Role of Toll-like receptor-4 in genetic susceptibility to lung injury induced by residual oil fly ash

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Cho, Hye-Youn, Anne E. Jedlicka, Robert Clarke, and Steven R. Kleeberger. Role of Toll-like receptor-4 in genetic susceptibility to lung injury induced by residual oil fly ash. Physiol Genomics 22: 108–117, 2005. First published March 22, 2005; 10.1152/physiogenomics.00037.2005.—The mechanisms of susceptibility to particle-induced lung injury are not clearly understood. To evaluate the contribution of genetic background to pulmonary pathogenesis, we compared the lung injury responses to residual oil fly ash (ROFA) in inbred mouse strains and calculated heritability estimates. Significant interstrain (genetic) variation was observed in ROFA-induced lung inflammation and hyperpermeability phenotypes; broad-sense heritability ranged from ~0.43 to 0.62, and the coefficient of genetic determination ranged from 0.28 to 0.45. C3H/HeJ (HeJ) mice were most resistant to the ROFA-induced injury responses. This was particularly important, as HeJ mice contain a dominant negative mutation in Toll-like receptor-4 (Tlr4). We then characterized ROFA-induced injury and TLR4 signaling in HeJ mice and its coisogenic strain C3H/HeOuJ (OuJ; Tlr4 normal) to understand the potential role of Tlr4 in this model. ROFA-induced lung injury was significantly greater in OuJ mice compared with HeJ mice. ROFA also significantly enhanced transcript and protein levels of lung TLR4 in OuJ but not in HeJ mice. Greater activation of downstream signal molecules (i.e., MYD88, TRAF6, IRAK-1, NF-κB, MAPK, AP-1) was observed in OuJ mice than in HeJ mice before the development of ROFA-induced pulmonary injury. Putative TLR4-dependent inflammatory genes that were differentially induced by ROFA in the two strains include interleukin-1β and tumor necrosis factor-α. Results support an important contribution of genetic background to particle-mediated lung injury, and Tlr4 is a candidate susceptibility gene.

Residual oil fly ash (ROFA) is a particle pollutant derived as a combustion product of fuel oil. ROFA contains soluble sulfates and substantial (~10% in mass) soluble transition metals, including iron, vanadium, and nickel (8). Intratracheal instillation of ROFA induces airway hyperreactivity and acute pulmonary injury characterized by neutrophilic inflammation, hyperpermeability and edema, and pulmonary fibrosis (8). Exposure to ROFA also enhanced allergic immune responses and susceptibility to microbial infections (25). Transition metals in ROFA have well-documented harmful health effects in human respiratory airways (27, 32). High content of these metals has been thought to account for the adverse pulmonary effects of ROFA by excess generation of reactive oxygen species in the lung (8, 14, 32). Furthermore, ROFA-induced increases in chemokine and Th1 cytokine release (3, 43) suggest that innate immune response mechanisms may be important in the pathogenesis of pulmonary ROFA toxicity. However, the precise molecular mechanisms through which ROFA causes lung injury and inflammation are uncertain.

Subpopulations that are susceptible to particle effects include the elderly and patients with preexisting heart disease, chronic obstructive pulmonary disease, and compromised immune systems (35, 40). Accumulating evidence suggests that genetic polymorphisms may also contribute to individual susceptibility to environmental pollutants and other inhaled stimuli (33). The present study was designed to investigate the role of genetic background in pulmonary susceptibility to the adverse effects of ROFA in mice. To address this objective, ROFA was instilled intratracheally in inbred strains of mice, and estimates of broad-sense heritability and coefficient of genetic determination for lung permeability and inflammatory responses were determined. Significant interstrain (genetic) differences in ROFA-induced injury were found, and the C3H/HeJ (HeJ) mouse was found to be the most resistant to injury. Importantly, HeJ mice bear a polymorphism in the coding region (exon 3) of Toll-like receptor-4 (Tlr4) on chromosome 4 (23) that renders them deficient in functional TLR4 compared with the coisogenic C3H/HeOuJ (OuJ) mice, which contain normal Tlr4 (36). TLR4 is required not only for endotoxin (gram-negative bacterial lipopolysaccharide; LPS) responsiveness (36, 37) but also for lung hyperpermeability by inhaled ozone (21) and innate immune response to respiratory viruses in mice (24, 45). We therefore determined the role of TLR4 in pulmonary ROFA toxicity by 1) comparing lung permeability and inflammatory responses between HeJ and OuJ mice and 2) characterizing differential TLR4 signaling and downstream inflammatory cytokine production in HeJ and OuJ mice.

A MAJOR COMPONENT OF urban air pollution is particulate matter (PM) from combustion sources, including fossil-fueled power plants. Epidemiological and clinical studies have correlated ambient air particle concentration and adverse health effects (6). Particles <10 μm in aerodynamic diameter (PM10) have been linked to increased mortality and morbidity, including increased respiratory symptoms and infection, pulmonary function decrements, and increased emergency room visits or hospitalization for respiratory disease (7).
MATERIALS AND METHODS

Animals and ROFA instillation. Male (6–8 wk old) DBA/2J, 129P3/J, C57BL/6J, Balb/cJ, A/J, HeJ, and OuJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in a virus- and antigen-free room. Water and mouse chow were provided ad libitum. Cages were placed in laminar flow hoods with high-efficiency particle-filtered air. Sentinel animals were examined periodically (titters and necropsy) for infection. All experimental protocols conducted in the mice were performed in accordance with the standards established by the United States Animal Welfare Acts, set forth in National Institutes of Health guidelines and the Policy and Procedures Manual, and were approved by the Johns Hopkins University Bloomberg School of Public Health Animal Care and Use Committee.

ROFA was obtained from a precipitator at a Boston Edison Mystic Power Plant Electrical Station (power unit no. 4; Boston, MA) and has been analyzed for metal constituents (1). The absence of LPS in this ROFA sample was previously reported (1) and was confirmed in the present study by the end-point chromogenic Limulus Amebocyte Lysate method using an Endosafe kit (Charles River Laboratories, Charlotte, SC); the assay determined 6 × 10⁻⁶ endotoxin units (EU)/μg ROFA (corresponding to 4 × 10⁻⁷ ng endotoxin/μg ROFA).

ROFA solution was prepared in saline (3 g/μl) as described previously (1). Mice were anesthetized by intraperitoneal injection with 1.39 mg of ketamine and 0.22 mg of xylazine (in 0.1 ml of saline), and ROFA was intratracheally instilled (6 mg/kg body wt, 150 μg in 50 μl/25 g) following the method of Walters et al. (44). Saline was instilled as vehicle control. Mice were euthanized 24 h after instillation for between-strain comparisons of lung injury. Additional HeJ and OuJ mice were euthanized 1.5, 3, and 6 h after instillation to compare TLR4-mediated early molecular events.

Bronchoalveolar lavage fluid analyses. ROFA-induced pulmonary injury responses in inbred strains of mice (n = 5–10/group) were assessed by total protein concentration and total and differential cell counts in bronchoalveolar lavage fluid (BALF) at 24 h after instillation. Bronchoalveolar lavage (BAL) and differential cell counts were performed following procedures described previously (4).

Total lung RNA isolation and RT-PCR. Total RNA was isolated from the left lung homogenate of each HeJ and OuJ mouse (n = 3/group), following published procedures (4), with the use of TRIzol reagent (Life Technologies, Gaithersburg, MD). RT-PCR was performed with mouse-specific forward and reverse primer sets (Table 1); cDNA bands were quantitated using a BioRad Gel Doc 2000 System (Hercules, CA) as described previously (4), with β-actin bands as internal control.

Nuclear protein isolation for nuclear factor-κB and activator protein-1 determination. Nuclear extracts were prepared from right lung lobes of HeJ and OuJ mice at 1.5, 3, and 6 h after ROFA instillation as described previously (4). An aliquot (6 μg) of nuclear proteins was incubated in binding buffer (19 μl) with 1 μl (3 × 10⁶ cpm) of [γ⁻³²P]dATP end-labeled oligonucleotide containing a nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) consensus sequence and electrophoresed in 4% SDS-PAGE for electrophoretic mobility shift assay (EMSA). Gels were autoradiographed at −70°C.

To analyze specific binding of NF-κB subunits, nuclear proteins (6 μg) were preincubated with either anti-p65 NF-κB antibody (sc-372X; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p50 NF-κB antibody (sc-1190X; Santa Cruz) and processed for gel shift assay as described above. To analyze specific binding of AP-1 proteins, transcription factor enzyme-linked immunosorbant assay (ELISA) was performed for c-Jun and c-Fos, following the manufacturer's instructions (AP-1 TransAM kit; Active Motif, Carlsbad, CA). Briefly, an oligonucleotide (AP-1 consensus sequence)-coated plate incubated with nuclear extracts (6 μg) was processed for sequential incubation with specific primary antibodies and anti-IgG horseradish peroxidase conjugate. Oligonucleotide-nuclear protein-antibody complex formation indicating activation of each transcription factor was assessed by chemiluminescent detection. Absorbance was measured at 450 nm using a BenchMark microplate reader (BioRad Laboratories).

Western blotting and immunoprecipitation analyses of TLR4 signaling. Cytoplasmic fraction was prepared from supernatants of lung homogenates during nuclear extraction from HeJ and OuJ mice (n = 3/group). An aliquot of cytoplasmic protein (20–100 μg) was utilized for Western blotting with specific primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Table 1. cDNA primer sequences and PCR conditions

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TLR, Toll-like receptor; LT, lymphotixin; IL, interleukin; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; Temp, temperature.
Biotechnology) to TLR4 (sc-10741), myeloid differentiation factor-88 (MYD88; sc-8197), tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6; sc-7221), interleukin (IL)-1 receptor-associated kinase-1 (IRAK-1; sc-7883), IκB (sc-371), phosphorylated IκB (p-IκB; sc-8404), phosphorylated c-Jun NH2-terminal kinase (p-JNK; sc-6254), phosphorylated extracellular signal-related kinase (p-ERK; sc-7383), IL-6 (sc-1265), and actin (sc-1615). To detect phosphorylated IRAK-1, 500 μg of anti-IRAK-1 antibody was used. ELISA analysis was performed using a mouse-specific ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Each cytokine quantity was calculated from absorbance at 450 nm, using a standard curve.

**Analysis of cytokine levels by ELISA.** Among the differentially regulated cytokines between HeJ and OuJ mice, quantities of the well-known TLR4-dependent cytokines TNF-α and IL-1β were determined in aliquots of cytoplasmic proteins (20–40 μg) using mouse-specific ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Each cytokine quantity was calculated from absorbance at 450 nm, using a standard curve.

**Statistics.** Data are expressed as group means ± SE. Two-way analysis of variance (ANOVA) was used to compare interstrain BAL responses and Western blotting, RT-PCR analyses, and ELISA data between HeJ and OuJ mice. Data from vehicle-treated mice at each time point were pooled for statistical analyses because no significant time effect was observed. Student-Newman-Keuls test was used for posteriori comparisons of means. All analyses were performed with the use of SigmaStat (SPSS, Chicago, IL). Statistical significance was accepted at P < 0.05.

Heritability, or the fraction of the phenotype variability that is due to gene effects, was estimated by calculating the intraclass correlation and the coefficient of genetic determination, following the methods outlined by Festing (10). Intraclass correlation, the proportion of total phenotype variation due to between-strain differences or heritability in the broad sense (11), was calculated by the following formula

\[
I = \frac{MSB - MSW}{n (MSB + n - 1) MSW}
\]

where \(I\) is the intraclass correlation estimate, \(MSB\) is the mean square of the between-strain comparison, \(MSW\) is the mean square of the within-strain comparison, and \(n\) is the number of animals tested per strain with corrections for differences in numbers of mice per strain (10, 11). The coefficient of genetic determination, which considers the doubling of the additive genetic variance with inbreeding and is a more conservative measure of heritability (10, 11), was calculated by the following formula

\[
g^2 = \frac{(MSB - MSW)(MSB + 2(n-1) MSW)}{n (MSB + n - 1) MSW}
\]

where \(g^2\) is the coefficient of genetic determination estimate, \(MSB\) is the mean square of the between-strain comparison, \(MSW\) is the mean square of the within-strain comparison, and \(n\) is the number of animals tested per strain.

**RESULTS**

**Strain distribution patterns of ROFA-induced lung injury and inflammation.** Interstrain variation in response to ROFA was evaluated 24 h after challenge. The mean total protein concentration and numbers of macrophages and polymorphonuclear leukocytes (PMNs) recovered from BALF were not significantly different between strains of mice exposed to vehicle (data not shown). However, ANOVA detected significant strain (genetic) effects on mean BALF protein concentrations and numbers of PMNs and macrophages after ROFA challenge (Fig. 1). ROFA caused the greatest increases of total BAL protein (Fig. 1A) and PMN inflammation (Fig. 1B) in DBA/2J mice among the six strains. The least responsive HeJ mice had significantly lower BAL protein levels than DBA/2J, 129P3/J, and C57BL/6J mice (Fig. 1A) and significantly lower concentrations and numbers of PMNs and macrophages after ROFA challenge (Fig. 1). ROFA caused the greatest increases of total
PMN numbers than DBA/2J, C57BL/6J, Balb/cJ, and 129P3/J mice (Fig. 1B). The numbers of BAL macrophages were significantly greater in A/J mice compared with all other strains after ROFA challenge (Fig. 1C).

Heritability estimates were calculated for BAL protein, macrophage, and PMN phenotypes. Intraclass correlation ($r_i$), or broad-sense heritability, was 0.62, 0.43, and 0.51 for total protein, macrophages, and PMNs, respectively. The coefficient of determination ($g^2$) was 0.45, 0.28, and 0.34 for the three phenotypes.

Differential ROFA-induced lung injury and variation of TLR4 mRNA/protein expression in HeJ and OuJ mice. ROFA-resistant HeJ mice have a unique sequence polymorphism in Tlr4 and lack functional TLR4 in response to LPS, ozone, Haemophilus influenzae, and respiratory syncytial virus (21, 24, 36, 37, 45). To determine whether Tlr4 is important in the pulmonary injury response to ROFA, we compared the permeability and inflammatory response in HeJ and Tlr4-normal OuJ mice. Statistically significant differences in total BAL protein and numbers of PMNs and macrophages were detected between resistant HeJ and susceptible OuJ mice (Table 2). To compare TLR4 activation in response to ROFA in these two strains, we first determined pulmonary TLR4 mRNA expression and protein levels. In HeJ mice, a significant decrease in TLR4 transcript level was found 1.5 h after ROFA instillation (Fig. 2A). However, ROFA caused significant upregulation of TLR4 mRNA expression over the control level from 1.5 h in OuJ mice with a peak induction at 6 h (∼4-fold). Although TLR4 mRNA expression was also enhanced in HeJ mice over the control levels at 6 h postinstillation, a statistically significant difference between strains was detected throughout the exposure. Steady-state expression level of β-actin mRNA was not affected by exposure or strain (Fig. 2A). ROFA instillation significantly enhanced TLR4 protein over its basal level in OuJ mice at 1.5 and 3 h after instillation, whereas no statistically significant ROFA-induced change in TLR4 level was found in HeJ mice. The TLR protein level of OuJ mice significantly exceeded (∼2–3 fold) that of HeJ mice at these time points (Fig. 2B).

Differential increases in TLR4 signaling transducers in HeJ and OuJ mice. To further investigate the role of TLR4 in pulmonary responses to ROFA, we compared differential activation of TLR4 downstream signal molecules in the lungs from HeJ and OuJ mice. MYD88 (35 kDa), an adaptor protein, is the most upstream component of the TLR4-mediated signaling cascade to interact directly with the cytoplasmic Toll/IL-1R homology domain of TLR4. ROFA caused production of lung MYD88 at 1.5–6 h after instillation in both strains. However, ROFA-induced MYD88 protein levels were greater (2.6-fold at peak time) in OuJ mice than in HeJ mice (Fig. 3A).

### Table 2. Differential pulmonary injury responses in C3H/HeJ and C3H/HeOuJ mice 24 h after instillation of ROFA

<table>
<thead>
<tr>
<th>BAL Phenotypes</th>
<th>C3H/HeJ</th>
<th>C3H/HeOuJ</th>
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<td>Vehicle Total protein (μg)/ml BAL</td>
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<td>ROFA 124±1 114±2</td>
<td>458±69±3.1†‡</td>
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<td>PMNs (×10³)/ml BAL</td>
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<td>Vehicle ROFA 18±1.7</td>
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<tr>
<td>Macrophages (×10³)/ml BAL</td>
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<tr>
<td>Vehicle ROFA 36±2.3</td>
<td>45±2.45†‡</td>
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Data are means ± SE; n = 4/group. ROFA, residual oil fly ash; BAL, bronchoalveolar lavage; PMN, polymorphonuclear leukocyte. *Significantly different from strain-matched vehicle controls (P < 0.05). †Significantly different from ROFA-exposed C3H/HeJ mice (P < 0.05).

Fig. 2. A: Toll-like receptor (TLR)-4 mRNA determined by RT-PCR in the lungs of HeJ and C3H/HeOuJ (OuJ) mice 1.5, 3, and 6 h after vehicle or ROFA instillation. Intensity of each TLR4 cDNA band was quantitated and normalized by that of corresponding β-actin cDNA, an internal control. Data are presented as group means ± SE (n = 4/group). Representative cDNA band images on an agarose gel are shown. *Significantly different from strain-matched vehicle controls (P < 0.05). †Significantly different from time-matched HeJ mice (P < 0.05). ‡Significantly different from ROFA-exposed C3H/HeJ mice (P < 0.05).
IRAK-1 (100 kDa), the downstream serine/threonine kinase of MYD88, was markedly increased 1.5 and 3 h after ROFA in both strains (Fig. 3A). ROFA stimulated induction and auto-phosphorylation of IRAK-1 in both strains as early as 1.5 h and by 6 h (Fig. 3A), while basal (40%) and ROFA-induced (25–50%) levels of activated IRAK-1 (p-IRAK-1) were greater in OuJ mice than in HeJ mice. TRAF6 interacts with activated p-IRAK-1 in TLR4 signaling. Lung accumulation of TRAF6 (55 and 60 kDa) over the control level was observed in OuJ mice at 1.5 and 3 h (2-fold) after ROFA challenge (Fig. 3A). ROFA-induced change of TRAF6 was relatively negligible in HeJ mice. Collectively, ROFA stimulated upregulation/activation of lung TLR4 signal transducers, MYD88, IRAK-1, and TRAF6, in the susceptible OuJ mice between 1.5–6 h after instillation. Relative to OuJ mice, these signal protein levels were marginally enhanced by ROFA in the resistant HeJ mice.

**Differential activation of NF-κB pathway in HeJ and OuJ mice.** TRAF6-mediated activation of downstream kinase cascade (e.g., NF-κB inhibitor kinase, IkB kinase) inactivates IkB through phosphorylation and degradation, which leads to nuclear translocation of NF-κB in the TLR4 signaling pathway. Western blot analyses indicated greater constitutive levels of IkB-α (37 kDa) in OuJ mice than in HeJ mice, and ROFA exposure suppressed IkB-α levels after 6 h in OuJ mice (Fig. 3B). In HeJ mice, the IkB-α level was slightly increased 1.5 h after ROFA and returned to basal level thereafter. As opposed to the kinetics of IkB-α degradation in OuJ mice, p-IkB-α increased (6- to 12-fold) in OuJ mice by 6 h after ROFA (Fig. 3B). Phosphorylation of IkB-α was suppressed in the lungs of HeJ mice exposed to either vehicle or ROFA.

Baseline activity of total NF-κB-DNA binding in HeJ mice was attenuated compared with OuJ mice (Fig. 3C). After ROFA challenge, total NF-κB DNA binding activity was enhanced over the constitutive level in OuJ mice at all postinstillation time points (Fig. 3C, top). ROFA caused a slight increase in total NF-κB DNA-binding in HeJ mice at 3 and 6 h. Specific p65- (Fig. 3C, middle) and p50- (Fig. 3C, bottom) κB binding activity was enhanced by ROFA over the respective control in each strain. However, basal and induced binding activities of each subunit were greater in the lungs of OuJ than HeJ mice. Additionally, the anti-p50 antibody led to a much stronger shift in the mobility of baseline and ROFA-inducible κB-DNA complex, whereas the anti-p65 antibody had a relatively modest effect in both strains under the same binding conditions.

**Fig. 3. A:** representative images of TLR4 signal transducers determined by Western blot analyses in the lungs of HeJ and OuJ mice 1.5, 3, and 6 h after vehicle or ROFA instillation. Mean band intensities from multiple analyses for myeloid differentiation factor-88 (MYD88), phosphorylated (p-) IL-1 receptor-associated kinase-1 (p-IRAK1), and tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) are shown. B: Western blotting analyses determined IkB-α and p-IkB-α. Mean band intensities of p-IkB-α are presented. C: NF-κB DNA binding activity in the lungs of HeJ and OuJ mice 1.5, 3, and 6 h after vehicle or ROFA instillation. Nuclear protein extracts (6 μg) were isolated from pooled right lung homogenates of each group of mice (n = 3/group) and were incubated with an end-labeled oligonucleotide probe containing NF-κB consensus sequence. Total NF-κB DNA binding was determined by gel shift analysis (top). To detect the specific binding activity of each NF-κB subunit by gel supershift analyses, either anti-p65 (middle) or anti-p50 (bottom) subunit antibody was added in the binding reactions. SB indicates shifted bands of total bindings (NF-κB motif-protein complex). Arrows indicate supershifted bands of specific bindings (NF-κB motif-protein-antibody complex). The results were reproducible by multiple analyses.
condition. This may suggest a predominance of the p50 NF-κB subunit in the lungs of these mice.

Differential activation of mitogen-activated protein kinase-AP-1 pathway in HeJ and OuJ mice. TLR4 has also been shown to signal through the mitogen-activated protein kinase (MAPK) cascade in pulmonary epithelial cells (15), immune cells (42), and tissues (2). Phosphorylated JNK1 was detected from 1.5 h and 3 h postinstillation in OuJ and HeJ mice, respectively (Fig. 4A). ROFA also induced phosphorylation of ERK1/2 at 1.5 and 3 h in both strains. ROFA-enhanced phosphorylation of these MAPKs (30–50%) and basal level of p-ERK1 (40%) were more abundant in OuJ mice than in HeJ mice. EMSA determined that basal AP-1-DNA binding activity was slightly higher in OuJ mice compared with HeJ mice (Fig. 4B). ROFA-increased total DNA binding activity of AP-1 protein was greater at 1.5–6 h in OuJ mice relative to HeJ mice. Accordingly, nuclear localization of total Jun proteins determined by Western blotting (Fig. 4C, blot image) was higher after ROFA (1.5–6 h) in OuJ compared with HeJ mice. Nuclear total Fos and Fra protein levels detected by Western blotting (Fig. 4C, blot image) were also more abundant in OuJ mice than in HeJ mice basally and after ROFA (3 h). Specific DNA binding activity of c-Jun and c-Fos AP-1 proteins determined by transcription factor ELISA was significantly higher in OuJ mice than in HeJ mice at 1.5/6 h and 1.5–3 h after ROFA, respectively (Fig. 4C).

Differential inflammatory/immune gene expression in HeJ and OuJ mice. Transcript levels of selected proinflammatory cytokine and immunity genes were determined in the lungs of HeJ and OuJ mice to identify potential downstream effectors of the TLR4-mediated response to ROFA. Genes were selected based on previous findings (23, 36) and a cytokine cDNA array analysis (data not shown). The constitutive expression levels (vehicle) of IL-1β, lymphotoxin (LT)-β, IL-1α, IL-7, IL-13, and IL-16 were significantly higher in OuJ mice than in HeJ mice (Fig. 5). ROFA-induced gene induction was characterized as early peak (IL-1β, TNF-α, IL-1α, IL-7), persistent (LT-β, Gro1, IL-13), or biphasic (macrophage inflammatory protein-2, IL-6) pattern. Significantly greater induction of each of these genes was found in OuJ mice relative to HeJ mice at one or more time points. IL-16 mRNA abundance was not significantly changed by ROFA in OuJ mice, although there was significant strain variation.

![Fig. 4. A: phosphorylated MAPK (p-JNK1, p-ERK) levels were determined by Western blot analyses in the lungs of HeJ and OuJ mice after vehicle or ROFA instillation. Representative images and mean band intensities from multiple analyses are presented. B: differential activator protein (AP)-1 DNA binding activity in the lungs of HeJ and OuJ mice 1.5, 3, and 6 h postinstillation. Nuclear protein extracts (6 μg) from pooled right lung homogenates of each group of mice (n = 3/group) were incubated with an end-labeled oligonucleotide probe containing AP-1 consensus sequence, and total AP-1 DNA binding was determined by gel shift analysis. C: to detect nuclear localization of AP-1 family proteins, Western blot was performed against an aliquot of nuclear protein (20 μg) with broadly detecting anti-Jun (total Jun proteins) or anti-Fos/Fra (c-Fos, Fos B, Fra-1, Fra-2) antibodies. Representative images are presented. Specific DNA binding activities of nuclear c-Jun and c-Fos were determined by transcription factor ELISA using AP-1 TransAM kit. Mean absorbance (A450) and SE from 2 independent assays are depicted in the graphs. *Significantly higher than strain-matched vehicle controls (P < 0.05). **Significantly higher than time-matched HeJ mice (P < 0.05).](http://physiolgenomics.physiology.org/10.220.33.5 downloading from June 20, 2017)
Differential inflammatory cytokine levels in HeJ and OuJ mice. Lung protein levels of TNF-α (Fig. 6A), IL-1β (Fig. 6A), and IL-6 (Fig. 6B) were enhanced by ROFA challenge with similar time-dependent patterns in both strains. However, the induced amount of each cytokine level was significantly higher in OuJ lungs compared with HeJ.

DISCUSSION

Results from the present study demonstrate that genetic background is an important determinant of pulmonary injury and inflammatory responsiveness to ROFA. Significant interstrain variation was found in protein hyperpermeability and cellular inflammatory responses to ROFA challenge in seven inbred strains of mice. Because these strains of mice are homozygous at essentially all loci, significant interstrain variation partitioned from intrastrain variation (environmental) implies a genetic contribution to the phenotype measured. For the ROFA-induced inflammatory phenotypes in the six inbred strains tested, broad-sense heritability estimates \( r_I \) ranged from 0.43 to 0.62, and the coefficient of determination \( g^2 \), a more conservative estimate of heritability) ranged from 0.28 to 0.45. Although estimates of heritability for pulmonary responses to particulate challenges have not been published previously, interstrain differences in pulmonary immune and inflammatory responses to environmental particles have been determined. For example, relative to Balb/c mice, greater airway allergic responses (e.g., eosinophilic inflammation and cytokine production) to inhaled diesel exhaust particles after ovalbumin sensitization were found in C3H/He mice (30). Ohtsuka et al. (33) demonstrated significant interstrain variation in alveolar macrophage function to acid-coated particle aerosol challenge assessed by Fc receptor-mediated phagocytosis in nine inbred strains of mice. Variation in ROFA-induced chronic lung injury was also observed among three strains of rats in a study by Kodavanti et al. (22). In that study, an earlier development of lung fibrosis accompanying upregu-
within the TLR4 cytoplasmic domain, which results in dysfunctional TLR4 in these mice (36). In humans, TLRs are emerging as key regulators of host responses to injury and infection (38). Unlike the receptors of the adaptive immune system (i.e., T-cell receptor and B-cell antibody receptor), cells of the innate immune system such as macrophages and dendritic cells discriminate between “self” and infectious “non-self” via constitutive receptors that identify pattern ligands synthesized exclusively by pathogens [e.g., LPS, bacterial lipoproteins (BLPs), bacterial DNA], so-called pattern recognition factors (PRFs). Many recent studies with mice have shown that immune cell activation by the PRFs of gram-negative bacteria requires a pattern recognition receptor, TLR4 (17, 36). Therefore, TLR4 has been known as an LPS receptor in mice, and altered TLR4 function by the mutation in HeJ mice is believed to confer resistance to LPS (endotoxin tolerance) in this strain. Moreover, studies with Tlr4-knockout mice (Tlr4<sup>−−</sup>) strongly supported the role of murine TLR4 in LPS responsiveness (17). Mutation of TLR4 also limited the pulmonary hyperpermeability induced by ozone exposure in mice (21). Furthermore, decreased innate immune responses (i.e., natural killer cell trafficking, cytokine expression, inflammation) were observed in the lungs of Tlr4<sup>−−</sup> mice after respiratory viral infection (24, 45). Our current findings suggest an additional important role for TLR4 signaling in pulmonary injury responsiveness to particles.

Consistent with our current observation in HeJ mice, dys-regulation of TLR4 mRNA expression has been addressed in Tlr4<sup>−−</sup> mice or cells (e.g., macrophages) after exposure to bacterial endotoxin (29, 36) or oxidative injury (20). Fan et al. (9) determined in rats that TLR4 protein levels in whole lung exposed to LPS were synchronous with changes in TLR4 message. However, ROFA challenge elicited distinct patterns of lung mRNA and protein expression for TLR4 in both strains. That is, no ROFA-induced changes in TLR4 protein were found in HeJ mice, although early downregulation of mRNA followed by its upregulation was found after ROFA. In OuJ mice, TLR4 protein level was increased by 3 h postinstillation and diminished at 6 h, whereas TLR4 mRNA levels gradually increased up to 6 h after ROFA. The reason for this discrepancy is not understood. It is possible that regulatory mechanisms of transcriptional/posttranscriptional and translational/posttranslational activation of Tlr4 may dissociate in the mouse lungs injured by ROFA, although further studies are required to confirm this concept.

LPS activates NF-κB via the distinct signal transduction molecules MYD88, IRAK-1, IRAK-2, and TRAF6. The importance of this signal cascade in TLR4-mediated LPS response has been corroborated by the absence of both IRAK-1 and NF-κB activation in Tlr4<sup>−−</sup> mice treated with LPS (17). Other studies in mice with targeted disruption of genes for TLR4 signal transduction confirmed their essential roles in endotoxin-TLR4 responsiveness (19, 28). An important role for the MAPK-AP-1 pathway in TLR4-mediated responses to LPS has also been reported (46). In addition, investigators determined positive cooperation between IKK and JNK in NF-κB activation (41). In pulmonary airways and cultured airway cells, MAPK-AP-1 and NF-κB activation by ROFA have been demonstrated previously (39). Results of the present study suggested prior activation of these two pathways to TLR4-mediated pulmonary injury and inflammation by ROFA.

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**Fig. 6.** TNF-α, IL-1β, and IL-6 protein levels in HeJ and OuJ mouse lungs after vehicle and ROFA challenge. A: TNF-α and IL-1β levels were determined in aliquots of cytoplasmic proteins by ELISA. Data are presented as group means ± SE (n = 3/group). B: lung cytoplasmic IL-6 amount was detected by Western blotting. Representative band images and data (means ± SE) from 3 independent assays were presented. *Significantly higher than strain-matched vehicle controls (P < 0.05). **Significantly higher than time-matched HeJ mice (P < 0.05).
Although it was not examined in the current study, an MYD88-independent TLR4 signaling mechanism was recently elucidated using MYD88-deficient mice and cells (19). This pathway is known to associate with newly defined TLR4 adaptors [e.g., Toll/IL-1 receptor resistance (TIR) adaptors protein (TRIF), TRIF-related adaptor molecule (TRAM)] (12) and activate interferon (IFN) regulatory factors (e.g., IRF-3) to induce IFN-α/β and subsequently IFN-inducible genes (e.g., IP-10) with delayed activation of NF-κB and MAPK during dendritic cell maturation (12, 19).

As may be predicted by differential NF-κB and AP-1 activity, multiple inflammatory and immune genes were differentially expressed in Ouj and Hej mice after ROFA instillation. Innate immune response genes induced through a TLR4-mediated signaling pathway include cytokines (e.g., TNF-α, IL-1β, IL-6, IL-8, and IL-12), adhesion molecules (e.g., CD54), oncosteines (e.g., Gro1), and inducible enzymes [e.g., cyclooxygenase (COX)-2 and inducible nitric oxide synthase] (20, 29). Importantly, most of these genes are known to be highly inducible by ROFA in airway cells or in rodent lungs (3, 31, 43). The role of individual cytokines in ROFA-induced pathology has not been investigated, although anti-TNF-α antibody treatment showed a trend for reduced lung inflammation in ROFA-exposed rats (26). Suppressed induction of inflammatory and immune genes in Hej mice relative to Ouj suggests that their regulation is at least in part through TLR4 signal transduction during the pulmonary pathogenesis induced by ROFA. Previous coexposure studies with LPS postulated involvement of TLR4 and its downstream cytokines in ROFA-induced lung injury and inflammation. Imrich et al. (18) reported that air particle exposure including ROFA augmented TNF-α production by LPS priming in rat alveolar macrophages. Similarly, exposure to diesel exhaust particles increased COX-2 and PGE2 expression stimulated by LPS in inflammatory cells (16). Moreover, Gao et al. (13) suggested that mycoplasma-induced IL-6 production in lung cells was potentiated by ROFA through TLR2, which shares the MYD88-dependent intracellular signaling cascade with TLR4.

The upstream mechanism through which ROFA initiates TLR4 signaling is unknown. In vitro studies have identified putative TLR4 ligands including heat shock proteins (e.g., HSP60, HSP70), lung surfactant protein-A, and cF nests (type III repeat extra domain A; ED A or EIIIA). They are "exogenous" (e.g., HSP60), from infected pathogens (5), or "endogenous," produced in response to stress or tissue injury (34), and initiate TLR signaling leading to inflammatory gene expression. Interestingly, critical ROFA-inducible genes include cF nests-EIIIA but not other Fn isoforms (22, 31), and its gene and protein expressions were correlated with rat strain susceptibility to chronic ROFA-induced lung fibrosis (22). In addition to these potential endogenous ligands, activation of the TLR4 receptor directly by oxidants (e.g., reactive oxygen species) cannot be excluded. Association of these putative ligands to in vivo TLR4 response remains to be determined.

In summary, we identified significant genetic variation in the ROFA-induced pulmonary injury among inbred strains of mice. Differences in ROFA-induced hyperpermeability and cellular inflammation between Hej and Ouj mice suggest a potential role of Tlr4. Differentially abundant expression of TLR4 signal transduction intermediates in the sensitive Ouj mice provided further support for this gene as a critical determinant of particle-induced pulmonary injury and innate immunity.

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