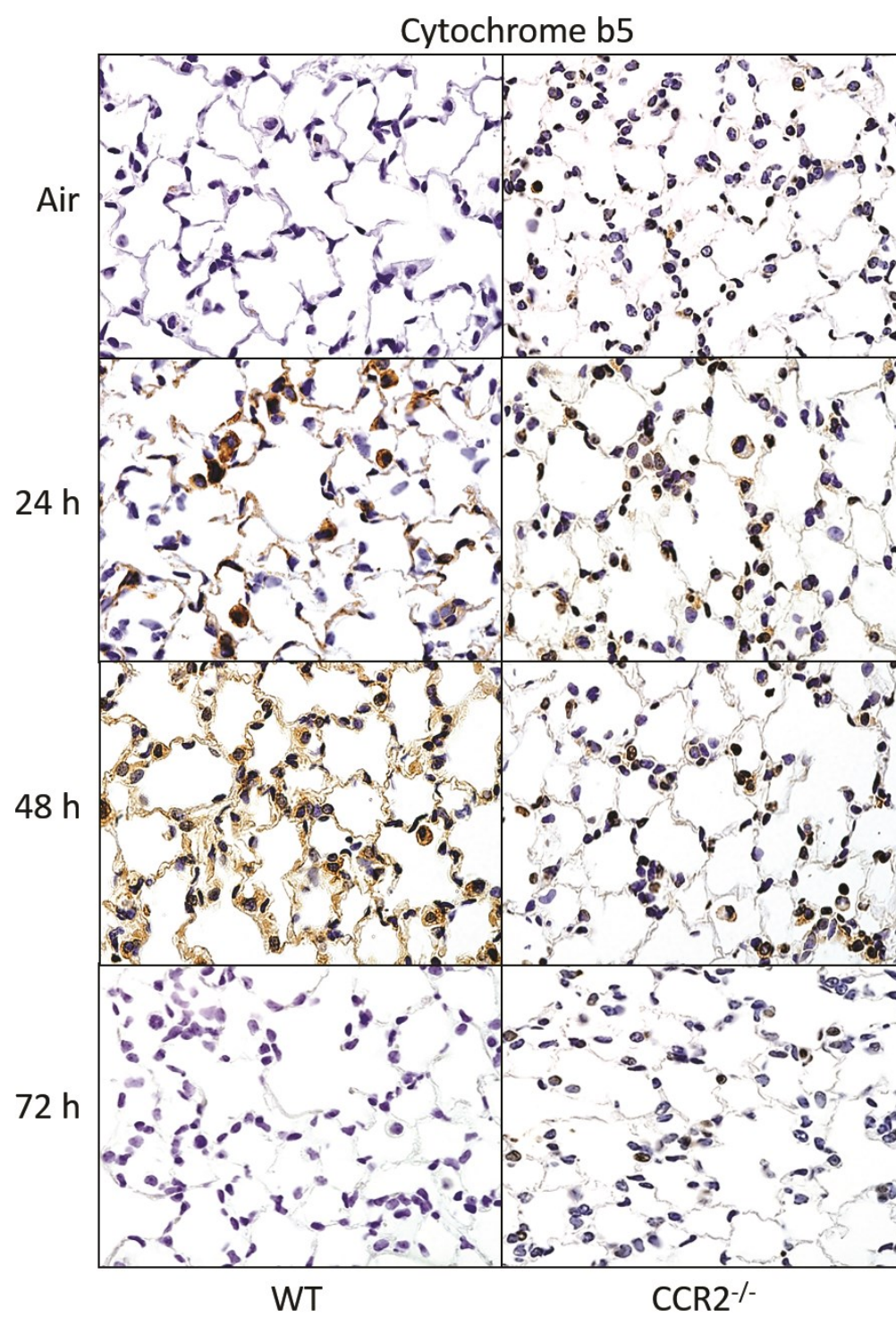


FIGURE 23. Effects of loss of CCR2 on ozone-induced expression of 4-HNE. Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to 4-HNE. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

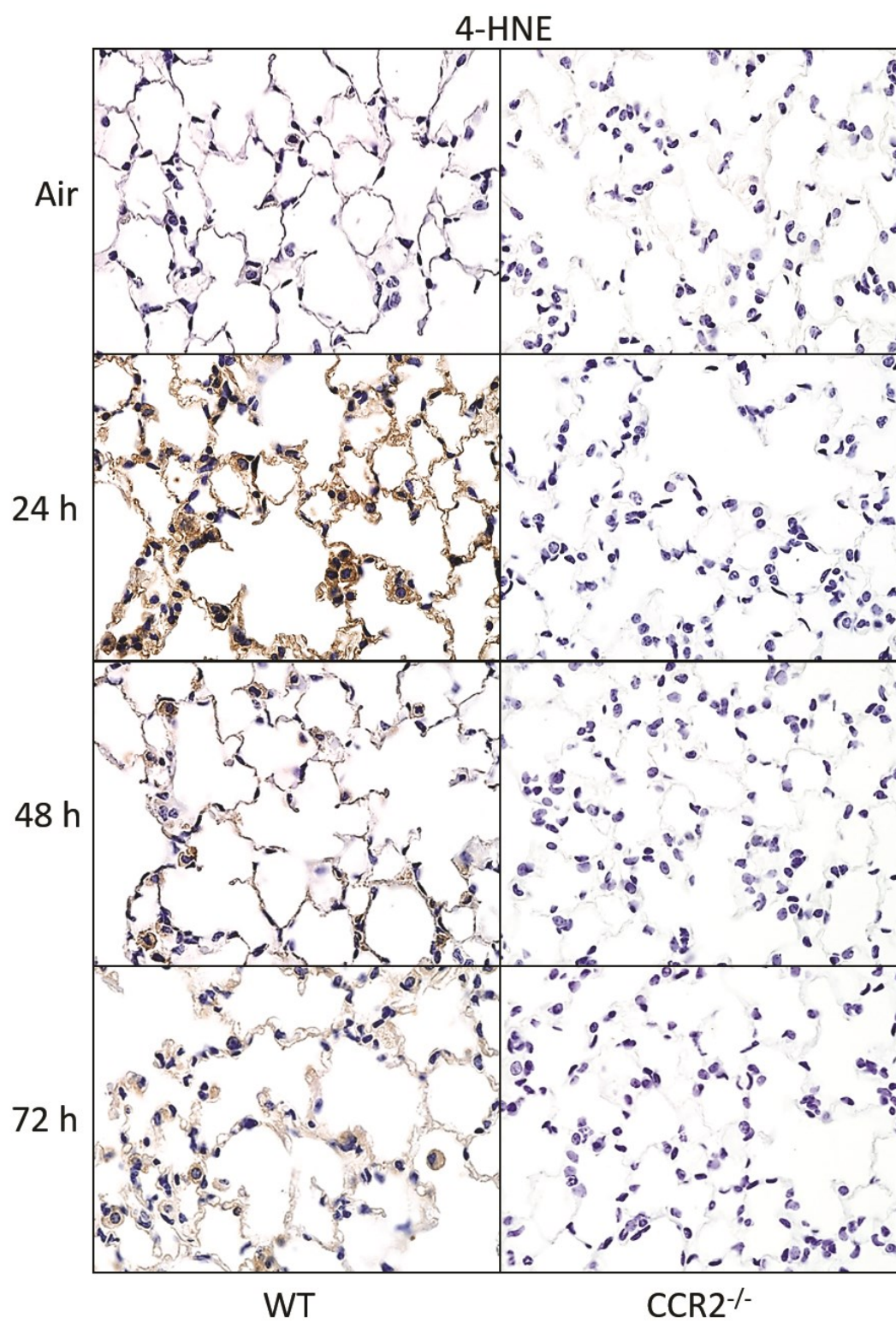
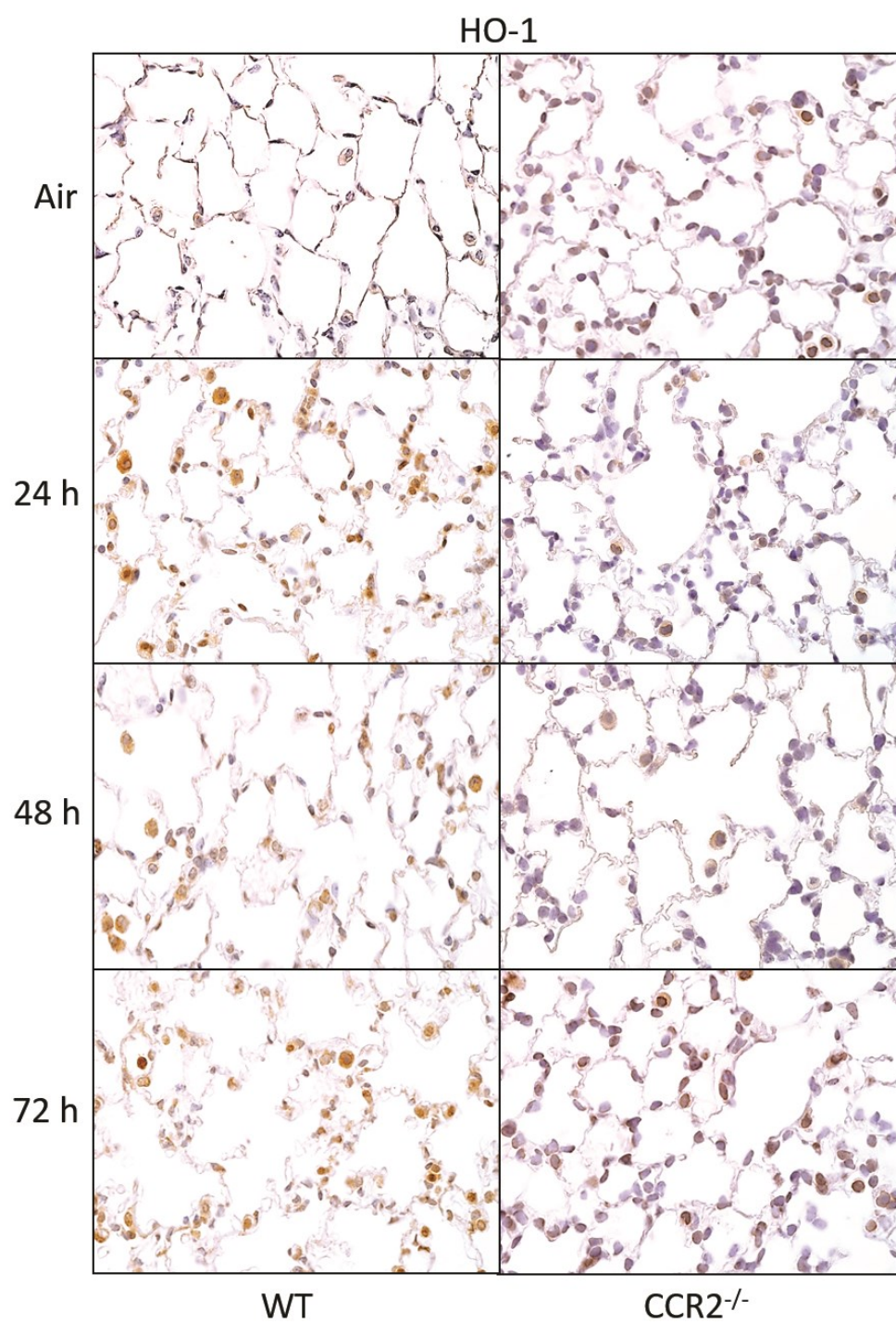


FIGURE 24. Effects of loss of CCR2 on ozone-induced expression of HO-1. Lung sections, prepared 24-72 h after exposure of WT and CCR2^{-/-} mice to air or ozone, were stained with antibody to HO-1. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.



PART III. TRACKING INFLAMMATORY MACROPHAGE ACCUMULATION IN THE LUNG DURING OZONE-INDUCED LUNG INJURY IN MICE

Ozone is a ubiquitous urban air pollutant that causes oxidative stress, inflammation, and decreased lung functioning (Mudway and Kelly, 2004; Savov *et al.*, 2004). Evidence suggests that inflammatory macrophages recruited to the lung following ozone induced injury contribute to oxidative stress and toxicity (Hiraiwa and van Eeden, 2013; Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011). These cells have been characterized as classically activated macrophages that release cytotoxic/pro-inflammatory mediators including reactive oxygen and nitrogen species, and tumor necrosis factor, which have been implicated in tissue injury (Fakhrzadeh *et al.*, 2002; Sunil *et al.*, 2015b; Sunil *et al.*, 2012b). Inflammatory macrophages have also been shown to play a protective role in the lung by clearing oxidized products and cellular debris after ozone-induced injury (Dahl *et al.*, 2007a). These cells, referred to as alternatively activated macrophages are highly phagocytic and release mediators that down regulate inflammation and initiate wound repair (Laskin *et al.*, 2011). Mechanistic studies have implicated chemotactic factors and other inflammatory mediators in macrophage-mediated induction or suppression of inflammation. In this regard, the accumulation of proinflammatory and anti-inflammatory macrophage subpopulations depends on chemokines released at injured sites and chemokine receptors present on responding cells (Duque and Descoteaux, 2015; Shi and Pamer, 2011). Thus, while proinflammatory macrophages trafficking is mediated in large part by the chemokine receptor CCR2 and its ligand CCL2, anti-inflammatory macrophage trafficking involves the chemokine receptor CX3CR1 and its ligand CX3CL1 (Yang *et al.*, 2014a).

Earlier studies demonstrated upregulation of the CCL2/CCR2 and CX3CL1/CX3CR1 signaling pathways in the lung following exposure of animals to pulmonary irritants including ozone, tobacco smoke, particulate matter, hypoxia, silica and/or bleomycin (Krishnaswamy *et al.*, 1999; McComb *et al.*, 2008; Tighe *et al.*, 2011a) (Okuma *et al.*, 2004; Johnston *et al.*, 2000; Provoost *et al.*, 2012). CCR2 has also been implicated in inflammation-driven lung disorders such as allergic asthma and idiopathic pulmonary fibrosis (Deng *et al.*, 2013; Moore *et al.*, 2005). The present studies were designed to track pro- and anti-inflammatory macrophage accumulation in the lung from the bone marrow following ozone exposure, and to assess the role of these chemokine signaling pathways in their migration. Results from these studies demonstrate that bone marrow-derived macrophage subpopulations that accumulate in the lung following ozone exposure consist of pro- and anti-inflammatory macrophage subpopulations that can be distinguished based on chemokine receptors they express. These findings are important as they provide insights into inflammatory mechanisms of ozone toxicity which may lead to the development of new therapeutic approaches for treating inflammatory lung diseases.

RESULTS

Effects of ozone on trafficking of GFP⁺ bone marrow cells to the lung

Approximately 90% of monocytes in bone marrow of chimeric mice were GFP⁺; this did not change after ozone exposure (Table 1). In contrast, a time related increase in GFP⁺ cells was observed in the lung following ozone exposure, a response which peaked at 24 h (Table 1). To analyze the phenotype of GFP⁺ lung macrophages responding to ozone, tissue sections were stained with antibodies to M1 (Ly6C, CCR2) and M2 (YM1, Nur77, HO-1, CX3CR1) markers and analyzed by confocal microscopy. Exposure of mice to ozone resulted in an increase in GFP⁺Ly6C⁺ and GFP⁺CCR2⁺ cells in the lung at 48 h (Figure 1). GFP⁺YM1⁺ and GFP⁺HO-1⁺ macrophages were also increased 48 h post ozone (Figure 1). In contrast, ozone had no major effect on GFP⁺CX3CR1⁺ or GFP⁺Nur77⁺ cells.

To further characterize GFP⁺ cells accumulating in the lung after ozone, we used techniques in flow cytometry. In these studies, lung macrophages were identified as CD11b⁺F4/80⁺CD11c⁺ and further characterized as pro-inflammatory (Ly6C^{Hi}) or anti-inflammatory (Ly6C^{Lo}) (Sunil et al., 2015). We found that 65-75% of GFP⁺ cells were CD11b⁺, consistent with an infiltrating myeloid cell phenotype (Kirby et al., 2006). In contrast, GFP⁻ cells were mostly CD11b⁻, indicative of a resident macrophage phenotype. Both pro-inflammatory GFP⁺CD11b⁺Ly6C^{Hi} and anti-inflammatory GFP⁺CD11b⁺Ly6C^{Lo} macrophages increased in the lung after 24 h post ozone (Figure 2). Conversely, ozone had no significant effect on GFP⁻ macrophages.

Effects of ozone on trafficking of CX3CR1^{+/GFP} macrophages to the lung

In our next series of studies we analyzed CX3CR1⁺ M2 macrophage trafficking to the lung using CX3CR1^{+/GFP} reporter mice (Tighe et al., 2011a). Following ozone exposure, we observed a time related increase in CX3CR1^{+/GFP} macrophages was observed in the lung, which reached a maximum at 96 h (Table 2). CX3CR1^{+/GFP} macrophages were found to co-express YM1 and Nur77, consistent with an M2 phenotype (Figure 3). After ozone exposure, there was a decrease in CX3CR1⁺ cells co-expressing YM1, with no effect on CX3CR1⁺ cells co-expressing Nur77. Flow cytometric analysis of isolated lung macrophages showed that the majority of CX3CR1^{+/GFP} macrophages were Ly6C^{Lo} (Figure 4). This is in contrast to CCR2⁺ macrophages, which expressed high levels of Ly6C. Treatment of mice with ozone resulted in time related increases in both CCR2⁺Ly6C^{Hi} and CX3CR1⁺Ly6C^{Lo} macrophages in the lung (Figure 4). Whereas increases in CCR2⁺Ly6C^{Hi} macrophages were rapid, peaking 24-48 h post ozone, CX3CR1⁺Ly6C^{Lo} macrophages increased more gradually for at least 96 h.

DISCUSSION

The bone marrow has been established as the major source of inflammatory monocytes that accumulate at sites of tissue injury. Migration of these cells is regulated by chemokines and chemokine receptors that direct their trafficking to sites of inflammation. The present studies demonstrate that the bone marrow functions as a source of both pro-inflammatory and anti-inflammatory macrophages that contribute to ozone toxicity in the lung. This is based on our findings that bone marrow-derived GFP⁺ macrophages accumulate in the lung follow ozone exposure. Furthermore, GFP⁺ macrophage subpopulations expressing chemokine receptors CCR2 or CX3CR1 were identified. Whereas CCR2⁺ macrophages were pro-inflammatory expressing high levels of Ly6C, CX3CR1⁺ macrophages were anti-inflammatory and expressed low levels of this antigen. These data confirm that macrophages that accumulate in the lung are derived from the bone marrow and that pro- and anti-inflammatory subpopulations can be distinguished based on chemokine receptors they express.

In these studies, we used *in vivo* tracking techniques to investigate the origin of infiltrating macrophage subpopulations. Initially, we used GFP⁺ chimeric mice. Our findings that >85% of bone marrow cells were GFP⁺, while only <5% of lung cells were GFP⁺ are consistent with findings in studies with chimeric mice in models of pulmonary fibrosis and emphysema (Hashimoto *et al.*, 2004; Ishizawa *et al.*, 2004; Nakashima *et al.*, 2013). Following ozone exposure, increased numbers of GFP⁺CD11b⁺ inflammatory cells were observed in the lung. These cells represent newly recruited macrophages which are distinct from GFP⁻CD11b⁻ resident macrophages. Resident alveolar macrophages are relatively long-lived cells that do not require replenishment from circulating monocytes (Hashimoto *et al.*, 2013; Janssen *et al.*, 2011; Yona *et al.*, 2013).

The fact that GFP⁺CD11b⁺ resident macrophages are abundant in the lungs of chimeric mice confirm that radiation-induced myeloablation caused minimal damage to the lung.

Confocal microscopic analysis of the lung tissue from GFP⁺ chimeric mice exposed to air confirmed that only a small percentage of macrophages expressed GFP. These cells were mainly localized in the alveoli which is typical for leukocytes (Hogan *et al.*, 2014). GFP⁺ macrophages were found to express the anti-inflammatory marker YM1, consistent with an M2-like phenotype. Following ozone exposure, an accumulation of GFP⁺ macrophages that co-expressed YM1 was noted, which is in accord with previous findings that anti-inflammatory macrophages accumulate in the lung after ozone exposure (Groves *et al.*, 2013; Sunil *et al.*, 2012). YM1⁺ macrophages have been observed in lung of mice postnatally, confirming that these cells represent a recruited subpopulation (Hung *et al.*, 2002). The induction of HO-1 is a cellular response to oxidative stress (Raval and Lee, 2010). HO-1 expression is also considered a marker of anti-inflammatory macrophages (Naito *et al.*, 2014). Following ozone exposure, increased numbers of GFP⁺ lung macrophages co-expressing HO-1 were observed which is in line with the idea that GFP⁺ macrophages are anti-inflammatory. Nur77 is a transcription factor known to control bone marrow monocyte differentiation and the survival of anti-inflammatory Ly6C^{Lo} monocytes (Hanna *et al.*, 2011). We found increased numbers of GFP⁺ co-expressing Nur77. Taken together, these data demonstrate that GFP⁺ macrophages accumulating in the lung after ozone, consist of a subpopulation of cells with anti-inflammatory/wound repair activity.

Ozone inhalation results in increased numbers of infiltrating CD11b⁺ cells in the lung (Sunil *et al.*, 2015; Tighe *et al.*, 2011a). We found that these CD11b⁺ cells were

largely derived from the bone marrow. Thus, the majority of these cells were also GFP⁺. However, a population of GFP⁺CD11b⁺ cells were identified. The spleen has been shown to act as an extramedullary source of inflammatory monocytes that differentiate into macrophages that contribute to tissue injury (Kim et al., 2014; Swirski et al., 2009). It may be that the GFP⁺CD11b⁺ macrophages are recruited from an extramedullary source such as the spleen. In this regard, we previously demonstrated that splenic macrophage precursors contribute to pro-inflammatory lung macrophage pools in response to ozone toxicity (Francis *et al.*, 2016).

In further studies we assessed the role of chemokine receptors in mediating the recruitment of bone marrow derived-monocytes to the lung after ozone exposure. We found that both CX3CR1⁺ and CCR2⁺ macrophages are present in the lungs of ozone-exposed mice, demonstrating that both populations are bone marrow-derived. The fact that CX3CR1⁺ cells were mainly Ly6C^{Lo}, while CCR2⁺ cells were Ly6C^{Hi} demonstrate that expression of these chemokine receptors can be used to distinguish between pro- and anti-inflammatory macrophages responding to ozone. These findings are consistent with early studies on blood monocytes (Geissmann *et al.*, 2003; Jacquelin *et al.*, 2013).

To track CX3CR1⁺ anti-inflammatory macrophages directly to the lung after ozone, we used CX3CR1^{+/GFP} reporter mice. Previous studies have shown that CX3CR1⁺ macrophages are distinct from resident lung macrophages, and are recruited to the lung following ozone exposure (Tighe et al., 2011a). Similarly, we found that CX3CR1⁺ macrophages were present in the lung after ozone exposure; however, this was evident at later times (96 h) after ozone; moreover, these cells were predominately anti-inflammatory Ly6C^{Lo} macrophages that expressed YM1 and Nur77. This is consistent

with reports of reduced numbers of Ly6C^{Lo} and CX3CR1⁺ macrophages in naïve Nur77^{-/-} mice (Hanna *et al.*, 2011) (Hamers *et al.*, 2016). Findings that there are differences in the kinetics of appearance of CX3CR1⁺YM1⁺ and CX3CR1⁺Nur77⁺ macrophage subpopulations in the lung after ozone is in accord with the idea that there are multiple subpopulations of alternatively activated macrophages responding to ozone (Laskin *et al.*, 2011).

Evidence suggests that CCR2 mediates pro-inflammatory macrophage accumulation at inflammatory sites and that these cells promotes tissue injury (Shi and Pamer, 2011). In this regard, CCR2 has been reported to direct the migration of iNOS- and TNF α -producing macrophages to the lung following bleomycin and hyperoxia induced injury (Okuma *et al.*, 2006; Osterholzer *et al.*, 2013). Our findings that CCR2⁺ macrophages express high levels of Ly6C and appear rapidly (within 24 h) after ozone exposure are consistent with other models of injury (Hammond *et al.*, 2014; Jiang *et al.*, 2016; Schumak *et al.*, 2015) and support the notion that they contribute to inflammatory lung injury. CCR2 also plays an important role in mobilizing monocytes from the bone marrow under pathological conditions (Serbina and Pamer, 2006). Our studies suggest that it plays a similar role during the pathogenesis of ozone toxicity.

In conclusion, the present studies demonstrate that the bone marrow is a source of macrophage pools that respond to ozone. Our findings that migration of these bone marrow-derived macrophages is regulated by chemokine receptors CX3CR1 and CCR2 supports the idea that multiple macrophage subpopulations contribute to the pathogenic response to ozone. The balance between pro- and anti-inflammatory macrophages is thought to influence the outcome of the response to ozone exposure; therefore,

identifying and selectively targeting macrophage subpopulations may prove beneficial in attenuating lung inflammation and injury.

TABLE 4. Effects of ozone on GFP⁺ monocytic cells in bone marrow and lung.

Cells were collected from bone marrow and lung 24-72 h after exposure of chimeric mice to air or ozone, as described in Methods and analyzed by flow cytometry. Cells were gated on monocytes/ macrophages and then analyzed for GFP expression. Data are mean \pm SE (n=3-4 mice). ^aSignificantly different (p <0.05) from air.

Time (h)	% GFP ⁺	
	Bone marrow	Lung
Air	87.2 ± 2.2	4.9 ± 0.8
24	90.1 ± 1.4	21.8 ± 1.3 ^a
48	88.3 ± 2.1	13.9 ± 1.9 ^a
72	85.2 ± 1.2	9.7 ± 0.3 ^a

FIGURE 25. Phenotype of bone marrow-derived lung macrophages. Lung sections, prepared 48 h after exposure of bone marrow chimeric mice to air or ozone, were stained with AF647-conjugated antibodies to Ly6C, CCR2, YM1, NUR77, CX3CR1, or HO-1. Green, GFP; Red, AF647-conjugated antibodies; Blue, DAPI. Original magnification, x630. *Left Panels:* Representative fluorescent images; *Right Panels:* Quantitation of co-localization. Bars, mean \pm SE (n = 3) of Pearson's correlation coefficient in nine 630x fields/group.

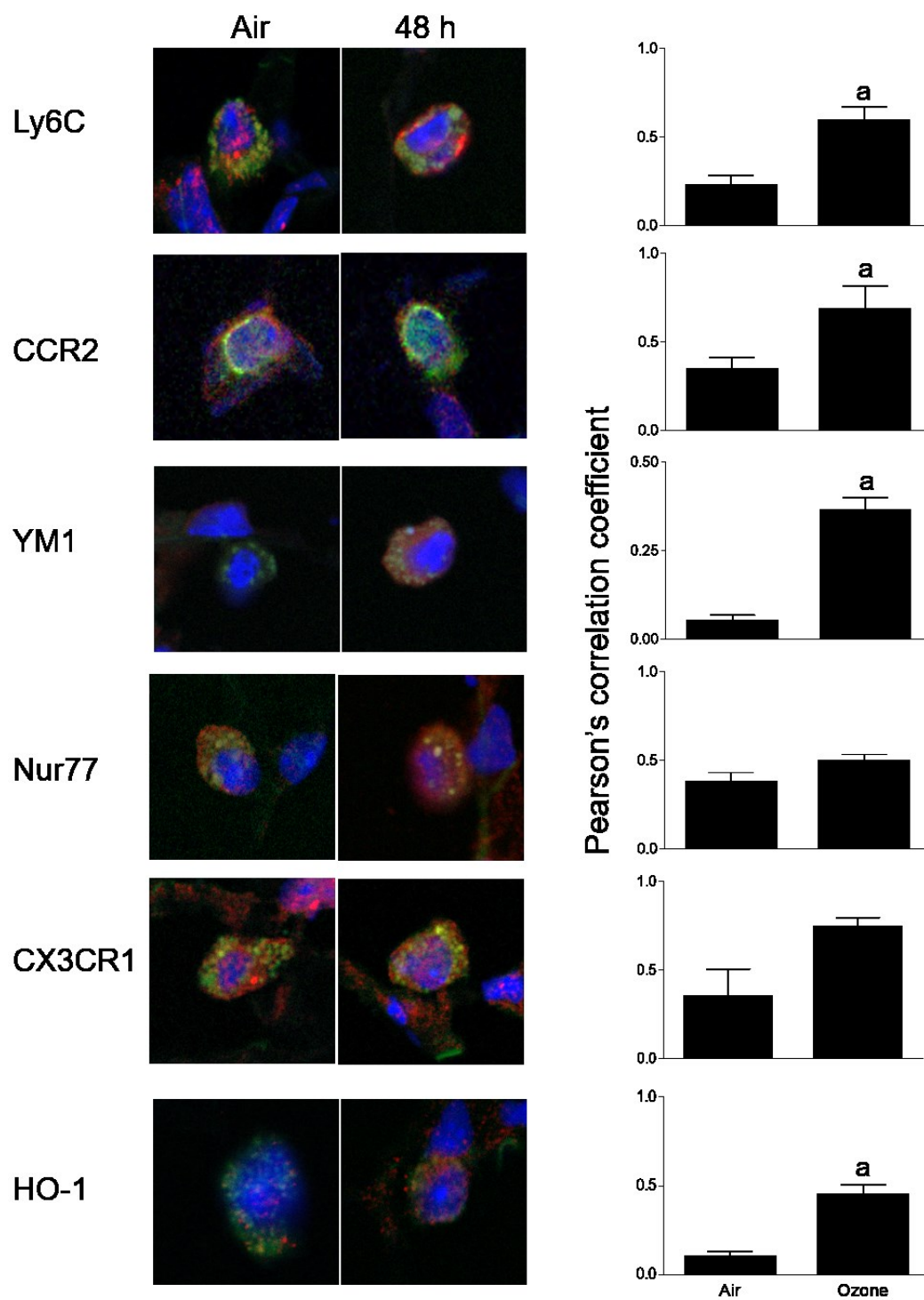


FIGURE 26. Flow cytometric analysis of lung macrophages responding to ozone.

Lung macrophages, collected 24-72 h after exposure of chimeric mice to air or ozone, were stained with antibodies to CD11b, Ly6C, F4/80, and CD11c or isotypic controls, and analyzed by flow cytometry. GFP⁺ and GFP⁻ macrophages were sequentially analyzed for CD11b, Ly6C, F4/80, and CD11c. Bars, mean \pm SE (n = 3-4).

^aSignificantly different (p <0.05) from air-exposed animals.

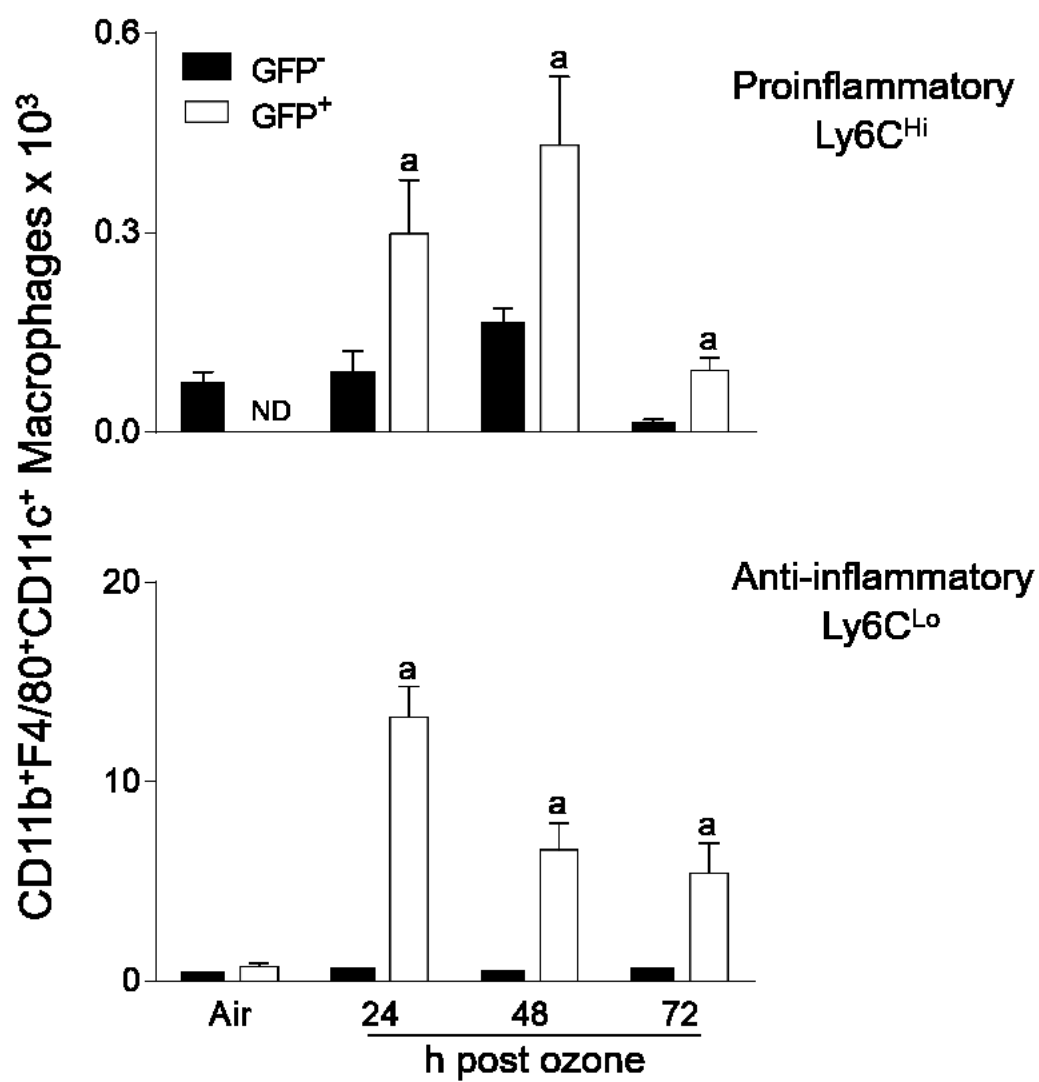


TABLE 5. Effects of ozone on total number of CX3CR1^{+GFP} cells in the lung. BAL cells, were collected 24-96 h after exposure of mice to air or ozone, and analyzed by flow cytometry. Cells were gated on macrophages and then analyzed for GFP expression. Data are the mean \pm SE (n = 3-4 mice). ^aSignificantly different (p <0.05) from air-exposed animals.

Time	CX3CR1⁺ Cells x 10³
Air	2.9 ± 0.3
24 h	8.2 ± 0.8
48 h	16.1 ± 3.0^a
72 h	39.7 ± 14.9^a
96 h	61.7 ± 16.1^a

FIGURE 27. Phenotype of CX3CR1^{+/GFP} lung macrophages. Lung sections, prepared 48 h after exposure of CX3CR1^{+/GFP} mice to air or ozone, were stained with AF647-conjugated antibodies to YM1 or NUR77. Green, GFP; Red, AF647-conjugated antibodies; Blue, DAPI. Original magnification, x630. *Left Panels:* Representative fluorescent images; *Right Panels:* Quantitation of co-localization. Bars, mean \pm SE (n = 3) of Pearson's correlation coefficient in nine 630x fields/group.

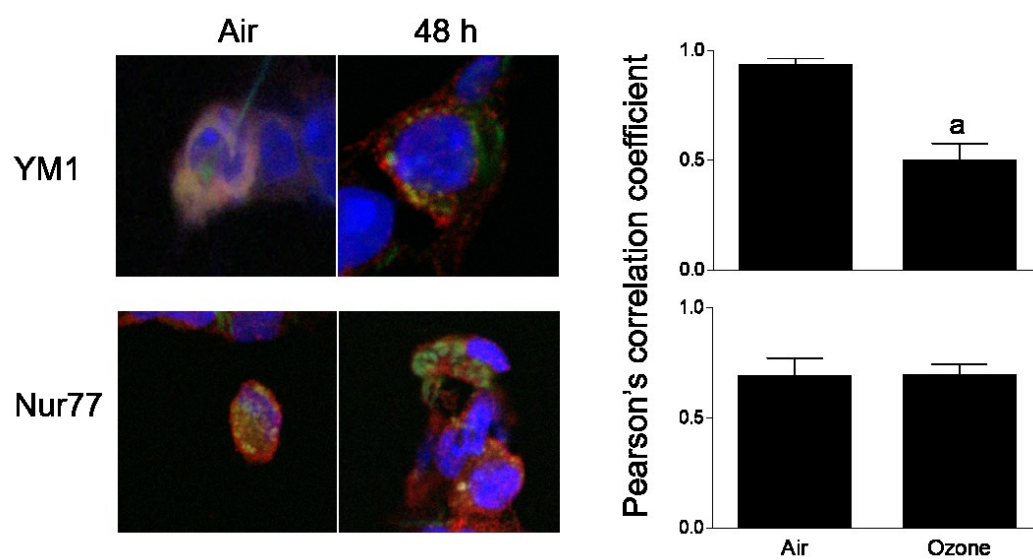
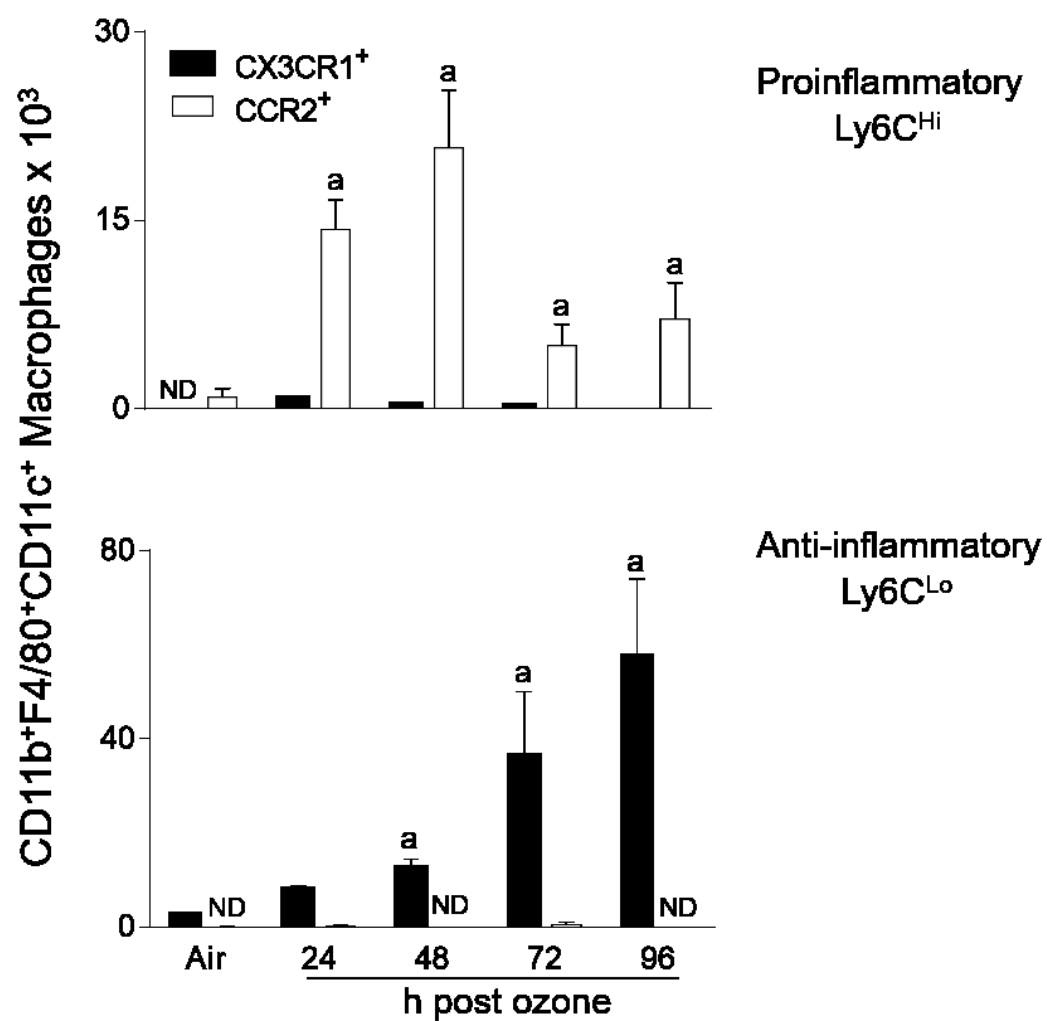


FIGURE 28. Trafficking of CCR2⁺ and CX3CR1⁺ macrophages to the lung after ozone exposure. Macrophages, collected 24-96 h after exposure of CX3CR1^{+/GFP} or WT mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, and F4/80 and in some experiments CCR2, or isotypic controls and analyzed by flow cytometry. CCR2⁺ or CX3CR1⁺ cells were sequentially analyzed for CD11b, Ly6C, F4/80, and CD11c. Bars, mean \pm SE (n = 3-4). ^aSignificantly different (p < 0.05) from air-exposed animals.



PART IV. REGULATION OF MACROPHAGE ACCUMULATION AND ACTIVATION IN THE LUNG FOLLOWING OZONE EXPOSURE BY THE FARNESOID X RECEPTOR

Ozone is a ubiquitous urban air pollutant known to cause injury to the lower lung. This is thought to be due to the generation of reactive oxygen and nitrogen species (RONS), which oxidize biomolecules in the respiratory tract including unsaturated lipids, nucleic acids, and proteins (Mustafa, 1990; Pulfer and Murphy, 2004; Uhlson *et al.*, 2002). Evidence suggests that activated macrophages and mediators they release including RONS contribute to ozone toxicity (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011). These cells accumulate in the lung in response to chemotactic factors released from damaged tissues and cells. Once localized at injured sites, macrophages become activated towards a proinflammatory/cytotoxic M1 phenotype or anti-inflammatory/wound repair M2 phenotype depending on mediators they encounter in the tissue microenvironment (Laskin *et al.*, 2011). It appears that the outcome of the response to ozone depends, in part, on the balance in the activity of these two macrophage subpopulations in the lung (Laskin *et al.*, 2011).

Macrophage activation is controlled, in part, by transcription factors such as NF- κ B and AP-1 which are activated by proinflammatory stimuli generated at sites of injury (Sharif *et al.*, 2007; Zhao *et al.*, 2011). Farnesoid x receptor (FXR) is a transcription factor important in regulating bile acid, lipid, and glucose homeostasis; as such, it is highly expressed by hepatocytes, mucosal intestine epithelial cells, and renal mesangial cells (Ding *et al.*, 2015; Jiang *et al.*, 2007; Li and Guo, 2015). It has also been identified in peritoneal macrophages and in pulmonary endothelial cells (Guo *et al.*, 2006a; Zhang

et al., 2012b). In addition to regulating lipid metabolism and transport, FXR has been shown to exert anti-inflammatory activity, a response thought to be due to deactivation of NF- κ B in macrophages, and down regulation of key pro-inflammatory genes (Lawrence, 2009; Stojancevic *et al.*, 2012). This is supported by findings of exaggerated inflammation and increased expression of proinflammatory proteins in lungs of FXR^{-/-} mice treated with bacterially-derived lipopolysaccharide (LPS) (Zhang *et al.*, 2012a)

In the present studies, we analyzed the role of FXR in regulating macrophage activity in the lung following ozone exposure. We hypothesized that by modulating lung lipids and NF- κ B activation, FXR would suppress ozone-induced lung inflammation and injury, and indeed this is what we observed. The results of these studies provide novel mechanistic insight into inflammatory mechanisms contributing to ozone toxicity.

RESULTS

Effects of ozone on FXR expression

In initial studies, we evaluated the effects of ozone on FXR expression in the lung. Low level FXR expression was noted in lung macrophages in air-exposed WT mice (Fig. 1). Treatment of the mice with ozone resulted in a time related increase in FXR expression in macrophages, as well as in type II cells, beginning within 24 h and persisting for at least 120 h after exposure.

Effects of loss of FXR on lung phospholipids

We next used FXR^{-/-} mice to assess the role of FXR in ozone-induced alterations in lung phospholipids. In WT mice, low levels of phospholipids were detected in BAL; these were unaltered by ozone (Fig. 2). In contrast, in FXR^{-/-} mice, ozone caused a significant increase in BAL phospholipids. SP-B, but not SP-D, was observed in BAL from air-exposed WT mice. Whereas ozone exposure caused a gradual increase in BAL SP-B levels beginning at 72 h and continuing for at least 120 h, increases in SP-D were transient occurring at 48 h and 72 h. Although constitutive SP-B levels were greater in air exposed FXR^{-/-} mice, when compared to WT mice, they were largely unchanged after ozone. Ozone also had no effect on SP-D levels in FXR^{-/-} mice.

Effects of loss of FXR on proteins involved in lipid metabolism and transport

Treatment of WT mice with ozone resulted in a rapid (within 24 h) and transient increase in expression of LXR α , a nuclear receptor important in lipid and cholesterol metabolism (Ory, 2004) (Fig. 3). In contrast, ozone caused a decrease in the inhibitory nuclear receptor, small heterodimer partner (SHP) or NR0B2 (Goodwin *et al.*, 2000), a

response evident at 96 h and 120 h post exposure. ApoE is important in lipoprotein metabolism and transport (Getz and Reardon, 2009). Following ozone inhalation, ApoE, as well as VLDLR, a transmembrane lipoprotein receptor involved in lipoprotein metabolism and cholesterol uptake, and the cholesterol efflux transporter, ABCG1, were increased in the lung.

The increase in VLDLR was most notable 24 h post ozone exposure, while ApoE and ABCG1 were upregulated at 96 h and 120 h; ApoE was also increased 72 h post ozone (Fig. 3). Ozone had no effect on expression of the cholesterol transporter, ABCA1. Loss of FXR resulted in a marked suppression in expression of each of these genes in air, as well as in ozone exposed mice (Fig. 3).

Effects of loss of FXR on lung macrophages and inflammatory gene expression

Consistent with previous studies (Fakhrzadeh *et al.*, 2002; Francis *et al.*, 2016), ozone exposure was associated with increases in pro-inflammatory iNOS⁺ and AT1R⁺ proinflammatory/cytotoxic M1 macrophages in the lung at 24 h; ATR1⁺ macrophages were also increased 48 h post ozone (Figs. 4 and 5); this was correlated with increased expression of the pro-inflammatory genes, TNF α and IL-1 β (Fig. 6). NF κ B, which regulates the transcription of these genes, was also activated after ozone, a response observed at 24 h and 48 h post exposure (Fig. 7). Ozone also caused an increase in expression of the chemokine receptor CCR2, which is involved in trafficking of proinflammatory macrophages to sites of injury, with no effect on its ligand, CCL2 (Fig. 6). Loss of FXR resulted in a persistent increase in AT1R⁺ and iNOS⁺ macrophages in the lung beginning at 24 h and 48 h, respectively. TNF α and IL-1 β were also increased in lungs of FXR^{-/-} mice, when compared to WT mice; this was noted in both air and

ozone treated mice. Loss of FXR also resulted in increased expression of CCL2 and CCR2 in response to ozone. This was evident at 24 h and 48 h for CCL2, and at 72 h for CCR2. Expression of CCL2 and CCR2 was also greater in FXR^{-/-} mice relative to WT mice after air exposure. Increases in pro-inflammatory macrophages and gene expression in the lungs of FXR^{-/-} mice treated with ozone were correlated with increases in NFκB p65 activity at 48-96 h post exposure (Fig. 7).

Following exposure of WT mice to ozone, anti-inflammatory MR⁺ and Arg-1⁺ M2 macrophages increased in the lung beginning at 24 h. While MR⁺ macrophages remained in the lung for at least 96 h post ozone, at this time, Arg-1⁺ macrophages were at control levels (Figs. 8 and 9). In contrast, ozone had no effect on YM1⁺ anti-inflammatory macrophages, which were present in the lungs of air-exposed WT mice (Fig. 10). Whereas loss of FXR had no major effect on MR⁺ lung macrophages, ozone-induced increases in Arg-1⁺ macrophages were significantly delayed in FXR^{-/-} mice relative to WT mice, and not evident until 96 h post exposure. The effects of loss of FXR on expression of genes associated with anti-inflammatory macrophages were also examined. In WT mice, ozone exposure resulted in increased expression of CX3CL1 at 24 h and CX3CR1 at 96 h and 120 h (Fig. 6). In the absence of FXR, expression of these genes was upregulated in air exposed mice and in ozone exposed mice at 24 -72 h.

To further characterize the effects of loss of FXR on macrophage subpopulations responding to ozone, we used techniques in flow cytometry (Sunil *et al.*, 2015). Mature (F4/80⁺CD11c⁺) infiltrating macrophages (CD11b⁺Ly6G⁻) were identified as Ly6C^{Hi} pro-inflammatory or Ly6C^{Lo} anti-inflammatory (Fig. 11). Increases in both Ly6C^{Hi} pro-inflammatory and Ly6C^{Lo} anti-inflammatory macrophages were observed in the lungs of

WT mice following ozone exposure; this was most prominent 48 h post exposure. Loss of FXR resulted in a significant increase in Ly6C^{Hi} pro-inflammatory macrophages at 24-96 h post ozone, while Ly6C^{Lo} anti-inflammatory macrophages were largely unaffected.

Effects of loss of FXR on ozone-induced oxidative stress and lung injury

Treatment of WT mice with ozone resulted in increased expression of markers of oxidative stress, including Cypb5 and the lipid peroxidation end product, 4-HNE at 24 h and 48 h post exposure (Figs. 12 and 13). Although ozone-induced increases in Cypb5 were delayed until 48 h in FXR^{-/-} mice, they remained elevated for at least 96 h. Loss of FXR also resulted in increased and more prolonged expression of 4-HNE in response to ozone. By 96 h, however, 4-HNE expression was at control levels. In contrast, ozone induced increases in BAL protein levels were significantly reduced in FXR^{-/-} mice relative to WT mice at 48 post exposure (Fig. 14).

Effects of loss of FXR on pulmonary mechanics

In our next series of experiments, we analyzed the effects of loss of FXR on ozone-induced changes in airway and parenchymal resistive and elastic properties by measuring responses to increasing PEEP. In both WT and FXR^{-/-} mice exposed to air, increasing PEEP from 0-9 cm H₂O resulted in a decrease in tissue damping and tissue elastance (Fig. 15). Treatment of the mice with ozone had no significant effect on tissue damping or tissue elastance in either WT or FXR^{-/-} mice. We next analyzed the effects of changes in quasi-static pressure volume (PV) curves at varying levels of PEEP in WT and FXR^{-/-} mice after exposure to ozone. In WT mice, ozone caused a significant increase in PV curves at 48 h post exposure; this response increased with increasing PEEPs (Fig. 16). In contrast, no changes in PV curves were noted in FXR^{-/-} mice at any PEEP examined.

DISCUSSION

Activated macrophages have been implicated in tissue injury induced by diverse pulmonary toxicants including ozone (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011). The present studies demonstrate that FXR is important in regulating macrophage inflammatory activation in the lung following ozone exposure, and that this involves alterations in lung lipids and the transcription factor, NF κ B. These data provide new insights into mechanisms regulating macrophage activation in the lung after ozone exposure and their potential contribution to ozone toxicity.

Treatment of mice with ozone resulted in increased numbers of FXR⁺ macrophages and type II cells in the lung. These findings are in accord with previous reports that FXR is upregulated in the lung in response to injury induced by LPS and monocrotaline (Zhang *et al.*, 2012b) (Ye *et al.*, 2015), as well as in idiopathic pulmonary fibrosis and asthma (Chen *et al.*, 2016; Shaik *et al.*, 2015). These findings suggest that upregulation of FXR may be a general response to inflammatory lung injury and disease.

FXR is known to regulate lipid metabolism and transport in the liver (Ding *et al.*, 2015). The present studies demonstrate that FXR plays a similar role in the lung. Thus, in the absence of FXR, increases in total phospholipids were noted in BAL following ozone exposure. Additionally, in contrast to increases in SP-B and SP-D levels observed in BAL from WT mice after ozone exposure, levels of these proteins were largely unchanged in FXR^{-/-} mice; however, constitutive SP-B levels were greater in FXR^{-/-} mice, when compared to WT mice. These findings are consistent with a derangement in lung lipids in FXR^{-/-} mice, most likely due to impaired lipid uptake or efflux. Earlier studies showed that FXR activation results in impaired surfactant production by isolated type II

cells (Yang *et al.*, 2014a). In contrast, the present studies suggest that after ozone exposure, FXR promotes surfactant production. Differences between these findings may be due to the unique aspects of the models used to assess type II cell activity and surfactant production. Bile acids are known to bind to FXR resulting in upregulation of ABCA3, a lipid transporter essential for the uptake and synthesis of pulmonary surfactant by type II cells (Stahlman *et al.*, 2007). It is possible that bile acids accumulate in the lung following ozone induced lung injury and that in the absence of FXR, SP-D and SP-B production is impaired; however, this remains to be determined.

Following ozone exposure, increases in LXR α , ApoE, VLDLR and ABCG1, which function to regulate lipid metabolism and transport (Calkin and Tontonoz, 2010) were observed, while expression of SPH, a negative regulator of nuclear receptors, was suppressed. Loss of FXR caused a marked reduction in these proteins, as well as in ABCA1, consistent with reports that FXR positively regulates their expression (Beyea *et al.*, 2007; Calkin and Tontonoz, 2010; Li *et al.*, 2013). Decreases in these proteins may contribute to aberrations in phospholipid metabolism in the lung following ozone exposure. Evidence suggests that lipid transporters also play a role in regulating lung inflammation and injury. Thus, following exposure of mice to LPS or *Mycobacterium tuberculosis* LXR α expression is upregulated in alveolar macrophages and type II cells (Gong *et al.*, 2009; Korf *et al.*, 2009; Smoak *et al.*, 2008); it is also upregulated in the lung in models of chronic obstructive pulmonary disease and aging (Botez *et al.*, 2015; Higham *et al.*, 2013). This may be important in limiting inflammatory tissue injury. Similarly, ApoE and VLDLR have been shown to protect against lung injury induced by LPS and house dust mite, respectively (Fredriksson *et al.*, 2014; Ni *et al.*, 2013).

Increases in toll-like receptor (TLR)4 signaling and inflammation have been described in mice lacking ABCA1 and ABCG1 (Yvan-Charvet *et al.*, 2007). Earlier studies showed that ozone induced injury and oxidative stress are due in part, increased in TLR4 signaling (Connor *et al.*, 2012). Down regulation cholesterol efflux transporters in lungs of FXR^{-/-} mice may lead to increases in TLR4 resulting in exacerbation of ozone toxicity. FXR has been shown to suppress inflammation and promote tissue repair in a number of models of lung injury (Vignozzi *et al.*, 2016) (Chen *et al.*, 2016) and infection (Zhang *et al.*, 2012b). Consistent with this activity, we found that loss of FXR was associated with an exaggerated inflammatory response to ozone. Thus, in the lungs of FXR^{-/-} mice treated with ozone, prolonged increases in numbers of iNOS⁺ and AT1R⁺ proinflammatory macrophages were noted, along with increases in expression of TNF α and IL-1 β , and NF- κ B activity. Similar increases in NF κ B activity and pro-inflammatory proteins have been observed in primary hepatocytes from mice lacking FXR after treatment with LPS (Wang *et al.*, 2008). FXR has been shown to block activation of NF- κ B and upregulate AT2R expression, resulting in decreases in AT1R-mediated effects (Cosentino *et al.*, 2005; Zhang *et al.*, 2007). Decreases in AT2R in FXR^{-/-} mice may also contribute to reduced numbers of AT1R⁺ macrophages in the lung. We also found that numbers of pro-inflammatory/cytotoxic Ly6C^{Hi} macrophages were increased in lungs of ozone treated FXR^{-/-} mice compared to WT mice, along with expression of CCL2/CCR2, which is important in their trafficking to the lung. Similar increases in Ly6C^{Hi} macrophages have been described in mice treated with a FXR agonist in a model of nonalcoholic fatty liver disease (McMahan *et al.*, 2013). Increases in pro-inflammatory macrophages in the lungs of ozone treated FXR^{-/-} mice were correlated with increased

oxidative stress as reflected by expression of Cyb5 and 4-HNE. Exacerbations in oxidative stress and the peroxidation of lipids has previously been described in mice deficient in FXR treated with high fat diet and ethanol (Gai *et al.*, 2016; Livero *et al.*, 2014; Nomoto *et al.*, 2009). These data provide additional support for the role of inflammatory macrophages in ozone toxicity.

Previous studies showed that the anti-inflammatory chemokine CX3CL1 and its receptor CX3CR1, contribute to oxidized lipid-driven activation of macrophages towards an alternatively activated M2 phenotype (Barlic *et al.*, 2006; Raoul *et al.*, 2008). We observed increases in CX3CL1/CX3CR1 in lungs of FXR^{-/-} mice when compared to WT. This may contribute to increased numbers of Ly6C^{Lo} macrophages in the lungs of these mice after ozone exposure. These cells may contribute to reduced BAL protein levels in the lung. Findings that Arg I⁺, YM1⁺, and MR⁺ M2 macrophages represents the impact of altered lipid handling in heterogeneity of macrophage activation. Previous studies showed that Arg I⁺, YM1⁺, and MR⁺ macrophages were unaffected by loss of FXR are consistent with the idea that there are multiple populations of M2 macrophages that vary in their phenotype and function (Laskin *et al.*, 2011).

We also analyzed the effects of loss of FXR on pulmonary mechanics using a forced oscillation technique. Consistent with previous studies in ozone exposed mice (Groves *et al.*, 2012; Groves *et al.*, 2013) (Rivera-Sanchez *et al.*, 2004), no effects of ozone were noted tissue dampening and elastance in either WT or FXR^{-/-} mice, suggesting that there is no effect on the alveolar parenchyma. In contrast, increases in PV curves were observed in WT mice following ozone exposure, but not in FXR^{-/-} mice. Increases in PV curves most likely reflects ozone-induced upregulation of SP-B

expression in WT mice, which were absent in FXR^{-/-} mice. SP-B contributes to the surface tension-lowering properties of surfactant (Weaver and Conkright, 2001). Our findings suggest that the loss of FXR does not induce changes in airflow following ozone exposure.

In summary, the present studies demonstrate a key role of FXR in regulating lipid transport and anti-inflammatory macrophage activities in the lung in response to ozone. Loss of FXR resulted in increased pro-inflammatory macrophage accumulation and surfactant dysfunction. Elucidating mechanisms in lipid handling in inflammatory cells that respond to ozone may lead to the development of more efficacious approaches for mitigating oxidant-induced pulmonary toxicity and disease pathogenesis.

FIGURE 29. Effects of ozone on expression of FXR in the lung. Tissue sections, prepared 24-120 h after exposure of WT mice to air or ozone, were stained with antibody to FXR. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group) (Original magnification, x1000).

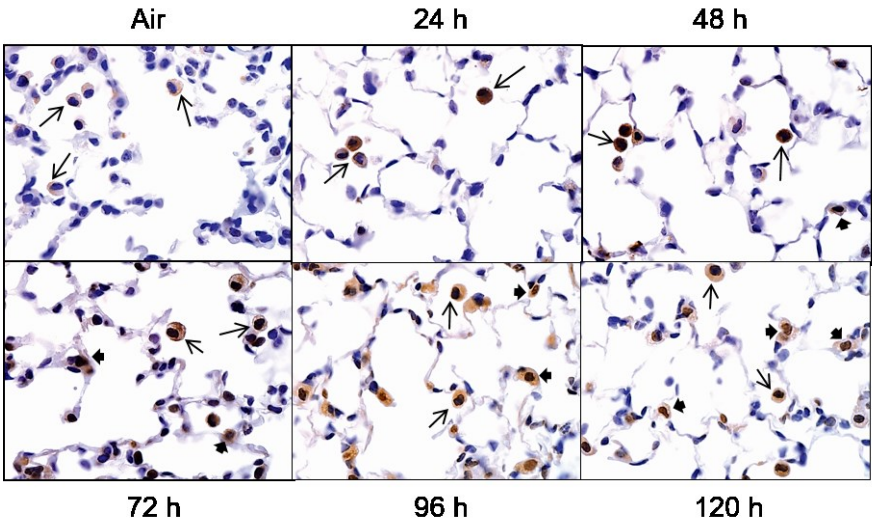


FIGURE 30. Effects of loss of FXR on ozone-induced alterations in phospholipids and surfactant proteins. BAL was collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone. Total phospholipid content (*upper panel*) and SP-D and SP-B levels (*lower panel*) were assessed as described in the Materials and Methods (n = 4-5 mice/treatment group). ^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from WT.

FIGURE 31. Effects of loss of FXR on ozone-induced expression of lipid transport genes. Lung tissue, collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone, were analyzed by real-time PCR. Data are presented as fold change relative to GAPDH. Bars, mean \pm SE (n = 3-4 mice/treatment group). ^aSignificantly different (p < 0.05) from air. ^bSignificantly different (p < 0.05) from WT mice.

FIGURE 32. Effects of loss of FXR on ozone-induced expression of iNOS. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to iNOS. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

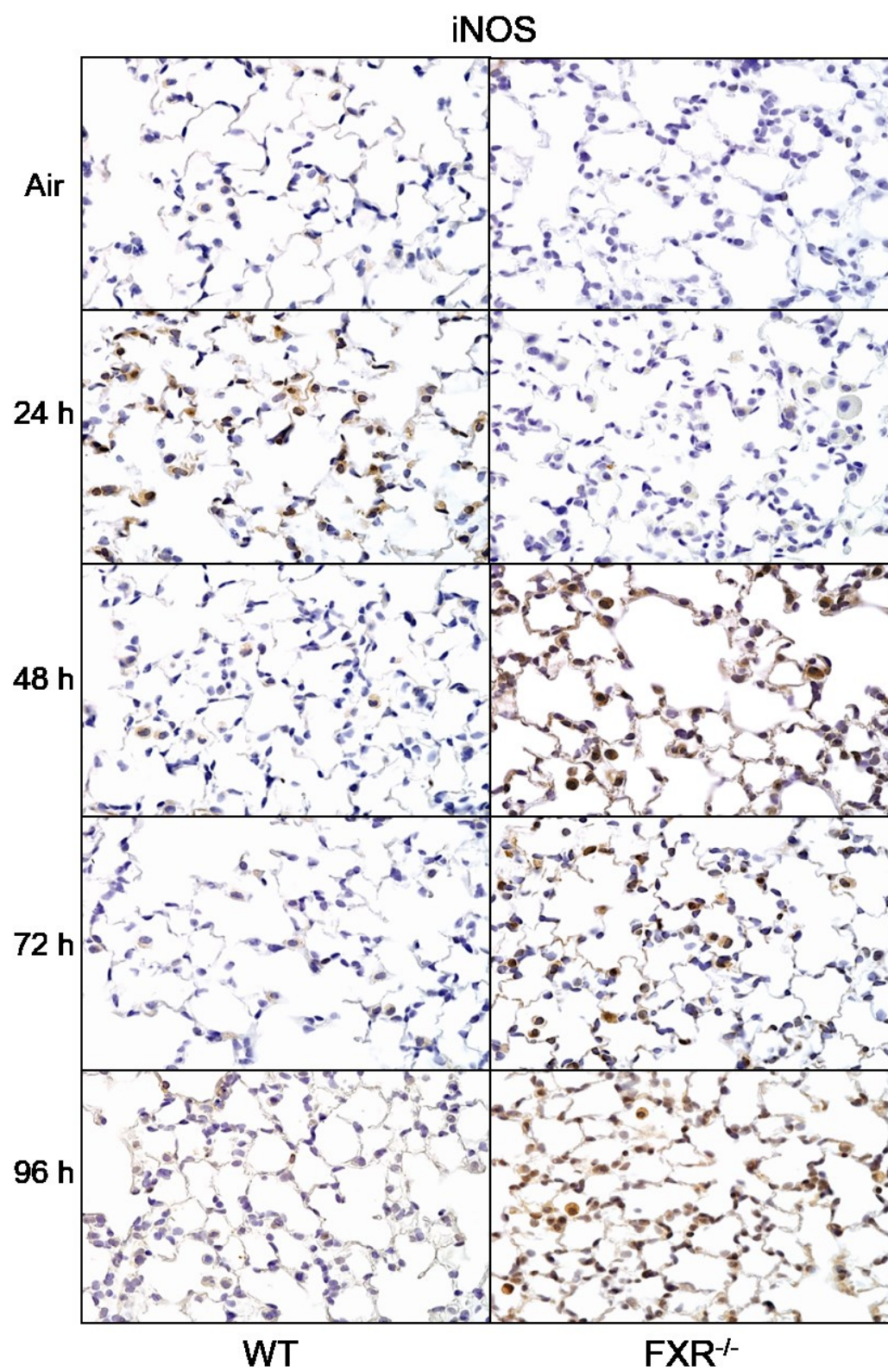


FIGURE 33. Effects of loss of FXR on ozone-induced expression of AT1R. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to AT1R. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

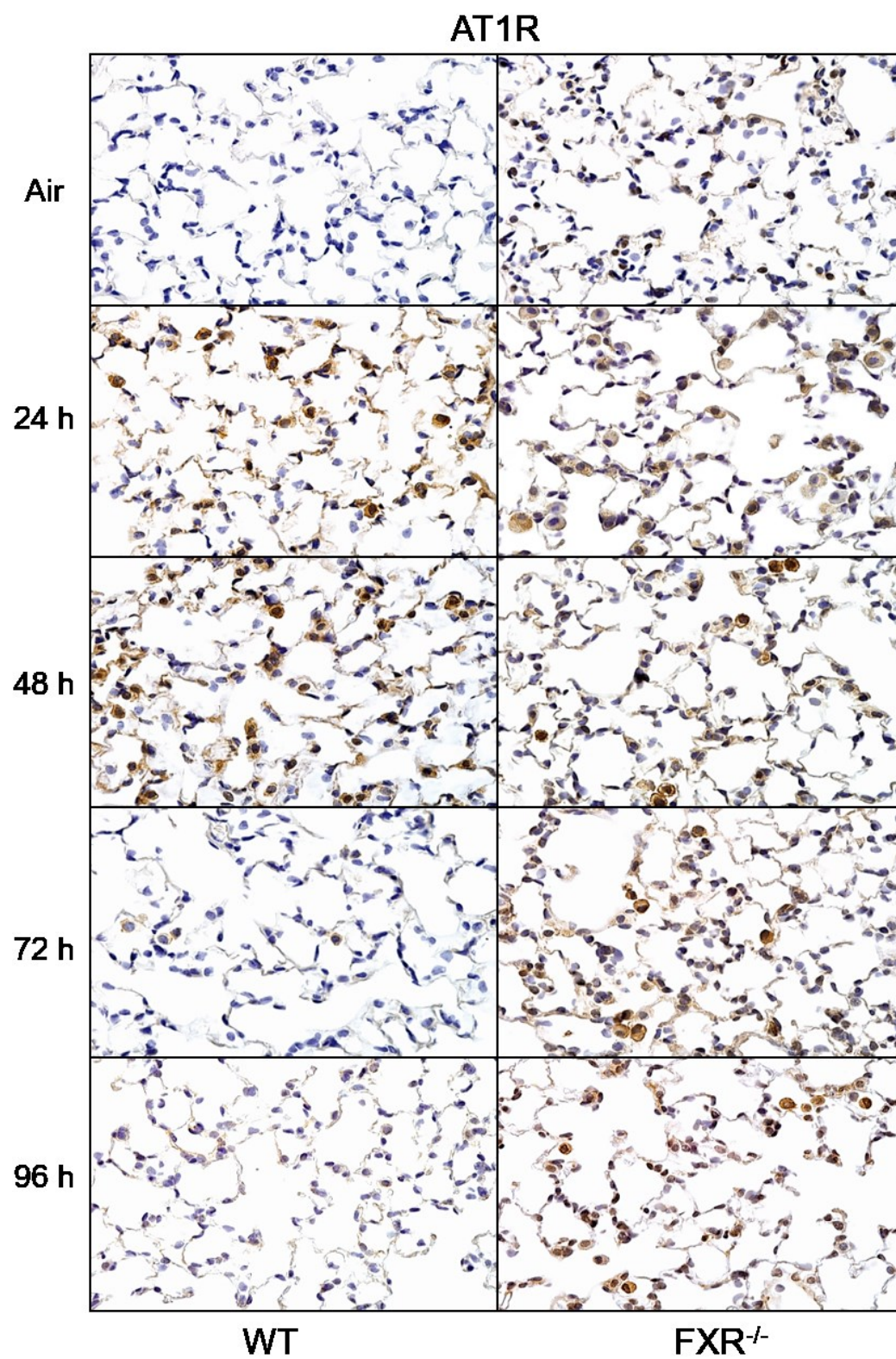


FIGURE 34. Effects of loss of FXR on ozone-induced expression of inflammatory genes. Lung, collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone, were analyzed by real-time PCR. Data are presented as fold change relative to GAPDH. Bars, mean \pm SE (n = 3-4 mice/treatment group). ^aSignificantly different (p < 0.05) from air. ^bSignificantly different (p < 0.05) from WT mice.

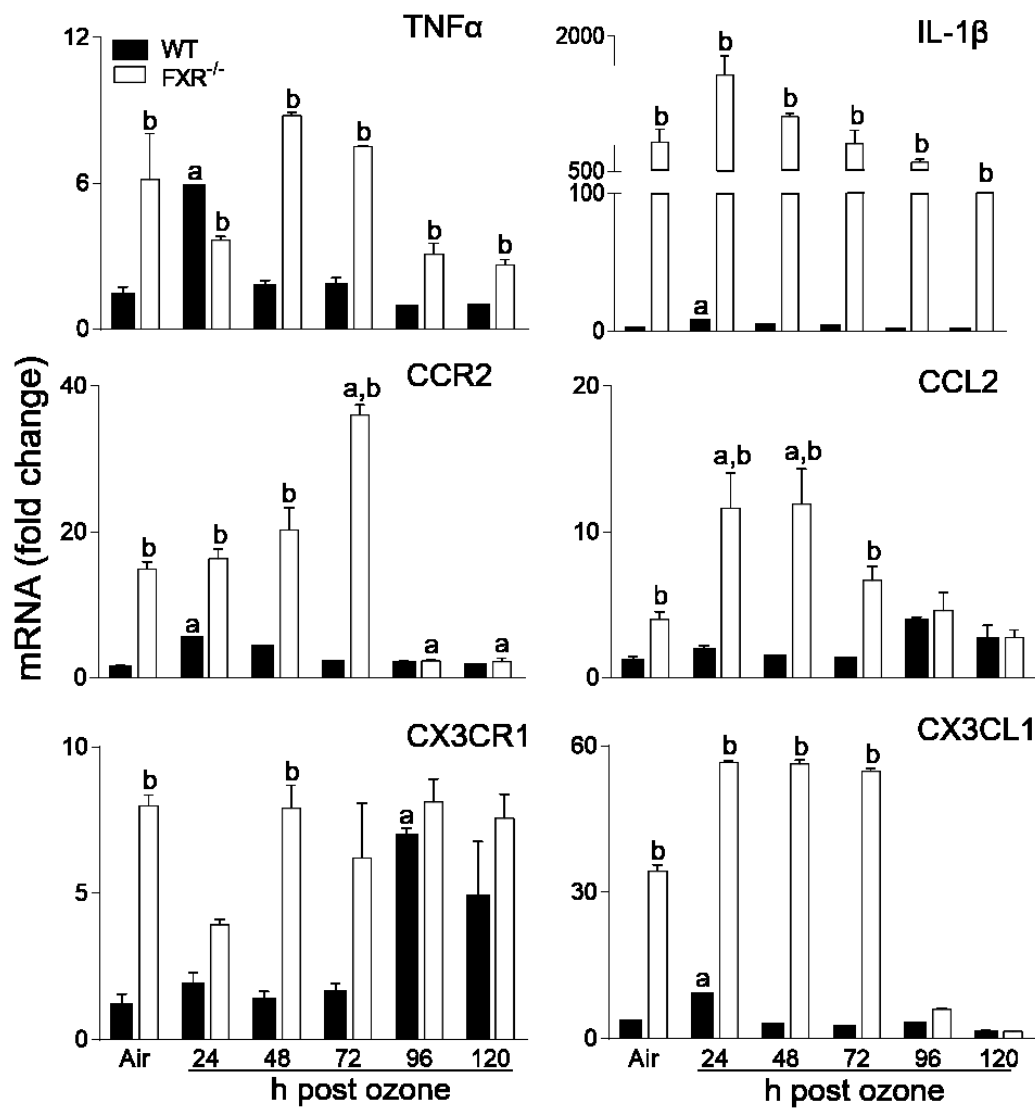


FIGURE 35. Effects of loss of FXR on ozone-induced NFκB nuclear binding

activity. Macrophages were collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone. Nuclear extracts were prepared and analyzed in duplicate for p65 NFκB activity using a TransAM NFκB kit. Data are presented as fold-change relative to air-exposed animals. Bars, mean \pm SE (n = 3-4 mice/treatment group). ^aSignificantly different (p <0.05) from air-exposed animals; ^bSignificantly different (p<0.05) from WT.

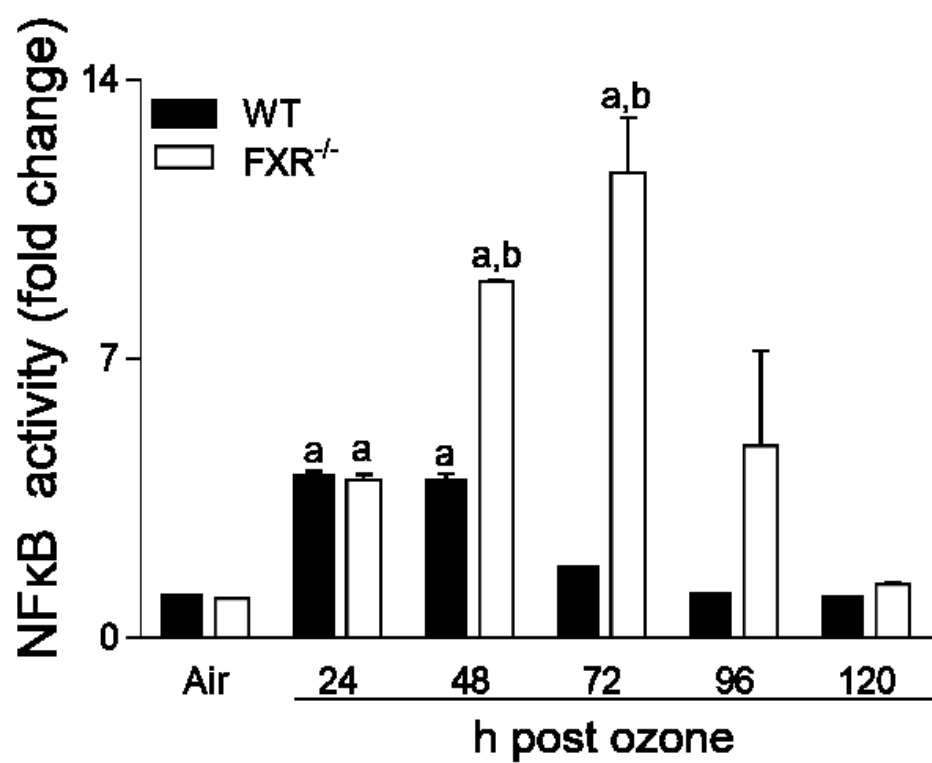


FIGURE 36. Effects of loss of FXR on ozone-induced expression of MR. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to MR. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

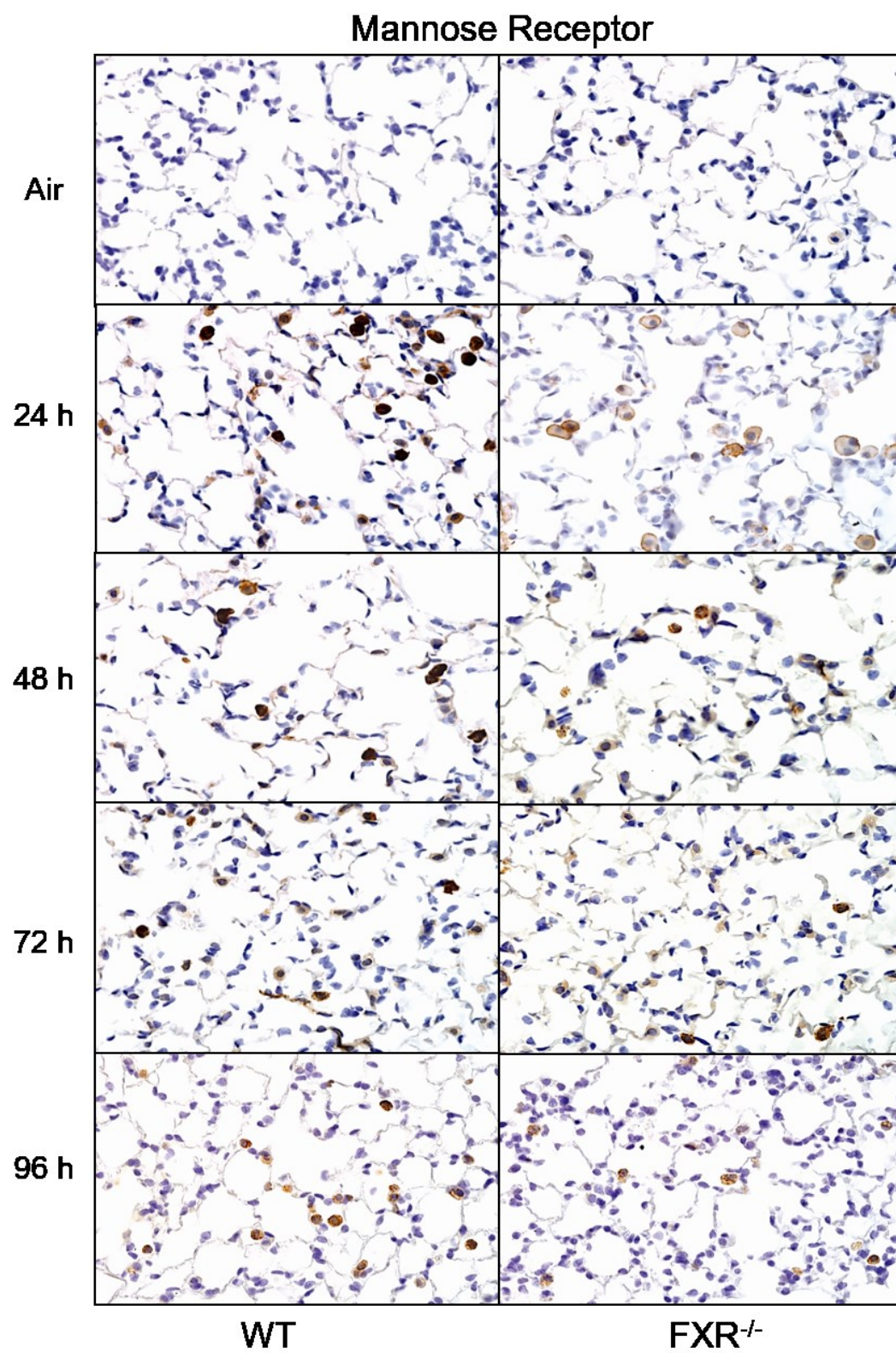


FIGURE 37. Effects of loss of FXR on ozone-induced expression of Arg I. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to Arg I. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

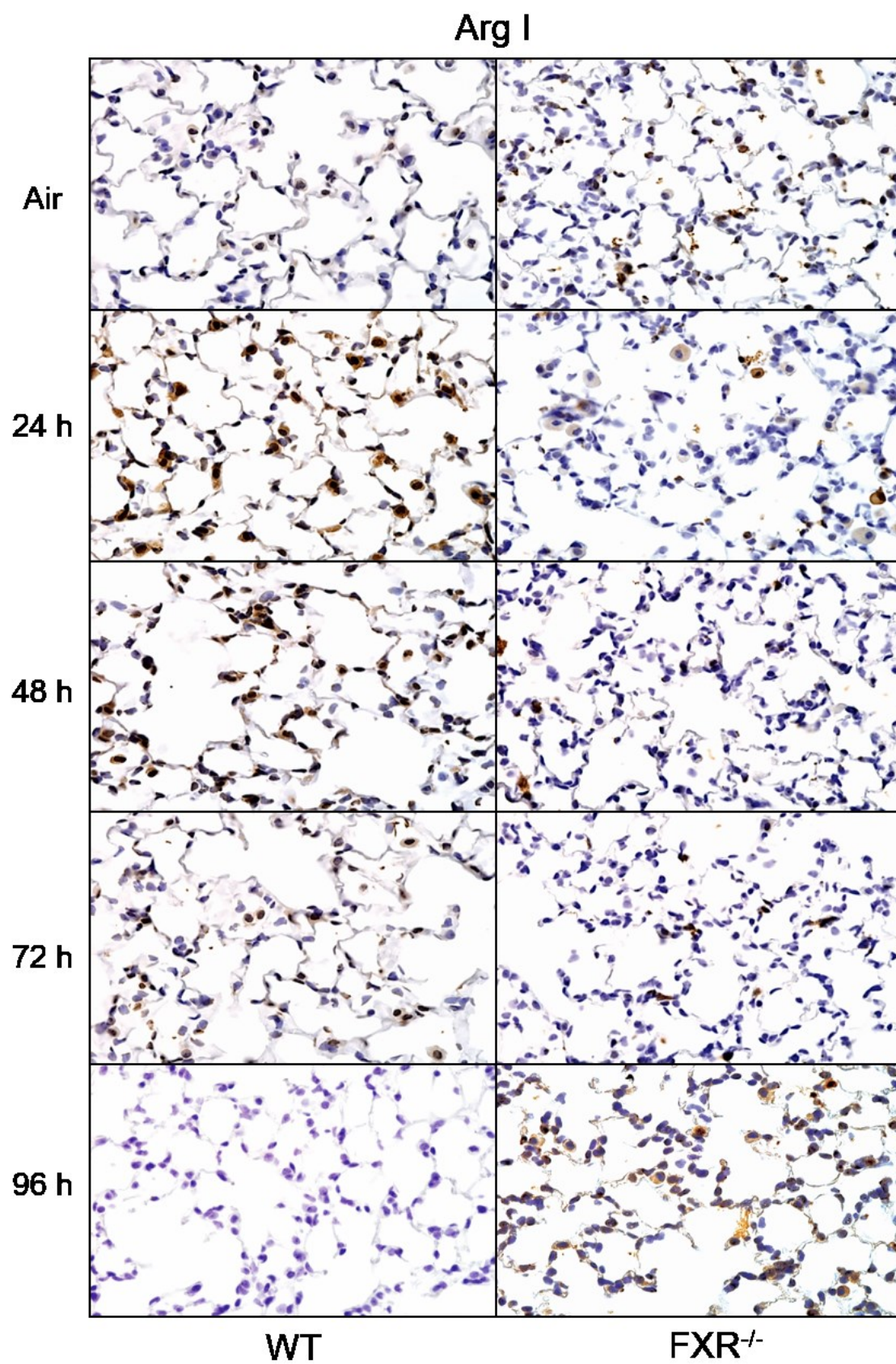


FIGURE 38. Effects of loss of FXR on ozone-induced expression of YM1. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to YM1. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

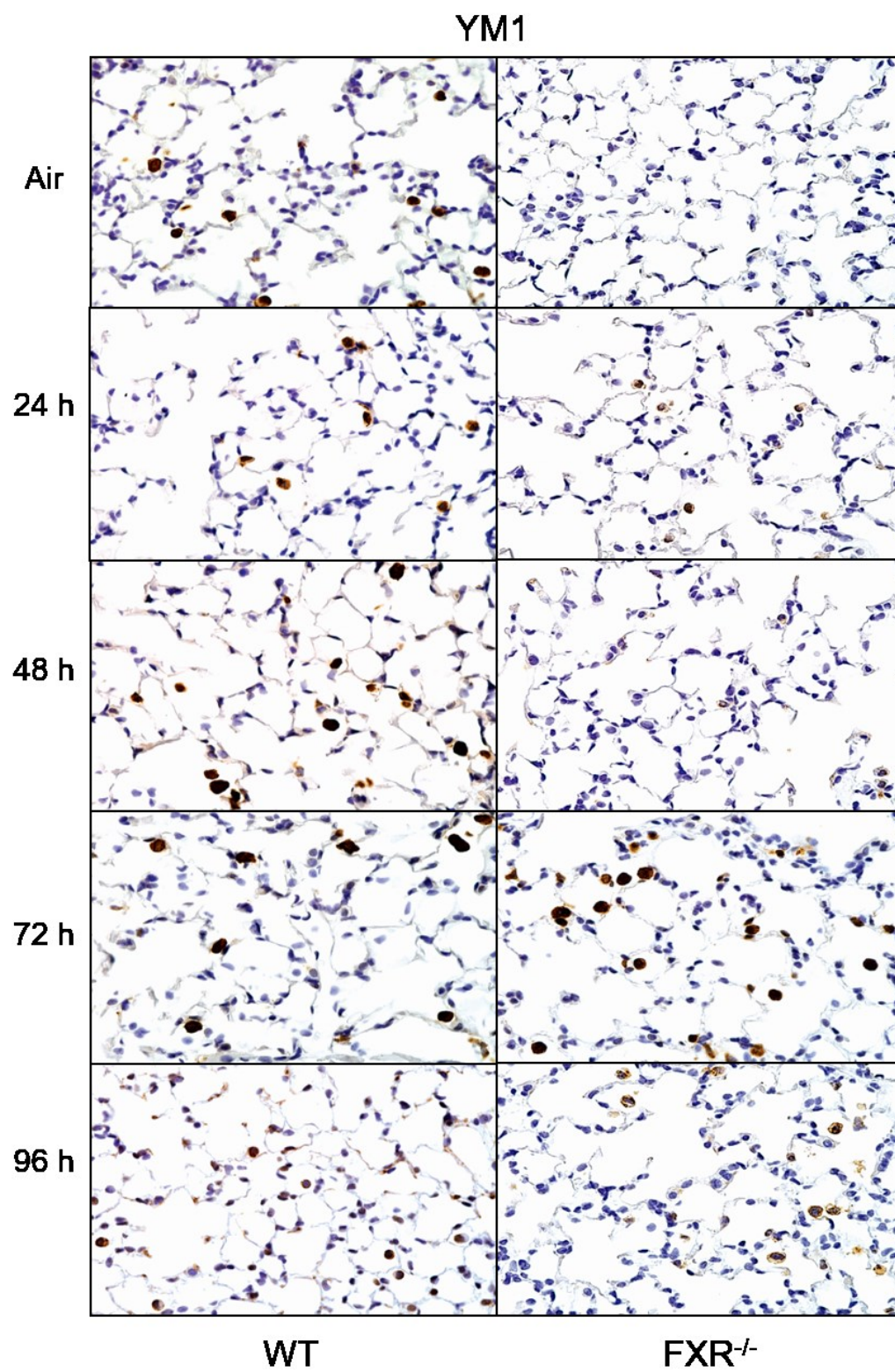


FIGURE 39. Effects of loss of FXR on lung macrophages responding to ozone.

Macrophages, collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, F4/80, and CD11c or isotypic controls, and analyzed by flow cytometry. Bars, mean \pm SE (n = 3-10). ^aSignificantly different (p <0.05) from air; ^bSignificantly different (p<0.05) from WT.

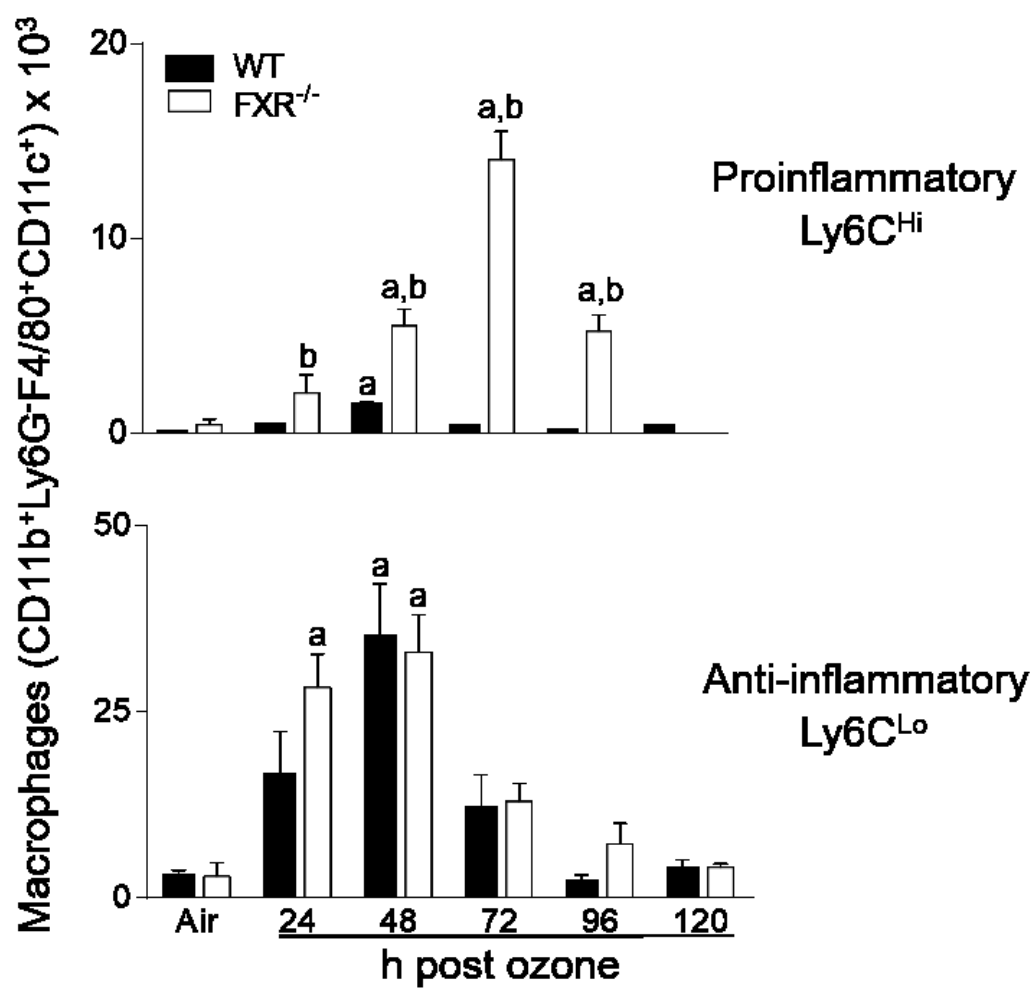


FIGURE 40. Effects of loss of FXR on ozone-induced expression of Cypb5. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to CypB5. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

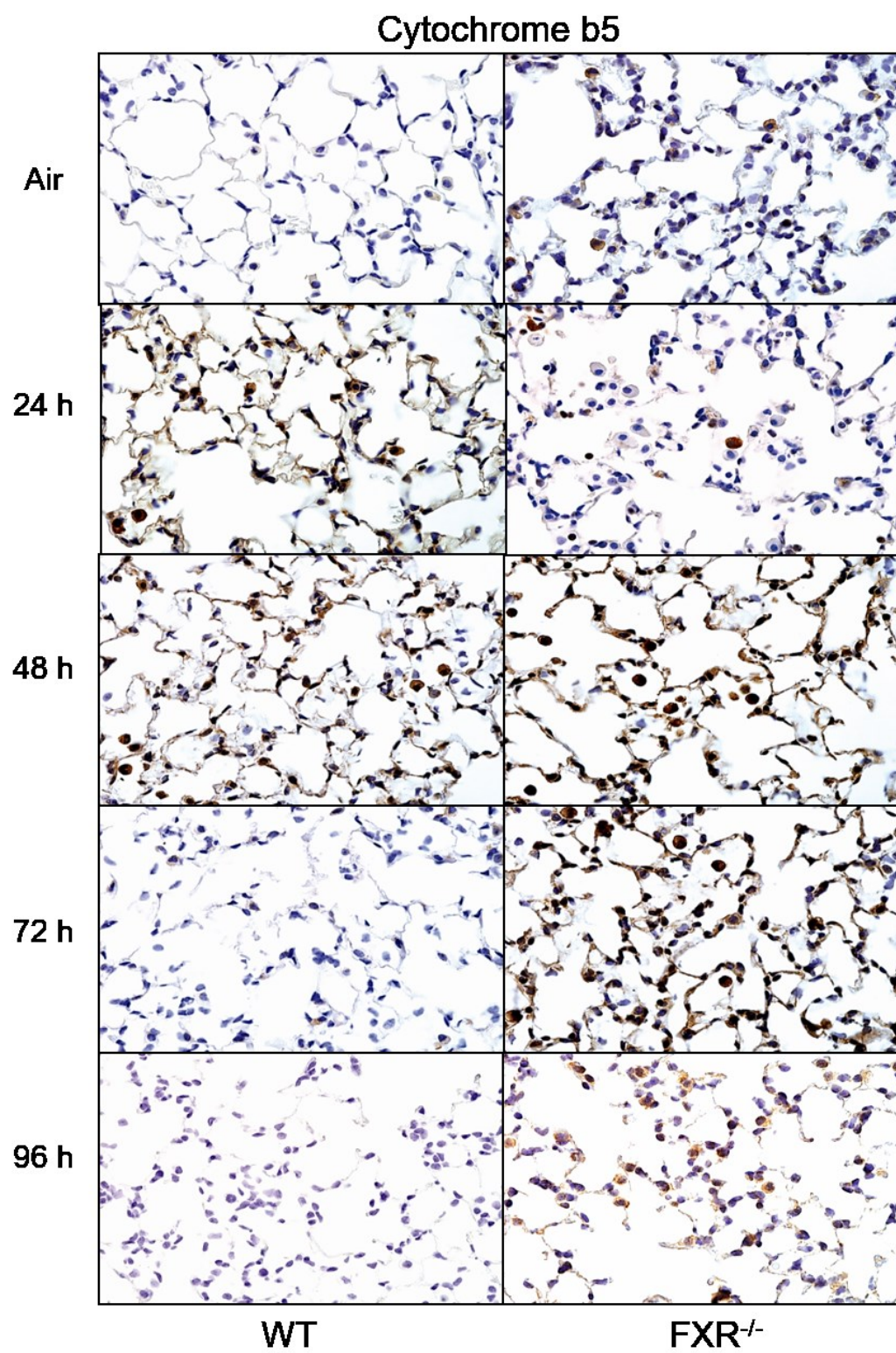


FIGURE 41. Effects of loss of FXR on ozone-induced expression of 4-HNE. Lung sections, prepared 24-96 h after exposure of WT and FXR^{-/-} mice to air or ozone, were stained with antibody to 4-HNE. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3 separate experiments is shown ($n = 3$ mice/treatment group). Original magnification, x600.

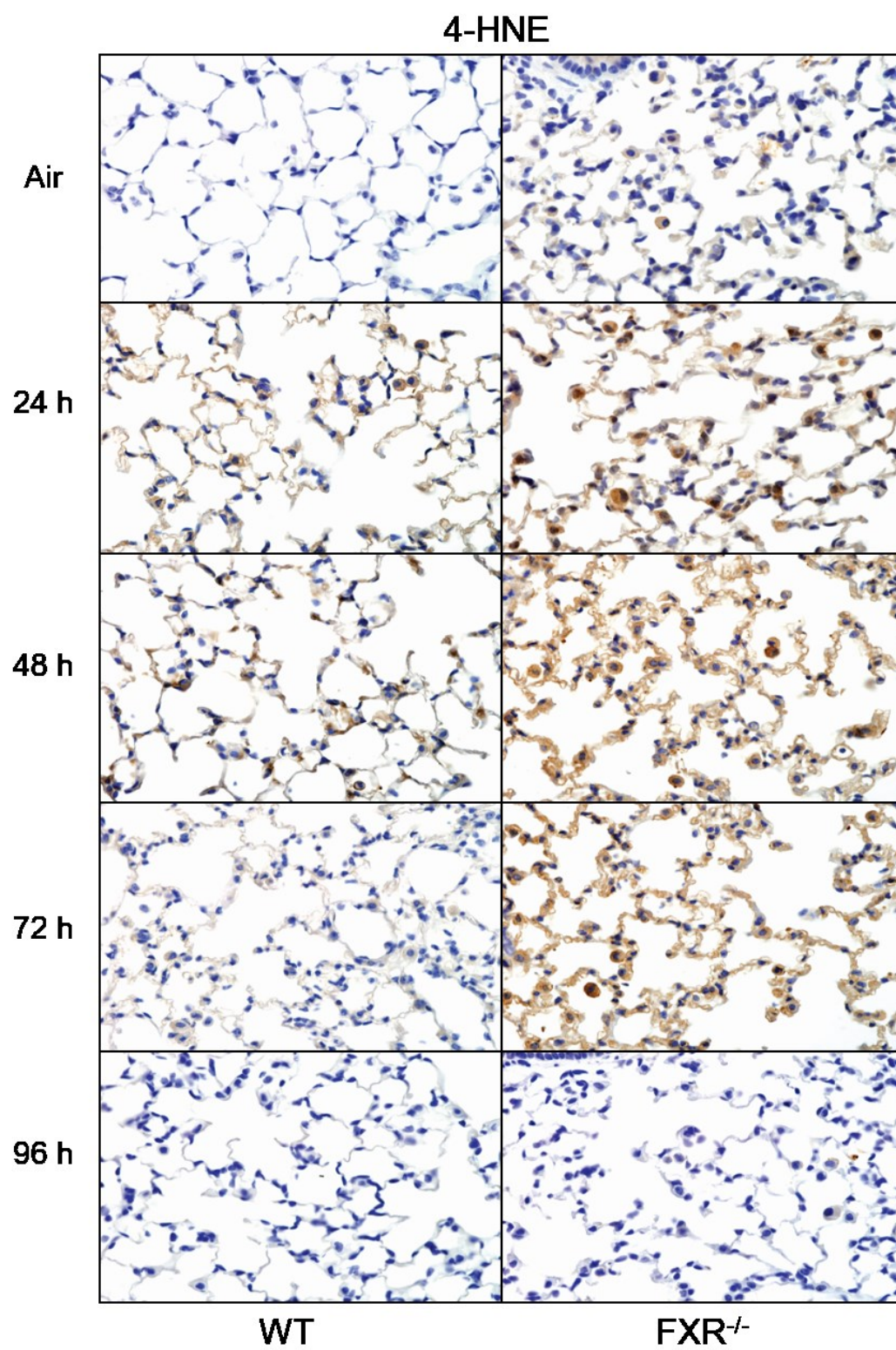


FIGURE 42. Effects of loss of FXR on ozone-induced alterations in BAL protein.

BAL was collected 24-120 h after exposure of WT and FXR^{-/-} mice to air or ozone and analyzed for protein content. Bars, mean \pm SE (n = 10-12 mice). ^aSignificantly different (p < 0.05) from air; ^bSignificantly different (p < 0.05) from WT.

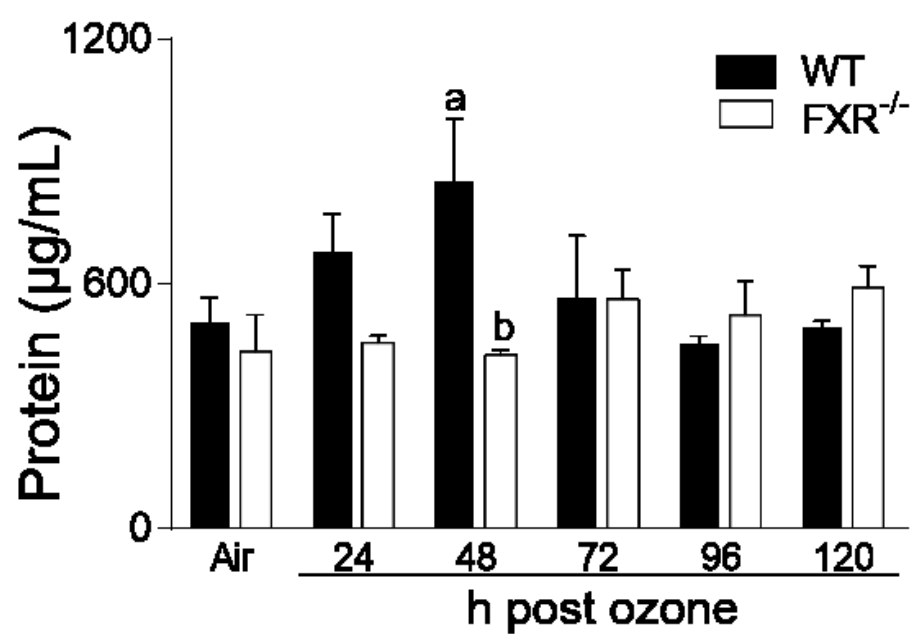


FIGURE 43. Effects of loss of FXR on pulmonary mechanics. Measurements of tissue damping (G) and tissue elastance (H) at PEEPs ranging from 0 cm to 9 cm H₂O, at 48 h, from mice treated with air or ozone. Data were normalized to 3 cm H₂O and presented as mean \pm SE (n=5/treatment group).

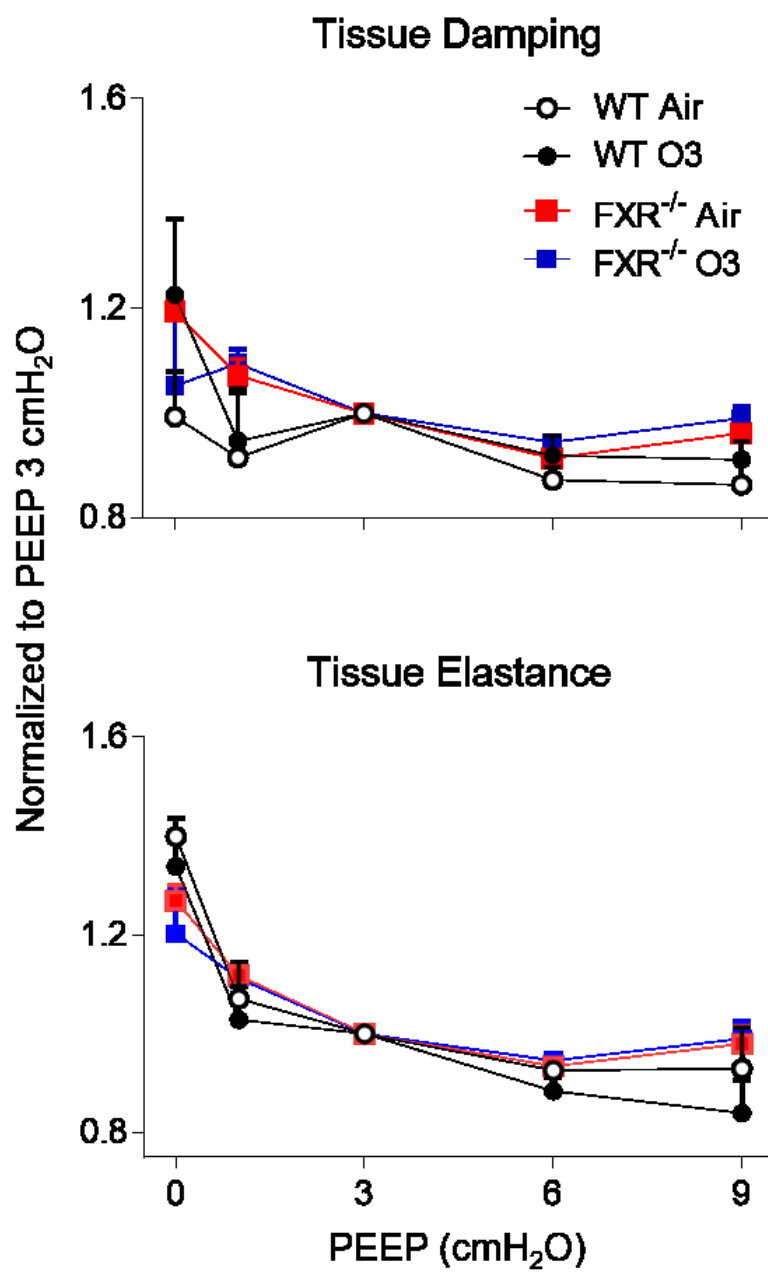
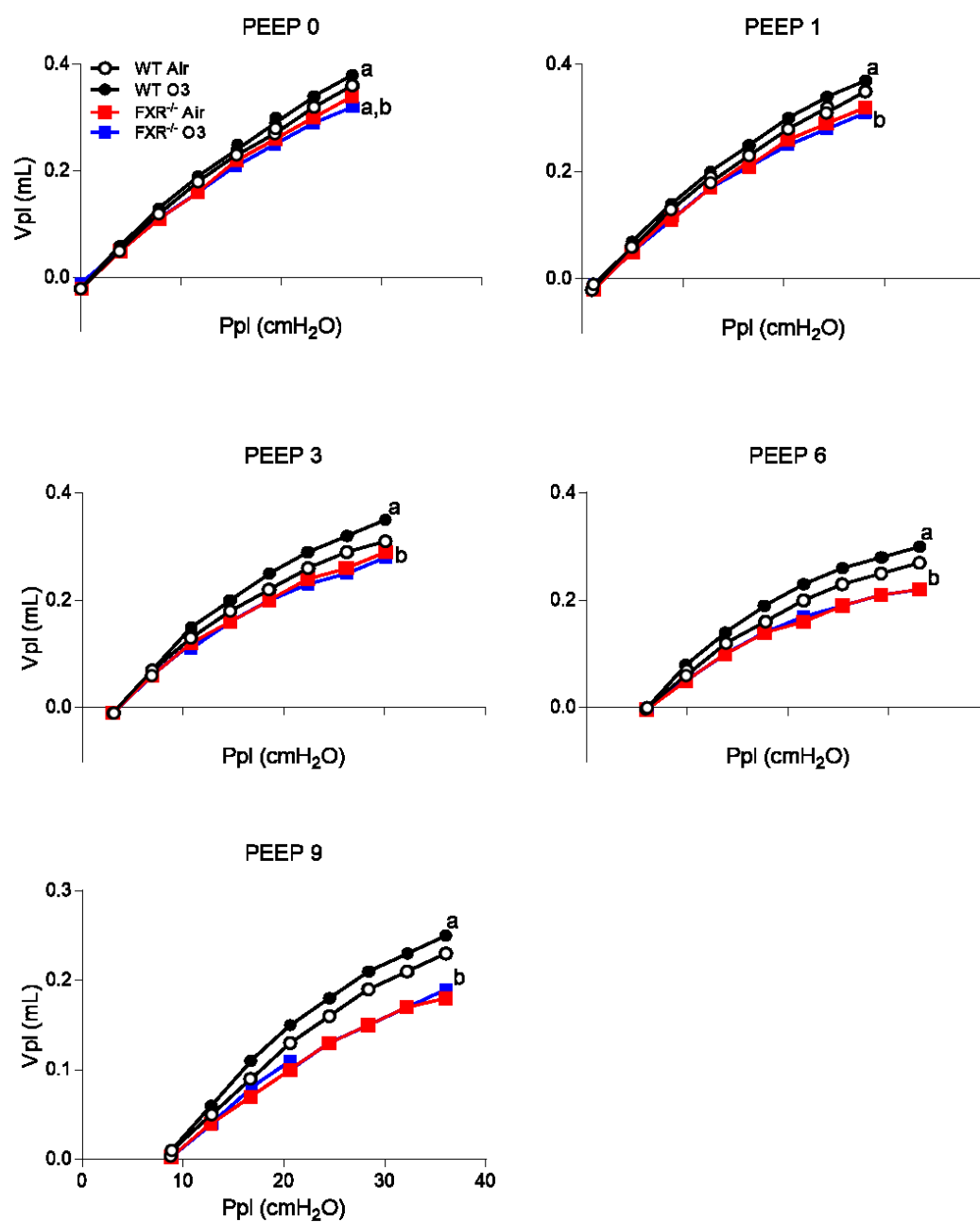


FIGURE 43. Effects of loss of FXR on ozone-induced alterations in pressure volume curves. A pressure volume curve was generated 48 h after exposure of WT and FXR^{-/-} mice to air or ozone at five different PEEP levels (in cm H₂O). Data are represented as best lines using linear regression analysis (n=3). ^aSignificantly different (p <0.05) from air; ^bSignificantly different (p<0.05) from WT



SUMMARY AND CONCLUSIONS

The overall objective of these studies was to characterize lung macrophages responding to ozone, identify their origin, and analyze potential mechanisms regulating macrophage activation with a focus on the nuclear receptor, FXR. We hypothesized that distinct activated macrophage subpopulations contribute to tissue injury and repair, and that these cells are derived from both bone marrow and spleen; moreover, lung macrophage activation following ozone exposure is mediated, in part, by FXR. The results of our studies provided strong support for this hypothesis. Thus, using SPX, CCR2^{-/-}, GFP⁺ chimeric, CX3CR1^{+/GFP} reporter and FXR^{-/-} mice, we demonstrated that activated lung macrophages accumulating in the lung after ozone exposure originate from both the spleen and bone marrow. Additionally, macrophages recruited to the lung following ozone traffic in response to chemokines CCL2 and CX3CL1 and their receptors CCR2 and CX3CR1. We also demonstrated that FXR plays a key role in dampening ozone induced inflammation and oxidative stress; FXR is also important in regulating lung lipids and surfactant function. These results are novel and point to potential new targets for the development of therapeutics aimed at reducing inflammatory lung injury.

To characterize macrophage subpopulations responding to ozone, we used techniques in immunohistochemistry and flow cytometry. Our studies established that both pro-inflammatory macrophages, characterized as iNOS⁺, CCR2⁺, MMP9⁺, and CD11b⁺Ly6C^{Hi}F4/80⁺CD11c⁺ and anti-inflammatory macrophages characterized as Arg I⁺, MR⁺, YM1⁺, and CD11b⁺Ly6C^{Lo}F4/80⁺CD11c⁺ accumulate in the lung following ozone exposure. Splenectomy resulted in a decrease in numbers of pro-inflammatory

macrophages accumulating in the lung after ozone. In contrast, numbers of anti-inflammatory macrophages increased. This was associated with decreased lung injury and oxidative stress, confirming a role of pro-inflammatory macrophages in ozone toxicity. Similar findings were observed using CCR2^{-/-} mice. Thus, following ozone exposure, CCR2⁺ macrophages accumulate in the lung. Additionally, ozone-induced lung injury was reduced in mice lacking CCR2, a response correlated with decreased expression of pro-inflammatory proteins and genes, including iNOS, TNF- α , and IL-1 β , as well as reduced numbers of pro-inflammatory macrophages. Taken together, these data indicate that pro-inflammatory Ly6C^{Hi} and anti-inflammatory Ly6C^{Lo} macrophages play distinct roles in tissue injury and repair following ozone exposure; moreover, the spleen and bone marrow are major sources of lung macrophages responding to ozone.

In vivo tracking techniques were used to identify the bone marrow-derived macrophages based on GFP expression. We found that bone marrow-derived lung macrophages responding to ozone-induced injury expressed pro-inflammatory proteins, including Ly6C and CCR2, and anti-inflammatory proteins, including YM1, Nur77, CX3CR1, and HO-1. These findings correlated with increased numbers of both pro-inflammatory CD11b⁺Ly6C^{Hi}F4/80⁺CD11c⁺ and anti-inflammatory CD11b⁺Ly6C^{Lo}F4/80⁺CD11c⁺ macrophages in the lung following ozone exposure. Using CX3CR1 reporter mice, we also found that ozone exposure resulted in increased CX3CR1⁺ macrophage trafficking to the lung; additionally, CX3CR1⁺ macrophages were mainly Ly6C^{Lo} and accumulated at later times after ozone. In contrast, CCR2⁺ macrophages were mostly Ly6C^{Hi} and peaked at earlier times after ozone. These findings

demonstrate that macrophage subpopulations can be identified based on their expression of chemokine receptors.

Following ozone exposure, FXR expression increased in alveolar macrophages and alveolar type II cells. This was associated with derangements in lung lipids and surfactants, a response correlated with altered expression of proteins involved in lipid metabolism and transport, including ABCA1, ABG1, ApoE, and VLDLR. Our studies demonstrated that loss of FXR resulted in increased expression of pro-inflammatory proteins, including iNOS, TNF- α , IL-1 β , CCR2, and CCL2 and increases in pro-inflammatory CD11b⁺Ly6C^{Hi}F4/80⁺CD11c⁺ macrophages in the lung. In contrast, reduced expression of MR, YM1, and Arg I was observed. However, increased expression of CX3CR1 and CX3CL1 and numbers of CD11b⁺Ly6C^{Lo}F4/80⁺CD11c⁺ macrophages were observed, suggesting that there are multiple subpopulations of anti-inflammatory macrophages responding to ozone induced injury.

In conclusion, these studies suggest that the outcome of ozone-induced lung injury depends, in part, on the balance between pro-inflammatory and anti-inflammatory macrophages that accumulate in the lung. Additionally, the spleen and bone marrow are sources of both pro- and anti-inflammatory macrophages and that chemokine receptors CCR2 and CX3CR1 are involved in the recruitment of distinct macrophage subpopulations to the lung following ozone exposure. In addition, we also identified FXR as an important nuclear receptor involved in lung macrophage activation. Elucidating specific subpopulations of inflammatory cells responding to ozone and mechanisms regulating their trafficking and activity may lead to the development of more efficacious approaches for mitigating oxidant-induced pulmonary toxicity and disease pathogenesis.

PERSPECTIVES

PART I. ROLE OF SPLEEN-DERIVED MACROPHAGES

Activated macrophages have been implicated in ozone toxicity; however, the origin of these cells is unknown. The present studies demonstrate that the spleen is a source of proinflammatory/cytotoxic macrophages that contribute to ozone-induced lung injury. Identification of the source of inflammatory macrophages responding to lung injury may be important in the development of novel therapeutics aimed at reducing inflammatory lung diseases.

PART II. CCR2 REGULATES INFLAMMATORY CELL

Exposure to toxic levels of ozone causes damage to the lower lung. This is associated with an accumulation of CCR2⁺ activated macrophages in the lung. Evidence suggests that this distinct macrophage subpopulation contributes to tissue injury. Loss of CCR2 resulted in reduced ozone-induced lung inflammation. These results demonstrate a key role of CCR2 in ozone-induced inflammatory cell emigration into the lung and toxicity.

PART III. TRACKING INFLAMMATORY MACROPHAGES

Macrophages contribute to both lung injury and repair following ozone inhalation. One aspect of these studies was identification of the origin of these cells. Our results demonstrate that following ozone exposure, inflammatory cells enter the lung from the bone marrow. In addition, the activity of the macrophages subpopulations in the lung can be distinguished based on chemokine receptors. While CCR2 plays a key role in pro-

inflammatory macrophage trafficking to the lung, CX₃CR1 is involved with anti-inflammatory macrophage migration. Taken together, these studies demonstrate distinct pathways regulating macrophage accumulation in the lung after ozone.

PART IV. REGULATION OF MACROPHAGE ACCUMULATION AND ACTIVATION IN THE LUNG FOLLOWING OZONE EXPOSURE BY THE FARNESOID X RECEPTOR

Farnesoid x receptor (FXR) is a nuclear receptor that is involved with lipid transport and NF- κ B suppression. In these studies, we analyzed the role of FXR in ozone-induced inflammation and macrophage activation. Activation of FXR has been shown to play an anti-inflammatory role in the liver and intestine. Our data suggest that FXR plays a role in limiting lung inflammatory responses to ozone. This represents a highly novel observation that provides new insights into inflammatory mechanisms contributing to ozone toxicity.

FUTURE STUDIES

The present studies demonstrated that CD11b⁺ macrophages are recruited to the lung following exposure to toxic doses of ozone and that they play a key role in inflammation and injury. Future studies could focus on further characterizing CD11b⁺ cells and distinguishing between their contribution to ozone toxicity and the contribution of resident macrophages. To accomplish this, studies could be performed to selectively ablate these cell populations. To ablate resident alveolar macrophages, liposome-encapsulated clodronate could be used; intratracheal administration of these liposomes has previously been reported to deplete resident macrophages (van Rooijen and Hendriks, 2010). To further characterize the contribution of infiltrating CD11b⁺ macrophages to ozone toxicity, these cells can be depleted by administering diphtheria toxin (DT) to CD11b-DTR transgenic mice prior to ozone (Duffield *et al.*, 2005). This would result in specific *in vivo* ablation of CD11b⁺ cells, which would be expected to reduce inflammatory macrophage accumulation in the lung. Findings that ozone-induced lung injury is reduced in these experiments would provide additional support for our hypothesis that infiltrating macrophages contribute to ozone-induced lung injury.

These present studies evaluated CCR2⁺ and CX3CR1⁺ macrophages that accumulate in the lung following ozone. In future studies, it would be valuable to simultaneously assess the accumulation of these cells in the lung. To accomplish this, a mouse model carrying both CX3CR1^{+/GFP} and CCR2^{+/RFP} reporters could be used (Mizutani *et al.*, 2012; Saederup *et al.*, 2010). Macrophages isolated from the lungs of these mice could also be evaluated for co-expression of CCR2 and CX3CR1. Findings that macrophages specifically express CCR2 or CX3CR1 would provide evidence that

these macrophages are distinct. Evidence suggests that CCR2⁺ and CX3CR1⁺ macrophages are bone-marrow derived. This can be investigated with bone marrow reconstitution experiments. In these studies, irradiated wild type mice would have their bone marrows reconstituted with CCR2^{+/RFP} and CX3CR1^{+/GFP} cells. This would allow quantification of recruited CCR2⁺ and CX3CR1⁺ macrophages following ozone exposure.

TLR4 has been reported to play a role in ozone-induced inflammation (Connor et al., 2012; Hollingsworth et al., 2007). Calcium binding proteins Mrp8 (S100A8) and Mrp14 (S100A9) have been shown to activate TLR4 (Vogl *et al.*, 2007). Mrp8 and Mrp14 can form a heterodimer that is localized in the cytosol of phagocytes. In pilot studies, we observed increased expression of Mrp8 and the Mrp8/14 heterodimer in the lung after ozone exposure. Studies using mice deficient in Mrp8, which would also lack the heterodimer, will provide insight into signaling pathways regulating macrophage activities following ozone exposure.

In further studies, molecular mechanisms regulating macrophage activity after ozone exposure can be investigated. Nur77 is a nuclear receptor that is prominent in anti-inflammatory Ly6c^{Lo} monocytes. Findings that Ly6c^{Lo} macrophages are decreased in the lungs of ozone-exposed Nur77^{-/-} mice while Ly6c^{Hi} macrophages increased would confirm that the Nur77 is involved in Ly6c^{Hi} macrophage differentiation; this would be expected to impair tissue recovery after ozone toxicity. Bone marrow reconstitution studies using Nur77^{-/-} bone marrow cells could also be performed. The observation that the bone marrow derived Nur77^{-/-} monocytes cause increased numbers of Ly6c^{Hi}

macrophages in the lung would provide new insight into mechanisms regulating monocyte maturation.

Studies could also be performed to evaluate the effects of high fat diet on ozone-induced lung inflammation. In pilot studies increased BAL protein content and foamy macrophages were observed in the lungs of mice fed a high fat diet. In future studies, we can characterize diet-related alterations in pulmonary inflammation and macrophage activity in mice following ozone exposure. Evidence has demonstrated that diet can influence the response to ozone (Shore *et al.*, 2009). Furthermore, CCR2 antagonism has been shown to improve lipid metabolism (Han *et al.*, 1999; Kang *et al.*, 2010). These findings suggest that following ozone exposure, macrophages may be influenced by lipids and diet. To further assess this, macrophage activity in mice with dysfunctional lipid metabolism (ApoE^{-/-} or LDLR^{-/-} mice) could be examined following ozone exposure; FXR expression in these mice could also be analyzed. These results would be expected to demonstrate that lipids and oxidized lipids play an important role in macrophage activity and the response to ozone.

Mice that lack SP-D exhibit increased lung inflammation following ozone exposure compared to WT mice (Groves *et al.*, 2012). In pilot studies, we observed that SP-D^{-/-} mice lack FXR expression in the lungs. In future studies, an FXR agonist can be administered intravenously to SP-D^{-/-} mice. Findings that FXR activation reduces inflammation in the mice exposed to ozone and air in mice that lack SP-D would provide insight on potential anti-inflammatory activities of FXR. Osteoclast-associate receptor (OSCAR) is recognized as a receptor for SP-D that is involved in the release of pro-inflammatory cytokines from CCR2⁺ monocytes (Barrow *et al.*, 2015). It would be of

interest to evaluate the expression of OSCAR in mice with reduced inflammatory cells (CCR2^{-/-}, SPX) and mice that have dysfunctional lipid transport (FXR^{-/-}, ApoE^{-/-}, LDLR^{-/-}).

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