Genetic Susceptibility to Ozone-Induced Lung Hyperpermeability

Role of Toll-Like Receptor 4

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The pollutant ozone (O_3) induces lung hyperpermeability and inflammation in humans and animal models. Among inbred strains of mice, there is a 3-fold difference in total protein (a marker of permeability) recovered in bronchoalveolar lavage (BAL) fluid after a 72-h exposure to 0.3 ppm O_3. To determine the chromosomal locations of susceptibility genes, we performed a genome screen using recombinant inbred (RI) strains of mice derived from O_3-susceptible C57BL/6J (B6) and O_3-resistant C3H/HeJ (HeJ) progenitors. Each RI strain was phenotyped for O_3-induced hyperpermeability, and linkage was assessed for 558 markers using Map Manager QTb27. A significant quantitative trait locus (QTL) was identified on chromosome 4. The likelihood ratio χ^2 statistic (16.6) for the peak of the QTL was greater than the significance threshold (16.3) determined empirically by permutation test. This QTL contains a candidate gene, Toll-like receptor 4 (*Tlr4*), that recently has been implicated in innate immunity and endotoxin susceptibility. The amount of the total trait variance explained by the QTL at *Tlr4*, the gene with the highest likelihood ratio statistic in the QTL, was approximately 70%. To test the role of *Tlr4* in O_3-induced hyperpermeability, BAL protein responses to O_3 were compared in C3H/HeOuJ (OuJ) and HeJ mice that differ only at a polymorphism in the coding region of *Tlr4*. Significantly greater protein concentrations (430 $\pm$ 35 μg/ml) were found in OuJ mice compared with HeJ mice (258 $\pm$ 18 μg/ml) after exposure to O_3. Furthermore, reverse transcriptase/polymerase chain reaction analysis demonstrated differential expression of *Tlr4* message levels between HeJ and OuJ mice after O_3 exposure. Together, results indicate that a QTL on mouse chromosome 4 explains a significant portion of the genetic variance in O_3-induced hyperpermeability, and support a role for *Tlr4* as a strong candidate susceptibility gene.

Acute exposures to the oxidant ozone (O_3) may elicit a number of physiologic effects, including airways inflammation and hyperpermeability, decrements in pulmonary function, and altered immune status (1–3). Although the prevalence of this highly reactive pollutant may be declining in some urban environments of the United States and other industrialized countries, there continues to be concern about the pulmonary health effects of O_3 and mixtures of O_3 and other pollutants such as particulates. Recent population and epidemiologic studies have associated O_3 exposure with exacerbation of asthma (4–6), altered lung function in adults and children (7–10), and mortality (11–13), thus underscoring the continued important detrimental effects of O_3 on the lung.

Whereas considerable progress has been made to understand the effects of O_3 in humans and animal models (14), the mechanisms of O_3 toxicity and factors that confer interindividual variation in response to O_3 have still not been clearly defined. Susceptibility to O_3-induced lung injury is almost certainly multifactorial, but it is becoming increasingly clear that genetic background is important in human populations. Significant interindividual variation in inflammatory (15–17) and pulmonary function (18, 19) responses to O_3 has been demonstrated in human subjects. Collectively, these studies of normal healthy human subjects provide strong evidence that there is a heritable component to the inflammatory and pulmonary function responses to O_3, although they do not exclude the possibility that intrinsic factors such as age may contribute to observed interindividual variation.

To further understand the mechanisms of O_3-induced lung injury, we performed a genome-wide linkage analysis for susceptibility quantitative trait loci (QTLs) to explain interstrain differences in hyperpermeability induced by an environmentally relevant concentration of O_3 (0.3 ppm). We previously identified a significant QTL on chromosome 17 and a suggestive QTL on chromosome 11 that control susceptibility to inflammation induced by subacute (0.3 ppm/72 h) exposures to O_3 in inbred C57BL/6J (B6) and C3H/HeJ (HeJ) mice (20). However, because there is an apparent dissociation between inflammatory cell infiltration and lung hyperpermeability induced by O_3 (21, 22), we hypothesized that different loci control the hyperpermeability response. Results of this study identify a significant QTL on murine chromosome 4 and suggestive QTLs on chromosomes 3 and 11 that control hyperpermeability responsiveness in the mouse. Toll-like receptor 4 (*Tlr4*), which determines responsivity to endotoxin (23, 24) and is located within the chromosome 4 QTL, was tested as a candidate gene for O_3-induced hyperpermeability. The response to O_3 was associated with *Tlr4* message levels in HeJ mice (homozygous for the mutant *Tlr4* allele) and C3H/HeOuJ (OuJ) mice (homozygous for the wild-type *Tlr4* allele).

Materials and Methods

Inbred Mice

Inbred B6, HeJ, and OuJ mice, as well as BXH recombinant inbred (RI) mice (6 to 8 wk, male), were purchased from Jackson

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Abbreviations: analysis of variance, ANOVA; bronchoalveolar lavage, BAL; mouse strain C57BL/6J, B6; complementary DNA, cDNA; mouse strain C3H/Hej, Hej; interleukin, IL; murine urinary protein 1, *Mup1*; nuclear factor κB, NF-κB; ozone, O_3; mouse strain C3H/HeOuJ, OuJ; polymorphonuclear leukocyte, PMN; quantitative trait locus, QTL; recombinant inbred, RI; reverse transcriptase/polymerase chain reaction, RT-PCR; strain distribution pattern, SDP; simple sequence length polymorphism, SSLP; Toll-like receptor 4, *Tlr4*; Toll-like receptor, TLR; tumor necrosis factor, TNF; TNF receptor-associated factor protein, TRAF.

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Laboratories (JAX, Bar Harbor, ME). An RI strain is derived by the systematic inbreeding (> 20 generations) of two randomly selected F_2 progeny from a cross between two unlike, inbred progenitor strains (25). A set of 12 BXH RI (B: B6; H: HeJ) strains has thus been developed.

All mice were housed in an antigen- and virus-free room at 22°C with a constant 14:10-h light-dark photoperiod. Water and mouse chow (Pro-Lab RMH 1000; Agway, Waverly, NY) were provided *ad libitum*. Cages were placed in laminar flow hoods with high-efficiency, particulate-filtered air (HEPA). Sentinel animals were examined periodically (titers and necropsy) to ensure that the animals had remained free of infection. All experimental protocols conducted in the mice were carried out in accordance with the standards established by the U.S. Animal Welfare Acts, set forth in National Institutes of Health guidelines and the *Policy and Procedures Manual* (Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee).

Ozone Generation and Exposures

All O_3 exposures for the proposed studies were performed in the inhalation facilities of the Johns Hopkins University School of Hygiene and Public Health (Baltimore, MD). Mice were placed individually in stainless-steel wire cages with free access to food and water during the exposures. The cages were set inside 700-liter laminar flow inhalation chambers (Baker, Sanford, ME) that are equipped with a charcoal- and HEPA-filtered air supply. During exposures, mice were maintained at 22°C with a constant 14:10-h light-dark photoperiod. Chamber air was renewed at the rate of approximately 20 changes per hour, with 50 to 65% relative humidity, and an ambient temperature of 20° to 25°C. O_3 was generated by directing dried and filtered 100% oxygen through an ultraviolet-light O_3 generator (Orec Corp., Phoenix, AZ) located upstream of the exposure chamber. The O_3-oxygen mixture was metered into the inlet air stream with computer-operated stainless-steel mass flow controllers. O_3 exposures were performed automatically using a control program and microcomputer that is interfaced with the O_3-generating system. O_3 concentration was monitored regularly at different levels within the chamber, using an O_3 ultraviolet photometer (Model 1003AH; Dasibi Environmental Corp., Glendale, CA) and recorded on a strip chart recorder. The Dasibi model 1003AH was calibrated regularly against a standard source (Model 1008-PC; Dasibi Environmental Corp.). Chambers were cleaned and food and water replaced daily; these procedures took 1 to 2 h.

Bronchoalveolar Lavage

Mice were killed by cervical dislocation and lungs were lavaged four times *in situ* with Hanks' balanced salt solution (HBSS; 0.35 ml/kg, pH 7.2–7.4, room temperature). Recovered bronchoalveolar lavage (BAL) fluid from each mouse was pooled and immediately cooled to 4°C. The HBSS contains the following (g/liter): 8.0 NaCl; 0.4 KCl; 0.06 KH_2PO_4; 0.05 Na_2HPO_4; 0.35 $NaHCO_3$; and 1.0 dextrose. For each mouse, the first BAL return was isolated from the remaining three BAL returns, which were pooled. The lavage returns were then centrifuged (500 × *g*, 4°C), and the supernatant of the first lavage return was decanted. The total protein concentration in this supernatant was measured and used as an indicator of lung permeability. The remaining supernatants were discarded. Numerous indicators of altered airspace-blood barrier function have been used in oxidant toxicity studies and each has its limitations. Many studies of changes in lung permeability have used total BAL protein, which includes albumin, immunoglobulins, and enzymes. Most of the added protein that accumulates after oxidant challenge originates from the serum (26). A bovine serum albumin protein assay kit (Pierce, Rockford, IL) was used that follows the method of Bradford (27) and is accurate from 10 to 2,000 μg/ml. The cell pellets from all four lavages were combined and resuspended in 0.8 ml RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% newborn calf serum, and cells were counted with a hemocytometer. Aliquots (50 μl) were cytocentrifuged (Shandon Southern Products, Pittsburgh, PA), and the cells were stained with Diff-Quik (Baxter Scientific Products, McGaw Park, IL) for differential cell analysis. Differential cell counts were done by identifying 300 cells according to standard cytologic techniques.

Experimental Protocol

Lung permeability and inflammation were assessed in mice after 24, 48, or 72 h of continuous exposure to 0.30 ppm O_3. Simultaneous exposures to filtered air were done in age- and strain-matched mice to serve as O_3 controls. Mice were killed within 1 h of removal from the exposure chambers. BXH RI mice were assessed for permeability and inflammatory changes after 72 h of exposure; B6 and HeJ mice were included in each experiment to serve as positive and negative controls.

Linkage Analyses

Genome-wide searches for QTLs were done using the mean hyperpermeability (BAL protein) and polymorphonuclear leukocyte (PMN) phenotypes for each RI strain in the BXH RI set. Interval analyses were performed by fitting a regression equation for the effect of a hypothetical QTL at the position of each simple sequence length polymorphism (SSLP) and other polymorphic markers, and at 1-cM intervals between markers, in the BXH RI strain distribution library. The markers have been typed for the BXH RI strains by numerous investigators and are archived in Map Manager (28). The dominance properties of each putative QTL were evaluated by performing interval analyses using an additive regression model. The regressions and significance of each association (likelihood ratio χ^2 statistic) were calculated by the Map Manager QTb27 program, which is distributed electronically and available at http://mcbio.med.buffalo.edu/mmQT.html (28). To establish empirically the significance thresholds of all QTL mapping results, permutation tests were performed on the phenotype and genotype data using Map Manager QTb27 and following the methods of Churchill and Doerge (29). For the genome scan, 10,000 permutations were performed to establish significant and suggestive linkage threshold values. These values corresponded to the genome-wide probabilities proposed by Lander and Kruglyak (30). To conform to assumptions of the linkage analyses, the BAL protein concentration and PMN (phenotype) data from B6, HeJ, and RI animals were tested for normality and homoscedasticity (homogeneity of variances).

Tlr4 Messenger RNA Expression

Total RNA was isolated from lung tissues of HeJ and OuJ mice exposed to 0.3 ppm O_3 for 72 h, as well as unexposed controls, by homogenizing in Trizol reagent (Life Technologies, Gaithersburg, MD) and following the manufacturer's recommended protocol. Complementary DNA (cDNA) was prepared by reverse transcribing 5 μg of total RNA primed with oligo(dT) using the SuperScript Preamplification System (Life Technologies). Amplification was done under the following conditions: 1.5 mM $MgCl_2$, 70°C annealing temperature, 2.5 min extension time, and 35 cycles. Primers, which generate a 2.6-kb fragment, were synthesized according to Poltorak and coworkers (23). β-Actin was simultaneously amplified as an internal (reference) control. Fragments were analyzed on 1.3% agarose gels. The amplified cDNA fragments were scanned and quantitated using a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY).

Statistics

Analyses of permeability and PMN changes in RI strains were done by two-way analysis of variance (ANOVA). The factors were strain (RIs and progenitors) and exposure (air, O_3). Dunnett's test was used to compare mean BAL protein concentrations and number of PMNs from RI strains with those of the progenitors (31). The minimum numbers of RI mice required for the analyses were determined by sample size calculation based on the variances in hyperpermeability and PMN responses to O_3 in the progenitor B6, HeJ, and OuJ strains (power was approximately 0.85 with 0.05 level of significance). Statistical analysis of O_3-induced hyperpermeability in HeJ and OuJ mice was done by three-way ANOVA (SuperANOVA statistical package; Abacus Concepts, Berkeley, CA). The factors for the analysis were strain, exposure (air, O_3), and time (6, 24, 48, 72 h). Student-Newman-Keuls (SNK) *a posteriori* tests were used for comparisons of mean responses between experimental groups. Statistical significance for all comparisons of means was accepted at $P < 0.05$.

Results

O_3-Induced Change in Lung Permeability and PMNs in BXH RI Strains

Lung permeability was assessed in 12 age- and sex-matched BXH RI strains after 72 h of exposure to air and 0.3 ppm O_3 (n = 5/strain). Relative to air-exposed animals, O_3 caused significant increases in the mean (± standard error [SE]) BAL protein concentrations of all RI strains except numbers 6 and 12. The mean (± SE) BAL protein concentrations in the RI strains after O_3 ranged from 150 ± 12 μg/ml BAL return (RI number 6) to 712 ± 83 μg/ml BAL return (RI number 11) (Figure 1). The mean BAL protein concentrations in BXH RI strains 2, 3, 4, 6, 7, 8, 9, and 12 were not significantly different ($P > 0.05$) from the mean BAL protein concentration from O_3-exposed HeJ mice (258 ± 18 μg/ml BAL return) (Figure 1). The mean BAL protein concentrations in BXH RI strains 10, 14, and 19 were not significantly different ($P > 0.05$) from O_3-exposed B6 mice (550 ± 36 μg/ml BAL return) (Figure 1). BXH RI number 11 had a hypersusceptible permeability phenotype as the mean BAL protein concentration was significantly ($P < 0.05$) greater than the mean protein concentration of B6 mice (Figure 1).

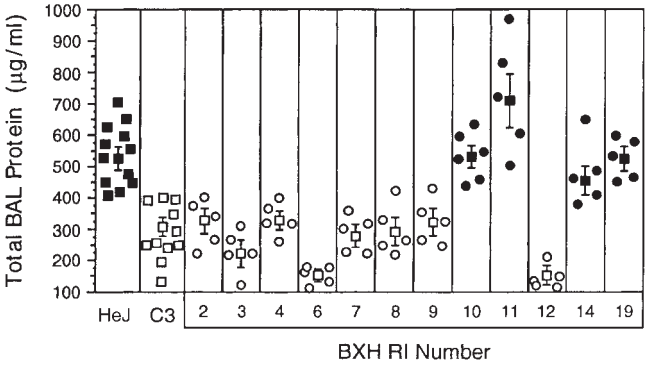

Figure 1. Total BAL protein recovered from BXH RI mice and B6 and HeJ progenitors after 72 h exposure to 0.3 ppm O_3. Means ± standard error of the mean (SEM) are presented. *Closed symbols,* "B6-like." *Open symbols,* "HeJ-like."

Compared with air-exposed control mice, O_3 caused significant increases in the mean (± SE) number of lavageable PMNs of all RI strains except number 6 (Table 1). The mean number of PMNs recovered from RI numbers 8 and 14 were not significantly different from B6 mice, and those from RI strains 2, 4, 6, 7, 9, 10, 11, 12, and 19 were not significantly different from HeJ mice. The mean number of PMNs from RI number 3 was significantly ($P < 0.05$) greater than (i.e., hypersusceptible) the mean from B6 mice. The mean PMN responses in RI strains 3, 8, 10, 11, and 19 were discordant with respect to the BAL protein responses. That is, both phenotypes were not qualitatively "B6-like" or "HeJ-like." The discordance of strain distribution patterns within the RI set suggests that the genetic mechanisms responsible for each phenotype are not identical.

Genome-Wide Scan for QTLs in the BXH RI Strains

Linkage analyses were initiated by scanning the entire genome for associations between the O_3-induced hyperpermeability phenotype and 558 polymorphic SSLPs and other markers. Significance thresholds of all QTL mapping results were determined by permutation tests performed on the phenotype and genotype data using Map Manager QTb27 (28). For the genome scan, 10,000 permutations were performed to establish significant and suggestive linkage threshold values. Interval analyses identified a significant QTL on chromosome 4 and suggestive QTLs on chromosomes 3 and 11 (Figure 2). Composite interval mapping was done to determine the potential influence of the suggestive QTLs on linkage of the O_3-induced hyperpermeability phenotype to chromosome 4. When the suggestive QTLs on chromosomes 3 and 11 were controlled, the significance of the QTL on chromosome 4 did not change. The peak likelihood χ^2 statistic in the chromo-

TABLE 1

Mean (± SE) number of PMNs recovered by BAL from C57BL/6J, C3H/HeJ, and BXH RI mice after 72 h exposure to 0.30 ppm O_3

		PMNs ($\times 10^3$/*ml BAL return*)	
Strain or RI	*n*	Mean ± SE	Range
C57BL/6J	16	10.1 ± 0.5	7.2–14.5
C3H/HeJ	14	2.4 ± 0.3*	0.5–4.1
RI 2	9	2.8 ± 0.7*	0.5–7.4
3†	8	15.3 ± 1.9*	7.5–18.5
4	10	2.1 ± 0.5*	0.4–4.7
6	9	1.7 ± 0.8*	0.4–7.6
7	13	2.3 ± 0.4*	0.6–4.8
8†	9	13.2 ± 1.8	7.9–24.2
9	9	1.6 ± 0.3*	0.4–2.9
10†	10	2.8 ± 0.4*	1.0–4.6
11†	11	1.3 ± 0.3*	0.2–3.4
12	6	2.7 ± 0.7*	0.7–5.5
14	14	10.8 ± 0.9	6.2–18.1
19†	8	1.9 ± 0.4*	0.5–3.3

*$P < 0.05$, statistical comparison of RI strains with C57BL/6J mice.
†BXH RI strains with phenotypes that are discordant compared with BAL protein responses.

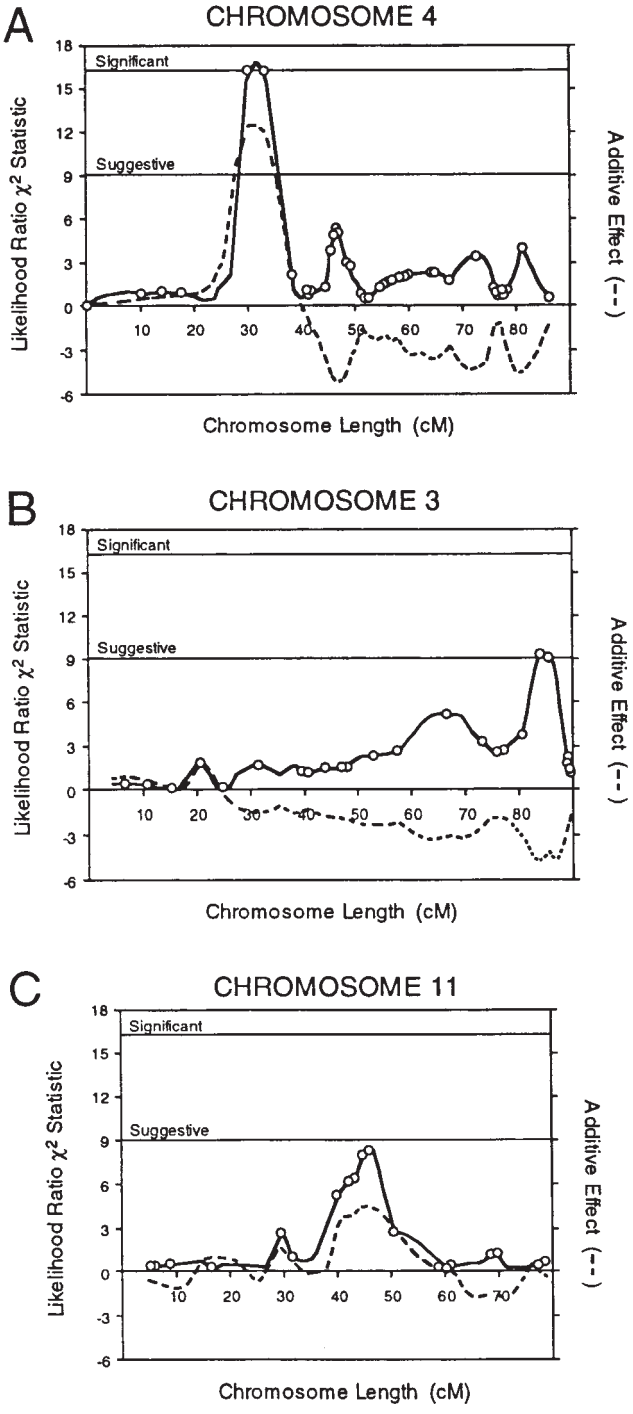

Figure 2. Plot of the likelihood ratio χ^2 statistic (*solid lines*) and additive regression coefficient (*dashed lines*) for the association of O_3-induced hyperpermeability phenotype with polymorphic markers on chromosome 4 (*A*), chromosome 3 (*B*), and chromosome 11 (*C*). RI mice used for these analyses were phenotyped after 72 h exposure to 0.3 ppm O_3. Interval mapping was done by simple linear regression with Map Manager QTb27 to generate χ^2 and regression coefficient values. The *upper* and *lower horizontal lines* in each plot represent significant and suggestive linkage thresholds, respectively, as determined by permutation test (*see* text).

some 4 QTL included the loci *Tlr4* and *Mup1* (murine urinary protein 1). The amount of the total trait variance explained by the QTL at *Tlr4* and *Mup1*, the genes with the highest likelihood ratio statistic in the QTL, was approximately 70%.

The hyperpermeability phenotype was also treated as a qualitative trait (i.e., RI strains were characterized as "HeJ-like" or "B6-like") and tested for linkage with the RI strain distribution pattern (SDP) library. There was complete concordance (logarithm of the odds $[LOD]_{Linkage}$ = 3.6) of the O_3 susceptibility SDP with the SDPs for *Tlr4* and *Mup1* (Figure 3). The LOD scores exceed the 95% probability of linkage of the three SDPs (28).

A linkage analysis was also done for the mean number of PMNs recovered from RI strains exposed to 0.3 ppm O_3 for 72 h. In contrast to previous analyses using B6 backcross and $B6C3F_2$ cohorts (20), no significant or suggestive QTLs were identified. The absence of linkage may be due to differences in phenotype between the studies. In the previous study, B6 backcross (108 meioses) and $B6C3F_2$ (230 meioses) mice were phenotyped after 48 h of exposure when the difference between mean numbers of PMNs in B6, HeJ, and OuJ mice was greatest. In the present study, the BXH RI strains were phenotyped after 72 h of exposure to O_3 when differences between progenitors was not as great. Another contributing factor may be that the PMN phenotype is not as penetrant as the permeability response, and the number of meioses represented in the BXH RI set was not sufficient to establish linkage for the PMN response. The B6 backcross and $B6C3F_2$ cohorts were more informative for this phenotype.

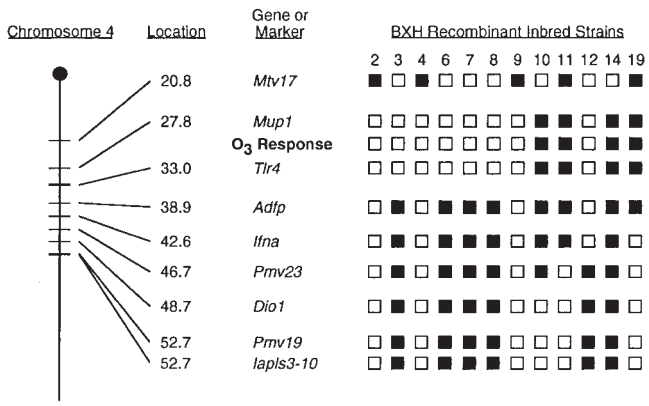

Figure 3. SDP for O_3-induced hyperpermeability phenotype and representative loci on chromosome 4 that have been typed in the BXH panel of RI strains. RI mice used for these analyses were phenotyped after 72 h exposure to 0.3 ppm O_3. *Solid squares* designate alleles inherited from the C57BL/6J strain and *open squares* designate those from the C3H/HeJ strain. Approximate location of the loci was obtained from the mouse genome database. *Mtv19*, mammary tumor virus 19; *Mup1*, murine urinary protein 1; *Tlr4*, Toll-like receptor 4; *Adfp*, adipose differentiation related protein; *Ifna*, interferon alpha gene family, leukocyte; *Pmv*, polytropic murine leukemia virus; *Dio1*, deiodinase, iodothyronine, type I; *Iapls3-10*, intracisternal A particle, lymphocyte specific 3–10.

Kinetics of O_3-Induced Hyperpermeability in HeJ and OuJ Mice

The *Tlr4* locus (formerly designated *Lps* for lipopolysaccharide) is of particular interest as homozygosity for the codominant mutant allele confers resistance to endotoxin in HeJ mice. Substrain C3H/HeN and OuJ, as well as B6, mice are homozygous for the wild-type, nonmutant allele and exhibit vigorous responsiveness to endotoxin challenge. Recent work by Poltorak and colleagues (23) and Qureshi and coworkers (24) has demonstrated that the mutant allele corresponds to a missense mutation in the third exon of *Tlr4*. To evaluate the candidate gene *Tlr4* in the differential O_3 susceptibility model, we compared the kinetics of O_3-induced change in BAL protein in mice homozygous for the mutant *Tlr4* allele (HeJ) with the kinetics in mice homozygous for the wild-type *Tlr4* allele (OuJ) (Figure 4). Statistical analysis by two-way ANOVA indicated that there was a statistically significant ($P < 0.05$) effect of strain, exposure, and time on the mean BAL protein concentration. There was also statistically significant ($P < 0.05$) interaction between strain and exposure, indicating that the strains responded differently to the exposure. Relative to respective air-exposed control mice, O_3 caused a significant (SNK; $P < 0.05$) increase in BAL protein in HeJ and OuJ mice after 24 h exposure (Figure 4). BAL protein concentrations remained elevated in both strains of mice after 48 and 72 h exposure (Figure 4). Furthermore, the mean BAL protein concentrations from O_3-exposed OuJ mice were significantly (SNK; $P < 0.05$) greater than those from HeJ mice after 24, 48, and 72 h exposure to O_3. Protein concentrations after air exposure or after 6 h exposure to O_3 were not significantly different between strains.

Tlr4 Gene Expression in C3H/HeJ and C3H/HeOuJ Mice after Exposure to O_3

Based on the linkage analyses and differential responsiveness to O_3 in HeJ and OuJ mice, we assessed expression of *Tlr4* by reverse transcriptase/polymerase chain reaction (RT-PCR). To establish baseline expression, RNA from lung tissue of six to eight unexposed mice of each strain was isolated, reverse-transcribed, and amplified by PCR. There was no difference in expression of *Tlr4* between the two strains (Figure 5). Bands in *lanes 1* and *2* were derived from amplified pools of cDNA and are representative of individual mice from each strain. After 72 h exposure to O_3, the expression level of *Tlr4* in OuJ mice was approximately 40% higher than baseline (Figure 5, *lane 4*). However, there was no detectable expression of *Tlr4* in O_3-exposed HeJ mice (Figure 5, *lane 3*). *Lanes 3* and *4* represent pooled cDNA from six to eight individual mice of each strain and are representative of individuals from each strain assessed independently. β-Actin was simultaneously amplified to serve as a reference internal control (Figure 5), and consistent expression was detected for all amplified pools.

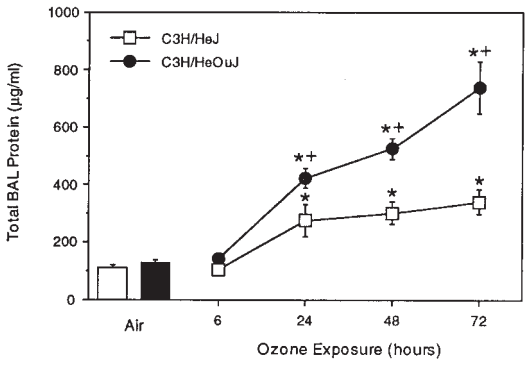

Figure 4. Total BAL protein responses in C3H/HeOuJ and C3H/HeJ mice after 6, 24, 48, and 72 h exposure to 0.30 ppm O_3. Air controls were not significantly different from each other and were pooled. Means ± SEM are presented. Group sizes are five to 16. Statistical comparison of air versus O_3 treatment groups: $^*P < 0.05$; C3H/HeJ versus C3H/HeOuJ: $^+P < 0.05$.

Discussion

It is well established that interindividual variation in pulmonary responses to environmental agents, including O_3, exists in human populations. Mechanisms that confer differential responsiveness are not completely understood, but likely include a number of host factors such as age, nutrition, sex, and pre-existing disease (e.g., asthma). Differential responsiveness in individuals otherwise controlled for known susceptibility factors has suggested that genetic background may have an important role in determining responsivity.

Our laboratory previously identified a significant QTL on chromosome 17 and a suggestive QTL on chromosome 11 that accounted for a portion of the genetic variance in an inflammatory cell phenotype (infiltrating PMNs) induced by exposure to 0.3 ppm O_3. Tumor necrosis factor

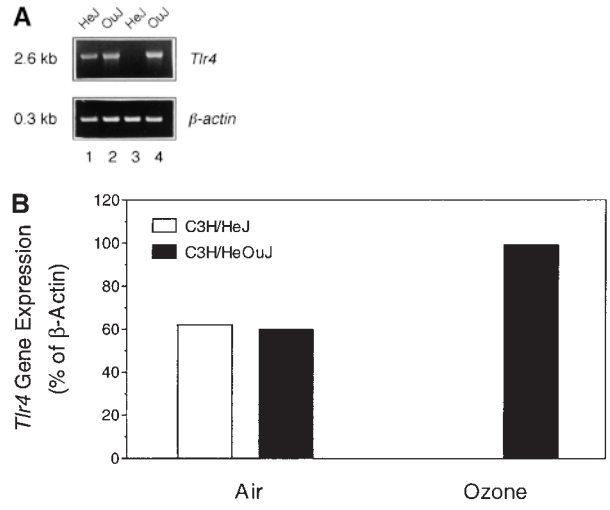

Figure 5. (*A*) Comparison of *Tlr4* gene expression in pooled lung tissues from HeJ and OuJ mice as detected by RT-PCR (*upper panel*). *Lanes 1* and *2* are from unexposed animals, and *lanes 3* and *4* are from animals exposed to 0.3 ppm O_3 for 72 h. Lanes represent pooled cDNA from six to eight individual mice of each strain. Simultaneous analysis of *β-actin* gene expression (*lower panel*) was done as a positive reference. (*B*) Relative *Tlr4* gene expression in C3H/HeJ and C3H/HeOuJ mice, expressed as a percent of *β-actin* gene expression. Expression of *Tlr4* was not detectable in C3H/HeJ mice after O_3.

(*Tnf*) was identified as a candidate gene in the chromosome 17 QTL. Treatment of susceptible B6 mice with monoclonal anti-TNF antibodies significantly attenuated the inflammatory response (20), thus providing support for *Tnf* as a candidate susceptibility gene in that model.

The current study was designed to identify additional genes that may have a role in determining susceptibility to O_3-induced injury. With hyperpermeability as the response phenotype, a genome scan was performed with BXH RI strains. Despite the small number of RI strains used in the genome screen, the algorithm for identification of QTLs within the Map Manager program identified the interval between *Tlr4* (formerly *Lps*) and *Mup1* on chromosome 4 as a statistically significant QTL. The χ^2 values for the interval exceeded the threshold levels for 95% confidence as determined by permutation test. Furthermore, there was perfect concordance between the qualitative hyperpermeability phenotype SDP and *Tlr4* and *Mup1* SDPs on chromosome 4 (Figure 3). The lack of stronger statistical significance is due to the limited number of available RI lines in the BXH set (n = 12). In any case, the QTL was sufficiently informative to identify a candidate gene for hyperpermeability susceptibility that has provided considerable insight to the mechanisms of acute lung injury induced by an environmentally relevant O_3 concentration (*see subsequent paragraphs*). The suggestive QTL on chromosome 11 was identical to the QTL characterized previously for the PMN phenotype (20). This broad QTL has also been identified independently for contribution to genetic variance in O_3-induced lethality (32) and particle-induced alveolar macrophage phagocytic dysfunction (33).

Based on the strength of association between the hypersusceptibility phenotypes and the chromosome 4 QTL, we initiated experiments to evaluate the importance of candidate genes within the QTL. *Tlr4* is a particularly intriguing candidate gene for susceptibility to O_3 because of its role in innate immunity in *Drosophila* and humans (34, 35), and its importance in modulating responses to endotoxin (23, 24, 36, 37). *Tlr4* belongs to a family of genes that code for Toll-like receptors (TLRs). TLRs activate intracellular signaling that results in the induction of a variety of effector genes (34). The cytoplasmic domain of the TLRs is homologous to the cytoplasmic domain of the interleukin (IL)-1 receptor family and shares signaling components. In particular, the transcription factor NF (nuclear factor)-κB is an important effector of *Tlr4* activation, as NF-κB has been shown to have critical involvement in a number of inflammatory processes (38-40). Further, HeJ mice have a C to A transversion at codon 712 in *Tlr4* that results in a single nonconservative amino acid substitution of the highly conserved proline by histidine within the cytoplasmic domain (24). Endotoxin-susceptible B6 and OuJ mice do not have the mutation. The mutation was shown to cosegregate with endotoxin responsiveness in backcross cohorts from HeJ X B6 and HeJ X DBA/2J progenitors, thus providing convincing evidence for *Tlr4* as the susceptibility locus (24). In the present study, the kinetics of O_3-induced hyperpermeability were evaluated in HeJ and OuJ mice, and OuJ mice had a significantly more responsive phenotype compared with the HeJ strain. The magnitude and time course of response in OuJ mice compared favorably with that of B6 mice (41). These novel results are consistent with a role for *Tlr4* in modulating the hyperpermeability response to O_3 in the mouse.

The mechanism through which *Tlr4* regulates the O_3 response is not known. Although the number of newly identified TLRs continues to increase (42), the ligands for all of the receptors remain largely uncharacterized (34). O_3 reacts with molecules in the epithelial lining fluid of the airways or with cell membranes to form a variety of substances, including lipid ozonation products. It has been proposed that these products are the most likely relay molecules of O_3 signaling (43) and may activate the TLR4. The downstream pathway through which activated TLR4 regulates O_3-induced hyperpermeability/inflammation is likely through NF-κB as this transcription factor has a critical role in the induction of key proinflammatory cytokines involved in O_3 responsiveness (44–46). In type II–like A549 respiratory epithelial cells, Jaspers and associates (45) demonstrated that O_3 induced activity of NF-κB (as well as NF–IL-6 and activator protein 1 [AP-1]) and IL-8, a potent chemotactic factor for neutrophils, which led the authors to suggest a potential link between them in an inflammatory cascade. Zhao and coworkers (46) demonstrated a correlation between the time course of NF-κB activity and monocyte chemoattractant protein-1 in B6 mice exposed to O_3, indicating a potential role of NF-κB in modulating inflammation in this model. It is thus conceivable that a mutation in a key regulatory element upstream of the NF-κB signaling pathway after O_3 exposure, such as TLR4, could modulate inflammatory and other responses.

To further investigate the role of *Tlr4* in this model, we evaluated *Tlr4* gene expression in HeJ and OuJ mice. As determined by RT-PCR, we observed no substantial differences in the basal level expression of *Tlr4* messenger RNA (mRNA) expression in lung homogenates from the two strains. This observation is consistent with findings by Poltorak and colleagues (23) who showed that *Tlr4* gene expression did not differ in macrophages obtained from endotoxin-resistant HeJ and endotoxin-susceptible C3H/HeN mice. However, we found *Tlr4* gene expression was markedly different between the two strains of mice after exposure to O_3. *Tlr4* gene expression was not detectable in lung tissue from HeJ mice after 72 h exposure to O_3, whereas expression increased by 40%, relative to controls, in OuJ mice (Figure 5). Suppression of *Tlr4* mRNA has been described previously in RAW 264.7 cells challenged *in vitro* with endotoxin (23), but to our knowledge, this is the first demonstration that oxidant exposure has similar effects *in vivo*. This suggests that downregulation of *Tlr4* gene expression may contribute to O_3 resistance in HeJ mice. Experiments are ongoing to understand the molecular mechanisms involved in the modulation of *Tlr4* gene expression by O_3 exposure in these strains.

The current work and previous studies (20) strongly implicate the Toll and TNF receptor signaling pathways in modulating the PMN and hyperpermeability responses to O_3 exposure. Whereas these two pathways have similar general organization that leads to activation of transcription factor NF-κB (34, 47), there are also important differences. Both pathways use TNF receptor-associated factor protein (TRAF) adapters and serine/threonine kinases

that link them to the protein kinase NF-κB–inducing kinase. However, the Toll pathway uses the TRAF6 protein and IL-1 receptor-associated kinase (IRAK), whereas TNF signaling requires TRAF2 and TRAF5, as well as receptor-interacting protein (34, 47). MyD88, another adapter protein in the Toll signaling pathway, recruits IRAK to the receptor complex and is critical to the signaling cascade that is mediated by Toll/IL-1 (48–50). TNF receptor associated death-domain-containing protein is an analogous adapter protein for the TNF receptor complex (34, 47). Even though the roles of these proteins in innate immunity and development are becoming clarified, their importance in modulating lung injury by nonspecific agents (such as oxidants) is largely unknown. It is likely that neither the Toll nor TNF signaling pathway alone accounts for the integrated lung response to O_3, but rather is a complex interaction of the two. We believe that an understanding of these intracellular pathways that lead to activation of transcription factors and subsequent proinflammatory mediators induced by O_3 and other oxidants should provide novel intervention strategies for modulating susceptibility.

In summary, we have identified a significant QTL (chromosome 4) and two suggestive QTLs (chromosomes 3 and 11) that contribute to the genetic variation in susceptibility to O_3-induced hyperpermeability. *Tlr4* is located in the chromosome 4 QTL and was tested as a candidate gene. Differential O_3 responsivity in resistant C3H/HeJ and susceptible C3H/HeOuJ and C57BL/6J mice is associated with a *Tlr4* polymorphism that was identified previously as an important determinant of endotoxin susceptibility in mice. Results suggest that there is a genetic commonality between signal transduction pathways involved in determining responsivity to O_3 and endotoxin.

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References

1. Balmes, J. R., L. L. Chen, C. Scannell, I. Tager, D. Christian, P. Q. Hearne, T. Kelly, and R. M. Aris. 1996. Ozone-induced decrements in FEV_1 and FVC do not correlate with measures of inflammation. *Am. J. Respir. Crit. Care Med.* 153:904–909.
2. Gielen, M. H., S. C. van der Zee, J. H. van Wijnen, C. J. van Steen, and B. Brunekreef. 1997. Acute effects of summer air pollution on respiratory health of asthmatic children. *Am. J. Respir. Crit. Care Med.* 155:2105–2108.
3. Peden, D. B., B. Boehlecke, D. Horstman, and R. Devlin. 1997. Prolonged acute exposure to 0.16 ppm ozone induces eosinophilic airway inflammation in asthmatic subjects with allergies. *J. Allergy Clin. Immunol.* 100:802–808.
4. Delfino, R. J., B. D. Coate, R. S. Zeiger, J. M. Seltzer, D. H. Street, and P. Koutrakis. 1996. Daily asthma severity in relation to personal ozone exposure and outdoor fungal spores. *Am. J. Respir. Crit. Care Med.* 154:633–641.
5. Romieu, I., F. Meneses, S. Ruiz, J. J. Sienra, J. Huerta, M. C. White, and R. A. Etzel. 1996. Effects of air pollution on the respiratory health of asthmatic children living in Mexico City. *Am. J. Respir. Crit. Care Med.* 154: 300–307.
6. Thurston, G. D., M. Lippmann, M. B. Scott, and J. M. Fine. 1997. Summertime haze air pollution and children with asthma. *Am. J. Respir. Crit. Care Med.* 155:654–660.
7. Brauer, M., J. Blair, and S. Vedal. 1996. Effect of ambient ozone exposure on lung function in farm workers. *Am. J. Respir. Crit. Care Med.* 154:981–987.
8. Delfino, R. J., A. M. Murphy-Moulton, R. T. Burnett, J. R. Brook, and M. R. Becklake. 1997. Effects of air pollution on emergency visits for respiratory illnesses in Montreal, Quebec. *Am. J. Respir. Crit. Care Med.* 155: 568–576.
9. Korrick, S. A., L. M. Neas, D. W. Dockery, D. R. Gold, G. A. Allen, L. B. Hill, K. D. Kimball, B. A. Rosner, and F. E. Speizer. 1998. Effects of ozone and other pollutants on the pulmonary function of adult hikers. *Environ. Health Perspect.* 106:93–99.
10. Naeher, L. P., T. R. Holford, W. S. Beckett, K. Belanger, E. W. Triche, M. B. Bracken, and B. P. Leaderer. 1999. Healthy women's PEF variations with ambient summer concentrations of PM_{10}, $PM_{2.5}$, SO_4^{2-}, H^+, and O_3. *Am. J. Respir. Crit. Care Med.* 160:117–125.
11. Abbey, D. E., N. Nishino, W. F. McDonnell, R. J. Burchette, S. F. Knutsen, W. L. Beeson, and J. X. Yang. 1999. Long-term inhalable particles and other air pollutants related to mortality in nonsmokers. *Am. J. Respir. Crit. Care Med.* 159:373–382.
12. Hoek, G., J. D. Schwartz, B. Groot, and P. Eilers. 1997. Effects of ambient particulate matter and ozone on daily mortality in Rotterdam, the Netherlands. *Arch. Environ. Health* 52:455–463.
13. Lee, J.-T., D. Shin, and Y. Chung. 1999. Air pollution and daily mortality in Seoul and Ulsan, Korea. *Environ. Health Perspect.* 107:149–154.
14. Bascom, R., P. A. Bromberg, D. A. Costa, R. Devlin, D. W. Dockery, M. W. Frampton, W. Lambert, J. M. Samet, F. E. Speizer, and M. Utell. 1996. Health effects of outdoor air pollution. *Am. J. Respir. Crit. Care Med.* 153: 3–50.
15. Aris, R. M., D. Christian, P. Q. Hearne, K. Kerr, W. E. Finkbeiner, and J. R. Balmes. 1993. Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. *Am. Rev. Respir. Dis.* 148:1363–1372.
16. Devlin, R. B., W. F. McDonnell, R. Mann, S. Becker, D. E. House, D. Schreinemachers, and H. S. Koren. 1991. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am. J. Respir. Cell Mol. Biol.* 4:72–81.
17. Schelegle, E. S., A. D. Siefkin, and R. J. McDonald. 1991. Time course of ozone-induced neutrophilia in normal humans. *Am. Rev. Respir. Dis.* 143: 1353–1358.
18. McDonnell, W. F., III. 1991. Intersubject variability in human acute ozone responsiveness. *Pharmacogenetics* 1:110–113.
19. Weinmann, G. G., S. M. Bowes, M. W. Gerbase, A. W. Kimball, and R. Frank. 1995. Response to acute ozone exposure in healthy men: results of a screening procedure. *Am. J. Respir. Crit. Care Med.* 151:33–40.
20. Kleeberger, S. R., R. C. Levitt, L.-Y. Zhang, M. Longphre, J. Harkema, A. Jedlicka, S. M. Eleff, D. DiSilvestre, and K. J. Holroyd. 1997. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nature Genetics* 17:475–478.
21. Kleeberger, S. R., and B. B. Hudak. 1992. Acute ozone-induced change in airway permeability: the role of infiltrating leukocytes. *J. Appl. Physiol.* 72: 670–676.
22. Pino, M. V., J. R. Levin, M. Y. Stovall, and D. M. Hyde. 1992. Pulmonary inflammation and epithelial injury in response to acute ozone exposure in the rat. *Toxicol. Appl. Pharmacol.* 112:64–72.
23. Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in the *Tlr4* gene. *Science* 282: 2085–2088.
24. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (*Tlr4*). *J. Exp. Med.* 189:615–625.
25. Taylor, B. A. 1978. Recombinant inbred strains: use in gene mapping. *In* Origins of Inbred Mice. H. Morse, editor. Academic Press, New York. 423–438.
26. Hu, P. C., F. J. Miller, M. J. Daniels, G. E. Hatch, J. A. Graham, D. E. Gardner, and M. K. Selgrade. 1982. Protein accumulation in lung lavage fluid following ozone exposure. *Environ. Res.* 29:377–388.
27. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
28. Manly, K., and J. M. Olson. 1999. Overview of QTL mapping software and introduction to Map Manager QT. *Mamm. Genome* 10:327–334. See also: http://mcbio.med.buffalo.edu/mapmgr.html
29. Churchill, G. A., and R. W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. *Genetics* 138:963–971.
30. Lander, E. S., and L. Kruglyak. 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nature Genetics* 11:241–247.
31. Sokal, R. R., and F. J. Rohlf. 1969. Biometry: The Principles and Practice of Statistics in Biological Research. Freeman Press, San Francisco.
32. Prows, D. R., H. G. Shertzer, M. J. Daly, C. L. Sidman, and G. D. Leikauf. 1997. Genetic analysis of ozone-induced acute lung injury in sensitive and resistant strains of mice. *Nature Genetics* 17:471–474.
33. Ohtsuka, Y., K. Brunson, A. E. Jedlicka, W. Mitzner, R. W. Clarke, L.-Y. Zhang, S. M. Eleff, and S. R. Kleeberger. Linkage analysis of susceptibility to particle-induced macrophage dysfunction in mice. *Am. J. Respir. Cell Mol. Biol.* (In press)
34. Kopp, E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol.* 11:13–18.
35. Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan.

1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* 95:588–593.
36. Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274:10689–10692.
37. Vogel, S. N., D. Johnson, P. Y. Perera, A. Medvedev, L. Lariviere, S. T. Qureshi, and D. Malo. 1999. Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an *Lps*^{n} gene: in vivo evidence for a dominant negative mutation. *J. Immunol.* 162:5666–5670.
38. Baeuerle, P. A. 1998. IκB-NF-κB structures: at the interface of inflammation control. *Cell* 95:729–731.
39. Baldwin, A. S. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649–683.
40. Lenardo, M. J., and D. Baltimore. 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58:227–229.
41. Kleeberger, S. R., R. C. Levitt, and L.-Y. Zhang. 1993. Susceptibility to ozone-induced inflammation: I. Genetic control of the response to subacute exposure. *Am. J. Physiol.* 264:L15–L20.
42. Takeuchi, O., T. Kawai, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Takeda, and S. Akira. 1999. TLR6: a novel member of an expanding Toll-like receptor family. *Gene* 231:59–65.
43. Kafoury, R. M., W. A. Pryor, G. L. Squadrito, M. G. Salgo, X. Zou, and M. Friedman. 1998. Lipid ozonation products activate phospholipases A_2, C, and D. *Toxicol. Appl. Pharmacol.* 150:338–349.
44. Haddad, E.-B., M. Salmon, H. Koto, P. J. Barnes, I. Adcock, and K. F. Chung. 1996. Ozone induction of cytokine-induced neutrophil chemoattractant (CINC) and nuclear factor-κb in rat lung: inhibition by corticosteroids. *FEBS Lett.* 379:265–268.
45. Jaspers, I., E. Flescher, and L. C. Chen. 1997. Ozone-induced IL-8 expression and transcription factor binding in respiratory cells. *Am. J. Physiol.* 272:L504–L511.
46. Zhao, O., L. G. Simpson, K. E. Driscoll, and G. D. Leikauf. 1998. Chemokine regulation of ozone-induced neutrophil and monocyte inflammation. *Am. J. Physiol.* 274:L39–L46.
47. Kopp, E., R. Medzhitov, J. Carothers, C. Xiao, I. Douglas, C. A. Janeway, and S. Ghosh. 1999. ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes Dev.* 13:2059–2071.
48. Muzio, M., G. Natoli, S. Saccani, M. Levrero, and A. Mantovani. 1998. The human toll signaling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* 187:2097–2102.
49. Wesche, H., W. J. Henzel, W. Shillinglaw, S. Li, and Z. Cao. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* 7:837–847.
50. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the *MyD88* gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143–150.