TLR4 Is Necessary for Hyaluronan-mediated Airway Hyperresponsiveness after Ozone Inhalation

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Rationale: Ozone is a common environmental air pollutant that contributes to hospitalizations for respiratory illness. The mechanisms, which regulate ozone-induced airway hyperresponsiveness, remain poorly understood. We have previously reported that toll-like receptor 4 (TLR4)-deficient animals are protected against ozone-induced airway hyperresponsiveness (AHR) and that hyaluronan (HA) mediates ozone-induced AHR. However, the relation between TLR4 and hyaluronan in the airway response to ozone remains unexplored.

Objectives: We hypothesized that HA acts as an endogenous TLR4 ligand for the development of AHR after ozone-induced environmental airway injury.

Methods: TLR4-deficient and wild-type C57BL/6 mice were exposed to either inhaled ozone or intratracheal HA and the inflammatory and AHR response was measured.

Measurements and Main Results: TLR4-deficient mice have similar levels of cellular inflammation, lung injury, and soluble HA levels as those of C57BL/6 mice after inhaled ozone exposure. However, TLR4-deficient mice are partially protected from AHR after ozone exposure as well as after direct intratracheal instillation of endotoxin-free low molecular weight HA. Similar patterns of TLR4-dependent cytokines were observed in the bronchial alveolar lavage fluid after exposure to either ozone or HA. Exposure to ozone increased immunohistological staining of TLR4 on lung macrophages. Furthermore, *in vitro* HA exposure of bone marrow-derived macrophages induced NF- κ B and production of a similar pattern of proinflammatory cytokines in a manner dependent on TLR4.

Conclusions: Our observations support the observation that extracellular matrix HA contributes to ozone-induced airways disease. Furthermore, our results support that TLR4 contributes to the biological response to HA by mediating both the production of proinflammatory cytokines and the development of ozone-induced AHR.

Keywords: environmental airways injury; asthma; toll-like receptor; macrophage; TNF- α

Urban air pollution with ozone is common in developed countries and leads to increased morbidity in susceptible individuals (1–4). Ambient ozone exposure contributes to 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). According

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Inhalation of the common environmental air pollutant ozone can lead to oxidant lung injury, which is associated with fragmentation of lung extracellular matrix, activation of innate immunity, and airway hyperresponsiveness.

What This Study Adds to the Field

We identify the role of toll-like receptor 4 in recognition of short fragments of hyaluronan contributing to ozone-induced airway hyperresponsiveness.

to population-based studies, each 10-ppb increase in 1-hour daily maximum level of ozone is associated with an increase in mortality risk of individuals with cardio-respiratory disease of 0.39 to 0.87% (2, 3, 6, 7). However, the biological mechanisms that regulate the response to ambient ozone exposure are not completely understood.

There is a growing body of evidence supporting the role of pulmonary innate immunity in the biologic response to ozone. Ozone exposure alters the lung transcriptome (8) and induces the secretion of many proinflammatory factors into the lung, including neutrophil elastase, complement, prostaglandins, IL-1, tumor necrosis factor (TNF)- α , IL-6, IL-8, and granulocyte macrophage-colony stimulating factor (9–11). Many proinflammatory factors play a key role in the biologic response to inhaled ozone including keratinocyte-derived chemokine (KC), IL-1B, IL-6, monocyte chemoattractant protein-1 (MCP-1), and TNF- α (12–18). These soluble factors are also recognized as downstream products of innate immune activation. Cumulatively, these data suggest that activation of innate immunity could play an important role in response to ambient ozone. However, the initial stimulus leading to activation of the innate immune response remains poorly described.

The innate immune response is mediated, in part, by surface receptors called toll-like receptors (TLRs), which can recognize foreign pathogen-associated molecular patterns (PAMPs) (19). PAMPs bind TLRs and then interact with intracellular adaptor molecules resulting in the activation of nuclear transcription regulators. This cascade results in the production of proinflammatory factors associated with the immediate inflammatory response. The endotoxin receptor, TLR4 was found to play a role in airway injury in response to ozone through fine-mapping of a quantitative trait locus in the endotoxin-resistant and ozone-resistant C3H/HeJ mouse (20, 21). Subsequently, ozone-induced airway hyperresponsiveness (AHR) was shown to be dependent on intact TLR4 using gene-targeted mice (22). Recent data also supports that ozone-induced AHR is additionally dependent on the downstream adaptor protein MyD88 (23) and that the

(Received in original form March 10, 2009; accepted in final form December 10, 2009)

Supported by the National Institute of Environmental Health Services (ES16347, ES16126, ES16659). Support is also provided, in part, by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences.

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Am J Respir Crit Care Med Vol 181, pp 666–675, 2010

Originally Published in Press as DOI: 10.1164/rccm.200903-0381OC on December 10, 2009
Internet address: www.atsjournals.org

Figure 1. Ozone-induced airway hyperresponsiveness (AHR) and proinflammatory factors in bronchoalveolar lavage fluid. Mice were exposed to either filtered air or ozone (2 ppm $\times$ 3 hrs) and invasive measurements of airway response to methacholine were determined by flexivent. (A) Toll-like receptor 4 (TLR4) is essential for the development of ozone-induced AHR ($n = 6$; $*P < 0.05$). (B) Proinflammatory cytokines were measured from the cell-free supernatant by luminex bead profiling. The level of keratinocyte-derived chemokine, IL-1 β , IL-6, monocyte chemoattractant protein-1, and tumor necrosis factor- α were increased with exposure to ozone and dependent on the presence of TLR4 ($n = 6$; $*P < 0.01$).

biological response to ozone is partly dependent on the nuclear transcription regulator nuclear factor (NF)- κ B (24). Based on these observations, it seems plausible that TLR4-dependent signaling can lead to MyD88-dependent activation of NF- κ B and transcription of downstream proinflammatory factors leading to ozone-induced AHR. However, the biologically active endogenous ligand to the TLR4 surface receptor after exposure to ambient ozone remains unknown.

We have previously demonstrated that short fragments of hyaluronan (HA) can mediate ozone-induced AHR (25). In this report, we investigated whether TLR4 is necessary for HA-mediated airway response to inhaled ozone. TLR4-deficient mice and C57BL/6 mice exposed to ozone demonstrated similarly elevated lung lavage fluid levels of HA. However, TLR4-deficient mice were protected from ozone-induced AHR, as well as AHR from direct intratracheal HA instillation. The level of TLR4 on alveolar macrophages was markedly increased after exposure to ozone. Both ozone and HA exposure led to NF- κ B activation in

alveolar macrophages and airway epithelia. HA-exposed cultured macrophages expressed a similar cytokine profile as both ozone-exposed and HA-exposed mice in a TLR4-dependent manner. Our observations thus support a critical role for a HA-TLR4 interaction in the airway response after ozone exposure.

METHODS

Mice

TLR4-deficient mice were generously provided by Shizuo Akira (26) and backcrossed onto C57BL/6J for ten generations. C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Experimental groups consisted of male mice (6–8 wk old), and all experiments were repeated at least once. NF- κ B reporter mice (luciferase and green fluorescent protein [GFP]) used in these experiments were generated by Timothy Blackwell (27). Experimental protocols were approved by the Animal Care Committee at Duke University.

Figure 2. Ozone enhanced soluble hyaluronan in the bronchoalveolar lavage fluid. (A) Lung lavage fluid hyaluronan (HA) levels were measured and elevated 24 hours after ozone exposure ($n = 10$; $*P < 0.05$). (B) Agar gel electrophoresis of concentrated lung lavage fluid HA, visualized with staining with Stains-All (Sigma, St. Louis, MO). Lane 1: low molecular weight HA ladder. Lane 2: high molecular weight HA ladder. Lane 3: high molecular weight HA (Healon). Lane 4: sonicated Healon (low molecular weight HA). Lane 5: Toll-like receptor-4^{-/-} (TLR4^{-/-}) mouse lavage fluid after ozone exposure. Lane 6: C57BL/6 mouse lavage fluid after ozone exposure. Lane 7: representative C57BL/6 and TLR4^{-/-} mouse lavage fluid after free air exposure. Lane 8: Ozone-exposed mouse lavage fluid after treatment with hyaluronidase.

Ozone Exposure Protocol

C57BL/6J or TLR4^{-/-} mice were exposed to either filtered air (FA) or ozone. Animals were housed in cages with low-endotoxin bedding and given water and chow *ad libitum*. Ozone exposures were 2 ppm for 3 hours. Our selection of ozone concentration levels in the mouse is based on similar biological response observed in human exposure studies and published deposition fraction data for O₃ in rodent models (28, 29). This level of ozone is recognized to cause moderate lung injury in mice and may help us understand the biological response to human environmental ozone inhalation. Exposures were performed in 55-L Hinner-style chambers. Air at 20 to 22°C and 50 to 60% relative humidity was supplied at approximately 20 exchanges per hour. Ozone was generated by directing 100% O₂ through an ultraviolet (UV) light generator and mixed with a filtered air supply. Chamber ozone concentration was monitored continuously with a UV light photometer (1003AH, Dasibi, Glendale, CA).

HA Challenge

Sterile, endotoxin-free (0.00008 EU/ml) high molecular weight HA (HMW-HA) (Healon, AMO, Santa Ana, CA) was reconstituted at 0.5 mg/ml in 0.02 M acetate per 0.15 M sodium chloride, pH 6.0. HA used in these experiments was sonicated on ice to produce low molecular weight HA. Sizes were confirmed by electrophoresis (30). In all *in vivo* experiments, 50 µl of low-molecular weight HA (0.5 µg/µl or total of 25 µg) or vehicle were instilled oropharyngeally into isoflurane-anesthetized mice and AHR was measured invasively 2 to 3 hours later.

Whole Lung Lavage

Whole lung lavage was performed as previously described (25). Supernatants were stored at -70°C. HA ELISA was performed

according to manufacturer's instructions (Echelon, Salt Lake City, UT). Total lung protein was measured by the Bradford method. Inflammatory cytokines/chemokines (KC, TNF-α, IL-1β, IL-6, and MCP-1) were measured by Luminex (BioRad, Hercules, CA) using reagents purchased from Upstate/Millipore (Billerica, MA).

Airway Physiology

Anesthesia was achieved with 60 mg/kg of pentobarbital sodium injected intraperitoneally. Mice were then given neuromuscular blockade (0.8 ml/kg pancuronium bromide) and ventilated with a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, QC, Canada), with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm H₂O. Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0, 10 mg/ml, 25 mg/ml, and 100 mg/ml) was determined by resistance measurements every 30 seconds for 5 minutes, ensuring 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 resistance measurements were then averaged at each dose and graphed (R_T, measured in cm H₂O/ml/s) along with the initial baseline measurement.

Immunohistochemistry

Formalin-fixed paraffin embedded lung tissue specimens were sectioned in 5-µm thick sections, and stained with PE-anti-mouse TLR4 (eBioscience, San Diego, CA) and biotinylated HA-binding protein (HABP) (Associates of Cape Cod, Falmouth, MA). A secondary streptavidine-488 fluorochrome (Molecular Probes/Invitrogen, Carlsbad, CA), was used to detect HA. Slides were mounted with ProLong Gold with DAPI (Invitrogen, Carlsbad, CA) for nuclear staining. A

laser-scanning confocal microscope (LSM 510 NLO mounted on Axiovert 200M microscope; Zeiss, Minneapolis, MN) was used for other images. The images were obtained simultaneously using the 488-nm and 543-nm lasers as the light source. The software used for acquisition was Zeiss LSM510 version 3, and for analysis, LSM Image Browser version 4.2. Colocalization of HA and TLR4 was analyzed using the colocalization tool of Zeiss software and measuring the colocalization coefficient. To detect GFP, tissue sections were immunostained with rabbit anti-GFP antibodies (Clontech, Mountain View, CA). A standard immunoperoxidase/avidin-biotin complex protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was used for immunodetection.

Luciferase Assay

All reagents were purchased from Promega (Madison, WI). Left lungs were harvested and homogenized in reporter lysis buffer. Protein concentration of homogenates were determined and adjusted to be equal among samples. 20 μ l of lung homogenates were then mixed with 75 μ l of luciferase reagents in a luminometer tube. Luciferase activity was detected by a TD 20/20 luminometer. Data were presented as increased fold over controls.

Cell Culture Experiments

Bone marrow cells were removed from long bones obtained from C57BL/6J or TLR4^{-/-} mice and equal number of cells cultured in RPMI, 10% fetal calf serum, and 1% penicillin/streptomycin. After 2 hours, adherent cells were cultured for 4 to 7 days in the presence of 20 ng/ml murine M-CSF (PeproTech, Rocky Hill, NJ). Cells were allowed to grow to 70% confluence and then challenged to short fragments of HA for 24 hours. Cell-free supernatant was removed and analyzed for cytokines/chemokines. For experiments with NF- κ B reporter cells, bone marrow macrophages were challenged to either short fragments of HA (50 μ g/ml) or *Escherichia coli* 0111:B4 LPS (Sigma, St. Louis, MO) (50 ng/ml) for 1 hour. Cells were harvested and analyzed for luciferase activity.

Statistics

Data are expressed as mean $\pm$ SEM. Significant differences between groups were identified by analysis of variance and the Student *t* test

unless otherwise stated using SPSS (Chicago, IL) and GraphPad (San Diego, CA) software. A two-tailed *P* value of less than 0.05 was considered significant.

RESULTS

Ozone Causes TLR4-dependent AHR and Inflammatory Cytokine Expression in the Alveolar Lavage Fluid

C57BL/6 and TLR4-deficient mice were exposed to 2 ppm of ozone for 3 hours and phenotyped 24 hours after exposure. The inflammatory cell influx and airway injury (as measured by total protein) in the bronchial alveolar lavage after exposure to ozone was similar in both C57BL/6 and TLR4-deficient mice (data not shown). We observed that the airway response to methacholine after exposure to ozone, invasively measured by FlexiVent, is partially dependent on TLR4 (Figure 1A). Next, we measured the lavage level of proinflammatory cytokines that have been previously implicated in the pathogenesis of ozone exposure (12–18). We found that the level of cytokines (KC, IL-1 β , IL-6, MCP-1, TNF- α) in the lavage fluid after ozone exposure was also partially dependent on the presence of TLR4 (Figure 1B). These data support that both ozone-induced AHR and release of cytokines associated with AHR are partially dependent on TLR4.

HA Causes TLR4-dependent AHR and Inflammatory Cytokine Expression in the Alveolar Lavage Fluid

Because ozone exposure causes sterile lung injury, it is reasonable that the TLR4-dependent inflammation and AHR described above could be due to an endogenous ligand that activates surface TLR4 receptors. Therefore, we next examined the hypothesis that an endogenous ligand of TLR4, HA (31–33), plays a role in the TLR4-dependent response to inhaled ozone. Exposure to ozone increased the levels of soluble HA in

Figure 3. Hyaluronan-induced airway hyperresponsiveness (AHR) and cytokines are dependent on toll-like receptor-4 (TLR4). (A) Direct instillation of hyaluronan (HA) into the lungs of mice resulted in AHR in C57BL/6J mice, when compared with vehicle instillation. TLR4-deficient mice were relatively protected from HA-induced AHR, when compared with C57BL/6J ($n = 5$; $*P < 0.05$). (B) Levels of proinflammatory cytokines in cell-free supernatant as measured by luminex bead profiling. The levels of keratinocyte-derived chemokine, IL-1 β , IL-6, monocyte chemoattractant protein-1, and tumor necrosis factor- α were increased with HA challenge and dependent on the presence of TLR4 ($n = 5$; $*P < 0.01$).

bronchial alveolar lavage fluid in a manner independent of strain (Figure 2A). In the lavage fluid, we detected predominantly short-fragment HA (Figure 2B), which can be immunostimulatory (34). We have previously reported that direct instillation of short-fragment HA into murine airways can induce AHR in a manner dependent on the major HA receptor (CD44) and inter- α -trypsin inhibitor (25). It is now also recognized that TLR4 can bind HA and that direct TLR4–HA interactions contribute to noninfectious lung injury (31, 35). To specifically address the role of TLR4 in response to HA in the development of airway hyperresponsiveness, mice were directly challenged by oropharyngeal aspiration of either HA (25 μ g) or vehicle. After challenge to HA, we did not observe

differences in cellular inflammation (data not shown). However, HA instillation into C57BL/6J mice induced AHR, when compared with vehicle (Figure 3A). In contrast, TLR4-deficient animals failed to develop HA-induced airway hyperresponsiveness (Figure 3A). These findings demonstrate that HA can directly induce airway hyperresponsiveness in a manner dependent on TLR4. We then investigated whether levels of proinflammatory cytokines after HA instillation parallel the TLR4-dependent response previously associated with ozone-induced AHR. Indeed, instillation of HA into the lungs of mice elicited elevated levels of TNF- α , KC, IL-1, KC, and MCP-1 in a manner partially dependent on TLR4 (Figure 3B). These observations demonstrate that both the cytokine

Figure 4. Hyaluronan and toll-like receptor 4 (TLR4) staining in the airways after ozone exposure. Confocal immunohistochemistry was evaluated on lung histology; HA is colored green, TLR4 is colored red, and colocalization is colored yellow (400 $\times$ magnification). (A) In air-exposed TLR4-deficient mice, there is some visible subepithelial HA. (B) In air-exposed wild-type mice, faint TLR4 staining is observed in bronchial epithelial cells. (C) No significant change in ozone-exposed TLR4-deficient mice. (D) Wild-type mice show up-regulation of TLR4 expression in bronchial epithelia. No significant colocalization of TLR4 with HA is observed.

response and AHR response to short-fragment HA are dependent on TLR4.

HA and TLR4 Distribution after Exposure to Ozone

To begin to understand the cell types important in TLR4 recognition of short-fragment HA, we stained murine lungs for both HA and TLR4. Both HA and TLR4 were detected in murine airways by confocal microscopy in both air-exposed and ozone-exposed wild-type mice, but no TLR4 was seen on TLR4-deficient mice, as expected (Figures 4A–4D). HA was predominantly located in the subepithelial region in both groups of mice (Figures 4A, 4D) and appeared increased in the ozone-exposed mice (Figure 4D). After ozone exposure, there was more intense TLR4 staining in airway epithelial suggesting that ozone exposure may lead to increased TLR4 expression in this cell type (compare Figures 4B and 4D). We did not observe significant TLR4-HA colocalization on airway epithelia. However, differences were observed in alveolar macrophages between groups (Figures 5A–5E). In air-exposed

mice, there was minimal or no HA staining (*green*) on either TLR4-deficient or wild-type macrophages and relatively faint TLR4 staining (*red*) in wild-type macrophages (Figures 5A and 5B). However, after ozone exposure there was clear HA staining on macrophage membranes for both strains of mice, and TLR4 staining appeared to be more pronounced in wild-type macrophages (Figure 5C–5D), as we have previously reported (36). TLR4 and HA colocalized on the cell membrane of alveolar macrophages (Figure 5D and 5E). Semiquantitative measurement of colocalization indicated enhanced overlay of TLR4 and HA after inhalation of ozone (Figure 5F). Consistent with a previous report (32), coimmunoprecipitation studies demonstrate direct binding of the major receptor for HA (CD44) and TLR4 in both alveolar macrophages and whole lung homogenates (Figure 5G). However, there was no observed difference in mRNA expression of TLR4 in C57BL/6J lungs after exposure to either ozone or HA (data not shown). These findings support that ozone exposure increases the level of HA in murine

Figure 5. Hyaluronan and toll-like receptor 4 (TLR4) costaining in the parenchyma after ozone exposure. Confocal immunohistochemistry was evaluated on lung histology; hyaluronan (HA) is colored *green*, TLR4 is colored *red*, and colocalization is colored *yellow* (1200 $\times$ magnification). In (A) air-exposed TLR4-deficient (B) and wild-type mice, there is very little HA staining (*green*) on alveolar macrophages. (B) Faint TLR4 staining (*red*) is observed on air-exposed alveolar macrophage. No significant colocalization of HA and TLR4 is observed. After ozone exposure, HA staining (*green*) is observed in the macrophage membrane (arrows) in both (C) TLR4-deficient and (D) wild-type mice. TLR4 staining on alveolar macrophages appears significantly enhanced after ozone exposure in (D) wild-type mice. (E) Close-up of merged image (D) shows colocalization of TLR4 and HA on macrophage membranes (arrows). (F) Semiquantitative analysis of TLR4-HA colocalization in murine airways and alveoli at 600 $\times$ magnification shows increased colocalization after ozone exposure. (G) Coimmunoprecipitation for CD44-TLR4 (Immunoprecipitation: CD44, blot: TLR4) shows presence of CD44-TLR4 complexes in alveolar macrophage lysates from lung lavage fluid (AM = alveolar macrophages) and whole-lung lysates. Each band represents a sample from a separate mouse.

airways, and that HA is in a location to interact with TLR4 on alveolar macrophages after exposure to ozone.

Macrophage Response to HA

Because HA colocalizes with TLR4 on lung macrophages and previous work supports macrophages as a critical cell type in the inflammatory response to short-fragment HA (34), we speculated that both ozone and HA could similarly activate lung macrophages. Previous work supports that both ozone (24) and HA (34) can activate NF- κ B. It is also recognized that NF- κ B is an important downstream signal after ligation and activation of surface TLR4 (37). Therefore, to determine the cell types activated by both ozone and HA, we used NF- κ B reporter mice that when activated express both luciferase and GFP. Measurement of whole lung luciferase determined high-level activation of NF- κ B 24 hours after ozone exposure, which correlated with the enhanced response to methacholine (Figure 6A). Twenty-four hours after exposure to ozone, NF- κ B was activated in many cell types in the lung, including airway epithelia and alveolar macrophages (Figure 6D–6E), when compared with air exposed animals (Figure 6B–6C). After direct challenge to HA, maximal activation of NF- κ B was measured 2 to 24 hours after instillation as measured by whole lung luciferase activity (Figure 7A). We observed robust stain-

ing for both airway epithelial and macrophage activation of NF- κ B after exposure to HA (Figure 7D–7E), as opposed to no activation with vehicle (Figure 7B–7C). These data suggest that both macrophages and airway epithelia are important cell types in response to both ozone and short-fragment HA. To demonstrate a direct effect of HA on macrophages, we harvested and cultured bone marrow macrophages from NF- κ B reporter mice. Cultured bone marrow macrophages demonstrate a dose-dependent activation of NF- κ B with direct exposure to HA (Figure 8A). To determine whether the macrophage response to HA is dependent on TLR4, bone marrow derived macrophages from wild-type and TLR4-deficient animals were exposed to short-fragment HA. We demonstrate the same profile of proinflammatory cytokines after direct stimulation of wild-type but not TLR4-deficient bone marrow macrophages with HA as was observed in lavage fluid after exposure to either ozone or HA (Figure 8B). To specifically determine whether HA-induced NF- κ B activation in macrophages is dependent on TLR4, we crossed the NF- κ B reporter mouse with the TLR4-deficient mouse. Stimulation of cultured bone marrow derived macrophages with HA demonstrates the activation of NF- κ B is dependent on TLR4 (Figure 9). These data support an important role of TLR4 in the functional response of macrophages to short-fragment HA.

Figure 6. Ozone activates nuclear factor- κ B (NF- κ B) in alveolar macrophages and airway epithelia. (A) Using NF- κ B reporter mice, we identify a significant increase in NF- κ B activity by luminescence in whole lung homogenates both 3 hours and 24 hours after exposure to ozone when compared with naive (unexposed mice). No increase in luminescence was detected immediately after completion of ozone exposure (time = 0 hr) ($n = 5$; $*P < 0.05$). To determine the specific cell types with NF- κ B activation, tissue histology was immunostained for green fluorescent protein (GFP)-reporter 24 hours after either air (B–C) or ozone (D–E) challenge. Immunostaining is present in many cell types in the lung only after exposure to ozone. RLU = Relative Luminescence Units.

Figure 7. Hyaluronan activates nuclear factor- κ B (NF- κ B) in alveolar macrophages and airway epithelia. (A) Using NF- κ B reporter mice, we identify a significant increase in NF- κ B activity by luminescence in whole lung homogenates both 1 hour and 12 hours after exposure to HA when compared with vehicle ($n = 6$ per group; $*P < 0.05$). To determine the specific cell types with NF- κ B activation, tissue histology was immunostained for green fluorescent protein (GFP)-reporter 2 hours after either vehicle (B–C) or HA challenge (D–E). Dramatically increased immuno-staining is present on alveolar macrophages and airway epithelia after exposure to hyaluronan (arrows).

Figure 8. Macrophages generate pro-inflammatory cytokines when exposed to hyaluronan. Bone marrow-derived macrophages were collected from C57BL/6J and toll-like receptor 4^{-/-} (TLR4^{-/-}) mice. (A) Bone marrow macrophages were directly challenged to increasing doses of hyaluronan (HR). HR can lead to a modest increase of tumor necrosis factor (TNF)-α in the supernatant from C57BL/6J macrophages ($n = 5$; $*P < 0.01$). (B) Level of proinflammatory cytokines in supernatant as measured by luminex bead profiling. The level of keratinocyte-derived chemokine (KC), IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), and TNF-α were increased with HA (100 μg/ml) challenge and dependent on the presence of TLR4 ($n = 5$; $*P < 0.01$).

DISCUSSION

We have previously shown that short fragments of HA mediate ozone-induced AHR (25). In this study we demonstrate that TLR4 is necessary for both ozone-induced AHR and HA-induced AHR. Furthermore, we show that after both ozone exposure and HA challenge, TLR4 is necessary for the production of a number of proinflammatory cytokines implicated in the pathogenesis of AHR. Our data suggest a central role for macrophage-derived TLR4 in the release of proinflammatory cytokines and likely the development of AHR. Our observations also suggest that activation of airway epithelia could contribute to the biological response to both ozone and HA. Our report therefore links innate immunity to the development of AHR after a relevant environmental challenge and further supports that endogenous ligands of TLR4 can act as innate immune activators in sterile environmental lung injury.

The role of innate immunity in the response to noninfectious injury has recently received some attention. Toll-like receptors can mediate the immunologic response to noninfectious cell damage such as ischemia-reperfusion injury (38), autoimmunity (39), bleomycin lung injury (31), and asbestos-related lung injury (35) through recognition of endogenous ligands released during tissue injury (danger-associated molecular patterns). Danger-associated molecular patterns include extracellular matrix components including fibronectin, fibrinogen, and HA. Of these, HA has been most frequently implicated in innate immune activation. Previous work supports the conclusion that oxidant tissue injury can lead to release of short-fragment HA, which acts as a proinflammatory mediator and enhances cellular inflammation in sterile lung injury (40, 41). Although in this model we did observe clear differences in both cytokines and

AHR in a manner dependent on TLR4, we did not observe notable differences in cellular inflammation. The role of TLR4 in HA-mediated cell recruitment remains unknown. However, our data support that TLR4 contributes to HA-induced activation of macrophages. Previous work suggests that TLR4 interaction with long-fragment HA can promote epithelial cell integrity and recovery from sterile lung injury (31), whereas short-fragment HA elicits immuno-stimulatory responses by macrophages. It is therefore plausible that the response to HA is ultimately dependent on the intensity of lung injury, HA fragment size, and the cell types that encounter this ligand.

TLR4 and other HA receptors, such as CD44, are located on several lung cell types that can regulate TLR4-dependent response to inhaled endotoxin, including both airway epithelia (42) and alveolar macrophages (43). Previous work suggests that each of these cell types have the potential to bind HA and mediate HA-induced effects. We observed that ozone induced activation in both airway epithelia and macrophages, and direct challenge to HA led to enhanced activation of NF-κB primarily in both cell types. Additionally, after exposure to ozone, we observed colocalization of HA and TLR4 on lung macrophages but not on bronchial epithelia. Furthermore, direct challenge of cultured macrophages with HA reconstituted a similar cytokine profile as observed with *in-vivo* ozone and HA challenge in the lung. Therefore, although we cannot rule out a direct effect of HA on airway epithelia in this model, we suspect that alveolar macrophages may play a major role in the development of ozone-induced and HA-induced AHR, whereas airway epithelia may be indirectly activated by downstream HA-induced factors (44). Interestingly, we also observed localization of HA on the cell surface of subepithelial smooth muscle cells. It has been previously recognized that biological stress can induce

Figure 9. Hyaluronan activation of nuclear factor- κ B (NF- κ B) in macrophages is dependent on toll-like receptor 4 (TLR4). Bone-marrow derived macrophages were collected from NF- κ B reporter mice (C57BL/6J) and TLR4^{-/-}. The intensity of NF- κ B activation was increased with either hyaluronan (50 μ g/ml) or LPS (50 ng/ml) challenge and dependent on the presence of TLR4 (n = 5; *P < 0.01).

production of HA in airway smooth muscle (45, 46). The role of both airway epithelia and airway smooth muscle in this context will be a focus of future investigation. In our model, HA alone only partially reconstituted the physiological response to ozone. We suspect that other factors, in addition to HA, contribute to the full response to ozone inhalation. However, our data do support that release of short-fragment HA contributes to TLR4-dependent activation of macrophages resulting in release of proinflammatory cytokines and the development of AHR.

We have previously shown that HA binding through inter- α trypsin inhibitor and CD44 is necessary for AHR after ozone exposure (25). Taylor and colleagues recently demonstrated that HA released during sterile tissue injury binds to a receptor complex consisting of TLR4, CD44, and MD-2 and initiates the innate immune response (32). We speculate, based on CD44-TLR4 coimmunoprecipitation in alveolar macrophages and whole lung lysates, that a similar receptor complex is operative in ozone-induced lung injury. However, the precise coreceptor mechanism between TLR4 and CD44 remains unknown. CD44 may act as a platform, stabilizing HA for TLR4 to bind. Alternatively, CD44 may facilitate dimerization of TLR4 and TLR4 signaling. Finally, the cytoplasmic tail of CD44 may bind to tyrosine kinases, which may in turn help activate the TLR4 signaling pathway. It is possible that other coreceptors (MD-2 and CD14) contribute to the full response to HA. A previous report from our group supports the observation that ozone exposure can prime innate immunity through trafficking of TLR4 to the cellular surface of lung macrophages, which can sensitize the organism to subsequent TLR4 activation (36). The current report demonstrates that TLR4 activation is also important for the immediate response to inhaled ozone. The binding of TLR4 to HA appears partially necessary for the development of ozone-induced AHR. However, the mechanism by which macrophage-derived soluble factors leads to AHR remains unknown. We speculate that TLR4 interaction with HA results in the generation of inflammatory cytokines like TNF- α , which can promote airway smooth muscle constriction (47, 48). In this way, ozone exposure could lead to enhanced AHR. The increased availability of both cell receptor (TLR4) and ligand (HA) significantly improves conditions for their interaction, and may explain, in part, the clinical observation that AHR peaks at 24 to 48 hours after inhaled ozone exposure. However, it is notable that CD44 and inter- α -trypsin inhibitor deficiency leads to complete abolition of ozone-induced AHR (25), whereas TLR4 deficiency only partially ameliorates in-

flammation and AHR. Therefore, it remains plausible that other HA-dependent but partially TLR4-independent pathways exist that lead to the development of ozone-induced AHR and inflammation.

In summary, we demonstrate that ozone-induced AHR is partially mediated by the endogenous innate immune ligand HA and the surface receptor TLR4. This report provides important insight into the pathogenesis of environmental airways disease and implicates innate immune activation through the endogenous ligand HA in the pathogenesis of AHR.

Conflict of Interest Statement: S.G. is an employee of the NIH, receiving more than \$100,001 in compensation. Z.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.N.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.Y.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.P.S. is employed by the NIEHS as a biologist, receiving \$50,001–\$100,000 in compensation. V.V.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.A.S. received \$10,001–\$50,000 from Wallace and Graham as an expert witness for workers compensation evaluations, \$5,001–\$10,000 from Brayton and Purcell as an expert witness for determination of asbestos induced lung disease, \$50,001–\$100,001 from Weitz and Luxemburg as an expert witness for determination of asbestos induced lung disease, and \$10,001–\$50,000 from Waters and Kraus as an expert witness for determination of asbestos induced lung disease. W.M.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.W.H. does not have a financial relationship with a commercial entity that has interest in the subject of this manuscript.

Acknowledgment: The authors appreciate the technical support provided by Holly Rutledge for image analysis. Healon hyaluronan was provided at no cost from the manufacturer.

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