Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6-dependent manner in the airway epithelium

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Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6-dependent manner in the airway epithelium

K. L. Bailey,[1] J. A. Poole,[1] T. L. Mathisen,[1] T. A. Wyatt,[1,2] S. G. Von Essen,[1] and D. J. Romberger[1,2]

[1]*Pulmonary, Critical Care, Sleep and Allergy Section, Department of Internal Medicine, University of Nebraska Medical Center; and* [2]*Research Service, Omaha Veterans Affairs Medical Center, Omaha, Nebraska*

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Bailey KL, Poole JA, Mathisen TL, Wyatt TA, Von Essen SG, Romberger DJ. Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6-dependent manner in the airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 294: L1049–L1054, 2008. First published March 21, 2008; doi:10.1152/ajplung.00526.2007.—Hog confinement workers are at high risk to develop chronic bronchitis as a result of their exposure to organic dust. Chronic bronchitis is characterized by inflammatory changes of the airway epithelium. A key mediator in inflammation is Toll-like receptor 2 (TLR2). We investigated the role of TLR2 in pulmonary inflammation induced by hog confinement dust. Normal human bronchial epithelial cells (NHBE) were grown in culture and exposed to hog confinement dust extract. Hog confinement dust upregulated airway epithelial cell TLR2 mRNA in a concentration- and time-dependent manner using real-time PCR. There was a similar increase in TLR2 protein at 48 h as shown by Western blot. TLR2 was upregulated on the surface of airway epithelial cells as shown by flow cytometry. A similar upregulation of pulmonary TLR2 mRNA and protein was shown in a murine model of hog confinement dust exposure. Hog confinement dust is known to stimulate epithelial cells to produce IL-6. To determine whether TLR2 expression was being regulated by IL-6, the production of IL-6 was blocked using an IL-6-neutralizing antibody. This resulted in attenuation of the dust-induced upregulation of TLR2. To further demonstrate the importance of IL-6 in the regulation of TLR2, NHBE were directly stimulated with recombinant human IL-6. IL-6 alone was able to upregulate TLR2 in airway epithelial cells. Hog confinement dust upregulates TLR2 in the airway epithelium through an IL-6-dependent mechanism.

chronic bronchitis; normal human bronchial epithelial cells

PULMONARY DISEASE IS a well-established occupational hazard of production agriculture (6, 10). Hog confinement workers have been shown to develop chronic bronchitis and asthma as a result of their exposure to organic dust (9, 22). Hog confinement dust has been shown to incite inflammation of the airway epithelium (18). Airborne microorganisms are present in the dust and are also thought to contribute to the inflammation (17).

The lung's first line of defense against airborne microorganisms is the airway epithelium. The airway epithelium expresses Toll-like receptors (TLRs), which play a critical role in triggering the inflammatory response directed against microbial insults. TLRs recognize invading microbial pathogens through pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved microbial components such as LPS, peptidoglycan, and flagellin. There are 10 known human TLRs and each recognizes a different PAMP (1).

Toll-like receptor 2 (TLR2), which recognizes peptidoglycan, lipoteichoic acid (20), and zymosan (8, 19), is expressed in airway epithelium and is a key mediator in lung innate immunity. When TLR2 is activated, it initiates the cellular inflammatory response to Gram-positive microbial invasion. In this study, we investigated the effect of hog confinement dust exposure on expression of TLR2 mRNA and protein in an in vitro model of airway epithelial cells and in an in vivo murine model of hog confinement dust exposure. We have previously shown that IL-6 is rapidly secreted by the airway epithelium in response to hog confinement dust stimulation (18). This led us to hypothesize that IL-6 may be a critical hog confinement dust mediator that stimulates TLR2 expression in the airway epithelium.

MATERIALS AND METHODS

Cell culture. Normal human bronchial epithelial cells (NHBE; Lonza; Walkersville, MD: lot numbers: 2F1341, 2F1678, and 4F1499) were grown in submerged culture using serum-free bronchial epithelial basal media (Lonza), supplemented with bronchial epithelial growth media (BEGM SingleQuots; Lonza). The cells were cultured in plates coated with 1% vitrogen solution (Cohesion; Paolo Alto, CA). Cells were maintained in a 37°C incubator with 5% carbon dioxide. Cells were fed every 24–48 h until they were ~80% confluent, at which time the experiments were performed. Experiments were performed on cells that were *passages 2–6*. Morphology of the cells was carefully observed before experiments. Cells demonstrating a squamous morphology were not used.

Hog confinement dust extract preparation. Hog confinement dust was collected from settled dust at two separate hog confinement buildings. Both dusts were used in experiments with similar results. Hog dust extract (HDE) was prepared as described previously (12). HDE was prepared by mixing 10 g of the dust and 10 ml HBSS without calcium. The mixture was incubated at room temperature for 1 h. The mixture was centrifuged for 10 min, and the supernatant was decanted and recentrifuged for 10 min. The supernatant was again decanted, sterile filtered, and used immediately.

RNA extraction. NHBE were grown to 60–70% confluency and then exposed to various concentrations of HDE (1, 2.5, and 5%) at various time points (1, 6, and 24 h). Cell monolayers were rinsed twice in HEPES solution and then trypsinized and stored in RNA Later (Applied Biosystems, Foster City, CA) until RNA extraction could be performed. RNA was extracted and genomic DNA was removed using the Magmax 96 kit (Applied Biosystems) according to the manufacturer's instructions. Concentration and purity of the RNA were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). All RNA samples had a A_{260}/A_{280} ratio of 1.9–2.0.

Address for reprint requests and other correspondence: K. L. Bailey, Pulmonary, Critical Care, Sleep and Allergy Section, Dept. of Internal Medicine, 985300 Nebraska Medical Center, Omaha, NE 68198-5300 (kbailey@unmc.edu).

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Real-time RT-PCR. First strand cDNA was synthesized using 100 ng of template RNA and the Taqman reverse transcription kit (Applied Biosystems). Reactions were prepared containing the following: 1× TaqMan RT buffer, 5.5 mM magnesium chloride, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl of MultiScribe reverse transcriptase. Samples were incubated in a thermocycler at 25°C for 10 min, then 48°C for 30 min, and then 95°C for 5 min.

Real-time PCR reactions were prepared using 1× TaqMan Master Mix (Applied Biosystems) and human TLR2 primers and probes (Applied Biosystems Hs00152932_m1). Ribosomal RNA was used as an endogenous control. PCR was performed using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Reactions underwent 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was carried out in duplicate. For relative comparison of TLR2 to the ribosomal RNA endogenous control, we analyzed the cycle threshold (C_t) value of real-time PCR data with $\Delta\Delta C_t$ method (11).

Western blot analysis. NHBE cells were grown to 60–70% confluency and then exposed to 5% HDE for 48 h. Cells were lysed on ice for 20 min with lysis buffer consisting of the following: 10 mM Tris, 150 mM NaCl, 3 mM EDTA, 100 μM leupeptin, 10 μM aprotinin, 20 μg/ml of soybean trypsin inhibitor, 1 mM PMSF, 5 mM benzamidine, and 1% Triton X-100. The concentration of protein in the samples was determined by spectrophotometry. Each well was loaded with 25 μg of total protein before PAGE and electroblotting to nitrocellulose. Rabbit anti-human TLR2 (H-175) antibody (SantaCruz Biotechnology, Santa Cruz, CA: sc-10739) and rabbit anti-human TLR4 (H-80; SantaCruz: sc-10741) were used in a 1:400 dilution. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Millipore, Temecula, CA) was used in a 1:10,000 dilution. The blots were imaged using the Visualizer Western blot detection kit (Millipore) and exposed to X-ray film. β-Actin loading controls were performed to ensure loading of equal amounts of protein. The blots were scanned and densitometry was performed using ImageJ software.

Fluorescence-activated cell sorting. NHBE were grown in submerged culture to 60–70% confluency and exposed to 1–5% HDE for 48 h. Cells were trypsinized and washed. They were incubated with mouse anti-human TLR2 (TL2.1; Abcam, Cambridge, MA: ab-9100) for 1 h, washed, and incubated for 1 h with an Alexa 647 conjugated secondary antibody (Invitrogen, Carlsbad, CA). Cells were washed twice and fixed with 1% paraformaldehyde in PBS (pH 7.4). Analysis by fluorescence-activated cell sorting (FACS) was performed using a FACSCaliber flow cytometer using BD CellQuest software (Becton-Dickinson, San Jose, CA) The mean fluorescence intensity was reported for 10,000 events for each experimental condition.

Mouse exposure to HDE. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Omaha Veterans Affairs Medical Center and the University of Nebraska Medical Center.

Female C57BL/6 mice, age 6–8 wk (Charles River, Wilmington, MA), were acclimated for 1 wk after shipping. During this time, they were group housed and fed commercial rodent chow and water ad libitum. The mice were then randomly assigned to a treatment group: no handling, PBS instillation, or 1, 5, or 12.5% HDE instillation. There were four mice in each group. Mice assigned to an instillation group received daily nasal instillations for 1 wk.

The procedure for nasal instillation of HDE is based on a previously established model using cigarette smoke extract (5, 14). The instillations were carried out as follows: before nasal instillations, the mice were anesthetized with isofluorane. Each mouse was held vertically while 50 μl of PBS or HDE was placed at the opening of the nare. The mice were held in the vertical position until the solution was inhaled through the nasal cavity and into the lungs. They were then monitored until awake and moving around normally after the treatment. No mice exhibited respiratory distress. All mice were weighed throughout the instillation time course. No weights of any group of mice were significantly different from another group.

Mice were killed at the end of the experiment, and the lung tissue was flash frozen. This tissue was homogenized and RNA extraction was performed using the MagMax kit as described in *Real-time RT-PCR*. The real-time PCR protocol was also the same, substituting mouse TLR2 primers and probe (Applied Biosystems: Mm00442346_m1). Protein was extracted from the lung homogenate, and Western blots were performed using the same protocol and antibodies described in *Real-time RT-PCR*.

IL-6 blocking experiments. To study the role of IL-6 in HDE-induced TLR2 expression, NHBE were preincubated with or without IL-6 neutralizing antibody (AB-206-NA; R&D Systems, Minneapolis, MN) at 5 μg/ml for 1 h. Cells were then stimulated with and without 1% HDE for 24 h. The anti-IL-6 antibody remained in the media throughout the 24-h stimulation. Cells were treated with goat IgG (Sigma, St. Louis, MO) at the same concentration to assess for nonspecific binding. Cell layers were harvested and RNA was extracted as described in *Mouse exposure to HDE*.

Recombinant human IL-6 experiments. NHBE were exposed to 5–20 ng of recombinant human IL-6 (rhIL-6; R&D Systems) for 24 h. These concentrations of IL-6 correspond to the amount of IL-6 that epithelial cells typically produce in response to HDE. Cell layers were harvested and RNA was extracted as described in *Mouse exposure to HDE*.

Lactate dehydrogenase assay. Cell viability was determined using an lactate dehydrogenase (LDH) assay. We used the "In vitro toxicology assay kit, lactate dehydrogenase based" (Sigma) according to the manufacturers instructions. It was performed on supernatants of cells exposed to varying concentrations of HDE. There was no cell death at the concentrations of HDE used in this study.

Statistics. Each quantitative experiment was repeated a minimum of three times with different lot and passage numbers of NHBE. Data are presented as mean values ± SE. Statistical significance was determined using Student's *t*-test. Differences were considered statistically significant at the 95% confidence interval if $P \leq 0.05$.

RESULTS

HDE increases TLR2 mRNA in a time- and concentration-dependent manner in airway epithelial cells. To determine the effect of HDE on expression of TLR2, NHBE were exposed to 5% HDE for 1, 6, and 24 h. We observed a statistically significant increase in TLR2 mRNA expression at each time point between 1–24 h. (Fig. 1*A*). Cell viability was documented by insignificant LDH release at all time points.

NHBE were also exposed to 1–5% HDE for 24 h, the time of peak TLR2 mRNA expression. TLR2 mRNA was upregulated in a concentration-dependent manner by HDE. There was a statistically significant increase in TLR2 mRNA expression with each concentration of HDE, with a maximum increase detected at 5% (Fig. 1*B*). Concentrations of HDE >5% did not cause further increase in TLR2 mRNA expression.

HDE increases TLR2 protein, which is expressed on the cell surface. To determine whether HDE also induced translation of TLR2 mRNA into protein, we performed Western blots and FACS. In these experiments, NHBE were exposed to 5% HDE for 48 h, and Western blots were performed. Compared with control, there was a fourfold increase in TLR2 protein in cells exposed to 5% HDE (Fig. 2, *A* and *B*). Because endotoxin is important in the agricultural setting, we also assessed the effect of HDE on TLR4. We saw no difference in TLR4 in stimulated vs. unstimulated cells (Fig. 2, *C* and *D*).

To determine whether TLR2 was transported to the cell surface after synthesis, FACS was performed. FACS revealed

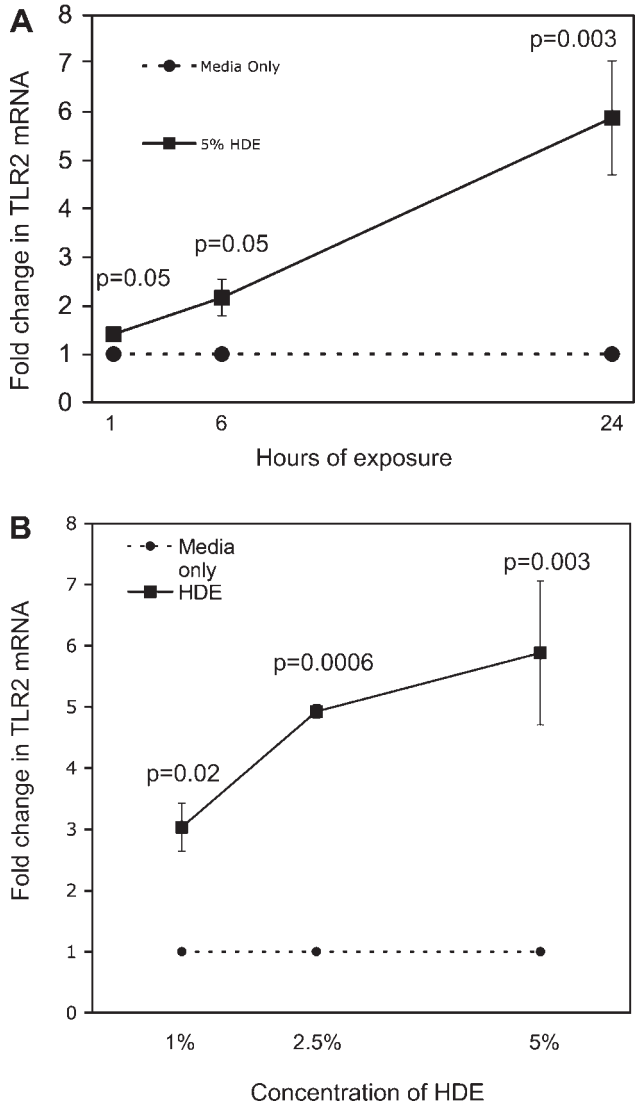

Fig. 1. Toll-like receptor 2 (TLR2) mRNA is upregulated by hog confinement dust in a time- and concentration-dependent manner in airway epithelial cells. *A*: normal human bronchial epithelial cells (NHBE) were incubated with (solid line) or without (dashed line) 5% hog dust extract (HDE) at 1, 6, and 24 h. Real-time PCR for TLR2 was performed. Data are expressed as fold change in TLR2 compared with control ± SE. ($n = 4$). *B*: NHBE were incubated with varying concentrations of HDE (solid line) or without (dashed line) for 24 h. Real-time PCR for TLR2 was performed ($n = 4$).

a concentration-dependent increase of TLR2 protein expressed on the cell surface, with a 1.6-fold increase with 1% HDE and a 3.8-fold increase with 5% HDE (Fig. 3). An isotype control showed negligible staining. HDE alone did not cause any autofluorescence.

HDE upregulates TLR2 in a murine model. Because our data revealed that HDE upregulates TLR2 in airway epithelium in vitro, we hypothesized that HDE upregulates TLR2 in vivo. To test this hypothesis, mice were exposed to intranasal PBS or varying concentrations of HDE for 1 wk. RNA was extracted from whole lung homogenate and real-time PCR was performed. Mice exposed to 5 and 12.5% HDE had statistically significant increases in TLR2 mRNA, which correlated with increasing HDE concentration (Fig. 4*A*). Likewise, there was a corresponding increase in TLR2 protein in the lung homogenates of HDE-treated mice as detected by Western blot (Fig. 4, *B* and *D*).

IL-6 mediates the upregulation of TLR2 by HDE. To determine whether IL-6 was participating in the modulation of TLR2, NHBE were preincubated with and without anti-IL-6 antibody for 1 h and then stimulated with or without 1% HDE. As in previous experiments, we saw a 3.5-fold increase in TLR2 mRNA in cells stimulated with 1% HDE. The upregulation of TLR2 was greatly attenuated when the cells were preincubated with anti-IL-6. Compared with control, unstimulated cells pretreated with IL-6 also had a statistically significant ($P = 0.01$) decrease in baseline TLR2 mRNA production (Fig. 5*A*). No change was observed in cells treated with an isotype control antibody at the same concentration (data not shown).

To further investigate the role of IL-6 in modulating TLR2, NHBE were directly stimulated with 5–20 ng/ml of rhIL-6 for 24 h. This represents the range of IL-6 concentrations detected in supernatants of cells stimulated with HDE. We found that rhIL-6 stimulates TLR2 mRNA production in a concentration-dependent manner (Fig. 5*B*).

DISCUSSION

In these experiments, we have demonstrated that hog confinement dust upregulates TLR2 in a concentration- and time-dependent manner in the airway epithelium. We have shown that an increase in TLR2 mRNA leads to an increase in synthesis of TLR2 protein and that this increase in TLR2 occurs on the airway epithelial cell surface. This upregulation of TLR2 not only occurs in vitro, but, importantly, it occurs in vivo using a murine model of inhaled hog confinement dust exposure.

We have shown TLR2 is upregulated in the airway epithelium by exposure to organic dust. Organic dusts have been shown to upregulate TLR2 in bone marrow dendritic cells (3). However, our finding is novel in airway epithelial cells. Importantly, this upregulation of TLR2 is dependent on the dust-induced production of IL-6 by the airway epithelium. The exact component(s) in hog dust that trigger TLR2 expression are not known.

Hog confinement dust is a complex mixture composed of many substances, including bacterial products such as peptidoglycan and LPS. It also contains particulate matter from grain particles and fecal matter (4). It is possible that many of these substances contribute to the production of IL-6 and subsequent upregulation of TLR2 to some degree. For instance, peptidoglycan is known to increase IL-6 production. Particulate matter has also been shown to upregulate TLR2 in airway epithelial cells. (2) Coarse particulate matter (>2.5 μm) has been shown to be the most potent stimuli. However, it is not likely to be a major contributor to the upregulation of TLR2 in our experiments, because coarse particulate matter was removed in our sterile filtering process. Another component of the dust that could possibly regulate TLR expression is LPS, a component of the Gram-negative cell wall. LPS is known to signal through TLR4. In vitro, TLR4 is not thought to play a large role in airway epithelial cell inflammation. Airway epithelial cells lack MD-2, an essential cofactor for TLR4 signaling (15, 16). However, clinically, LPS has been associated with respiratory symptoms in hog confinement workers (21). Be-

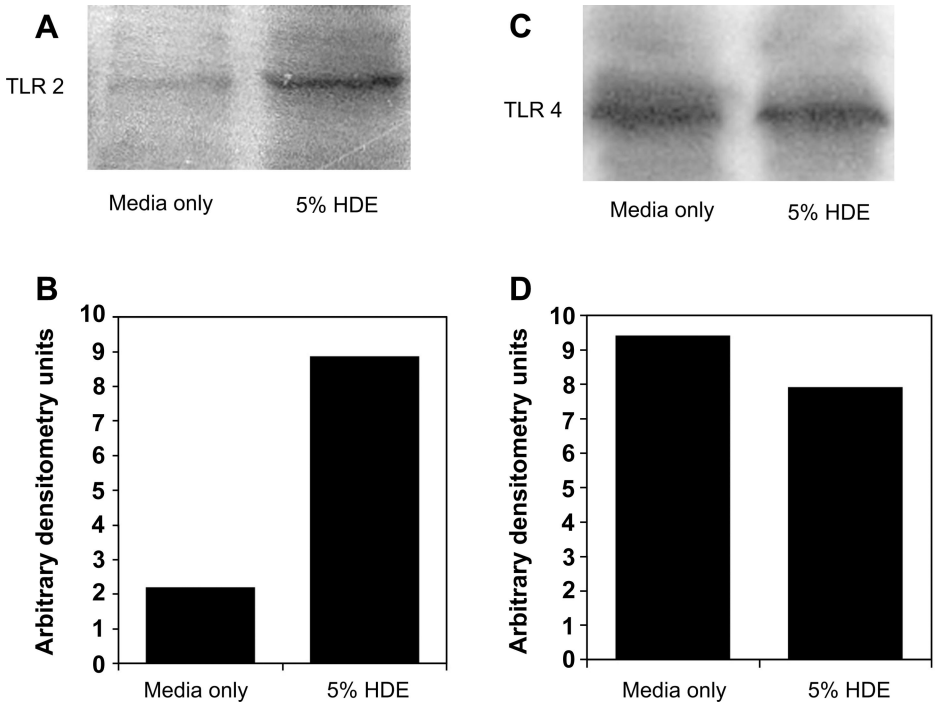

Fig. 2. TLR2 protein is upregulated by hog confinement dust in the airway epithelium. *A*: NHBE were incubated with or without 5% HDE. Cells were lysed, and Western blot for TLR2 is shown. TLR2 was detected at its expected molecular mass of 86 kDa. This is a representative blot of 4 separate experiments. *B*: densitometry was performed showing a >4-fold increase in TLR2 protein expressed in the HDE-treated cells compared with the media-treated cells. *C*: in the same experiments, we also probed for TLR4. *D*: densitometry showed no increase in TLR4 in response to HDE.

cause of this correlation between LPS and respiratory symptoms, we also looked at the role of LPS and TLR4. In experiments stimulating NHBE with HDE, we did not see any upregulation of TLR4 mRNA (data not shown) or protein (Fig. 2*B*). Likewise, removing endotoxin from the HDE did not diminish the upregulation of TLR2 (data not shown). Therefore, it is not likely that LPS is playing a large role in the upregulation of TLR2 that we have observed.

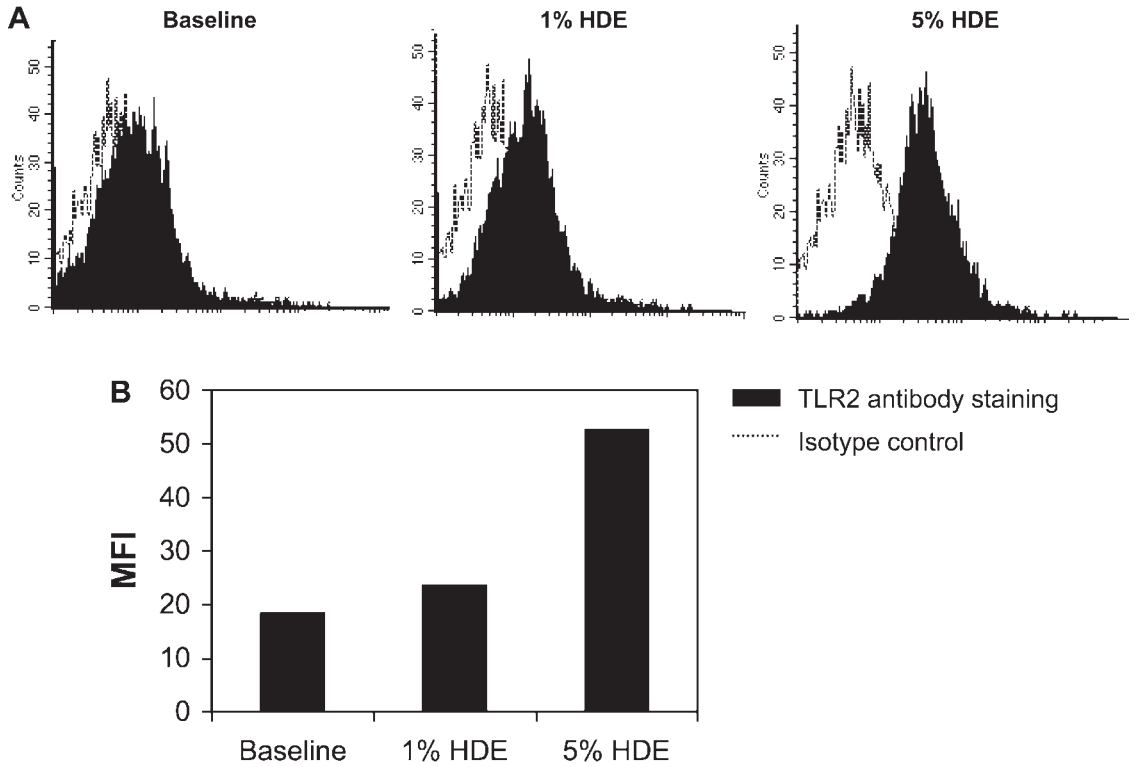

Fig. 3. TLR2 receptors are upregulated on the plasma membrane by hog confinement dust in the airway epithelium. NHBE were incubated with media only, 1% HDE and 5% HDE for 48 h. Fluorescence-activated cell sorting staining for TLR2 was performed. *A*: representative histogram of 3 separate experiments. Open histogram represents isotype control, and solid histogram represents TLR2 staining. *B*: summary bar graph shows an increasing mean fluorescence intensity (MFI) with increasing concentrations of HDE.

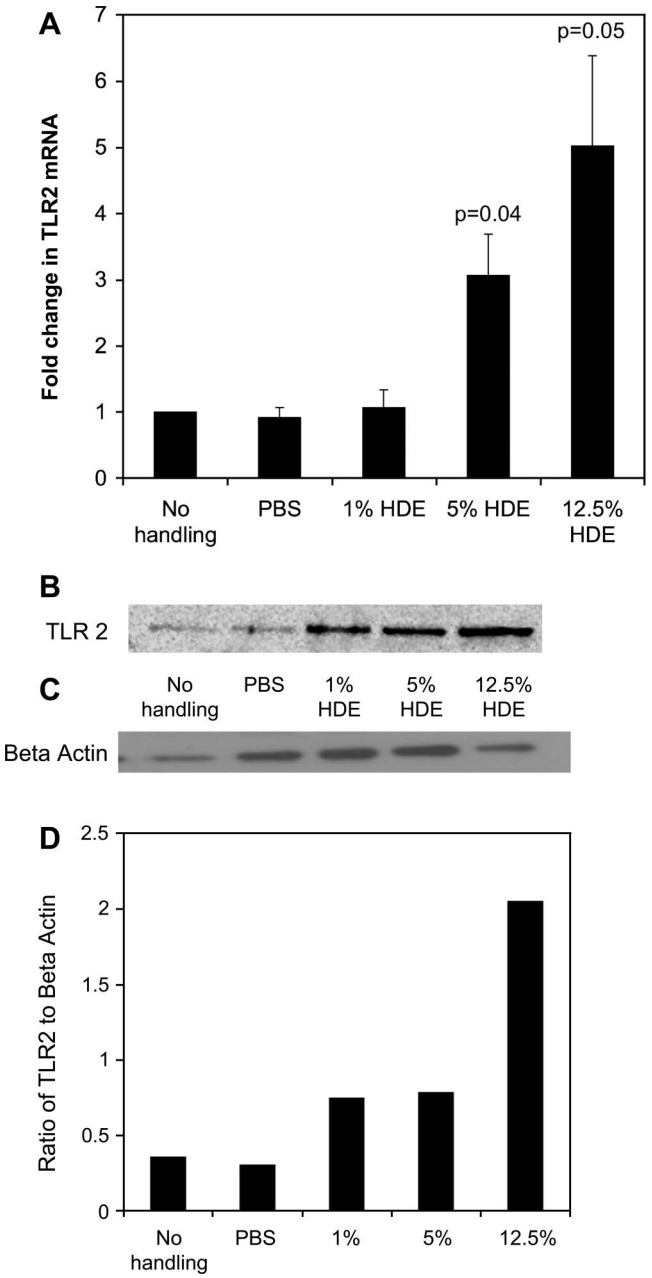

Fig. 4. TLR2 mRNA and protein are upregulated in the lung of a murine model of hog confinement dust exposure. *A*: mice (n = 4/group) were exposed to PBS and 1, 5, and 12.5% HDE daily for 1 wk. RNA was extracted from total lung homogenate and real-time PCR for TLR2 was performed. Data are expressed as mean fold change from mice with no handling ± SE. *B*: Western blot for TLR2 from a representative mouse lung from each group demonstrates the 86-kDa TLR2 protein band is upregulated in response to increasing concentrations of instilled HDE. *C*: β-actin loading control lane. *D*: ratio of the densitometry of the TLR2 Western blot to the β-actin loading control demonstrates a 4- to 5-fold increase in TLR2 protein in response to mouse lung instillation with 1–12.5% HDE.

We were also able to show that HDE upregulates TLR2 in an in vivo model of exposure. In mice exposed to hog confinement dust intranasally for 1 wk, HDE upregulates TLR2 in the lung. This was true both for TLR2 mRNA and protein. Because we performed our RT-PCR and Western blots on total lung homogenates, we cannot conclude that the source is solely the airway epithelium, although we speculate that is a major contributor to whole lung TLR2 expression.

Perhaps our most interesting finding is that IL-6 is an essential modulator in the regulation of TLR2 in the airway epithelium. Other cytokines have been shown to regulate TLR2 in airway epithelial cells. TNF-α and INF-γ have been shown to synergistically enhance expression of TLR2 in the airway epithelium (7). In macrophages, IL-12, IL-15, IL-1β, TNF-α, and INF-γ have been shown to increase TLR2 mRNA (13). It is not entirely unexpected then that IL-6, an important inflammatory cytokine in the airway epithelium, would participate in mediating TLR2 expression. We were able to demonstrate the importance of IL-6 in the regulation of TLR2 in 2 ways. First,

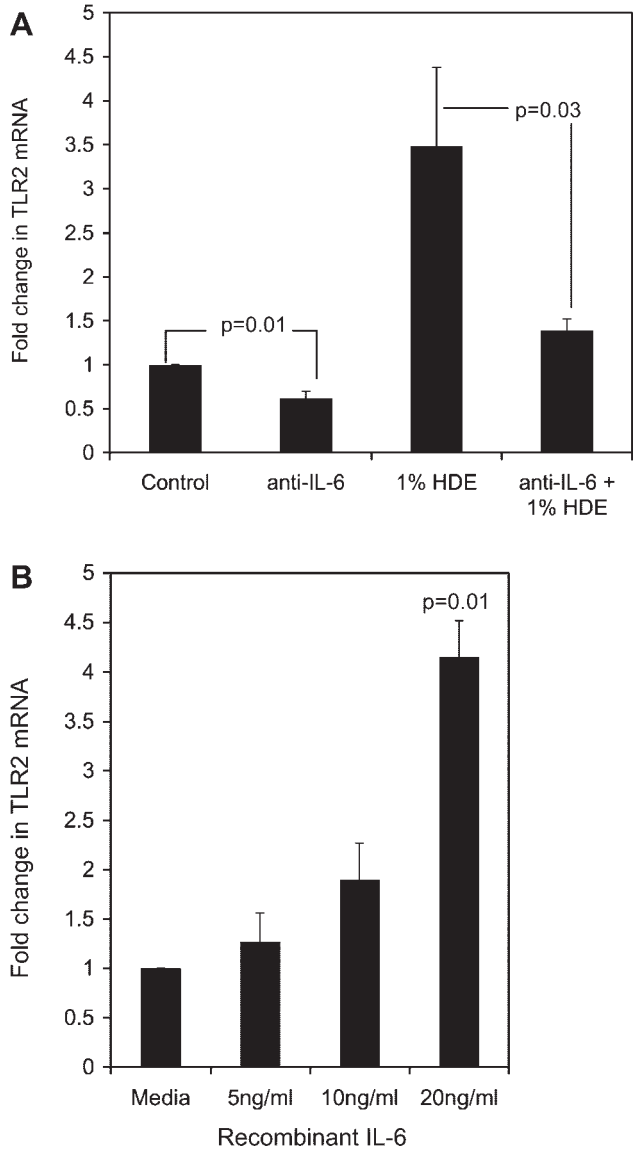

Fig. 5. IL-6 plays an important role in the upregulation of TLR2 in airway epithelial cells. *A*: NHBE were preincubated with or without anti-IL-6 for 1 h and then stimulated with or without 1% HDE for 24 h. Real-time PCR for TLR2 was performed. Data are expressed as fold change in TLR2 normalized to ribosomal RNA ± SE (n = 4). *B*: NHBE were stimulated for 24 h with varying concentrations of recombinant human IL-6. Real-time PCR for TLR2 was performed (n = 4) showing that 20 ng/ml of recombinant human IL-6 significantly (P = 0.01) increases TLR2 mRNA expression.

we showed that blocking IL-6 production dramatically attenuates the upregulation of TLR2 both at baseline and after HDE stimulation. In addition, stimulation of NHBE with recombinant human IL-6 alone led to an increase in TLR2 mRNA that is similar to what we see with HDE stimulation. The exact mechanism of how IL-6 upregulates TLR2 is unknown. We speculate that IL-6 binds to its receptor and activates the JAK/STAT pathway. This could induce the MAPK cascade, leading to the production of transcription factors, which cause increased transcription of TLR2.

In vivo, the role of IL-6 in the regulation of TLR2 may be even more important. Monocytes and macrophages present in the lung frequently produce IL-6 in response to infection early in the inflammatory process, compared with airway epithelium. This early IL-6 production may prime the airway epithelium for a more vigorous response by initiating an earlier upregulation of TLR2. Further studies into the role of IL-6 in the regulation of TLR2 in vivo are necessary.

Although these experiments show that it is likely that IL-6 plays a role in the upregulation of TLR2, we are not able to conclude that this is the only cytokine that helps to regulate TLR2. Other cytokines that are known to enhance TLR2 expression such as TNF-α and IFN-γ may also play a role (7). However, hog confinement dust, even at high concentrations, does not stimulate production of large amounts of these cytokines. Hog confinement dust does stimulate airway epithelial cells to produce large amounts of IL-8 (18). When we blocked the production of IL-8, this did not diminish TLR2 mRNA (data not shown). Likewise, when we stimulated cells with recombinant IL-8, we did not see an increase in TLR2 mRNA (data not shown). Based on this, we do not feel it is likely that IL-8 plays a large role in the regulation of TLR2 in airway epithelial cells. It is possible that other cytokines are also produced and may play a role in TLR2 regulation. Further research is necessary to determine this.

Taken as a whole, this series of experiments demonstrates that hog confinement dust upregulates TLR2 in the airway epithelium through an IL-6-dependent mechanism. This upregulation of TLR2 likely leads to increased inflammation in the airway epithelium, which could lead to, or exacerbate, diseases of the airway such as chronic bronchitis. TLR2, along with IL-6, likely plays a role in the development of airway disease in hog confinement workers.

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