The Role of Toll-like Receptor 4 in Environmental Airway Injury in Mice

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Inhalation of toxins commonly found in air pollution contributes to the development and progression of asthma and environmental airway injury. In this study, we investigated the requirement of toll-like receptor 4 (TLR4) in mice for pulmonary responses to three environmental toxins: aerosolized lipopolysaccharide, particulate matter (residual oil fly ash), and ozone. The physiologic and biologic responses to these toxins were evaluated by the extent of airway responsiveness, neutrophil recruitment to the lower respiratory tract, changes in inflammatory cytokines, and the concentration of protein in the lavage fluid. Genetically engineered, TLR4-deficient mice (C57BL/6$^{TLR4-/-}$) were unresponsive to inhaled lipopolysaccharide, except for minimal increases in some inflammatory cytokines. In contrast, C57BL/6$^{TLR4-/-}$ mice did not differ from wild-type mice in their airway response to instilled residual oil fly ash or acute ozone exposure; however, we found that, despite a robust inflammatory response, C57BL/6$^{TLR4-/-}$ mice are protected against the development of airway hyperresponsiveness after subchronic ozone exposure. These data demonstrate in the mouse that the requirement of TLR4 for pulmonary inflammation depends on the nature of the toxin and appears specific to toxin and exposure conditions.

Keywords: toll-like receptor; innate immunity; endotoxin; ozone; residual oil fly ash

The lung is constantly exposed to a broad spectrum of environmental toxins, including microbiologic pathogens and their products, particulate matter, and ozone. Both epidemiologic and *in vitro* data suggest that these inhaled environmental toxins impact the severity of asthma. A common feature of the host response to these toxins is both acute neutrophilic inflammation and upregulation of proinflammatory cytokines. Although this response facilitates the clearance of pathogens, it can also lead to tissue injury and compromise lung function. Accordingly, it is of considerable importance to identify the molecular mechanisms that initiate and regulate neutrophilic inflammation and consequent tissue damage. In this study, we investigated the *in vivo* requirement of TLR4 for responses to lipopolysaccharide (LPS), residual oil fly ash (ROFA), and ozone.

Endotoxin or LPS is a structural component of membranes of gram-negative bacteria and a potent proinflammatory agent. Epidemiologic reports indicate that exposure to endotoxin can cause inflammatory airway diseases in agricultural workers (1, 2) and can exacerbate reactive airway disease in those with asthma and in wheezing children (3, 4). Experimental investigations of humans (5, 6) and mice (7–9) are consistent with these epidemiologic data and further show that a single exposure to aerosolized LPS can induce airflow obstruction that commences within minutes of challenge and persists for up to 48 hours (10). In addition to airflow obstruction, inhaled LPS also leads to neutrophil recruitment and the release of proinflammatory molecules, including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 and the chemokines macrophage inflammatory protein-2, keratinocyte-derived chemokine, and IL-8 (5, 6, 8).

Like LPS, inhalation of coarse, ambient particulate matter may also contribute to the exacerbation of reactive airways disease. Epidemiologic studies have shown that asthma-related hospital emergency room visits increase during periods of increased particulate matter less than 10 micrometers in diameter (PM$_{10}$) levels (11–13). In experimental studies, human volunteers exposed to instilled PM$_{10}$ develop increased pulmonary concentrations of polymorphonuclear leukocytes, protein, and chemokines such as IL-8 (14). In addition, exposure of human alveolar macrophages to PM$_{10}$ *in vitro* causes an increase in oxidant radical generation (15) and proinflammatory cytokines, including TNF-α, IL-6, and IL-1β (16). ROFA produced from the combustion of residual fuel oil contributes significantly to the ambient air particle burden. In human airway epithelial cells, ROFA induces IL-6, IL-8, and TNF-α (17, 18). Inhalation of ROFA by experimental animals has been widely used as a model to study the biologic effects of air pollution (19). ROFA, like PM$_{10}$, causes increased airway hyperreactivity (20), neutrophilic inflammation, and hyperpermeability in rats (19) and can enhance allergic inflammation in a mouse model of asthma (21).

Ozone is another ubiquitous urban air pollutant that can significantly contribute to increased morbidity in human populations (11). Despite the demonstrated clinical relevance of ambient ozone, the biological mechanisms of ozone-induced airway injury remain unclear. Humans exposed to ozone develop neutrophilic inflammation, increased expression of proinflammatory cytokines, and decrements in pulmonary function (22–25). The biologic response to ozone varies considerably among different human subjects (26, 27) and among inbred strains of mice, suggesting a genetic basis for susceptibility to ozone (28, 29).

Several lines of evidence demonstrate that a member of the TLR family, TLR4, is required for innate immune responses to LPS from gram-negative bacteria. Inbred mice having either deletions in TLR4 (C57BL/10ScCr) or mutations in this gene (C3H/HeJ) are unresponsive to systemic LPS (30), as are genetically engineered, TLR4-deficient mice (31). In human subjects, a polymorphism of TLR4 (Asp299Gly) is associated with a blunted response to endotoxin *in vitro* and also with diminished airway obstruction after inhaled endotoxin (32).

Particulate matter can contain biologically significant amounts of LPS (33, 34), suggesting that part of the inflammatory response to this pollutant might be due to biologic material. *In vitro*, low levels of microbial products within particulate matter

contribute to the inflammatory response, which appears to be TLR dependent (35). Furthermore, increased expression of an endogenous ligand of TLR4, fibronectin (36), has been related to differences in development of ROFA-induced pulmonary fibrosis in rats. These findings suggest that TLR4 might participate in the biologic response to ROFA.

TLR4 may also be important in the response to inhaled ozone. Endotoxin-resistant C3H/HeJ mice have reduced neutrophilic inflammation (28) and airway permeability (29) after inhalation of ozone. Endogenous ligands of TLR4, including fibronectin (36), are upregulated after exposure to ozone (37). Furthermore, studies using recombinant inbred mice suggest that a locus containing TLR4 is important in ozone-induced hyperpermeability (29, 38). Although C3H/HeJ mice have been phenotyped for their response to ozone (28), genetically engineered TLR4-deficient mice have not yet been studied. It is possible that genetic changes in genes other than TLR4 could, in part, contribute to the airway phenotype observed in those animals.

We hypothesized that intact TLR4 is required to maintain the physiologic and biologic response in mouse lungs to three inhaled environmental respiratory toxins, including LPS, ROFA, and ozone. To test this hypothesis, we separately challenged TLR4-deficient mice with *Escherichia coli* LPS, ROFA, and ozone and then measured their airway physiology, neutrophil recruitment, cytokine production, and total protein in the lung lavage fluid.

METHODS

Inbred Mice

Genetically engineered TLR4−/− mice were provided by Dr. Takeuchi from Osaka University (31) and were backcrossed onto a C57BL/6 background for at least six generations (39). C57BL/6$^{TLR4-/+}$ mice were interbred to create age-matched, littermate wild-type (C57BL/6$^{TLR4+/+}$) and C57BL/6$^{TLR4-/-}$ male animals used in these experiments. Animals were challenged at 8–9 weeks of age. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center or Man Tech Environmental Technology and performed in accordance with the standards established by the U.S. Animal Welfare Acts.

Inhaled LPS Protocol

LPS was purchased as lyophilized, purified *E. coli* 0111:B4 (Sigma, St. Louis, MO). LPS aerosol was generated and monitored as previously described (40). The concentrations of LPS aerosol generated in these experiments were 3.94–7.94 μg/m^3, as measured by limulus amebocyte lysate. Fourteen C57BL/6$^{TLR4+/+}$ and 12 C57BL/6$^{TLR4-/-}$ mice were exposed to either inhaled LPS or an aerosol of sterile Hank's balanced salt solution. Whole-body plethysmography was performed before and immediately after a 4-hour LPS challenge. Mice were sacrificed 2 hours after the completion of assessment of pulmonary function. Airway pressure time index was performed on 8 C57BL/6$^{TLR4+/+}$ and 11 C57BL/6$^{TLR4-/-}$ mice immediately after a 4-hour LPS challenge.

ROFA Protocol

Six C57BL/6$^{TLR4+/+}$ mice and six C57BL/6$^{TLR4-/-}$ mice were anesthetized with methoxyflurane vapor and instilled intratracheally via oropharyngeal aspiration either with 50 μL of a 1-μg/ml solution of ROFA resuspended in sterile dH20 or sterile dH20 alone. Whole-body plethysmography was performed before and both 6 and 24 hours after ROFA instillation. Mice were sacrificed immediately after whole-body plethysmography.

Acute Ozone Protocol

Six C57BL/6$^{TLR4+/+}$ and six C57BL/6$^{TLR4-/-}$ mice were exposed to either ozone (2.0 ppm) or filtered air in 55-L Hinners-style exposure chambers for 3 hours. Chamber air at 20–22°C and 50–60% relative humidity was supplied at a rate of approximately 20 changes/hour. O$_3$ was generated by directing 100% oxygen through an ultraviolet light ozone generator. Ozone concentration was monitored continuously within the chamber

with an ozone ultraviolet light photometer (Dasibi model 1003AH; Dasibi Environmental Corp., Glendale, CA). Whole-body plethysmography was performed before and at 6 hours and 24 hours after challenge. Mice were sacrificed 24 hours after exposure.

Subchronic Ozone Protocol

Fifteen C57BL/6$^{TLR4+/+}$ and 16 C57BL/6$^{TLR4-/-}$ mice were exposed to a lower ozone concentration for a longer duration (0.3 ppm ozone for 72 hours, 24 hours/day). Animals were placed in individual stainless-steel wire cages located within a large exposure chamber equipped with a charcoal and high-efficiency particulate air–filtered air supply with free access to both food and water. The dose of ozone generated is continually monitored (Dasibi Environmental Corp.). At the completion of a 72-hour exposure, 12 animals from each genotype were phenotyped within 8 hours for airway hyperreactivity. Whole lung lavage was performed on all animals. Six C57BL/6$^{TLR4+/+}$ and six C57BL/6$^{TLR4-/-}$ unexposed mice were phenotyped as control subjects.

Whole-body Plethysmography

Unrestrained whole-body plethysmography (Buxco Electronics, Inc., Troy, NY) was conducted (41–43), and measurements were obtained at baseline and after stimulation with inhaled methacholine (0, 5, 10, and 20 mg/ml) as previously described (40, 44).

Airway Pressure Time Index

Mice were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneally. Airway pressure is measured by a differential pressure transducer connected to the side port of a surgically inserted tracheostomy cannula used to ventilate the animals with 6–8 ml/kg at 125 breaths/minute. Neuromuscular blockade was accomplished with doxacurium chloride (0.25 mg/kg) and assessed by spontaneous respiratory efforts. Intravenous methacholine (25, 100, and 250 μg/kg) was administered into the jugular vein.

Whole Lung Lavage

Whole lung lavage was performed as previously described (7, 40, 44). Lavage cells were spun onto a glass slide using a cytocentrifuge (Cytospin-2; Shanden Southern, Sewickley, PA) and stained using a Diff Quick Stain Set (Harleco, Gibbstown, NY). Cell-free lavage supernatants were stored at −70°C.

Cytokine and Protein Assays

ELISA kits for TNF-α, IL-1β, macrophage inflammatory protein-2, IL-6, IL-10, and IL-12p70 were purchased from R&D Systems (Minneapolis, MN). The ELISA kit for transforming growth factor-β1 was purchased from Promega (Madison, WI). Kits were used according to the manufacturer's instructions. The lower limit of detection for each protein was as follows: 5.1 pg/ml for TNF-α, 3.0 pg/ml for IL-1β, 1.5 pg/ml for macrophage inflammatory protein-2, 10 pg/ml for IL-6, 4 pg/ml for IL-10, 2.5 pg/ml for IL-12p70, and 15.6 pg/ml for transforming growth factor-β1. Total protein concentrations in BAL fluid were determined using the Lowry Assay (BioRad, Hercules, CA).

Statistical Analysis

Data are expressed as mean ± SEM. Significant differences between groups were identified by analysis of variance. Individual comparisons between groups were confirmed by the two-tailed Student's *t* test or the Mann-Whitney U test (45). We report only the least significant p value. Statistical calculations were performed using SPSS (SPSS, Inc., Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

Lower Airway Response to Inhaled LPS

We first investigated whether the absence of TLR4 affects the response to methacholine in the absence of other stimuli. The airway response to methacholine in naïve mice was measured noninvasively by whole-body plethysmography (enhanced pause [Penh]) and was not significantly different between unchallenged

C57BL/6$^{TLR4-/-}$ mice and their genetically matched wild-type counterparts (Figure 1A). This demonstrates that TLR4 does not affect baseline estimates of airway function or responses to methacholine. After exposure to aerosolized LPS, wild-type mice had heightened airway responsiveness to methacholine (Figure 1B); however, LPS-exposed C57BL/6$^{TLR4-/-}$ mice failed to display this heightened responsiveness and were, in fact, indistinguishable from unexposed mice. To determine whether the difference in airway responsiveness as measured noninvasively between C57BL/6$^{TLR4-/-}$ and wild-type mice was related to changes in the lower airway, the airway pressure time index was measured in mice after challenge with LPS. Similar to the Penh results, after inhalation of LPS, C57BL/6$^{TLR4-/-}$ mice had a significantly blunted response to methacholine when compared with the wild-type mice (Figure 1C).

We next investigated the requirement of TLR4 for the inflammatory response to inhaled LPS. Wild-type mice displayed a robust inflammatory response in whole lung lavage, which was comprised of approximately 90% neutrophils. In contrast, LPS-challenged C57BL/6$^{TLR4-/-}$ mice failed to recruit inflammatory cells to the lower airway, and their lavage cellularity was indistinguishable from saline-challenged, wild-type mice (Figure 2). Moreover, after exposure to LPS, wild-type mice had marked increases in TNF-α, IL-1β, IL-6, macrophage inflammatory protein-2, and total transforming growth factor-β1 in lavage fluid, whereas C57BL/6$^{TLR4-/-}$ mice had significantly less but detectable concentrations of these proteins (Table 1). IL-10 and IL-12p70 were undetectable in all groups of mice. These data demonstrate that TLR4 is required for the proinflammatory cytokine response in the airspace to LPS.

Total protein levels in lung lavage are increased in the injured lung and are frequently used as an index of airway injury. To determine the role of TLR4 in LPS-induced airway injury, we evaluated protein levels in the whole lung lavage fluid after exposure to LPS. LPS-challenged, wild-type mice had significantly more total protein in whole lung lavage fluid than saline-exposed control mice (Figure 3), whereas LPS-challenged C57BL/6$^{TLR4-/-}$ mice did not differ from the saline-exposed, wild-type mice. Thus, acute LPS-induced injury of the lower respiratory tract is clearly dependent on an intact TLR4 receptor.

Lower Airway Response to Oropharyngeal Aspiration of ROFA

To determine whether TLR4 was also required for the physiologic and biologic responses to inhaled particulate matter, we challenged mice with oropharyngeal aspiration of ROFA at doses that are known to cause airway injury. ROFA had no effect on methacholine sensitivity, as measured by Penh in either C57BL/6$^{TLR4-/-}$ or wild-type mice at either time point evaluated (Figure 4A). Also, both wild-type and C57BL/6$^{TLR4-/-}$ mice exhibited similar increases in total cell number and neutrophils within the lavage fluid 24 hours after exposure to ROFA (Figure 4B). Similarly, no differences were observed between wild-type and C57BL/6$^{TLR4-/-}$ mice in protein concentrations of whole lung lavage fluid (Figure 4C).

Lower Airway Response to Inhaled Ozone

To determine the requirement of TLR4 in the response to ozone, we challenged animals with an acute exposure (2 ppm × 3 hours). Based on measures of Penh, both wild-type and C57BL/6$^{TLR4-/-}$ mice had significant elevations in their sensitivity to inhaled methacholine at both 6 (Figure 5A) and 24 hours (Figure 5B) after acute ozone exposure. In addition, there were significant increases in total cells and neutrophils in the lavage from both C57BL/6$^{TLR4+/+}$ and C57BL/6$^{TLR4-/-}$ mice as compared with free air–exposed control subjects at 24 hours (Figure 5C); however, protein in the whole lung lavage was unaffected by acute exposure to ozone
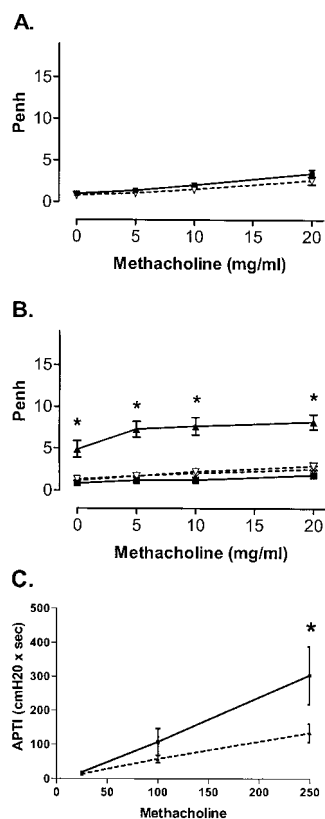

Figure 1. Airway responsiveness in mice before and after challenge with inhaled endotoxin. (*A*) Baseline enhanced pause (Penh) values with methacholine challenge were similar in unexposed animals (*closed squares*, TLR4+/+; *inverted triangles*, TLR4−/−). (*B*) After exposure to LPS, TLR4+/+ developed significant increased airway obstruction at all doses of methacholine when compared with saline control subjects (*closed squares*, TLR4+/+ saline; *closed triangles*, TLR4+/+ LPS; *inverted triangles*, TLR4−/− saline; *open diamonds*, TLR4−/− LPS) (*p < 0.001). (*C*) Observed differences in airway reactivity were confirmed by airway pressure time index (*solid line*, TLR4+/+; *dashed line*, TLR4−/−). After exposure to inhaled LPS, TLR4+/+ animals demonstrate significant increased airway responsiveness when compared with TLR4−/− (*p < 0.05).
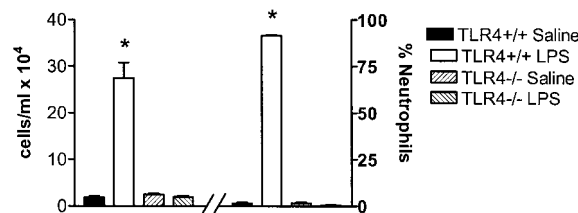

Figure 2. Inflammation in the whole lung lavage after exposure to inhaled saline and endotoxin. Significant increase in total cells (*p < 0.001) and polymorphonuclear leukocytes (*p < 0.001) were recovered in the bronchoalveolar lavage fluid in TLR4+/+ animals when compared with saline-exposed control subjects. TLR4−/− animals remained unresponsive to inhaled endotoxin and were no different than saline control subjects.

TABLE 1. TOLL-LIKE RECEPTOR 4–DEPENDENT PRODUCTION OF CYTOKINES/CHEMOKINES IN RESPONSE TO INHALED ENDOTOXIN

Genotype	Exposure	TNF-α	IL-1β	IL-6	MIP-2	IL-10	IL-12p70	TGF-β (*Total*)	TGF-β (*Active*)
TLR4+/+	Saline	ND	ND	ND	ND	ND	ND	ND	ND
	LPS	122.1 ± 28.8	23.8 ± 3.0	85.8 ± 9.0	49.3 ± 6.2	ND	ND	52.7 ± 6.4	5.1 ± 2.3
TLR4−/−	Saline	ND	ND	ND	ND	ND	ND	ND	ND
	LPS	1.8 ± 1.8	2.0 ± 0.7	27.9 ± 9.6	3.2 ± 1.8	ND	ND	31.6 ± 4.4	8.5 ± 3.5
Significance		$p = 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS	NS	$p = 0.015$	$p = 0.425$

Definition of abbreviations: IL = interleukin; LPS = lipopolysaccharide; MIP-2 = macrophage inflammatory protein-2; ND = not detected; NS = not significant; TGF-β1 = transforming growth factor-β1; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor-α.

Cytokine levels in bronchoalveolar lavage fluid supernatants were evaluated by ELISA and reported as pg/ml concentrations. Levels of cytokines in saline exposed animals remained below the limits of detection by our assays (ND). There were significant increases in many markers of inflammation detected in the LPS-exposed TLR4+/+ animals when compared with LPS-exposed TLR4−/− animals.

(Figure 5D). Thus, TLR4 does not appear to be required for a pathophysiologic response to inhalation of acute ozone.

Next we challenged animals with a subchronic exposure to ozone to determine the effects of both the intensity and duration of challenge to this environmental toxin. Although C57BL/6$^{TLR4+/+}$ animals developed a significant increase in airway hyperreactivity at all doses of methacholine after exposure to subchronic ozone, TLR4-deficient animals did not develop airway hyperreactivity in response to subchronic ozone exposure and were no different than unexposed animals (Figure 6A). Both groups of exposed animals demonstrated increases in neutrophilic inflammation in response to inhaled ozone, which did not appear to be dependent on TLR4 (Figure 6B). When comparing lavage fluid protein concentrations between animals exposed to acute ozone and subchronic ozone, there were significant increases in protein concentration after a 72-hour exposure to ozone (Figure 6C); however, lavage fluid protein concentrations were not dependent on TLR4.

DISCUSSION

Our results demonstrate that intact TLR4 is required for the acute physiologic and biologic response to inhaled LPS. TLR4-deficient mice were unresponsive to inhaled endotoxin with the exception of a low but detectable increase in the concentration of whole lung lavage cytokines. In contrast, there was no observable difference in physiologic or biologic responses to either ROFA or acute ozone inhalation when comparing wild-type and TLR4-deficient mice. Interestingly, our results suggest that TLR4 is important in mediating the physiologic (but not biologic) changes observed after subchronic inhalation of ozone. In aggregate, our findings indicate that the requirement of TLR4 for airway hyperreactivity and pulmonary inflammation depends on the nature of the toxin and appears specific to LPS and subchronic inhalation of ozone.

Unlike the response to inhaled LPS, both wild-type and TLR4-deficient mice were able to recruit neutrophils to the airways in response to an oropharyngeal aspiration of ROFA. Prior *in vitro* studies provide evidence that particulate matter may contain microbial products that play a role in the inflammatory response (35). These data support the hypothesis that intact
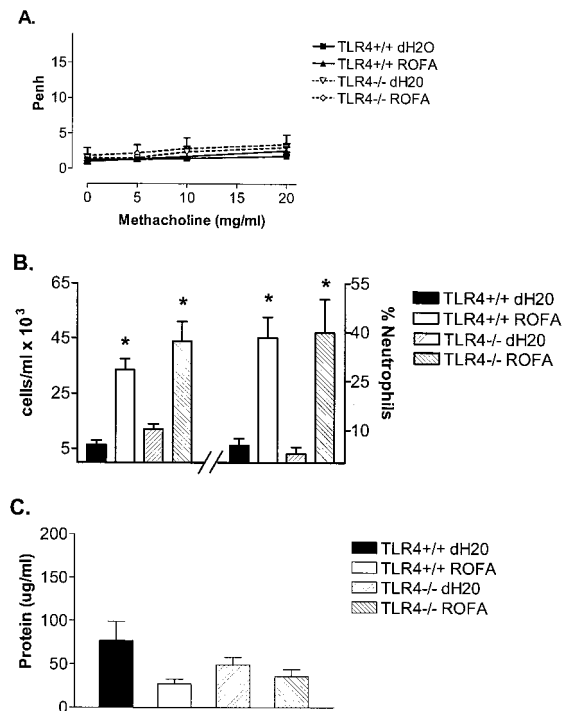
Figure 4. Response to oropharyngeal aspiration of residual oil fly ash (ROFA). (*A*) Animals exposed to intratracheal ROFA did not demonstrate increased Penh at 24 hours when compared with control subjects. (*B*) Significant differences in inflammation were observed in total cells (*$p < 0.01$) and polymorphonuclear leukocytes (*$p < 0.01$) in response to ROFA when compared with control subjects. No significant differences were observed when comparing ROFA-exposed TLR4+/+ and TLR4−/− animals. (*C*) There were no observed differences in bronchoalveolar lavage fluid protein concentration after exposure to ROFA.
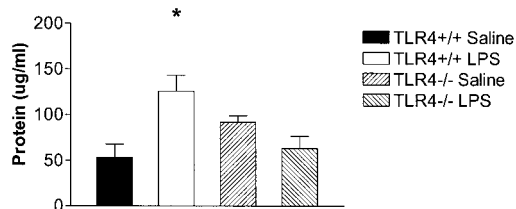
Figure 3. Total protein concentrations in bronchoalveolar lavage fluid were evaluated as an indicator of airway injury. There were significant increases in bronchoalveolar lavage fluid protein in TLR4+/+ exposed to inhaled LPS when compared with saline (*$p < 0.05$). TLR4−/− animals were unresponsive to endotoxin when compared with saline control subjects.
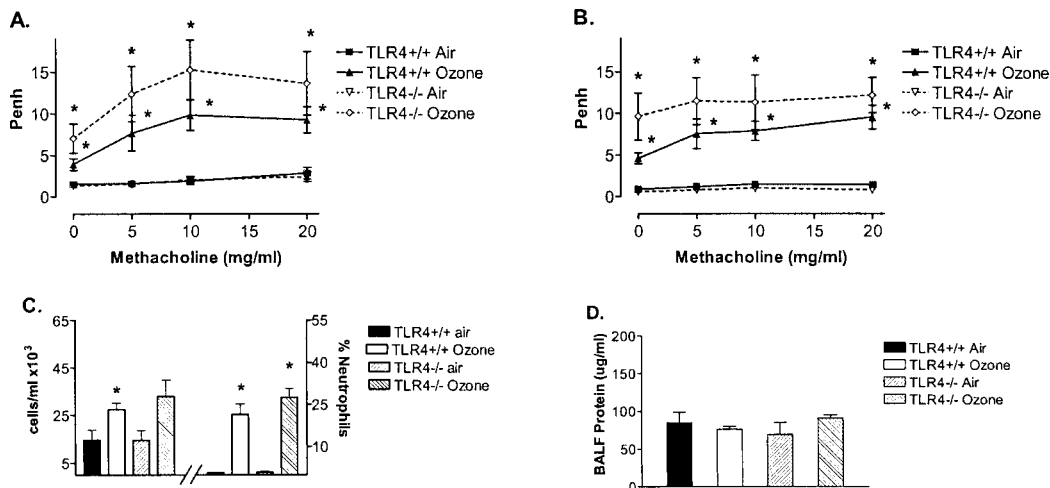
Figure 5. Response to acute ozone exposure. (*A*) Penh values were significantly increased 6 hours after exposure to ozone in both TLR4+/+ (*$p < 0.05$) and TLR4−/− (*$p < 0.05$) when compared with air-exposed control subjects. There were no observed differences dependent on the presence of TLR4 after exposure to ozone. (*B*) The significantly increased airway obstruction persisted at 24 hours after exposure to ozone in both TLR4+/+ (*$p < 0.01$) and TLR4−/− (*$p < 0.001$) when compared with air exposed control subjects. Significant differences in airway physiology were independent of intact TLR4. (*C*) When comparing inflammatory response to ozone or air exposure, TLR4+/+ animals recruited significantly more total inflammatory cells (*$p < 0.05$) and polymorphonuclear leukocytes (*$p < 0.01$), whereas TLR4−/− animals demonstrated a marginally higher number of total cells ($p = 0.08$) and a significantly greater number of polymorphonuclear leukocytes (*$p < 0.01$). There was no difference in observed response to ozone dependent on TLR4. (*D*) There were no observed differences in bronchoalveolar lavage fluid protein concentrations after exposure to ozone when compared with air.

TLR4 is potentially important in the inflammatory response to ROFA. Surprisingly, despite the fact that our ROFA contained low detectable levels of endotoxin (2.9 endotoxin units/ml by limulus amebocyte lysate), the *in vivo* pulmonary inflammatory response was no different between ROFA-exposed wild-type and TLR4−/− mice. This finding suggests that components of ROFA, such as heavy metals (20, 46), play a more prominent role in the pulmonary response to acute ROFA exposure.

Previous evidence suggests a potential role for TLR4 in the response to ozone. Specifically, C3H/HeJ mice (with a defective TLR4 gene) have reduced lung permeability and neutrophilic inflammation after ozone exposure (29, 38). Moreover, linkage analysis in recombinant inbred strains of mice has suggested an association between the lung permeability response to ozone and a region of mouse chromosome 4, which contains the TLR4 locus (29). Although we found that TLR4-deficient mice maintained both a physiologic and biological response to acute ozone when compared with wild-type mice, we observed the presence of TLR4 to be critical to the development of airway hyperreactivity after subchronic ozone challenge. Surprisingly, there was no difference in either neutrophil recruitment or airway permeability in response to subchronic ozone challenge dependent on TLR4. These observations indicate that genes other than TLR4 contribute to the blunted inflammatory response to ozone in C3H/HeJ mice. Given prior observations by Kleeberger and colleagues (29, 38), it remains likely that a novel gene on mouse chromosome 4, other than TLR4, regulates the airway permeability (but not the airway hyperreactivity) in response to subchronic inhalation of ozone.

Although intact TLR4 appears critical for neutrophil recruitment into the lung in response to inhaled LPS, TLR4-deficient mice maintain preserved inflammation in response to airway injury by both ROFA and ozone. Our findings suggest that airway injury by inhaled LPS requires specific signaling through TLR4 to initiate a cascade of events resulting in neutrophilic inflammation. In contrast, ROFA, which contains transition metals, appears to induce neutrophilic inflammation through Fenton-like chemical reactions and the production of reactive oxygen species independent of TLR4 (19). Likewise, exposure to acute ozone directly causes oxidative airway injury, resulting in a neutro-

philic inflammatory response. If reactive oxygen species are the primary cause of airway injury, it is less likely that a single transmembrane receptor would be responsible for the biological response to each of these agents. Although the acute inflammatory response to either ROFA or acute ozone appears to be independent of TLR4, we speculate that endogenous ligands of TLR4, which are upregulated after oxidant-induced airway injury, play a role in subchronic exposures to either of these inhaled environmental toxins. For example, fibronectin, which is an endogenous ligand of TLR4 (36), is upregulated in both ROFA-induced fibrosis (47) as well as ozone exposed lung cells (37). Further investigation of the role of TLR4 in subchronic exposure to each of these environmental toxins is warranted.

The isolated cytokine response to inhaled LPS was somewhat surprising and suggests that TLR4-independent mechanisms may play a minor role in the response to inhaled LPS. Alternatively, this response could be the result of low levels of contamination of our commercial-grade *E. coli* LPS by a TLR4-independent signaling stimulus. With regard to the latter possibility, TLR2 can contribute to signaling after stimulation with unpurified forms of LPS (48). Furthermore, the RP105 (CD180) receptor, which is expressed primarily in B cells, requiring CD19 for its function, can also contribute to the response to LPS (49). It is possible that the low level of cytokine production in the lung results either from RP105-mediated signaling in pulmonary B cells or that this receptor is present at a low level in cells other than B cells. Moreover, other receptors (50) and signaling/effector proteins (51) may prove to play an important role in LPS responsiveness, apart from the TLR4 receptor.

In summary, the physiologic and biologic response to LPS and the airway hyperreactivity observed after subchronic ozone inhalation requires intact TLR4. Aerosolized LPS challenge results in TLR4-dependent airway hyperreactivity, neutrophilic inflammation, increases in proinflammatory cytokines, and increased airway hyperpermeability; however, genetically engineered TLR4-deficient mice are able to recruit neutrophils to the lower airways in response to inhaled environmental toxins other than LPS, including both acute ozone and ROFA. Finally, the observation that TLR4 is critical to the development of airways hyperresponsiveness after subchronic ozone exposure
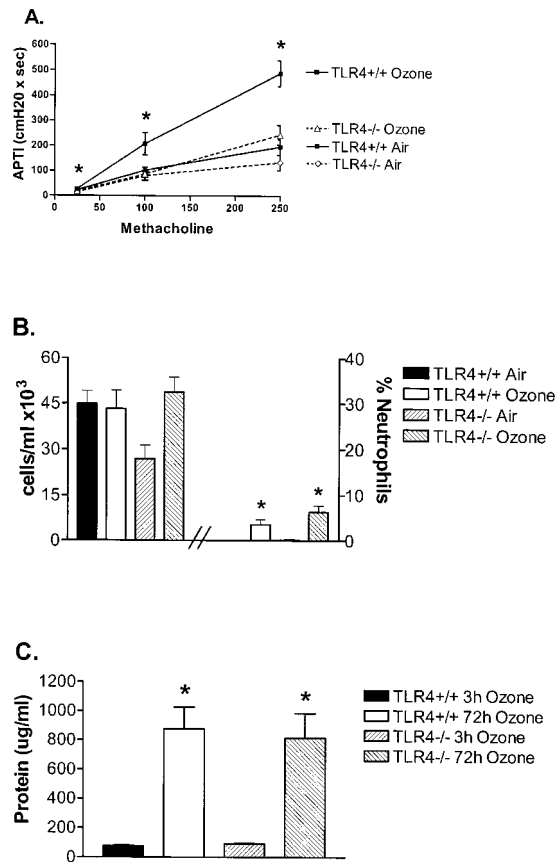
Figure 6. Response to subchronic ozone exposure. (*A*) Increased airway pressure time index values in ozone exposed C57BL/6 animals when compared with TLR4−/− were seen at all doses of methacholine, including 25 μg/ml (*p < 0.01), 100 μg/ml (*p = 0.02), and 250 μg/ml (*p = 0.001). Exposed TLR4−/− animals were no different than unexposed animals. (*B*) Significant increases in percent neutrophils were observed in both groups exposed to ozone (*p = 0.001) when compared with unexposed control subjects. (*C*) Significant increases in lavage protein were observed in both groups of animals exposed to subchronic ozone when compared with animals exposed to acute ozone (*p < 0.001).

highlights the potential role of innate immune genes in the physiologic response to other inhaled environmental toxins.

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