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Ambient Ozone Primes Pulmonary Innate Immunity in Mice

John W. Hollingsworth,‡ Shuichiro Maruoka,† Zhuowei Li,* Erin N. Potts,* David M. Brass,† Stavros Garantziotis,*† Alan Fong,‡ W. Michael Foster,* and David A. Schwartz‡

Exposure to ozone in air pollution in urban environments is associated with increases in pulmonary-related hospitalizations and mortality. Because ozone also alters clearance of pulmonary bacterial pathogens, we hypothesized that inhalation of ozone modifies innate immunity in the lung. To address our hypothesis, we exposed C57BL/6J mice to either free air or ozone, and then subsequently challenged with an aerosol of Escherichia coli LPS. Pre-exposure to ozone resulted in enhanced airway hyperreactivity, higher concentrations of both total protein and proinflammatory cytokines in lung lavage fluid, enhanced LPS-mediated signaling in lung tissue, and higher concentrations of serum IL-6 following inhalation of LPS. However, pre-exposure to ozone dramatically reduced inflammatory cell accumulation to the lower airways in response to inhaled LPS. The reduced concentration of cells in the lower airways was associated with enhanced apoptosis of both lung macrophages and systemic circulating monocytes. Moreover, both flow cytometry and confocal microscopy indicate that inhaled ozone causes altered distribution of TLR4 on cells of the lower airways was associated with enhanced apoptosis of both lung macrophages and systemic circulating monocytes. These observations indicate that ozone exposure increases both the pulmonary and the systemic biologic response to inhaled LPS by priming the innate immune system.


Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the U.S., and it is the only common cause of death that is increasing in incidence. Exposure to ambient air pollutants can lead to exacerbations of COPD associated with both enhanced pulmonary inflammation and loss of lung function. Ozone is a common urban air pollutant that significantly contributes to increased pulmonary mortality, especially among those with chronic lung disease (1–4). It is known that inhalation of ambient ozone can modify adaptive immune function (reviewed in Ref. 5) in both rodents (6–9) and humans (10, 11). However, COPD is characterized by increased numbers of macrophages, neutrophils, and cytotoxic CD8 lymphocytes, suggesting a role of innate immune function in pathogenesis of this disease. Ozone is known to alter macrophage function (12, 13). Alveolar macrophages exposed to ozone in vitro demonstrate reduced phagocytosis in both rodent (14) and humans (15), which in part is related to cytotoxicity (16). In addition, exposure to ozone is associated with impaired clearance of multiple live organisms, including the following: Streptococcus zooepidemicus (17), Streptococcus pyogenes (12), Staphylococcus aureus (18), Klebsiella pneumonia (19), Mycobacteria tuberculosis (20), and Listeria monocytogenes (21). Altered vulnerability to these bacterial pathogens suggests that ozone could specifically impair innate immune function in the lung and provide an underlying mechanism for the enhanced morbidity and mortality observed in human populations exposed to higher concentrations of ambient ozone. Understanding the environmental factors that regulate macrophage and neutrophil recruitment and function will improve our understanding of the factors that contribute to the pathogenesis of COPD. To address this possibility, we hypothesized that inhalation of ozone would modify the innate immune response in the lung.

Strict regulation of innate immune function can affect the progression and outcome of several diseases, including bacterial pneumonia, chronic bronchitis, and environmental airways disease. Specific activation of innate immunity in the lung can be achieved by exposure to aerosolized bacterial endotoxin (22). Inhalation of bacterial endotoxin can contribute to the development and progression of occupational lung disease (23). Furthermore, inhaled LPS can modify allergic asthma (24, 25) and cause severe inflammatory disease in both humans and mice (26–28). Although this immediate inflammatory response plays an important role in host defense, uncontrolled inflammation can contribute to the progression of pulmonary (and systemic) disease (29). Accordingly, it is of considerable interest to identify environmental factors, which may modify innate immune responsiveness.

By focusing on the effect of ozone on innate immunity in the lung, we have found that ambient exposure to ozone can prime both local and systemic innate immune responsiveness by altering the cellular distribution of TLR4 and increased signaling by alveolar macrophages, leading to cellular apoptosis. These findings provide a biological mechanism for the epidemiological relationshipship between ozone and increased pulmonary mortality and morbidity.

Materials and Methods

Mice

Six- to 8-wk-old male C57BL/6J mice were purchased from The Jackson Laboratory. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and were

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3 Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; AHR, airway hyperresponsiveness; FA, filtered air; LTB4, leukotriene B4; PMN, polymorphonuclear cell; ppm, parts per million.
conducted in accordance with the standards established by the U.S. Animal Welfare Acts.

**Exposure protocol**

Six- to 8-wk-old male C57BL/6J mice (The Jackson Laboratory) were exposed to either filtered air (FA) or ozone (2 parts per million (ppm) × 3 h), and then challenged with an aerosol of purified 0111:B4 *Escherichia coli* LPS (LPS; 4 µg/ml × 2.5 h), as previously described (22). This dose of LPS models the inflammatory response experienced by grain mill workers during a typical 8-h workday (28, 30). All animals were evaluated 4 h after initiation of the LPS aerosol. The mean level of LPS measured in aerosol was 3.58 µg/ml (2.19–4.96 µg/ml), and serum was 0.66 EU/ml (0.28–1.41 EU/ml) by the *limulus* amebocyte lysate assay (BioWhittaker).

The level and duration of ozone exposure (2 ppm × 3 h) used in this protocol are an accepted murine model of ozone/oxidant injury in comparison with effective ambient exposure levels in humans. Level of ozone exposure for ex vivo analysis of macrophage function was 1 ppm × 3 h. Ozone or filtered air exposures were performed in 55-L Hinner-style exposure chambers for 3 h. Chamber air at 20–22°C and 50–60% relative humidity was supplied at a rate of 20 changes/hour. Ozone was generated by directing 100% oxygen through a UV light ozone generator. Ozone concentration was monitored continuously within the chamber with an ozone UV light photometer (Dasisi model 1003AH; Dasisi). In vivo experiments represent 10 mice per group with at least two repeats.

**Airway physiology**

Direct measurements of respiratory mechanics in response to methacholine were made using the flexivent system (Scireq) and reported as total pulmonary resistance (R_{pulm}) cmH2O/ml/s. Anesthesia was achieved with 60 mg/kg pentobarbital sodium injected i.p. and ventilated with a computer-controlled small animal ventilator (flexiVent; Scireq) with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm H2O. The mice were then given a neuromuscular blockade (0.8 ml/kg pancuronium bromide). Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0, 10, 25, and 100 mg/ml) was determined by resistance measurements every 30 s for 5 min, ensuring the parameters calculated had peaked. Total lung capacity breaths were given after each dose, keeping the airways open and returning the measurements back to baseline. The resistance measurements were then averaged at each dose and graphed (R_{pulm} cmH2O/ml/s) along with the initial baseline measurement.

**Lung samples**

Whole lung lavage and cell differentials were determined, as previously described (22). Luminex (Bio-Rad) was used to evaluate protein concentrations of keratinocyte cytokine, IL-6, MIP-1α, IL-10, IL-12(p70), and IL-17 with a commercially available immunoassay (Linco Research). Total protein concentrations in lung lavage fluid were determined using the Lowry assay (Bio-Rad).

**Chemotaxis assays**

Fresh polymorphonuclear cells (PMN) were harvested from either alveolar lavage or peripheral blood from animals exposed to either FA-LPS or ozone-LPS. First, live alveolar neutrophils were harvested from animals exposed to either FA-LPS or ozone-LPS. RBCs were removed with lysis buffer (0.14 M NH4Cl and 0.015 M Tris (pH 7.2)) for 5 min. A total of 1 × 10^6 cells suspended in RPMI 1640 with 10% FCS was placed in upper Transwell with 5 µm pore size (Costar). Cells were incubated in the presence of leukotriene B4 (LTB4; 30 µM) for 1 h at 37°C. Cells were collected from the lower well and stain for neutrophils (Gr-1^+) and quantified by flow cytometry. Second, circulating neutrophils were isolated with Histopaque 1083, per manufacturer’s recommendations (Sigma-Aldrich), and analyzed for chemotaxis to LTB4. Third, lavage supernatants from pre-exposed animals were evaluated for chemotactic factors. A total of 1 × 10^6 peritoneal neutrophils was placed in the upper well, and then 600 µl of supernatants from either FA-LPS or ozone-LPS mice was used as chemoattractant. Neutrophils (Gr-1^+) were quantified in lower well by flow cytometry.

**Western blots**

Lung tissues were homogenized and lysed in ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 157 mM NaCl, 25 mM β-glycerophosphate (pH 7.4), 2 mM EDTA (pH 7.4), 1% Triton X-100, 10% glycerol, 1 mM PMSE, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin, 1 mM Na3VO4, and 1 mM DTT. After centrifugation, tissue extracts were resolved by SDS-PAGE and were analyzed by immunoblot. The membranes were probed with Abs to phospho-JNK, phospho-p38MAPK, phospho-ERK, cleaved caspase-3, and β-actin as loading control (Cell Signaling Technology). Blots were developed with ECL plus (Amersham Biosciences).

**Apoptosis**

Fresh lung alveolar cells were pooled from five animals (5 × 10^6 fresh cells) for each group and were analyzed. Fresh whole blood was drawn from the inferior vena and pooled from five animals (1 × 10^6 fresh cells) for each group. After blocking with Fc block (BD Biosciences), murine IgG, and rat IgG (Jackson ImmunoResearch Laboratories), cells were stained with mAbs, including the following: FA/40-allophycocyanin (Scirocco), Gr-1-FITC (BD Biosciences), 7-aminoactinomycin D, and annexin V-PE (BD Biosciences). Analysis was performed using FACS Vantage SE (BD Biosciences), and counts were calculated automatically by FlowJo software (Tree Star). For the TUNEL assay (DeadEnd Colorometric TUNEL System; Promega), paraffin-embedded sections were deparafinized by immersing slides in xylene and then through graded ethanol washes (100, 95, 85, 70, 60, and 50%), followed by a wash in 1× PBS. The tissue sections were then fixed by immersing the slides in 4% paraformaldehyde for 15 min. A proteinase K solution of 20 µg/ml was placed on the slides for 2 min to help permeabilize the tissue. The slides were then
washed with 1× PBS and fixed in 4% paraformaldehyde for another 5 min and washed again. In situ nick end labeling of nuclear DNA fragmentation was performed in a humid chamber for 1 h in the dark at 37°C. A positive control slide was prepared by treating the tissue sections with RNase-free DNase for 10 min before the above labeling. A negative control slide was prepared by omitting the rTdT from the above labeling step. The labeling reaction was stopped by immersing the slides in 2× SSC for 15 min, followed by a 1× PBS wash. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide in PBS for 5 min and then washing with 1× PBS. Color was developed by diaminobenzidine.

The slides were then mounted with Permount mounting medium (Fisher Scientific) and observed under a light microscope.

**TLR4 immunohistology and flow cytometry**

Whole lung lavage was performed to obtain three pools of two animals 24 h after exposure to ozone or FA. After blocking for 10 min on ice with Fc block, murine IgG, and rat IgG (Jackson ImmunoResearch Laboratories), live lavage cells were stained for surface expression of F4/80 (Serotec) and TLR4 for 30 min and washed. Alveolar macrophages were identified as F4/80+ TLR4−. Surface expression of TLR4 was determined using PE-conjugated mAbs to TLR4 (clone UT41; eBioscience). Cells were fixed with 1% ultrapure paraformaldehyde (Polysciences); flow cytometry analysis was performed using BD Biosciences LSR II flow cytometer; and data were analyzed with BD FACSDiva software (BD Biosciences). A laser-scanning confocal microscope (LSM 510 UV mounted on Axiowert 200M microscope; Zeiss) was used to obtain the fluorescence and differential interference contrast images. The images were obtained simultaneously using the 488- and 543-nm lasers as the light source, and the Zeiss Plan-Apo 63 oil N.A. 1.4 as objective lens. For fluorescence, either a 505- to 550-nm or a long pass 560-nm filter was used for the emission, with a pinhole of 1 Airy unit, corresponding to a z-resolution of 0.8 μm. The software used for acquisition was Zeiss LSM510 version 3, and for analysis, LSM Image Examiner version 3.2.

**Statistics**

Data are expressed as mean ± SEM. Significant differences between groups were identified by ANOVA. Individual comparisons between groups were confirmed by Student’s t test, unless otherwise stated. Statistical calculations were performed using SPSS. A two-tailed p value of <0.05 was considered statistically significant.

**Results**

Ozone enhances airway hyperresponsiveness in response to inhaled LPS

To determine whether ozone could modify the innate immune response to LPS, we primarily exposed mice to either filtered air or inhaled ozone for 3 h (2 ppm), followed by secondary exposure to...
Ozone pre-exposure enhances total protein in the lung lavage and systemic IL-6 after exposure to LPS. Bronchial alveolar lavage fluid was measured for total protein at each time point (A). We did not observe significant increases in total protein in the lavage fluid after FA, ozone, or FA-LPS inhalation. Pre-exposure to ozone dramatically increased the level of total protein in the lavage when compared with FA-LPS at 24, 48, and 72 h (*, p < 0.05; FA-LPS vs ozone-LPS). Systemic inflammatory response was determined by measurement of serum IL-6 (B). LPS increased systemic IL-6 when compared with either FA or ozone; however, this was dramatically increased in the ozone-LPS group (*, p < 0.05; FA-LPS vs ozone-LPS).

FIGURE 4. Ozone pre-exposure enhances total protein in the lung lavage and systemic IL-6 after exposure to LPS. Bronchial alveolar lavage fluid was measured for total protein at each time point (A). We did not observe significant increases in total protein in the lavage fluid after FA, ozone, or FA-LPS inhalation. Pre-exposure to ozone dramatically increased the level of total protein in the lavage when compared with FA-LPS at 24, 48, and 72 h (*, p < 0.05; FA-LPS vs ozone-LPS). Systemic inflammatory response was determined by measurement of serum IL-6 (B). LPS increased systemic IL-6 when compared with either FA or ozone; however, this was dramatically increased in the ozone-LPS group (*, p < 0.05; FA-LPS vs ozone-LPS).

Either saline or inhaled LPS at 24 h, 48 h, 72 h, or 7 days. All experiments follow the same experimental protocol for coexposures. Previously, both ozone and LPS inhalation have independently been shown to cause airway hyperresponsiveness (AHR) to inhaled methacholine. Ozone exposure alone caused enhanced AHR at early time points (24 and 48 h), and this response peaked at 24 h and returned to baseline by 72 h. However, 48 and 72 h after ozone exposure, the airway response to inhaled LPS was synergistically enhanced over the AHR observed following either exposure alone (Fig. 1). By 7 days after ozone exposure, the physiologic response to LPS was independent of ozone pre-exposure (data not presented). This observation suggests that the effects of ozone priming response to LPS have, in part, resolved by 7 days after exposure. Therefore, ozone can enhance LPS-induced airway hyperresponsivity to methacholine up to 72 h after exposure.

Ozone attenuates cellular inflammation in response to inhaled LPS

To determine whether ozone priming of the LPS response in the airways was dependent on cellular inflammation, we characterized recruitment of cells into the alveolar compartment after exposure. Unexpectedly, we observed significantly reduced LPS-induced pulmonary inflammation at both 24 and 48 h after pre-exposure to ozone (Fig. 2). We have previously demonstrated that mice develop airways hyperresponsiveness after inhalation of ozone (22). Because it was conceivable that enhanced ozone-induced bronchoconstriction could minimize the effects of inhaled LPS by reducing the deposition and distribution of the aerosol, we challenged animals pre-exposed to FA or ozone to doses of LPS similar to aerosol by oropharyngeal aspiration (1.7 μg/50 μl saline). LPS delivered to the airways in this manner is largely independent of ozone-induced changes in air flow. The differences we observed in response between the groups using this method were identical with those using aerosolized LPS. Importantly, reduced concentrations of total cells (ozone-LPS, 6.1 × 10⁴ vs FA-LPS, 16.5 × 10⁴), macrophages (ozone-LPS, 1.0 × 10³ vs FA-LPS, 1.7 × 10³), and neutrophils (ozone-LPS, 5.0 × 10⁴ vs FA-LPS, 14.7 × 10⁴) persisted with this method of LPS administration. Therefore, we conclude that ozone pre-exposure can attenuate cellular inflammation in the lungs in response to aerosolized LPS independent of changes in airway hyperresponsiveness.

Ozone enhances production of inflammatory markers in response to inhaled LPS

Although reductions in inflammatory cells in the lung caused by sequential exposure to ozone and LPS could have been explained by reduced production/release of cytokines and chemokines in the lower airway, this was not observed. In contrast, we found dramatic increases in the concentration of several proinflammatory proteins after pre-exposure to ozone, including the following: KC, MIP-1α, and IL-6 (Fig. 3). Minimal, but significant increases in IL-10, IL-12(p70), and IL-17 were also seen with ozone pre-exposure at 24, 48, and 72 h (data not shown), TNF-α was significantly increased after ozone pre-exposure only at 72 h (1811 ± 459 vs 3614 pg/ml ± 521, p < 0.05). To specifically address whether any undetected secreted factors contribute to altered cellular migration, we harvested zymosan-elicited peritoneal neutrophils from naive animals. No significant differences were observed in the chemotactic response to lavage fluid obtained from ozone vs FA pre-exposed mice to explain an absolute reduction in recruitment of inflammatory cells (chemotaxis index; FA-LPS, 5.8 vs ozone-LPS, 6.7). This further suggests that alteration in secreted
factors in the alveolus did not significantly contribute to reduced cell populations in the lung associated with ozone pre-exposure.

Similarly, we found that markers of LPS-induced lung injury were substantially increased in mice that had been previously exposed to ozone. Enhanced LPS-induced lung injury in ozone-exposed mice was supported by dramatic increases in the concentration of total protein in the lavage fluid (Fig. 4A), a higher percentage of circulating neutrophils (FA, 2.2%; ozone, 1.1%; FA-LPS, 10.8%; ozone-LPS, 20.2%), and higher serum concentrations of IL-6 (Fig. 4B). Disruption of epithelial integrity and enhanced systemic inflammation could be associated with increased translocation of inhaled LPS into the systemic circulation. However, we

FIGURE 6. LPS caused robust neutrophil recruitment and ozone-enhanced apoptosis in macrophages. Histology was evaluated at 24 h after pre-exposures, including the following: FA (A and B), ozone (C and D), FA-LPS (E and F), and ozone-LPS (G and H). We present representative H&E staining (A, C, E, and G) and TUNEL staining (B, D, F, and H) at ×400 power. Moderate perivascular and subepithelial neutrophils are observed with exposure to LPS. Alveolar epithelial septal thickening is appreciated with exposure to ozone. Airway macrophages stain positive for TUNEL in both ozone and ozone-LPS groups (*, arrows).

FIGURE 7. Ozone activates MAPKs immediately after exposure. Western blot analysis on lung homogenates was performed on animals before ozone exposure, 4 h after initiation of exposure, and 24 h after exposure (A). Densities of bands are quantified as normalized to B-actin in phospho-JNK (B), phospho-p38 (C), phospho-ERK (D), and cleaved caspase 3 (E). Statistically significant differences are identified (*, p < 0.05; two-tailed Mann-Whitney U test). Densitometry data represent mean ± SEM from three repeats.
were unable to detect significant differences in the concentration of LPS in the serum of coexposed animals (24-h values; FA-LPS, 0.73 EU/ml vs ozone-LPS, 0.96 EU/ml; \( p < 0.31 \)).

**Inhaled ozone reduces neutrophil spontaneous cytokinesis and induces apoptosis of macrophages in the lung and circulating blood monocytes**

Reduced LPS-induced inflammatory cell recruitment into the lungs associated with ozone pre-exposure could be the result of either impaired cellular motility or enhanced cell death. To determine whether ozone directly impaired cellular migration, we harvested lung neutrophils from animals exposed to inhaled LPS with or without ozone pre-exposure. Cells were then analyzed for their migratory potential using Transwells (31). In the absence of any chemotactic signal, there was a 7.2-fold decrease in nonspecific migration (chemokinesis) in ozone-treated cells compared with those exposed to FA (data not shown). When a chemotactic gradient was added (LTB4), similar increases in PMN migration from each of these groups were observed (chemotaxis index; FA-LPS, 1.5 vs ozone-LPS, 2.8). However, the absolute number of ozone-treated cells that migrated toward LTB4 was reduced. Thus, a reduction in spontaneous neutrophil cytokinesis after ozone exposure resulted in an absolute reduction in the number of PMNs that migrated to LTB4. This observation suggested that mice pre-exposed to ozone had a population of PMNs that were functionally unable to migrate to chemotactic stimulus.

To determine why subset of neutrophils demonstrated reduced spontaneous cytokinesis and to better understand the reduced numbers of macrophages recruited into the lung associated with pre-exposure to ozone, we explored the possibility that ozone exposure was inducing apoptosis or necrosis of inflammatory cells. To address this question, we performed flow analysis to discriminate populations of inflammatory cells in the lower airway and circulating blood 24 h after pre-exposure to ozone that might be undergoing cell death. Neutrophils recruited into the lung after inhaled LPS demonstrated a 40% increase in apoptosis when pre-exposed to inhaled ozone (ozone, 4.2%; LPS, 3.6%; ozone-LPS, 6.0%). Interestingly, ozone exposure by itself had a profound effect on macrophages. We found that ozone alone can induce apoptosis in alveolar macrophages (FA, 7.8% vs ozone, 27%), whereas inhaled LPS alone did not have a measurable impact on macrophage apoptosis at this time point (FA, 7.8% vs FA-LPS, 8.0%) (Fig. 5A). Mice were phenotyped at an early time point after exposure to LPS, which most likely explains the lack of LPS-induced apoptosis observed. However, LPS exposure in the context of ozone pre-exposure substantially increased the percentage of macrophages in the lung undergoing apoptosis (ozone, 27% vs ozone-LPS, 51%). Ozone-induced apoptosis of airway inflammatory cells in the lung was confirmed by TUNEL staining (Fig. 6) and an increase in cleaved caspase 3 (Figs. 7E and 8E). Secondary necrosis was observed after either ozone or LPS exposure (FA,

**FIGURE 8.** Ozone pre-exposure enhances LPS-dependent downstream signaling. Western blot analysis on lung homogenates was performed to determine the level of phosphorylation of proteins related to LPS-dependent signaling and apoptosis at 24 h after pre-exposure to either FA or ozone (A). For each gel, ozone pre-exposure increased the level of phospho-JNK1, phospho-p38, phospho-ERK, and cleaved caspase 3 when compared with LPS alone. Densitometry of the bands revealed the following: FA + LPS and O3 + LPS were statistically significantly higher, when compared with either FA or O3 for phospho-JNK1 (B), phospho-p38 (C), phospho-ERK (D), and cleaved caspase 3 (E) (comparisons not specifically identified; \( p < 0.05 \); two-tailed Mann-Whitney \( U \) test). Significant differences between FA + LPS vs O3 + LPS are labeled (*, \( p < 0.05 \); two-tailed Mann-Whitney \( U \) test). Densitometry data represent mean ± SEM from three repeats.
TLR4 (G) and increased the median fluorescent intensity (H). We increased the percentage of macrophages with increased surface expression of TLR4 on alveolar macrophages after ozone exposure (F), when compared with FA controls (C). Three pools of two animals of live bronchial alveolar lavage cells were obtained 24 h after pre-exposure to either FA or ozone. Flow cytometric analysis was performed and gated on alveolar macrophages (F4/80 positive). Surface expression of TLR4 was determined by staining. Ozone increased the percentage of macrophage with increased surface expression of TLR4 (G) and increased the median fluorescent intensity (H).

FIGURE 9. Ozone modifies expression and cellular distribution of TLR4 on macrophages. Bronchial alveolar cells were analyzed by confocal microscopy. We identified alveolar macrophages by light microscopy (A and D) and the presence of the alveolar macrophage marker F4/80 (B and E). We demonstrate enhanced surface expression of TLR4 on alveolar macrophages after ozone exposure (F), when compared with FA controls (C). We did not observe differences in either mRNA or protein expression of TLR4 at this time point (Fig. 7). We did not observe ozone-dependent differences in TLR4 protein expression or in mRNA expression of known negative regulators of TLR4 signaling, including IRAK-M, A20, or tollip at this time point (data not shown).

Ozone exposure modifies the cellular distribution of TLR4

We did not observe differences in either mRNA or protein expression of TLR4 at 24 h after exposure to ozone that would explain ozone-induced enhanced TLR4 signaling (data not shown). For that reason, we considered that the spatial distribution of this receptor might account for the enhanced TLR4 signaling. In fact, 24 h after exposure to ozone, we observe increased intensity and altered distribution of TLR4 on alveolar macrophages by fluorescent microscopy (data not shown). Alveolar macrophages were identified by confocal microscopy (F4/80−) and demonstrate robust enhanced expression of TLR4 after exposure to ozone (Fig. 9, A–F). This was quantified by a shift in the percentage of alveolar macrophages that express a high concentration of TLR4 as detected by flow cytometry and a shift in the median fluorescent intensity (Fig. 9, G and H). Moreover, confocal microscopy images indicate that inhaled ozone results in altered spatial distribution of TLR4 on alveolar macrophages. These observations suggest that enhanced TLR4 signaling is related to trafficking of TLR4 within alveolar macrophages in response to inhaled ozone.

Ozone enhances LPS signaling in the lung

To determine whether ozone initiates intracellular signaling through innate immune pathways, we investigated the individual and combined effects of ozone and LPS on downstream signaling in whole lung homogenates. Ozone alone transiently increased total IL-1R-associated kinase-M (data not shown), as well as phosphorylation of JNK, ERK, and p38 4 h after exposure (Fig. 8). This increase was statistically significant for p38. However, the enhanced signal associated with acute ozone exposure returned to baseline by 24 h after exposure. All proteins were significantly higher in mice exposed to either FA + LPS or O₃ + LPS, when compared with mice exposed to either FA or O₃ (p < 0.05; Mann-Whitney U test). Mice pre-exposed to ozone showed enhanced phospho-JNK, phospho-ERK, and phospho-p38 immunoreactivity, when compared with either exposure alone at the 24-h timepoint (Fig. 7). We did not observe ozone-dependent differences in TLR4 protein expression or in mRNA expression of known negative regulators of TLR4 signaling, including IRAK-M, A20, or tollip at this time point (data not shown).

Ozone exposure modifies the cellular distribution of TLR4

Ozone exposure modifies the cellular distribution of TLR4

Ozone enhances LPS-induced production of proinflammatory cytokines in the whole lung, increased apoptosis of alveolar macrophages, and was associated with redistribution of TLR4 on alveolar macrophages. To specifically determine whether exposure to ozone modified the functional response of alveolar macrophages to LPS, we isolated lung macrophages from ozone-exposed animals by density centrifugation. These cells were subsequently challenged to LPS in vitro, and levels of TNF-α from the supernatant were measured. Pre-exposure to ozone does prime the innate immune response to endotoxin under these conditions (Fig. 10). This observation further supports the association between ozone-induced altered distribution of cellular TLR4 and enhanced biological response to LPS.

Discussion

Our findings indicate that inhalation of ozone substantially enhances innate immune responsiveness. Prior exposure to ozone results in greater LPS-induced airway hyperreactivity, higher concentrations of total protein, higher concentrations of proinflammatory cytokines, and an enhanced systemic inflammatory response...
to inhaled LPS. Ozone inhalation was associated with an enhanced biologic response to LPS, which was associated with enhanced proinflammatory signal and enhanced programmed cell death. Our results suggest that the enhanced biologic response to LPS is caused by altered cellular distribution of TLR4 following inhalation of ozone. In aggregate, these observations indicate that inhalation of ozone increases local and systemic LPS-induced injury by priming the innate immune system through altered special distribution of TLR4 and enhanced signaling.

It has long been appreciated that strict control of the biologic response to bacterial toxins is critical to host survival. This is highlighted by the fact that TLR4-deficient animals are protected against LPS-induced shock (32), yet are vulnerable to overwhelming live Gram-negative infections (33). Although it is essential to recognize, respond to, and clear pathogens, enhanced (acute or persistent) response to microorganisms can also lead to detrimental effects. In this study, we have demonstrated that ozone can substantially affect the response to LPS by priming the innate immune system through TLR4 cell surface expression. These findings are entirely consistent with the observed effect of ozone on decreased clearance of live bacterial pathogens (12–17). Moreover, these findings provide a biological hypothesis for the epidemiological relationship between ozone and increased pulmonary morbidity and mortality. Loss of lung function in chronic obstructive pulmonary disease is related to exposure to environmental air pollutants (34–37) and frequency of exacerbations (38). Our observations support that exposure to ozone can significantly enhance both lung injury and macrophage apoptosis after exposure to inhaled endotoxin. These findings suggest that ozone priming of response to inhaled endotoxin could contribute to both loss of lung function and defective antibacterial host defense.

Our results demonstrate that inhaled ozone can alter the spatial distribution of TLR4 and results in an enhanced response to LPS. This observation is consistent with previous in vitro observations, which demonstrate after stimulation trafficking of TLRs to the surface membrane of HEK293 cells (37). Recent observations in a model of hemorrhagic shock demonstrate that H2O2 can induce surface expression of TLR4 on monocytic cells (38). Furthermore, previous studies using transgenic animals demonstrate that the level of TLR4 RNA expression correlates with biologic function, suggesting a dose effect dependent on TLR4 expression (39). Our in vivo observations clearly demonstrate the pathophysiologic importance of exposure to inhaled environmental air pollutants and support that exposure to ambient ozone may have a profound effect on innate immune responsiveness. However, it remains possible that other members of the receptor complex or downstream adaptor molecules could also be altered by inhaled ozone. For example, an increase in soluble CD14 after exposure to ozone has been observed in an experimental study in humans (40). CD14, a critical component of the TLR4 complex, can either enhance the biologic response to LPS or act as a sink attenuating this response. Improved understanding of the environmental factors that can regulate TLR4-dependent signaling will improve our understanding of host defense.

Precise regulation of TLR-dependent signal is required to optimize normal inflammation and resolution of injury. Although inhaled ozone activates many proinflammatory pathways, we found that it also accelerates apoptosis. Thus, enhanced apoptosis may serve to regulate uncontrolled innate immune response. It remains plausible that this is a protective mechanism, in that robust monocyte and neutrophil recruitment, in the absence of apoptosis, could magnify the severity of lung damage. Attenuated inflammatory cell recruitment after a severe oxidative lung injury could prove protective. We speculate that in the absence of macrophage apoptosis, there might have been even higher levels of proinflammatory cytokines, neutrophil recruitment, and lung injury. In fact, ozone-induced apoptosis could also explain how pre-exposure to ozone can be protective with influenza infection (41), a disease in which acute morbidity is associated with an uncontrolled inflammatory response. In contrast, recent work supports that low-level TLR4 signaling appears protective in other forms of oxidative lung injury (42–44). Combined, these observations support divergent mechanisms of strict regulation of innate immune response to limit oxidative lung injury dependent on the level of activation.

It is noteworthy to highlight that the physiologic response of the airways was independent of the concentration of inflammatory cells. However, secreted cytokines/chemokines were clearly upregulated with coexposure, as are other markers of lung injury. Many factors, including the cytokines IL-1β (45), TNF-α (46–48), and IL-6 (49), have been associated with ozone-related AHR. In our model of coexposure, IL-6 was dramatically increased in both the lavage fluid and serum at all time points tested. Although it remains unclear whether this specific cytokine accounts for the differences in TLR4-dependent AHR, this study further supports the dichotomy between cellular inflammation and AHR. Further studies are needed to identify the pathogenesis of airway hyperresponsiveness, which remains a hallmark of asthma.

Ozone is a common urban air pollutant that significantly contributes to increased pulmonary morbidity and mortality, especially among those with chronic lung disease (1–4). We have demonstrated that ozone can prime the biologic response to inhaled LPS, leading to enhanced airway injury and apoptosis of inflammatory cells in the lung. TLR4-dependent signaling in the lung appears to be a double-edged sword. A controlled response is critical for effective clearance of bacterial pathogens. However, an exaggerated response can be associated with an increase in airway hyperresponsiveness, airway injury, and reduced numbers of functional inflammatory cells. If we are to gain a better understanding of disease etiology and pathogenesis, we need a more detailed understanding of the complex interaction between common environmental exposures and fundamental homeostatic mechanisms that regulate innate immunity.

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Disclosures
The authors have no financial conflict of interest.

References


Corrections


Following an inquiry at Duke University, we were informed that FlexiVent data provided to us by the animal pulmonary physiology laboratory at Duke University may have been unreliable. We therefore repeated the experiments described in Fig. 1 of the published article and were unable to replicate the airway hyperresponsiveness findings. Therefore, we have concerns about the integrity of the data published in Fig. 1. We hereby retract Fig. 1 and its legend from the published article. The other data presented in the article are not affected by the unreliable FlexiVent data, and retraction of the FlexiVent data described in Fig. 1 does not impact the overall conclusions of the published article.

Retraction of Fig. 1 means that the following changes need to be made to the text of the published article:

In the fourth sentence of the Abstract, the words “enhanced airway hyperreactivity” need to be omitted.

The first section of the Results, entitled Ozone enhances airway hyperresponsiveness in response to inhaled LPS, should be removed.

In the Discussion section, the following text should be removed: “airway hyperreactivity” in the second sentence; “in TLR4-dependent AHR, this study further supports the dichotomy between cellular inflammation and AHR” in the second to last sentence of the fifth paragraph; and “of airway hyperresponsiveness” in the last sentence of the fifth paragraph.

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