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Lung macrophages: current understanding of their roles in Ozone-induced lung diseases

Sonika Patial, **Yogesh Saini**

Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA

Abstract

Through the National Ambient Air Quality Standards (NAAQS), the Clean Air Act of the United States outlines acceptable levels of six different air pollutants considered harmful to humans and the environment. Included in this list is ozone (O_3), a highly reactive oxidant gas, respiratory health hazard, and common environmental air pollutant at ground level. The respiratory health effects due to O_3 exposure are often associated with molecular and cellular perturbations in the respiratory tract. Periodic review of NAAQS requires comprehensive scientific evaluation of the public health effects of these pollutants, which is formulated through integrated science assessment (ISA) of the most policy-relevant scientific literature. This review focuses on the protective and pathogenic effects of macrophages in the O_3-exposed respiratory tract, with emphasis on mouse model-based toxicological studies. Critical findings from 39 studies containing the words O_3, macrophage, mice, and lung within the full text were assessed. While some of these studies highlight the presence of disease-relevant pathogenic macrophages in the airspaces, others emphasize a protective role for macrophages in O_3-induced lung diseases. Moreover, a comprehensive list of currently known macrophage-specific roles in O_3-induced lung diseases is included in this review and the significant knowledge gaps that still exist in the field are outlined. In conclusion, there is a vital need in this field for additional policy-relevant scientific information, including mechanistic studies to further define the role of macrophages in response to O_3.

Keywords

Ozone; macrophages; airspaces; lung; epithelial lining fluid

CONTACT Yogesh Saini ysaini@lsu.edu Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, 1909 Skip Bertman Drive, Baton Rouge 70803, LA, USA.

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Ozone: a respiratory health hazard

Ozone (O_3), a highly reactive oxidant gas, is a respiratory health hazard and one of the most common environmental air pollutants at ground level. Approximately, 90% of Earth's O_3 is confined to the stratosphere where it is generated when oxygen is exposed to ultraviolet radiation, electrical discharges, and heat (Aucamp 2007). In contrast, ground level (tropospheric) O_3 is formed during a photochemical reaction between oxides of nitrogen and volatile organic hydrocarbons that originate from automobile emissions and other high-heat combustion-based industrial processes (Moller 2004). While stratospheric O_3 is beneficial to life as it prevents the entry of harmful ultraviolet radiation into the earth's atmosphere, tropospheric O_3 causes serious health problems.

Elevated levels of ground-level O_3 contribute to a significant increase in hospitalization resulting in substantial health and economic burdens (Schwartz 1994; Medina-Ramon et al. 2006; Lin et al. 2008; Moore et al. 2008). Increases in tropospheric O_3 levels are associated with reduced pulmonary function, exacerbations of asthma and chronic obstructive pulmonary disease (COPD), and mortality (Halonen et al. 2010; Kariisa et al. 2015). Children, the elderly, and individuals with preexisting respiratory diseases are particularly vulnerable to elevated levels of ambient O_3 (Burnett et al. 2001; Gent et al. 2003; Bell et al. 2004; Gryparis et al. 2004; Ito et al. 2005). According to one estimate, a 25-ppb increase in the levels of ambient O_3 causes an approximately 4% increase in mortality (Parodi et al. 2005). Further, the unto-ward effects of O_3 are worsened when other pollutants, such as particulate matter, allergens, and noxious gases are simultaneously inhaled (Jakab and Hemenway 1994; Linn and Gong 1999; Bernard et al. 2001; Schelegle et al. 2003; Wong et al. 2018).

Risk-assessment paradigm for ozone

O_3 is one of six air pollutants, the others being carbon monoxide, lead, particulate matter, nitrogen dioxide, and sulfur dioxide identified under Section 109 of the Clean Air Act. The Clean Air Act requires the Environmental Protection Agency (EPA) to set National Ambient Air Quality Standards (NAAQS) for all criteria air pollutants, including O_3. In the USA, the atmospheric levels of these air pollutants are closely monitored to attain NAAQS status. According to 2015 NAAQS, a level of O_3 below 70 ppb (annual 4th highest daily maximum 8-h concentration averaged over 3 years) is required to achieve NAAQS attainment status (McClellan et al. 2009).

The exposure and risk assessment paradigm for O_3 is employed by EPA administrators to make informed revisions of NAAQS (Figure 1). Studies focused on the toxicodynamic aspects of O_3 toxicity are critical components of risk assessment and as a result, risk management. In particular, studies employing rodent models have continuously increased the mechanistic understanding of O_3 toxicity. Here, we review critical advancements in understanding of the contribution of macrophages to O_3-induced airway diseases.

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Lifecycle of ozone in the respiratory tract

Luminal surfaces of the airways and alveolar spaces are lined with an aqueous epithelial lining fluid (ELF) layer. The ELF layer acts as a physical barrier that prevents the direct onslaught of epithelial cells by inhaled entities including O_3. Thus, before interacting with the resident cells in airspaces, the inhaled O_3 interacts with the ELF layer of the conducting airways and alveolar spaces (Pryor 1992; Gerrity et al. 1995). The low solubility of O_3 in the ELF layer limits its rate of diffusion toward the apical surfaces of the epithelial cells (Gerrity et al. 1995). During its diffusion toward the epithelial cell surfaces, a portion of the dissolved O_3 is detoxified by antioxidants, including urate, ascorbate, vitamin E, and reduced glutathione, normally found in the ELF (DeLucia et al. 1975; Housley et al. 1995; Duan et al. 1996; Kelly et al. 1996; Mudway et al. 1996; Mudway and Kelly 1998; Mudway et al. 1999). Kelly et al. (1995) previously provided a detailed overview of the antioxidant defense system against O_3.

While the ELF layer serves to protect the mucosal surfaces of the respiratory tract from inhaled agents, molecular interactions between O_3 and constituents of the ELF layer result in the generation of harmful ozonation products. Studies employing radioactive oxygen (^{18}O) labeled O_3 revealed a higher concentration of ^{18}O in the ELF suggesting its incorporation into ELF biomolecules (Hatch et al. 1994). The incorporation of O_3 into the unsaturated carbon backbone of ELF constituents, including phospholipids, cholesterol, epoxy cholesterol, proteins, and hyaluronic acids (HA), leads to a rapid drop in its levels within the ELF layer before it reaches the epithelial cells (Johnson 1980; Gordon et al. 1981; Sharman and Mudd 1981; Friedman et al. 1985; Madden et al. 1987). Through the Criegee reaction, ozonation of unsaturated fatty acids yields aldehydes, such as hexanal, nonanal, heptanal, and carbonyl oxide. These aldehydes have been shown to be elevated in the BALF of O_3-exposed humans and rats (Pryor et al. 1996; Frampton et al. 1999a, 1999b). Among these, carbonyl oxide, being a reactive species, further combines either with aldehydes to form ozonide (incorporation of O_3 in carbon–carbon chain) or with water to form hydroxy hydroperoxide in aqueous environments (Santrock et al. 1992).

Ozonation of proteins at aliphatic amino acids results in the formation of nitrates, ammonia, carbonyl, and carboxyl byproducts (Mudd et al. 1969; Pryor and Uppu 1993; Kotiaho et al. 2000; Kelly and Mudway 2003). In addition, ozonation of proteins at aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine), also results in alteration of the folding abilities of proteins, which in turn alters their tertiary structures and biologic activities (Berlett et al. 1996). For instance, O_3 is known to reduce α1-proteinase activity (Johnson, 1980, 1987; Nadziejko et al. 1995), presumably through misfolding. The enzyme α1-proteinase is an endogenous inhibitor of neutrophil elastase, an enzyme that causes alveolar wall destruction resulting in pulmonary emphysema (Johnson 1980, 1987). The negative effect of O_3 on α1-proteinase activity suggests that O_3 may contribute to the alveolar space enlargement by negatively affecting α1-proteinase activity.

Given the poor solubility of O_3 in the ELF layer, its significant detoxification by ELF antioxidants, and the molecular reactions of O_3 with the constituents of the ELF layer, it is highly likely that for the most part, ozonation products produced within the ELF layer, rather

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than the O_3 itself, interact directly with epithelial and immune cells, particularly macrophages. Since resident macrophages patrol the ELF layer, these cells are likely the first to respond to both dissolved O_3 and ozonation products produced within the ELF (Figure 2). In the following sections, we will focus on the current understanding of lung macrophages and their responses to O_3 exposure.

Lung macrophages

Macrophages are present in all vertebrate tissues where they play critical roles in tissue development, homeostasis, and pathogenesis of diseases (Epelman et al. 2014). Based on their anatomic locations, macrophages have been assigned organ-specific nomenclature including lung alveolar macrophages (AM), liver Kupffer cells, bone osteoclasts, and brain microglial cells (Davies et al. 2013; Hoeffel and Ginhoux 2015). Despite their distinct anatomical locations, macrophages throughout the vertebrate body act as professional phagocytes to clear pathogens, abiotic entities, and dead host cells. In addition, fine-tuned by their distinct tissue microenvironment, macrophages also possess varied functions including antigen-presentation, pathogen killing, tissue repair, tissue destruction, and scavenging (Wynn and Vannella 2016).

Due to their ability to sense molecular cues in the extra-cellular microenvironment and mount a wide array of functional responses, macrophages are regarded as a remarkably plastic cell type. In addition, macrophage populations within a tissue are heterogenous. While plasticity refers to the capability of a cell to acquire a distinct molecular and functional phenotype, macrophage heterogeneity refers to the ability of a macrophage population to exhibit a wide range of diverse molecular and functional phenotypes. This heterogeneity arises either due to the presence of cells that are in various stages of differentiation or due to differential responses to diverse extrinsic cues present in the immediate vicinity of the cells. Due to the presence of a multitude of surface receptors, including Toll-like receptors (TLRs), scavenger receptors, C-type lectin receptors, and cytokine receptors, macrophages possess extraordinary abilities to sense the presence of aberrant entities of both biotic and abiotic nature in the tissue microenvironment (Taylor et al. 2005; Ley et al. 2016).

Lung macrophages patrol the epithelial surfaces of the pulmonary airway and alveolar spaces and, thus, face a continued onslaught from a plethora of inhaled environmental insults (Hussell and Bell 2014). Alongside epithelial cells, lung macrophages constitute the first line of cellular host defense against inhalational exposure to aberrant biotic and abiotic agents. According to the nature and degree of the insult, macrophages respond in a variety of ways including functional modulation (activation), release of chemical mediators of inflammation that dictate recruitment of immune cells, and modulation of epithelial responses (Mosser and Edwards 2008). Macrophage-derived inflammatory mediators communicate with neighboring cells including neighboring naïve macrophages, other immune cells, and epithelial cells. The net overall response generally encompasses a wide variety of responses ranging from localized activation of macrophages, in the case of mild exposure, to large-scale systemic responses, in the case of severe exposure where increased macrophage

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recruitment and proinflammatory classical macrophage activation responses play critical roles in conjunction with epithelial- and other immune cell-driven responses.

Macrophage types in the lung

Broadly, lung macrophages are classified into two major categories, alveolar macrophages (AM) and interstitial macrophages (IM). AM reside in the two anatomically distinct airspace compartments, alveolar airspaces, and the airway lumen. IM are localized to the interstitial spaces between the alveolar epithelium and the endothelium of septal blood vessels. These two cell types are distinguished by the differential expression of surface receptors including CD11b, CD11c, and sialic acid-binding immunoglobulin-like lectin F (SIGLEC-F) (Misharin et al. 2013; Zaynagetdinov et al. 2013). AM and IM are defined as CD11b^{-}CD11c^{high}SIGLEC-F^{high} and CD11b^{int}CD11c^{-}SIGLEC-F^{-}, respectively. In addition, AM express CD206 and CD204R at higher levels than IM (Misharin et al. 2013; Zaynagetdinov et al. 2013).

Origins of lung macrophages under steady state

At embryonic stage E18.5 (day of observance of vaginal plug is set at E0.5) in mice, fetal liver-derived monocytes start differentiating into the pre-alveolar macrophages (preAM) within the alveolar septa. At birth, these preAMs start migrating to the alveolar spaces as immature macrophages, where, under the action of granulocyte-macrophage colony-stimulating factors (GM-CSF), they develop into long-lived AM. This process carries on during the first week of postnatal life (Guilliams et al. 2013). Tan and Krasnow (2016) carried out detailed studies and identified three distinct waves of macrophage development in the lung. At E10.5 the first wave originates from the yolk sac resulting in a population of F4/80+ macrophages in the interstitial spaces. At E12.5, a second wave originates from the fetal liver and populates interstitial spaces with Mac2^{+}macrophages. These macrophages then enter the alveolar spaces at the time of birth and give rise to self-renewing long-lived AM (Guilliams et al. 2013). Finally, the third wave starts with the recruitment of circulating monocytes into the interstitial spaces to give rise to F4/80^{+}definitive IM (Tan and Krasnow 2016). Thus, these findings indicate that the AM in healthy lungs are a self-renewing population of macrophages originated from the fetal liver.

Origin of lung macrophages during inflammation and stress

Inhalation of aberrant entities into airspaces causes varied macrophage responses including pro-inflammatory, anti-inflammatory, and phagocytic responses as well as proliferation, apoptosis, and death. These responses likely contribute to the heterogeneity of alveolar macrophage populations *via* perturbed self-renewal and recruitment responses. In macrophage depletion models, circulating monocytes have been regarded as a central source of macrophages in the alveolar spaces. However, Landsman et al. (2007) showed that Gr1^{low}CX3CR1^{high}CCR2^{-} blood monocytes are the precursors of interstitial lung macrophages. These IM were later identified as precursors of AM (Landsman and Jung 2007). Using fate mapping and parabiosis studies in both steady state and macrophage depletion models, circulating monocytes were later ruled out as precursors for AM and a self-renewal mechanism was found to be responsible for the maintenance of resident alveolar macrophages (Hashimoto et al. 2013).

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Along similar lines, local proliferation, rather than recruitment from blood, was also reported to be the primary mechanism responsible for the increased number of alternatively activated (M2) macrophages in the pleural cavity of helminth-infected mice (Jenkins et al. 2011). In allergen-exposed mice, the self-renewal mechanism was also found to be responsible for maintenance of the resident AM pool during the early stages of inflammation (Zasłona et al. 2014). Misharin et al. (2017) reported that monocyte-derived macrophages are recruited during various lung fibrotic stages, and that expression of caspase-8, a cysteine protease, on these macrophages contributes to the worsening of lung pathology. Acute exposure to lipopolysaccharide (LPS), a potent inflammatory insult, results in a drastic reduction in the number of AM, which are restored *via* self-renewal mechanisms (Dong et al. 2018). Simultaneously, monocyte-derived macrophages also appear in the alveolar spaces, albeit at a slower rate and to a relatively lesser extent than occurs with self-renewal mechanisms (Dong et al. 2018). These studies point to the involvement of insult-dependent mechanisms for the maintenance and turnover of macrophages in the lungs.

Macrophages in ozone-exposed lungs

To review the current literature relevant to lung macrophages in mouse model-based O_3 toxicity studies, we identified relevant studies published in the PubMed database using the EndNote citation manager. Our scheme for this literature search is outlined in Figure 3. We retrieved 142 references containing four keywords, O_3, lung, mice, and macrophage, within any field of reference. Through manual screening, we selected 39 references that reported macrophage-relevant findings in O_3-exposed mouse lungs. Most of the studies excluded used the term “macrophage” only in reference to increased levels of macrophage inflammatory protein 2 (MIP2/CXCL2) chemokines or increased numbers of macrophages in O_3-exposed mice. Select studies are discussed in this review, while the exposure conditions and salient findings from all 39 studies are presented in Table 1.

Effect of ozone on macrophage phagocytosis

Phagocytosis relies on cell surface receptors that have been evolutionarily selected to recognize particles including microbes, apoptotic and necrotic debris, and foreign abiotic substances. For example, antibody/complement-coated (opsonized) bacteria are recognized by Fc-gamma (Fcγ)/complement receptors (Allen and Aderem 1996). Ligand bound Fcγ/complement receptors signal substantial reorganization of the macrophage actin cytoskeleton, which, in turn, triggers the formation of membrane extensions called pseudopodia that surround and finally engulf recognized particles (Allen and Aderem 1996). Critical known receptors in opsonin-dependent phagocytosis include FcγRI (CD64), FcγRIIa (CD32), FcγRIIIa (CD16), FcαR1 (CD89), FcεR1, CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18 complex), CR4 (CD11c/CD18 complex), C5a (CD88), and surfactant protein A (SPA) (Gordon 2016; Rosales and Uribe-Querol 2017). In contrast to bacteria, environmental toxicants devoid of microbial contents are scavenged through scavenger receptors (Palecanda and Kobzik 2001). Mechanistically, these receptors scavenge inflammatory products, such as oxidized lipids, that are also potent neutrophil chemoattractants.

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Goldstein et al. (1974) reported that AM from O_3-exposed rats are defective in bacterial clearance. Later, McAllen et al. (1981) and Sherwood et al. (1986) reported compromised macrophage mobility and lysosomal enzyme contents in O_3-exposed rats. Further, Pearson and Bhalla (1997) reported that O_3-exposed rat macrophages exhibit increased adherence to epithelial cells. O_3-exposed rat macrophages have also been found to show diminished capabilities to produce superoxide radicals, which in turn, limits their bactericidal activities (Amoruso et al. 1981). Along these lines, *in vitro* exposure of cultured human AM to O_3 results in reduced phagocytosis (Becker et al. 1991). Together, these studies indicate that there is a negative impact of O_3 on macrophage function.

Several mouse-based studies have also linked O_3 exposure to poor phagocytic abilities of alveolar macrophages. AM from mice exposed to 2 ppm O_3 for 3 h exhibited poor ability to phagocytose *Klebsiella pneumonia* (Mikerov, Gan, et al. 2008; Mikerov, Haque, et al. 2008). Similarly, in another study, macrophages from O_3-exposed mice (1.5 ppm O_3 for 4 h) were found to exhibit compromised phagocytosis of carbon black (Jakab and Hemenway 1994). In yet another study, macrophages from mice exposed to 0.4–0.8 ppm for 3 h had reduced ability to engulf bacteria, i.e. *Streptococcus zooepidemicus* (Gilmour et al. 1993).

The mechanisms by which O_3 compromises phagocytosis are, however, not understood. Interestingly, *S. zooepidemicus* was found to develop a virulence factor, antiphagocytic capsulation, within 3 h of O_3 exposure at 0.4–0.8 ppm that resisted phagocytosis by AM (Gilmour et al. 1993). O_3 is known to impair the structure and function of SPA, an opsonin that is important for the phagocytic ability of macrophages (Stringer and Kobzik 1996; Benne et al. 1997; Tenner 1998; Schagat et al. 2001), thus compromising opsonin-mediated phagocytosis in the alveolar spaces (Oosting et al. 1991; Su and Gordon 1996). In fact, O_3-exposed SPA-deficient mice exhibit severely compromised clearance of *K. pneumonia* (Mikerov, Gan, et al. 2008; Mikerov, Haque, et al. 2008) suggesting that SPA acts as a critical modulator of host defense against bacterial infections. Haque and colleagues reported that SPA plays a critical role in scavenging O_3-induced reactive oxidants, thus mitigating oxidative stress, a protective response that was found to be absent in SPA knockout mice (Haque, Umstead, Ahn, et al. 2009). However, the effects of O_3 on other opsonins and phagocytosis-related receptors remain largely unknown.

Effect of ozone on the production of reactive species in macrophages

Macrophages recognize, engulf, and kill bacteria inside phagolysosomes where NADPH-dependent oxidases produce reactive superoxides, which are further converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (Bedard and Krause 2007). Earlier studies conducted on mice exposed to 0.1 ppm O_3 for 3 h revealed that AM from these mice are poor producers of superoxide anions, substrates required for the production of bactericidal H_2O_2 (Ryer-Powder et al. 1988). Superoxide and nitric oxide (NO) species also react to produce highly reactive peroxynitrite ($ONOO^-$). While superoxides, NO, and $ONOO^-$ are essential for creation of the bactericidal microenvironment of lysosomes, they have also been implicated in causing tissue injury (Bedard and Krause 2007).

AM from O_3-exposed mice (0.8 ppm O_3 for 3 h) exhibit higher expression of inducible nitric oxide synthase (iNOS/NOS_2) and elevated production of NO and $ONOO^-$ compared to

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control mice (Laskin et al. 2002). In contrast, O_3-exposed iNOS-deficient mice exhibit attenuated epithelial injury, and AM from these mice do not produce NO or $ONOO^-$ (Laskin et al. 2001). In a more long-term study, iNOS-deficient mice exposed to 1 ppm O_3 for 8 h per night for three consecutive nights had elevated lung injury as compared to O_3-exposed wild-type mice (Kenyon et al. 2002). The difference in the outcomes of iNOS deletion in the two studies is most likely due to differences in the O_3 exposure paradigm. Furthermore, overexpression of superoxide-dismutase (SOD), an antioxidant enzyme, was shown to confer protection against oxidative stress and O_3-induced lung injury (Fakhrzadeh, Laskin, Gardner, et al. 2004). Overexpression of SOD also reduced the production of NO and $ONOO^-$ and expression levels of iNOS (Fakhrzadeh, Laskin, Gardner, et al. 2004).

While the elevated production of reactive nitrogen species in macrophages from O_3-exposed mice was found to be associated with increased lung injury, its impact on the bactericidal potential of macrophages following O_3/bacteria co-exposure is not known. While earlier studies reported that the worsening of bacterial infection in O_3-bacteria co-exposure models is attributed to the poor phagocytic ability of macrophages (Gilmour et al. 1993; Mikerov, Gan, et al. 2008; Mikerov, Haque, et al. 2008), in depth analyses of the effects of O_3 on iNOS and SOD enzyme activity and generation of their products is still awaited.

Effect of ozone on macrophage activation

O_3 exposure leads to activation, i.e. enhanced function, of macrophages. Macrophages have also been suggested to react to products released from epithelial cells injured by O_3 resulting in classical macrophage activation and release of products such as reactive oxygen species (ROS) and TNFα, which further promote lung injury (Pendino et al. 1995; Cho et al. 2001; Fakhrzadeh et al. 2002; Toward and Broadley 2002; Fakhrzadeh, Laskin, et al. 2004; Fakhrzadeh, Laskin, Gardner, et al. 2004). AM from O_3-exposed mice show elevated expression of iNOS (Fakhrzadeh et al. 2002), a bonafide marker of M1 macrophages. Further, iNOS deficiency is protective against O_3-induced (0.8 ppm for 3 h) tissue injury implicating M1 activation as a factor responsible for tissue injury (Fakhrzadeh et al. 2002). These reports clearly highlight the role of classically activated macrophage-derived nitrosative and oxidative stress as a promoter of acute O_3-induced lung injury (Fakhrzadeh et al. 2002; Fakhrzadeh, Laskin, Gardner, et al. 2004; Hollingsworth, Kleeberger, et al. 2007; Laskin et al. 2011).

Alternatively activated (M2) macrophages are known to release anti-inflammatory mediators and actively participate in wound repair, resolution of inflammation (Reinhart et al. 1999; Backus et al. 2010), and clearance of apoptotic/necrotic cells (Ishii et al. 1998; Dahl et al. 2007). Mathew and colleagues showed that γδT cell-derived IL17A induces alternative activation of macrophages, which promotes clearance of apoptotic cells and resolution of O_3-induced lung inflammation (Mathews et al. 2015).

Effect of ozone on macrophage survival

O_3 is known to induce cell death *via* autophagy, apoptosis, as well as necroptosis (Sunil et al. 2012). Macrophages from O_3 exposed lungs were reported to undergo cell death as indicated by induction of markers of apoptosis (cleaved caspase-9) and autophagy (beclin-1)

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(Sunil et al. 2012). ROS-induced apoptosis-like cell death (oxeiptosis) has been reported in O_3-exposed mice lungs (Holze et al. 2018). Further, it was reported that the abolition of mitochondrial, serine/threonine protein phosphatase (PGAM5), a major player in oxeiptosis pathway, resulted in exaggerated oxidative stress and inflammation. While oxeiptosis have been observed in a wide range of cell types, macrophages appear to be insensitive to oxeiptotic response (Moriwaki et al. 2016). Since activated macrophages express a variety of heat-shock proteins (HSP), including HSP60, the γδT cells in O_3-exposed airspaces most likely recognize these HSPs and target macrophages for apoptotic cell death (Hirsh and Junger 2008; Mathews et al. 2015). Despite a growing list of cell death pathways, our current understanding of macrophage cell death in O_3-exposed lungs is still limited.

Cell surface receptors involved in the recognition of ozonation products

Toll-like receptors (TLRs)

TLRs, including TLR2 and 4, are expressed on AM (Fan et al. 2002; Droemann et al. 2003), and their expression increases following O_3 exposure (Oakes et al. 2013). The increased expression of TLR4 on AM likely occurs through an increase in lipid raft colocalization and peripheralization (Garantziotis et al. 2010; Li et al. 2010). O_3-induced inflammatory responses in TLR4 knockout mice have been investigated in multiple studies. Using C3H/HeJ mice with a missense mutation in the *Tlr4* gene, Kleeberger et al. (2000) and Kleeberger et al. (2001) showed that TLR4 mediates *Nos2* mRNA expression, which causes ozone-induced epithelial injury and TNFα expression. Another study revealed that knockdown of TLR4, TLR2 or the intracellular adaptor protein, MyD88, abrogates O_3-induced (3 ppm for 3 h) airway hyper-responsiveness in mice (Williams et al. 2007). Interestingly, the absence of Myd88, but not TLR2 or TLR4 individually, inhibited O_3-induced lung neutrophilia (Williams et al. 2007) suggesting two possibilities: 1) Redundancy of TLR2 and TLR4, 2) Presence of additional triggers, such as IL33 or IL1α/β, that also work through MyD88 signaling (Griesenauer and Paczesny 2017). A second study revealed that although the absence of TLR4 on the C57BL6 background did not provide protection against O_3-induced (0.8 ppm for 3 h) lung injury, it did confer partial protection against airway hyperresponsiveness (Garantziotis et al. 2010). In contrast, Connor et al. (2012) reported that mutation of TLR4 in C3H/HeJ mice results in protection against O_3-induced (2 ppm for 3 h) inflammation, oxidative stress, and classical macrophage activation. The discrepancy in these findings may be attributed to differences in strain backgrounds, O_3 concentrations, or both.

Both TLR2 and TLR4 bind to multiple endogenous ligands including HMGB1, HSP60, HSP70, hyaluronan, monosodium urate crystals, and biglycans (Erridge 2010). In addition, other endogenous molecules, including β-defensin, fibronectin, S100 protein, and SP-A, specifically bind only TLR4. Garantziotis et al. (2009) found increased levels of hyaluronan (HA) in BALF from O_3-exposed mice and mice deficient in CD44, a major receptor for HA, were protected from O_3-induced airway hyper-responsiveness (AHR). Li and colleagues reported that O_3 causes the conversion of HA, a glycosamino-glycan-rich macromolecule found in lung epithelial cell basement membranes, to low molecular weight (LMW)-HA. LMW-HA has been shown to interact with macrophage surface receptors, CD44 and TLR4,

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resulting in proinflammatory cytokine production (Li et al. 2010, 2016; Li et al. 2011). Further, HSP70, another endogenous ligand for TLR4, was elevated in the lungs of O_3-exposed (0.3 ppm for 6–72 h) mice both at the level of mRNA and protein, and HSP70-deficient mice were protected from O_3-induced inflammation (Bauer et al. 2011). Whether other TLR ligands are elevated in O_3-exposed lungs remains unexplored.

Scavenger receptors

O_3 has also been implicated in the production of unique ozonation products including 1-palmitoyl-2-(9′-oxo-nonanoyl)-glycerophosphocholine (PON-GPC) as well as nonspecific auto-oxidation products such as 5β, 6β-epoxy-cholesterol (β-epoxide) and secosterols (Seco A and B) (Almstrand et al. 2015). PON-GPC is known to compromise macrophage viability (Uhlson et al. 2002) and induce release of proinflammatory mediators *via* activation of the 5-lipoxygenase pathway (Zemski Berry and Murphy 2016). Macrophage receptors of the SRA family (scavenger receptor A-I/II (SRA-I/II) and MARCO) have been implicated in the scavenging of oxidized lipids. Dahl and colleagues mechanistically established that β-epoxide and PON-GPC fail to induce neutrophil recruitment in MARCO-deficient mice implicating MARCO as a likely receptor for β-epoxide and PON-GPC. They further reported that mice deficient in MARCO as well as SRA-I/II had more robust O_3-induced lung injury suggesting a protective role for MARCO and SRA-I/II in O_3-induced lung injury (Dahl et al. 2007). CD36, a class B macrophage scavenger receptor, is known to bind to a number of oxidized lipoproteins and phospholipids, and CD36 expression is elevated in mice upon O_3 exposure (Valacchi et al. 2007). Moreover, genetic deletion of CD36 protects against lung inflammation (Robertson et al. 2013; Mumaw et al. 2016).

Ozone and ozonation products: effect on macrophage population size

O_3 exposure results in an increase in total numbers of airspace macrophages; however, the source of this increase is not fully known. Francis and colleagues reported that O_3 exposure induces the recruitment of pro- as well as anti-inflammatory macrophages *via* CCL2(MCP1)-CCR2 ligand-receptor interactions. The recruited pro-inflammatory macrophages promoted lung injury and oxidative stress (Francis, Groves, et al. 2017). In a subsequent study, Francis and colleagues identified the spleen as a source of pro-inflammatory macrophages in mice exposed to 0.8 ppm O_3 for 3 h (Francis, Sun, et al. 2017). These splenic macrophage populations appeared to contribute to O_3-induced inflammation and oxidative stress. In contrast, Tighe and colleagues reported that exposure of mice to 2 ppm O_3 for 3 h did not induce recruitment of either CD11b + exudative macrophages (ExMacs) or CCR2-dependent macrophages (Tighe et al. 2011). Instead, a novel subset of cells derived from CX3CR1-dependent resident macrophages appeared to be protective against O_3-induced pulmonary responses. Thus, the authors concluded that a higher dose of O_3 causes proliferation of local macrophages rather than extrapulmonary mobilization into the lung airspaces. Therefore, further dose-response and time-course studies are required to fully understand macrophage recruitment in response to O_3.

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Macrophage response to ozone and ozonation products

Recently, Ordija et al. (2017) reported that free actin, possibly a necrotic product, is elevated in the BALF of O_3-exposed mice. These authors went on to show that this free actin blocks recognition of bacterial products, likely, *via* competitive binding to SRA-I/II and MARCO on macrophages. Although not yet fully established, it is likely that ozonation/oxidation-induced disruption of multiple surface receptors accounts for poor bacterial clearance.

Cell–cell interactions in ozone-exposed airspaces

Studies using macrophage/epithelial co-culture models are critical to our understanding of how O_3-induced stress is communicated between various cell-types in airspaces and then translated to airway disease. Earlier studies in mice exposed to O_3 (2 ppm for 3 h) resulted in increased expression of MIP2, a potent neutrophil chemoattractant, and CCL2 (MCP1), a potent monocyte chemoattractant, in macrophages (Driscoll et al. 1993; Zhao et al. 1998).

Manzer et al. (2008) studied the effect of mediators derived from O_3-exposed macrophages on epithelial cells. *In vitro* exposure of macrophages to O_3 (100 ppb for 1 h) induces release of IL1α, IL1β, IL6, IL18, and GM-CSF into the culture medium. Moreover, an alveolar epithelial cell culture stimulated with macrophage-conditioned media for 24 h was shown to release CCL2 (MCP1) and CXCL1 (KC), key chemoattractants for monocytes and neutrophils, respectively. These *in vitro* and *in vivo* studies suggest there is a flow of information between O_3-exposed macrophages and epithelial cells; however, whether these interactions are macrophage→epithelium (unidirectional) or macrophage←→epithelium (bidirectional) is not clear. Further, it remains to be tested whether macrophage-derived inflammatory mediators communicate in an autocrine or paracrine manner with neighboring cells including neighboring naïve macrophages, other myeloid cells, and epithelial cells.

Bauer and colleagues studied the effect of O_3-exposed epithelial-derived mediators on macrophages (Bauer et al. 2015). This study revealed an interesting interaction between epithelial cells and macrophages involving the release of hyaluronic acid (HA; also known as hyaluronan), a collagen synthesis marker, shown to be elevated in the BALF from volunteers exposed to O_3 as well as O_3-exposed mice (Garantziotis et al. 2009, 2016; Garantziotis et al. 2010). While the levels of HA did not increase in O_3-exposed epithelial monocultures, HA levels were significantly elevated in O_3-exposed epithelial/macrophage co-cultures. The study also revealed that, in contrast with macrophages in monoculture, macrophages co-cultured with airway epithelial cells had increased alternative activation, poor phagocytosis, and enhanced cytotoxicity to O_3 (Bauer et al. 2015). Further, comprehensive profiling of macrophage- and epithelium- (airway *versus* alveolar) derived mediators in *in vivo* models is critical to understand the roles of macrophages and the epithelium in modulating airspace responses to O_3. Since paracrine, autocrine, and contact-dependent cell–cell interactions likely orchestrate O_3-induced airspace perturbations, the co-culture model should be customized to identify the nature of the cell–cell interactions in airspaces.

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Although airspace macrophage populations are composed primarily of motile subpopulations, a subpopulation of sessile macrophages bound to alveolar epithelium has also been reported (Westphalen et al. 2014; Bhattacharya and Westphalen 2016). Using an LPS-induced lung injury model, Westphalen and colleagues identified connexin 43 (Cx43)-mediated syncytial communication channels between sessile macrophages and alveolar epithelial cells that suppress the release of proinflammatory cytokines (Westphalen et al. 2014; Bhattacharya and Westphalen 2016). Whether such interactions play a role in O_3-induced airway pathogenesis is currently unclear.

Conclusions

While major cellular and molecular players in O_3-induced airway diseases have been identified, little is known about the exact sequence in which they are involved in airway disease pathogenesis. Between the two primary responders in airspaces, epithelial cells and macrophages, the exact cascade of events resulting in pulmonary pathology is poorly understood. Since diffusion of O_3 across the ELF towards epithelial cells is a rate-limiting step, and because macrophages are motile cells, it is highly likely that macrophages encounter O_3 and/or ozonation/oxidation products well before epithelial cells do; however, this notion remains to be tested.

It is fairly well understood that macrophage populations in airspaces are highly plastic and manifest remarkable heterogeneity in the inflammatory microenvironment. The macrophages localized to the airspace compartment with the highest concentration of dissolved O_3, and therefore the highest concentration of ozonation products, most likely differ from the macrophages localized in airspace compartments with lower levels of O_3 deposition. This heterogeneity is further compounded during airspace inflammation when macrophage precursors in various stages of differentiation populate the airspaces. Thus, further studies to understand the anatomic distribution of O_3 and its products in airspaces (trachea, airways, and alveoli) may aid in our understanding of macrophage responses. Comprehensive studies, including acute *versus* chronic and low-dose *versus* high-dose of O_3 exposure, should be conducted to understand macrophage recruitment or localized proliferative responses following O_3 exposure.

Future studies with an intense focus on the cell-specific roles of receptors, cytokines, and inflammatory mediators will provide further information on the initiation, progression, and resolution of O_3-induced airway diseases. Controlled experimental models, such as co-culture models and gene-knockout mice, may be employed to further our understanding of the complex interactions between O_3 and lung airspaces. Together, these studies will have a transformative impact on the development of therapeutic strategies against O_3-induced airway diseases.

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Abbreviations:

O3	Ozone
NAAQS	National Ambient Air Quality Standards
ppm	parts per million
COPD	chronic obstructive pulmonary disease
AM	alveolar macrophage
IM	interstitial macrophage
ELF	epithelial lining fluid
iNOS	inducible nitric oxide synthase
SOD	superoxide dismutase
ROS	reactive oxygen specie

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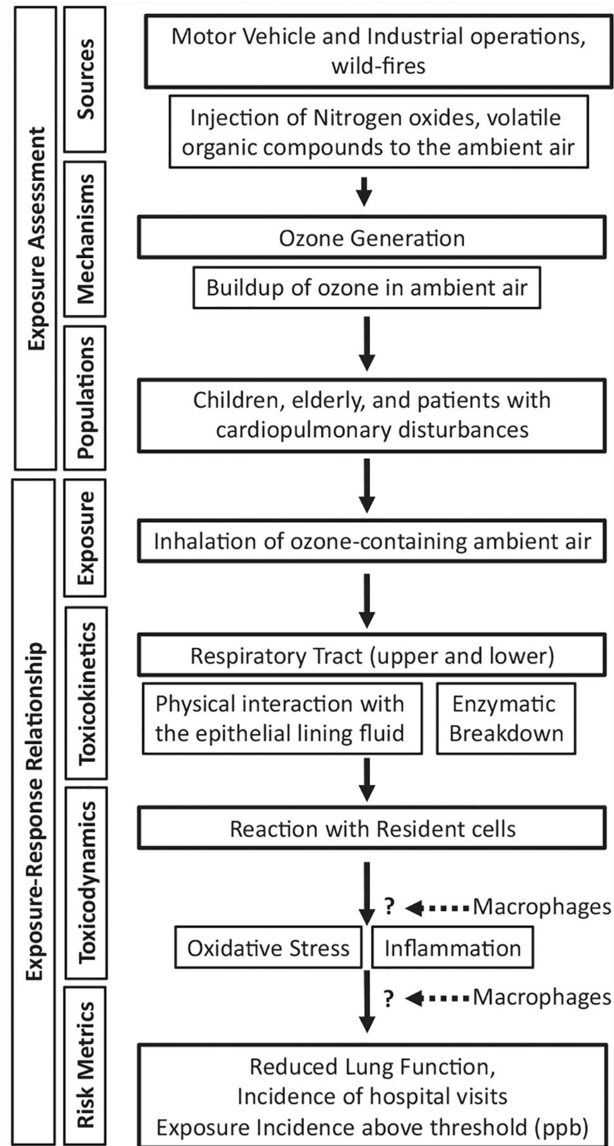

Figure 1.
Risk assessment paradigm for ozone.

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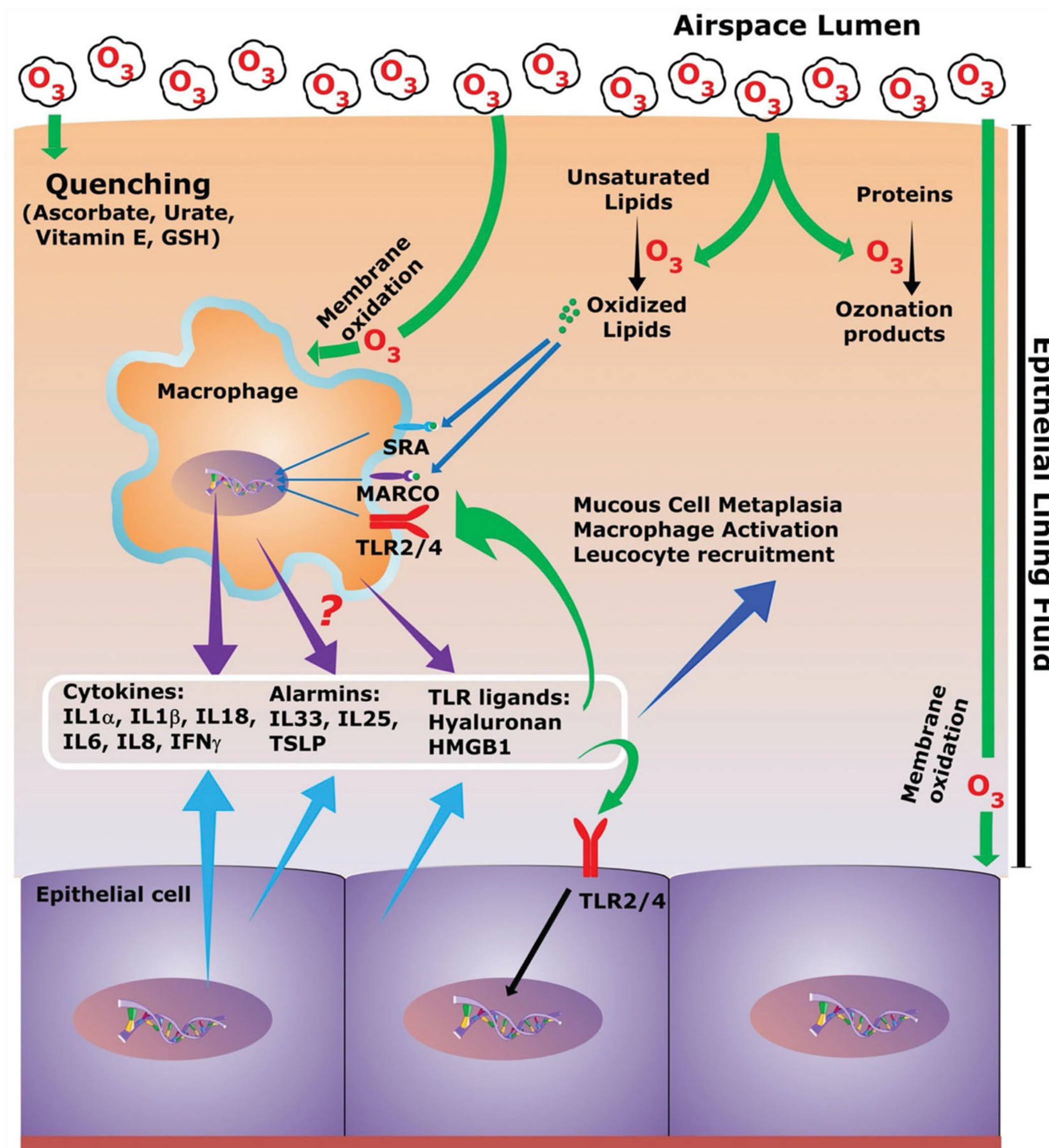

Figure 2.

Schematic of the ozone life cycle in airspaces and the potential effects on airspace cells. Inhaled ozone molecules interact with the epithelial lining fluid (ELF) in the airway and alveolar compartments. Soluble ozone is either quenched by the antioxidant system of the ELF or reacts with lipids and proteins to form ozonation products. Ozone molecules also oxidize membrane proteins and lipids of epithelial cells and macrophages. Surface receptors such as TLRs, MARCO, and SR-A on macrophages are known to bind oxidized lipids. Resident cells, macrophages, and epithelial cells respond to the presence of ozone and ozonation products through the release of cytokines, alarmins, and Toll-like receptors (TLRs) ligands, which leads to multiple outcomes including immune cell recruitment, macrophage activation, and epithelial remodeling.

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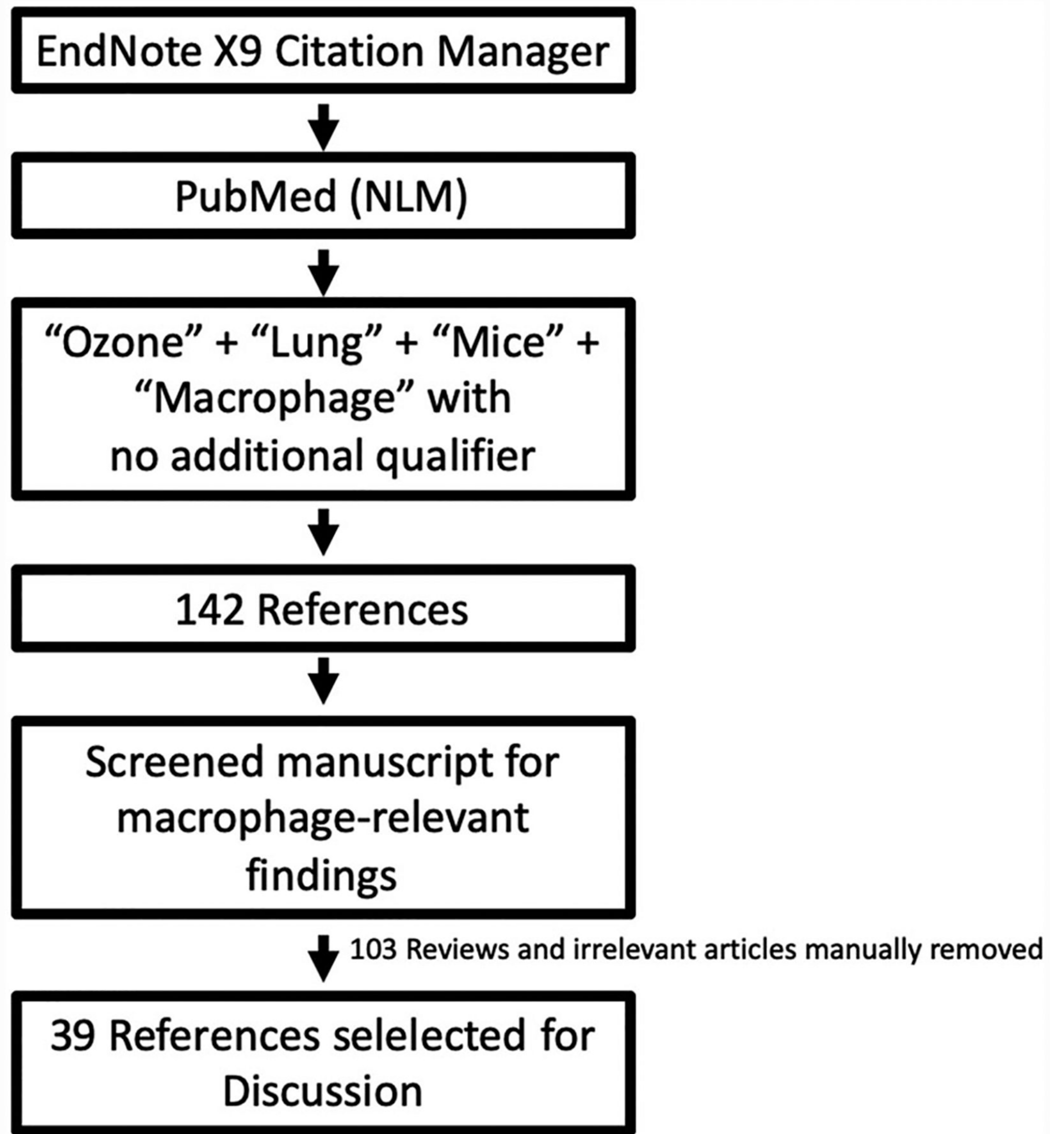

Figure 3.
Screening strategy for review of literature.

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Table 1.

Selected mouse model-based studies with focus on macrophage responses to ozone exposure.

	Dose (ppm)	Duration	Endpoint (h/d post exposure)	Findings	References
1	0.1–3	3 h	0h	Ozone exposure decreases superoxide anion radical production in mouse alveolar macrophages	Ryer-Powder et al. (1988)
2	0.4–0.8	3 h	20 d	Alveolar macrophages from ozone-exposed mice exhibit poor clearance of *Streptococci*	Gilmour et al. (1993), Gilmour and Selgrade (1993)
3	0.1–2	3 h	6h	Ozone exposure induces expression of MIP2 in macrophages	Driscoll et al. (1993)
4	1.5	4 h	24 h	Ozone suppresses phagocytic responses of macrophages	Jakab and Hemenway (1994)
5	2	3 h	4–72 h	Monocyte influx in ozone-exposed airways is mediated through MCP1	Zhao et al. (1998)
6	0.8	3h	24–48 h	Macrophages from ozone-exposed mice with iNOS deficiency do not produce nitric oxide or nitrotyrosine in lungs	Laskin et al. (2001)
7	1	6 h/d × 3d	24–48 h	The deficiency of iNOS does not protect against ozone-induced tissue injury	Kenyon et al. (2002)
8	0.8	3h	24–48 h	Ozone exposure induces nitric oxide production and iNOS expression in alveolar macrophages	Laskin et al. (2002)
9	0.5	24 h	2h	Ozone exposure compromises release of IL6 from LPS-challenged macrophages	Yu et al. (2002)
10	0.8	3h	24–48 h	iNOS expression is elevated in alveolar macrophages from ozone-exposed mice and iNOS deficiency protects against ozone-induced tissue injury	Fakhrzadeh, Laskin, et al. (2004)
11	0.8	3h	24–48 h	Overexpression of superoxide-dismutase (SOD), an antioxidant enzyme, confers protection against oxidant stress and ozone-induced lung injury	Fakhrzadeh, Laskin, Gardner, et al. (2004)
12	0.3	48 h	0h	Scavenger receptors (MARCO and SR-AI/II) on macrophages scavenge oxidized lipids and mitigate inflammatory response to ozone	Dahl et al. (2007)
13	2	3h	4h	Alveolar macrophages from ozone-exposed mice show elevated apoptosis and functional response to endotoxin	Hollingsworth, Maruoka, et al. (2007)
14	0.8	3h	12 h	TNFα suppresses caveolin 1 expression in alveolar macrophages	Fakhrzadeh et al.(2008)
15	2	3h	0h	Ozone exposure reduces phagocytic capabilities of macrophages	Mikerov, Haque, et al. (2008)
16	2	3h	0h	Ozone-exposed mice with SP-A deficiency exhibit poor macrophage phagocytic potential in *Klebsiella pneumonia* infection	Mikerov, Gan, et al. (2008)
17	3	3h	21 –24 h	Ozone-induced macrophage recruitment to lungs is attenuated in IL13 and IL4/13 knockout mice	Williams et al. (2008)
18	2	3h	24 h	Macrophages from ozone-exposed mice express CD44, a hyaluronan (HA) receptor	Garantziotis et al. (2009)
19	2	3h	4h	Surfactant protein A (SP-A) plays a critical role in scavenging ozone-induced reactive oxidants and mitigates oxidative stress	Haque, Umstead, Freeman, et al. (2009)
20	2	3h	24 h	Ozone exposure increases TLR4 expression on lung macrophages	Garantziotis et al. (2010)
21	1	3h	24 h	Ozone exposure induces TLR4 peripheralization in macrophages and enhances their responsiveness to LPS	Li et al. (2010)

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	Dose (ppm)	Duration	Endpoint (h/d post exposure)	Findings	References
22	2	3h	24 h	Short-term exposure of mice to 2 ppm ozone for 3 h induces appearance of a novel subset of resident-intermediate derived macrophages that appear to be protective against ozone-induced pulmonary responses	Tighe et al. (2011)
23	0.8	3h	0.5, 12, 24, 48 h	Macrophage activation is compromised in the absence of TLR4	Connor et al. (2012)
24	0.8	3h	72 h	Mice deficient for surfactant protein D (SP-D) exhibit exaggerated macrophage recruitment and activation responses upon ozone exposure	Groves et al. (2012)
25	1	4h	24 h	Deletion of CD36, a macrophage scavenger receptor, protects against ozone-induced lung inflammation	Robertson et al. (2013)
26	2	3h	4/24 h	Ozone induces expression of TLR1/2/4 on alveolar macrophages	Oakes et al. (2013)
27	0.3	24–72 h	24 h	Γδ T cell-derived IL17A induces alternative activation of macrophages that promotes clearance of apoptotic cells and resolution of inflammation	Mathews et al. (2014)
28	0.8	3h	24 h	Galectin-3 expressing proinflammatory macrophages accumulate in ozone-exposed lungs and promote lung toxicity	Sunil et al. (2015)
29	0.7	72 h	24 h	Alveolar macrophages from ozone-exposed mice exhibit higher levels of mitochondrial ROS, elevated cytosolic mtDNA, increased caspase-1 activation, and increased IL-1β production	Che et al. (2016)
30	2	3h	4/24 h	Ozone-induced lung injury is comparable between wildtype and plasminogen activator inhibitor-1 (PAI-1) knockout mice	Elkhidir et al. (2016)
31	3	3 h/twice a week (6 weeks)	24 h	Ozone exposure induces macrophage migration inhibitory factor (MIF) release, which promotes lung inflammation	Russell et al. (2016)
32	0.8	3h	24 h	Spleen acts as a source of pro-inflammatory macrophages in ozone-exposed lungs, which contribute to inflammation and oxidative stress	Francis, Groves, et al. (2017)
33	0.8	3h	24 h	Ozone induces recruitment of macrophages *via* CCL2/MCP1-CCR2 ligand-receptor interactions, which promotes lung injury and oxidative stress	Francis, Sun, et al. (2017)
34	2	3h	4 and 18h	Surfactant protein A2 provides microRNA-mediated protection against oxidative stress in ozone-exposed males, but not females	Noutsios et al. (2017)
35	2	3h	24 h	Ozone induces release of free-actin into the ELF, which competitively inhibits microbial interactions with SRA/MARCO receptors	Ordija et al. (2017)
36	1	1h	24 h	Acute ozone exposure recruits IL-33 expressing macrophages to the lungs. Absence of IL-33 receptor increases macrophage recruitment to the lungs	Michaudel et al. (2018)
37	2	3h	4h	Alveolar macrophages from females are more susceptible to ozone-induced oxidative stress. SP-A provides protection in males *via* regulation of microRNAs involved in protection against oxidative stress	Thorenoor et al. (2019)
38	1 and 2	3h	21 h	Acute ozone exposure alters gene expression in alveolar macrophages	Tovar et al. (2020)