RESPONSES OF MOUSE MACROPHAGES TO OXIDATIVE STRESS

By

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ABSTRACT OF THE DISSERTATION

Responses of Mouse Macrophages to Oxidative Stress

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Tissue injury induced by infections or xenobiotics is associated with oxidative stress and inflammation, which are thought to contribute to pathogenic response. We hypothesized that toll-like receptor 4 (TLR4) is important in macrophage responsiveness to oxidative stress. To test this, we compared the response of TLR4 mutant C3H/HeJ mice and control C3H/HeOuJ mice to ozone and to bacterially-derived lipopolysaccharide (LPS).

Exposure of C3H/HeOuJ mice to ozone (0.8 ppm for 3 h) resulted in increases in bronchoalveolar lavage lipocalin 24p3, 4-hydroxynonenal, surfactant protein-D, macrophage and protein content. Increased nuclear binding activity of NF-κB and expression of TNFα mRNA was also noted in lung macrophages. Findings that these responses to ozone were reduced in C3H/HeJ mice demonstrate that functional TLR4 contributes to ozone-induced injury, inflammation, and oxidative stress.

We next determined if lung and liver macrophage responses to LPS are also mediated by TLR4. Treatment of control C3H/HeOuJ mice with LPS (3 mg/kg) resulted in increased numbers of macrophages in liver and lung after 48 h. In liver, but not lung macrophages, a rapid increase in mRNA expression of MnSOD and HO-1, as well as

COX-2 and microsomal prostaglandin E synthase-1 was also observed. Conversely, macrophage COX-2 protein expression increased in both macrophage populations. The effects of LPS were significantly reduced in C3H/HeJ mice indicating TLR4 is also involved in LPS-induced oxidative stress, inflammation and macrophages activation in the liver and lung.

To investigate mechanisms regulating macrophage responses, we evaluated the effects of hypoxia-induced oxidative stress on LPS-induced activation of macrophages using RAW 264.7 murine macrophages. Hypoxia augmented the effects of LPS on iNOS, COX-2, IL-1β, GLUT-1 and VEGF-A mRNA expression. Hypoxia also upregulated LPS-induced protein expression of iNOS and COX-2, as well as MnSOD, lipocalin 24p3, and MMP-9. Some of these responses were dependent on p44/42 mitogen activated protein kinase signaling.

Taken together, these studies demonstrate a key role of TLR4 in both sterile and infection driven inflammatory responses. These findings may be important in the development of effective therapeutics for treating diseases associated with prominent macrophages inflammatory responses.

DEDICATION

To my family for their unconditional love and infinite support, my parents, Zdzisław and Danuta Jankowski, and my beloved children Veronica, Mark, and Christian Connor

QUOTATIONS

The Lord is my shepherd; I shall not want (Psalm 23:1)
Zelo zelatus sum pro Domino Deo exercituum (1 Kings 19:10)

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ABBREVIATIONS

4-HNE	4-hydroxynonenal
AHR	airway hyperresponsiveness
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CLR	C-lectin receptors
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
DAMPs	damage-associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOC	deoxycholate
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ELF	epithelial lining fluid
EMSA	electrophoretic mobility shift assay
ETX	endotoxin
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIF	hypoxia inducible transcription factor
$HMGB_1$	high mobility group protein-1

HO-1	heme-oxygenase-1
HSP	heat shock protein
IFN	interferon
IL	interleukin
IL-1R	interleukin-1 receptor
JNK	c-Jun N-terminal kinases
KDO	2-keto-3-deoxyoctonic acid
LBP	LPS binding protein
LOP	lipid ozonation products
LOX	lipoxygenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MIP-2	macrophage inflammatory protein-2
MMP-9	matrix metalloproteinase-9, gelatinase-B
MnSOD	manganese superoxide dismutase
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NOD	nucleotide-binding oligomerization-domain protein
NOS	inducible nitric oxide synthase
NRL	NOD-like receptors
PAF	platelet-activating factor
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PG	prostaglandin

PGDH	15-hydroxyprostaglandin dehydrogenase
PGES	prostaglandin E synthase
PHDs	prolyl hydroxylases
PRRs	pattern-recognition receptors
PUFA	polyunsaturated fatty acids
RIG	retinoic acid-inducible gene
RLR	RIG-I-like receptor
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
RSV	severe respiratory syncytial virus
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SP	surfactant protein
TEA	triethylamine
TGF	transforming growth factor
TIR	Toll/IL-1 receptor
TLR	toll-like receptor
TNFR1	TNF receptor 1
TNFα	tumor necrosis factor alpha
TX	thromboxane
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau factor

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INTRODUCTION

LUNG

The lung provides molecular oxygen to the body eliminating carbon dioxide in the process. While the extensive surface area of the deep lung optimizes gas exchange, it predisposes the lung to injury either by inhalation of air pollutants, such as ozone, or toxins in the blood, like endotoxin (Connor et al., 2012; Sunil et al., 2007). Although the lung is equipped with defense mechanisms including mucociliary clearance and alveolar macrophages, it often sustains injury. Major lung injury is caused by excessive oxidative burden mediated by free radicals generated from reactive gases like ozone or pulmonary immune cells (Connor et al., 2012; Fakhrzadeh et al., 2004; Fakhrzadeh et al., 2008; Pendino et al., 1995; Sunil et al., 2009; Wizemann and Laskin, 1994). Severe lung injury can lead to diseases including chronic bronchitis, fibrosis, emphysema, and cancer (Rahman et al., 2006). The extent of the injury depends on the location, physicochemical properties of the toxicant, the dose, and the metabolic capabilities of the lung's cellular components (Laskin et al., 2011).

The main task of the lung is to bring into the body essential oxygen, an electron acceptor in aerobic production of the high energy molecule adenosine triphosphate (ATP), and to clear the blood of carbon dioxide, a toxic by-product. Gas exchange occurs in three stages: ventilation, perfusion, and diffusion (Maina, 2011). During ventilation, air enters the lung though the nasal and oral cavity in humans, but exclusively though the nasal passage in rodents, where it is warmed and humidified. Air then moves though the conducting airways into the gas-exchange region of the alveoli. There, blood from the right ventricle enters the pulmonary capillary bed. After the oxygen diffuses into the blood across the entire alveolar surface, carbon dioxide diffuses into alveoli and is exhaled. The oxygenated blood returns to the left atrium and ventricle though pulmonary veins and is pumped into systemic arteries though the aorta.

Human lung has five lobes: the superior and inferior left lobe and the superior, middle, and inferior right lobe (Ukil and Reinhardt, 2009). In contrast, in rodents the lung consists of a single left lobe and four right lobes: cranial, middle, caudal, and ancillary (Thiesse et al., 2010). Air enters the lung though the nasal and oral cavity, then though the pharynx, then directly to the trachea. The trachea subsequently divides into bronchi; bronchi become bronchioles, and finally the alveoli, the gas exchange regions of respiratory bronchioles (Casarett, 2010). Whereas inhaled materials are prevented from direct access to the brain by metabolically active nasal epithelium, pollutants and cell debris are trapped by mucociliary lining. The mucus is thought to contain protective antioxidants and free-radical scavengers (Behndig et al., 2009). Synchronized beating of the respiratory tract cilia moves the mucus layer towards the pharynx where it is swallowed or expectorated (Casarett, 2010). The trachea is populated by mixture of ciliated columnar and mucus secreting goblet cells, while the lower airways are dominated by highly metabolic Clara cells (You et al., 2002). To aid in collection of gases and particles on airway walls, the total surface area increases from trachea to the distal airway though characteristic bifurcating structure (Casarett, 2010). The cartilaginous bronchi turn into non-cartilaginous bronchioles, which turn into respiratory bronchioles, and ultimately alveoli.

Approximately 90% of alveoli surface is covered by attenuated, highly differentiated type I epithelial cells (Hackett et al., 2011). Type I cells prevent leakage of fluid and proteins across the alveolar wall into the air space though tight junctions. Because of their vast surface area, type I epithelial cells are highly susceptible to injury. Damaged type I epithelial cells are replaced by proliferation and differentiation of type II epithelial cells (Demaio et al., 2009). These cuboidal cells are located at the corners of the alveoli between capillaries. Type II epithelial cells function to synthesize, store and secrete surfactant, an oily film that lowers alveolar surface tension reducing the pressure

difference required by the lung to inflate (Casarett, 2010). Surfactant is composed of 90% phospholipids and 10% protein; it includes hydrophilic surfactant protein (SP)-A and SP-D, as well as hydrophobic SP-B and SP-C (Demaio et al., 2009). All four surfactant proteins are synthesized and secreted by alveolar type II epithelial cells (Boggaram, 2003). SP-A, SP-B, and SP-D are also synthesized by airway Clara cells and submucosal cells. After being packaged into intracellular organelles called lamellar bodies and tubular myelin, surfactants are secreted into the alveolar space. Lowering of the surface tension also keeps the alveolar space dry by reducing the draw of fluids from the capillaries. Capillaries cover one-fourth of the alveoli lined by endothelial cells (Warburton et al., 2000). In many places, endothelial cells along type I epithelial cells lie on a fused continuous basement membrane. This forms an extremely thin blood/air barrier of approximately 0.45 μm. An increase in thickness above 0.8 μm results in insufficient gas exchanges (Perlman and Bhattacharya, 2007). In other places these cells are separated by an interstitium. Mesenchymal interstitial cell population includes fibroblasts, neutrophils, lymphocytes, plasma cells, mast cells and macrophages. Interstitial macrophages, as they enter alveoli, become alveolar macrophages. Macrophages participate in lung defense, inflammation, and immune responses (Laskin et al., 2011).

LIVER

The liver maintains metabolic homeostasis in the body by aiding in digestion and detoxification of toxins, xenobiotics and bacterial-derived products. Nutrients are absorbed, stored or metabolized and distributed to blood and/or bile (Malarkey et al., 2005). In the liver, xenobiotics are generally changed from lipophilic (favoring absorption) to hydrophilic (water soluble which favors urine or fecal excretion) (Casarett, 2010). This process of biotransformation usually limits the systemic bioavailability of oral

xenobiotics; however, some compounds are converted to reactive metabolites, which contribute to hepatotoxicity. The liver also contributes to the systemic response to local inflammation, clearance of particles and soluble molecules from the circulation, and defense against invading pathogens (Parker and Picut, 2005).

The liver is a heterogeneous tissue and consists of parenchymal cells (hepatocytes) and non-parenchymal cells (endothelial cells, stellate cells, pit cells, and Kupffer cells) (Mescher, 2010). The liver tissue is designed to maximize contact of blood with the surface of the hepatocytes, which are arranged in plates of cords one cell thick that intersect at different angles (Casarett, 2010). The spaces between these plates are the hepatic sinusoids. The portal vein (draining from the mesenteric, gastric, splenic, and pancreatic veins) and hepatic artery enter sinusoids in the portal triad and terminate in all the branches of the central vein. The portal triad consists of portal vein, bile duct, and hepatic artery.

Hepatocytes represent 60% of total liver cells. They are metabolically highly active and contain vast numbers of lysosomes, peroxisomes, Golgi complexes, and mitochondria (Casarett, 2010). Specific metabolic pathways that are active in the liver include the urea cycle, regulation of lipid metabolism, production of bile, and glycogenolysis and gluconeogenesis. Hepatocytes also produce albumin and acute-phase proteins. As compared to centrilobular region, periportal hepatocytes normally have higher levels of oxygen saturation, activity, urea cycle activity, bile acid uptake, glutathione content, and glycogen synthesis.

The primary barrier between blood and hepatocytes are the sinusoidal endothelial cells, which represent about 20% of total liver cells (Casarett, 2010). Unlike vascular endothelial cells, they have no basement membrane and possess processes containing fenestrae, generally 150-175 nm in diameter, thus functioning as a selective semipermeable barrier (Braet and Wisse, 2002). The fenestrae in sinusoidal endothelial

cells are greater in number and diameter near the central vein. The sinusoidal endothelial cells filter fluids, solutes, and particles between sinusoidal lumen and space of Disse (perisinusoidal space). Sinusoidal endothelial cells have high endocytotic capacity, which allows them to clear the blood from different molecular waste products. Sinusoidal endothelial cells also play a role in immunological defense by producing cytotoxic and proinflammatory mediators (Crispe, 2009).

Hepatic stellate cells, located between basolateral surface of hepatocytes and the anti-luminal side of sinusoidal endothelial cells, comprise about 5% of liver cells (Friedman, 2008). In normal liver, stellate cells, previously called Ito or fat-storing cells, store and transport retinoids (vitamin A compounds) stored in cytoplasmic droplets as retinyl esters (Handharyani et al., 2001). Depending on the diet, these droplets also contain triglycerides, phospholipids, cholesterol, and free fatty acids. Stellate cells are smaller in the periportal regions, have perisinusoidal processes, and smaller volume of lipid droplets. In midzone region, they are elongated with larger volume of lipid droplets and greater abundance of desmin. In the centrilobular region they have intercellular processes, relatively more vitamin A, and reduced amounts of desmin (Casarett, 2010). During inflammation, stellate cells produce chemoattractants to induce infiltration of leukocytes (Maher, 2001), secrete lipoproteins, growth factors, and cytokines (Gao and Brigstock, 2003). When activated, stellate cells begin proliferating, become fibrogenic and contractile. Excessive stellate cell activity remains the most dominant pathway in development of hepatic fibrosis (Bataller et al., 2005; Friedman et al., 2000; Gressner et al., 2002; Kisseleva and Brenner, 2006).

Kupffer cells, about 15% of all liver cells, are the resident macrophages of the liver. They adhere to the surface of hepatic sinusoidal endothelial cells (Wisse, 1974). Ameroid-shaped Kupffer cells bear microvilli and lamellipodia. In periportal regions, Kupffer cells are about twice as abundant and larger, possess greater lysosomal

enzyme activities but generate less superoxide anion than cells in centrilobular regions (Bowers et al., 1986; Laskin et al., 2001; Sleyster and Knook, 1982). Their major function is to clear particulate and foreign materials from the portal circulation (Bykov et al., 2003).

MACROPHAGES

Macrophages originate from bone-marrow monocyte precursors (Gordon and Taylor, 2005). In most adult tissue macrophages are derived from circulating monocytes, but studies have shown that renewal and maintenance of these cells also depends on local proliferation (Davies et al., 2011; Wohl et al., 2010). These widely distributed cells include monocyte precursors, which migrate into the tissue in steady state or in response to inflammation and mature into macrophages. Resident macrophages include Kupffer cell in the liver, alveolar macrophages in the lung, free or fixed macrophages in the spleen, histiocytes in the skin and connective tissue, osteoclasts in bone, microglia in the brain, and macrophages of other tissues (Gordon and Taylor, 2005). Macrophages maintain tissue homeostasis through clearance of senescent cells (Gordon, 1998). These phagocytic cells also play a critical role in innate immune response. Following injury or infections, macrophages phagocytize microbes, viruses, cell debris, and some tumor cells, as well as actively secrete various mediators, which kill the pathogens and regulate host defense (Gordon and Taylor, 2005; Mosser and Edwards, 2008). These various functions are possible due to plastic, rapid, and fully reversible macrophage phenotypes, which differentiate under changing microenvironments (Porcheray et al., 2005).

Activated macrophages polarize into heterogeneous cell populations, which vary in differentiation, tissue distribution and responsiveness to stimuli (Gordon and Taylor,

2005; Mosser and Edwards, 2008). They are generally classified into two groups: classically activated M1 and alternatively activated M2 macrophages. M1 macrophages respond to inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα) and interferon (IFN)-γ, microbial products, and secrete proinflammatory mediators, reactive oxygen, and nitrogen intermediates (Laskin et al., 2011). M1 macrophages protect the host during acute infectious disease. M2 macrophages are further divided into three subsets according to functional expression of alternative activation markers: M2a, induced by interleukin (IL)-4 or IL-13; M2b, induced by immune complexes and agonists to toll-like receptors (TLRs) or IL-1 receptors; and M2c, induced by IL-10 and glucocorticoid hormones (Mantovani et al., 2004). M2 macrophages generally produce low levels of proinflammatory cytokines, including IL-1, TNFα and IL-6, except for M2b macrophages, which protect mice against LPS toxicity while promoting Th2 differentiation and humoral antibody production (Mosser and Edwards, 2008). M1 macrophages drive type I responses, kill microorganisms and tumor cells and produce copious amounts of proinflammatory cytokines. Whereas M2a and M2b macrophages exert immunoregulatory functions and drive type II responses, help in parasite clearance and allergy, M2c macrophages suppress immune responses, scavenge debris and promote angiogenesis, tissue remodeling and repair (Mantovani et al., 2004; Martinez et al., 2009).

MACROPHAGES IN PULMONARY TOXICITY

Pulmonary macrophages include alveolar macrophages residing in air spaces and interstitial macrophages located in the lung connective tissue. Macrophages have been implicated in the pathogenesis of various pulmonary diseases including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome, and

asthma (Tetley, 2002). Whereas hyperresponsiveness of classically activated pulmonary M1 macrophages may promote acute injury, overactive alternatively activated M2 macrophages contribute to chronic disease such as pulmonary fibrosis, and exacerbate cytotoxic and allergic responses (Laskin et al., 2011).

Exposure to toxicants such as ozone, lipopolysaccharide (LPS), bleomycin, or vesicants, results in increased numbers of macrophages in the lower lung and the generation of increased quantities of cytotoxic reactive oxygen species (ROS), reactive nitrogen species (RNS), IL-1, TNFα, bioactive lipids, and proteases (Fakhrzadeh et al., 2004; Fakhrzadeh et al., 2008; Inghilleri et al., 2006; Malaviya et al., 2010; Pendino et al., 1995; Wilson et al., 2010). Excessive production of inflammatory mediators by classically activated M1 macrophages exacerbates lung injury (Laskin et al., 2011). An involvement of M1 macrophages in pulmonary pathology has been demonstrated experimentally where toxicant-induced lung damage was reduced by blocking or depleting macrophages or macrophage-derived inflammatory mediators (Elder et al., 2004; Haddad et al., 1995b; Wigenstam et al., 2009).

Persistence of irritants such as allergens or toxic chemicals results in disruption of normal lung architecture, recruitment of macrophages and ultimately wound healing (Moodley et al., 2003). However, overproduction of cytokines may result in a chronic pulmonary disease. Chronic allergic airway disease in asthmatics and pulmonary fibrosis are associated with elevated M2 macrophage-generated type 2 cytokines: IL-4, IL-13, IL-10, and transforming growth factor (TGF)-β (Baran et al., 2007; Emad and Emad, 2007; Holgate, 2008; Ishida et al., 2008; Trujillo et al., 2008). Further evidence for the involvement of M2 macrophages is demonstrated in mice overexpressing IL-10 and IL-13, which results in increased lung fibrosis (Barbarin et al., 2005; Lee et al., 2001). In contrast, preventing monocyte chemoattractant protein (MCP)-1 function,

responsible for macrophage recruitment, or inhibition of monocyte differentiation into macrophages, attenuates pulmonary fibrosis in mice (Inoshima et al., 2004; Murray and Wynn, 2011).

MACROPHAGES IN HEPATOTOXICITY

Kupffer cells, strategically located in the lumen of hepatic sinusoids, mainly clear particulate and foreign materials from the portal circulation by phagocytosis. Increased numbers of Kupffer cell in the liver following exposure to hepatotoxicants is associated with hepatotoxicity (Holt et al., 2008a; Laskin et al., 2010). Treatments with hepatotoxicants like acetaminophen, carbon tetrachloride or thioacetamide, which cause centrilobular hepatic necrosis, or hepatotoxicants like endotoxin, phenobarbital or galactosamine, which affect the whole liver, are characterized by accumulation of M1 macrophages at sites of injury (Chen et al., 2007; Gardner et al., 2010; Laskin et al., 2010). Blocking or depleting liver macrophages, using hydrocortisone, gadolinium chloride or dextran sulfate, abrogates acetaminophen- or carbon tetrachloride-induced hepatotoxicity (Abdel-Zaher et al., 2007; Muriel et al., 2005). Moreover, acetaminophen- or galactosamine-induced hepatotoxicity increases when macrophages are activated by pretreatment with LPS or polyinosinic-polycytidylic acid (Al-Tuwaijri et al., 1981; Kalabis and Wells, 1990; Maddox et al., 2010).

Depending on the stimuli, Kupffer cells are either classically activated into M1 or alternatively activated into M2 macrophages. Activated M1 macrophages release increased amounts of proinflammatory mediators, such as TNFα, proteolytic enzymes, ROS and RNS, which are all implicated in hepatotoxicity (Masubuchi et al., 2009; Orfila et al., 1999). In contrast, activation of M2 macrophages, involved in wound repair and suppression of inflammation by producing mediators like TGFβ and vascular endothelial

growth factor (VEGF), protects against hepatotoxicity (Matsukawa et al., 2000; Yee et al., 2007). However, prolonged hyperactivity of M2 macrophages contribute to chronic liver disease including hepatic fibrosis (Heymann et al., 2009; Karlmark et al., 2009; Seki et al., 2009).

INFLAMMATION

Inflammation induced by pathogens, surgical trauma, caustic chemicals, immune responses, or ischemic damage, leads to release of soluble mediators, vasodilatation, increased blood flow, extravasation of fluid, cellular influx, and elevated cellular metabolism. This inflammatory response was early described by Celsius as heat, redness, swelling, and pain (Casarett, 2010). The main role of inflammation is to protect the host from infection, resolve an injury, and restore homeostasis (Medzhitov, 2008). Resolution of inflammation involves anti-inflammatory and pro-resolving mediators (Hang et al., 2003; Serhan et al., 2008). However, dysregulated inflammation may result in pathology and chronic diseases including septic shock, obesity, type 2 diabetes, cancer, atherosclerosis, asthma, fibrosis and neurodegenerative diseases (Barnes, 1999; Coussens and Werb, 2002; Libby, 2002; Medzhitov, 2008; Nathan, 2002; Serhan et al., 2008).

Pathogen-induced acute inflammation is triggered by structures conserved by microbial species called pathogen-associated molecular patters (PAMPs), which are recognized by germline-encoded pattern recognition receptors (PRRs) (Kawai and Akira, 2010). There are four classes of PRRs: TLRs, C-lectin receptors (CLRs), Retinoic acid-inducible gene-I-like receptors (RLRs) and nucleotide-binding oligomerization-domain protein (NOD)-like receptors (NRLs). These receptors are not only expressed on innate immune cells, including macrophages and dendritic cells, but also on nonprofessional cells, like epithelial cells, endothelial cells and fibroblasts (Kawai and Akira, 2010). TLRs

are responsible for sensing pathogens outside of the cell and in intracellular endosomes and lysosomes. There are 10 known TLRs in humans and 12 in mice. Whereas TLR1, 2, 4, 5, and 6, localized on the plasma membrane, recognize PAMPs derived from bacteria, fungi and protozoa, TLR3, 7, 8 and 9, mainly present on the endoplasmic reticulum membrane, recognize nucleic acid as well as PAMPs derived from various viruses and bacteria. The RLRs, consisting of RIG-I, MDA5, and LGP3, are localized in the cytoplasm and sense genomic RNA from dsRNA viruses (Kawai and Akira, 2010; Kumar et al., 2011). NRLs, comprised of 23 members in humans and about 34 in mice, are cytoplasmic receptors which recognize various microbial PAMPs. CLRs are transmembrane receptors which recognize carbohydrates on viruses, bacteria and fungi (Geijtenbeek et al., 2009). Activation of these receptors results in production of proinflammatory mediators and production of type I interferons in case of viral infection.

Inducers of inflammation trigger production of various inflammatory mediators including vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines, and proteolytic enzymes (Geijtenbeek et al., 2009). Vasoactive amines, released from mast-cells and platelets as histamine and serotonin, increase vascular permeability and vasodilation. Vasoactive peptides released from sensory neurons cause mast-cell degranulation, vasodilation and vascular permeability. Complement fragments promote recruitment of granulocytes and monocytes, and induce mast-cell degranulation. Eicosanoids and platelet-activating factor (PAF), derived from phospholipids present in the cellular membrane. Eicosanoids are synthesized from arachidonic acid by cyclooxygenases (COX)-1 and COX-2, which generate prostaglandins (PGs) or thromboxane. Prostaglandins, PGE_2 and PGI_2, cause vasodilation. PGE_2 is also a potent fever inducer (Blatteis et al., 2005; Funk, 2001). PAF, derived from acetylation of lysophosphatidic acid, recruits leukocytes, causes either vasodilation or vasoconstriction, increases vascular permeability and platelet

activation (Vadas et al., 2008). Proinflammatory cytokines, including TNFα, IL-1 and IL-6, produced rapidly by macrophages and mast-cells, upregulate adhesion molecules and stimulate endothelial cells to produce chemokines, and induce macrophages to release proinflammatory mediators such as IL-6, PAF, PGs, matrix metalloproteinases (MMPs), and various chemokines. Moreover, TNFα not only sensitizes leukocytes to produce ROS and RNS, but also directly induces cytotoxicity and apoptosis (Liu et al., 2004). Chemokines produced by many cell types in response to inflammation, control leukocyte extravasation and chemotaxis. C-C chemokines induce migration and activation of lymphocytes, monocytes and macrophages, while C-X-C chemokines attract neutrophils (Holt et al., 2008b). Proteolytic enzymes, elastins, cathepsins, and MMPs, degrade extracellular matrix (ECM) and basement-membrane proteins (Medzhitov, 2008).

Release of inflammatory mediators at the site of infection results in formation of local exudates as plasma proteins and neutrophils enter extravascular tissue. In order to kill invading pathogens, activated neutrophils release ROS, RNS, proteinase 3, cathepsins G and elastase (Kumar and Sharma, 2010). If the infectious agent is eliminated, resident and recruited macrophages initiate tissue-repair, reduction or removal of leukocytes and debris from inflamed site, and the return of homeostasis (Serhan et al., 2008). Macrophages release anti-inflammatory lipid mediators including lipoxins, which inhibit recruitment of neutrophils and promote recruitment of monocytes, and release of TGFβ, resolvins, and protectins (Serhan et al., 2008). If the pathogen is not eliminated, inflammation becomes chronic and infiltrating neutrophils are replaced by monocytes and T-cells. If macrophages fail to engulf and destroy pathogens, layers of macrophages surround the intruder and form granulomas (Fujiwara and Kobayashi, 2005).

STERILE INFLAMMATION

Sterile inflammation is caused by trauma, mechanically- or chemically-induced injury, generally in absence of microorganisms (Chen and Nunez, 2010; Matzinger, 2002). Sterile inflammation, similarly to pathogen-induced inflammation, is characterized by an innate immune response, which consists of vasodilation, recruitment of neutrophils and macrophages, production of proinflammatory cytokines and chemokines as well as release of ROS, RNS, proteases and growth factors. Whereas controlled inflammation repairs the injury and removes debris, unresolved inflammation, in cases when the trigger of inflammation is not removed, results in tissue destruction, fibroblast proliferation, fibrosis, and chronic inflammation. Sterile inflammation is involved in pulmonary interstitial fibrosis caused by inhalation of asbestos, silica, gout, and pseudogout, caused by deposition of monosodium urate and calcium pyrophosphate dihydrate crystals in the joints, Alzheimer's disease associated with activated microglia cells adjacent to β-amyloid containing plaques, atherosclerosis involving engulfment of cholesterol crystals by macrophages, cancer characterized by leukocyte infiltration, rejection of tissue transplants, and autoimmunity diseases (Chen and Nunez, 2010; Rock et al., 2010; Stewart et al., 2010).

Sterile inflammation resembles pathogen-induced inflammation and involves the same receptors. PRRs recognize not only PAMPs but also non-infectious endogenous materials released during cellular injury termed damage-associated molecular patters (DAMPs) (Matzinger, 2012). TLRs recognize endogenous ligands including mammalian DNA, heat shock proteins (HSP), high-mobility group box 1 protein (HMGB1), IFNα, IL-1β, CD40L, and breakdown product of hyaluronan (Lorne et al., 2010). Animal studies of acute tissue injury in hemorrhagic shock as well as cardiac, renal, and hepatic ischemia and reperfusion, show TLR4-mutant animals have reduced injury and

inflammation compared to control animals (Barnes, 1999; Oyama et al., 2004; Tsung et al., 2005; Wolfs et al., 2002; Zhai et al., 2004). TLR4 recognizes not only bacterial LPS but also endogenous Hsp70, HMGB1 and extracellular hyaluronan (Fang et al., 2011; Termeer et al., 2002; Yu et al., 2006). TLR2 binds hyaluronan, Hsp60 and HMGB1, while TLR9 binds to DNA CpG sequences (Hemmi and Akira, 2005; Round et al., 2011; Yu et al., 2006). Moreover, NOD receptors bind both injury and pathogen-related signals (Kumar et al., 2011).

OXIDATIVE STRESS

Oxidative stress refers to an imbalance between oxidants and antioxidant defense mechanisms resulting in an overabundance of oxygen or nitrogen derived free radicals (Droge, 2002; Halliwell, 2007; Pacher et al., 2007). Production of free radicals is promoted by various exogenous toxicants, including bacterial LPS or reactive ozone, either by damaging the mitochondria or by promoting inflammation and production of ROS or RNS. Furthermore, macrophages and neutrophils accumulating at sites of inflammation and tissue injury may contribute to oxidative stress by undergoing the respiratory burst, a critical host defense against invading microbes (Bedard and Krause, 2007). Whereas at moderate levels, free radicals, like nitric oxide or superoxide anion, play an important role in signaling and physiological processes, excessive generation of ROS or RNS, either as by-product of molecular oxygen metabolism or by specialized enzymes, have a potential to damage cellular components. This damage includes formation of isoprostanes and ethane from lipids, nitrotyrosine and bromotyrosine from proteins, or single-strand break in DNA and generation of 8-hydroxyguanosine (Cheng et al., 2003; Droge, 2002; Valko et al., 2007). Oxidative stress plays a pathological role in malignant diseases, diabetes, atherosclerosis, chronic inflammation, human immunodeficiency virus infection, ischemia-reperfusion injury, sleep apnea, and aging

(Droge, 2002; Thompson et al., 2010). The delicate balance between beneficial and deleterious effects of free radicals, the “redox homeostasis”, is achieved by “redox regulation” (Droge, 2002).

Free radicals are molecules or molecular fragments containing one or more unpaired electrons (Valko et al., 2007). Majority of free radicals are ROS, which include molecular oxygen-derived free radicals like superoxide anion, hydroxyl, peroxyl, and alkoxy radicals. A major source of ROS is mitochondria where 1-2% of consumed molecular oxygen is converted to ROS mainly though both complex I and III of the respiratory chain, and through Krebs cycle enzyme complexes, including α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Tretter and Adam-Vizi, 2004). Univalent reduction of molecular oxygen, mediated either enzymatically by NAD(P)H oxidase and xanthine oxidase, or non-enzymatically by redox-reactive compounds such as semi-ubiquinone compound of mitochondrial electron transport chain, forms superoxide anion radical, the most abundant ROS (Valko et al., 2007). Superoxide anion radical can further interact with other molecules, directly or indirectly though enzyme- or metal-catalyzed processes, to generate secondary ROS. Superoxide anion radical is dismutated by superoxide dismutase (SOD) into non-radical hydrogen peroxide (Zelko et al., 2002). Cellular hydrogen peroxide is also generated and stored in peroxisomes (Fritz et al., 2007). Hydrogen peroxide, if not scavenged by catalase or glutathione peroxidase, through the Fenton reaction catalyzed by reduced transitional metals, such as ferrous or cupreous ions, can break down into highly reactive hydroxyl radical (Thomas et al., 2009). Superoxide anion can further aid in production of hydroxyl radical by reducing transitional metal for the Fenton reaction though the Haber-Weiss reaction (Kehrer, 2000).

Hydroxyl radical reacts close to its site of formation due to the extremely short half-life (10^{-9} s) (Valko et al., 2007). The only effective protection from hydroxyl radical is

eliminating its precursor, hydrogen peroxide, and sequestering of transitional metals by various metal-binding proteins (Kakhlon and Cabantchik, 2002). The hydroxyl radical reacts with all components of DNA resulting in permanent modification of genetic material leading to mutagenesis, carcinogenesis, and ageing (Beckman and Ames, 1998). Hydroxyl radical can also initiate destruction of cellular membrane through lipid peroxidation by abstracting hydrogen from polyunsaturated fatty acids (Casarett, 2010). This result in production of lipid radical, which can further react with molecular oxygen and produce lipid peroxyl radical. Lipid peroxyl radicals, if not reduced by antioxidants, continue lipid peroxidation process with hydrogen abstraction and formation of lipid hydroperoxide, which by the Fenton reaction becomes lipid alkoxy radical. Lipid alkoxy radicals undergo cyclisation reactions to form six-membrane ring hydroperoxides, which by undergoing further reactions form reactive aldehydes including 4-hydroxy-nonenal (4-HNE) and malondialdehyde. These reactive aldehydes induce significant damage to cellular proteins, lipids and DNA (Valko et al., 2007)

RNS include nitrogen containing oxidants such as nitric oxide (MacMicking et al., 1997). Overproduction of RNS results in nitrosative stress, which may lead to nitrosylation reactions and alteration of structure and function of various proteins (Klatt and Lamas, 2000). Nitric oxide is formed in the cells by oxidation of L-arginine to citrulline catalyzed by nitric oxide synthase (NOS) (Alderton et al., 2001). Activated macrophages express inducible NOS (iNOS) regulated at the transcriptional and posttranscriptional level by signaling pathways involving mitogen-activated protein kinases (MAPKs) and transcription factor nuclear factor-kappa B (NF-κB) (Pautz et al., 2010). Nitric oxide can be converted into other RNS including nitrosonium cation, nitroxyl anion or peroxynitrite anion (Pacher et al., 2007). Nitric oxide plays a role in variety of physiological processes such as neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. Nitric oxide,

with the half-life of few seconds in aqueous environment and over 15 seconds in lower oxygen concentrations, is soluble in both aqueous and lipid media and readily diffuses through cytoplasm and plasma membranes (Pacher et al., 2007). Nitric oxide reacts with extracellular oxygen and water to form stable nitrite and nitrate anion. Many of physiological effects of nitric oxide are exerted by readily binding to transitional metal ions in enzymes including guanylate cyclase. However, evidence suggests that nitric oxide toxicity can also result from its ability to bind superoxide anion generating peroxynitrite anion, a more long-lived potent oxidizing agent capable of inducing DNA fragmentation and lipid oxidation (Pacher et al., 2007).

Protective responses against oxidative stress involve restoring "redox homeostasis" (Abbas et al., 2011). Concentrations of free radicals and reactive non-radicals present in biological cells and tissues are determined by the balance between the rate of their production and the rate of their inactivation, such as removal by various antioxidant compounds and enzymes. Antioxidants, efficient at low concentrations, include enzymes like SOD, catalase, glutathione peroxidase, and nonenzymatic compounds like glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E), and β-carotene (Sies and Stahl, 1995). ROS can also be scavenged, although at low efficiency and high enough concentrations, by free amino acids, peptides and proteins (Stadtman, 1993). One mechanism involved in maintenance of "redox homeostasis" is feedback inhibition. For example, nitric oxide inactivates NOS leading to feedback inhibition of iNOS (Aktan, 2004). After temporary exposure to increased levels of ROS, RNS, or decrease in the activity of antioxidant system, "redox homeostasis" can be restored by redox signaling. This involves increased expression of antioxidant enzymes or increased cystine transport system, which augments intracellular glutathione (Droge, 2002). Overwhelming production of ROS and RNS or damage to antioxidant defense

system can lead to permanent changes in signal transduction and gene expression, which may lead to tissue injury.

Oxidative stress is associated with pathogenesis of pulmonary diseases including asthma, acute respiratory distress syndrome (ARDS), COPD, and interstitial fibrosis (Rahman, 2005). In the lung, oxidative stress, induced by exposure to oxidant air pollutants such as ozone, results primarily in lipid peroxidation, which includes reaction of free radicals with polyunsaturated fatty acids in the cell membrane (Bowler and Crapo, 2002; Connor et al., 2012). One of the end products of lipid peroxidation is 4-HNE, known to cause further lipid peroxidation, depletion of intracellular glutathione, induction of peroxide products, and cell death (Rahman et al., 2002). Oxidative stress may also lead to modification and impairment of proteins. This includes peptide bond cleavage, protein-protein cross linking, or amino acid modification, especially cysteine and methionine residues found in various proteins including α-1-trypsin and SP-B (Manzanares et al., 2007). Oxidative stress was found to increase risk of lung cancer after exposure to either diesel particles or sulfur dioxide (Tokiwa et al., 1999, Xie, 2007 #387).

Oxidative stress is also involved in all inflammatory models of hepatotoxicity including endotoxemia, ischemia-reperfusion, hemorrhage-resuscitation, and acetaminophen or diquat-induced injury (Bautista and Spitzer, 1990; Dambach et al., 2006; Jaeschke et al., 1992; Knight et al., 2003; Laskin et al., 2010; Lehnert et al., 2003; Morio et al., 2001). Oxidative stress, generally occurring in the vasculature of the liver, is associated with M1 activated Kupffer cells (Dambach et al., 2006; Gardner et al., 2010; Laskin et al., 2010; Laskin et al., 2011). Although, some studies show the mechanism of cell death is lipid peroxidation, other studies point to oncotic necrosis involving opening of mitochondrial permeability transition pore, breakdown of membrane potential and depletion of ATP (Knight et al., 2003; Nieminen et al., 1997).

OZONE

Ozone is a highly reactive oxidizing gas composed of three oxygen atoms. Present in the stratosphere, ozone protects the earth surface from harmful ultraviolet radiation. In contrast, ozone in the troposphere is a toxic air pollutant and a component of a photochemical smog (Stanek et al., 2011). Los Angeles' smog in the 1940's was characterized by significant levels of ozone peaking at midday during high temperature (75-90°F) and low humidity (Jacobson, 2012). Photochemical smog forms from a mixture of oxidant compounds (including volatile hydrocarbons, halogenated organics and oxides of nitrogen, which are mainly generated from motor vehicles) reacting with ultraviolet irradiation from the sun (Mudway and Kelly, 2000). Photochemical reactions which generate ozone involve photodisassociation of nitrogen dioxide yielding atomic oxygen and nitric oxide. Atomic oxygen reacts with molecular oxygen to form ozone, which in absence of competing reactions, combines with nitric oxide to regenerate molecular oxygen and nitrogen dioxide (Kleffmann, 2007). Steady-state conditions do not allow ozone concentrations to increase unless most of the nitric oxide is converted to nitrogen dioxide by additional reactions. Oxidant compounds in photochemical smog, including hydroxyl and hydroperoxyl radicals formed from photodisassociation of nitrous acid and formaldehyde, undergo photodecomposition, which may contribute to conversion of nitric oxide to nitrogen dioxide. The concentration of the oxidant compounds in smog varies with the time of day. In the morning, photochemical reactions favor oxidizing nitric oxide to nitrogen dioxide allowing ozone concentrations to peak at midday. Later, the light intensity drops and termination reactions dominate as hydroxyl radical and nitrogen dioxide or nitric oxide form nitric acid, which slows down the generation of ozone. Ozone levels go up during sunny anticyclonic weather conditions where photolytic reactions increase in the photochemical smog and remain stagnant, for example during temperature inversion (Ma et al., 2011). Therefore,

elevated ozone concentrations depend on seasonal and geographical factors: stable air masses, magnitude of vehicle emissions or other pollutants, and weather conditions including intensity of sunlight (Mudway and Kelly, 2000). Generally, higher ozone concentrations occur in high emission sites of rural areas. During hot anticyclonic conditions ozone concentration can exceed safety guidelines of the annual daily maximum U.S. National Ambient Air Quality Standard for ozone of 0.075 ppm for an 8 h period (EPA, 2012).

Harmful concentrations of ozone affect an estimated 40% of the total United States population (Bell et al., 2004). Exposure to ozone has detrimental health effects including increased mortality and morbidity, increased rates of hospital admissions, and exacerbation of pulmonary diseases like asthma, COPD and pneumonia (Delfino et al., 1998; Peel et al., 2005; Trasande and Thurston, 2005). Subpopulations most vulnerable to the harmful effects of ozone are children and the elderly, as well as those with preexisting cardiopulmonary diseases, immune deficits, poor nutrition, and obesity (Medina-Ramon and Schwartz, 2008). Ozone is a pulmonary irritant, which causes constriction of the airways, decrease in lung function, sterile inflammation, alters immune function, increases bronchial reactivity, alters airway permeability, and may lead to airway remodeling (Connor et al., 2012; Fakhrzadeh et al., 2004; Kleeberger et al., 2000; Mudway and Kelly, 2004). Acute ozone inhalation results in necrosis of ciliated cells, degranulation of secretory cells in the conducting airways, and necrosis of type I epithelial cells and ciliated cells in the bronchioles. Maximum tissue injury appears in the lower lung, distal airways, bronchiole-alveolar duct junctions, and alveoli (Postlethwait et al., 2000). In 1950s, welders exposed to high levels of ozone experienced shortness of breath, pain on deep inspiration, and cough with measurable reduction in lung volume parameters (FEV_1 and FVC) (Challen et al., 1958). Chronic

exposure to ozone results in proliferation of type II pneumocytes, fibrosis, and bronchiolar metaplasia of alveolar ducts (Connor et al., 2012; Mudway and Kelly, 2000).

Highly reactive ozone does not transit epithelial lining fluid (ELF), therefore the cellular responses are mediated by secondary mediators, including free radicals and ozonation products (Pryor and Church, 1991). The alveolar and small airway ELF consists of thin aqueous layer composed of 10% protein and 90% lipid, which includes polyunsaturated fatty acids (PUFA). Ozone reacts with all hydrocarbons, especially PUFA. ROS are generated by lipid ozonation products (LOPs) which include aldehydes, hydroxyl hydroperoxides, and some Criegee ozonides (Mudway and Kelly, 2000). LOPs are relatively stable, small and diffusible. Exposure to LOPs activates phospholipase A_2, C, D, and inflammatory mediators including PAF, PGE_2, IL-6, and IL-8 (Kafoury et al., 1999). In vitro, oxidized phospholipids from ozone-exposed lung supernatants cause apoptosis in macrophages and necrosis in epithelial cells (Uhlson et al., 2002). Ozone toxicity includes reactions with ELF's proteins, directly or through generated ROS, causing oxidation of polypeptide backbone, peptide bond cleavage, protein-protein linking, and modification of amino acid side chains, especially cysteine and methionine residue (Kelly and Mudway, 2003). Ozone readily reacts with antioxidants found in ELF, which includes urate, α-tocopherol, ascorbic acid, and reduced glutathione. However, whereas treatment with antioxidants provides protection from ozone-induced injury in animals, this was not as affective against decreased lung function in humans (Mudway and Kelly, 2004; Pryor and Church, 1991).

Ozone-induced tissue injury is associated with an accumulation of macrophages in the lower lung (Connor et al., 2012; Fakhrzadeh et al., 2004). Ozone modifies alveolar macrophage function by impairing phagocytosis and superoxide production, but increases release of proinflammatory mediators and highly reactive ROS and RNS (Becker, 1991, Li, 2010, Laskin, 2011). Ozone exposure also exacerbates

acetaminophen-induced liver injury and primes hepatocytes to produce more nitric oxide (Laskin et al., 2011). Moreover, ozone interacts with adaptive immunity through modulation of innate immunity, increase in the number of antigen presenting cells in the lung, as well as expression of major histocompatibility complex II and co-stimulatory molecules (Koike et al., 2004).

HYPOXIA

Hypoxia is defined as a low oxygen tension. Whereas hypoxia itself can induce inflammation, as observed in individuals with mountain sickness who have increased levels of circulating proinflammatory cytokines and develop pulmonary or cerebral edema (Grocott et al., 2009; Semenza, 2007), it is also evident in tissues characterized by extensive inflammation (Ye et al., 2007). This may result from increased oxygen consumption by proliferating bacteria, vasoconstriction, damaged microvasculature, increased growth, or increased oxygen demand by infiltrating phagocytes (Murdoch et al., 2005). In vitro, hypoxia affects numerous macrophage functions including phagocytosis, cell surface marker expression, secretion of cytokines, chemokine receptor levels, adhesion, migration, and cell survival (Lewis et al., 1999). Previous studies have shown macrophages adapt to hypoxic environment by producing ATP mainly by anaerobic glycolysis (Kempner, 1939). Inhibition of glycolysis in phagocytes suppresses chemotaxis, aggregation, and invasion (Cramer et al., 2003). These observations led to the discovery that a basal regulator of energy metabolism in macrophages is hypoxia inducible transcription factor (HIF)-1α. HIF-1α has been found to be essential for hypoxia-induced increases in glycolysis and angiogenesis (Semenza, 2001). HIF is a heterodimeric transcription factor consisting of constitutively expressed HIF-1β subunit (ARNT) and HIF-α subunit (HIF-1α, HIF-2α, or HIF-3α), typically only

detected under low oxygen concentrations due to rapid degradation under normoxia (Mowat et al., 2010; Semenza, 2001). Under normoxia, prolyl residues on HIF-α subunits are hydroxylated by oxygen- and iron-dependent prolyl hydroxylases (PHDs). This creates a binding site for the von Hippel-Lindau factor (VHL), a component of the E3 ubiquitin ligase complex, which then degrades HIF-α subunits. Under hypoxia or iron deprivation, PHDs are inhibited and HIF-α subunit translocates into the nucleus and bind HIF-1β, which results in transcription of the target genes. These target genes include glycolytic enzymes, glucose transporters, erythropoietin and the angiogenic factor VEGF. Inactivation of HIF-1α in macrophages causes greatly reduced motility, invasiveness, and adhesion. In addition, whereas deletion of VHL results in hyperinflammatory response, loss of VEGF eliminates tissue edema (Cramer and Johnson, 2003).

HIF-1α can also be activated under conditions of normoxia. HIF-1α transcription is upregulated by LPS (Blouin et al., 2004), HIF-1α protein expression is stabilized by ROS (Cash et al., 2007), and HIF-1α protein accumulates when PHDs are inhibited by reduced cellular iron (Hartmann et al., 2008; Knowles et al., 2006). It appears that hypoxia and inflammation are linked though PHD-HIF pathway interaction with members of NF-κB signaling (Taylor, 2008). Moreover, HIF-1α transcription is regulated by NF-κB before and after inflammation (Rius et al., 2008).

LIPOPOLYSACCHARIDE

In 1879-80, Louis Pasteur is credited with the discovery of bacteria in blood from patients suffering from puerperal septicaemia, infection after childbirth caused by poor antiseptic techniques leading to sepsis (Annane et al., 2005). LPS is an endotoxin, first coined by Richard Pfeiffer in 1892, confined inside the microorganism and released only

when the microorganism is disrupted or dies (Beutler et al., 2003). LPS is generally released into the blood during bacterial cell division or lysis. LPS activates monocytes and macrophages to generate TNFα, IL-1, IL-6, IL-8, and IL-12, PAF, PGs, ROS and RNS (Cohen et al., 2002). LPS in the lung causes bronchoconstriction, recruitment and extravasation of neutrophils, injury of the alveolar epithelium, disruption of pulmonary capillary integrity, and hyperpermeability (Lefort et al., 2001). LPS-induced inflammation may lead to acute lung injury or acute respiratory distress syndrome (Rojas et al., 2005). LPS-induced inflammation is also associated with acute and chronic liver diseases, development of liver failure, and liver fibrosis (Szabo et al., 2007). LPS-induced local inflammation can lead to systemic inflammation, sepsis, severe sepsis, or septic shock. Sepsis is defined as infection characterized by systemic inflammation including increased or decreased temperature or leukocyte count, tachycardia, and rapid breathing (Abraham et al., 2000). Between 2000 and 2008, the rate of hospitalizations for sepsis doubled increasing with age, and ending with death for 17% of those hospitalized (NCHS, 2011). Sepsis is initially characterized by an increase in inflammatory mediators, but as the disease persists, it changes into anti-inflammatory immunosuppression, indicating that immune system is severely compromised and unable to eradicate pathogens (Hotchkiss and Karl, 2003).

LPS is found in the outer membrane lipid bilayer of Gram-negative bacteria (Raetz and Whitfield, 2002). LPS is composed of a hydrophilic polysaccharide moiety consisting of O antigen, an outer and inner core, and the toxic hydrophobic lipid A. Extending from the bacterial surface, O antigen consists of common sugars of various compositions and lengths attached to the terminal sugar of the outer core. The outer core consisting of three sugars with one or more covalently bound sugars as side chains. The outer core is attached to the inner core composed of two or more 2-keto-3-deoxyoctonic acid sugars linked to the glucosamine sugars of lipid A. Lipid A, two-

phosphorylated glucosamine sugars linked to six or more fatty acid residues, anchors LPS to the bacterial lipid bilayer.

TOLL-LIKE RECEPTOR 4

Bacterial LPS, other microbial PAMPs, as well as DAMPs, are detected by phagocytic leukocytes though germline-encoded PRRs (Kawai and Akira, 2010). One specific PRR for LPS is TLR4, a mammalian homolog of the *Drosophila melanogaster* protein Toll (Lemaitre et al., 1996). TLR4 is a type I transmembrane protein, which belongs to the IL-1 receptor (IL-1R) family containing an N-terminal leucine-rich repeat domain for binding, a transmembrane domain, and a C-terminal intracellular signaling domain homologous to the IL-1R, the Toll/IL-1R (TIR) domain. TLR4 was discovered as the defective gene in LPS-unresponsive C3H/HeJ and C57BL/10ScCr mice (Poltorak et al., 1998). The hyporesponsiveness to LPS in C3H/HeJ mice is due to a point mutation in TLR4 cytoplasmic proline residue 712 of TIR domain, which interferes with the recruitment of downstream effectors. LPS, released from damaged or dead bacteria, is tightly engaged by plasma LPS-binding protein (LBP) (Schumann et al., 1990). LBP transports LPS to the surface of the cell to membrane-bound CD14 (Wright et al., 1990). If soluble, CD14 can also convey LPS to the cell surface (Haziot et al., 1996). At the cell membrane, LPS is transferred to MD-2, which associates with TLR4. Activation of TLR4 initiates a cell signaling cascade. There are two TLR4 signaling pathways: MyD88-dependent (CD14, TLR4, MyD88, IRAK, TRAF, TAK1, IκB, NF-κB) and TRIF–dependent (CD14, TLR-4, TRIF, TRAF, TAK1, IκB, NF-κB or CD14, TLR-4, TRIF, TBK1, IRF3 and NF-κB). Negative regulators of TLR signaling include IRAK-M, SOCS1, and TOLLIP (Kawai and Akira, 2010).

Mutations in TLR4 signaling lead to increased susceptibility to infection (Vogel et al., 2005). Although rare in humans, three mutations have been identified, mutation within TLR4, IRAK-4, and NF-κB complex (IKKγ/NEMO and IκBα). Mutation within TLR4 is associated with diminished airway responsiveness to inhaled LPS, increased susceptibility to Gram-negative bacteria, septic shock, and severe respiratory syncytial virus (Arbour et al., 2000; Lorenz et al., 2002; Tal et al., 2004). Conversely, mutations within TLR4 are also associated with protection from atherogenesis and rheumatoid arthritis (Edfeldt et al., 2004; Kiechl et al., 2002; Radstake et al., 2004). Mutation within IRAK-4 is associated with repeated, life-threatening bacterial infections (Currie et al., 2004). Mutations in both NEMO and IκBα result in severe infections including ectodermal dysplasia (Puel et al., 2005).

Ozone-induced sterile inflammation can also modify complex immunological responses though TLR4 recognition of DAMPs, such as hyaluronan or other damage signaling molecules (Matzinger, 2012). The importance of TLR4 in ozone-injury was discovered by fine mapping of a quantitative trait locus for ozone-induced hyperpermeability in TLR4 mutant C3H/HeJ and control C3H/HeOuJ mice (Kleeberger et al., 2000). TLR4 mRNA and BAL protein concentrations were found significantly higher in control C3H/HeOuJ mice. Later studies showed ozone exposed TLR4deficient mice did not develop airway hyperresponsiveness suggesting an important role for TLR4 in ozone lung injury (Hollingsworth et al., 2004). TLRs are present on the surface of lung epithelial cells and innate immune cells including macrophages.

NUCLEAR TRANSCRIPTION FACTOR-KAPPA B (NF-κB)

Discovered in 1986 in B cells, NF-κB is a ubiquitous transcription factor known as a major regulator of innate and adaptive immunity and inflammatory responses (Lenardo

et al., 1989). NF-κB is activated by bacterial and viral infection, inflammatory mediators, antigen receptor ligands, oxidative stress, cell differentiation, proliferation and survival (Hayden and Ghosh, 2011). NF-κB is a homo- or heterodimer consisting of five proteins: NF-κB1 (p50 and precursor p105), NF-κB2 (p52 and precursor p100), RelA (p65), c-Rel and RelB. All NF-κB proteins contain an N-terminal motif with two immunoglobulin-like folds responsible for dimerization and sequence specific DNA binding called Rel homology domain (Vallabhapurapu and Karin, 2009). C-terminal domain of p65, c-Rel, and RelB, but not p52 or p50, contains transcriptional activation domain (TAD). Without TAD, relying on interactions with other factors to positively regulate transcription, p52, or its precursor p100, preferentially heterodimerizes with RelB, and p50 with p65 or c-Rel (Karin and Delhase, 2000; Senftleben et al., 2001).

NF-κB DNA binding and transcriptional activities are modified by posttranslational modifications of NF-κB proteins by phosphorylation and acetylation (Perkins, 2006). Under homeostatic conditions, NF-κB dimers are sequestered in the cytoplasm by binding to the inhibitory IκB proteins, which include IκBα (Ruland, 2011). IκBs are characterized by multiple ankyrin repeats, which mediate binding to NF-κB and interfere with the nuclear localization signals. IκB-like function is also present on the C-terminal regions of p100 and p105 (Dobrzanski et al., 1995). Activation of the NF-κB pathway involves the trimeric IκB kinase (IKK) composed of two catalytic subunits IKKα and IKKβ, and regulatory IKKγ (NEMO) (Karin and Delhase, 2000). Activated IKK phosphorylates IκB, which targets IκB for ubiquitination and proteasomal degradation, freeing NF-κB dimer to translocate into the nucleus. Activation of NF-κB includes the classical and the alternative pathways (Oeckinghaus et al., 2011; Vallabhapurapu and Karin, 2009). The classical pathway is induced by signals from cytokine receptors (TNFR and IL-1R), antigen receptors and PRRs (including TLR4). The classical

pathway depends on NEMO, IKKβ phosphorylation of IκBα, and translocation of most p65-containing heterodimers into the nucleus. As part of a negative feedback loop, after resynthesis, IκBα binds deacetylated p65:p50 dimers in the nucleus, and shuttles them out into cytoplasm terminating classical NF-κB activation (Vallabhapurapu and Karin, 2009). The alternative pathway is induced by specific members of TNF cytokine family, including CD40 ligand, LTα/β, and TNFα (Coope et al., 2002). The alternative pathway depends on NF-κB-induced kinase-dependent activation of IKKα, which phosphorylates and partially processes p100, this generates RelB:p52 dimer, which translocates into the nucleus (Novack et al., 2003). In the nucleus NF-κB binds to κB regulatory elements and activates target genes. Whereas the classical pathway regulates genes such as inflammatory cytokines, chemokines, adhesion molecules and inhibitors of apoptosis, the alternative pathway regulates lymphoid organogenesis, and B cell development and survival (Bonizzi et al., 2004).

SPECIFIC AIMS

Oxidative stress results from an imbalance between the generation of cytotoxic oxidants and protective antioxidants. The lung is a major barrier between the outside environment and the body. It is susceptible to variety of xenobiotics that induce oxidative stress including bacterial-derived LPS, pollutant gases such as ozone, and hypoxia. Macrophages are key components of host defense in the body. Previous studies have demonstrated that tissue injury associated with oxidative stress in the lung and the liver is due, in part, to cytotoxic inflammatory mediators released from activated macrophages (Laskin et al., 2011). Following bacterial infection or exposure to oxidants, macrophages produce a number of inflammatory mediators with cytotoxic potential including TNFα, superoxide anion, nitric oxide, and PGE_2. Each of these mediators has been implicated in tissue injury. A major receptor involved in macrophage production of inflammatory mediators is TLR4, a member of a family of pattern recognition receptors important in macrophage responsiveness to pathogens. We hypothesize that TLR4 signaling is also involved in macrophage responsiveness to oxidative stress and studies designed in this thesis were aimed at testing this using three different experimental models representing infection-driven and sterile lung inflammation. Our Specific Aims were to:

1. <u>Analyze the role of TLR4 in lung injury and inflammatory mediator production induced by exposure to inhaled ozone</u>. Ozone is a ubiquitous urban air pollutant known to damage the lower lung. This is associated with macrophage accumulation in the lung and the release of cytotoxic and proinflammatory mediators which contribute to oxidative stress and tissue injury. We speculated that TLR4 contributes to this sterile inflammation response. To test this hypothesis, we compared the cytotoxic and inflammatory effects of ozone in control C3H/HeOuJ mice and C3H/HeJ TLR4 mutant mice. Expression of markers of oxidative stress, lipid peroxidation, and lung

permeability in bronchoalveolar lavage (BAL) was assessed. We also measured TNFα transcription and NF-κB binding activity in lung macrophages, as well as BAL expression of SP-D, known regulator of macrophage inflammatory response.

2. Analyze the role of TLR4 in LPS-induced oxidative stress in lung and liver. LPS is a bacterially-derived product known to activate macrophages in the lung, as well as the liver. Exposure to excessive amounts of LPS results in increased production of ROS and RNS by macrophages leading to oxidative stress. We speculated that the response of macrophages to LPS was mediated by activation of TLR4. To test this hypothesis, we compared the effects of LPS on expression of antioxidants (MnSOD, CuZnSOD, HO-1) by lung and liver macrophages from control C3H/HeOuJ mice and C3H/HeJ TLR4 mutant mice, which possess a nonfunctional TLR4. Expression of enzymes mediating pro- and anti-inflammatory eicosanoid metabolism including COX-2, mPGES-1, and 12/15-lipoxygenase (LOX) were also assessed.

3. Analyze hypoxia-induced oxidative stress on macrophages and their responsiveness to LPS. The disruption of blood supply to injured or inflamed tissue results in decreased oxygen tension or hypoxia. Tissue injury caused by hypoxia is accompanied by increased production of ROS and oxidative stress. TLR4 signaling culminates in activation of transcription factors and expression of genes important in antioxidant defense and inflammation. We hypothesize that hypoxia alters transcription of these genes as well as macrophage activity. To test this hypothesis we used a mouse RAW 264.7 macrophage cell line to assess the effects of hypoxia on proinflammatory and growth factor gene expression including IL-1β, IL-6, TNFα, MIP-2, GLUT-1 and VEGF-A. Antioxidants and inflammatory enzymes including MnSOD, COX-2, iNOS, MMP-9 and lipocalin 24p3 were also evaluated. Specific inhibitors were used to assess the role of MAPKs pathways on expression on these proteins.

MATERIALS AND METHODS

Mice

Male TLR-4 mutant C3H/HeJ and control C3H/HeOuJ mice (11-12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolation cages and provided with autoclaved food and water *ad libitum*. Animal care was 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.

Animal Treatments

LPS

Acute endotoxemia was induced by i.p. injection of mice with 3 mg/kg *Escherichia coli* LPS (E. coli serotype 0128:B12 Sigma L4255, Sigma Chemical Co., St. Louis, MO) suspended in 0.2% triethylamine (TEA) (Sigma, T0886). Animals were sacrificed 3, 12, 24, and 48 h after LPS administration. LPS was re-purified using modified phenol extraction protocol to eliminate endotoxin lipoprotein. LPS (2.5 mg) dissolved in 500 μl of 0.2% TEA, 0.5% deoxycholate (DOC) (Sigma, D2510) and 500 μl of water-saturated phenol (Qbiogene, aquapho) was vortexed for 5 min and allowed to separate for 5 min at room temperature, 5 min on ice and centrifuged at 4 ºC for 2 min at 10,000 x g. The top aqueous layer was saved and bottom phenol layer re-extracted with 50 μl of 0.2% TEA/0.5% DOC. All aqueous layers were pooled and re-extracted with 1 ml of water-saturated phenol, adjusted to 75% ethanol with 30 mM sodium acetate, allowed to precipitate at -20 ºC for 1 h then centrifuged for 10 min at 4 ºC at 10,000 x g. Precipitated pellet was washed with 1 ml cold 100% ethanol and allowed to evaporate leaving LPS as a white powder.

Ozone

Mice were exposed to ozone or air for 3 h in a whole body Plexiglass exposure chamber. Ozone was generated from oxygen gas via ultraviolet (UV) light ozone generator (Orec Corp., Phoenix, AZ) and mixed with the inlet air of the exposure chamber. Ozone concentrations in the chamber were manually stabilized to 0.8 ppm by adjusting both the intensity of the UV light and the flow rate of ozone into the chamber. Concentrations were continuously monitored using an ozone monitor (Model 1008 AH, Dasibi, Glendale, CA). Lungs were collected 0.5 to 48 h after exposure.

Cell Isolation

Liver Macrophages

The modified methods described previously were used (Chen et al., 2007). Briefly, mice were sedated with Nembutol (200 mg/kg, i.p.). After midline incision of the peritoneal cavity, the hepatic portal vein was cannulated using Abbocath-T 24G x 19 mm (3/4") catheter for *in situ* perfusion of the liver. The liver was perfused with 10 ml of Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 0.5 mM EGTA and 4.4 mM $NaHCO_3$ (pH 7.3) at 37 ºC for 2 min at rate of 10 ml/min and inferior vena cava cut distal to the liver. The diaphragm was cut and aorta/vena cave clipped with hemostat to protect the lung from the next digestion step. The liver was perfused for 1 min with Leibovitz L-15 and 25 mM HEPES (pH 7.3) at 37 ºC containing 100 U/ml collagenase type IV under a heating source (60 Watt light bulb). Digested liver was removed and placed in a 100 mm petri dish. The liver was gently combed and filtered through 220-μm nylon mesh. Hepatocytes were separated from the non-parenchymal cells by centrifugation at 50 x g for 5 min. Non-parenchymal cells were washed with Leibovitz buffer with 0.04% DNase. Non-parenchymal cells recovered from the supernatants were centrifuged five times at 330 x g for 7 min. Non-parenchymal cells

were separated according to size and density in a Beckman J-6 elutriator (Beckman Instruments Inc., Fullerton, CA) equipped with a rotor speed set at 2500 rpm and a pump. Cells were mixed in Ca+2/Mg+2-free HBSS/2 g/L bovine serum albumin (BSA), 4 Mm sodium bicarbonate, 10 mg/L gentamycin (pH 7.3). The pump speed was set at 12 ml/min for loading cells, and 33 ml/min to collect macrophages, which were further purified using 1:2 gradient to sample ration with Nycodenz 265 mOsm (1.077 g/ml density), and centrifuged at 550 x g for 5 min. Cells were identified morphologically by Giemsa staining and electron microscopy and were >85% pure (Chen, 2007).

Lung Macrophages

Lung macrophages were isolated by bronchoalveolar lavage (BAL) as described previously (Connor et al., 2012). After liver perfusion, the diaphragm was removed and the ribcage cut in the midline and carefully forced open. The trachea was cannulated and the lung removed from the chest cavity. BAL was collected by slowly instilling and withdrawing 1 ml of HBSS. This was repeated until 10 ml of BAL was collected. After centrifugation (300 x g for 8 min), BAL proteins were quantified in the first ml of BAL fluid, which was stored at -80 ^{o}C until further analysis. The remaining collected BAL fluid was centrifuged (300 x g for 8 min). Cell pellets were washed 4 times with HBSS containing 2% fetal bovine serum (FBS) and then enumerated using a hemocytometer. Viability was 98% as determined by trypan blue dye exclusion, and cell purity >97% macrophages as assessed morphologically after Giemsa staining.

Cell Culture and Exposures

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 ºC in 5% CO_2 in Dulbecco modified Eagle medium (DMEM) containing high glucose (Invitrogen, Grand Island, NY),

supplemented with 10% FBS (Atlanta Biological, Norcross, GA), 0.1% penicillin-streptomycin, 2 mM L-glutamine and 0.1 mM Na-pyruvate. Viability was assessed by trypan blue dye exclusion. Cells were inoculated into 6-well plates (1 x 10^6 cells/well) in DMEM containing 1% FBS. After overnight incubation, the cells were treated with LPS (1 μg/ml) or PBS control and then placed in a MC-101 Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA). The chamber was purged with 1% O_2, 5% CO_2 balanced with nitrogen for 7 min at 20 L/min, sealed and incubated at 37 ºC. Control cells were cultured in standard 5% CO_2 incubator. Plates were removed 0.25-48 h later. Collected culture medium was stored in -80 ºC. For inhibitor studies, cells were pretreated with DMSO or 10 μM U0126, 5 μM SB203580, or 1 μM SP600125 for 3 h. Collected culture supernatants and cell lysates were stored in -80 °C for further analysis. SB203580 (Sigma, St. Louise, MO) is a highly specific and cell-permeable inhibitor of p38 MAPK (Barancik et al., 2001). U0126 (Cell Signaling, Danvers, MA) has been shown to be highly selective inhibitor of p44/42 MAPK (Duncia et al., 1998). SP600125 (Calbiochem, La Jolla, CA) is a selective, reversible ATP competitive c-Jun N-terminal kinase (JNK) inhibitor (Bennett et al., 2001).

Western Blot Analysis

Primary Cells

BAL fluid (400 μl) was concentrated by centrifugation at 14,000 x g for 33 min using a 10K centrifugal filter (Millipore, Billerica, MA). Equal volumes (12-15 μl) were then fractionated on sodium dodecyl sulfate (SDS) 10.5-14% Tris-HCl polyacrylamide Criterion™ Precast gels (Bio-Rad, Hercules, CA). After transferring to Trans-Blot pure nitrocellulose membranes (Bio-Rad, Hercules, CA), non-specific binding was blocked by incubation of the membrane with 5% FBS for 1 h at room temperature. Blots were

incubated overnight with 1:500 dilution of rabbit anti-lipocalin 24p3 or mouse monoclonal anti-4-hydroxynonenal (HNE, Abcam, Cambridge, MA), or 1:2000 dilution of rabbit anti-SP-D (Millipore, Billerica, MA) antibodies in 5% FBS. This was followed by incubation with a 1:20,000 dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA) in 5% FBS for 1 h at room temperature. Bands were visualized using a SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL). Densitometry was performed using Image Processing and Analysis in Java (ImageJ) gel analyzer software.

Cultured Cells

Culture supernatants were concentrated by centrifuging at 14,000 x g for 33 min using 10K filter units (Millipore, Billerica, MA) and protein concentrations were measured using the BCA assay (Pierce, Rockford, IL) with bovine serum as standards. However, cells were rinsed with cold PBS and suspended in lysis buffer (1% Triton, 20 mM Trizma base at pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 2.5 mM Na-pyrophosphate, 1:100 Protease and Phosphatase Inhibitor Cocktail from Sigma, St. Louise, MO). After 1 h incubation on ice, lysates were centrifuged at 14,000 g for 10 min at 4 °C and supernatants collected. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL) with bovine serum as standard. Equal amounts of protein (10-30 μg) were boiled at 95 ºC for 10 min and fractionated on SDS 10.5-14% Tris-HCl polyacrylamide Criterion™ Precast gels (Bio-Rad, Hercules, CA). After transferring to Trans-Blot pure nitrocellulose membranes (Bio-Rad, Hercules, CA), non-specific binding was blocked by incubating the membranes 1 h room temperature in 5% non-fat dry milk or 5% FBS. Blots were probed with primary antibodies in 5% milk or 5% FBS overnight. Binding was detected using horseradish peroxidase-conjugated

secondary antibody in 5% milk or 5% FBS for 1 h at room temperature. Bands were visualized using a SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL). Densitometry was performed using Image Processing and Analysis in Java (ImageJ) gel analyzer software.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BAL cells using Nuclear Extraction Reagent (NER) (Pierce, Rockford, IL) supplemented with 1:50 Protease Inhibitor Cocktail (Sigma, St. Louise, MO) following the manufacturer's instructions. EMSA was performed as described previously (Sunil, 2002) with some modifications. Binding reactions were carried out at room temperature for 30 min in a total volume of 15 μl containing 5 μg nucleic extracts, 5 μl 5X gel shift binding buffer (37.5% glycerol, 5 mM MgCl2, 0.25 mM DTT, 175 mM NaCl, 37.5 mM HEPES, pH 8.0), 0.1% BSA, 1 μg poly dI-dC and 2 μl γ[32P]ATP (3000 Ci/mmol at 10 mCi/ml)-labeled NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') consensus oligonucleotide (Santa Cruz Biotechnologies). Protein-DNA complexes were separated on 7% non-denaturing polyacrylamide gels at 150 V in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The gels were dried and autoradiographed. For competitor reactions, 50-fold excess unlabeled NF-κB oligonucleotide was added to the mixture 2 h prior to the labeled probe.

Immunohistochemistry

Tissues were fixed in 10% formalin buffer overnight at room temperature, followed by 50% ethanol. Sections (6 μm) were deparaffinized, then incubated overnight at 4 ºC with rabbit antibody to heme oxygenenase-1 (HO-1, 1:1000; Stressgen/Assay Designs, Ann Arbor, MI), proliferating cell nuclear antigen (PCNA,

1:250, Abcam, Cambridge, MA), pro-SP-C (1:2000, Millipore, Bellerica, MA), COX-2, (1:400, Abcam) or normal rabbit serum followed by a 30 min incubation with biotinylated secondary antibody (Vector Labs, Burlington, CA). Binding was visualized using a VECTASTAIN® Elite ABC kit (Vector Labs).

Relative RT-PCR

DNase I treated total RNA was extracted from BAL cells using RNeasy Mini kit (QIAGEN Inc, Valencia, CA) according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm. For cDNA synthesis, RNA (200 ng) in 9 μl of water was denatured at 65 °C for 4 min, rapidly cooled on ice and then resuspended in a 20 μl final volume containing 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 100 mM DTT, 10 mM dNTP, 200 μM random hexamers and 200 units Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). After 1 h incubation at 37 °C, 2 units RNase H^- was added and samples incubated at 37 °C for additional 20 min. The samples were denatured at 95 °C for 5 min and chilled on ice. Each sample reaction (20 μl) contained 1 μl cDNA template, 1.6 μl of 0.8 mM mouse TNFα primer pair and 1:9 ratio of 18S rRNA competimer/primer (Ambion, Austin, TX), 2 μl of 10X PCR buffer, 0.2 μl of 10 mM dNTP, 0.1 μl α[^{32}P] dCTP (10 mCi/ml; >3000 Ci/mmol), 0.1 μl of 0.5 units Taq DNA polymerase (Invitrogen), and 15 μl of distilled water. Using GeneAmp PCR System 9600 (Perkin, Elmer), amplification was initiated at 94 °C for 1 min, followed by 23 cycles at 94 °C for 15 sec, 58 °C for 25 sec and 72 °C for 90 sec. The amplified PCR products were then run on a 5% denaturing polyacrylamide gel. The gel was dried and radioactive bands from the PCR products excised and counted in a scintillation counter. Amplifications for all samples were performed at the same time and run on the same gel to minimize variability.

Real-time RT-PCR

Total RNA was isolated using a QIAshredder and RNeasy Miniprep kit (Qiagen, Valencia, CA). RNA concentrations were determined by absorbance at 260 nm. RNA (1-2 μg) was converted to cDNA using a High-Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Each reaction contained 2.5 μl target primer pair (300 nM final concentration) with 10 μl of 1:10 cDNA template and 12.5 μl Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), and was amplified on a 7900HT thermocycler initiated for 2 min at 50 °C, followed by 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Data were normalized to 18S rRNA. PCR primers were designed using Primer Express Software for Real-Time PCR version 3.0 (Applied Biosystems, Foster City, CA). Refer to Table 1 for forward and reverse primer sequences.

Gelatinase Activity

Culture medium was concentrated 25 fold using Amicon Ultra Centrifugal 10K Ultracel Filters (Millipore, Billerica, MA) by centrifugation at 14,000 g for 33 min at 4 °C. Concentrated protein (12 μg) was run on 10% Gelatine Zymogram Gels (Invitrogen, Carlsbad, CA) which were developed according to the manufacturer's protocol and stained with SimplyBlue^{TM} SafeStain (Invitrogen, Carlsbad, CA).

Statistics

All experiments were repeated at least three times. Data were analyzed by one-way ANOVA (Holm-Sidak method) using SigmaStat statistical analysis software. A p-value of ≤ 0.05 was considered significant.

Table 1

PCR Primers set for target genes. Gene sequences for mouse specific target genes for amplification were identified from NCBI Genbank database. PCR primers were designed using Primer Express Software for Real-Time PCR version 3.0 (Applied Biosystems, Foster City, CA).

PCR primer sets for target genes (Forward 5'-3' and Reverse 5'-3')

12/15-LOX	TCGGAGGCAGAATTCAAGGT and CAGCAGTGGCCCAAGGTATT
COX-2	CATTCTTTGCCCAGCACTTCAC and GACCAGGCACCAGACCAAAGAC
mPGES-1	GGCCTTTCTGCTCTGCAGC and GCCACCGCGTACATCTTGAT
CuZnSOD	ACCAGTGCAGGACCTCATTTTAA and TCTCCAACATGCCTCTCTTCATC
cyclin D1	AAAATGTTCTCCCTGCCCCT and CATCCTGAACGGATCCATGC
HPRT	GTTGGATACAGGCCAGACTTTGTTG and GAAGGGTAGGCTCTATA-GGCT
HO-1	CCTCACTGGCAGGAA-ATCATC and CCTCGTGGAGACGCTTTACATA
MnSOD	CACATTAA-CGCGCAGATCATG and CCAGAGCCTCGTGGTACTTCTC
p21	AAGGCCTGAAGACTCCACCA and TGTGACCAATGA-AGGGCAAG
18S	CGG CTA CCA CAT CCA AGG AA, GCT GGA ATT ACC GCG GCT
GLUT-1	CTG GGC AAG TCC TTT GAG ATG, CCG CAG TAC ACA CCG ATG AT
IL-1β	CCA AAA GAT GAA GGG CTG CT, TCA TCT GGA CAG CCC AGG TC
IL-6	TTC CAT CCA GTT GCC TTC TT, ATT TCC ACG ATT TCC CAG AG
iNOS	CCT GGT ACG GGC ATT GCT, GCT CAT GCG GCC TCC TTT
Lipocalin 24p3	AGG AAC GTT TCA CCC GCT TT, TGT TGT CGT CCT TGA GGC C
MIP-2	ACT GAC CTG GAA AGG AGG AGC, TGG TTC TTC CGT TGA GGG AC
MMP-9	AAA ACC TCC AAC CTC ACG GA, GCG GTA CAA GTA TGC CTC TGC
TNFα	AAA TTC GAG TGA CAA GCC GTA, CCC TTG AAG AGA ACC TGG GAG TAG
VEGF-A	CCC ACG TCA GAG AGC AAC ATC, TGG CTT TGG TGA GGT TTG ATC

PART I. ROLE OF TLR4 IN LUNG INJURY AND INFLAMMATORY MEDIATOR PRODUCTION INDUCED BY EXPOSURE TO INHALED OZONE

Ozone is a highly reactive gas present in a photochemical smog. Inhalation of toxic levels of ozone results in constriction of the airways, increased bronchial reactivity and decreased lung functioning (Mudway and Kelly, 2004; Savov et al., 2004). Ozone also disrupts alveolar epithelial integrity (Fakhrzadeh et al., 2004; Kleeberger et al., 2000). This leads to proliferation of type II pneumocytes which replace damaged epithelium and an accumulation of macrophages in the lung. In response to oxidative stress and products released from injured tissues, these cells are classically activated to release cytotoxic/ proinflammatory mediators such as tumor necrosis factor alpha (TNFα) and highly reactive oxygen and nitrogen species which contribute to the pathogenic response (Laskin et al., 2011). This is supported by findings that pulmonary damage induced by ozone is prevented or ameliorated by blocking macrophage activation or the production of inflammatory mediators (Cho et al., 2001; Fakhrzadeh et al., 2004; Haddad et al., 1995a; Pendino et al., 1995).

Toll-like receptor 4 (TLR4) belongs to a family of pattern recognition receptors, which are rapidly upregulated in macrophages in response to pathogens, proinflammatory cytokines and environmental stress (Chen et al., 2007; Kono and Rock, 2008; Li et al., 2011; Park et al., 2009). Engagement of TLR4 leads to recruitment of adaptor proteins and triggering of downstream signaling molecules culminating in activation of nuclear factor-kappa B (NF-κB) and upregulation of proinflammatory genes including TNFα and inducible nitric oxide synthase (iNOS) (Akira and Takeda, 2004). TLR4 has previously been shown to play a role in ozone-induced hyperpermeability and inflammation induced by ozone has previously been reported to involve activation of

TLR4 signaling (Kleeberger et al., 2001). This appeared to be due to upregulation of iNOS and altered HSP70 activity in lung macrophages (Bauer et al., 2011; Kleeberger et al., 2001). The present studies demonstrate that ozone-induced oxidative stress, lipid peroxidation, and macrophage accumulation and activation in the lung are also dependent on functional TLR4. These findings are important as they suggest a general role of TLR4 signaling in sterile inflammatory responses to tissue injury.

RESULTS

Initially we analyzed the role of TLR4 in lung injury and oxidative stress induced by acute exposure to ozone. Treatment of control C3H/HeOuJ mice with ozone resulted in a significant increase in BAL protein which peaked 12-24 h post exposure, demonstrating alveolar epithelial injury (Fig. 1, upper panel). Subsequently protein levels began to decline. This was correlated with significant increases in BAL levels of lipocalin 24p3, a marker of oxidative stress and the lipid peroxidation product, 4-HNE, as indicated by the appearance of a Mr = 50,000 modified protein (Rahman et al., 2002; Roudkenar et al., 2007) (Fig. 2). As observed with BAL protein, these were most prominent 12-24 h post exposure. Ozone-induced lung injury and oxidative stress were followed by an accumulation of inflammatory cells in the lung, as measured by increased BAL cell content (Fig. 1). Differential analysis revealed that the majority of these cells (>98%) were macrophages.

SP-D is a pulmonary collectin known to play a role in regulating macrophage inflammatory responses (McCormack and Whitsett, 2002). Following ozone exposure, increased levels of SP-D were also detected in BAL of C3H/HeOuJ mice. In contrast, C3H/HeJ mice were significantly less sensitive to ozone; no changes in BAL protein content or inflammatory cell accumulation were evident in these animals (Fig. 3). Moreover, changes in BAL SP-D, 24p3 and 4-HNE modified protein in these animals were diminished following ozone exposure.

We next analyzed role of TLR4 in ozone-induced activation of the transcription factor NF-κB, a marker of classically activated proinflammatory macrophages, shown to contribute to ozone toxicity (Cho et al., 2007; Fakhrzadeh et al., 2008). Consistent with previous studies in C57BL/6 mice (Fakhrzadeh et al., 2004), in C3H/HeOuJ mice, a time-related increase in NF-κB nuclear binding activity following ozone inhalation was

evident within 30 min and remained elevated for at least 24 h after exposure (Fig. 4). Competition experiments using 50-fold excess unlabeled NF-κB blocked NF-κB binding demonstrating the specificity of NF-κB DNA binding activity. Although ozone inhalation also induced NF-κB nuclear binding activity in macrophages from C3H/HeJ mice, this was reduced when compared to C3H/HeOuJ mice and maximal activity was delayed until 12 h post exposure.

TNFα is a proinflammatory cytokine under the transcriptional control of NF-κB. It is released by classically activated macrophages and is thought to play an important role in the pulmonary toxicity of ozone (Cho et al., 2007; Fakhrzadeh et al., 2008). Macrophage expression of TNFα is regulated by NF-κB. In C3H/HeOuJ mice ozone inhalation resulted in a significant increase in TNFα mRNA expression in alveolar macrophages, which was evident after 12 h (Fig. 5). In contrast, ozone effect on TNFα mRNA expression in macrophages from TLR4 mutant C3H/HeJ mice was blunted and delayed till 24 h.

In response to ozone-induced tissue injury, type II cells begin to proliferate in order to replace damaged epithelium. ProSP-C is a marker of proliferating type II epithelial cells (Weaver and Conkright, 2001). In C3H/HeOuJ mice, but not C3H/HeJ mice, ozone inhalation resulted in a time-dependent increase in pro-SP-C expression in type II epithelial cells beginning 24 h after exposure and increasing for at least 72 h (Fig. 6).

DISCUSSION

Recent studies have shown that pattern recognition receptors including TLR4, are upregulated on macrophages, not only in response to microbial products, but also following exposure to cell-derived danger-associated molecular patterns and other products released by injured cells and tissues (Lorne et al., 2010; Matzinger, 2002). Like inflammatory responses to microbial products, the resulting “sterile” inflammatory response, is characterized by an accumulation of neutrophils and macrophages in the tissue, and the generation of chemokines and pro-inflammatory cytokines, such as TNFα, as well as reactive oxygen and nitrogen species (Chen and Nunez, 2010). The present studies demonstrate that functional TLR4 plays a role in lung inflammation, injury and oxidative stress induced following acute exposure to toxic doses of ozone. These data provide additional support for the essential contribution of TLR signaling in sterile inflammatory diseases (Lin et al., 2011).

Ozone is a strong oxidizing agent that reacts with cell membranes damaging epithelial cells in the lower lung leading to increased vascular permeability (Broeckaert et al., 2003). Consistent with this observation, we found that exposure of control C3H/HeOuJ mice to ozone resulted in a significant increase in BAL protein (Kleeberger et al., 2000). Findings that this response was blunted in TLR4 mutant C3H/HeJ mice support the idea that TLR4 signaling is important in ozone-induced lung hyperpermeability (Kleeberger et al., 2000). In contrast, studies with TLR4 knockout mice have suggested that TLR4 is not involved in ozone-induced alveolar epithelial barrier dysfunction (Hollingsworth et al., 2004; Williams et al., 2007). These differences may be due to differential responses of distinct mouse strains to pulmonary irritants (Ewart et al., 2000; Savov et al., 2004; Takeda et al., 2001). We found that ozone-induced increases in BAL protein were correlated with elevated levels of lipocalin 24p3, a marker of oxidative stress (Roudkenar et al., 2007). Lipocalin 24p3 has been detected

in the lung after exposure of rodents to endotoxin or particulate matter, as well as in sputum from COPD patients, and in blood from Cynomolgus monkeys exposed to ozone (Andre et al., 2006; Hicks et al., 2010; Keatings et al., 1997; Sunil et al., 2009; Sunil et al., 2007). Our findings that lipocalin 24p3 is increased in the lung after ozone are novel and suggest that it may be a sensitive marker of acute lung injury induced by this oxidant. In TLR4 mutant mice, ozone exposure had minimal effects on BAL lipocalin 24p3 content indicating that TLR4 signaling is also important in oxidative stress induced by this pulmonary irritant.

Ozone-induced oxidative stress is associated with lipid peroxidation resulting in the generation of toxic aldehydes such as 4-HNE, which is known to induce cytotoxicity and apoptosis (Hamilton et al., 1998; Kirichenko et al., 1996; Li et al., 1996; Pryor and Church, 1991). 4-HNE can also react with amino acids such as Cys, Lys and His, resulting in the formation of protein adducts and altered protein function (Grimsrud et al., 2008). Following exposure of C3H/HeOuJ mice to ozone, 4-HNE-protein-adducts were identified in BAL. These findings are in accord with previous observations in humans and rodents exposed to ozone (Hamilton et al., 1996; Hamilton et al., 1998; Kirichenko et al., 1996). The fact that levels of BAL 4-HNE and protein peaked at the same time suggests a potential pathway leading to alveolar epithelial injury following ozone exposure. Only low levels of protein and 4-HNE protein-adducts were present in BAL from C3H/HeJ TLR4 mutant mice after ozone exposure, which indicates a role of TLR4 in ozone-induced lipid peroxidation. A similar contribution of TLR4 to lipid peroxidation has previously been described in the murine liver during the development of steatosis or ischemia, in the heart after ischemia-reperfusion injury, doxorubicin-induced cardiomyopathy, and in the brain after stroke (Caso et al., 2007; Spruss et al., 2009; Tsung et al., 2007).

Inhalation of ozone by C3H/HeOuJ mice also resulted in increased numbers of alveolar macrophages in BAL, which was evident 48 h after exposure. This was not observed in C3H/HeJ mice, providing additional support for TLR4 signaling in sterile lung inflammation induced by ozone. Previous studies have shown no effects of either acute or subchronic ozone on macrophage accumulation in BAL 24 h after exposure in mice with defective TLR4 signaling (Williams et al., 2007). Our findings that BAL macrophage numbers are only increased 48 h post exposure are consistent with these reports and indicate that the macrophage response to ozone is delayed in these mice.

Accumulating evidence suggests that macrophage functioning in the lung is regulated by the pulmonary collectin SP-D (McCormack and Whitsett, 2002). SP-D has been reported to bind to a soluble form of TLR4 and its adaptor proteins MD-2 (Nie et al., 2008; Ohya et al., 2006). This results in suppression of macrophage activation (Liu et al., 2010; Yamazoe et al., 2008). Following ozone exposure, we noted a significant increase in BAL SP-D levels. This may reflect an attempt by the host immune system to limit ozone-induced inflammation and tissue injury. This is supported by findings that loss of SP-D results in exacerbated inflammation in response to ozone (Kierstein et al., 2006). Loss of functional TLR4 was associated with decreased SP-D protein in the lungs of after ozone exposure. This is most likely a consequence of reduced lung injury and inflammation in C3H/HeJ mice.

Engagement of TLR4 results in sequential activation of cytoplasmic Toll/IL-1 receptor (TIR) domain, adaptor molecules, mitogen activated protein kinases and IκB kinase (Akira and Takeda, 2004). This culminates in activation of the transcription factor NF-κB and expression of proinflammatory genes such as TNFα (Lin et al., 2011). In control C3H/HeOuJ mice, but not C3H/HeJ TLR4 mutant mice, ozone inhalation resulted in a time-related increase in NF-κB nuclear binding activity which peaked after 24 h.

This was correlated with increased TNFα gene expression. Similar coordinate increases in NF-κB and TNFα have been described in inflammation sensitive C57BL/6J mice following ozone exposure (Fakhrzadeh et al., 2004). Findings that mice lacking NF-κB p50 are protected from ozone toxicity suggest that TLR4 dependent activation of NF-κB is an important mechanism leading to oxidant-induced inflammatory mediator production and toxicity. This is supported by reports that loss of functional TLR4 results in a blunted NF-κB response, reduced production of proinflammatory mediators, and decreased sensitivity of mice to hyperoxia (Ogawa et al., 2007).

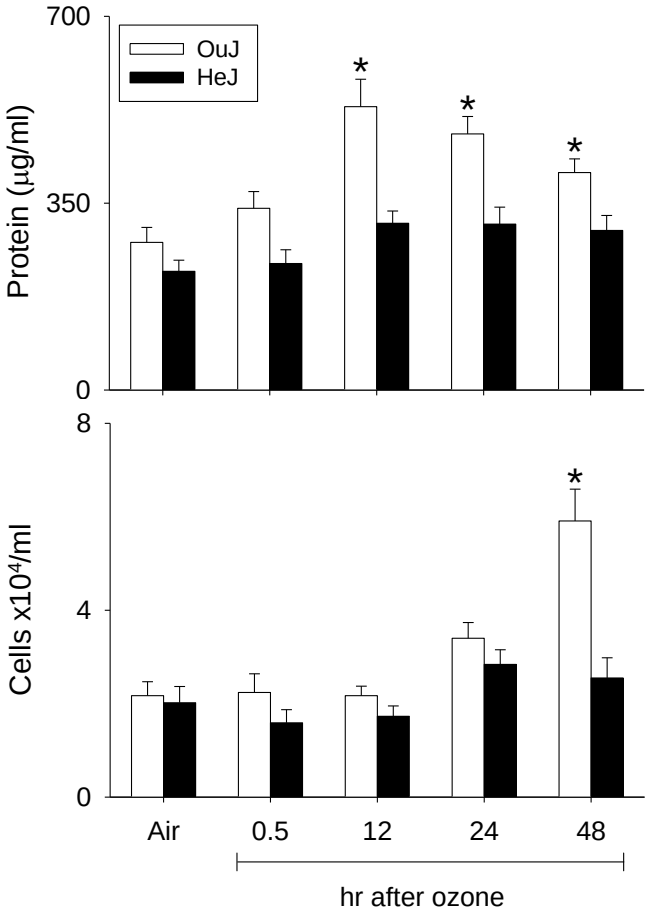
Previous studies have shown that excessive production of TNFα by alveolar macrophages after ozone inhalation contributes to tissue injury (Cho et al., 2007; Cho et al., 2001; Fakhrzadeh et al., 2004; Fakhrzadeh et al., 2008). Moreover, TNFα polymorphisms in humans are associated with exacerbation of ozone-induced alterations in lung functioning (Yang et al., 2005). The present studies demonstrate that ozone-induced increases in TNFα mRNA expression are significantly reduced in C3H/HeJ mice relative to C3H/HeOuJ mice. These findings are in line with reduced NF-κB activity in the TLR4 mutant mice. Similar decreases in TNFα have been described in the lungs of TLR4 mutant mice exposed to hyperoxia (Ogawa et al., 2007). These data indicate that TLR4 signaling is critical for production of TNFα by alveolar macrophages following irritant exposure.

In response to ozone-induced tissue injury, type II cells begin to proliferate to repair damaged epithelium (Fehrenbach, 2001). In accord with this response, we noted increased staining of Type IIs with proSP-C, a marker of proliferation, in lungs of control C3H/HeOuJ mice following ozone exposure. Our findings that proSP-C staining remained elevated for 72 h after ozone is histological evidence that injury was resolved and suggests this might be sensitive marker of tissue repair. In contrast to C3H/HeOuJ

mice, proSP-C staining was not evident in C3H/HeJ mice. These data are consistent with reduced injury and oxidative stress in these mice.

The role of TLR4 signaling in ozone-induced inflammation and lung injury is controversial (Bauer et al., 2011; Hollingsworth et al., 2004; Kleeberger et al., 2000; Williams et al., 2007). The present studies support the idea that TLR4 contributes to ozone-induced injury and inflammation. Data are also presented that TLR4 contributes to ozone-induced oxidative stress and macrophage infiltration into the lung. The reason for the disparate findings between the present studies and previous reports may be due to different mouse strains utilized and/or different ozone exposure protocols. It is also likely that multiple genetic polymorphisms contribute to oxidant induced lung injury, and this remains to be investigated.

Figure 1. Effects of ozone on markers of lung inflammation and injury. BAL was collected 0.5-48 h following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel:* Protein content was analyzed by the BCA assay with bovine serum as the standard. Each bar represents the mean ± SE (n=14-18). *Lower panel:* Viable cells were enumerated using trypan blue exclusion. Each bar represents the mean ± SE (n=7-11). *Significantly different ($p \leq 0.05$) from air control.


700
350
0
OuJ
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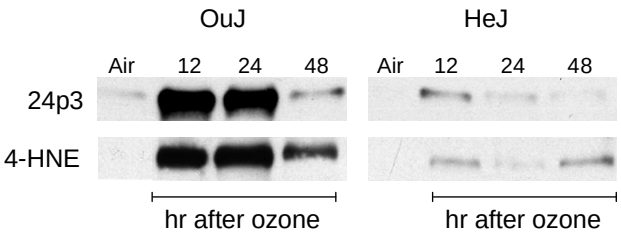
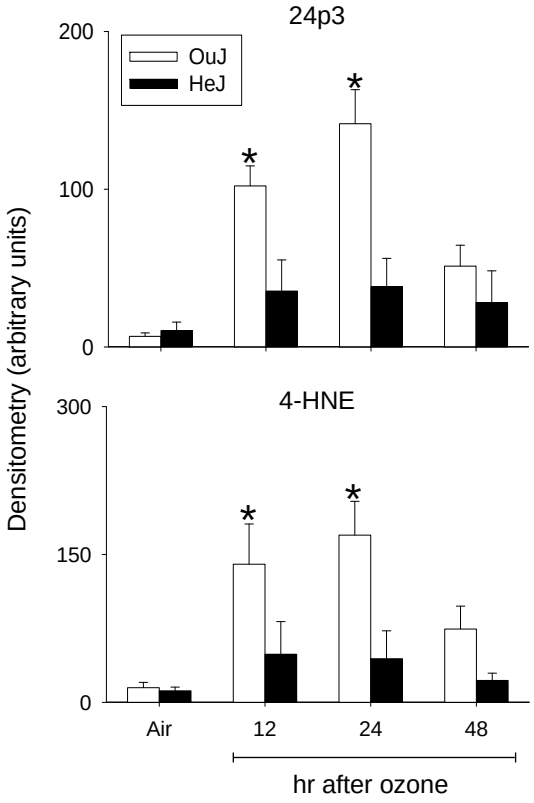
Protein (µg/ml)
*
*
*
8
4
0
Cells x10⁴/ml
*
Air
0.5
12
24
48
hr after ozone

Figure 2. Effects of ozone on markers of oxidative stress. BAL was collected 12-48 h after exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel*: BAL was analyzed for the presence of lipocalin 24p3 and 4-HNE-modified proteins by western blotting. Note that only a single 50 kDa molecular weight protein was detected in BAL following ozone inhalation. *Lower panel*: Gels were scanned and analyzed by densitometry using ImageJ. Each bar represents the mean ± SE in (n=3-5). *Significantly different ($p \leq 0.05$) from air control.


OuJ
HeJ
Air
12
24
48
Air
12
24
48
24p3
4-HNE
hr after ozone
hr after ozone


24p3
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100
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4-HNE
300
150
0
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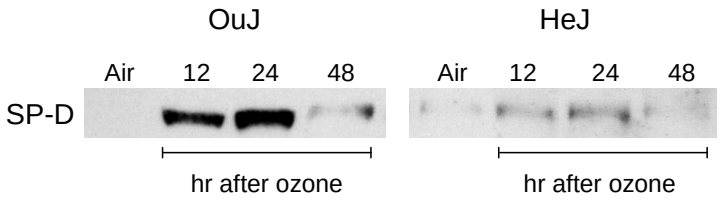
Densitometry (arbitrary units)
Air
12
24
48
hr after ozone

Figure 3. Effects of ozone on SP-D levels. BAL was collected 12-48 h after exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel*: BAL was analyzed for the presence of SP-D by western blotting. *Lower panel*: Gels were scanned and analyzed by densitometry using ImageJ. Each bar represents the mean ± SE in (n=3-5). *Significantly different ($p \leq 0.05$) from air control.


OuJ
HeJ
Air
12
24
48
Air
12
24
48
SP-D
hr after ozone
hr after ozone

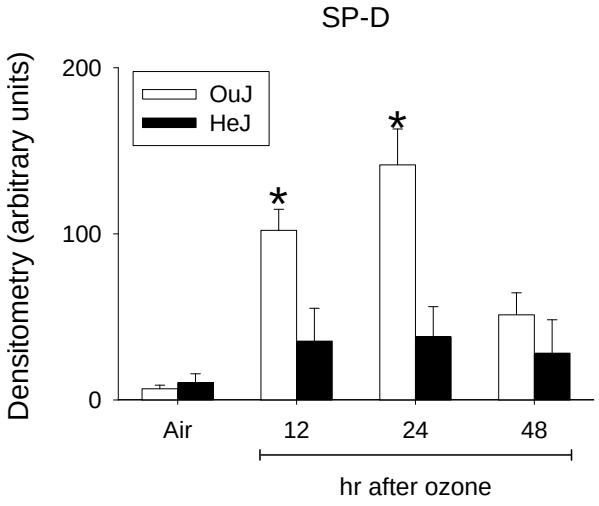

SP-D
Densitometry (arbitrary units)
200
100
0
OuJ
HeJ
*
*
Air
12
24
48
hr after ozone

Figure 4. Effects of ozone on NF-κB nuclear binding activity. Alveolar macrophages were isolated 0.5-24 h following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. NF-κB nuclear binding activity was quantified by EMSA. One representative of three independent experiments is shown. Cold competitor (CC) oligo was incubated with nucleic extracts 2 h before addition of ^{32}P-labeled NF-κB consensus oligonucleotide.

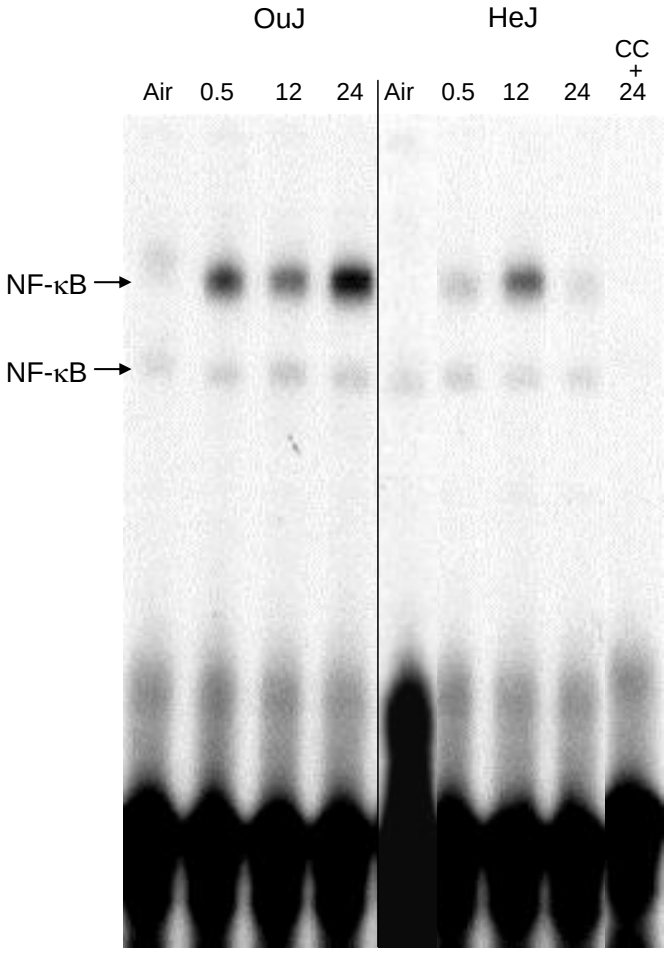
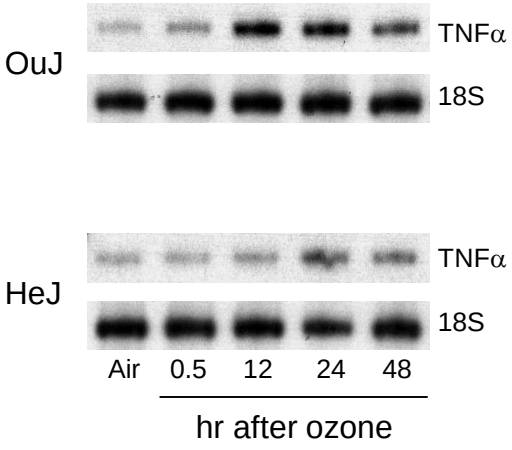

OuJ
HeJ
Air
0.5
12
24
Air
0.5
12
24
CC
+
24
NF-κB
NF-κB

Figure 5. Effects of ozone on TNFα mRNA expression. Alveolar macrophages were isolated 0.5-48 h following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. TNFα mRNA was quantified by relative quantitative RT-PCR. *Upper panel*: Representative PCR gel. *Lower panel*: TNFα was quantified by scintillation counting of excised bands. Each bar represents the mean ± SE (n=3). *Significantly different ($p \leq 0.05$) from air control.


OuJ
TNFα
18S
HeJ
TNFα
18S
Air
0.5
12
24
48
hr after ozone

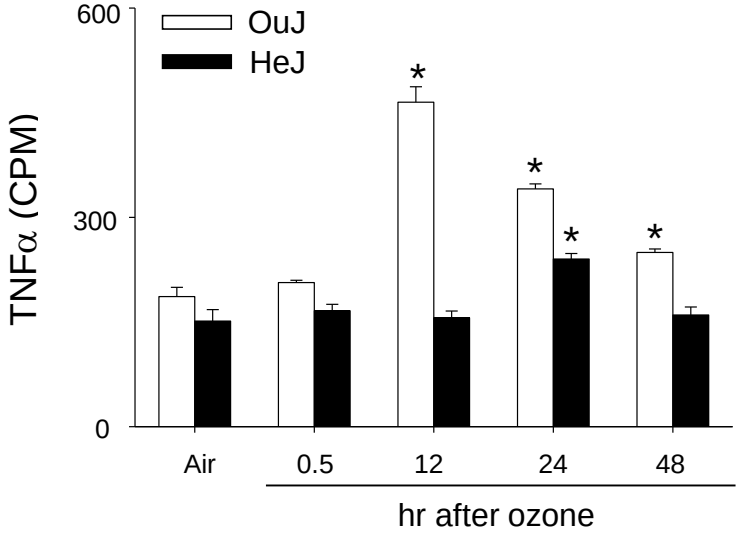
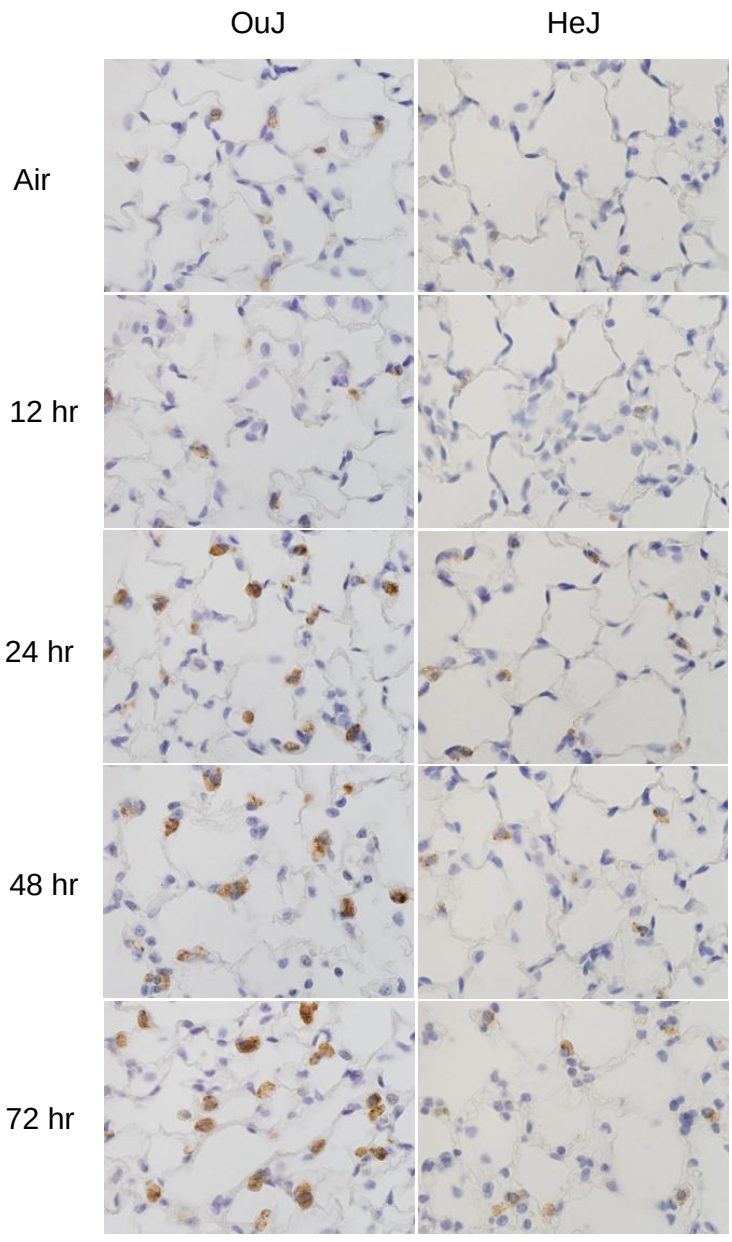

600
300
0
TNFα (CPM)
OuJ
HeJ
*
*
*
*
Air
0.5
12
24
48
hr after ozone

Figure 6. Effects of ozone on proSP-C expression. Lung sections were collected 12-72 h following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. Lung sections were stained with anti-proSP-C antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 1000x.


OuJ
HeJ
Air
12 hr
24 hr
48 hr
72 hr

PART II. ROLE OF TLR4 IN LPS-INDUCED OXIDATIVE STRESS IN LUNG AND LIVER MACROPHAGES

Lipopolysaccharide (LPS), typically found in the gastrointestinal tract, is a major component of the cell wall of Gram-negative bacteria. It is a glycolipid predominantly composed of oligo- and polysaccharides, and lipid A endotoxin (Raetz and Whitfield, 2002). LPS is primarily cleared from the body by Kupffer cells in the liver (Protzer et al., 2012; Vazquez-Torres et al., 2004). However, excessive levels of LPS can readily overwhelm this clearance mechanism resulting in acute endotoxemia. This is associated with systemic inflammation which can lead to septic shock, multiple organ failure and death (Annane et al., 2005). The lung is particularly sensitive to endotoxin and is usually the first organ to fail, followed closely by the liver (Ciesla et al., 2005). A characteristic feature of acute endotoxemia is an accumulation of macrophages in target tissues (Ahmad et al., 2002; Chen et al., 2007; McCloskey et al., 1992; Pilaro and Laskin, 1986; Wizemann and Laskin, 1994). These cells are activated by LPS to release reactive oxygen and nitrogen species, proinflammatory cytokines, proteases, and bioactive lipids, which are thought to contribute to tissue injury and the pathogenesis of organ failure (Laskin et al., 2011; Murray and Wynn, 2011).

A number of receptors have been identified on macrophages that are involved in LPS responsiveness. These include CD14 and toll-like receptor 4 (TLR4) (Kawai and Akira, 2010; Schumann et al., 1990). Following its release from dividing or damaged bacteria, LPS is sequestered by an LPS-binding protein in serum, which transports it to CD14 on the surface of macrophages (Akira and Takeda, 2004; Wright et al., 1990). Subsequently LPS is transferred to MD2, a soluble protein which associates with the extracellular domain of TLR4. Activation of TLR4 initiates a cell signaling cascade leading to translocation of NF-κB into the nucleus and transcription of proinflammatory

genes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor alpha (TNFα), proteins implicated in lung and liver injury (Kawai and Akira, 2010; Laskin et al., 2011). Previous studies have shown protection against LPS-induced inflammation and tissue injury in animals with TLR4 deficiency (Guo et al., 2008). Similar protection effects of loss of TLR4 have been described in sterile inflammatory responses to tissue injury induced by various target-organ specific toxicants (Connor et al., 2012; Lin et al., 2011; Matzinger, 2002). The present studies demonstrate that macrophage accumulation and responsiveness in the lung and the liver following acute endotoxemia depend on functional TLR4. These data provide additional evidence for an essential contribution of TLR signaling to inflammatory pathologies (Jiang et al., 2005; Matzinger and Kamala, 2011; Ostuni and Natoli, 2011).

RESULTS

Response of lung and liver macrophages to acute endotoxemia

Initially we analyzed the effects of acute endotoxemia on macrophage accumulation in the lung and the liver. Approximately 0.6 x 10^6 lung macrophages and 2 x 10^6 liver macrophages were recovered from control C3H/HeOuJ mice (Fig.7). Induction of acute endotoxemia resulted in a two-fold increase in the number of macrophages in both tissues, a response observed after 48 h. Evidence suggests that local proliferation of macrophages at sites of injury contributes to increased numbers of these cells in tissues (Davies et al., 2011; Isbel et al., 2001; Nakata et al., 1999; Wohl et al., 2010). To assess this, we measured markers of proliferation in lung and liver macrophages. Following LPS administration, expression of PCNA increased significantly in both lung and liver macrophages (Figs. 8 and 9). This response was noted within 12 h and was maintained for at least 48 h. We also observed a trend toward increased cyclin D1 mRNA expression in liver macrophages 3-24 h after LPS, with no effect on the lung macrophages (Fig. 10). Interestingly, the cell cycle inhibitory protein p21 also increased in liver, but not lung macrophages, within 3 h of LPS administration, remaining elevated for at least 24 h (Fig. 10).

To determine if acute endotoxemia was associated with oxidative stress in the macrophages, we analyzed expression of the antioxidants SOD and HO-1. In liver macrophages, but not lung macrophages, a rapid (within 3 h) increase in MnSOD mRNA expression was observed after LPS administration (Fig. 11). This persisted for 24 h, although at reduced levels. LPS had no effect on expression of CuZnSOD in either macrophage type. HO-1 mRNA also increased in liver macrophages after LPS treatment of the mice at 3-24 h. In contrast, LPS caused a transient decrease in constitutive expression of HO-1 protein in these cells at 3 h (Fig. 12). Whereas in

lung macrophages LPS administration had no effect on HO-1 mRNA expression, HO-1 protein increased after 3 h persisting for at least 24 h (Figs. 11 and 13). We also observed increased expression of HO-1 in pulmonary epithelium and interstitium, which was most prominent 24 h after LPS.

Macrophages activated by LPS are known to release proinflammatory eicosanoids implicated in endotoxin-induced tissue injury (Funk, 2001; Laskin et al., 2011). We next analyzed the effects of acute endotoxemia on lung and liver macrophage expression of enzymes mediating the generation of eicosanoids. Low constitutive levels of COX-2, mPGES-1, and 12/15-LOX mRNA were detected in lung and liver macrophages from control C3H/HeOuJ mice (Fig. 14). While induction of acute endotoxemia had no major effect on mRNA expression of these enzymes in lung macrophages, in liver macrophages a rapid (within 3 h) and persistent increase in COX-2 and mPGES-1 mRNA was observed, with no significant effect on 12/15-LOX. COX-2 protein expression also increased at 3 h following LPS administration in liver, as well as lung macrophages (Figs. 15 and 16). COX-2 protein expression also increased in alveolar epithelial cells (Fig. 16).

Effects of loss of functional TLR4 on macrophage responses to acute endotoxemia

In our next series of studies, we analyzed the role of TLR4 in lung and liver macrophage responsiveness to acute endotoxemia using C3H/HeJ mice, which possess a mutated nonfunctional TLR4 (Poltorak et al., 1998). In contrast to control C3H/HeOuJ mice, in TLR4 mutant C3H/HeJ mice, LPS administration had no effect on the number of macrophages recovered from the lung or the liver (Fig. 7). LPS-induced lung and liver macrophage proliferation, as measured by PCNA expression, was also reduced (Figs. 8 and 9), along with increases in cyclin D1 and p21 expression in liver macrophages (Fig. 10). Additionally, in TLR4 mutant C3H/HeJ

mice, the effects of LPS on liver macrophage expression of HO-1 mRNA and protein, and MnSOD mRNA, were diminished (Figs. 11 and 12), with no major effect on HO-1 protein in lung macrophages (Fig. 13). Loss of functional TLR4 also resulted in an attenuated response of liver macrophages to LPS-induced increases in expression of COX-2 mRNA and protein, and of mPGES-1 mRNA (Figs. 14 and 15). In contrast, COX-2 protein expression increased in macrophages as well as type II cells in TLR4 mutant C3H/HeJ mice after LPS administration, a response which was most prominent after 24 h (Fig. 16). In TLR4 mutant C3H/HeJ mice, 12/15-LOX mRNA also increased in lung macrophages 3-24 h after LPS administration, with no effects on mPGES-1 or COX-2 mRNA (Fig. 14).

DISCUSSION

Exposure to excessive levels of circulating endotoxin is associated with both acute and chronic inflammatory diseases (Miller et al., 2005; Qin et al., 2007; Rojas et al., 2005; Xu et al., 2010). Macrophages and cytotoxic/proinflammatory mediators they release play a key role in the development of these pathologies (Jiang et al., 2005; Laskin et al., 2011; Murray and Wynn, 2011). Of particular importance are macrophages located in the lung and liver which are highly responsive to endotoxin (Ahmad et al., 2002; Chen et al., 2007; Protzer et al., 2012; Sunil et al., 2002; Wizemann and Laskin, 1994). Evidence suggests that binding of LPS to TLR4 is a major triggering event in the signaling pathway leading to endotoxin-induced tissue injury (Akira and Takeda, 2004; Kawai and Akira, 2010). In the present studies we characterized the response of lung and liver macrophages to acute endotoxemia, with the goal of assessing the role of functional TLR4 in their response.

Acute endotoxemia is associated with an accumulation of macrophages in the lung and the liver, key target organs (Ahmad et al., 2002; Chen et al., 2007; McCloskey et al., 1992; Pilaro and Laskin, 1986; Sunil et al., 2002). Consistent with these reports, the present studies demonstrate increased numbers of macrophages in both the lung and the liver following LPS administration to mice, a condition which causes acute endotoxemia; this was most prominent 48 h after exposure. Inflammatory macrophages accumulating in tissues in response to injury or infection are thought to be derived mainly from blood and bone marrow precursors (Gordon and Taylor, 2005). Our observations that macrophages in lung and liver of LPS treated mice stained positively for PCNA, and that cyclin D1 expression increased in liver macrophages, suggest that local proliferation may also contribute to increases in the numbers of these cells in the tissues. These findings are in accord with previous studies showing local proliferation of macrophages following injury or infection in the

lung, liver, kidney, eye, and peritoneum (Davies et al., 2011; Isbel et al., 2001; Nakata et al., 1999; Vadiveloo, 1999; Wohl et al., 2010). We also observed LPS-induced increases in the cell-cycle inhibitor p21 in liver macrophages. Previous studies have shown that deficiency in p21 renders mice more susceptible to septic shock (Scatizzi et al., 2009; Trakala et al., 2009), potentially due to loss of p21-induced suppression of macrophage NF-κB (Coqueret, 2003; Lloberas and Celada, 2009; Vadiveloo, 1999). Increases in p21 in the liver may represent a compensatory response to limit excessive inflammatory responses and minimize tissue injury. The fact that this was not evident in lung macrophages suggests distinct regulation of macrophage proliferation and inflammatory activity in the lung and the liver following acute endotoxemia. This is supported by our observation of increased cyclin D1 in liver, but not lung macrophages, in LPS treated mice.

We have previously demonstrated that acute endotoxemia leads to increased production of reactive oxygen and nitrogen species by macrophages in the lung and the liver (Ahmad et al., 2002; Feder and Laskin, 1994; McCloskey et al., 1992; Wizemann and Laskin, 1994). These cytotoxic mediators cause oxidative stress which is thought to contribute to tissue injury and multiple organ failure (Ciesla et al., 2005). Markers of oxidative stress including F2-isoprostanes, isofurans, peroxiredoxin 4, and lipocalin 24p3 have been identified in blood from septic patients (Kumpers et al., 2010; Schulte et al., 2011; Ware et al., 2011). Expression levels of 24p3 are also increased in lung and liver macrophages following induction of acute endotoxemia in rodents (Sunil et al., 2007). In response to oxidative stress, cells upregulate antioxidants including SOD and HO-1 (Kinnula and Crapo, 2003). Consistent with an oxidative stress response, we observed a rapid induction of MnSOD and HO-1 mRNA in liver macrophages following LPS administration, and increased HO-1 protein expression in lung macrophages. In contrast, HO-1 protein,

which is constitutively expressed at relatively high levels in liver macrophages, decreased. Similar decreases in constitutive expression of antioxidants have been described in liver macrophages in response to other hepatotoxicants known to induce oxidative stress (Gardner et al., 2010). High levels of constitutive HO-1 in liver, but not lung macrophages, are likely due to continuous exposure of these cells to LPS in the portal circulation (Protzer et al., 2012). Previous studies have demonstrated that in addition to its antioxidant activity, HO-1 has anti-inflammatory functions suppressing macrophage NF-κB and promoting an alternatively activated wound repair phenotype in these cells (Paine et al., 2010; Weis et al., 2009). Upregulation of HO-1 in lung macrophages may reflect an attempt to reduce their proinflammatory activity.

Prostaglandins, synthesized from membrane derived arachidonic acid via COX-2 and mPGES-1, are known to be involved in inflammation and tissue injury (Funk, 2001). The present studies show that acute endotoxemia leads to a rapid upregulation of COX-2 and mPGES-1 mRNA in liver macrophages. Deficiency in mPGES-1 has been reported to result in a 95% decrease in the synthesis of prostaglandin E_2 (PGE_2) (Trebino et al., 2005). These data suggest that liver macrophages are major producers of PGE_2 during acute endotoxemia. This is supported by previous studies in endotoxemic rats demonstrating increased COX-2 expression and PGE_2 production by liver macrophages (Ahmad et al., 2002). In contrast to liver macrophages, LPS had no effect on mRNA expression of COX-2 or mPGES-1 in lung macrophages. However, COX-2 protein expression increased, suggesting distinct mechanisms regulating expression of COX-2 and PGE_2 production in lung and liver macrophages in response to endotoxin. LPS-induced COX-2 protein expression was also noted in alveolar epithelial cells, providing support for the idea that these cells play a role in pulmonary inflammatory responses

to tissue injury (Fehrenbach, 2001; Punjabi et al., 1994; Sunil et al., 2002). Eicosanoids generated via 12/15-LOX are thought to promote the resolution of inflammation (Levy et al., 2001; Serhan et al., 2008). Our findings that expression of 12/15-LOX mRNA was not altered in either lung or liver macrophages after LPS administration to the mice are in accord with prolonged inflammation in these tissues during acute endotoxemia (Chen et al., 2007; Rojas et al., 2005).

TLR4 mutant C3H/HeJ mice have been reported to be hyporesponsive to LPS (Chen et al., 2007; Poltorak et al., 1998). In agreement with these findings, the present studies demonstrate that loss of functional TLR4 leads to reduced responsiveness of lung and liver macrophages to acute endotoxemia. Thus, in TLR4 mutant C3H/HeJ mice, LPS had no effect on macrophage accumulation in the lung or the liver. LPS-induced increases in macrophage proliferation, as measured by PCNA and cyclin D1 expression, were also decreased. Reduced numbers of inflammatory cells have also been described in lungs of TLR4 deficient mice after exposure to bleomycin, Mycobacterium tuberculosis, or ozone (Abel et al., 2002; Connor et al., 2012; Hollingsworth et al., 2005). Whether this is due to decreased recruitment of inflammatory cells from blood and bone marrow precursors or reduced proliferation of these cells remains to be determined.

In liver macrophages, LPS-induced upregulation of MnSOD mRNA and HO-1 mRNA and protein was also attenuated in TLR4 mutant mice relative to control mice. Our findings that in lung macrophages, LPS-induced HO-1 protein expression was unaltered by loss of functional TLR4 suggest that TLR4 is not a major regulator of antioxidant responses in these cells which is in accord with previous reports (Mandal et al., 2010). Interestingly, HO-1 protein expression in pulmonary epithelium and interstitium was significantly reduced in TLR4 mutant mice, relative to control

C3H/HeOuJ mice. These data indicate that antioxidant defense is regulated by distinct pathways in different lung cell populations.

We also found that LPS-induced effects on COX-2 mRNA and COX-2 protein expression, and mPGES-1 mRNA were significantly reduced in liver macrophages in TLR4 mutant mice, relative to control mice. Conversely, loss of functional TLR4 resulted in an increase in COX-2 protein in lung macrophages, as well as type II cells; however this was delayed for 12-24 h. We also found that lung macrophages from TLR4 mutant mice express increased levels of 12/15-LOX mRNA following LPS administration. This may contribute to reduced inflammation and oxidative stress in the lung in these mice. In this regard, recent studies have demonstrated that 12/15-LOX plays a key role in macrophage clearance of apoptotic cells from tissues and in the maintenance of immunological tolerance, which may contribute to its anti-inflammatory activity (Uderhardt et al., 2012).

Macrophages exhibit a diverse spectrum of activation states in response to pathogens and microenvironmental signals they encounter in the lung and the liver (Laskin et al., 2011; Mosser and Edwards, 2008). The present studies demonstrate that while TLR4 signaling plays a role in promoting inflammatory responses of macrophages to LPS, this activity is tissue specific, which may be due, at least in part, to different expression levels of TLR4 on these cells. This is supported by findings that TLR4 expression is reduced on lung macrophages relative to liver macrophages, which may account for their attenuated response to LPS (Fan et al., 2002; Maris et al., 2006). Understanding pathways regulating the proinflammatory responses of lung and liver macrophages to LPS maybe important in the design of novel therapies to treat multiple organ failure.

Figure 7. Effects of loss of TLR4 on the number of macrophages in the lung and liver following LPS administration in mice. Macrophages were isolated from the lung and liver 12-48 h after treatment of C3H/HeOuJ or TLR4 mutant C3H/HeJ mice with control or LPS. Viable cells were counted on a hemocytometer using trypan blue exclusion. *Upper panel:* Each bar represents the mean ± SE (n=3-21 mice). *Lower panel:* Each bar represents the mean ± SE (n=11-72 mice). *Significantly different ($p \leq 0.05$) from control.

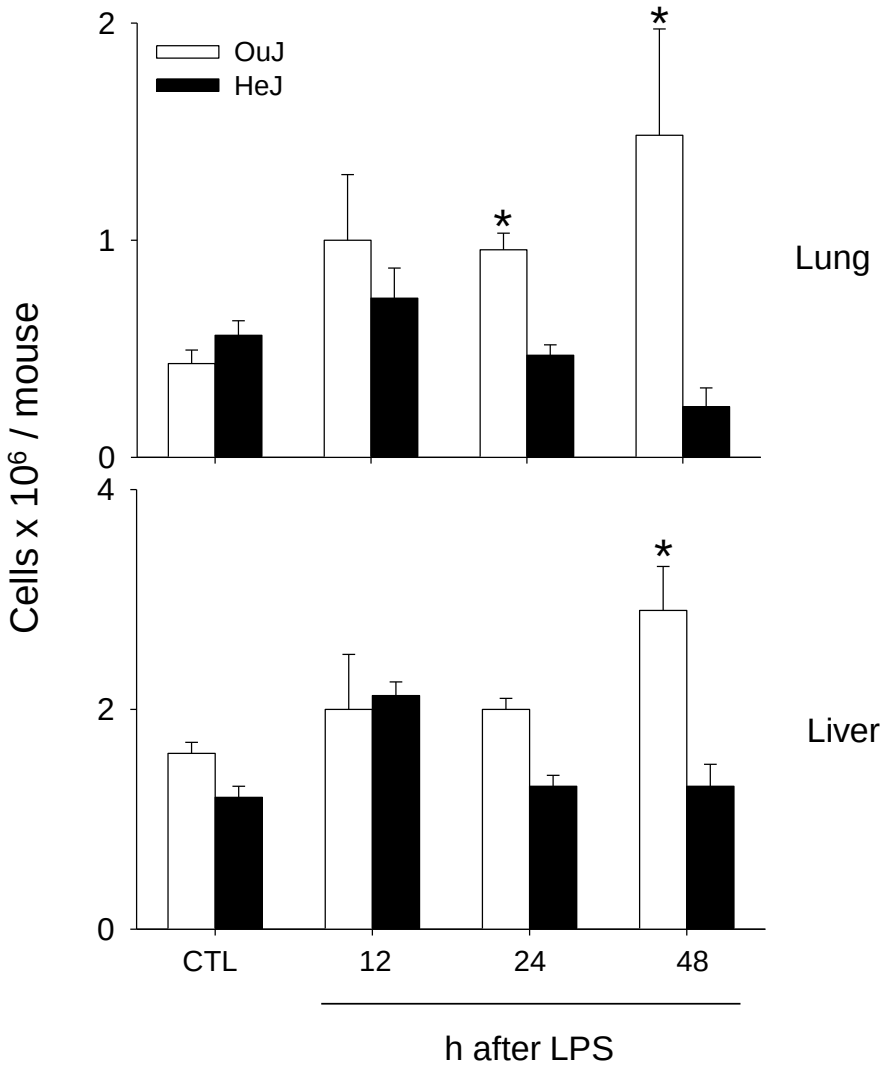

OuJ
HeJ
Cells x 10⁶ / mouse
Lung
Liver
CTL
12
24
48
h after LPS
*

Figure 8. Effects of loss of TLR4 on LPS-induced PCNA expression in the lung. Sections, prepared 12-48 h following treatment of C3H/HeOuJ and C3H/HeJ mice with control or LPS, were stained with anti-PCNA antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.

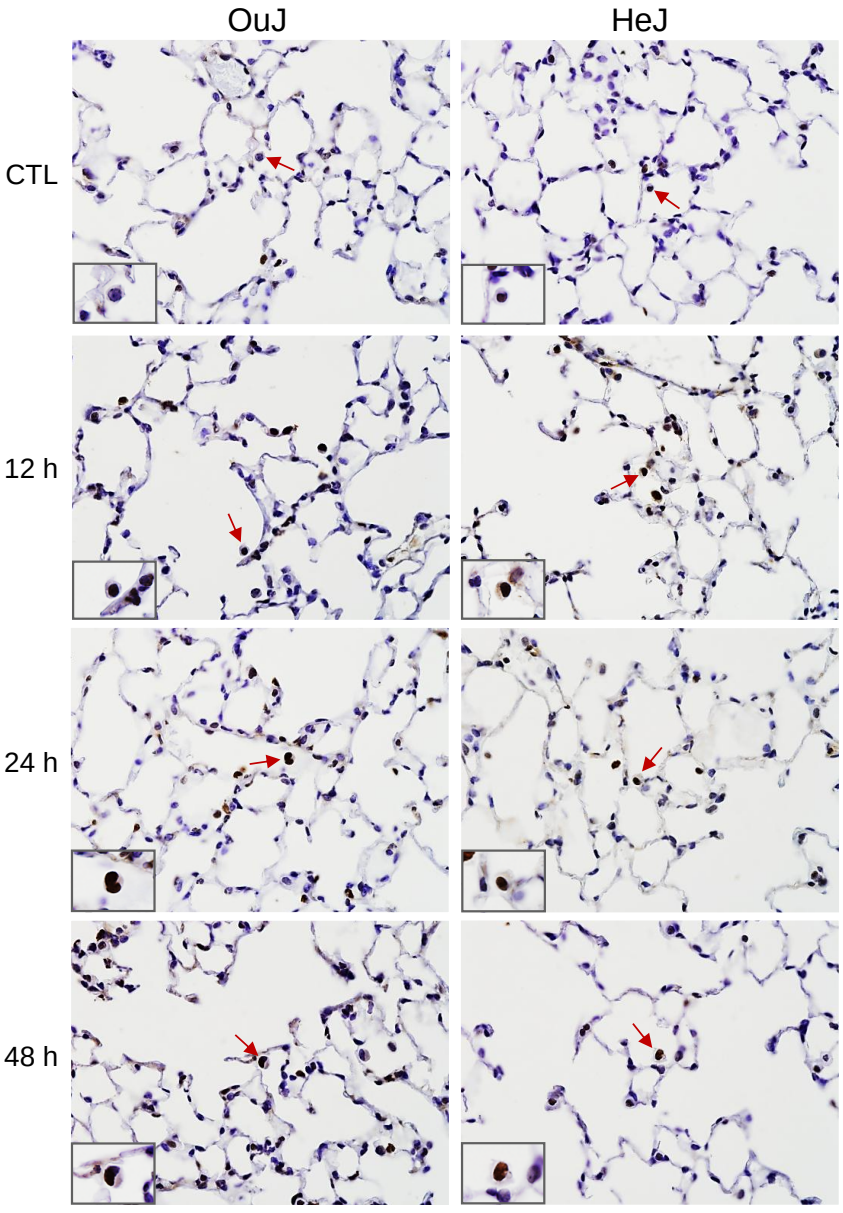

OuJ
HeJ
CTL
12 h
24 h
48 h

Figure 9. Effects of loss of TLR4 on LPS-induced PCNA expression in the liver. Sections were prepared 12-48 h following treatment of C3H/HeOuJ and C3H/HeJ mice with control or LPS. Sections were stained with anti-PCNA antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.

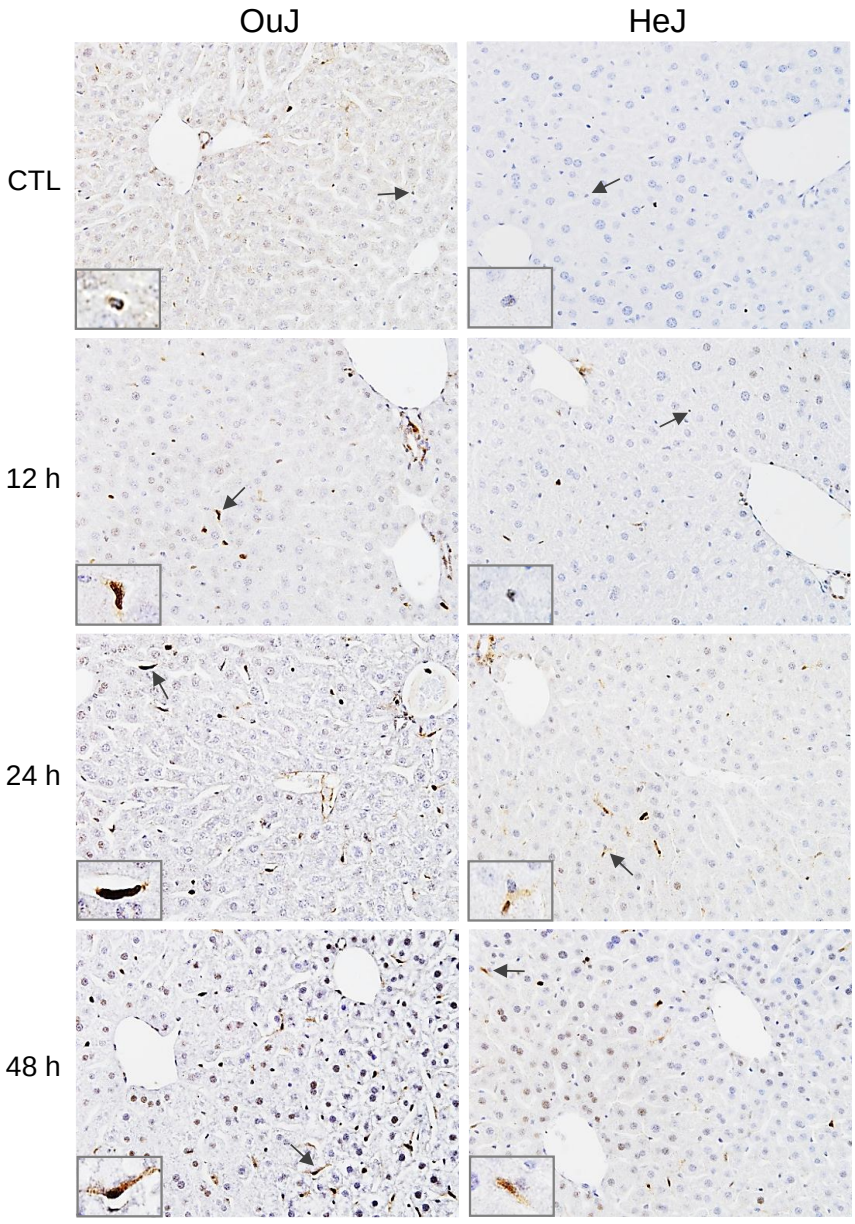

OuJ
HeJ
CTL
12 h
24 h
48 h

Figure 10. Effects of loss of TLR4 on LPS-induced expression of proliferation markers. Lung and liver macrophages were isolated 3-24 h after treatment of C3H/HeOuJ and C3H/HeJ mice with control or LPS. mRNA expression was quantified by real-time RT-PCR. Data were normalized to HPRT and expressed as fold change relative to controls. Each bar represents the mean ± SE in (n=3-8 mice). *Significantly different from control ($p \leq 0.05$).

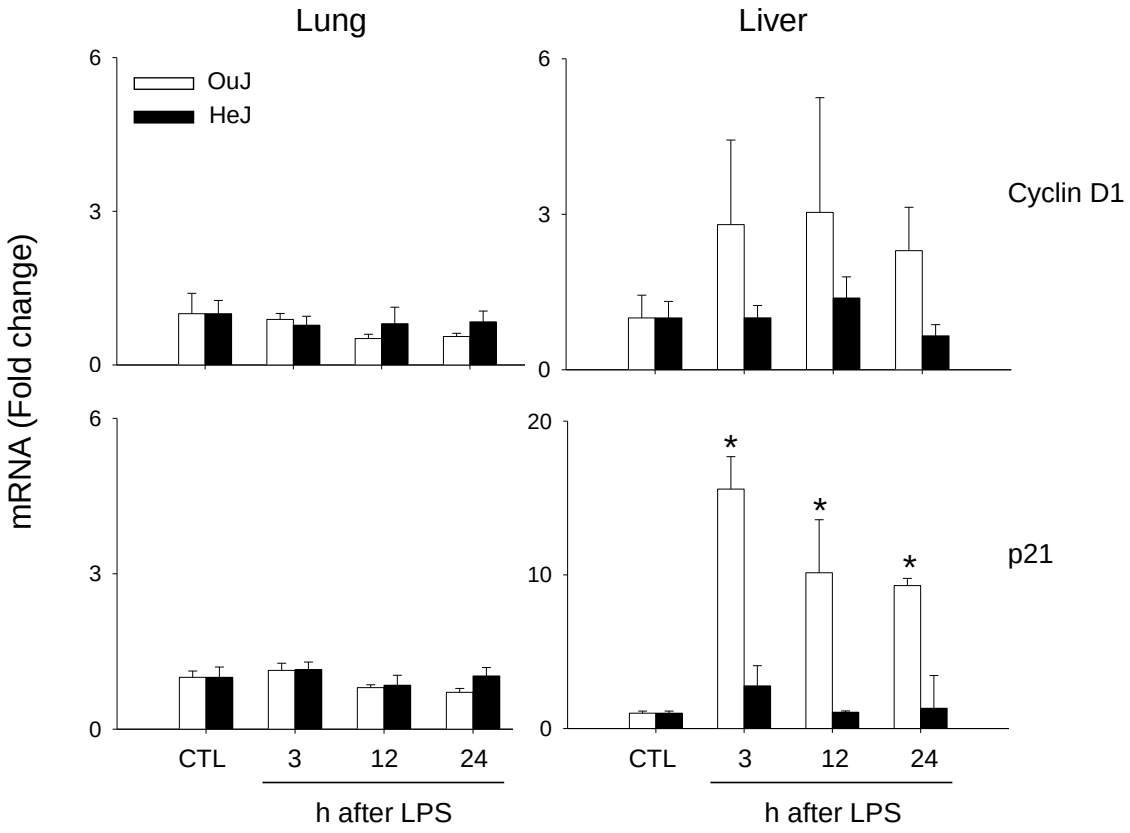

Lung
Liver
OuJ
HeJ
mRNA (Fold change)
Cyclin D1
p21
6
3
0
20
10
*
CTL
3
12
24
h after LPS

Figure 11. Effects of loss of TLR4 on LPS-induced expression of antioxidant enzymes. Lung and liver macrophages were isolated 3-24 h after treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS. mRNA expression was quantified by real-time RT-PCR. Data were normalized to HPRT and expressed as fold change relative to controls. Each bar represents the mean ± SE in (n=3-8 mice).
*Significantly different from control ($p \leq 0.05$).

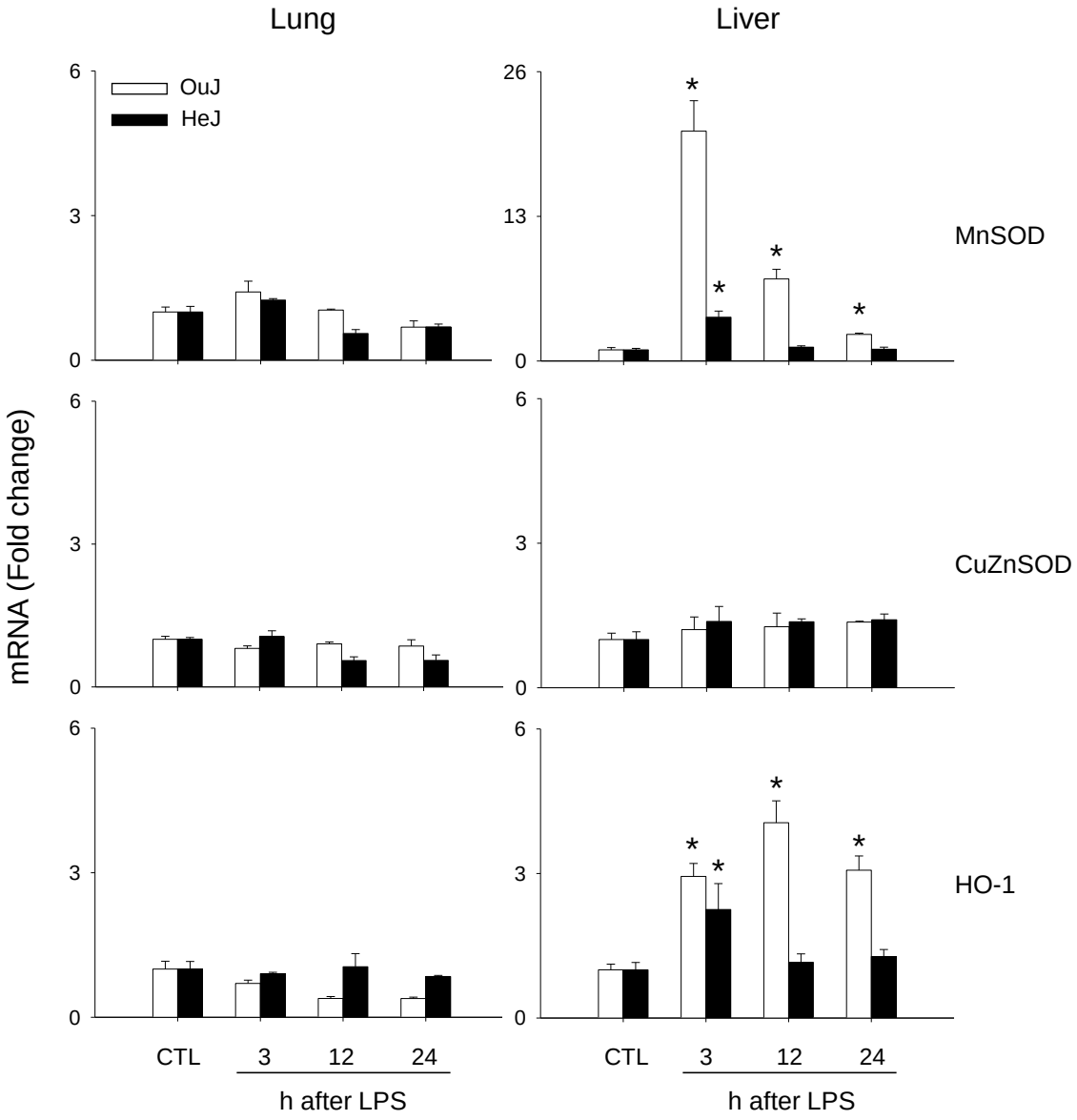

Lung
Liver
OuJ
HeJ
mRNA (Fold change)
MnSOD
CuZnSOD
HO-1
CTL
3
12
24
h after LPS

Figure 12. Effects of loss of TLR4 on LPS-induced HO-1 expression in the liver. Sections, prepared 3-24 h following treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS, were stained with anti-HO-1 antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.

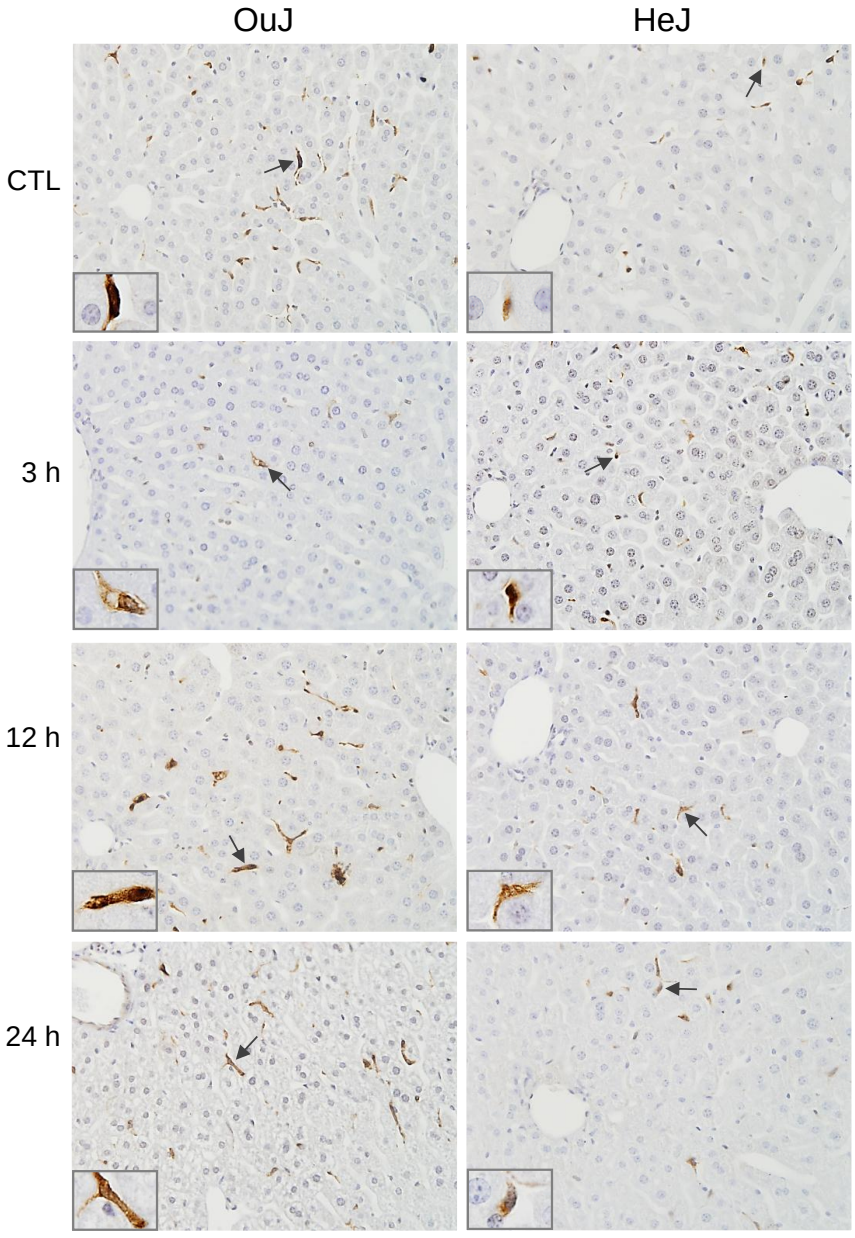

OuJ
HeJ
CTL
3 h
12 h
24 h

Figure 13. Effects of loss of TLR4 on LPS-induced HO-1 expression in the lung. Sections, prepared 3-24 h following treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS, were stained with anti-HO-1 antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.

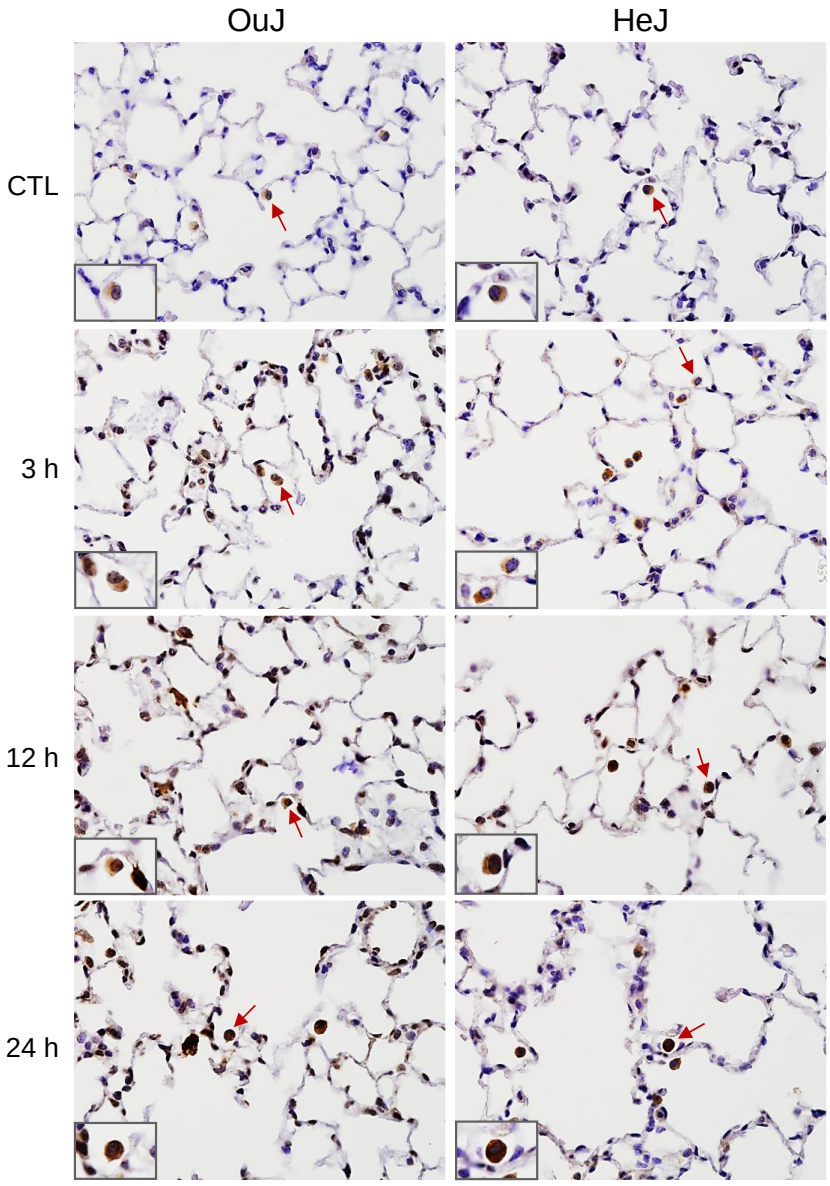

OuJ
HeJ
CTL
3 h
12 h
24 h

Figure 14. Effects of loss of TLR4 on LPS-induced expression of enzymes mediating eicosanoid metabolism. Lung and liver macrophages were isolated 3-24 h after treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS. mRNA expression was quantified by real-time RT-PCR. Data were normalized to HPRT and expressed as fold change relative to controls. Each bar represents the mean ± SE in (n=3-8 mice). *Significantly different from control ($p \leq 0.05$).

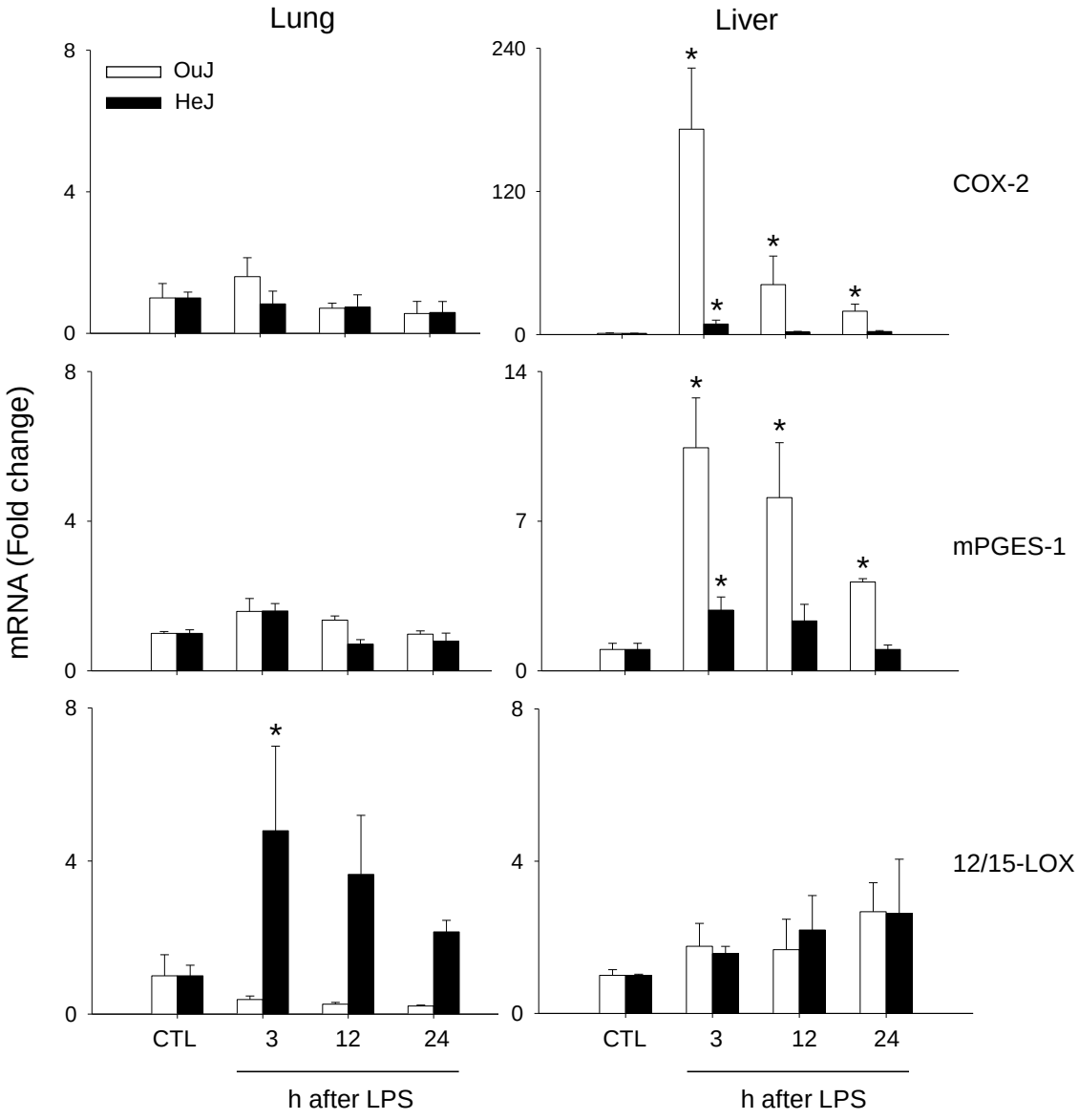

Lung
Liver
OuJ
HeJ
mRNA (Fold change)
COX-2
mPGES-1
12/15-LOX
CTL
3
12
24
h after LPS
h after LPS

Figure 15. Effects of loss of TLR4 on LPS-induced expression of COX-2 protein in the liver. Sections, prepared 3-24 h following treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS, were stained with anti-COX-2 antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.

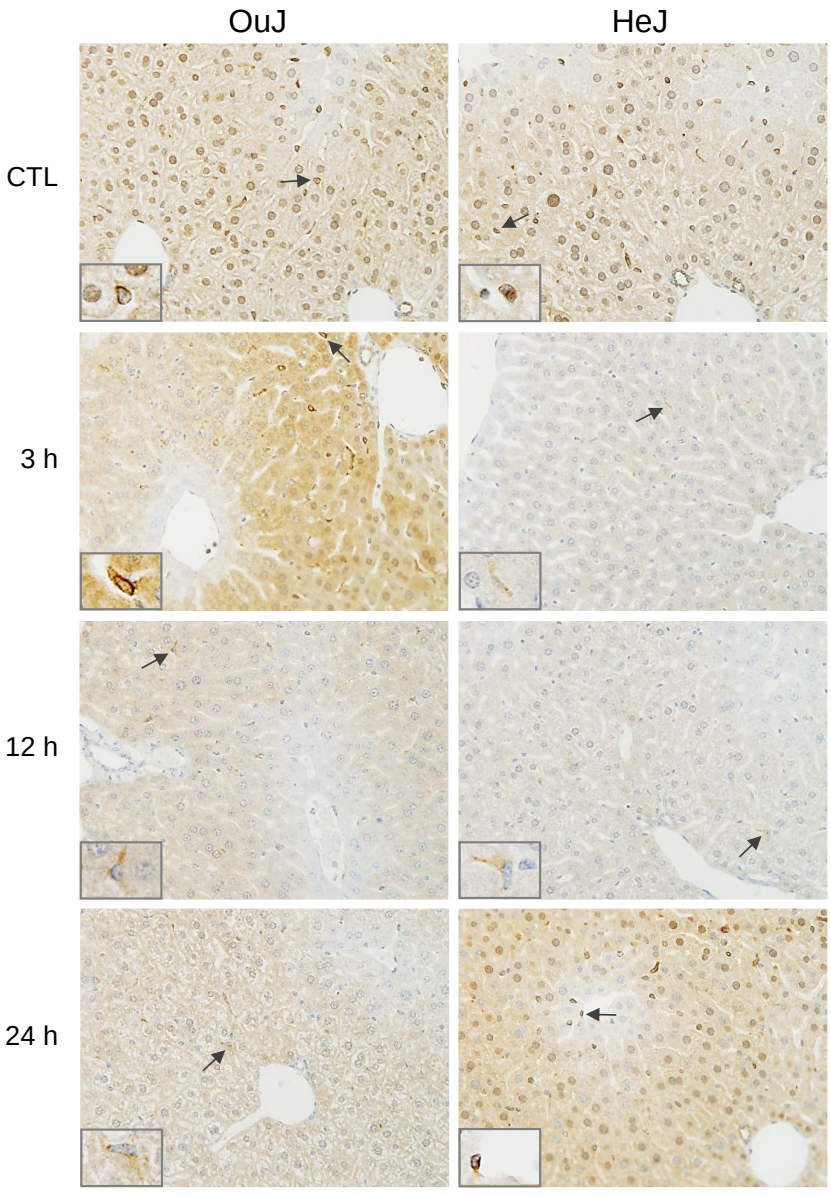
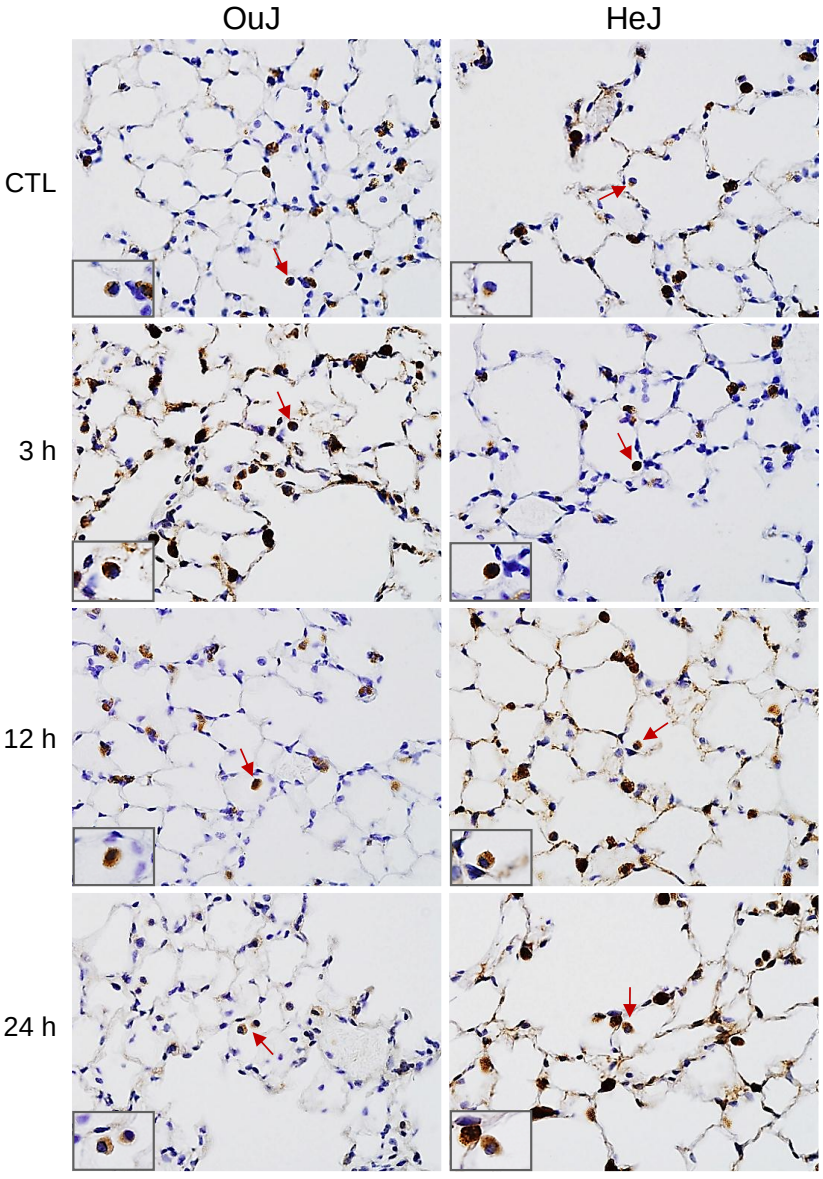

OuJ
HeJ
CTL
3 h
12 h
24 h

Figure 16. Effects of loss of TLR4 on LPS-induced expression of COX-2 protein in the lung. Sections, prepared 3-24 h following treatment of C3H/HeOuJ or C3H/HeJ mice with control or LPS, were stained with anti-COX-2 antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification 600x, inserts, 1000x. One representative section from three experiments is shown.


OuJ
HeJ
CTL
3 h
12 h
24 h

PART III EFFECT OF HYPOXIA-INDUCED OXIDATIVE STRESS ON MACROPHAGES AND THEIR RESPONSIVENESS TO LPS

Hypoxia represents a condition of reduced oxygen tension, which aggravates tissue damage and inflammation, and stimulates angiogenesis and fibrogenesis (Rosmorduc and Housset, 2010). Hypoxia is a consequence of inadequate blood supply to an injured tissue combined with increased oxygen consumption by localized inflammatory macrophages, and it has been shown to alter macrophage survival, mobility, expression of cell surface markers, phagocytosis, metabolic activity, and production of inflammatory mediators (Chao et al., 2009; Lewis and Pollard, 2006; Nizet and Johnson, 2009; Sawyer et al., 1991; Semenza, 2001). As a consequence, hypoxia contributes to the development of a number of pathologies associated with inflammatory macrophages including atherosclerosis, myocardial infarction, chronic obstructive pulmonary disease, pneumonia, hepatitis, and multiple organ failure (Chao et al., 2009; Dean and Wilcox, 1993; Fuhrmann et al., 2010; Lewis and Pollard, 2006; Murdoch et al., 2005; Nizet and Johnson, 2009; Swenson et al., 2002).

In response to bacterially-derived products such as lipopolysaccharide (LPS), macrophages are activated to release proinflammatory and cytotoxic mediators including reactive oxygen and nitrogen species, proteases, cytokines and bioactive lipids (Bode et al., 2012). Uncontrolled or dysregulated release of these mediators by macrophages can contribute to tissue injury (Laskin et al., 2011). Macrophage production of inflammatory mediators depends on activation of transcription factors such as NF-κB, AP-1, HIF-1 and Nrf2 which have been shown to be sensitive to hypoxia (Nizet and Johnson, 2009; Taylor, 2008). These transcription factors are controlled by mitogen activated protein (MAP) kinases including p42/44, p38 and JNK, which are activated following LPS binding to macrophages (Bode et al., 2012). In the present studies, we

evaluated the effects of hypoxia on LPS-induced inflammatory responses in mouse macrophages, and the role of MAP kinases in this activity. Using RAW 264.7 macrophages, hypoxia was found to cause marked increases in of several key inflammatory mediators. Moreover, this was associated with increased expression of enzymes important in the synthesis of nitric oxide and prostaglandins, a response dependent upon p44/42 MAP kinase signaling. Our results provide additional support for the idea that the responses of activated macrophages to bacterial-derived products is controlled, at least in part, by local tissue oxygen tension (Eltzschig and Carmeliet, 2011; Murdoch et al., 2005; Sitkovsky and Lukashev, 2005). Currently, few effective drugs target activated macrophages, thus a more complete understanding of the mechanism of bacterial-derived products on these cells is key to identifying potential therapeutic targets.

RESULTS

Effects of hypoxia on LPS-induced macrophage activation

Initially we analyzed the effects of hypoxia on LPS-induced expression of the pro-inflammatory cytokines, IL-1β, IL-6, TNFα, and MIP-2, the angiogenic protein, VEGF-A, and the glucose transporter, GLUT-1. LPS treatment of macrophages resulted in increased mRNA expression of each of these proteins (Table 2). Exposure of the cells to hypoxia enhanced LPS-induced expression of IL-1β, VEGF-A, and GLUT-1, with no significant effects on IL-6, TNFα, or MIP-2. Hypoxia alone also upregulated macrophage mRNA expression of MIP-2, VEGF-A and GLUT-1.

We next analyzed the effects of hypoxia on LPS-induced expression of iNOS and COX-2, enzymes mediating the generation of reactive nitrogen species and eicosanoids, respectively (Eliopoulos et al., 2002; Kim et al., 2004). Treatment of macrophages with LPS resulted in time related increases in expression of iNOS and COX-2 mRNA and protein (Figs. 17 and 18A). Whereas iNOS mRNA and protein expression remained elevated for at least 48 h, increases in COX-2 mRNA and protein expression were transient, peaking at 6 h. Hypoxia markedly enhanced the effects of LPS on iNOS and COX-2 mRNA and protein. While this was observed at 16-48 h for iNOS, it was only evident at 48 h for COX-2. Treatment of macrophages with hypoxia alone also resulted in increased iNOS and COX-2 protein expression at 16-48 h and 1-6 h, respectively, with no effect on mRNA expression.

We also evaluated the effects of hypoxia on markers of oxidative stress in macrophages including MnSOD and lipocalin 24p3. Low levels of MnSOD and lipocalin 24p3 mRNA and protein were detected in control macrophages (Figs. 17 and 18A-C). LPS treatment of the cells resulted in a rapid induction of MnSOD mRNA and protein which was observed within 6 h. Lipocalin 24p3 mRNA expression also increased in

response to LPS, but this was delayed for 24-48 h. Lipocalin 24p3 protein was also detected in the culture medium from LPS treated macrophages at these times. Whereas hypoxia upregulated LPS-induced MnSOD and lipocalin 24p3 protein expression at 24 h and 48 h, mRNA levels decreased. Exposure of macrophages to hypoxia alone had no significant effect on expression of MnSOD or lipocalin 24p3 mRNA and protein expression; however, lipocalin 24p3 protein increased in the culture medium at 3-48 h (Fig. 18B).

Migration of macrophages though tissues to inflammatory sites requires degradation of extracellular matrix mediated by matrix metalloproteinases such as MMP-9 (gelatinase-B) (Visse and Nagase, 2003). In further studies, we assessed the effects of hypoxia on LPS-induced MMP-9 expression and activity. Whereas LPS treatment of macrophages resulted in increased expression of MMP-9 mRNA at 16-48 h, there were no effects on protein expression (Figs. 17 and 18B). However, increased release of MMP-9 by macrophages was observed after 24 h and 48 h (Fig. 18C). This was correlated with increases in gelatinase activity in the culture medium (Fig. 18D). While LPS-induced MMP-9 release from macrophages was increased by hypoxia, gelatinase activity was decreased. Hypoxia alone also increased MMP-9 release from macrophages after 48 h, but had no significant effects on MMP-9 mRNA or protein expression, or on gelatinase activity.

The role of MAP kinases in hypoxia-induced alterations in macrophage expression of inflammatory mediators

MAP kinases are known to play a key role in regulating macrophage inflammatory activity (Rao, 2001). We found that p44/42, but not p38 or JNK MAP

kinases, was constitutively activated in RAW 264.7 macrophages (Fig. 19). LPS treatment of the cells resulted in increased expression of phosphorylated-p44/42, as well as p38 and JNK MAPK. Exposure to hypoxia augmented the effects of LPS on expression of phophorylated p44/42 after 0.25-0.5 h, with no major effect on phophorylated p38 or JNK MAPK. Hypoxia alone also had no effect on expression of phosphorylated MAP kinases. To investigate the role of these MAP kinases in macrophage responses to hypoxia, we used specific inhibitors of p44/42 (U0126), p38 (SB 203580) or JNK (SP600125). Inhibition of p38 or JNK had no major effect on LPS-induced expression of iNOS, COX-2 or MnSOD (Fig. 20A). In contrast, inhibition of p44/42 resulted in decreased release of MMP-9 and lipocalin 24p3 into the culture medium in response to LPS, as well as reduced gelatinase activity (Figs. 20B and 20C).

DISCUSSION

LPS is a membrane component of Gram-negative bacteria and a potent macrophage activator (Raetz and Whitfield, 2002). Exposure to excessive levels of LPS is associated with acute endotoxemia and septic shock, which can lead to multiple organ failure and death (Annane et al., 2005). This is thought to be due to dysregulated release of inflammatory mediators by activated macrophages (Laskin et al., 2011). Macrophages recognize LPS in large part via CD14 and toll-like receptor 4 (TLR4) (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Engagement of these receptors leads to activation of NF-κB, AP-1, Nrf2, and HIF-1, and transcription of genes involved in inflammation, cell survival, and angiogenesis (Fitzpatrick et al., 2011; Nizet and Johnson, 2009). Previous studies have demonstrated that hypoxia, a common feature of inflamed and infected tissues, can synergize with LPS in upregulating macrophage inflammatory responses (Bruning et al., 2012; Kim et al., 2010; Mi et al., 2008). This is thought to be due to activation of redox-sensitive transcription factors (Fitzpatrick et al., 2011; Murdoch et al., 2005; Taylor and Cummins, 2009). Consistent with this, the present studies demonstrate that exposure of macrophages to hypoxia enhanced LPS-induced mRNA expression of the proinflammatory cytokine IL-1β. This may be due to hypoxia-stabilized accumulation of HIF-1, which has previously been shown to be critical for IL-1β expression (Fang et al., 2009). In contrast, hypoxia had no effect on LPS-induced mRNA expression of TNFα, which is in accord with prior publications (Lahat et al., 2008; Liu et al., 2008; Ndengele et al., 2000). LPS-induced IL-6 and MIP-2 expression were also unaffected by hypoxia, suggesting distinct mechanisms regulating expression of IL-1β and other proinflammatory cytokines Hypoxia alone was also found to induce mRNA expression of MIP-2, but not IL-1β, IL-6 or TNFα, which is consistent with previous studies (Zampetaki et al., 2004). MIP-2 is a potent neutrophil

chemoattractant, as well as an inducer of endothelial cells proliferation (Moldobaeva and Wagner, 2005). Upregulation of MIP-2 may be important in hypoxia-induced angiogenesis. MIP-2 may also play a role in recruiting neutrophils into hypoxic areas (Naidu et al., 2002; Wang et al., 2000).

VEGF is a potent angiogenic growth factor associated with migration, survival, and proliferation of endothelial cells and the generation of new blood vessels (Perez-Ruiz et al., 1999). GLUT-1 is important in glucose transport into cells which is required for macrophage survival (Cramer et al., 2003; Weisdorf et al., 1982). Both of these proteins are transcriptionally regulated by HIF-1α (Blouin et al., 2004; Pugh and Ratcliffe, 2003). In accord with the previous studies (Blouin et al., 2004; Fukuzumi et al., 1996; Xiong et al., 1998), we found that LPS upregulated VEGF and GLUT-1 mRNA expression in macrophages. Moreover, this response was increased by hypoxia. This may be due to the combined effects of hypoxia and LPS on HIF-1α activation (Mi et al., 2008). This is supported by findings of increased HIF-1α in LPS treated macrophages exposed to hypoxia (Rius et al., 2008). Hypoxia alone also upregulated VEGF and GLUT-1 mRNA expression in macrophages, which is consistent with previous reports (Burke et al., 2003; Shweiki et al., 1992). Hypoxia has been reported to regulate VEGF expression by activating HIF-1α, and also by stabilizing VEGF mRNA in mouse peritoneal macrophages, mouse liver and rat pheochomocytoma cells (Cramer et al., 2003; Levy et al., 1995; Ramanathan et al., 2003; Shima et al., 1996). Additionally, in bone marrow-derived macrophages, hypoxia-induced VEGF and GLUT-1 transcription was shown to occur via NF-κB dependent regulation of HIF-1α mRNA (Rius et al., 2008).

Macrophages activated by LPS generate reactive nitrogen species and proinflammatory eicosanoids via iNOS and COX-2, respectively (Bogdan, 2001; Rouzer and Marnett, 2009; Xia and Zweier, 1997). Excessive production of these mediators at inflammatory sites is associated with tissue damage (Laskin et al., 2011). The present studies demonstrate that hypoxia augments the effects of LPS on both mRNA and protein expression of iNOS and COX-2. Upregulation of iNOS and COX-2 by hypoxia and LPS has been shown to be dependent on HIF-1α and NF-κB (Bruning et al., 2012; Fitzpatrick et al., 2011; Mi et al., 2008). It remains to be determined if RAW 264.7 macrophage responses to these stimuli are controlled by similar signaling mechanisms. Our findings that hypoxia alone had no significant effect on iNOS and COX-2 expression suggests that induction of these proteins in RAW 268.7 macrophages requires additional inflammatory signals (Lewis et al., 1999).

MnSOD is a potent antioxidant enzyme that protects against LPS induced oxidative stress (Choumar et al., 2011; Gonzalez et al., 1995). Previous studies have reported upregulation of MnSOD expression by LPS in mouse peritoneal macrophages (Tsan et al., 2001). Presently we found that LPS treatment of RAW 264.7 macrophages resulted in increased expression of MnSOD mRNA and protein. Interestingly, while LPS-induced MnSOD mRNA expression was reduced by hypoxia, protein levels were unchanged suggesting that hypoxia may regulate MnSOD transcription or post-translational stability. Hypoxia alone had minimal effects on MnSOD mRNA or protein expression in macrophages. These findings are contrary to previous observations in alveolar type II epithelial cells and lung fibroblast exposed to hypoxia (Jackson et al., 1996; Russell et al., 1994). These findings suggest differential responsiveness of these cells to hypoxia possibly by negative regulation of oxidative stress (Tang et al., 2007).

Lipocalin 24p3 is an oxidative stress marker induced by ROS (Roudkenar et al., 2007). Recent studies indicate that lipocalin 24p3 exhibits antioxidant activity (Roudkenar et al., 2011). We have previously demonstrated increased expression of lipocalin 24p3 in lung and liver macrophages after exposure of mice to LPS (Sunil et al., 2007). The present studies show that LPS induces lipocalin 24p3 mRNA expression, as well as protein release by macrophages. Similar release of lipocalin 24p3 has been described in LPS treated mouse PU5.1.8 macrophages (Meheus et al., 1993). Lipocalin 24p3 participates in the innate immune response by limiting bacterial growth (Flo et al., 2004). We found that while hypoxia attenuated LPS-induced 24p3 mRNA expression, it increased protein expression and release of lipocalin 24p3 by macrophages. Perhaps, reduced LPS-induced lipocalin 24p3 mRNA expression resulted from a negative autocrine feedback triggered by increased release of this protein in the cultured medium. In contrast, hypoxia alone had little effect on lipocalin 24p3 expression. Increased lipocalin 24p3 mRNA expression has been reported in mouse J774 macrophages exposed to hypoxia followed by reoxygenation (Hemdahl et al., 2006). These data suggest that low oxygen tension might not be sufficient for lipocalin 24p3 induction in macrophages.

MMP-9 is a key enzyme in the turnover and degradation of extracellular matrix, a process important in wound healing and tissue remodeling (Park et al., 2009). Predominantly expressed by activated macrophages (Coussens and Werb, 2002), MMP-9 is involved in the migration of immune cells to sites of injury (Opdenakker, 1997; Van den Steen et al., 2002). Consistent with previous studies (Rhee et al., 2007), we found LPS upregulated MMP-9 mRNA and protein expression, and increased gelatinase activity in RAW 264.7 macrophages. This may be important in the response of macrophages during sepsis (Dubois et al., 2002). Whereas hypoxia had no significant

effect on LPS-induced MMP-9 mRNA expression, protein expression and release increased, suggesting oxygen sensitive mechanism. In contrast, LPS-induced gelatinase activity was reduced suggesting oxygen tension plays a role in MMP-9 activity. Hypoxia has been reported to upregulate intracellular expression of TNFα-induced MMP-9 protein without altering mRNA expression in human monocytes; however, in contrast to our findings, secretion of MMP-9 was suppressed (Rahat et al., 2006). Differences in release of MMP-9 in our studies might be due to more complex signaling pathway induced by LPS, relative to TNFα. Hypoxia alone had no significant effect on MMP-9 expression suggesting that additional signals are required for its induction in these cells. This is consistent with previous findings using HeLa cells (Himelstein and Koch, 1998).

Translocation of proinflammatory transcription factors, such as NF-κB and HIF-1α into the nucleus depends on activation of MAPK signaling (Kawai and Akira, 2010; Mylonis et al., 2006). In macrophages, LPS signaling though TLR4 leads to phosphorylation of MAPKs including p44/42, p38 and JNK (Kawai and Akira, 2010; Rao, 2001). Consistent with this, we found significant increases in expression of p44/42, p38 and JNK MAPKs after LPS treatment of RAW 264.7 macrophages. Hypoxia exposure augmented p44/42, but not p38 or JNK expression, suggesting that p44/42 is involved in both NF-κB and HIF-1α signaling. Similar increases in LPS-induced phosphorylation of p44/42 by hypoxia have also been observed in human monocytes (Frede et al., 2006). Phosphorylation sites for p44/42 on HIF-1α have been identified in HeLa cells (Mylonis et al., 2006). Surprisingly, whereas inhibition of p44/42 reduced the synergistic effect of hypoxia on LPS-induced protein expression for COX-2, iNOS protein expression increased. This suggests that LPS-induced iNOS and COX-2 expression are regulated by distinct signaling pathways. In contrast, LPS-induced MnSOD protein expression

increased in the presence of a p44/42 MAPK inhibitor indicating a negative regulatory effect on this protein. We also observed that inhibition of p44/42 reduced LPS-induced release of MMP-9 and lipocalin 24p3 alone and following hypoxia exposure. These data demonstrate that p44/42 is critical in LPS regulation of these proteins. Induction of MMP-9 by LPS has similarly been found to be p44/42-dependent in human astrocytes and heart microvascular endothelial cells (Lee et al., 2003; Moshal et al., 2006).

Macrophage responses to bacteria are regulated by pattern recognition receptors and consequent activation of cell signaling cascades (Kawai and Akira, 2010). However, recent studies show that these responses vary with the target tissue (Matzinger and Kamala, 2011). A characteristic change in the tissue microenvironment following infection is a decrease in local-tissue oxygen tension or hypoxia. Accumulating evidence shows hypoxia alters the activities of innate immune cells (Bosco et al., 2008; Murdoch et al., 2005; Sitkovsky and Lukashev, 2005). A question arises as to how hypoxia regulates the biological effects of LPS on macrophages. Evidence presented herein indicates that at least some of the activity is mediated by p44/42 MAP kinase. Further studies are required to more precisely define the mechanisms by which p44/42 MAP kinases control gene expression in macrophages, as well as to identify additional signaling pathways involved in regulating macrophage responses to hypoxia in the setting of infection.

Table 2. Effects of hypoxia on proinflammatory and growth factor gene expression. Macrophages were exposed to air control, LPS (L), hypoxia (H) or LPS and hypoxia (L+H) for 24 h. mRNA expression was quantified by real-time PCR. Data were normalized to 18S and expressed as fold change relative to time-matched air controls. Data represent the mean ± SE (n=3-4). [a]Significantly different ($p \leq 0.05$) from air control; [b]Significantly different ($p \leq 0.05$) from LPS.

	Air	LPS	Hypoxia	L+H
IL-1β	1.0 ± 0.7	31.3 ± 2.2[a]	4.8 ± 3.0	85.9 ± 17.3 [a,b]
IL-6	1.0 ± 0.9	9.8 ± 2.9[a]	1.0 ± 0.7	9.4 ± 4.7[a]
TNFα	1.0 ± 0.2	4.6 ± 1.4[a]	1.4 ± 0.5	3.1 ± 0.3[a]
MIP-2	1.0 ± 0.1	60.0 ± 24.3[a]	5.4 ± 1.6[a]	66.3 ± 15.4[a]
GLUT-1	1.0 ± 0.1	4.0 ± 0.6[a]	4.6 ± 0.5[a]	8.8 ± 1.1[a,b]
VEGF-A	1.0 ± 0.2	3.1 ± 0.3[a]	3.7 ± 0.6[a]	5.9 ± 0.8[a,b]

Figure 17. Effects of hypoxia on LPS-induced expression of inflammatory and antioxidant genes. Macrophages were exposed to air control, LPS (L), hypoxia (H) or LPS + hypoxia (L+H) for 1 to 48 h. mRNA expression was quantified by real-time PCR. Data were normalized to 18S and expressed as fold change relative to time-matched air controls. Each bar represents the mean ± SE in (n = 3-4). *Significantly different from air control ($p \leq 0.05$); **Significantly different from LPS ($p \leq 0.05$).

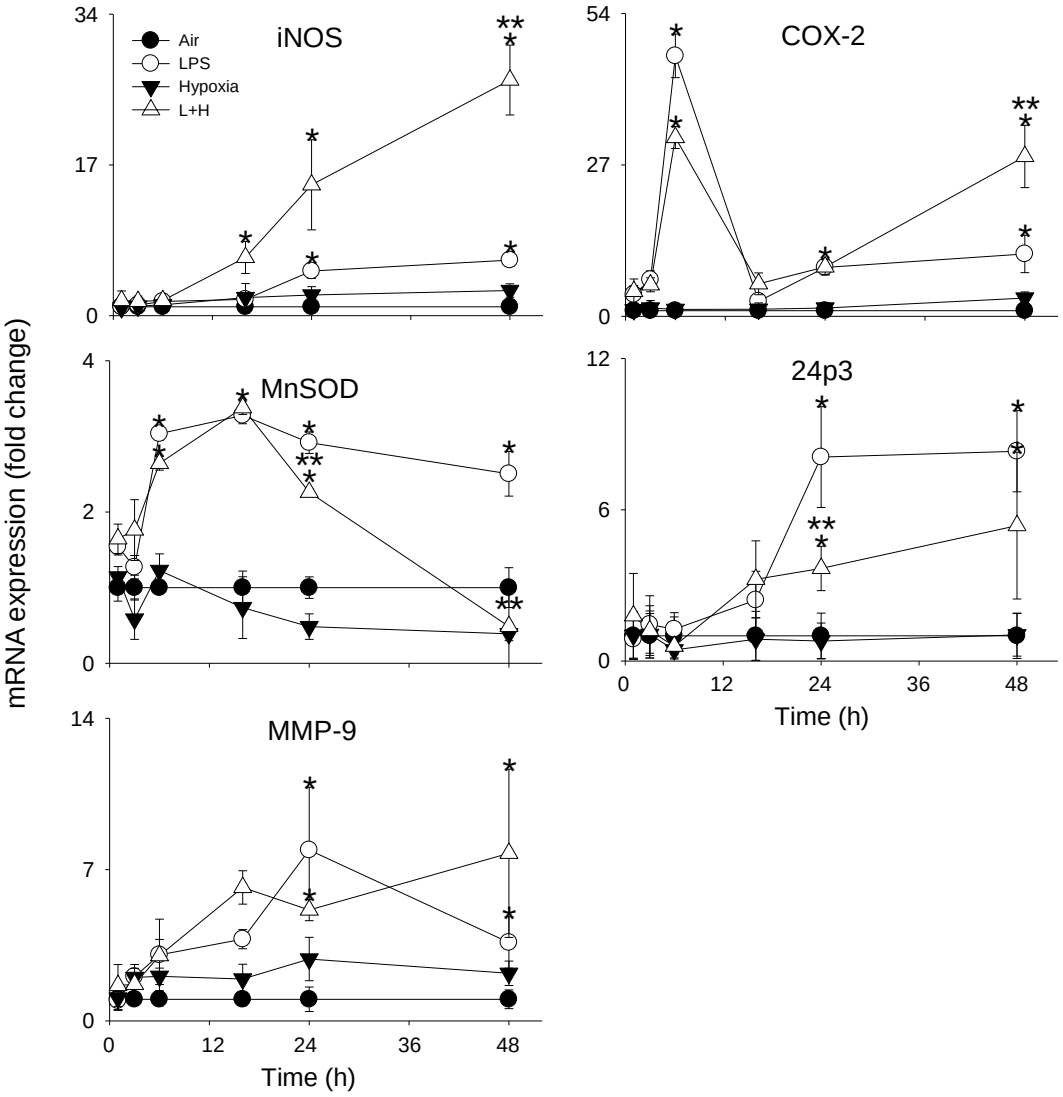

iNOS
COX-2
MnSOD
24p3
MMP-9
Air
LPS
Hypoxia
L+H
mRNA expression (fold change)
Time (h)
34
17
0
54
27
4
2
12
6
14
7
0
12
24
36
48

Figure 18. Effects of hypoxia on expression of inflammatory mediators, and proteins that can produce mediators or regulate inflammation. Macrophages were exposed to air control, LPS (L), hypoxia (H) or LPS + hypoxia (L+H). Cell lysates (panels A and B) and culture supernatants (panel C) were analyzed for expression of mediators 0.25 to 48 h later by Western blotting. *Panel D*; MMP-9 activity in culture supernatants were assayed by Zymography.

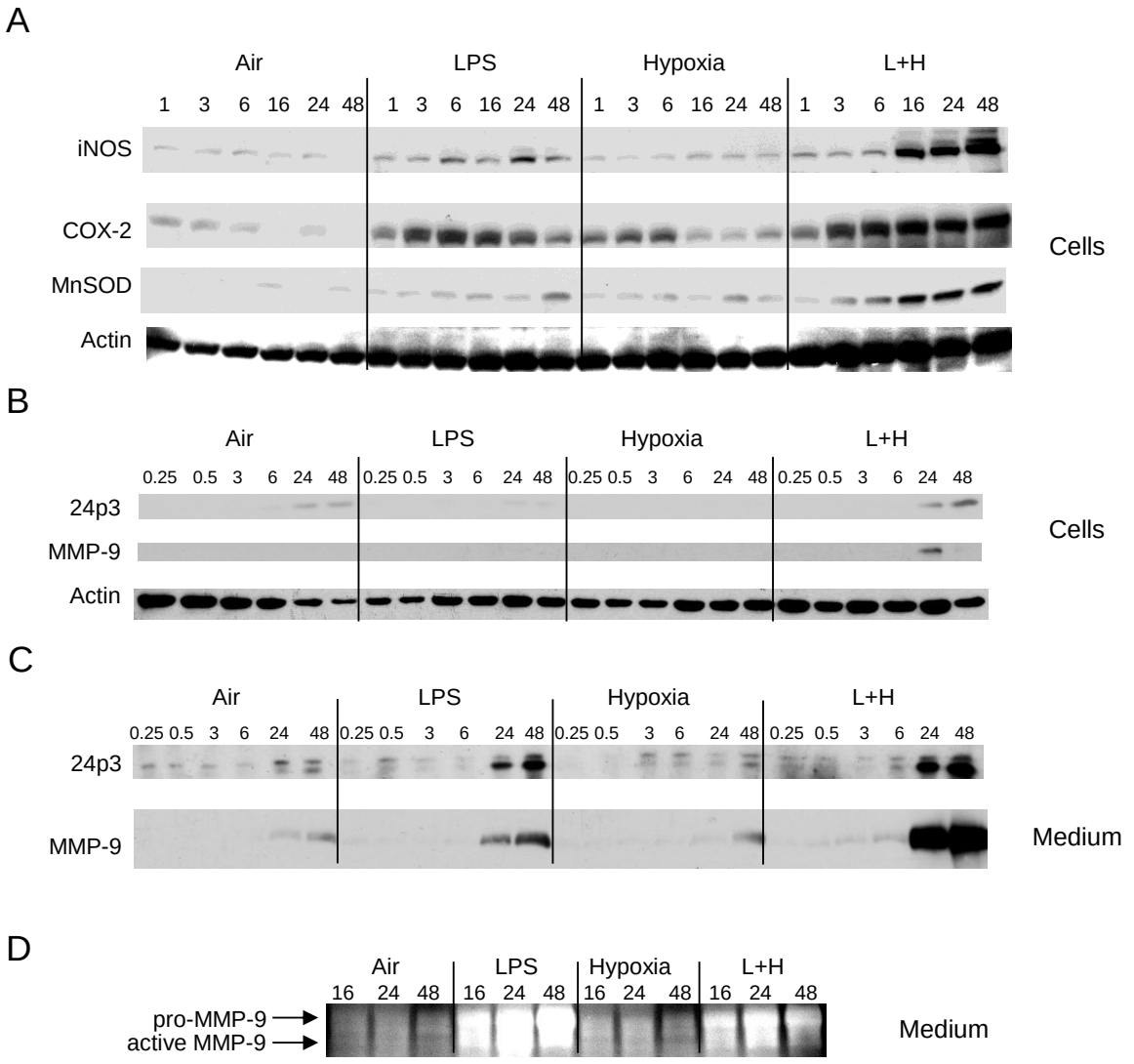
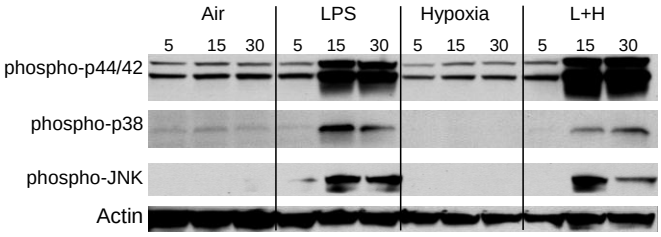

A
Air
LPS
Hypoxia
L+H
1 3 6 16 24 48 1 3 6 16 24 48 1 3 6 16 24 48 1 3 6 16 24 48
iNOS
COX-2
MnSOD
Actin
Cells
B
Air
LPS
Hypoxia
L+H
0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48
24p3
MMP-9
Actin
Cells
C
Air
LPS
Hypoxia
L+H
0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48 0.25 0.5 3 6 24 48
24p3
MMP-9
Medium
D
Air
LPS
Hypoxia
L+H
16 24 48 16 24 48 16 24 48 16 24 48
pro-MMP-9
active MMP-9
Medium

Figure 19. Effects of hypoxia on MAP kinase actvity. Macrophages were exposed to air control, LPS (L), hypoxia (H) or LPS + hypoxia (L+H) for 5 to 30 min. Cell lysates were then prepared and analyzed by Western blotting using antibodies to activated MAP kinases. One representative of 3-4 experiments is shown. ImageJ was used for densitometry and the sum of two bands is represented on the graph for phopho-p44/42 and JNK. Each bar represents the mean ± SE in (n = 3-4). *Significantly different from air control ($p \leq 0.05$); **Significantly different from LPS ($p \leq 0.05$).


Air
LPS
Hypoxia
L+H
5 15 30 5 15 30 5 15 30 5 15 30
phospho-p44/42
phospho-p38
phospho-JNK
Actin

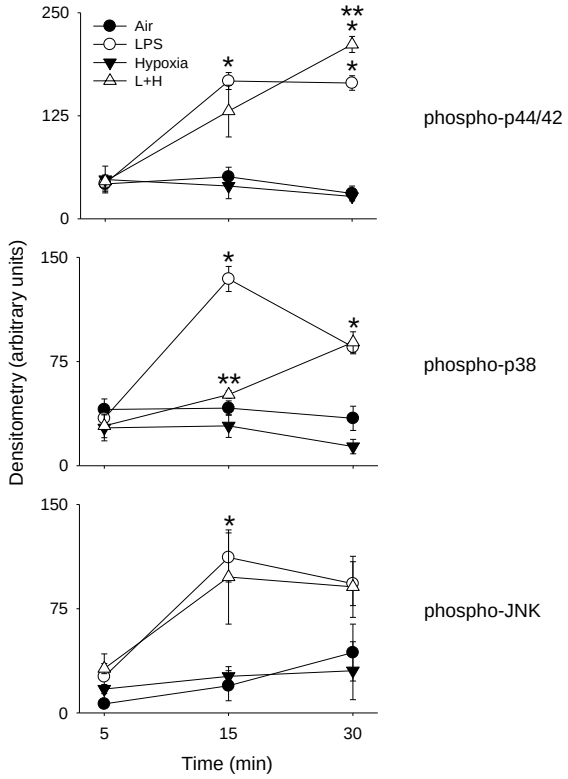
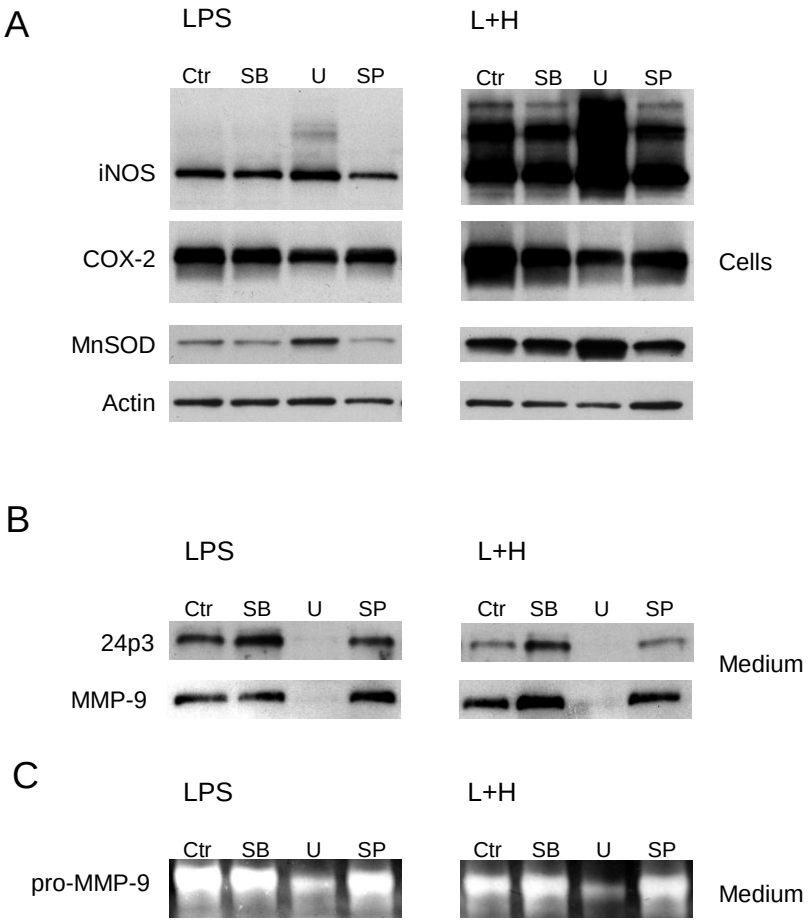

Air
LPS
Hypoxia
L+H
Densitometry (arbitrary units)
250
125
0
150
75
0
150
75
0
5
15
30
Time (min)
phospho-p44/42
phospho-p38
phospho-JNK

Figure 20. Regulation of mediator expression by MAP kinase inhibitors. Macrophages were pretreated for 3 h with control or with inhibitors of JNK (SP600125), p38 (SB 203580) or p44/42 (U0126) MAP kinase and then exposed to LPS (L) or LPS + hypoxia (L+H) for 24 h. Cell lysates (panel A) or culture supernatants (panel B) or were prepared and protein visualized by Western blotting. *Panel C;* MMP-9 activity in culture supernatants was assayed by Zymography.


A
LPS
L+H
Ctr SB U SP
Ctr SB U SP
iNOS
COX-2
MnSOD
Actin
Cells
B
LPS
L+H
Ctr SB U SP
Ctr SB U SP
24p3
MMP-9
Medium
C
LPS
L+H
Ctr SB U SP
Ctr SB U SP
pro-MMP-9
Medium

SUMMARY AND CONCLUSIONS

The main objective of these studies was to investigate mechanisms regulating macrophage activation and production of inflammatory mediators following oxidative stress induced by exposure to bacterial products (e.g., LPS) or irritants which cause sterile inflammation (e.g., ozone). We hypothesized that TLR4, which recognizes pathogen-associated molecular patterns found in LPS, is also involved in lung macrophage responsiveness to oxidative stress induced by ozone. Using control C3H/HeOuJ mice, we found acute ozone inhalation was associated with classical macrophage activation, as measured by NF-κB binding activity and upregulation of TNFα mRNA. Ozone exposure also resulted in increased lung macrophage cell number, BAL protein concentration, and SP-D expression, as well as oxidative stress and lipid peroxidation markers, lipocalin 24p3 and 4-HNE. These responses were not evident in TLR4 mutant C3H/HeJ mice suggesting that functional TLR4 contributes to ozone-induced sterile inflammation and macrophage activation.

To determine if macrophage inflammatory and oxidative stress responses in the lung were similar in the liver, we investigated the role of TLR4 in activation of lung and liver macrophages during acute endotoxemia and oxidative stress induced by LPS administration to mice. We found that acute endotoxemia resulted in an accumulation of activated macrophages in the lung and the liver as evidenced by increased cell number and expression of PCNA, suggesting local proliferation contributed to the response. Liver macrophages were generally more sensitive to acute endotoxemia than lung macrophages. Thus, a rapid (within 3 h) increase in liver, but not lung, macrophage mRNA expression of antioxidants, MnSOD and HO-1, as well as expression of enzymes mediating the biosynthesis of eicosanoids, COX-2 and mPGES-1 was observed. In contrast, COX-2 protein expression increased in both liver and lung macrophages. The

effects of acute endotoxemia were significantly reduced in TLR4 mutant C3H/HeJ mice demonstrating TLR4 was key to LPS-induced oxidative stress, inflammation and macrophage activation.

Further studies focused on analyzing the effects of oxidative stress induced by exposure to hypoxia on LPS-induced activation of macrophages. For these studies, we used the murine macrophage cell line, RAW 264.7. We found that hypoxia synergized with LPS in induction of mRNA expression of IL-1β, VEGF and GLUT-1, with no effect on TNFα, IL-6 or MIP-2. Additionally, although hypoxia augmented LPS-induced protein expression for iNOS, COX-2, MnSOD, lipocalin 24p3, and MMP-9, but gelatinase activity decreased. Similarly, previous studies have found synergistic effects of hypoxia on LPS-induced iNOS mRNA and protein expression did not correlate with decreased nitric oxide production (Daniliuc et al., 2003; McCormick et al., 2000). Using specific MAPK inhibitors, we found that protein expression for MMP-9 and lipocalin 24p3 were p44/42 MAPK dependent. Taken together, these studies suggest that hypoxia modulates macrophage function, survival and production of inflammatory mediators during inflammatory reactions.

In conclusion, the present studies show TLR4 plays a critical role in macrophage responses to oxidative stress induced by pathogen-driven and sterile inflammation. Moreover, these responses are modulated by the local microenvironment, specifically reduced oxygen tension. Results from these studies are important as they demonstrate LPS-induced activation of macrophages in the liver is not the same in the lung. This suggests that the macrophage response to an injury is specifically determined by each tissue. This implies that the immune response in each organ has to be carefully reevaluated and considered as its own entity.

FUTURE STUDIES

Our studies on ozone-induced lung injury in TLR4 control C3H/HeOuJ mice confirm a key role of TLR4 in sterile inflammation. We found that ozone-induced oxidative stress, lipid peroxidation, and macrophage accumulation and activation are TLR4 dependent. However, the ligand for TLR4 signaling generated by ozone-induced sterile inflammation remains to be determined. Recent studies show hyaluronan, a product released from degraded matrix, is released following ozone exposure (Li et al., 2011). Future studies designed to mimic ozone-induced inflammation by intratracheal instillation using hyaluronan, other matrix degradation products, or proteins released from necrotic cells, may provide clues about possible endogenous TLR4 ligands. Our studies revealed an increase in SP-D in BAL following ozone exposure. SP-D is known to downregulate TLR4 signaling on macrophages (Nie et al., 2008; Ohya et al., 2006). It would be interesting to see if pre-treatment of mice with SP-D would attenuate ozone-induced macrophages activation. Additionally, we found that ozone-induced increases in lipocalin 24p3 are TLR4 dependent. At present, little is known about the function of lipocalin 24p3 in lung injury. Future studies on ozone-induced lung injury using lipocalin 24p3 knockout mice might help to elucidate its function in sterile inflammation. In our analysis of the BAL from TLR4 control mice following ozone exposure, we detected a 50 kDa molecular weight 4-HNE-modified protein as a lipid peroxidation by-product. Further studies designed to identify this protein using 2D-gel electrophoresis might help us to better understand lung responses to ozone.

Following acute endotoxemia, we found that lung and liver macrophages respond distinctly. Although LPS-induced TLR4-dependent classical activation was identified in liver macrophages, the response of lung macrophages was more complex. Our in vitro studies revealed synergistic effects of hypoxia on LPS-induced expression of MnSOD

and COX-2. It is possible that the difference in LPS-induced macrophage activation in the liver and lung in vivo is due to reduced oxygen tension in the liver, which could lead to increased MnSOD and COX-2 mRNA and protein expression. Staining liver sections from LPS treated mice with pimonidazole, a hypoxia marker, may help to determine if hypoxia is present in this liver injury model. In addition, as seen in the ozone-induced lung injury, BAL analysis for SP-D levels, a known TLR4 signaling modulator, might help in understanding reduced transcriptional activation in lung macrophages when compared to liver macrophages.

Another interesting finding in these studies was increased PCNA staining in both lung and liver macrophages from following acute endotoxemia, which correlated with increased numbers of these cells in the tissues. It would be interesting to determine if these proliferating macrophages are recruited from the bone marrow and proliferate upon entering the tissue, or if these are the resident macrophages induced to proliferate in response to LPS or tissue injury. Further studies assessing specific surface markers may aid in addressing this question.

LPS-induced increases in 12/15-LOX expression in lung macrophages from TLR4 mutant mice suggest that TLR4 may play a negative role in regulation of this enzyme. To explore this possibility, future in vitro studies using siRNA transfection designed to silence TLR4 in macrophages could be performed. These studies would help us determine if LPS treatment under these conditions induces 12/15-LOX mRNA expression.

The present studies demonstrate that hypoxia is a modulator of macrophage responses to LPS. We showed LPS-induced MMP-9 gelatinase activity is reduced by hypoxia even though MMP-9 mRNA expression and protein release into the culture

medium was increased. The conclusion from these studies is that hypoxia is important in down regulating macrophage responses to LPS. Future studies investigating the activity of proinflammatory cytokines, not just their expression would provide information on the extent to which hypoxia can regulate macrophage function.

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