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Ambient ozone and pulmonary innate immunity

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Abstract

Ambient ozone is a criteria air pollutant that impacts both human morbidity and mortality. The effect of ozone inhalation includes both toxicity to lung tissue and alteration of the host immunologic response. The innate immune system facilitates immediate recognition of both foreign pathogens and tissue damage. Emerging evidence supports that ozone can modify the host innate immune response and that this response to inhaled ozone is dependent on genes of innate immunity. Improved understanding of the complex interaction between environmental ozone and host innate immunity will provide fundamental insight into the pathogenesis of inflammatory airways disease. We review the current evidence supporting that environmental ozone inhalation: (1) modifies cell types required for intact innate immunity, (2) is partially dependent on genes of innate immunity, (3) primes pulmonary innate immune responses to LPS, and (4) contributes to innate-adaptive immune system cross-talk.

Keywords

environmental; toll-like receptor; TLR4; CD44; hyaluronan; asthma; genetic; gene x environment

AMBIENT OZONE IS AN IMPORTANT PUBLIC HEALTH CONCERN

Ozone is an ambient gas, composed of three oxygen atoms, that is formed through a chemical reaction between oxides of nitrogen and volatile organic compounds in the presence of sunlight. Since the Clean Air Act of 1970, the EPA has identified ambient ozone as a criteria pollutant with adverse health effects. Inhalation of ozone significantly contributes to both human morbidity and mortality and is estimated to account for approximately 800 premature deaths, 4,500 hospital admission and 900,000 school absences annually. It causes more than a million days of restricted activity with an annual economic burden estimated at \$5 billion¹. Particularly vulnerable populations include; individuals with underlying respiratory disease (asthma, COPD), children, and adults over the age of 65. There does not appear to be a threshold level of ozone below which there are no detrimental health effects^{2–4}. Each 10ppb increase in daily ozone is associated with an approximately 0.87% increase in total mortality⁵. Therefore, stricter regulatory standards would not be predicted to completely eradicate the adverse health effects of ambient ozone. Furthermore, it is anticipated that the levels of ambient ozone will increase with climate change^{6–7}, which will present new challenges to enforcing current regulatory standards and could have substantial impact on global health. Independent of our ability to lower ground-level ozone concentrations, understanding mechanisms by which inhalation of ambient ozone adversely impacts human health will greatly improve our ability to treat airways disease.

AMBIENT OZONE AND IMMUNOLOGY

The lung is uniquely vulnerable to ambient toxicants by virtue of its continuous exposure to the external environment. Inhalation of ambient ozone exacerbates respiratory disease and can modify both the innate and adaptive immune responses. A growing body of evidence exists supporting the ability of ambient ozone to not only cause direct tissue injury through generation of reactive oxygen species, but also to modify complex immunological responses. Ambient ozone can modify the function of many cell types within the lung which are critical regulators of both innate and adaptive immunological responses.

The pulmonary immune surveillance system is highly efficient in eradicating inhaled foreign material and pathogens. Protection of the host is facilitated through a complex interaction between effective barrier function, innate immunity, and acquired/adaptive immunologic responses. Early studies of health effects of ozone inhalation were focused on either defects in epithelial barrier function or adaptive/acquired immunity. Recent evidence supports a role for genes of innate immunity in the response to inhaled ambient ozone. We now appreciate that the response to ambient ozone is dependent on a complex interaction between both innate and adaptive immunity. The innate immune system is a highly conserved evolutionary system used to identify threatening microorganisms. The innate immune system is activated through a combination of direct pathogen recognition, by pathogen-associated molecular patterns (PAMP), and through recognition of host factors associated with tissue damage or damage-associated molecular patterns (DAMP). PAMPs and DAMPs are recognized by germ-line encoded receptors of innate immunity and are broadly classified as pattern recognition receptors (PRR). The primary focus of this review is to understand the interaction between inhalation of ambient ozone and host innate immunity (Figure 1).

MULTIPLE CELL TYPES REQUIRED FOR INTACT INNATE IMMUNITY ARE MODIFIED BY OZONE INHALATION

Pulmonary innate immunity is dependent on a complex signaling network between many cell types⁸. Our basic understanding of this signal network has been derived primarily by studies analyzing the effects of bacterial lipopolysaccharide (LPS) binding to the surface PRR, toll-like receptor 4 (TLR4). LPS stimulation through TLR4 causes activation of NF- κ B and directs a cascade of pro-inflammatory events directed towards effective antibacterial host defense. It is increasingly evident that many cell types within the lung express TLR4 and can respond to stimulation by TLR4 ligands⁹. Hematopoietic-derived, TLR4-expressing cells in the lung include; macrophages¹⁰, neutrophils¹¹, lymphocytes¹², and dendritic cells¹³. In addition, non-hematopoietic-derived cells, such as airway epithelia^{14–16} and endothelia¹⁷ both express TLR4 and can respond to stimulation by LPS. While the complete signaling network required for intact innate immunity remains elusive, it is clear that inhalation of ozone can modify many cell types required for this signaling network. To facilitate a better understanding of the potential mechanisms by which ozone can modify pulmonary innate immunity, we review the current understanding of the effects of ozone on each relevant cell type.

Airway Epithelia

Airway epithelium is of critical importance to innate defense in the lung. It functions as both a barrier and as a regulator of immune response. Acute inhalation of ozone causes direct damage to airway epithelia resulting in loss of ciliary function¹⁸, increased airway epithelial permeability^{19–20}, and defects in mucociliary clearance²¹. These structural alterations in the respiratory epithelium contribute to impaired clearance of foreign material and microbes, thereby enhancing vulnerability to pulmonary infections.

In addition to its barrier function, the epithelium has a direct role in regulating the innate immune response^{22–23}. Toll-like receptors are expressed on the surface of epithelial cells^{24–25}. Despite this expression, the functional role of TLR receptors on airway epithelia remains somewhat unclear. There appear to be both direct and indirect mechanisms of activating airway epithelia dependent on the type of environmental challenge. Ozone can activate innate immune signaling in airway epithelia but also involves multiple mechanisms. Similar to human exposures²⁶, *in vitro* stimulation of immortalized human epithelial cell lines with ozone resulted in production of interleukin 6 (IL-6) and interleukin 8 (IL-8) under most^{27–31}, but not all experimental conditions^{32–33}. Given the high reactivity of ozone and low solubility in media, it remains unclear whether the effects of ozone on epithelia are direct or indirect³⁴. Ozone-induced modification of lipids can act as signal transduction molecules^{35–36} and more recent observations support an indirect effect of ozone on epithelia through macrophage-derived IL-1 α ³⁷. The latter findings support cross-talk between macrophages and epithelial cells in responses to inhaled ozone. Regardless of the specific mechanism, it is clear that airway epithelia are an important cell type for innate immune response and can be affected by exposure to ozone.

Macrophages

Growing evidence supports the importance of macrophages in pulmonary innate immunity. Macrophages appear exquisitely sensitive to ozone³⁸. Early results demonstrated that *in vitro* exposure of human alveolar macrophages to ozone resulted in decreased phagocytosis of particulate immune complexes, enhanced production of prostaglandin E2, increased superoxide production³⁹, and impaired antimicrobial host defense^{40–41}. However, additional studies of direct ozone exposure to alveolar macrophages, generate inconsistent results with either enhanced^{42–44} or reduced cytokine production^{27, 45–46}. It is unclear whether *in vitro* exposures reconstitute the complete biological response to ozone, as many factors observed in the bronchial alveolar lavage (BAL) fluid after ozone inhalation are derived from other cell types or the extracellular environment. For example, our recent studies support that ozone-induced lung injury is associated with increased levels of the damage-associated molecular pattern (DAMP), hyaluronan, which can induce macrophage-derived production of pro-inflammatory cytokines⁴⁷. Together, these findings suggest that macrophages are not only sensitive to direct exposure to ozone and but can also undergo indirect stimulation through soluble mediators released after inhalation of ozone.

Ozone inhalation can also enhance the expression of macrophage pattern recognition receptors. For example, macrophage receptor with collagenous structure (*marco*) is increased on alveolar macrophages after ozone exposure and functions to promote the uptake of surfactant-derived oxidation products in the epithelial lining fluid^{48–49}. Deficiency of *marco* resulted in enhanced oxidative stress and worsening airway hyperresponsiveness (AHR). This study suggest that inhalation of ozone can enhance lung macrophage antioxidant function through a new population of macrophages that express the scavenger receptor *marco*. This finding suggests a protective role for lung macrophages after ozone exposure in contrast to the adverse effect suggested by ozone-induced macrophage cytokine production.

Neutrophils

Neutrophils are recruited into the airways within 6-hours of exposure to ambient ozone⁵⁰. Despite this observation, the functional importance of pulmonary neutrophils in response to ozone remains unclear. Neutrophils generate both reactive oxygen species and neutrophil elastase, which help facilitate the clearance of bacterial pathogens. Despite increased numbers of neutrophils after ozone inhalation, bacterial clearance is impaired after ozone exposure under some⁵¹ but not all conditions⁵². Furthermore, the role of neutrophils in the

development of ozone-induced AHR remains inconsistent. Some studies support that neutrophils are required for ozone-induced AHR^{53–55}, while others do not^{56–60}. These observed discrepancies may, in part, reflect differences in the neutrophil depletion methods and the ozone exposure protocols. For example, one study demonstrated that the early AHR response to ozone is neutrophil independent, while the later response is neutrophil dependent⁵⁰. Clarification of the functional role of the neutrophil after ozone inhalation is necessary.

Lymphocytes

Lymphocytes are important to both immune function and the biological response to ambient ozone. Lymphocytes are generally regarded to contribute to adaptive immunity, but can additionally contribute to the innate immune response⁶¹. Inhalation of ozone has been associated with reduced weights of lymphoid organs^{62–64} and suppressed T-lymphocyte dependent immunologic response^{65–66} suggesting a toxic effect of ozone on lymphocytes. Lymphocytes additionally modulate the complete response to ozone as supported by the altered response observed in animals deficient in CD4+ lymphocytes⁶⁷. More recent evidence support a role of both $\gamma\delta$ T cells and invariant natural killer T (iNKT) cells in the response to ozone. $\gamma\delta$ T cells reside in the epithelial layer of the lung and are critical for innate host defense against pathogens and tumor cells⁶⁸. $\gamma\delta$ T cell-deficient mice exposed to ozone exhibit increased epithelial injury⁶⁹ and reduced AHR⁷⁰. The development of AHR in $\gamma\delta$ T cell-deficient mice was dependent on TNF- α ⁷⁰. Additionally, iNKT-cells are increased after ozone inhalation and are an important source of pro-inflammatory cytokines⁷¹. iNKT cells are required for murine ozone-induced AHR in a manner dependent on IL-17⁷². Together, these observations suggest that both $\gamma\delta$ T cells and iNKT cells can contribute to the response to ozone through production of pro-inflammatory cytokines including either TNF α or IL-17. The role of specific cytokines in response to ozone is discussed in more detail later in the article. Evidence supports that lymphocytes are sensitive to the toxic effects of ozone and both $\gamma\delta$ T cells and iNKT are important in the complete response to ozone.

GENES OF INNATE IMMUNITY AND AMBIENT OZONE

It is clear that many cell types contribute to the complete response to inhaled ozone and that a complex signaling network between cells is required. However, the extracellular factors which are either released or modified by ozone which then alter the pulmonary innate immune system remain poorly understood. We will review evidence supporting that activation of innate immunity contributes to intracellular signaling and the production of soluble pro-inflammatory factors associated with the complete response to inhaled ozone. Overall, the complete response to ozone is dependent on a complex interaction with the innate immune system.

Surfactant Protein A (SP-A)

Collectins are a class of proteins present in the airspace that contain both collagen-rich domains and lectin domains including; mannose binding lectin (MBL), surfactant protein A (SP-A), and surfactant protein D (SP-D). Collectins generally have dual innate immune functions in the airspace, either a direct function as an opsonin to facilitate clearance of foreign material/pathogens or indirectly to attenuate production of pro-inflammatory cytokines⁷³. Both SP-A and SP-D are recognized as potent inhibitors of lipid peroxidation and oxidative cellular injury⁷⁴. Previous work suggests that ozone-induced modification of lipids can act as signal transduction molecules^{35–36}. Therefore, it is not necessarily surprising that susceptibility to ozone-induced airway inflammation is associated with low levels of *sp-d*⁷⁵ and that *sp-a* deficient mice are more susceptible to detrimental effects after

inhalation of ozone⁷⁶. While there is likely an important role for SP-D in response to ozone, the majority of recent work has focused on SP-A.

The role of SP-A in pulmonary innate immunity is diverse including; macrophage chemotaxis⁷⁷, bacterial phagocytosis^{78–82}, and suppression of pro-inflammatory cytokines⁸³. Studies in *sp-a* deficient animals identify a central role of SP-A in both anti-viral and anti-bacterial host defense^{76, 84–89}. *sp-a* deficient mice are more susceptible to ozone-induced lung injury^{88, 90}. However, the precise mechanisms by which SP-A protects the lungs from oxidant-induced cellular injury remain unclear. Interestingly, we now recognize that in addition to the protective effects of SP-A against oxidant-induced cellular damage, the function of SP-A itself can be impaired by oxidation^{91–93}. Inhalation of ozone impairs the functional interaction between SP-A and either lung macrophages or alveolar epithelia^{94–95}. Similarly, oxidation of SP-A impairs binding to LPS and bacterial pathogens⁹⁰ and hence leads to ineffective macrophage phagocytosis of bacterial pathogens^{76, 79–80, 90}. Individuals with the *SP-A2* gene variant seem to be more susceptible to the effects of oxidation⁹⁰. Together these findings support that SP-A can provide protection against ozone-induced lung injury. However, inhalation of ozone can itself modify SP-A function and host genetic differences in SP-A may contribute to the variation in response of individuals to inhaled ambient ozone.

Toll-like Receptor 4 (TLR4)

Toll-like receptors are a highly evolutionarily conserved family of pattern recognition receptors (PRR). The role of toll receptors in the innate immune response was initially identified in the anti-fungal host defense of the *Drosophila* Toll⁹⁶. The mammalian homologue, *tlr4*, was recognized to be the exclusive bacterial LPS PRR in mice⁹⁷. The original observation that *tlr4* may play a role in ozone-induced lung injury was made through a forward genetic approach using the C3H/HeJ mouse⁹⁸. Additionally, studies in genetically engineered *tlr4*-deficient mice¹⁰ identified that *tlr4* contributes to ozone-induced AHR^{99–100}. We speculated that inhalation of ozone would result in release of endogenous ligands of TLR4. One such candidate was the glycosaminoglycan, hyaluronan, which is present in relatively high levels in the lung. Previous work supported that fragmented hyaluronan could be immunostimulatory to macrophages¹⁰¹ and could additionally function as an endogenous ligand of *tlr4*¹⁰². Hyaluronan fragments are important in other forms of oxidative lung injury including both bleomycin^{101, 103} and asbestos¹⁰⁴. We identified that hyaluronan fragments were increased in the airspace after ozone inhalation and contribute to ozone-induced AHR⁴⁷. Furthermore, ozone-induced hyaluronan fragments function as an endogenous ligand of the *tlr4* induction of AHR¹⁰⁵. Additional putative endogenous TLR-ligands are also induced by exposure to ozone. The functional consequences of many of these molecules in context of response to ozone are largely unknown. However, preliminary data suggests a potential role for hsp70 in regulation of ozone induced inflammation in a manner dependent on *tlr4*¹⁰⁶. Future studies will help to further characterize these endogenous factors which activate surface receptors of pulmonary innate immunity in context of ozone exposure.

Surface ligation of the TLR4 receptor results in activation of intracellular signaling¹⁰⁷. After inhalation of ozone, *tlr4*-dependent intracellular signaling is partially dependent on the intracellular adaptor molecule MyD88¹⁰⁰. A growing body of work supports the role of NF- κ B-dependent signaling in the complete response to ozone^{108–110}. Ozone-induced NF- κ B signaling appears important in the production of reactive nitrogen intermediates and TNF- α , thereby contributing to tissue injury¹¹¹. After inhalation of ozone, there is an increased binding of the p50 subunit of NF- κ B to DNA within the nucleus of both alveolar macrophages and epithelial cells. Targeted disruption of the p50 subunit of NF- κ B resulted in reduced levels of peroxynitrite and protection from ozone-induced lung injury¹¹².

Additionally, nitric oxide synthase 2, (*nos2*), an important source of reactive nitrogen species, contributes to the response to ozone^{98,113} and the expression of *nos2* is partially dependent on NF- κ B^{110, 113}. It is therefore, intriguing that ozone-induced expression of inducible nitric oxide synthase (iNOS) is reduced in the C3H/HeJ mouse (*tlr4*-mutant strain)⁹⁸. This finding suggested that ligation of the surface receptor TLR4 results in NF- κ B dependent gene transcription. We now know that short fragments of hyaluronan appear to drive the *tlr4*-dependent activation of NF- κ B¹⁰⁵.

Together these findings support that inhalation of ozone results in the release or production of endogenous ligands to TLR4, the prototypic receptor of innate immunity. Surface ligation of this receptor results in recruitment of intracellular adaptor proteins, including MyD88, required for TLR4-dependent signaling. TLR4-dependent signaling results in translocation of NF- κ B to the nucleus and transcription of numerous pro-inflammatory factors associated with ozone-induced lung disease. The specific mechanisms that lead to ozone-induced AHR remain an area of considerable interest.

Tumor Necrosis Factor α

TNF α is a prototypic secreted factor released during activation of the innate immune system. The role of TNF α in response to ozone was initially identified through a genome-wide linkage analysis utilizing divergent strains of mice¹¹⁴. This important study supported TNF α contribution to both neutrophil recruitment into the lung and epithelial proliferation after exposure to ozone. Subsequent studies demonstrate a role of TNF α in ozone-induced AHR in mice^{115–116}. This cytokine appears to play a central role in intracellular signaling after exposure to ozone. Loss of the TNF α receptor impairs ozone-induced activation of NF- κ B and MAPK/AP-1 pathways, which are each important to the complete biological response to ozone¹¹⁷. The functional role of TNF α in the human response to ozone remains unknown but is suggested as a common variant of TNF α was associated with response to ozone in human subjects¹¹⁸. Collectively, these data further support the role of innate immune activation in the response to ozone. Clear understanding of the mechanisms, by which, TNF α modifies the complete response to ozone in both animal models and human subjects will provide considerable insight into the pathogenesis of ozone-induced airways disease.

Interleukin-1

IL-1 is a pro-inflammatory cytokine that contributes to both acute and chronic inflammation. There are three members of the IL-1 gene family, IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1Ra). The available evidence supports a role for IL-1 signaling both in the development of ozone induced lung injury and also ozone induced AHR. IL-1 β appears to have a central role in the development of inflammatory responses. IL-1 β is primarily derived from cells of a monocytic lineage¹¹⁹ and is released by macrophages after direct exposure to ozone⁴². IL-1 β is recognized to increase both cyclooxygenase (COX)-2 expression and prostaglandin E₂ resulting in decreased β -adrenergic responsiveness of smooth muscle cells providing a potential link to AHR¹²⁰. Administration of an IL-1 receptor antagonist protected mice against ozone-induced AHR, cellular inflammation, epithelial injury, production of several pro-inflammatory cytokines, and ozone-enhanced airway smooth muscle responses^{121–122}. The specific disruption of the type 1 IL-1 receptor attenuated ozone-induced AHR^{123–124}. Collectively, these findings support a central role of IL-1 dependent signaling in response to ozone.

Interleukin-6

The pleiotropic cytokine, IL-6, is reported to have either pro-inflammatory or anti-inflammatory effects depending on the context^{125–126}. In the lungs, transgenic over-

expression of IL-6 in the absence of any exposure results in a decrease in airway hyper-responsiveness¹²⁷, which appears related to airway structural changes¹²⁸. After inhalation of ozone, IL-6 is released into the airspace^{129–131} and is synthesized by both macrophages and epithelial cells^{27, 42}. In context of ozone inhalation, IL-6 appears to contribute to airway epithelial injury¹³², recruitment of neutrophils into the airspace, and expression of TNF α ⁵⁰. These findings support that IL-6 contributes to ozone-induced lung inflammation and epithelial injury, but not AHR.

KC

KC, the mouse counterpart to human IL-8, is a member of the family of chemotactic cytokines (chemokines). KC is thought to play a role in the recruitment and activation of leukocytes during inflammatory responses. KC expression in the lung can be induced by either TNF α ¹³³ or exposure to ozone¹²². Interestingly, ozone-induced expression of KC is dependent on TLR4, TLR2, and MyD88¹⁰⁰. Only indirect evidence suggests that KC contribute to the response to ozone. Previous work supports that CXCR2, the receptor for KC and MIP2, is required for PMN recruitment and AHR after exposure to ozone⁵⁰.

Interleukin 17

IL-17 is pro-inflammatory cytokine commonly associated with allergic responses and induction of many other cytokines (including; IL-6, IL-1 β , TNF α)¹³⁴. IL-17 has been found to play a role in the biological response to ozone. It was demonstrated that the number of IL-17 producing α NKT cells in the lung is increased after ozone exposure and ozone-induced AHR is dependent on both IL-17 and α NKT cells⁷².

Complement

Complement activation is another highly evolutionarily conserved component of the innate immune system. Human exposure to ozone resulted in an increased level of C3a in BAL fluid¹³⁵. Depletion of complement during ozone challenge attenuates both neutrophil recruitment into the lung, pro-inflammatory cytokine production, and AHR⁵⁸. Interestingly, the effect of complement on AHR was independent of neutrophil recruitment into the airspace⁵⁸. While we recognize that both C3a and C5a can activate mast cells^{136,137}, the effect with ozone appears to be independent of mast cells^{58, 138}. The mechanisms and cell types affected by complement after exposure to ozone remain largely unknown.

OZONE PRIMES PULMONARY INNATE IMMUNITY

We have focused on the importance of genes of innate immunity in the biological response to ozone. However, recent evidence support that inhalation of ozone can additionally modify subsequent innate immune response to microbial pathogens. This is particularly important because we typically encounter pathogens in context of multiple inhaled toxicants. Effective clearance of microbial pathogens requires both recognition of foreign antigens and a controlled inflammatory response. Exaggerated initial responses to pathogens can result in enhanced lung injury and adverse clinical outcomes. Thus, a considerable interest exists to understand the complex interplay between commonly encountered inhaled environmental toxins and the pulmonary innate immune system.

Inhalation of ozone can directly impair antibacterial clearance and pathogen killing. Previous studies demonstrate that inhalation of ozone can impair clearance of multiple pathogens including; *Staphylococcus aureus*¹³⁹, *Streptococcus pyogenes*¹⁴⁰, *Streptococcus zooepidemicus*⁴¹, *Klebsiella pneumoniae*¹⁴¹, *Mycobacterium tuberculosis*¹⁴², and *Listeria monocytogenes*⁶⁶. Altered vulnerability to these bacterial pathogens suggests that ambient ozone could directly modify innate immune function in the lung. Clear understanding of the

mechanisms, by which, ozone modifies antibacterial host defense could provide insight into the mechanisms which regulate the enhanced morbidity and mortality observed in human populations after exposure to higher ambient ozone concentrations.

Previously we outlined that ozone modifies alveolar macrophage function by impairing phagocytosis, superoxide production, and increasing levels of secreted pro-inflammatory cytokine production^{45, 143}. However, pre-exposure to ozone can enhance the subsequent biological response to inhaled LPS including; increased cytokine production, increased lung injury, and increased airway hyperresponsiveness. Seemingly a paradox, pre-exposure to ozone resulted in a reduced number of macrophages in the lungs. However, consistent with the other phenotypes, pre-exposure to ozone enhanced LPS response, which was manifested as an increase in macrophage apoptosis¹⁴⁴. Fewer functional macrophages and enhanced lung injury would be anticipated to be associated with impaired microbial pathogen clearance. The enhanced response of macrophages to LPS after ozone exposure resulted from ozone-induced trafficking of TLR4 to the surface of alveolar macrophages¹⁴⁴. We now recognize that inhalation of ozone results in fragmentation of hyaluronan⁴⁷ and these fragments of hyaluronan contribute to the increased macrophage surface expression of TLR4 and enhanced functional response to LPS¹⁴⁵.

TLR4-dependent signaling in the lung appears to be a double-edged sword. A controlled response appears to be critical for effective clearance of bacterial pathogens, but an exaggerated response can be associated with an increase in AHR, airway injury, and reduced numbers of functional inflammatory cells. Understanding the fundamental homeostatic mechanisms regulating pulmonary innate immunity and the complex relationship with common environmental exposures will inevitably help us gain a better understanding of the etiology and pathogenesis of inflammatory airways disease.

INHALATION OF OZONE CAN MODIFY ADAPTIVE IMMUNITY

Asthma affects a total of 300 million worldwide contributes to an estimated 250,000 deaths per year. In the USA, approximately 10% of the population is asthmatic and approximately 4,000 deaths per year are attributed to asthma^{146–147}. The increased prevalence of asthma in urban environments with high ozone levels, suggests that ozone may contribute to the pathogenesis of asthma. The overall effects of ambient ozone on allergic airways disease appears variable and depends on the timing, dose, and duration of exposure in both rodents^{148–151} and humans^{152–153}. Ozone appears to have a dual effect of both exacerbating existing disease as well as to function as an adjuvant facilitating sensitization to potential allergens. Therefore, it is likely that high levels of ambient ozone contribute to both the severity and prevalence of allergic airways disease in urban environments.

Evidence from both animal models and humans with pre-existing asthma support the concept that inhalation of ozone contributes to exacerbation of allergic airways disease¹⁵⁴. Numerous studies utilizing animal models of allergic airways disease support that secondary challenge to ozone results in enhanced inflammation, airway injury, and AHR. Some evidence support that ozone can suppress Th1 response and favor Th2 reactions^{155–156}. Similarly numerous controlled ozone exposure studies in humans with pre-existing asthma demonstrate an exacerbation of asthma-related phenotypes. These findings are further supported by epidemiological studies which consistently demonstrate exacerbations in pre-existing asthma related to ambient levels of ozone¹⁵⁷.

The mechanisms that ozone contributes to exacerbations of pre-existing asthma are an area of considerable interest. Controlled human exposure studies with asthmatic subjects demonstrate increased numbers of neutrophils, monocytes, and mast cells in the airspace after inhalation of ozone. Additionally, inhalation of ozone increased the levels of allergen-

specific antibodies, the number of antigen presenting cells in the lung, and the expression of both MHC II and co-stimulatory molecules (B7.1, B7.2, CD11b or CD11c) necessary for antigen presentation^{158–162}. Recent evidence from ozone exposed human asthmatics suggests that one mechanism of exacerbated disease is through modification of the innate immune response¹⁶³. It is well known that generalized activation of innate immunity can exacerbate existing allergic airways disease^{164–169}. Not only does human inhalation of ozone increase surface markers associated with antigen presentation (CD86, HLA-DR), but ozone additionally can induce monocyte expression of cell surface molecules associated with innate immunity (mCD14, CD11b, CD16)^{163, 170}. These data suggest that ozone-induced priming of innate immunity may contribute to exacerbations of pre-existing asthma. The current evidence supports that ozone can exacerbate pre-existing asthma through enhanced cellular inflammation, more efficient DC-T cell interactions, and likely through priming monocyte-derived innate immunity.

Considerable evidence from animal studies support that exposure to ozone can also enhance sensitization to antigen in the lung. Ozone exposure has been shown to enhance sensitization to OVA in divergent strains of mice¹⁷¹. When rodents are co-exposed to OVA aerosol and ozone, then subsequently challenged to systemic OVA, there was a significant increase in the level of sensitization as supported by enhanced antigen-dependent fatal shock^{172–173} and increased IgE-containing cells in the lung¹⁷⁴. In monkeys, ozone exposure enhances the development of allergy to both house dust mite allergen¹⁷⁵ and inhaled platinum¹⁷⁶. Cumulatively, these data support that ambient ozone promotes airway sensitization to airborne allergens through a previously unrecognized mechanism.

We now know that genes of innate immunity can activate adaptive immunity¹⁷⁷ and contribute to airway sensitization to some antigens. For example, LPS can act as an adjuvant in a model of airway sensitization to ovalbumin in a manner dependent on TLR4¹⁶⁵. Similarly, we have recently reported that ozone can function as a weak adjuvant during airway sensitization to ovalbumin in a manner dependent on TLR4¹⁷⁸. These observations suggest that inhalation of ozone may additionally facilitate sensitization to otherwise inert antigens. There are currently no human data to support the potential role of ozone in sensitization to allergic antigens. If these observations are validated, this mechanism could partially account for the increased prevalence of allergic disease observed in urban environments.

Ozone impacts the pathogenesis of allergic airways disease. Ambient ozone can contribute to exacerbations of pre-existing asthma. Animal studies suggest that ozone can additionally function as a weak adjuvant during airway exposure to antigen. Interestingly, it appears that the innate immune system is important in both ozone-induced exacerbations of asthma and airway sensitization to antigens. Future studies in both animal models and humans are necessary to help clarify the complex interaction between ozone, allergic asthma, and the innate immune system.

CONCLUSIONS

Ambient ozone is a ubiquitous urban pollutant recognized to have adverse health effects. From a public health perspective, it is anticipated that climate change will increase the global burden of ground level ozone and further contribute to adverse health outcomes. Therefore, while ozone provides an environmentally relevant model of non-infectious lung injury to understand the pathogenesis of lung disease, improved understanding of the biological response to ozone could additionally improve future therapeutic approaches to non-allergic and allergic airways disease.

Current evidence supports a complex interaction between inhalation of environmental ozone and the pulmonary innate immune system. Inhalation of ozone can alter many cell types in the lung required for intact immediate host defense. The biological response to ozone is dependent on genes of innate immunity including; surface receptors, intracellular signaling molecules, and the production of downstream pro-inflammatory cytokines. Interestingly, inhalation of ozone can additionally prime innate immune response through inducing trafficking of *tlr4* to the surface of macrophages. Given the role of innate immunity in subsequent adaptive immune response, it is perhaps not surprising that ozone can interact with adaptive immune response through activation of innate immunity. Understanding the mechanisms that regulate the response to ozone can provide fundamental insight into gene x environment interactions and will broadly impact our understanding of inflammatory lung disease.

Many challenges remain to understand the biological response to ozone. Quite large gaps remain in our current understanding of the complex interaction between inhalation of ozone and innate immunity. The specific genes of innate immunity and cell types required for the complete biological response to ozone remain unknown. We have a very limited understanding of the complex signaling networks between cell types required for the response to ozone. It remains unknown whether inhalation of ozone modifies the response to toll-like receptor ligands other than those to *tlr4*. The specific innate immune mechanisms required for ozone to modify adaptive immunity remain unknown. Finally, there is only limited evidence supporting that mechanisms required for the response to ozone in animal models are relevant to human disease. Mechanistic studies in human cohorts are required to translate observations from animal models.

Overall, studies of ambient ozone have provided novel insight into the pathogenesis of airways disease. Investigations of non-infectious lung injury associated with ozone have provided fundamental insight into mechanisms that a common inhaled environmental exposure can interact with the host innate immune system. Observations from this line of investigation could lead to the development of new approaches to therapy for many individuals suffering from inflammatory airways disease.

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Figure 1. Ozone alters many cell types in the lung and modifies pulmonary innate immunity (A) Many cell types are modified by inhalation of ozone. Inhalation of ozone can lead to increase numbers of macrophages, neutrophils, and dendritic cells in the lung. Ozone disrupts airway epithelia cells including; impaired barrier function, injury to cilia, impaired mucociliary clearance, and alters production of cytokines. Ozone can impair macrophage phagocytosis, induce cytokine expression, induce apoptosis, and alter expression of genes of innate immunity. (B) The response to ozone is dependent on genes of innate immunity and can enhance surface expression of TLR4 on macrophages. Current evidence supports a role of both macrophages and airway epithelia in the complete innate immune response to ozone. Ozone induces fragmentation of extracellular matrix hyaluronan (HA) into low molecular weight (LMW). HA fragments result in TLR4-dependent and CD44-dependent production of pro-inflammatory cytokines. The response to ozone supports a role of both MyD88 and NF-κB in the production of pro-inflammatory cytokines.