Ozone and Pulmonary Innate Immunity

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Ambient ozone (O₃) is a commonly encountered environmental air pollutant with considerable impact on public health. Many other inhaled environmental toxicants can substantially affect pulmonary immune responses. Therefore, it is of considerable interest to better understand the complex interaction between environmental airway irritants and immunologically based human disease. The innate immune system represents the first line of defense against microbial pathogens. Intact innate immunity requires maintenance of an intact barrier to interface with the external environment, effective phagocytosis of microbial pathogens, and precise detection of pathogen-associated molecular patterns. We use ambient O₃ as a model to highlight the importance of understanding the role of exposure to ubiquitous air toxins and regulation of basic immune function. Inhalation of O₃ is associated with impaired antibacterial host defense, in part related to disruption of epithelial barrier and effective phagocytosis of pathogens. The functional response to ambient O₃ seems to be dependent on many components of the innate immune signaling. In this article, we review the complex interaction between inhalation of O₃ and pulmonary innate immunity.

Keywords: Toll-like receptor; tlr4; environmental airways injury; macrophage; epithelia

IMPACT OF AMBIENT OZONE ON PUBLIC HEALTH

Ozone (O₃) is a commonly encountered urban air pollutant that significantly contributes to increased morbidity in human populations (1–4). Ambient O₃ was highlighted as an important ambient air pollutant in human health in the Clean Air Act of 1970 by the U.S. Environmental Protection Agency (EPA). In 1997, the EPA reduced the O₃ standard from 120 to 80 ppb based on evidence that supported detrimental health effects. Since that time, multiple epidemiologic studies have supported the health-related benefits of adherence to established regulatory standards. Environmental exposure to ambient O₃ has a significant impact on human health, which can lead to a significant economic burden. It has been estimated that each year strict adherence to the established 8-hour O₃ standard would result in reductions in the 800 premature deaths, 4,500 hospital admissions, 900,000 school absences, and more than 1 million restricted activity days with an estimated \$5 billion annual economic burden (5). Children (6, 7) and adults older than 65 years of age (8) are particularly vulnerable to low levels of inhaled O₃. However, population-based studies support a global impact on minimal increases in ambient levels of O₃. One study found a 4% increase

in mortality for each 25-ppb measured increase in the level of ambient O₃ (9). Recent estimates suggest that, for each 10-ppb increase in 1-hour daily maximum of O₃, there is an increase in mortality between 0.39 and 0.87% (2, 3, 10, 11). The association with increased mortality persists even at levels of ambient O₃ of 15 ppb, which is well below the current EPA standard (12). Increased levels of O₃ can lead to increased severity of respiratory illness, but it remains unclear whether this is secondary to primary alterations in airway mechanics or dysregulation of immune function (13). O₃ can modify immune function (14). We speculate that altered morbidity and mortality associated with ambient O₃ is, in part, dependent on a complex interaction with the innate immune system. In this article, we provide background and updated evidence that supports the relationship between exposure to ambient O₃ and innate immune function.

O₃ AND PULMONARY IMMUNITY

Many previous studies suggest that preexposure to O₃ can reduce host antibacterial defense. Altered host vulnerability to live pathogens after O₃ exposure is supported by multiple previous rodent studies that demonstrate impaired clearance of live microbial organisms, including *Streptococcus zooepidemicus* (15), *Streptococcus pyogenes* (16), *Staphylococcus aureus* (17), *Klebsiella pneumoniae* (18), *Mycobacterium tuberculosis* (19), and *Listeria monocytogenes* (20). The mechanisms that alter host vulnerability to each of these bacterial pathogens remain poorly understood. O₃ susceptibility seems, in part, dependent on genetic background in humans (21, 22) and mice (23–26). Current literature supports a complex interaction between O₃ and host response (27, 28).

The immune system is broadly divided into two categories: the innate immune system and the adaptive immune system. The adaptive immune system relies on an antigen-specific response and activation of T and B lymphocytes. Previous studies of ambient O₃ have primarily focused on the effects on adaptive immune function. O₃ exposure has been associated with reduced lymphoid organ weights (29, 30) and suppressed T-lymphocyte-dependent immunologic response (31, 32). The overall effects on allergic asthma are variable and seem to depend on the timing, dose, and duration of exposure in rodents (33–36) and humans (37, 38). In contrast to the adaptive immune response, the innate immune response is a highly evolutionarily conserved, first-line defense against microbial pathogens. The innate immunity broadly consists of structural barriers, microbial phagocytosis, and specific microbial pattern recognition. Since the sentinel discovery that *Drosophila* Toll receptors play a fundamental role in host detection of microbial pattern recognition (39), Toll-like receptors (TLRs) in mammalian species have been recognized to play a fundamental role in the detection of invasive pathogens and in many other inflammatory diseases. Disruption of any component of the innate immune response could have a profound impact on immune function and host antibacterial defense. We review the evidence suggesting that exposure to ambient O₃ modifies epithelial barrier integrity and microbial phagocytosis. In addition, we review a growing body of evidence to suggest that pattern recognition receptors and their downstream

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Figure 1. Ozone (O_3) modifies antibacterial defense in many cell types in the lung. O_3 can disrupt epithelial tight junctions and mucociliary clearance and can induce production of proinflammatory factors. O_3 is directly cytotoxic to macrophages. O_3 can modify macrophage phagocytosis, intracellular killing, and levels of secreted factors. O_3 can impair neutrophil phagocytosis and intracellular killing.

signaling play a fundamental role in the biologic and physiologic response to O_3 .

The lung is constantly exposed to a broad spectrum of environmental toxins, including microbiological pathogens, bacterial products, and ambient O_3 . Epidemiologic evidence and animal studies suggest that these inhaled environmental toxins can affect the severity of airways disease. A common feature of the host response to each of these inhaled toxins is neutrophilic inflammation, up-regulation of proinflammatory cytokines, and decrements in lung function (40–46). Although these responses help

facilitate the clearance of pathogens, they can lead to tissue injury and compromised lung function. Accordingly, it is important to understand the molecular mechanisms that initiate and regulate neutrophilic inflammation and consequent tissue damage. Many cell types in the lung are involved in pulmonary innate immune response, including macrophages (47, 48), neutrophils (49), endothelia (50), and airway epithelia (51). Evidence suggests that the biologic function of many of these cell types can be modified by exposure to ambient O_3 (Figure 1).

O_3 DISRUPTS EPITHELIAL INTEGRITY

A primary innate immune defense mechanism is through maintenance of intact epithelial barrier. This is achieved by intact airway epithelia and effective mucociliary clearance. The presence of cellular and biochemical markers of inflammation in lung lavage fluids suggested that the integrity of respiratory epithelia is impaired by O_3 (52, 53). Acute exposure to O_3 causes damage to airway epithelial cells and alveoli. Human studies suggest that exercise in combination with exposure to moderate levels of O_3 can increase airway epithelial permeability (43). Furthermore,

Figure 2. Ozone disrupts epithelial barrier function to protein. Lung clearance of soluble radiolabeled albumin in a subject evaluated after exposure to filtered air (A) or ozone (B) when compared with baseline. Lung retention is expressed as natural log of activity level in right lung at time zero with retention points fitted to a linear regression. Data points represent lung retention of soluble marker at the indicated times. Lung retention is expressed as natural log of the fractional retention of the activity level of the radiomarker deposited at time zero. Superimposed color images represent scintigraphy scans of the lung acquired from the posterior aspect and demonstrate dynamics of the clearance process with color intensity equivalent to radiolabel activity levels: blue > green > yellow. As activity clears the lung from epithelial airway and alveolar surfaces by transport via paracellular pathways into the systemic and pulmonary vasculatures, bilateral accumulation is visualized below the diaphragms, representing cleared activity filtered by the kidneys. (Adapted by permission from Reference 46.)

exposure to ambient levels of O₃ can impair selective permeability of the epithelium (46). The loss of epithelial integrity after O₃ parallels the inflammatory changes in the lower respiratory tract (52), but they are not necessarily codependent. Inhalation of hydrophilic low-molecular-weight ^{99m}Tc-DTPA (MW 492, radius 0.57 nm) allows investigation of transepithelial transit via paracellular channels, which are primarily limited by structural pores. O₃-induced hyperpermeability through paracellular pathways is of considerable clinical interest because impaired epithelial barrier function can facilitate access of xenobiotic molecules to basolateral surfaces of submucosal tissues and then subsequently the systemic circulation. O₃-induced defects in airway epithelial barrier function could contribute to the increased severity of pulmonary infections observed after high levels of ambient O₃ (Figure 2).

Mucociliary clearance provides another first-line defense against pulmonary infections. This important aspect of epithelial barrier defense facilitates effective clearance of foreign material from the airways. Mucociliary clearance is dependent on the interplay among several factors, including mucin secretion, mediator release, epithelial permeability, and ciliary beat frequency. Acute exposure to O₃ in humans demonstrated that the smaller peripheral bronchioles were more sensitive than lobar bronchi to ambient levels of O₃ (54). Subsequent studies replicated these observations and suggest that the response to O₃ is transient (55). Oxidant gases can also react with cross-linking bonds of mucus glycoprotein structures, resulting in lower viscosity of respiratory secretions and impaired mucociliary clearance (56). The effects of O₃ on secretory cells in culture have not been well characterized; however, *in vivo* animal models have shown that O₃ exposure can increase levels of tracheal glycoconjugates (57). Of interest is the rodent model of mucus cell hyperplasia developed by Fannuchi and Harkema in which mRNA levels of rMuc5ac (the major component of respiratory mucins) become elevated in transitional epithelium after exposure to ambient O₃ levels followed by nasal instillation of endotoxin (58). In addition, loss of cilia in the terminal bronchioles is considered an early indicator of acute oxidant-induced injury (59). These data suggest that O₃ can modify many components required for effective mucociliary clearance of foreign material or microbial pathogens.

In addition to defects in epithelial barrier and defects in mucociliary clearance, airway epithelial cells are functionally altered after exposure to O₃. *In vitro* studies of airway epithelia cultured in liquid-air interface demonstrate that O₃ exposure can induce the secretion of many proinflammatory factors (60). More recent murine studies demonstrate that exposures to O₃ can attenuate the transcription of IL-1 mRNA in response to secondary endotoxin challenge (61). The later observation suggests that primary damage to airway epithelia, as a result of O₃ exposure, can impair the subsequent response to TLR ligands.

IMPAIRED MACROPHAGE FUNCTION AFTER EXPOSURE TO O₃

Macrophages play a critical role in orchestrating pulmonary innate immune response to inhaled endotoxin (47, 48). Substantial evidence suggests that inhalation of O₃ can directly modify alveolar macrophage function. *In vitro* studies of O₃ effects on human alveolar macrophages demonstrate impaired phagocytosis, superoxide production, and increased levels of secreted cytokines (62). Human alveolar macrophages seem to be more sensitive to the cytotoxic effects of ambient O₃ when compared with cultured airway epithelia (60). Murine studies demonstrate that O₃-induced impaired antibacterial defense to *S. aureus* and *S. zooepidemicus* was associated with impaired phagocytic capac-

ity of alveolar macrophages (63, 64). Although O₃ can impair macrophage phagocytosis and superoxide production in the lung, *in vitro* studies of peripheral human blood monocytes demonstrate that O₃ can also directly stimulate the production of tumor necrosis factor (TNF)-α and IFN-γ (65). Furthermore, preexposure to O₃ can enhance pulmonary macrophage production of nitric oxide with subsequent *in vitro* treatment by endotoxin or IFN-γ (66). These findings demonstrate that O₃ can directly impair antibacterial clearance through reduced phagocytosis and superoxide production. Furthermore, *in vitro* studies of coexposure suggest that O₃ can prime some aspects of subsequent pulmonary innate immune response.

NEUTROPHILS AND ENVIRONMENTAL AIRWAY INJURY WITH O₃

Similar to inhaled endotoxin, acute response to inhalation of O₃ results in airway injury and neutrophil influx into the airspace. It remains unclear whether recruited neutrophils directly contribute to the biologic response to inhaled O₃. Previous reports suggest that accumulation of neutrophils in the airways is critical to the physiologic response to O₃ (67–69), whereas other similarly conducted studies have not substantiated this observation (70–72). It is possible that differences in O₃ exposure protocols or techniques of neutrophil depletion used in these experiments contribute to the divergent observations. Regardless, recruited neutrophils maintain the capacity to generate proinflammatory cytokines and reactive oxygen species, which can contribute to acute lung injury (73). Enhanced recruitment of neutrophils into the airspace conceptually would prove beneficial in rapid clearance of secondary exposure to bacterial pathogens. However, this was not observed with models of live pathogen challenge after preexposure to O₃ and could be related to impaired function of recruited neutrophils. Early work by Peterson and colleagues demonstrates that human exposure to low levels of ambient O₃ lead to defective neutrophil phagocytosis and intracellular killing (74). More recent *in vitro* studies indicate that O₃ impaired the capacity of human neutrophils to produce superoxide radicals (75). These observations suggest that exposure to ambient levels of O₃ can impair the functional ability of neutrophils to respond to bacterial pathogens and provide further evidence that O₃ can modify the innate immune response.

SECRETED FACTORS AND BIOLOGIC RESPONSE TO O₃

It has been well documented in animal models and human studies that exposure to O₃ induces secretion of inflammatory factors into the lung. Analysis of alveolar lavage fluid reveals biochemical evidence of O₃-induced inflammation with increases in many secreted factors, including fibronectin, elastase, plasminogen activator, tissue factor, factor VIII, C3a fragment of complement, prostaglandins, IL-1, TNF-α, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (44, 60, 76). Many of these secreted factors are recognized downstream products of activation of the innate immune system. The critical role of downstream secreted factors in the biologic response to O₃ was initially identified through a genome-wide linkage analysis study. In that study, a quantitative trait locus on chromosome 17 was identified, and fine mapping identified TNF-α as a candidate gene (23). The fundamental role of TNF-α in neutrophil recruitment and epithelial cell proliferation in response to O₃ was confirmed with TNF-α-neutralizing antibodies. Subsequent studies have identified that TNF-α contributes to O₃-induced airway hyperresponsiveness in mice (77, 78) and humans (79). Additional studies have identified that many of the up-regulated downstream proinflammatory factors play a role in the biologic response to inhaled

O₃, including neutrophil elastase (80), complement (70), IL-1 (81), and IL-6 (82). Additionally, the neutrophil chemokines, KC and macrophage inflammatory protein-2, are expressed in the lungs of mice after exposure to O₃ (83). Studies demonstrate that the receptor for these chemokines (CXCR2) is essential for complete response to O₃ (84). These data suggest that downstream activation of proinflammatory factors play an important role in response to ambient O₃. The initial stimulus leading to activation of these proinflammatory factors remains poorly described.

SURFACE RECEPTORS AND RESPONSE TO O₃

The innate immune system consists of epithelial barriers, phagosomes, and the recognition of pattern-associated molecular patterns. The discovery of surface receptors that immediately recognize foreign material has dramatically enhanced our understanding of host immunity. Mammalian TLRs play a critical role in detecting invading pathogens and triggering subsequent inflammatory and immune responses. The surface receptors bind pattern-associated molecular patterns and interact with intracellular adaptor molecules, resulting in activation of nuclear transcription regulators. This cascade results in the production of many proinflammatory factors associated with the immediate inflammatory response. The discovery that the LPS receptor *tlr4* plays a role in airway injury in response to O₃ was made through fine mapping of a quantitative trait locus and characterization of the C3H/HeJ mouse (24, 85) (Figure 3A). Subsequently, O₃-induced airway hyperresponsiveness was identified to be dependent on intact *tlr4* (86) (Figure 3B). Recent unpublished data presented at the 2006 American Thoracic Society International Conference suggest that O₃-induced airway hyperresponsiveness is dependent on the downstream adaptor protein MyD88 (87). Furthermore, previous work suggests that the biologic response to O₃ is, in part, dependent on the transcription regulator, nuclear factor (NF)- κ B p50 (88). Based on these observations, it is plausible that *tlr4*-dependent signaling can lead to MyD88-dependent activation of NF- κ B and transcription of downstream proinflammatory factors, leading to O₃-induced airway hyperresponsiveness. Ozonolysis products (eicosanoid release and generation of peroxy radicals) of epithelial membrane fatty acids can function as early transducers of O₃ reactions at the epithelial surface (89). Lipid ozonation products (hydrogen peroxide and aldehydes) have been suggested to act as signal transduction molecules in the lung and in extrapulmonary tissues (90, 91). Recent evidence suggests that ozonation of low-density lipoprotein can inhibit NF- κ B and IL-1 receptor-associated kinase-1-associated signaling (92). These observations suggest that O₃ could modify lipid products and modify subsequent systemic innate immune response. For this reason, host genetic factors related to oxidative stress/redox balance are of considerable interest, including glutathione S-transferase M1, glutathione S-transferase P1, nicotinamide adenosine dinucleotide (phosphate) reduced:quinone oxidoreductase, glutathione peroxidase-1, glutathione reductase, and superoxide dismutase-2. The mechanisms that link the biologic response to O₃ and innate immunity remain poorly understood.

To further elucidate the mechanism through which *tlr4* modulates responsiveness to O₃, Dahl and colleagues (93) used microarrays to determine gene expression profiles in the lungs of C3H/HeJ (*Tlr4* mutant, O₃-resistant) and C3H/HeOuJ (*Tlr4* normal, O₃-susceptible) mice after exposure to 0.3-ppm O₃. *Marco* (macrophage receptor with collagenous structure) was highly up-regulated by O₃ in the lungs of C3H/HeJ mice, whereas no changes were found in C3H/HeOuJ mice. Targeted disruption of *Marco* significantly enhanced O₃-induced lung inflammation

Figure 3. Functional response to ozone is dependent on Toll-like receptor 4 (TLR4). Mice were exposed to 300 ppb ozone (O₃) for 72 hours. (A) Airway injury was determined by level of protein in the bronchoalveolar lavage (BAL) fluid in the C3H/OuJ (*tlr4*-sufficient) and the C3H/HeJ (*tlr4*-deficient) over time. Statistical comparison: air versus O₃ treatment groups (**p* < 0.05); C3H/HeJ versus C3H/HeOuJ (**p* < 0.05). (B) After completion of exposure to O₃, physiologic response to intravenous methacholine in wild-type and *tlr4* knockout mice were evaluated by direct measurements of tracheal pressures. Increased airway pressure-time index (APTI) values in O₃ exposed C57BL/6 animals when compared with *tlr4*^{-/-} were seen at all doses of intravenous methacholine, including 25 µg/ml (**p* < 0.01), 100 µg/ml (**p* = 0.02), 250 µg/ml (**p* = 0.001). (B) TLR4^{+/+} O₃, upper solid line, solid squares; TLR4^{-/-} O₃, dotted line, open triangles; TLR4^{+/+} air, lower solid line, solid squares; TLR4^{-/-} air, dotted line, open diamonds. (Adapted by permission from References 24 and 86.)

compared with wild-type mice, and inflammation induced by the instillation of β -epoxide and PON-GPC (surfactant-derived ozonation products) was significantly greater in *Marco*-deficient mice compared with wild-type mice. These results suggest an important role for scavenger receptors in decreasing inflammation after O₃ by scavenging proinflammatory oxidized lipids (93). Additionally, recent human data suggest that O₃ exposure can enhance surface expression of the *tlr4* coreceptor CD14 on airway macrophages and monocytes (94). The role of TLR4 in the biologic response to O₃ in humans has not been adequately examined. These data strongly suggest that the biologic response to O₃ is dependent on a complex interaction with innate immune signaling.

CONCLUSIONS

The functional innate immune system consists of an intact epithelial barrier, effective phagocytosis of pathogens, and precise

activation of innate immune signaling pathways. Exposure to ambient O₃ can disrupt epithelial integrity and impair mucociliary clearance. O₃ impairs effective phagocytosis of microbial pathogens in macrophages and neutrophils. The functional response to O₃ is dependent on TLRs, likely the adaptor molecule MyD88, and many downstream proinflammatory secreted factors. Understanding the fundamental mechanisms that regulate the biologic response to commonly encountered inhaled environmental toxins will provide a better understanding of the increased morbidity and mortality associated with high levels of ambient air pollution. Furthermore, with improved understanding of the complex interaction between inhaled toxicants and innate immunity, we will be able to provide more effective therapeutic intervention for patients with environmental airways disease.

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