Hyaluronan Fragments Contribute to the Ozone-Primed Immune Response to Lipopolysaccharide

Zhuowei Li, Erin N. Potts, Claude A. Piantadosi, W. Michael Foster and John W. Hollingsworth

*J Immunol* 2010; 185:6891-6898; Prepublished online 29 October 2010;
doi: 10.4049/jimmunol.1000283
http://www.jimmunol.org/content/185/11/6891

References  This article cites 38 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/185/11/6891.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Errata  An erratum has been published regarding this article. Please see next page or:
/content/196/5/2426.full.pdf

The *Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Hyaluronan Fragments Contribute to the Ozone-Primed Immune Response to Lipopolysaccharide

Zhuwei Li,* Erin N. Potts,* Claude A. Piantadosi,* W. Michael Foster,* and John W. Hollingsworth*†

Hyaluronan is a high-molecular mass component of pulmonary extracellular matrix, and lung injury can generate a low-molecular mass hyaluronan (HA) fragment that functions as endogenous ligand to cell surface receptors CD44 and TLR4. This leads to activation of intracellular NF-κB signaling and proinflammatory cytokine production. Based on previous information that ozone exposure causes increased HA in bronchial alveolar lavage fluid and ozone pre-exposure primes immune response to inhaled LPS, we hypothesized that HA production during ozone exposure augments the inflammatory response to LPS. We demonstrate that acute ozone exposure at 1 part per million for 3 h primes the immune response to low-dose aerosolized LPS in C57BL/6J mice, resulting in increased neutrophil recruitment into the airspaces, increased levels of protein and proinflammatory cytokines in the bronchoalveolar lavage fluid, and increased airflow hyperresponsiveness. Intratracheal instillation of endotoxin-free HA (25 µg) enhances the biological response to inhaled LPS in a manner similar to ozone pre-exposure. In vitro studies using bone marrow-derived macrophages indicate that HA enhances LPS responses measured by TNF-α production, while immunofluorescence staining of murine alveolar macrophages demonstrates that HA induces TLR4 peripheralization and lipid raft colocalization. Collectively, our observations support that ozone primes macrophage responsiveness to low-dose LPS, in part, due to HA-induced TLR4 peripheralization in lung macrophages. The Journal of Immunology, 2010, 185: 6891–6898.

Epidemiological studies have shown that increased ambient levels of the air pollutant ozone are associated with increased respiratory morbidity and all-cause mortality (1–3). Laboratory studies support that exposure to ozone can modify antibacterial host defense (4, 5), and we have identified that inhalation of ozone (2 parts per million [ppm] for 3 h) can enhance surface expression of TLR4 on lung macrophages, resulting in enhanced functional response to LPS (6). In that model, we observe enhanced lung injury and increased apoptosis of macrophages with coexposures. Priming of pulmonary innate immunity and enhanced lung injury could contribute to impaired antibacterial host defenses reported in association with inhalation of ozone. However, our previous study was limited by a relatively high dose of ozone exposure, and the mechanisms that lead to ozone-induced priming of response to LPS remain unclear. The goal of this study was to better understand the mechanisms of how inhalation of ozone can modify pulmonary innate immune response. We recently reported that exposure to ozone results in the formation of biologically active short fragments of hyaluronan (HA) that contribute to airway hyperresponsiveness (AHR) (7). Additionally, previous work has suggested that HA interaction with the surface receptor CD44 can stabilize macrophage TLR4 (8). We therefore hypothesized that exposure to environmentally relevant levels of ozone would result in release of short-fragment HA, which would enhance the biological response to LPS through enhanced surface expression of TLR4.

HA is synthesized as a high-molecular mass nonsulfated glycosaminoglycan found in many cell types, including epithelial, neural, and soft connective tissues (9). HA is a major component of extracellular matrix, important for cell proliferation and migration. In tissue injury and inflammation, HA undergoes a degradation process induced by reactive oxygen species, enzymatic cleavage, or abnormal synthesis, and low-molecular mass HA fragments can be immunostimulatory (10). CD44 is the major cell surface receptor for HA, and it significantly contributes to the clearance of HA fragments and mediates cell signaling. However, it has been reported in CD44-deficient mice that the HA-induced inflammatory response is only partially blocked (11), suggesting the participation of other surface receptors for HA. HA has been identified as an endogenous ligand of TLR4 (12), and the HA interaction with TLRs was identified as a factor in bleomycin-induced sterile lung injury (11). HA also contributes to the pulmonary biological response to both asbestos fibers (13) and ozone (7, 14), but its role in priming pulmonary innate immunity remained unexplored.

Understanding the mechanisms that regulate the intensity of innate immune response could have implications in many common lung diseases. We demonstrate that exposure to the common urban air pollutant ozone can both prime innate immune response to LPS and increase levels of soluble HA. Ozone-primed immune response to LPS is partially blocked by HA-binding peptide, which supports the idea that HA plays a role in ozone priming of innate immunity. Direct administration of HA fragments into the lung of C57BL/6J mice augments the immune response to inhaled LPS. Previ-
ous reports have supported a central role of lung macrophages in the response to inhaled LPS (15, 16). We demonstrate that HA fragments can increase surface expression of TLR4 on murine macrophages, which is associated with enhanced functional response to LPS. Our results support that HA fragments associated with sterile lung injury can prime pulmonary innate immune response to LPS.

Materials and Methods

Mice

Male C57BL/6J mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at Duke University Medical Center.

Inhalation exposures

Animals were placed into separate cages and exposed in a chamber to filtered air (FA) or 1 ppm of ozone for 3 h and then allowed to recover for 24 h under normal housing condition. The lower level of ozone exposure (1 ppm for 3 h) used in this protocol produced a minimum lung injury in terms of total and inflammatory cells counted in bronchoalveolar lavage fluid (BALF) compared with the more typical murine ozone injury model (2 ppm for 4 h). Our selection of ozone concentration levels in the mouse is based on similar biological responses observed in human exposure studies and published deposition fraction data for ozone in rodent models (17, 18). Animals were exposed in the chamber with air at 20–22°C and 50–60% relative humidity supplied at a rate of 20 exchanges per hour. Ozone generated by directing 100% oxygen through a UV light ozone generator was monitored continuously with an ozone analyzer (Winguard, FA). The concentration of ozone in the exposure chamber was monitored continuously by an ozone UV light photometer (model 1003A; Dasibi Environmental, Glendale, CA). Preexposed animals were then challenged with aerosolized LPS purified from 0111:B4 Escherichia coli (Sigma-Aldrich, St. Louis, MO) for 2.5 h. All animals were evaluated at 4–7 h after the initiation of LPS exposure. LPS at 0.9 μg/ml in PBS was placed in a jet nebulizer (TSI, Shoreview, MN) to generate a mean level of aerosolized LPS of 4.3–4.8 μg/m3 in a 55-liter Hanniers-style exposure chamber. The dosage is similar to that experienced by gain mill workers during a typical 8-h work day that causes an inflammatory response in the lower respiratory tract. In blocking experiments, C57BL/6J mice were anesthetized with isoflurane and then 0.22 mg hyaluronic acid-binding peptide (HABP) or scrambled-binding peptide (SBP) (GenScript, Piscataway, NJ) were oropharyngeally instilled into the mouse immediately prior to ozone exposure. Experimental groups consisted of 6–10 mice with at least two repeats.

HA challenge

Endotoxin-free HA fragments were prepared as described (7). Briefly, high-molecular mass HA (Healon GV; Abbott Medical Optics, Santa Ana, CA) was reconstituted to 0.5 mg/ml in 0.02 M acetate/0.15 M sodium chloride (pH 6.0). For the production of low-molecular mass HA, Healon GV was sonicated 3 × for a total of three times on ice. Sizes of HA were confirmed by agarose gel electrophoresis. For in vivo experiments, 50 μl HA or vehicle control were instilled into isoflurane-anesthetized mice by oropharyngeal aspiration. LPS exposure, as described above, was initiated 2 h after instillation of HA.

Whole lung lavage

As previously described (7), mice were euthanized with CO2 and the lungs were exposed and fully inflated three times serially to 25 cmH2O of 6.9% NaCl. Cell counts were performed using a hemocytometer, and differentials were performed by H&E-stained cytopsins. Cytokines/chemokines IL-1β, IL-6, KC, MCP-1, and TNF-α were determined by Luminex (Bio-Rad, Hercules, CA) using five-plex reagents from Millipore (Billerica, MA). Assay sensitivities are 1.8 pg/ml for IL-6, 2.0 pg/ml for IL-1β, 1.4 pg/ml for KC, 5.3 pg/ml for MCP-1, and 1.0 pg/ml for TNF-α. Quantikine or DuoSet TNF-α ELISA kits from R&D Systems (Minneapolis, MN) were used for some in vitro experiments. Total protein concentrations in lung lavage fluid were measured by the lowry Assay (Bio-Rad). HA level in BALF was measured by ELISA (Echelon, Salt Lake City, UT).

Airway physiology

Anesthesia was achieved with 60 mg/kg sodium pentobarbital injected i.p. Mice were then given neuromuscular blockade (0.8 ml/kg pancuronium bromide) and ventilated with a computer-controlled small animal ventilator (FlexiVent; Scireq, Montreal, Quebec, Canada), with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cmH2O. 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 that the parameters calculated had peaked. The lungs were inflated to total lung capacity after each dose of methacholine, maintaining open airways and returning the measurements back to baseline. The measurements were averaged at each dose and graphed (measured in cmH2O/ml/s) along with the initial baseline measurement.

Isolation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMMs) were obtained from M-CSF–induced differentiation of bone marrow cells. Briefly, mouse bone marrows were harvested from femurs and tibias and passed through a 10-cc syringe with an 18-gauge needle to generate single-cell suspensions in bone marrow culture medium (RPMI 1640 with 10% FBS and 1% streptomycin/penicillin). Then cells were centrifuged and seeded into 24-well plates by 3 × 104 cells/well. Cells were allowed to attach to the bottom for 4 h before a complete change of media with fresh culture medium containing 20 ng/ml M-CSF (R&D Systems). After 5–7 d culture in M-CSF–containing medium, nonadherent cells were washed away and adherent cells were treated with different doses of HA fragments and LPS in RPMI 1640 with 10% heat-inactivated FBS.

Immunofluorescence staining for TLR4 expression

Murine alveolar macrophage (MH-S) cells were used to study TLR4 surface expression. Cells plated in eight-well chamber slides were cultured in RPMI 1640 with 10% PBS and 10 mM HEPES to 60–70% confluency at 37°C with 5% CO2. Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma-Aldrich and used to inhibit NF-κB activation. After treatment, cells in the chamber were washed once with cold PBS and then fixed with 4% ultrapure paraformaldehyde for 30 min at room temperature. Following three washes with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min. Lipid rafts in the cell were labeled by incubation with 8 μg/ml PE-conjugated cholera toxin B on ice for 30 min. TLR4 staining, the slides were washed three times with PBS, blocked with 5% PBS in PBS for 1 h at room temperature, and then incubated with 1/50 diluted goat anti-TLR4 Ab (L-14; Santa Cruz Biotechnology, Santa Cruz, CA) or IgG control in 3% PBS in PBS for another 1 h. TLR4 expression was detected by incubation with Alexa 594-labeled donkey anti-goat secondary Ab for 30 min in the dark. Nucleus was counterstained with DAPI in PBS for 2 min. After unlabeled, dyes were washed off with PBS, slides were mounted with ProLong Gold (Invitrogen, Carlsbad, CA) and sealed with nail polish for later observation. Images were captured by a Zeiss LSM510 inverted confocal microscope (Carl Zeiss Light Microscopy, Göttigen, Germany) with ×100 or ×63 oil objective, using 405-, 488-, and 594-nm lasers for excitation light sources. Fluorescence was generated through a 505–550-nm or 560–600-nm filter, and a Zeiss LSM Image Browser version 4.0 (Carl Zeiss Light Microscopy) was used for image analysis.

Flow cytometry

Alveolar macrophages were collected by whole lung lavage. BALFs from two mice were pooled to get enough cells for flow cytometry analysis. Cell pellets were collected by centrifuge and then resuspended in 100 μl cold PBS, blocked for 10 min on ice with Fc blocker, rat and mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Surface expression of TLR4 and macrophage surface marker CD11c were cultured in RPMI 1640 with 10% heat-inactivated FBS and 10 mM HEPES. Cells were plated in eight-well chamber slides and allowed to attach to the bottom for 4 h before a complete change of media with fresh culture medium containing 20 ng/ml M-CSF (R&D Systems). After 5–7 d culture in M-CSF–containing medium, nonadherent cells were washed away and adherent cells were treated with different doses of HA fragments and LPS in RPMI 1640 with 10% heat-inactivated FBS.

Statistics

Data are expressed as means ± SEM. Software from SPSS (Chicago, IL) was used for statistical analysis. Significant difference among multiple groups with two variants was determined by two-way ANOVA. The Student t test was used for individual comparisons between groups. A two-tailed p value <0.05 was considered statistically significant.
Results

Biological effects of ozone exposure

Inhalation of ozone at 2 ppm for 3 h enhances the immune response to inhaled LPS in the lung (6). This level of ozone induces significant neutrophilic inflammation and causes lung injury. To minimize lung injury and more closely model human exposures, we reduced the level of ozone to 1 ppm for 3 h. We observed increases in total cells, macrophages, and neutrophils with both levels of ozone; however, the lower dose of ozone increased the number of total cells, macrophages, and neutrophils in BALF only slightly compared with air-exposed animals. Compared with the higher dose of ozone, mice exposed to 1 ppm ozone had significantly reduced lung inflammation and lavage total protein (Fig. 1). The lower level of ozone was associated with a reduction in the number of neutrophils by 90% and in the lavage protein by 62% of the level observed after 2 ppm ozone (Fig. 1A, 1B). Both levels of ozone exposure induced similar enhancement of AHR to methacholine when compared with air (Fig. 1C). These results support a dose response to ozone in the development of lung inflammation and epithelial injury. However, similar degrees of ozone-induced AHR were observed with both concentrations of ozone.

Ozone exposure enhances pulmonary response to inhaled low-dose LPS

Animals were pre-exposed to ozone (1 ppm for 3 h) and then 24 h later were exposed to low-dose aerosolized LPS. This level of LPS exposure led to 50–60% neutrophils in lavage fluid and significantly increased AHR to methacholine challenge, but did not increase lavage protein levels. Ozone pre-exposure significantly increased LPS-induced neutrophil recruitment into the airspace (Fig. 2A). Coexposed animals had enhanced lung injury as measured by the level of lavage protein, which were increased by 5-fold compared with LPS and 2.5-fold compared with ozone (Fig. 2B). Additionally, pre-exposure to ozone significantly augmented LPS-induced AHR at highest dose of methacholine challenge (Fig. 2C) and the production of proinflammatory cytokines (Fig. 2D). Collectively, these observations support the idea that inhalation of ozone primes the biological response to low level of aerosolized LPS.

Inhalation of ozone increases the level of low-molecular mass. HA in the lung

Elevated levels of HA within the lung have been observed in many forms of oxidative stress. We previously reported that 2 ppm ozone exposure for 3 h significantly increased HA level in BALF (7, 14), and we now demonstrate that 1 ppm ozone for 3 h also increases HA levels in BALF. The average molecular mass of HA is ~100–200 kDa by agarose gel electrophoresis, which is consistent with previous report (data not shown). We can successfully prevent ozone-induced increased soluble HA by supplementing the animal water with the antioxidant N-acetylcysteine (data not shown). This finding suggests that fragmentation of HA is related to ozone-

FIGURE 1. Comparison of biological effects of ozone exposure at different doses. Ozone-induced inflammatory response in the lung (A) and level of protein in the lung lavage (B) are exposure dose-dependent. C. One part per million and 2 ppm of ozone did not produce different level of AHR. *p < 0.05, compared with FA group; #p < 0.05, 2 ppm versus 1 ppm; n = 6.

FIGURE 2. Ozone exposure primed pulmonary response to inhaled LPS. Animals were exposed to either FA or ozone at 1 ppm for 3 h. After 24 h, mice were then exposed to aerosolized LPS for 2.5 h. At 4 h postexposure, animals were phenotyped. Ozone pre-exposure significantly increased LPS inhalation-induced lung inflammation (A) and epithelial injury (B). C. AHR was significantly augmented in the coexposed group when compared with the single exposed groups. D. LPS-induced IL-6, MCP-1, and TNF-α production were enhanced by ozone pre-exposure. *p < 0.05; n = 6.
induced oxidant stress. Inhalation of LPS did not significantly increase HA levels in lavage fluid. Coexposure to both ozone and LPS resulted in a doubling of air space HA over ozone alone (ozone: 65.90 ± 11.46 ng/ml versus ozone-LPS: 152.1 ± 24.48 ng/ml) (Fig. 3).

HA blockade attenuates ozone priming of innate immunity

To determine whether HA is necessary for the priming effects of ozone in the lung, the blocking peptide HABP or control SBP was directly administered into the lungs of mice immediately prior to ozone exposure. Again, it was observed that pre-exposure to ozone enhances the biological response to secondary challenge to LPS. This finding was supported by enhanced cellular inflammation, lavage protein, AHR, and cytokines. Treatment with SBP had no effect on the response to coexposure. However, HABP pretreatment significantly attenuated the ozone-priming response to LPS as measured by inflammatory cell recruitment, AHR, and level of proinflammatory cytokines (Fig. 4). We did not observe differences in the level of lavage total protein with HABP treatment. Similar experiments were performed by administration of HABP or SBP through i.p. injection. In those experiments, we observed similar reduced ozone priming LPS response with HABP treatment as measured by AHR and cytokines, but not cellular recruitment (data not shown). These results indicate that HA is necessary for the complete biological response to ozone–LPS coexposures.

HA is sufficient to prime pulmonary immune response to LPS

To determine whether HA is sufficient to prime pulmonary innate immune response to inhaled LPS, 25 μg HA was directly instilled into the lungs of C57BL/6J mice. HA treatment did not cause pulmonary inflammatory cell infiltration when compared with vehicle (Fig. 5). HA treatment alone also did not increase the level of protein in the lung lavage but did cause an increase in both AHR and IL-6 production. When HA-treated mice received a secondary exposure to low-level aerosolized LPS, a significant increase in total cells and neutrophils was observed in BALF compared with LPS-alone exposed mice (Fig. 5A). Similarly, lavage protein, BALF IL-6 levels, and AHR were also augmented in coexposed mice (Fig. 5B–D). HA pretreatment replicated the phenotype observed with ozone-primed pulmonary response to LPS. Therefore, these data indicate that HA is sufficient to prime immune response to inhaled LPS.

HA primes macrophage for augmented immune response to LPS stimulation

Our previous studies suggest that alveolar macrophages play a role in ozone-induced lung disease (14). HA instillation triggers NF-κB activation in lung macrophages. Additionally, macrophages play an important role in the biological response to LPS (15, 16). These observations suggest that the HA-primed response to LPS could be related to alterations in macrophage innate immune response. This possibility was tested by first determining whether soluble factors released into the airspace after inhalation of ozone were sufficient to prime cultured BMDM response to LPS. Cell-free lung lavage supernatant from animals exposed to air or ozone

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Ozone exposure increased HA level in lavage fluid. Lavage fluid from ozone-exposed groups contained higher levels of HA when compared with the FA group. Although low-dose LPS exposure did not increase HA level, HA was significantly enhanced in ozone/LPS coexposed group. **p < 0.01; n = 8 for FA and ozone groups; n = 5 for LPS and coexposure groups.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Ozone-enhanced LPS responsiveness was partially blocked by HABP. Oropharyngeal aspiration of HABP was performed immediately prior to ozone exposure. Twenty-four hours later, animals were then exposed to LPS aerosol. A, HABP partially inhibited inflammatory cell recruitment. B, HABP did not affect the lavage level of total protein. C, Enhanced AHR was completely blocked by HABP pretreatment. D, HABP pretreatment blocked ozone-augmented IL-6, MCP-1, and TNF-α production in response to LPS. *p < 0.05; n = 6. RT, resistance.
with or without HABP pretreatment was placed on BMDMs and then cells were challenged with LPS. We found that inhalation of ozone resulted in the release of soluble factors that can prime macrophage response to LPS. BALF from HABP-pretreated mice failed to prime macrophage response to LPS. This observation further supports a role for soluble HA in ozone-induced priming of macrophage response to LPS (Fig. 6, A, B). However, many soluble factors are released into the airspace after inhalation of ozone. To directly determine the effects of HA, BMDMs were stimulated with HA and then the cells were exposed to LPS. HA alone induced low levels of TNF in a dose- and time-dependent manner (Fig. 6C). HA-pretreated cells demonstrated an enhanced response to LPS exposure (Fig. 6D). These in vitro data demonstrate that HA is sufficient to prime innate immune response of BMDMs to LPS.

**HA enhances macrophage TLR4 expression in lipid rafts**

TLR4 is the LPS surface receptor, and it is known that the level of expression contributes to the intensity of host immune response to LPS (19, 20). Furthermore, we have previously demonstrated that inhalation of ozone (2 ppm) can enhance surface expression of TLR4 on lung macrophages (6). Accordingly, we observed a small significant increase in intensity of TLR4 staining on alveolar macrophages in BALF after exposure to low-dose ozone (Fig. 7; FIGURE 5. Hyaluronan fragments enhanced pulmonary response to inhaled LPS. Fifty microliters HA at 0.5 mg/ml was intratracheally instilled into the lung 2 h prior to LPS inhalation. A, HA pretreatment significantly enhanced inflammatory cell infiltration in response to LPS exposure. B, HA instillation enhanced LPS-induced lung injury marked by increased lavage protein. C, HA-LPS coexposed group demonstrated increased airway reactivity to 100 mg/ml methacholine challenge compared with LPS-exposed group. D, HA instillation increased LPS-induced IL-6 production. *p < 0.05; n = 10.

**FIGURE 6.** HA primed macrophages for increased response to LPS. BMDMs were treated with BALF from air/ozone-treated mice or HA for 2 h prior to LPS exposure. Supernatants were collected after 4 h LPS stimulation and evaluated for TNF-α production. BALF from ozone-treated animals but not HABP-pretreated animals significantly increased TNF-α production in BMDMs in response to 0.1 mg/ml LPS (A) or 1 mg/ml LPS (B) stimulation. *p < 0.05; n = 6. C, Dose and time response of BMDMs to HA treatment. *p < 0.05, compared with vehicle control; #p < 0.05, compared with 4 h incubation; n = 6. D, HA enhanced LPS-induced TNF-α production in BMDMs in response to LPS stimulation. *p < 0.05; n = 6.
p < 0.05, versus FA-exposed group). To directly determine the role of HA in macrophage expression of TLR4, a murine alveolar macrophage cell line was stimulated with HA (25 μg/ml) for 1 h and then stained for TLR4 and GM-1. Confocal images in Fig. 8A demonstrated that TLR4 was evenly distributed in untreated control cells. However, after HA treatment, TLR4 staining was significantly increased along the cell membrane, suggesting peripheral aggregation of the TLR4 molecule. Additionally, this peripheralized TLR4 colocalized with GM-1, a marker for lipid rafts. Previously, we reported that HA-induced NF-κB activation contributes to ozone-induced AHR (14). By using a commonly used NF-κB inhibitor, PDTC, we blocked HA-induced TLR4 peripheralization. This observation suggests that NF-κB activation may contribute to TLR4 trafficking. Next, we determined the functional consequence of redistribution of TLR4. Treatment of MHS cells with HA alone for 2.5 h did not result in a detectable release of TNF-α into the supernatant. However, pretreatment of MHS cells with HA enhanced functional response to LPS as measured by TNF-α (Fig. 8B). These data demonstrate that HA led to redistribution of TLR4 on macrophages, which was associated with enhanced functional response to LPS.

**Discussion**

Ambient ozone exposures contribute significantly to pulmonary morbidity, resulting in increased emergency room visits and hospitalizations at 24–48 h after ground levels exceed regulatory standards (2, 21). However, the mechanisms by which ozone inhalation results in enhanced morbidity remain poorly understood. We now recognize that ozone-induced fragmentation of HA contributes to AHR and the release of proinflammatory cytokines (7, 14). The present study builds on previous observations by demonstrating that after inhalation of ozone, HA is necessary to enhance the innate immune response to inhaled LPS and that HA alone is sufficient to explain the enhanced innate immune response to inhaled LPS. Mechanistically, our data indicate that HA can enhance both macrophage surface expression of TLR4 and the functional response to LPS. Our data suggest that HA activation of NF-κB may contribute to the cellular distribution of TLR4.
Overall, we identify that low-molecular mass HA, as an intermediate product of ozone exposure, contributes to priming of pulmonary innate immune response to LPS. Modification of pulmonary innate immunity may alter host susceptibility either to pathogens or to other environmental toxicants and therefore contributes to morbidity associated with inhalation of ambient ozone.

The alveolar macrophage is a front-line cell of the immune system in the recognition of environmental pathogens and inhaled toxicants. Immediate recognition of bacterial endotoxin by the macrophage is dependent on the expression of the TLR4 surface receptor (22, 23), and macrophage-derived TLR4 generates the early-phase inflammatory response to bacterial LPS (15, 16). Although we have previously shown that inhalation of ozone can prime the lung macrophage response to LPS (6), the mechanisms by which ozone leads to an enhanced response to LPS was not understood. In this study, using the information that HA stimulation of macrophages causes the release of proinflammatory cytokines, we have been able to identify a central role of HA in priming lung macrophages to ozone that was not dependent on a significant increase in the number of alveolar macrophages in the lungs of animals challenged with HA. This finding suggested that HA exposure affected a qualitative change in the macrophages, which we subsequently observed. However, we suspect that if the mice had been phenotyped at later time points after HA challenge, additional macrophages might have been recruited into the lungs of these animals as a result of the chemokine gradient. Furthermore, the possibility of indirect effects of proinflammatory cytokines on the macrophage distribution of TLR4 expression cannot be ruled out, but the in vitro studies demonstrate translocation of TLR4 in response to HA stimulation, suggesting a direct effect. Thus, HA can modify the distribution of TLR4 on macrophages, which is associated with enhanced functional responses to LPS.

Strict and precise regulation of TLR-dependent signaling is required to optimize normal inflammation and resolution of tissue injury. It appears that the level of TLR4 activation can produce divergent effects on the host. For example, low-level TLR4 signaling appears protective in some forms of oxidative lung injury (24–26) and moderate signaling facilitates the clearance of pathogens (27, 28), whereas excessive prolonged TLR4 signaling can augment lung injury (29). The level of expression of TLR4 can contribute to the intensity of response to TLR4-ligands (19, 30); however, immediate regulation of TLR4 signaling intensity can also be achieved through trafficking of surface receptors (31, 32). In vitro observations support trafficking of TLRs to the surface membrane of HEK 293 cells after stimulation (8, 33). Treatment of MHS cells with LPS alone, which results in NF-κB activation and production of intracellular reactive oxygen species (ROS), resulted in enhanced surface expression of TLR4 (data not shown). Understanding the factors that regulate trafficking of TLRs on resident alveolar macrophages in the context of common environmental exposures will improve our understanding of pulmonary innate immunity. Previous studies have suggested that the generation of ROS contribute to TLR4 trafficking from endoplasmic reticulum to lipid raft. For example, in a mouse model of hemorrhagic shock, it was demonstrated that H$_2$O$_2$ can induce surface expression of TLR4 on monocytes cells (34). In contrast, TLR4 trafficking to the cell surface can be inhibited by exposure to CO, which may depend on suppression of ROS generation (35).

Our results suggest that macrophage exposure to fragments of HA induce TLR4 trafficking in a manner dependent on NF-κB. However, it remains unclear whether the effects of PDTC in this study are the result of attenuated activation of NF-κB or reduced intracellular ROS. Previous work has supported the idea that inhaled oxidant stress can result in activation of NF-κB. Additionally, activation of NF-κB can be associated with intracellular ROS production. Therefore, we cannot exclude the possibility that intracellular release of ROS associated with NF-κB activation accounts for HA-induced TLR4 trafficking. The intracellular signaling, which regulates trafficking of TLR4 in this context, will be an area of future investigation. Prior to this study, it was not clear whether extracellular factors contribute to the distribution of TLR4 in the context of an inhaled environmental oxidant stress. However, recent observations by Taylor et al. (8) demonstrated that extracellular HA treatment of macrophages enhanced surface colocalization of TLR4-CD44. Our observations support the functional consequences of increased levels of HA in the airspace in the context of a clinically relevant environmental exposure.

The levels of HA in the airspace are relevant to a number of common lung diseases, and increased levels of HA have been identified in the airspaces of both asthmatic and chronic obstructive pulmonary disease patients (36–38). However, the role of HA in these lung diseases remains poorly understood, although our finding that HA can modify the intensity of TLR4-dependent signaling may be relevant. TLR4-dependent signaling in the lung appears to be a double-edged sword. A controlled response is critical for effective clearance of bacterial pathogens, but an exaggerated innate immune response can be associated with increases in AHR, airway injury, and in the numbers of functional inflammatory cells. It remains plausible that blocking HA-dependent signaling could be an effective way to control exacerbations of diseases of airways without necessarily impairing effective pathogen clearance. This study provides a more detailed understanding of the complex interaction between a common environmental exposure and fundamental homeostatic mechanisms that regulate the intensity of pulmonary innate immune response. Specifically, HA fragments produced after exposure to ambient ozone contribute to priming of macrophages for increased responsiveness to LPS. Collectively, these data demonstrate that inhalation of common inhaled ambient toxicants can modify host innate immunity and could impact the severity of several common diseases of airways.

Acknowledgments

We thank the members of the ONES External Advisory Committee, including Rashmin Savani, Timothy Blackwell, Kent Pinkerton, and Debra Laskin, for insightful comments.

Disclosures

The authors have no financial conflicts 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 may have been unreliable. We therefore repeated the experiments described in Fig. 5C of the published article and were unable to replicate the airway hyperresponsiveness findings. Therefore, we also have concerns about the integrity of the data published in Figs. 1C, 2C, and 4C. We hereby retract Figs. 1C, 2C, 4C, and 5C 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 Figs. 1C, 2C, 4C, and 5C does not impact the overall conclusions of the published article.

Retraction of Figs. 1C, 2C, 4C, and 5C means that the following changes need to be made to the text of the published article:

In the abstract, the words “... and increased airway hyperresponsiveness...” need to be omitted.

In the legend to Fig. 1C, the sentence “One part per million and 2 ppm of ozone did not produce different level of AHR” should be removed.

In the legend to Fig. 2C, the sentence “AHR was significantly augmented in the coexposed group when compared with the single exposed groups” should be removed.

In the Results section, under the subheading “Ozone exposure enhances pulmonary response to inhaled low-dose LPS,” the words “... AHR at highest dose of methacholine challenge (Fig. 2C) and the...” should be removed.

In the Results section, under the subheading “HA blockade attenuates ozone priming of innate immunity,” the words “… inflammatory cell recruitment, AHR, and…” as well as the next instance of “… AHR and…” should be removed.

In the Results section, under the subheading “HA is sufficient to prime pulmonary immune response to LPS,” the words “… and AHR…” should be removed.

In the legend to Fig. 4C, the following sentence needs to be deleted: “Enhanced AHR was completely blocked by HABP pretreatment.”

In the legend to Fig. 5C, the following sentence needs to be deleted: “HA-LPS coexposed group demonstrated increased airway reactivity to 100 mg/ml methacholine challenge compared with LPS-exposed group.”

In the first paragraph of the Discussion, the following sentence needs to be deleted: “We now recognize that ozone-induced fragmentation of HA contributes to AHR and the release of proinflammatory cytokines (7, 14).”

In the last paragraph of the Discussion, the following needs to be deleted: “… AHR…”

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1600004